SCRIBE

Science Chronicles in Research and Investigation Based Education

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(supported by DBT Star Status)

Sophia College (Autonomous)

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We are delighted to bring to you the second issue of SCRIBE (Science Chronicles in Research and Investigation Based Education), our annual in-house inter-disciplinary science journal, now under the aegis of DBT Star Status.

We sincerely thank our Principal, Dr. (Sr.). Ananda Amritmahal, for her kind support.

The first issue of SCRIBE was inaugurated in a formal ceremony in our College and published on National Science Day, 28th February 2020. Since then, despite the challenges posed by the COVID-19 pandemic, our dedicated team of student editors has worked hard for the release of this issue of SCRIBE.

We are happy to announce that the distinctive feature of this issue is that all articles are peer-reviewed by a panel of experts in the field. This, we believe, is a step towards bringing in more rigour and critical appraisal, making SCRIBE a journal of greater credibility. We have tried to maintain a double-blind system to keep the process unbiased. We express our deepest gratitude to the peer reviewers who have taken the work of reviewing the articles very seriously.

In this issue, we have also restricted ourselves to Research and Review articles, steering clear of articles that do not fall under either category and in a format that we hope is more formal and visually appealing.

We bring for you an invited article by our Vice Principal (Science), who has shared her research journey on 'Zebrafish' as a model system. The Research articles are on topics related to Environmental Microbiology and Cognitive neuroscience and Review articles are on fields as varied as Cell biology, Developmental biology, Ecology, Endocrinology, Immunology, Microbiology, and Neurobiology.

Undoubtedly, this issue would be incomplete without addressing the elephant in the room – COVID-19, thus, it encompasses Review articles on SARS-CoV2.

Besides these, articles on 'Women in Science' and 'Nobel Prizes 2020' have been included to commemorate and celebrate their contributions to science.

We wish you happy reading!

Invited article

Can zebrafish (*Danio rerio)* replace rodents as a vertebrate model?

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Abstract

Zebrafish (*Danio rerio*) is a freshwater fish that is native to the south –eastern Himalaya region and is naturally found in parts of India, Pakistan, Bangladesh, Nepal and Burma. Originally discovered in the Ganges river in the late 19th century, it is proving to be an excellent vertebrate model for a variety of studies. Here we give an overview of the uses of zebrafish as a model organism and then provide glimpses of the research studies carried out in our Zebrafish facility.

Introduction

If you do a PubMed search for 'Zebrafish', an interesting graph of the number of papers published over the years is visible. The earliest reference starts from 1948, however, in 1990 there were 48 publications with zebrafish; in 2020 that number stands at 4282 (shown in Fig.1).

Originally, zebrafish was used as a model for early developmental biology studies, due to the large size of the transparent embryos that were obtained by external fertilization. It also proved to be amenable to genetic modifications and was used for study of genetic diseases. There are several other benefits that zebrafish offers as a model organism. It has high fertility with a single breeding pair yielding on average 300 fertilized eggs at regular intervals over a period of one year or more. This makes their maintenance low cost compared to other vertebrate model organisms. *In vitro* fertilization and egg transparency allows in vivo analysis of organogenesis and embryogenesis. Zebrafish development happens very rapidly. All major organs develop within 36 hour of fertilization and hatching of embryos take place 8 to 12 hours later (Sarvaiya et al., 2014).

Fig. 1: Screenshot from PubMed search for 'Zebrafish'

The zebrafish genome has been sequenced in 2001. Seventy percent protein –coding genes are related to humans. There are 84 % human disease related genes found in zebrafish, therefore it is a good model for genetic screening (Arend et al., 2017). *Danio rerio* is an emerging vertebrate model for screening of chemical libraries for therapeutics (pharmacogenomics), to study the mode of action of drugs and toxins (toxicogenomics), for analysis of gene function and to determine teratogenicity. It is an excellent model for drug toxicity testing and allows whole animal screening with high throughput especially in the embryonic stages (Sarvaiya et al., 2014). Testing of new drugs can be done simply by absorption of small molecules from the water by the embryos. Zebrafish is also used to investigate novel anticancer drugs. Recently a popular model used to study cancers is xenotransplant, where an animal can be injected by cells from another species. During the first 4 -5 days after fertilization, zebrafish lack a functional immune response making it an ideal model for xenotransplant studies (Nicoli & Presta, 2007). Using genetic engineering tools several transgenic fish are available which give the opportunity to study various processes by visualizing live embryos/larvae under a fluorescence microscope. Zebrafish are also capable of regenerating several organs, for example fin, heart, spinal cord and several others (Poss et al., 2002; Poss et al., 2003). This makes it a useful vertebrate model for regenerative medicine. The zebrafish can be used to study gene expression by using mutation, knockout and knockdown techniques. In situ hybridization in zebrafish helps to study specific gene expression.

Zebrafish, being a vertebrate animal, also lends itself to studying complex behaviour such as learning and memory, aggression, sleep and anxiety. By isolating behavioural mutants it is also possible to study neural circuits that control behaviour. Zebrafish models have been established to study cognitive impairment, seizures, and other abnormal behaviors (Grone et al., 2017).

Our lab has been using zebrafish for a variety of studies. Here we give a glimpse into 3 such projects with diverse applications.

Fin regeneration in zebrafish and its modulation: Regeneration is the regrowth of a damaged or missing organ from the remaining tissue. An adult zebrafish has 5 different types of fin, while the larva only has the caudal and pectoral fins at day 3 post fertilization. The caudal fin, which is the largest and is most amenable to amputation is often the one used for study. An adult fin completely regenerates within 13-15 days while an amputated larval fin regenerates in 2 -3 days post amputation (Poss et al., 2003; Kawakami et al., 2004) (Fig 2).

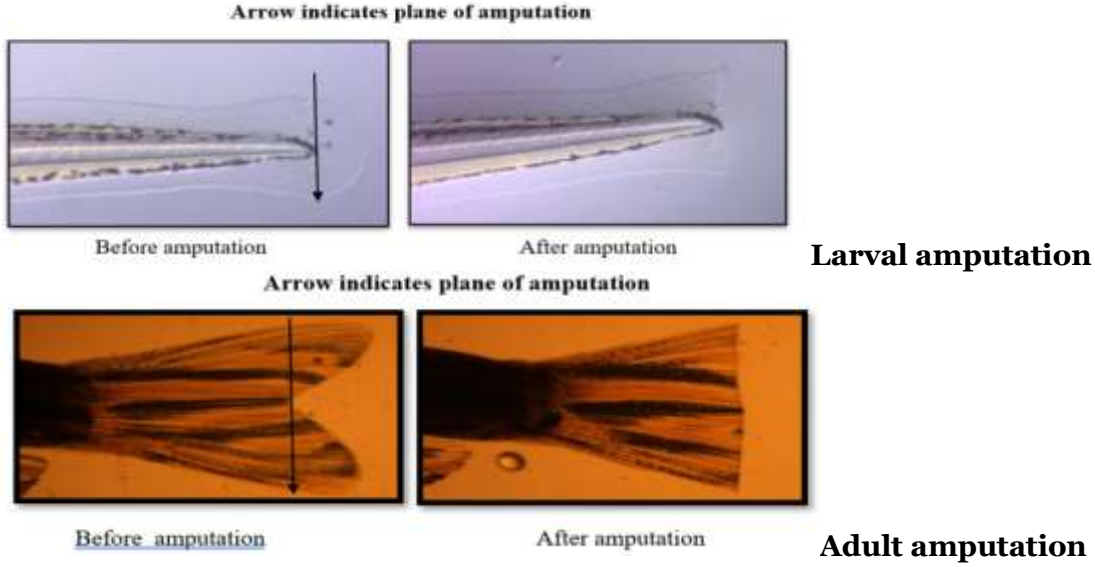

Fig.2: The method of amputation in the larva and adult zebrafish caudal fin

Our lab is studying the effect of valproic acid (VPA), a drug used to treat depression, but also known to inhibit histone deacetylation (hence causing epigenetic changes) on fin regeneration using the larval model. The small size of the larvae and the rapid regeneration of the fn allows for several sets to be performed within a short period of time. Results showed that VPA was able to almost completely inhibit regeneration of the caudal fin in larvae at very low doses (Fig 3).

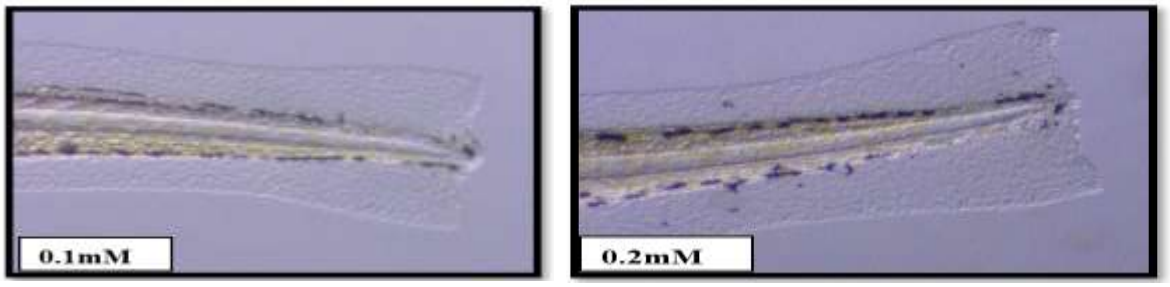

Fig. 3: Effect of varying doses of VPA on regeneration of the larval caudal fin

While the control larvae showed significant regeneration within 2 days post amputation, larvae treated with 0.1 and 0.2 mM VPA showed a complete inhibition of regeneration. The regenerated area can be measured using the ImageJ software.

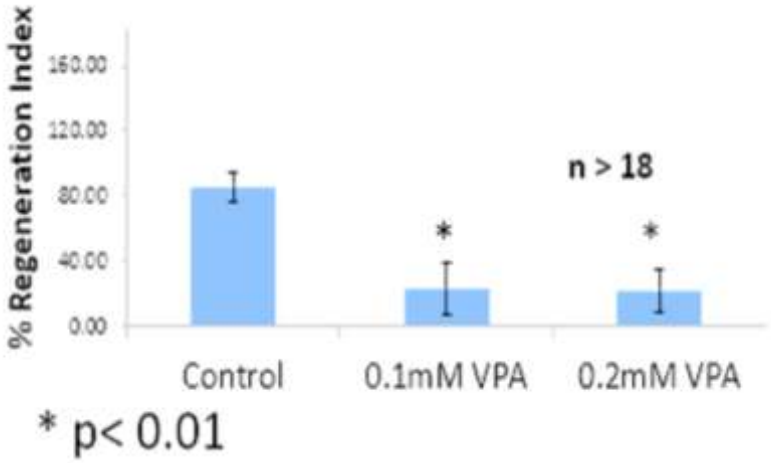

Fig. 4: The above graph shows the measurement of the effect of VPA (Valproic acid) on the caudal fin area

Using a similar assay various drugs can be tried for increased regeneration. The zebrafish larvae also lend itself to studying mechanisms underlying the process of regeneration. Insights gained from such experiments can lead to novel drugs that could be used to enhance regeneration in humans.

Using zebrafish for toxicity assays: For toxicity screening, it is essential that in vivo experiments are done, which would require a large number of organisms. Zebrafish larvae, due to their small size and availability of large numbers permit the testing of several compounds at the same time with high throughput. Zebrafish larvae have been used to screen toxicity that can be assessed using different endpoints like measurement of heart rate, presence of edema, organ morphology, body shape and other morphological defects and motility (Spitsbergen & Kent, 2003). Zebrafish also show measurable behaviour patterns that can be monitored and used to check for

drugs that affect the nervous system (Ramlan et al., 2017).

Several studies have reported that exposure to alcohol has a toxic effect on zebrafish development affecting several parameters, including viability, developmental morphology, heart rate and motility (Ramlan et al., 2017). We decided to ascertain if another stress inducer added along with the alcohol would alter the effect of alcohol on zebrafish. Maternal alcohol consumption during pregnancy often leads to varying degrees of abnormalities in the foetus that are termed as Fetal Alcohol Syndrome Disorder (FASD). Often during delivery, the foetus may not get sufficient oxygen leading to hypoxia (Bilotta et al., 2004). To simulate this model, we decided to study the synergistic effect of hypoxia and alcohol. Alcohol was added to the water directly and hypoxia was induced by addition of sodium sulphite, a chemical that scavenges oxygen, thus reducing the oxygen content of the water (Alef et al., 2018).

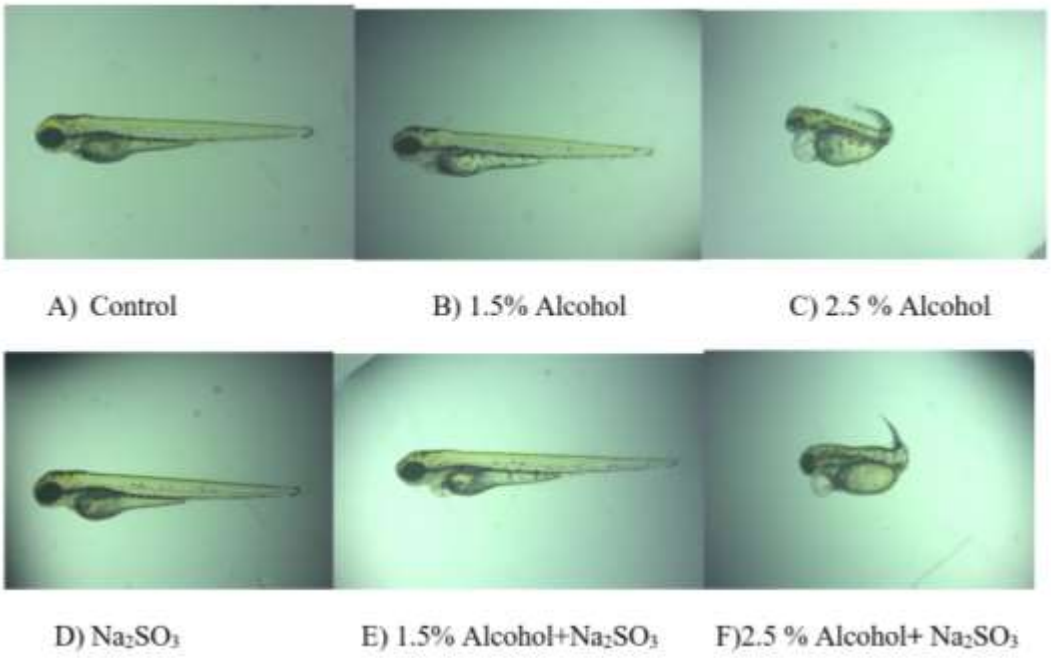

Fig. 5: shows the morphology of embryos that were exposed to the various treatments at 5 hours after fertilization and the images were taken at 72 hpf.

The results were very interesting. Alcohol at a concentration of 1.5% showed minimal effect, however, a slight increase to 2.5% led to severe abnormality in morphology. Sodium sulphite alone did not adversely affect the larvae and also did not show any synergistic detrimental effect along with alcohol. Besides morphology all the other parameters tested, such as viability, mobility and heart rate were affected by alcohol and alcohol with hypoxia.

Use of Zebrafish as a Xenograft model: A Xenograft model is useful to study metastasis *in vivo*. Since zebrafish lack an effective immune system for the first 4 - 5 days they readily accept xenotransplants, which are cells injected from another species. In our study we transplanted rat glioma cells in the yolk sac region of the 2-day old zebrafish larvae. Cells were labeled with Chloromethylbenzamido (CM- Dil). 100-200 cells were injected into the middle of the embryonic yolk sac region using a Pneumatic Pico-Pump Injector with a capillary needle. Injected embryos were incubated at 33°C for 5 dpi (day post injection). At 0 dpi and 5 dpi tricaine – anaesthetized xenotransplanted animals were examined for migration of the cells.

Comparison of the images (in Fig. 6) shows that the cells have replicated *in vivo* as the number of cells at the injection site are definitely more in number. Migration of more than 5 cells is considered metastasis.

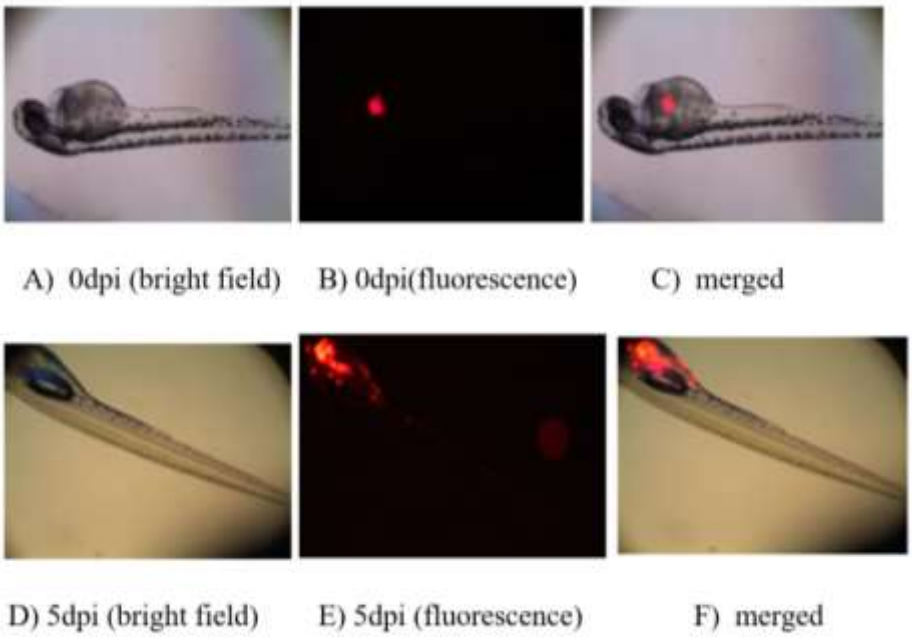

Fig. 6. shows the C6 glioma cell injected at 0 dpi and the cells that have started migrating toward the tail region at 5dpi.

The zebrafish xenograft model allows the *in vivo* analysis of angiogenesis and cell migration that occurs during metastasis (Nicoli & Presta, 2007). Due to the permeability of the embryos to small molecules, zebrafish allows drug target identification following a xenograft as a method for novel anti-cancer therapy and elucidation of the underlying mechanisms.

Besides the versatility of zebrafish as a vertebrate model for study of complex processes, it is also an excellent teaching tool. With use of a simple stereo microscope the complete early development of the embryo can be monitored and imaged. Over the years we have seen the fascination of students actually observing cell division in a one cell fertilized egg to give rise to a blastula, something that they had only read and seen images of in a developmental biology textbook. Further, using zebrafish, several undergraduate students have carried out minor projects themselves.

Zebrafish is an animal model and permission is required for its use from the Ethics Committee. All protocols used for zebrafish were as per guidelines of IAEC (Institutional Animal Ethics Committee)

Acknowledgements

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Research article

Duration of Unconscious Exposure does not affect Logo Retention

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Abstract

Brands recruit advertisements as means of increasing awareness and sales. These advertisements consist of varied components such as appealing colors and words, gratifying textures and odors, entertaining ambassadors, etc. Amongst them, logos are one of the most utilized advertising icons, especially in product placements. This creates a need to decipher the time period of unconscious exposure required to ensure retrieval. We have tested brand logo recognition at three different times (milliseconds or ms) of unconscious exposure (100ms, 70ms, and 50ms) of 85 subjects using a psychoanalytical test created on 'OpenSesame'. There was no significant difference in the recognition of logos at all exposure times. Implications of these findings are discussed.

Introduction

Brand identity consists of a plethora of factors such as packaging dynamics like the texture, shape, color, associated sounds, slogans, logos, and many more. Advertisements based on these influence consumer purchase decisions. They significantly help increase the product's value in the minds of the consumers (Rai, 2013). This relative value for each product is what compels consumers to buy or reject it. Increasing the frequency of exposure to advertisements also has a positive effect on the buying behavior (Burton et al., 2019).

However, combined usage of many identity constituents may lead to cluttering. This visual clutter translates into cognitive clutter and has an impact on the decision-making of the consumers, thus reducing the efficacy of advertisements (Abdul et al., 2009). Therefore, it is imperative to understand the components that precisely cause the desired effect.

Amongst the many components used, logos serve as static and most frequently used identity icons. Each logo conveys an intention in its pictographic or typeface glory. For example, brands like Nike tightly link their taglines ('Just do it') along with the logo. Or the huge yellow 'M' of Mc Donald's flaunts happiness and cues hunger (Singh, 2006). These efforts create an association between the consumer and the brand. So, the next time, a consumer is looking for an eatery, chances are that he is looking for a yellow 'M' subconsciously.

Merriam-Webster defines logo as an identifying symbol that carries the weight of distinguishing and characterizing a brand. Such recognition is built using only

shapes, symbols, and a limited number of words (Salgado-Montejo et al., 2014). Logos act as visual cues and use almost no other sensory modality to create an impression. Therefore, a sound knowledge of the contributing factors is indispensable in order to create new logos or ameliorate already existing brand icons.

Logos are extensively used in product placements. They are placed in the background or are involved in the scene or pasted on billboards such that the viewers can easily get primed by them (High et al., 2019). This is very effective in terms of creating brand memory and enabling successful recall (Chaney et al., 2018). Efficient brand recall influences better recognition. It is therefore essential to ascertain the optimal visual cue duration for maximal brand recognition. In this study, we aim to understand the minimum time of unconscious exposure to logos in order to facilitate accurate retrieval.

Methodology

Sample

81 participants (average age = 23.5 years) belonging to diverse backgrounds were invited for the test. Working knowledge of computers and basic proficiency in English formed the basis of selection. No other screening was conducted.

Ethics statement

Prior to the task, each participant was thoroughly briefed about the requirements and the risks associated. The task, as well as the task setting, were non-invasive and only involved usage of computers. Post briefing, an informed consent was taken from the candidates. All the participation were voluntary subject to the declaration of their consents.

Stimuli

The stimulus was provided in the form of a computerized test generated on 'OpenSesame 3.2.8'. Primary task displayed the chosen animal images. The major or secondary task consisted of the chosen logo images. All the images were obtained under creative commons license from the internet and were normalized with regard to size. During the test, images were presentedcentrally against a black background.

Test paradigm

During each trial of the primary task, the participants were presented with an initial trial run with neutral cues to accustom them to the pattern of the test. Each run started with a screen consisting of a fixation of 5000ms to aid the participants in locating the exact region where the images will be displayed. Just before the beginning of the primary task, the question, 'Is the mouth of the animal open?' was presented on the screen for 7000ms. 20 photographs of various wild animals and birds were used in the primary task. Each photograph appeared 5 times and hence a total of 100 runs were conducted. After each animal picture, one of the chosen logo images was displayed for the same duration. 20 well-known brand logos were chosen from finance and communication sectors. This ensured that all logos did not cater extensively to anybody's interests, thus reducing familiarity biases. In order to expose the logos conditionally, they were flashed exactly after each animal image cue without any time lag. The order in which individual animal images and logos appeared was randomized to eliminate any bias caused due to default strategic responses adopted by participants. A blank screen was flanked post each cue until a response was given.

Participants were divided into three groups of 27 named A, B, and C. Group 'A' was exposed to the animal and logo images for *50*ms, 'B' for *70*ms, and 'C' for *100*ms. The three durations of exposure were chosen to be well below the threshold of conscious perception as used in the IAT (Implicit Association Test) (Lin et al., 2014). Keypress responses were obtained using 'Y' key on the keyboard for the '*Yes*' response and 'N' key for the '*No*' response. The task was programmed to continue only after a response was given for each (animal) image. The secondary task factor, i.e., the logos displayed were not discussed during the instructions. Participants were

solely spoken to about the primary task and the responses.

A post-test was conducted after the completion of 100 runs by each participant. A virtual sheet consisting of 50 logos inclusive of the ones displayed during the test was presented. The participants were asked to mark the 'logos' they *remember seeing during the test*. This facilitated recollection of the logos observed due to unconscious exposure (Elgendi et al., 2018). The significance of the number of correctly marked logos by each group was compared by performing a one-way ANOVA analysis. MATLAB 2017α and GraphPad Prism8 were used for the same.

Results

Pre test

In the primary animal mouth open-closed task, more than 75% of the participants gave correct responses to the images displayed. Figure 1 gives a graphical depiction of the same.

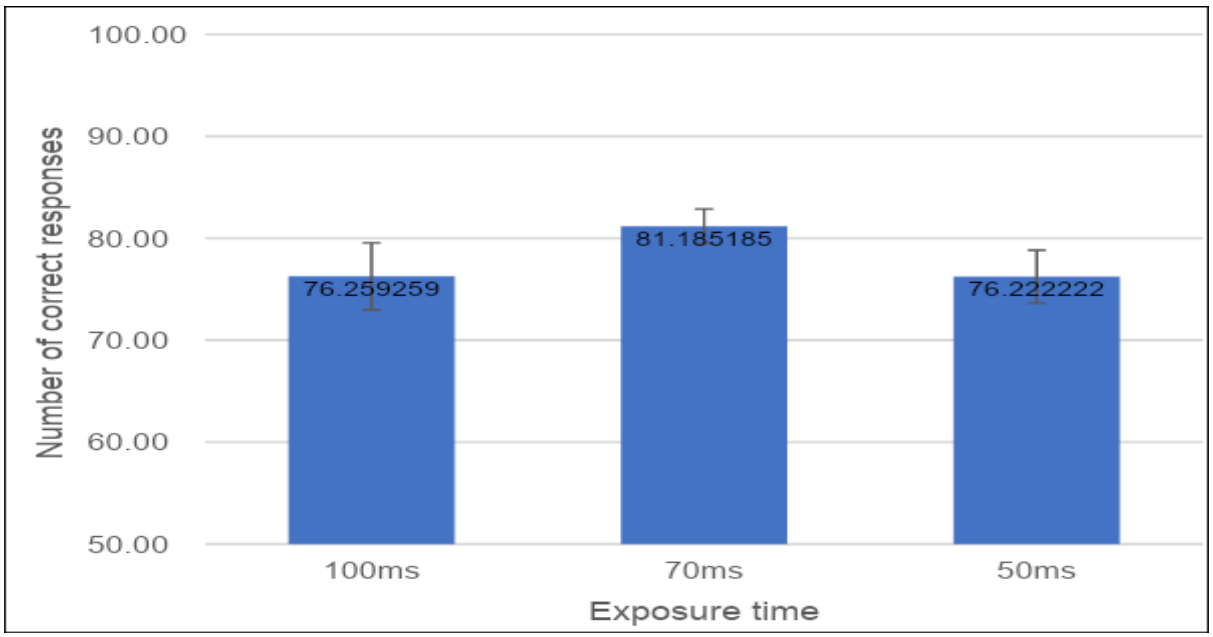

Fig.1: The above graph illustrates the average number of correct responses given in the primary task at different exposure times.

There was no correlation found between the number of wrong responses and the correct number to logos recognized at all exposure timings (Figure 2.1, 2.2& 2.3).

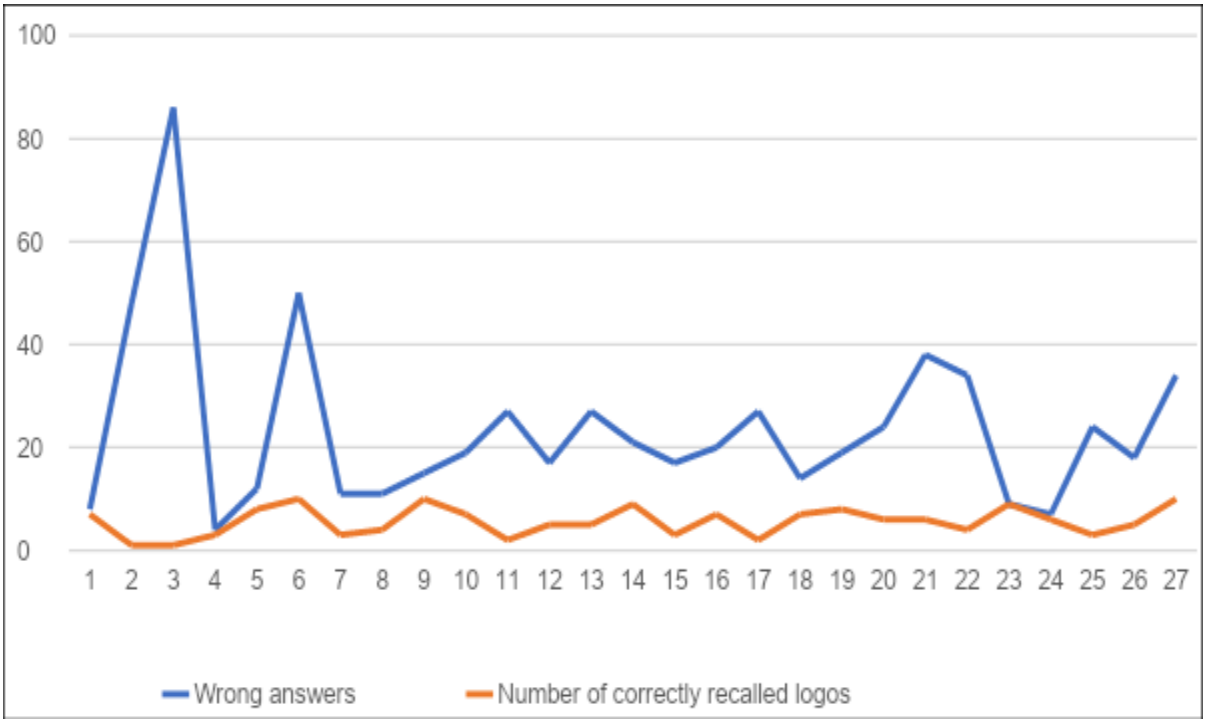

Fig.2.1: This graph illustrates the number of wrong answers and correctly recognized logos at exposure of *100ms*.

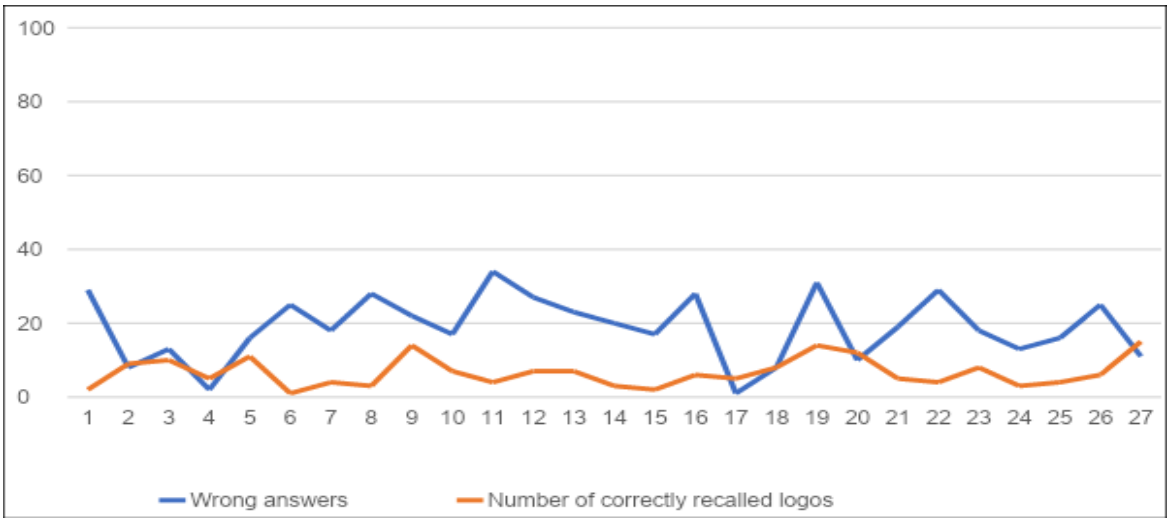

Fig.2.2: This graph illustrates the number of wrong answers and correctly recognized logos at exposure of *70ms*.

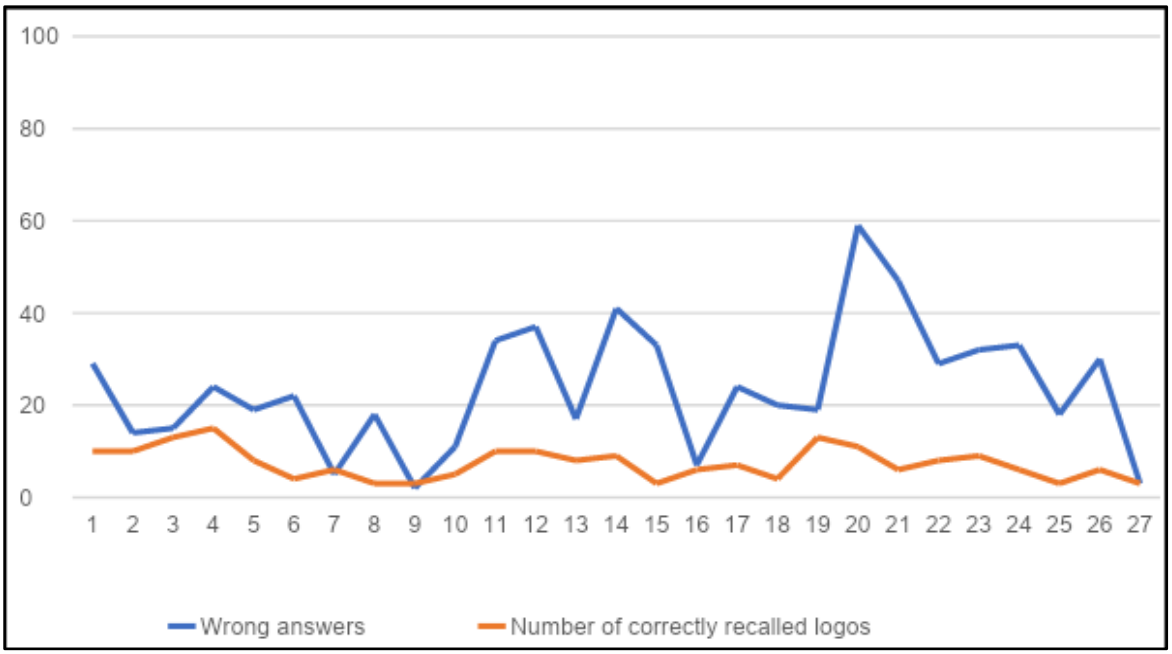

Fig.2.3: This graph illustrates the number of wrong answers and correctly recognized logos at exposure of *50ms*.

Post-test

A one-way ANOVA test was conducted to compare the effect of different times of exposure i.e., 100ms, 70ms, and 50ms on the number of correctly recognized logos using GraphPad Prism 8.4.3.
The test yielded an insignificant effect of duration of exposure on the logos recognized correctly at the $p< 0.05$ level for the three conditions [$F(2,82) = 2.745$, $p= 0.07$]. These results suggest that the duration of logo exposure has no significant effect on their immediate recognition for these times of exposure.

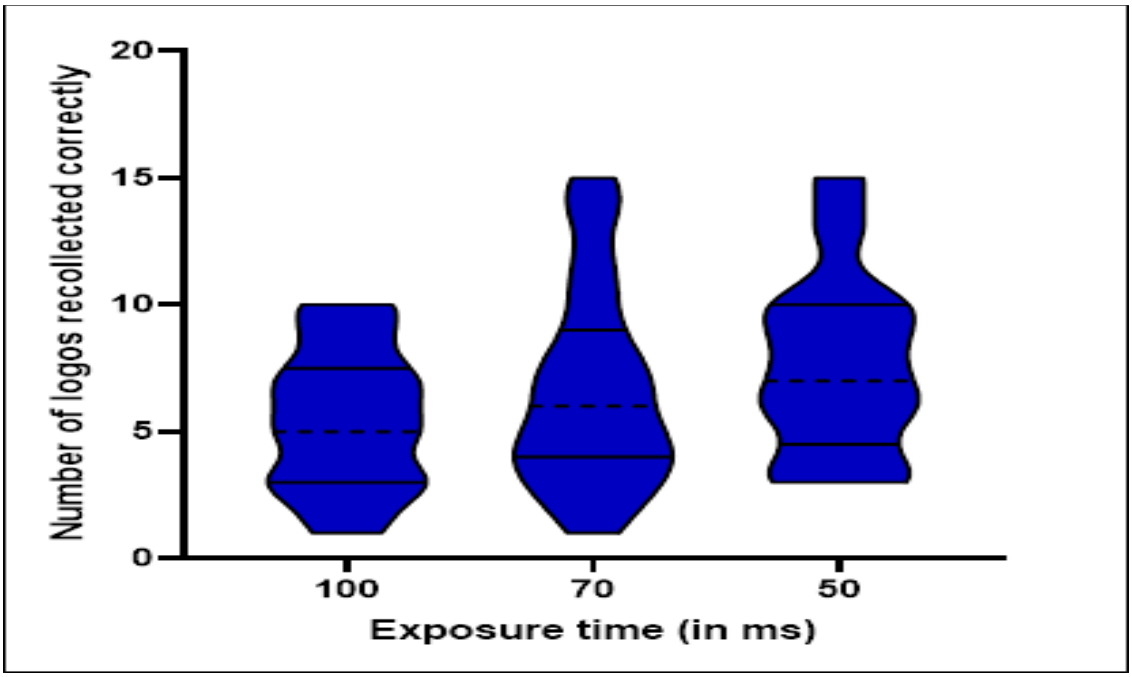

Fig.3: This violin plot displays the distribution of a one-way ANOVA analysis comparing the number of logos 'correctly' recollected at the three exposure times i.e., *100ms*, *70ms,* and *50ms*.

Twenty brand logos were used in the test. Amongst them, the Finance based Brand logo (FB) 1 has a complex design with edges and a pink color. It was only recollected at 50ms exposure. FB 2 logo has soft corners with a yellow-blue scheme. FB 3 also has soft corners with white-orange scheme. Both were recognized the least at 100ms exposure. FB 4 with a violet and sharp-edged logo. FB 5, the logo with curved contour and blue-red scheme displays a marginal difference in the recognized with the highest being at 50ms of exposure. FB 6 has soft edges and a blue-white scheme. FB 7 also has a curved contour and blue-white scheme but has been recognized fewer times than FB 6. FB 8 has blue-white-red color scheme and a mixed contour. It was least recognized at 100ms exposure. FB 9 was highly recognized, most at an exposure of 70ms. It has a red-yellow-white color scheme. FB 10 logo has been recognized maximally at 50ms compared to retrieval at 100ms and 70ms. It has a red-blue scheme with sharp contours.

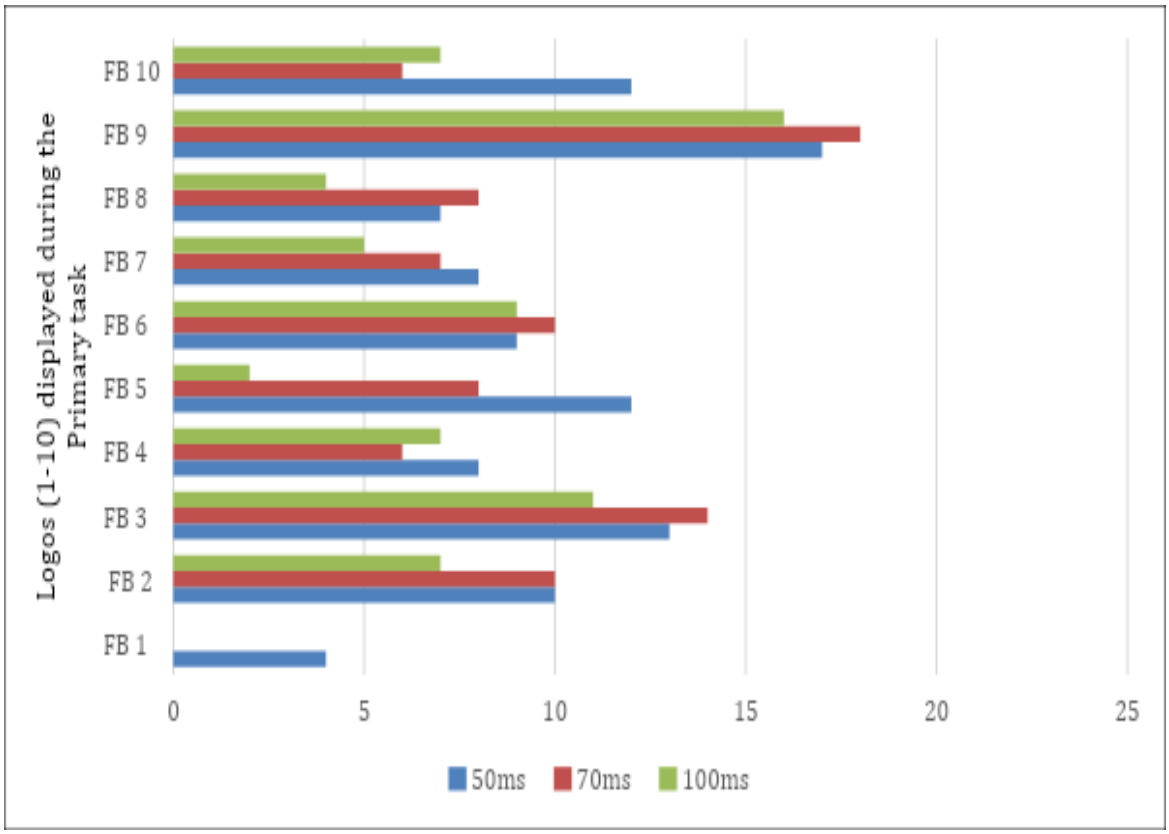

Fig.4: This graph illustrates the number of correctly recognized logos of Finance based Brands (FB) by all 27 subjects of each time group.

Communication-based Brand logo 1 has an alphabet as a representative. It is curved and is red, blue, green, and yellow-colored. The group exposed to CB 1 for 50ms showed maximum recognition. CB 2 logo also showed maximum recognition at 50ms of exposure. This logo has a red-blue scheme with a curved contour. CB 3 has a red curved logo and participants showed no recognition at 50ms exposure. CB 4 also has displayed recognition only at 70ms and 100ms of exposure. The logo has a red-yellow scheme with an edgy contour. CB 5 has a green-white scheme with curves as well as edges. Participants showed the least amount of recognition at 70ms. CB 6 has a mixed contour with a blue-white scheme. It has been most recognized with 70ms of exposure. CB 7 has a yellow-white scheme, again with

mixed contour. It was minimally recognized with 50ms exposure. CB 8 was most recognized with 70ms of exposure and had a white-red scheme with a curved contour. CB 9 with pointed edges and a red, yellow, blue, and green color scheme was highly recognized with an exposure of 70ms. CB 10 logo is curved with various shades of violet and orange. It was also maximally recognized at 70ms of exposure.

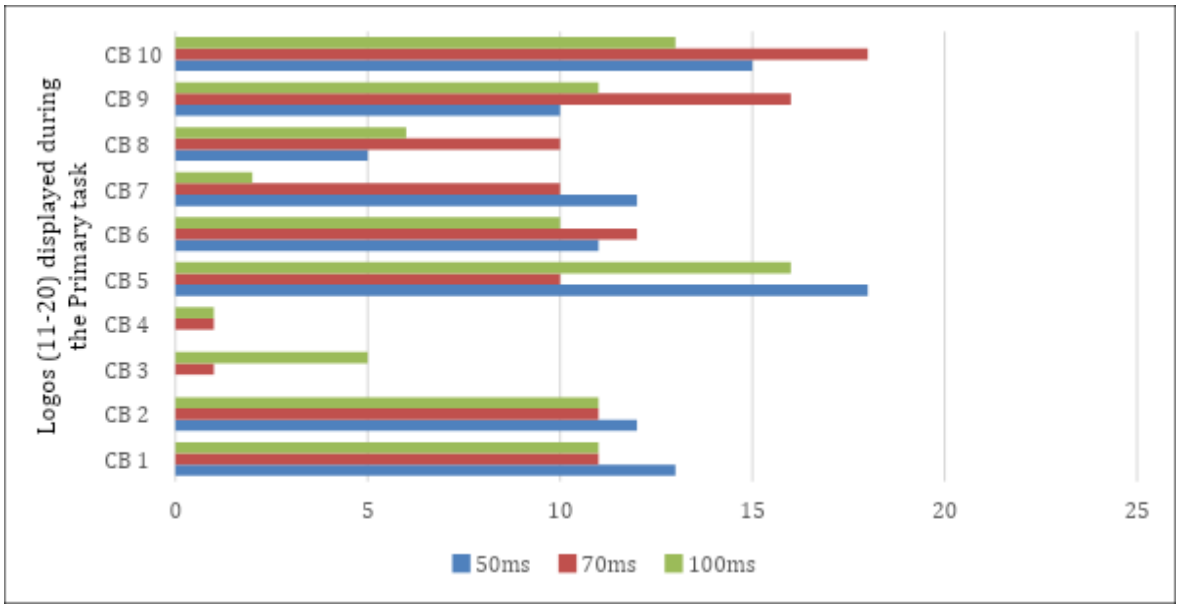

Fig.5: This graph illustrates the number of correctly recognized logos of Communication-based Brands (CB) by all 27 subjects of each time group.

Discussion

The primary task data shows that all the subjects gave at least 75% correct responses. This indicates that their attention was focused on the primary task and hence, they were less likely to consciously notice the logos being displayed (Perkins et al., 2008). Since, they weren't informed regarding the post-test prior to the commencement of the session, and based on the number of correct responses in the primary task, we can conclude that their attention was focused on the primary task itself. This increased the chances of them marking the logos they were successfully primed by in the limited periods of exposure (100ms, 70ms, and 50ms). This unconscious exposure (Dijksterhuis, 2019) to the logos was examined in the post-test.

The comparison between the number of wrong responses and the number of correctly marked logos displays no relationship. This lack of trend suggests that the wrong responses weren't due to the subjects' shift of attention towards the logos. Also, the subjects were likely on a goal-directed pursuit (Laran, 2016) of getting as many responses correct as possible which further lessens the probability of conscious logo awareness.

Statistical analysis of post-test data indicates that there is no significant difference in the logo retrieval at 100ms, 70ms, and 50ms. Longer exposure times, therefore don't necessarily have a huge impact on immediate recognition. It is however, important to note that all logos recognized at each exposure time were not uniform. Some logos peaked at 100ms while others at 50ms. This could be attributed to the factors comprising them. Ascertaining the patterns and testing recognition at even lower exposure times may pave a path to define the lowest unconscious exposure time.

Conclusion

This study brings into light one of the core factors of unconscious advertising-exposure span. In our study, brand

placement of 50mswas ample enough for a consumer to get exposed to the product and recognize it on probing. There is paucity of publications that indicate that an exposure of less than 50ms yields significant retrieval, hence it is necessary to undertake a study regarding the same. The possibility of using this exposure time can be explored for causes like blood donations or voting encouragement. An unconscious, but effective projection of the causes can increase efficacy without the audience deeming it as a nuisance.

We can further try to discern the physiological activity of the brain to understand the underlying processing of the unconscious stimuli.

Disclaimer

None of the logos used in the study are sponsored by the trademark owner. All the logos have been used purely for research purposes with no intention of advertising them. All the logos have been sourced from the 'Creative commons' website under Creative commons license.

Conflict of interest

The authors declare no conflict of interest.

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Research article

Isolation of chlorpyrifos degrading bacteria and comparative analysis of methods to estimate extent of chlorpyrifos degradation

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Abstract

Chlorpyrifos (O, O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate) is a broad-spectrum organophosphorus insecticide that plays a key role in pest management around the world. Due to its excessive utilization, high concentrations have been found in the surface and groundwater in a study conducted in three districts of Maharashtra. Owing to long half-life and non-specific mode of action, further accumulation may negatively impact the health and environment. Such consequences could be avoided through routine testing and effective reduction of the existing pesticide in the soil and water bodies. Bacterial strains capable of degrading chlorpyrifos were isolated from soil samples procured from a local plant nursery (Borivali, Mumbai) where chlorpyrifos had been routinely applied for over a period of two years. The soil sample was enriched in minimal medium by using chlorpyrifos as a sole carbon source. Three potential isolates were obtained. Based on tests conducted to compare tolerance level and ability to retain chlorpyrifos degrading capacity of the isolated strains, colony 1 (CP C1) was determined to be a suitable degrader. Sensitivities of two methods-colorimetric and UV-Vis method were compared to detect chlorpyrifos in the range of 0.01 – 0.1 mg/mL for determination of the rate of degradation. The biochemical tests indicate the possibility of CP C1 belonging to *Aeromonas* spp.

Introduction

Pesticides are synthetic compounds employed to protect crops from pests, thereby boosting agricultural production. In order to meet the demands of the ever-increasing population, this has now become an essential product in this sector (Chambers et al., 2001). In India, 80 % of pesticides are used in the form of insecticides, 15 % are herbicides, 2 % are fungicides (Agarwal et al., 2015). Organophosphorus (OP) compounds, known for their broad-spectrum and effectiveness at low concentrations, are found to be the largest class of insecticides primarily used in industrial countries (Rogers et al., 1999).

Chlorpyrifos (O, O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate) also represented as CP, CPY, or CPF; belongs to the group of organophosphorus compounds and is routinely applied to crops such as cotton, rice, potato, corn, and wheat to control insect pests. They also have residential and indoor applications for pest control, especially for

cockroaches and termites (Saunders et al., 2012). The mode of action is initiated by inhibition of acetylcholinesterase (AChE) by the metabolized product chlorpyrifos-oxon (CPO). The resultant accumulation of acetylcholine in the synapse induces death by disrupting the nervous system function (Mackay et al., 2014). Studies conducted by Yiran Liang et al shows that chlorpyrifos intake promotes obesity and insulin resistance by affecting the gut and gut microbiota and disrupting the integrity of the gut barrier, leading to increased lipopolysaccharide entry into the body (Kopjar et al., 2018).

Replacing endosulphan (banned in the year 2011 due to the development of deformities in children in contact with the pesticide over a long period), chlorpyrifos has now been excessively utilized in the country (Menezes et al., 2017). Sampling of surface water and groundwater in three districts in Maharashtra revealed that chlorpyrifos is present in the highest concentration in surface water with an average value (0.34 μg/ml) exceeding the permissible limits provided by the EU (European Union). Prolonged exposure to chlorpyrifos can cause toxicity to non-targeted organisms like bees which are known to play a crucial role in the pollination of commercial crops (Lari et al., 2014).

Due to its long half-life and non-specific mode of action, any further accumulation may negatively impact the health and environment. Such consequences could be avoided through routine testing and effective reduction of the existing pesticide in the soil and water bodies. Bacteria and fungi are frequently used for bioremediation of pesticide polluted soil and water, owing to their versatile ability for utilizing a wide array of substrates and subsequent degradation to inactive compounds. In addition, exploitation of the potential degrader for the fabrication of biosensors enables in achieving both targets (concentration determination and bioremediation) simultaneously without the requirements of additional methods or apparatus.

Few strains of *Ochrobactrum, Delftia, Stenotrophomonas, Bacillus, Cupriavidus, Sphingomonas, Achromobacter* in the consortium are capable of complete degradation of the pesticide to pyruvic acid. Bacterial communities of the families *Xanthomonadaceae, Comamonadaceae, Pseudomonas, Burkholderiaceae, and Rhodospirillaceae* have been discovered that are capable of utilizing toluene which is a chemical component in the formulations of chlorpyrifos. (Chen et al., 2012; Fang et al., 2008; Sasikala et al., 2012) The enzymes required for degradation of chlorpyrifos are encoded by an organophosphate-degrading gene (opd). These genes were found to be plasmid-based and have similar DNA sequences in most chlorpyrifos degrading organisms. (Singh et al., 2004) Most bacterial strains tend to lose opd genes when grown in a complete medium lacking chlorpyrifos as a carbon source due to the burden or fitness cost associated with carrying and/or expressing the extra piece of genetic material (Price et al., 2016). The analytical techniques utilized for screening the aforementioned potential degraders were GC-MS, HPLC, and radioactive isotope. (Nasiri et al., 2016; Wang et al., 2019)

These techniques, although sensitive, are high-priced, thereby making it difficult to conduct analysis of numerous samples. N.V.S. Venugopal et al and Y. Makino et al have described Spectrophotometric determination of chlorpyrifos using diazotized aniline as a reagent and UV spectrophotometric determination of the concentration of chlorpyrifos (Makino et al., 2009; Venugopal et al., 2012). These procedures are economical and can be performed using devices available in the college laboratory.

The objective of this experiment is concerned with preliminary isolation and identification of chlorpyrifos-degrading bacterial strain that could be further employed as a potential degrader and for the fabrication of a biosensor, using two cost-effective analytical techniques - Colorimetry and UV-Vis spectrophotometry.

Materials and Methods

Chemicals and media:

Chlorpyrifos (technical grade, purity 99.5 %) and 20 % Emulsion Concentration (EC) of chlorpyrifos were obtained from Hiranba Industries Ltd. (Mumbai, India). Chlorpyrifos wettable powder (concentration ≈0.2 %) and soil sample used for primary enrichment were procured from a local plant nursery where chlorpyrifos had been routinely applied for over a period of two years (Mumbai, India). Mineral Salt Medium (MSM) contained the following (g/L)- $(NH_4)_2SO_4$- 1.3 g, K_2HPO_4- 0.15 g, KH_2PO_4- 0.05 g, NaCl- 0.02 g, $MgSO_4$- 0.02 g). Diluents- isopropanol and acetonitrile used for the study were of analytical grade.

Enrichment and isolation of chlorpyrifos degrading bacterial strains:

1 g of weighed soil sample was added to 9ml saline and vortexed. 5ml of the vortexed solution was inoculated in an Erlenmeyer flask containing 100ml of sterile minimal medium- MSM (Mineral Salt Medium). 1 g of chlorpyrifos wettable powder was added to the medium as a source of carbon for primary enrichment. The flask was incubated in a shaker at room temperature for 7 days. 1ml of the primary enrichment medium was further inoculated in a second enrichment mineral salt medium containing 50 µL (10 mg/L) of chlorpyrifos 20 % EC as a carbon source and incubated in conditions as mentioned previously. After 5 days, a loopful of the second medium was isolated on MSM agar containing 10 µL of chlorpyrifos 20 % EC. The plates were incubated at room temperature for 48 hours. The bacterial isolates that were obtained were termed as CP C1 (colony 1), CP C2 (colony 2), and CP C3 (colony 3).

Since the emulsions used in chlorpyrifos 20 % EC are made up of hydrocarbons, it is crucial to confirm the utilization of chlorpyrifos as the sole carbon source by the isolates. 1 ml of culture suspension of each bacterial isolates (OD adjusted to 0.1 at 540 nm) was inoculated in two Erlenmeyer flasks, one containing 100ml MSM and stock solution (5µg/mL of chlorpyrifos) (test) and the other containing MSM and 50 µL of isopropanol (control). The flasks were incubated in a shaker at room temperature for 24 hours. Isopropanol was selected as the solvent due to its high solubilizing property and leaves no turbidity in MSM medium thereby eliminating the possibility of false-positive results. After incubation, the loopful from the flasks (test and control) of each strain was further isolated on MSM agar containing 10 µL of chlorpyrifos 20 % EC (each labelled as test and control) and incubated at room temperature for 24 hours.

Selection of potential degrader:

For determination of tolerance for a high concentration of the pesticide, the stock solution of chlorpyrifos (chlorpyrifos technical dissolved in isopropanol to attain a concentration of 1 g/l) was prepared. Culture suspension of each isolate was prepared in saline with density adjusted OD 0.1 at 540 nm and the cell count (cell/mL) was determined using the Direct Microbial Count (DMC) method. 1 mL of each culture suspension was inoculated in three flasks containing 100 mL MSM and stock solution of concentration of 0.1 mg/mL, 1 mg/mL, and 2 mg/mL respectively and incubated in the shaker at room temperature for a period of 48 hours.

In addition, the retention of pesticides degrading ability of the bacterial strains was simultaneously analyzed. Each isolate was subcultured after 7 days in nutrient agar for 2 weeks. After the incubation period, the culture suspension of each isolate (OD adjusted to 0.1) obtained from nutrient agar was inoculated separately in 100 mL of MSM containing 50 µg of chlorpyrifos and 100 mL of MSM containing 50 µL of isopropanol (control) and incubated in conditions as mentioned for 24 hours.

Biochemical Tests:

The following biochemical tests (Table 1) were conducted for preliminary

identification of the potential degrader (Palumbo et al., 2006; Wang & Gu, 2005).

Table 1: Biochemical tests

Biochemical Test	Justification
Oxidase Test	This test was done to differentiate between *Enterobacteriacae spp* and *Aeromonas spp, Vibrio, Pseudomonas spp*.
Catalase	For identification of *Pseudomonas spp., Vibrio spp., Aeromonas spp.*
Nitrate Reduction	For identification of *Pseudomonas spp., Vibrio spp., Aeromonas spp.*
Citrate	For identification of *Pseudomonas spp., Vibrio spp., Aeromonas spp.*
Growth on Mac Conkeys Agar	For identification of *Pseudomonas spp., Vibrio spp., Aeromonas spp.*
Indole	To distinguish between *Pseudomonas spp* and *Vibrio spp., / Aeromonas spp*.
Triple Sugar Ion	To differentiate between *Pseudomonas spp., Vibrio spp., Aeromonas spp*.
H_2S Production	For identification of *Pseudomonas spp., Vibrio spp., Aeromonas spp.*
Methyl Red	To differentiate between *Pseudomonas spp., Vibrio spp., Aeromonas spp*.
Voges Proskauer	To differentiate between *Pseudomonas spp., Vibrio spp., Aeromonas spp*.
pH TEST (Growth Observed In pH 8)	To distinguish between *Aeromonas and Vibrio spp*.

Spectrophotometric determination of chlorpyrifos using diazotized aniline as a reagent:

A study conducted by N.V.S Venugopal et al showed a new reaction system for spectrophotometric determination of chlorpyrifos pesticide. This is based on the reaction of chlorpyrifos with diazotized anthranilic acid in an alkaline medium to form an orange-red colour. The absorbance maximum was observed at 450 nm. The Beer's law is obeyed up to 8.18 ppm for chlorpyrifos standard solution (Venugopal et al., 2012).

The stock was prepared by dissolving chlorpyrifos technical in isopropanol to attain a concentration of 0.1 mg/ml. Using isopropanol as a diluent a working standard of 0.01 – 0.1 mg/ml was prepared since the volume of the diluent used is large (approx. 40 mL each set); one additional standard with an identical concentration range was prepared using a lower volume of diluent. 2ml of 2M NaOH was added to each test tube and the temperature was maintained at 5^oC and incubated for 30 minutes to which 1mL of diazotized aniline which was maintained at 5^oC was added producing an orange-red colour. Using the reagent as a blank the absorbance was measured using colorimeter the absorbance was measured at 540nm. A control containing isopropanol and 2mL of 2M NaOH was subjected to similar conditions as mentioned.

UV spectrophotometric determination of the concentration of chlorpyrifos:

Y. Makino et al. had suggested a non-destructible UV method for detection and quantification of chlorpyrifos. The study showed that absorbance of chlorpyrifos dissolved in acetonitrile was positively proportional to its concentration at 229 and 290 nm at limits of detection (LOD) of 0.0233 and 0.0113 mg kg^{-1}, and limits of quantification (LOQ) of 0.0778 and 0.0378 mg kg^{-1}, respectively (Makino et al., 2009).

The stock solution of chlorpyrifos of concentration 10mg/mL in acetonitrile was prepared. Standards ranging from concentration 0.01, 0.05, 0.1, 0.2, 0.5, 1, 2, 5 and 10 mg/mL was prepared by utilizing acetonitrile as a diluent. 1.0 mg/ml was utilized for the determination of λmax from 200nm to 400nm. The absorbance was measured by using diluent as a blank.

Results and Discussion

Enrichment and isolation of chlorpyrifos degrading bacterial strains:

Four bacterial isolates were initially obtained; however, one lost its ability to grow in chlorpyrifos after subsequent enrichments. The remaining were termed as CP C1 (colony 1), CP C2 (colony 2), and CP C3 (colony 3).

Further, the three strains isolated (CP C1, CP C2, and CP C3) showed turbidity in the test flasks and no turbidity in control. It was further confirmed on obtaining colonies on the test agar plates and no colonies in the control agar plates. This indicated that all the three isolates could utilize chlorpyrifos as a sole carbon source and did not require the hydrocarbons present in the emulsion concentrate to grow. Therefore, CP C1, CP C2, and CP C3 were selected for further studies.

Selection of potential degrader:

The initial cell count of CP C1, CP C2, and CP C3 before inoculation was determined to be 2.8 x 10^6 cells/mL, 2.9 x 10^6 cells/mL and 2.1 x 10^6 cells/mL respectively. The cell counts of each isolate after incubation at varying concentrations are represented in the table below (Table 2).

After subculturing in nutrient agar for 2 weeks and inoculating in respective flasks, no growth (turbidity) was observed in both flasks (test and control) that were inoculated with CP C2 and CP C3. Whereas growth was observed in the test flask and no turbidity was obtained in the control flask (100 mL MSM and 50 µL of isopropanol) for CP C1. From the conducted tests CP C1 was found to withstand a high concentration of chlorpyrifos (2mg) and did not lose its ability to utilize the pesticide as a sole carbon source.

The biochemical tests revealed that CP C1 is a Gram-negative bacterium, produces oxidase, and is capable of utilizing lactose, glucose, and sucrose as a carbon source. The pH Test shows low tolerance to alkaline conditions. The pH tests could indicate its application is limited to bioremediation of freshwater systems and less alkaline soil.

Further, the pH test helps in differentiating between *Aeromonas spp.* and *Vibrio spp.*, where *Aeromonas spp.* cannot tolerate high pH. As no growth was observed, it could indicate that CP C1 could belong to *Aeromonas spp* (Table 3).

Results obtained in Table 2 indicate that CP C1 can tolerate concentration as high as 2 mg/ml. However, there is a 10-fold decrease in the bacterial count in concentrations above 0.1mg/mL. Therefore, in order to study the optimum degrading capacity of the isolate, 0.1 mg/mL is a preferable upper limit for the determination of the rate of degradation of chlorpyrifos (working range 0.01 mg/mL – 0.1 mg/mL).

Table 2- cell count of CP C_1, CP C_2, and CP C_3 after incubation in MSM containing 1.0, 1 and 2 mg/mL of chlorpyrifos

Concentration of chlorpyrifos (mg/mL)	Colony 1 (Cells/ml)	Colony 2 (Cells/ml)	Colony 3 (Cells/ml)
0.1	6.50 x 10^8	5.94 x 10^7	6.50 x 10^8
1	2.01 x 10^7	2.17 x 10^7	2.24 x 10^7
2	2.23 x 10^7	2.6 x 10^6	2.21 x 10^7

Table 3 – Results of Biochemical tests

BIOCHEMICAL TEST	OBSERVATION
OXIDASE TEST	+
CATALASE	+
NITRATE REDUCTION	+
CITRATE	+
Isolation on Mac Conkeys agar	White colonies obtained
INDOLE	+
TSI	Acid butt, Alkaline slant
H_2S Production	-
MR	+
VP	-
pH Test (growth observed in pH 8)	-

Key: + indicates a positive test,
- indicates a negative test

Table-4 Colony Characteristics of CP C_1

Characteristics	**CP C_1**
Size	Medium
Shape	Irregular
Colour	white
Margin	Irregular
Elevation	Raised
Surface	Wrinkled
Consistency	Mucoid
Opacity	Opaque
Gram Nature	Gram negative
Morphology	Bacilli in pair

Fig. 1: Colony if CP C_1 studied for colony characteristics. Also mentioned in table-2

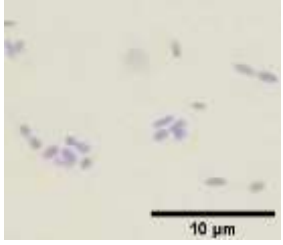


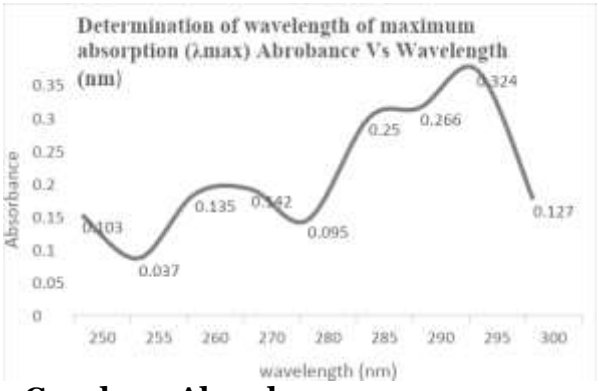
Fig. 2: Gram staining of CP C_1 showing Gram negative bacilli in pairs. Also mentioned in table-2

Table-5 – Absorbance values obtained at wavelengths 250 nm- 300nm

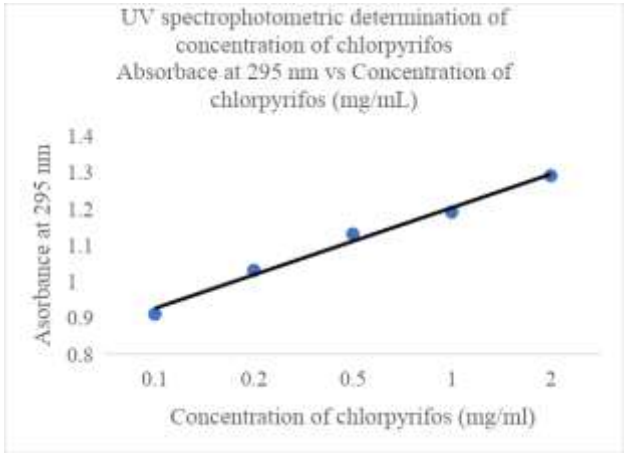
Wavelength (nm)	Absorbance
250	0.103
255	0.037
260	0.135
270	0.142
280	0.095
285	0.250
290	0.266
295	0.324
300	0.127

Table-6 – Absorbance values at 295 nm obtained for concentration range 0.1 mg/mL to 2 mg/mL

Standard concentration (mg/mL) (Working stock – 10mg/mL)	Absorbance at 295 nm
0.1	0.91
0.2	1.03
0.5	1.13
1.0	1.19
2.0	1.29



Graph 1 – Absorbance vs. Wavelength showing λ_{max} at 295 nm



Graph-2 – Absorbance vs. Wavelength at 295 nm showing linear range from 0.1 to 2 mg/mL

Spectrophotometric determination of chlorpyrifos using diazotized aniline as a reagent:

A total of 10 sets each with the concentration of chlorpyrifos ranging from 0.01 mg to 0.1 mg was prepared under identical conditions. The absorbance at 540 nm was determined for the colored solutions obtained. The OD values obtained for each set was random and showed no linear relationship. Precipitation was observed in the second set where the volume of diluent for each standard was below 4mL thereby making it unsuitable for colorimetric analysis. The random readings obtained could arise due to two factors- concentration of the reagent and stability of the coloured solution formed. The reagent (diazotized aniline) is highly unstable at temperatures above 5^{o}C, therefore in addition to the cold-water bath (maintained at 1^{o}C), it requires frequent addition of ice cubes resulting in variation in the concentration of the reagent that is prepared in each set. The coloured solution was observed to be stable for a very short amount of time, therefore the test could be further conducted using an instrument capable of measuring the absorbance during or immediately after the formation of the product.

UV spectrophotometric determination of the concentration of chlorpyrifos:

Standards (0.01 mg/ml - 2.0mg/ml) of chlorpyrifos were prepared using acetonitrile as a diluent. 1.0mg/ml was utilized for the determination of λmax from 250 nm to 325 nm. The wavelength of maximum absorption was confirmed to be 295nm (Graph-1).

The method is suitable for the determination of chlorpyrifos concentration above 0.1mg/ml as the absorbance measured at 295nm showed a linear relationship from 0.1 mg/ml to 2.0 mg/ml (Graph-2). The method showed no linear relationship in the range of 0.01 mg/ml to 0.1 mg/ml, the required working range for the determination of the rate of degradation.

Conclusion

In the present study, the isolation and identification of chlorpyrifos degrading bacteria was carried out using the soil samples that were collected from a nursery at Borivali (Mumbai, India). The nursery had been using the pesticide. The primary screening of the chlorpyrifos degrading bacteria was carried out using enrichment and isolation using a Mineral salt medium. The sole source of carbon in the medium was chlorpyrifos and any organism growing in the medium indicated that it had the ability to degrade it. After enrichment and isolation, three isolates were obtained. Selection of the potential degrader was done using plate and flask tests and upon purification of the isolates it was found that two of the isolates have lost their ability to degrade chlorpyrifos. Therefore, CP C1 (colony1) was identified as the isolate with the potential to degrade chlorpyrifos and the biochemicals indicated that CP C1 is Gram-negative bacterium and is oxidase positive and cannot tolerate alkalinity.

To experimentally quantify the degrading capacity of the isolate (CP C1) two chemical assays were performed. One assay was performed for the detection of degraded TCP and the other for the detection of chlorpyrifos that was being consumed. For detection of TCP using diazotized aniline reagent, the OD values that were obtained had no linear relationship, also random OD values were observed for all the sets and therefore could not be considered for analysis. One of the sets also showed precipitation and therefore the OD readings were unsuitable for analysis. The second spectrophotometric method was the determination of chlorypyrifos using UV-VIS spectrophotometry. This method was found to be suitable for the detection of chlorpyrifos above 0.1 mg/ml i.e., in the range of 1-10 mg/ml and hence this technique showed lower sensitivity between 0.01-0.1 mg/ml which was the required working range for the study.

The chlorpyrifos degrading capacity of the potential degrader isolated can be further determined experimentally using various

other biochemical assays like enzyme assays, fluoresence assays, etc.

Future Prospects

This present research was conducted chiefly to isolate and identify a suitable microorganism that could degrade chlorpyrifos hence lending a hand in the bioremediation of chlorpyrifos contaminated soil. Chlorpyrifos is one of the most common pesticides used in many parts of India and the permitted levels of water contamination due to this pesticide have been crossed many years ago. Chlorpyrifos has a very long half-life and therefore remediation cannot depend upon self-degradation. These reasons indicate that an efficient bioremediation method needs to be developed. The microorganisms that were isolated could be identified using 16s rRNA sequencing and further tested for their efficiency and overall impact on the plants and soil microflora so that it can be practically put to use. The efficiency can be quantified through various methods e.g., by developing a biosensor that can detect either the pesticide or the degraded product. The future objective of developing a biosensor will allow the detection of microliter differences in chlorpyrifos degradation carried out by each colony that has been isolated. This research can thus hold great potential to be a boon to the farmers who are struggling with deteriorating soil quality and also contaminated water for irrigation. So, the detection, isolation and identification followed by necessary testing for toxicity, and efficiency can, in turn, lead to better soil and water quality and therefore better crop production.

Conflict of interest

The authors declare no conflict of interest.

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Review article

Cell migration:
Machinery and Regulatory Signaling Networks

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Abstract

Cell migration is found across simple unicellular organisms such as amoebae, to multi-cellular organisms such as mammals. Research over the decade has focused on elucidating an understanding pertaining to the complexities underlying cell migration. The generation of temporal-spatial cues resulting in cell adhesion, and polarization in cells, have been extensively studied. In this review article, an overview of the basic machinery of cell migration is provided, wherein the role of different proteins and complexes during cell migration has been elucidated. A brief overview as to how adhesions serve as signalingcenters and contact points is provided with an account of the role played by the complexes and various proteins during actin polymerization, nuclear positioning, and retraction in the trailing edge during cell movement. An understanding of the mechanism of cell migration and the signaling networks that regulate it, has provided insights into the development of therapies for the treatment of cell migration-related disorders.

Cell Migration

Cell migration is a fundamental process, which is essential for embryonic development and for maintaining adult health. Cell migration is an integral part of many branches of biology such as cell biology, immunology, biochemistry, neuroscience, embryology, and molecular biology. Cells can migrate individually or in groups. Cell migration is essential for morphogenesis in the animal body (Vicente-Manzanares & Horwitz, 2011). In an adult organism, cell migration plays a role in eliciting an immune response in the body and repairing wounds. During migration, the cells migrate in response to some specific chemical and mechanical signals. Proteins play a central role in the regulation of cell migration. In the case of damaged cells, new cells often migrate and replace them from the underlying tissue (Trepat et al., 2012).

Cell migration involves a coordinated response of cytoskeletal dynamics and re-organization, adhesion in cells, and signal transduction. Migrating cells have an internal organization that helps them to sense and move along the gradients of soluble attractants and repellents, a process commonly called chemotaxis (Devreotes & Horwitz, 2015).

Single-cell migration can be seen in the case of Primordial germ cells, leucocytes, migration of fibroblasts during wound

healing, and hematopoietic stem cells. Migration of hematopoietic stem cells to bone marrow during transplantation requires regulation of the activities of adhesion and chemo-attractants. In the amoeba *Dictyostelium,* cell migration is essential for seeking nutrients, cell-cell aggregation, and morphogenesis (Vicente-Manzanares & Horwitz, 2011).

Cells can migrate in groups as well, in a process called collective cell migration. During the process of collective cell migration, the cells maintain cell-cell contacts and exhibit both morphological and behavioral polarization. The cells also interact with neighboring cells within the collective, and thereby affect each other's behavior (Olson &Nechiporuk, 2018). In the case of vertebrates, collective cell migration is seen in the gastrulation and development of neural crest cells, and during the development of the sensory lateral line in the case of aquatic vertebrates. It is also seen in tracheal development and border cell migration in the case of *Drosophila* (Devreotes & Horwitz, 2015).

Under the pathological condition, the abnormal migratory signals may lead to the migration of a given cell type to the wrong place, causing a disbalance in homeostasis in the tissues and system. This can be reported in the case of rheumatoid arthritis, wherein abnormal migratory signals cause certain white blood cells to migrate to the joints (Ridley et al., 2003). De-regulation or dysregulation of cell migration plays a role in disorders such as chronic inflammation, autoimmune syndromes, and cancer.

Machinery of Cell Migration

A cell initiates movement in response to an external signal in the surrounding environment, which is detected by transmembrane proteins like G-protein coupled receptors, located on the cell membrane. The process of single-cell migration through the interstitial tissue can be broadly categorized into five stages comprising of- polarization of cell and protrusion of the leading edge driven by the actin cytoskeleton, attachment of the leading edge with the substrate, degradation of the components of the tissue confining the cell body (proteolytic degradation), contraction in actomyosin, finally leading to the forward sliding of the cell rear (Friedl & Wolf, 2009).

The process of cell migration involves constant restructuring in the actin cytoskeleton. The actin cytoskeleton is a network of actin and actin-binding proteins, which co-ordinates cellular processes, with microtubules and intermediate filaments. The actin network is re-organized at the leading edge of the cell, helping in propulsion. The cell is then adhered onto the substrate at the leading edge and detaches at the rear and cell body. The contractile forces generated by the actin-myosin network pull the cell forward. After the signal is sensed, the cell starts moving by actin polymerization. Actin polymerizes in the region closest to the signal, when the signal is a chemoattractant. However, if the signal is a chemorepellent, the cell moves away by polymerizing actin on the opposite side. As and when the extending edge moves forward, the signal direction is monitored. This signal tracking is seen in a cell that chases an object when attempting to engulf it; or by a cell moving in response to a chemoattractant (Devreotes & Horwitz, 2015).

After the leading edge has begun protruding, the adhesion molecules such as Vinculin, talin, and integrin, present in the extending region, help in the attachment of the leading edge to the substrate. These cell-substrate attachments are formed at the leading edge when the actin bundles have linked the cytoskeleton to the substrate at certain sites. The attachments thereby prevent the leading edge that is protruding, from retracting. As and when the cell continues to adhere at the leading edge, it detaches at the rear by the disassembly of its actin bundles (attachments). The rest of the cell is pulled forward by the contractile forces generated by the sliding of myosin motors on actin filaments present in the cell body and rear. As the cell moves on the substrate, the actin cytoskeleton is transitioned between a solid-like elastic material (gel) and a solution-like viscous

material (sol) (Ananthakrishnan & Ehrlicher, 2007). These gel-sol transitions are important for cell movement. Also, these transitions are likely caused by the constant net actin polymerization and network assembly at the leading edge (depolymerization and disassembly at the rear of the cell). The process of cell movement is spatially coordinated by proteins via the mechanical changes in the cytoskeleton (Ananthakrishnan & Ehrlicher, 2007).

Protrusions are the extensions from the cell membrane. A leading protrusion points in the direction of the movement. Protrusions occur in response to the chemoattractant signals in the extracellular medium. The actin filaments are made of monomeric G-actin subunits. During polymerization, ATP bound to G-actin gets hydrolyzed, and the bond is formed between two monomers. The polarised actin filaments thereby show a fast-growing barbed end where new monomers are added and a pointed end where the growing filament originates (Devreotes & Horwitz, 2015).

The globular actin molecules are polymerized to form filamentous actin. This results in the production of oriented filaments which grow at the barbed end and thereby push the leading edge (front end) of the cell forward. Formins (examples- mDia1 and mDia2) and the Arp2/3 complex regulate the process of actin nucleation and polymerization. Not only do the formins initiate unbranched actin filaments but also remain associated with the elongating barbed end, which is seen in the case of yeast. (Vicente-Manzanares & Horwitz, 2011).

The formins also regulate the growth of the actin filaments (linear). The capping proteins sequentially add monomers of actin while remaining attached weakly to the barbed end (rapidly growing) of the filaments (progressive elongation). The formins are regulated by the Rho-family small GTPases (RhoA and Cdc42). ABI1 and Gα12 seem to direct mDia1 to actin filaments as well as adhesions. The Arp2/3 complex nucleates new actin filaments by binding to the existing filaments from the sides. The Arp2/3 complex encompasses seven proteins, including two actin-related proteins (Arp2 and Arp3) and subunits ranging from ARPC1 to ARPC5 (Devreotes & Horwitz, 2015).

WASP family members (WAVE and WASPs) are the targets of the Rho- family GTPases Rac1 and Cdc42, and they interact with the Arp 2/3 complex and thereby regulate its activity. The actin filaments are stabilized by capping at the barbed end that inhibits de-polymerization. The anti-capping proteins of the Mena/Vasp family regulate capping (Bear & Gertler, 2009). Cofilin is a major regulator for the stability of actin filament. Being an essential actin regulatory protein, Cofilin increases the off-rate of monomers of actin at the non-polymerizing end. Thereby, Cofilin accelerates actin assembly dynamics by increasing the number of filament ends via which actin monomers are added or removed. LIM kinase stimulates cofilin activity, and itself is regulated by Cdc42 and Rac1, that act via the Kinase PAK. The polymerization rate is increased by the binding of profilin to the actin monomers (Padrick & Rosen, 2010).

The myosin II motor proteins generate the contraction forces. These forces are well coordinated with actin polymerization at the leading edge. Myosin II promotes the retrograde movement in actin filaments, away from the zone of actin polymerization that is active in the lamellipodium. This is seen in the case of fibroblasts and epithelial cells. Therefore, the net protrusion rate is reduced (Ponti et al., 2004). Myosin II activity is often regulated by phosphorylation, wherein the RLC in the myosin (Regulatory light chain) is phosphorylated. The assembly into filaments is also regulated by phosphorylation in the heavy-chain tail region. The kinases that phosphorylate the RLC include MLCK (Myosin light-chain kinase) and ROCK (Rho-associated protein kinase) (Oakes et al., 2012). Over the years, research has shown the key involvement of the actin cytoskeleton in migration, and how its fine regulation is needed for maintaining cellular integrity. The points of molecular interaction

between the cell and substrate are called adhesions. Adhesions are known to regulate cell motility. Adhesion is the physical interaction of a cell with another cell or the extracellular matrix. Cell-cell adhesion helps in maintaining epithelial tissues and provides support for the functional contacts between cells. Adhesions serve as traction points as well as signalingcenters during cell migration. When acting as traction points, adhesions transmit forces to the substrate, which aids in actin polymerization causing protrusion at the cell front. The traction points are released at the cell rear as it retracts and the cell thereby moves forwards. There is sufficient adhesion for traction at the cell front which allows efficient release at the rear (Trepat et al., 2012). Cell-ECM adhesions include focal adhesions, focal complexes, and close contacts.

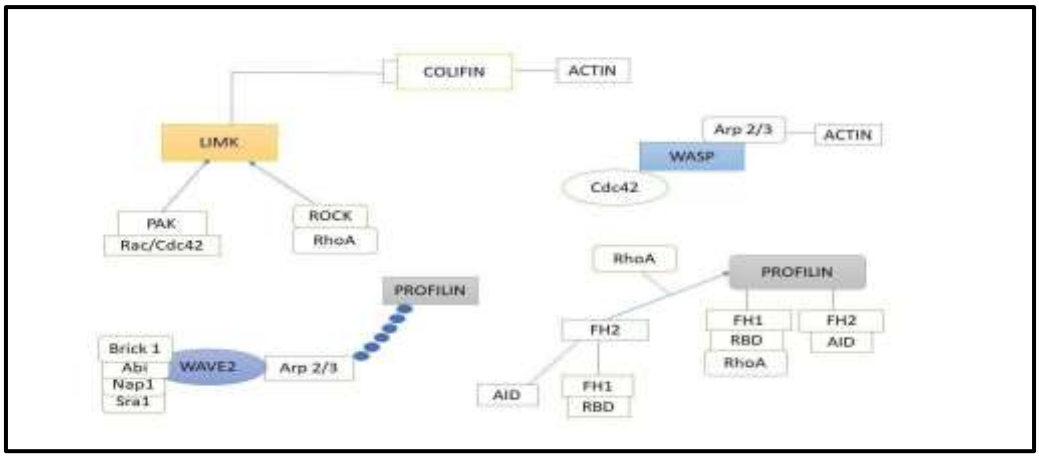

Fig.1: Regulation of Actin Dynamics during Protrusion

The adhesions in their early stages of maturation are termed focal complexes. They are larger than nascent adhesions and are found at the boundary of the lamellum and lamellipodium. They grow or elongate into focal adhesions. This elongation occurs along with a template of bundled actin. The presence of nascent adhesions and focal complexes are seen in motile cells and their appearance represents high velocities of protrusion and movement. The matured adhesions evolving slowly are termed focal adhesions. They are linked to large contractile actomyosin stress fibres. The regulation of actin dynamics during Protrusion is shown in Figure 1.

Podosomes are ring-shaped adhesions found in cells like macrophages and dendritic cells. They form dot-like structures like suction cups. A podosome has a core containing proteins involved in actin polymerization like WASP, Arp 2/3 complex. A surrounding ring with integrin receptors and adhesion proteins (Paxillin and FAK/Pyk2) is also present. The adhesion receptors (for example- the Integrin family) are the physical link between the cell and the extracellular matrix. Integrins consist of an α and a β subunit. Both subunits are transmembrane proteins that associate to form a heterodimer. Integrins organize the signaling molecules through association with the C-terminal, cytoplasmic tails.

Integrins are activated by inside-out signaling. Talin and kindlin bind at the intracellular region of the integrin receptor (bind to the cytoplasmic tail), causing a conformational change that leads to extension in the molecule, and the sites of ligand binding are exposed. Integrins form large clusters which increase the avidity thereby modulating the adhesiveness of a cell. The conformation change in integrin also creates a high-affinity state (Devreotes& Horwitz, 2015).

A few proteins binding to the cytoplasmic tail of the integrins, also bind to actin (examples- talin, α-actinin, vinculin). α-

actinin binds to actin and also binds to β-1 integrins. Vinculin binds to the integrins through talin. Vinculin also binds to actin and the actin nucleator Arp 2/3. Filamin is an actin cross-linker that binds to β-1 and β-7 integrin tails. Talin regulates integrin avidity by controlling the integrin clustering (through its association with actin). Vinculin and α-actinin also control integrin clustering. The integrins modulate their activation, by themselves, in response to mechanical forces (Friedland et al., 2009).

The signaling molecules associated with integrin-containing adhesions include kinases, scaffolds and phosphoproteins. FAK is a tyrosine kinase that is recruited early to adhesions. It is activated by autophosphorylation. It is involved in the recruitment and phosphorylation of adhesion proteins like paxillin. It controls Rho activation through the binding of GEFs and GAPs. Example- FAK binds to p190RhoGEF (A Rho activator) via p120RasGAP. Paxillin is a phosphoprotein that is localized to adhesions. Through phosphorylated Tyr, it creates binding sites for recruiting other signalling regulators. It recruits effectors that are implicated in activation of Rac (Friedland et al., 2009).

Tensin, a phosphoprotein is seen only in mature adhesions and interacts with protein phosphatases. Zyxin, a scaffold protein interacts with α-actinin and plays a role in regulating actin polymerization close to adhesions, through the association to VASP. As signalling centers, the adhesions in protrusions regulate actin polymerization and myosin II activity via Rho-family GTPases. The adhesions elongate along with the actin filament bundles, at the interface of dendritic actin (present in the lamellipodium) and actin bundles (present in the adjacent lamellum) (Oakes et al., 2012)

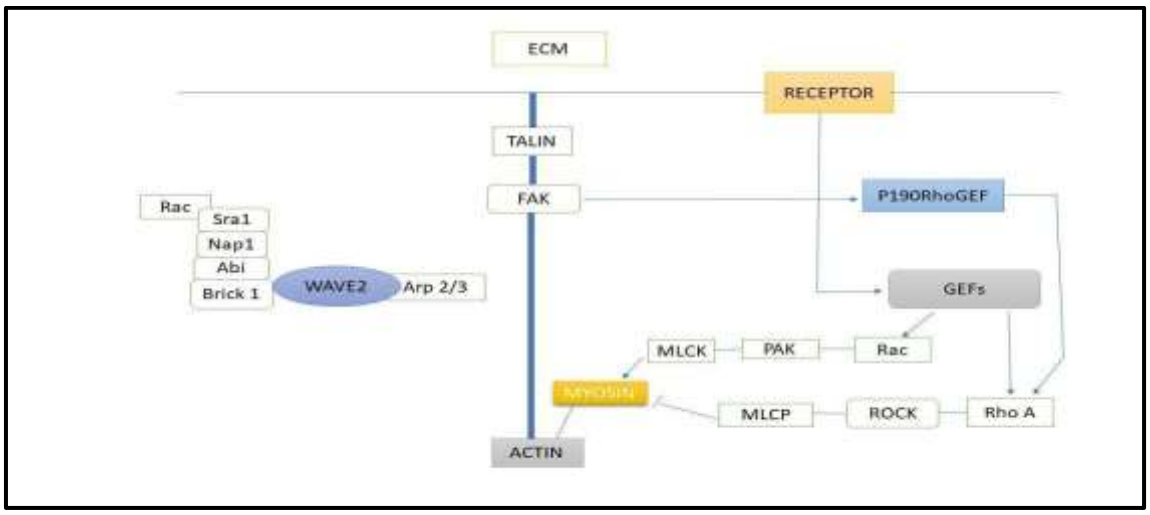

Fig.2: Adhesion, serve as contact points and signaling centers

Figure2 **s**hows adhesions serving as contact points and signalingcenters. The integrin-based adhesions are large assemblies which link the substratum to actin and thereby generate signals which regulate Rho GTPases and cell migration. Talin, vinculin, and α-actinin mediate the structural linkage to actin. Signaling is basically mediated by adhesion-associated complexes. The paxillin/FAK and its linkage to Rac GEFs and Rho GEFs is shown as an example in this figure. The activity of Arp 2/3 complex and myosin II, is regulated by Rho and Rac, is also shown in this figure (Devreotes& Horwitz, 2015).

Often cells migrate, proliferate, differentiate in response to given conditions around them (called the 'microenvironment'). The factors that trigger such responses include nutrient availability, gradients of growth factors, the pliability of the surrounding tissue, etc. In the case of *Dictyosteliumdiscoideum*, the cells divide actively when there is plenty of nutrient supply. The cells are actively chemotactic,

looking for cues to the environment (nutrient-rich). When the nutrients are scarce, cells aggregate, form multicellular bodies, and undergo dissemination (Willard & Devreotes, 2006). The microenvironment directs the behaviour in cells by triggering the activation of various signalling networks which would direct an appropriate cellular response. Cell migration is a response regulated by multiple interlinked signalling networks that are organized and initiated at the cell surface.

Initiation of signalling can be done through GPCR, integrins, growth factors, cadherins, immune receptors, etc. Many receptors usually aggregate and organize signalling scaffolds in response to extracellular stimulus. At the level of receptor, responses include an intrinsic kinase activity in growth factor receptors, resulting in phosphorylation of the proximal effectors. Also, in some cases, activation can occur through conformational change on binding to the ligand, which triggers the binding of downstream effectors. The initial signals are then passed along one or multiple branches of a complex (interconnected) network of intermediates which finally lead to the Rho family of small GTPases. This in turn regulates the machinery of migration (protrusion, polarity, adhesion, etc).

The growth factor and adhesive signalling work synergistically. Integrin-mediated migration recruits multiple proteins to adhesions. FAK recruits GEFs and GAPS (GEFs turn on signaling whereas GAPs terminate signaling by inducing GTP hydrolysis), that regulate the Rho GTPases (examples- p190RhoGEF, p190RHOGAP). Paxillin recruits Rac effectors like CAS/CRKII/DOCK180, PI3K, and β-PIX (Tomar & Schlaepfer, 2009).

With Rho GTPases, small other GTPases are also implicated in migration. Ras is seen to activate PI3K, which is involved in chemotaxis. Rap is an analog of Ras, wherein Rap controls the integrin affinity (thereby controls cell adhesion) via its effector RapL. The ARF/Rab family is seen to control the vesicular traffic, and plays a role in the recycling of adhesion receptors to and from the plasma membrane during migration (Caswell et al., 2009). Rab GTPases play an important role in the process of internalization and recycling of the migratory receptors. Small Rho GTPases act via effectors on a small number of end-points which include polymerization of actin, organization, contraction; polymerization of microtubule; and regulation (transcriptional) of motogenic gene products. Rho GTPases are small Ras-like proteins, around 20 in number, distributed into RhoA-like, Rac-like, atypical Rho, Cdc42-like. GEFs are multiple GTP exchange factors that activate specific Rho GTPases. GAPs are GTPase activating proteins that are capable of catalysing GTP hydrolysis by one/more Rho proteins. GDP-dissociation inhibitors (GDIs) isolate specific Rho GTPases in the cytoplasm (Vicente-Manzanares & Horwitz, 2011).

Research based on cell migration has provided insights for understanding fundamental biological processes, and migration-related disorders. It has provided the framework for developing therapeutics and diagnostics for various disorders. Combinations of various branches of medical science have revealed exciting insights which explain how cells adhere and move, how the migration of cells is coordinated and regulated, and how the cells can interact with the neighboring cells or react to any changes in their microenvironment.

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Review article

Hox Genes- The Master Regulator of Body Patterning: A mini review

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Abstract

The *hox* or *homeotic complex* genes play an important role in the establishment of the anterior-posterior axis during embryogenesis. Discovered in the year 1983 by the team working with Thomas Kaufman, the *homeotic* complex genes were first identified with the help of *antennapdia* and *ultrabithorax* mutations in the fruit fly *Drosophila*. *Hox* genes are vertebrate homologs of *homeotic complex* genes in *Drosophila*. These genes encode the proteins that specify the positional identity of the segments in the embryo, thus allowing the correct part to form at the right location. The vertebrate *hox* genes are organized in clusters along the axis and show spatial and temporal correspondence during transcription. These genes also regulate other genes, although the function of the protein encoded by them remains conserved across species. Evolution of the *hox* genes leads to morphological diversity amongst organisms.

Introduction

The establishment and patterning of the axes during embryogenesis has always been a fascinating phenomenon in Developmental Biology. *Homeotic genes* or *Hox genes* play an important role in demarcating the positions of segments in the organism along the anterior-posterior axis. The homeodomain proteins play a fundamental role in controlling regulation of many other genes and thus are also known as master regulator genes. The *Hox* genes are activated and inactivated by a cascade of regulatory proteins (Driever et al., 1988).

These genes encode for the protein that specifies the location or the positional identity in the embryo rather than development of a part. A mutation or mis-expression of the *hox* gene results in the structures forming at the wrong location. In humans, a mutation in *HoxD13* causes *Synpolydactyly*, which is a rare genetic disease that results in extra digits on the hands and feet (Malik et al., 2008).

Discovery

In 1983, two groups of scientists, Ernst Hafen, Michael Levine, William McGinnis, working in Walter Gehring's lab at the University of Basel, Switzerland; and Matthew P. Scott, Amy Weiner, who were then working with Thomas Kaufman at Indiana University in Bloomington, independently discovered the existence of homeotic genes in *Drosophila* (Kaufman et al., 1990; Mcginnis et al., 1992). The homeotic genes

were identified in *Drosophila*, through mutations in genes which led to transformation of one segment into another. These genes are hence named homeotic, as the word '*homeos'* in Greek means similar.

Hox genes are vertebrate homologs of homeotic complex genes in the fruit fly *Drosophila*. They were first identified when two mutations in *Drosophila* were observed, the *Antennapedia* and the *Ultrabithorax* mutation. These mutations caused transformation of certain parts of one segment into another. A mutation in the *antennapedia* gene as shown in fig. 2(a) caused it to express itself in the head region instead of the second segment which results in the second-segment leg to develop in the place of the antenna.

The other observation was pertaining to the *ultrabithorax* gene. A mutation in the *ultrabithorax* gene caused formation of a second set of wings instead of halteres (balancing organ) in the third segment (as shown in fig.2 (b)) as well as in the second segment. The function of the *ultrabithorax* gene is to repress the second segment identity and formation of wings in the third segment (Lewis, 1978).

Organization of *Hox* genes:

Drosophila has two complexes comprising of eight *Hox* genes. The two complexes are the Antennapedia complex (Kauffman et. al., 1990) and the Bithorax complex (Lewis, 1978), that act on different regions of the body. The order of the *Hox* genes on the chromosomes and their position of expression in the developing organism show remarkable correlation (Kmita & Duboule, 2003).

The location and time of induction of *hox gene's* transcription shows spatial and temporal correspondence within the clusters of the gene. For example, the genes towards the 3'end of the cluster are expressed earlier and more anteriorly than the genes located towards the 5' end (Dolle et al., 1989; Kmita & Duboule, 2003).

Hox genes in vertebrates are similarly organized in clusters that are arranged in an anterior to posterior pattern (Krumlauf, 1994). In vertebrates there are four clusters of 9 to 11 *Hox* genes aligned in 13 sets. These clusters are based on their sequence, organization and homology to the *Drosophila*. All genes in the vertebrate cluster are not seen in all organisms and some seem to have undergone duplication since the divergence of the invertebrates and the vertebrates. The gene similar to the *Drosophila* Abdominal-B has been duplicated several times and therefore exists in multiple forms in three clusters out of the four major clusters(A,C and D) indicating that it was expanded before the process of duplication took place (Lappin et al., 2006; Duboule, 1992).

Regulation and Functions of *Hox genes*

Hox genes play critical role in the formation of limbs, organization of the hindbrain segments (rhombomeres), eye formation, etc. They regulate genes that in turn regulate other networks of genes that are involved in the formation of specific parts. The targets of the *hox* genes often lead to cell division, cell death, cell adhesion and migration as they are involved in setting up segmentation boundaries, defining structures to be formed, etc (Pearson et, al. 2005).

In flies, the *hox genes* are regulated by the pair rule and gap genes; which are regulated by the maternal mRNAs. The mRNAs regulate the pair rule genes via protein concentration gradient. In vertebrate Retinoic Acid signalling regulates the expression of the *hox genes* along the anterior-posterior axis (Sanes et al., 2012).

Evolution of *Hox genes* across species

Hox genes are of ancient origin highly conserved in evolution, and appear to have arisen through duplication of an original *Hox* gene long ago. This ancestral gene underwent a duplication forming a cluster of 4 *hox genes,* which further underwent another 4 rounds of

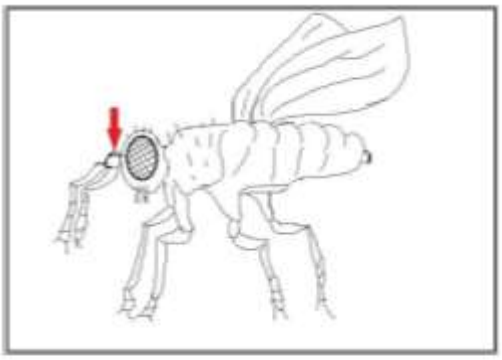

Fig. 2(a): The *Antennapedia* mutation in *Drosophila*

Above diagram shows an *antennapedia* mutation in *Drosophila;* the arrow indicates the second-segment leg that develops in place of the antennae.

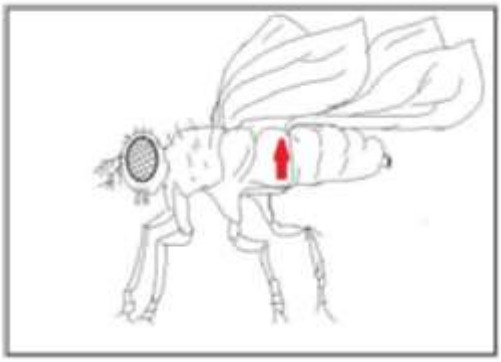

Fig. 2(b): The *Ultrabithorax* mutation in *Drosophila*

The above diagram shows an *ultrabithorax* mutant. The arrow indicates the formation of a second set of wings instead of halteres (balancing organ) in the third segment.

duplication in the vertebrates. *Hox* genes are an attractive candidate to understand the diverse morphology in organisms as they direct the formation of certain structures or body segments in all bilateral organisms (Carroll, 1995).

The conservation of the *Hox* genes seems conflictory at first; as animals having the same *Hox* genes show such morphological differences. For example, the flies and the beetles have the same complements of the *Hox* genes and yet show morphological differences. Mammals and teleost fishes both have four clusters of *Hox* and these genes express themselves along the anterior-posterior axis in an orderly manner. This can be explained because *Hox* genes only demarcate relative positions in animals, not specify any particular structure. These genes control morphology of different body regions within the species. Within one species morphology of the regions of the body is controlled by different *Hox* genes (McGinnis & Kuziora, 1994). The same *Hox* gene regulates the homologous body segments between the species in different ways.

Evolution of *Hox* genes leads to morphological diversity amongst organisms. But the genes are still conserved and code for the homeodomain proteins in all organisms. This conserved characteristic is often used in analysing the expression of *Hox* genes during craniofacial development, limb formation, rhombomere organization, etc., to study and identify developmental defects (Carroll, 1995).

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Review article

A reflection on the impact of anthropogenic actions on the future of life on Earth

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Abstract

Extinction is the cessation of a kind of an organism or a particular species of plant or animal. It is referred to as the death of the last individual of a species, although the capacity to breed and recover may have been lost before this point. Although extinction is a natural phenomenon, anthropological activities have accelerated the process by a thousand-fold. Global warming is one of the major causes that contributes to extinction. Although it's a part of a natural phenomenon, researchers have agreed to the fact that global warming is mainly caused by human activities. Amongst all the species which exist today, about 10,000 of their relative ancestors are extinct and several are on the verge of extinction. The current article reflects upon the anthropogenic activities which are leading to the sixth mass-extinction and emphasizes how certain preventive measures can ameliorate the current situation.

Introduction

The "Every day on this planet some species that doesn't draw the attention of humans goes extinct"- Liu Cixin-Chinese (science-fiction writer).

Extinction is the termination of a kind of an organism or a particular species of plant or animal. Generally, it is referred to as the death of the last individual of a species, although the capacity to breed and recover may have been lost before this point. Although extinction is a natural phenomenon, anthropological activities have accelerated the process by a thousand-fold. Among all the species which exist today, about 10,000 of their relative ancestors are extinct or are on the verge of extinction. According to the 2017 International Union for Conservation of Nature (IUCN) Red List of threatened species, 25% of the mammals and 40% of the amphibians are threatened to be extinct in the near future (Ceballos et al., 2020).

Historically, extinction has been divided into five segments which include the Ordovician-Silurian Extinction which occurred 440 million years ago where small marine organisms died and massive glaciation resulted in large amounts of water in south polar masses (Drake et al., 2014). This led to depletion in carbon dioxide content in the atmosphere, drastically cooling the earth's temperature. As a result, the water levels increased and simultaneously the oxygen levels decreased making the water toxic due to metal depositions. The second mass extinction which is known to humans is Devonian Extinction which was 365 million years ago where many tropical

marine species like corals, shelled brachiopods and trilobites went extinct. During this time oxygen levels dropped severely making it difficult for shelled brachiopods and reef-building organisms to survive and caused them to perish. The third extinction was Permian-Triassic Extinction which occurred 250 million years ago, this major mass extinction affected a range of species, including many vertebrates. About 96% percent of marine life got wiped out during this era caused by the Siberian traps, the volcanic eruptions led to spread of lava over the land which is currently known as Siberia. Eventually, this led to a rise in the temperature, weathering of rocks increased rapidly, sulphur from the volcanic eruptions led to acid rains, these reasons gave way to species curbing to the weather conditions. The fourth extinction that followed was around 210 million years ago, the Triassic-Jurassic Extinction, where the extinction of other vertebrate species on land allowed dinosaurs to go extinct. In this era archosaurs reptiles (crocodiles, pterosaurs, nonavian) flourished a lot but a sudden loss of land and marine species had a major blow on this era. The land temperatures increased making it difficult for land species to thrive and the carbon dioxide content in oceans also increased leading to cooling of marine water making it difficult for marine creatures to keep their shells hard enough due to calcium carbonate deficiency. The last extinction: Cretaceous-tertiary Extinction, occurred 65 Million Years Ago, the most recent one was due to a major asteroid impact (Veron, 2008).

The major difference between the extinctions that happened in the past and what is happening now is that, in the past extinctions occurred due to catastrophic natural phenomenon like volcanic eruptions, asteroid strikes, and violent changes in the climatic conditions, while the current extinction is almost entirely the result of anthropogenic activities. Human activities occur at a faster rate as compared to the natural phenomenon and cause higher extinction rates. Increased energy usage, mainly when using nonrenewable sources like coal, leads to increased emissions of CO2, CO, SO2, oxides of Nitrogen and Mercury leading to acid rain, air pollution, and negative health effects. The biggest concern world-wide is with the CO2 and greenhouse gas emissions that would lead to further global warming. Carbon dioxide contributes to play a major role in causing warming than the other gases Methane, Nitrous Oxide, Ozone, and several Chlorofluorocarbons (CFCs) combined. It has been appraised that about 9.5 billion metric tons of carbon gets released into the atmosphere each year just by burning fossil fuels, and another sum of 1.5 billion is added up through deforestation and other land cover changes. Humans are cutting down rainforests that majorly contribute in balancing the carbon dioxide. These forests if not cut down by humans can absorb around 3.2 billion metric tons per year, whereas the ocean absorbs about 2.5 billion metric tons per year. A net 5 billion metric tons of human-produced carbon remains in the atmosphere each year, raising the global average carbon dioxide concentrations by about 2.3 parts per million per year. Since 1750, humans have amplified the copiousness of carbon dioxide in the atmosphere by approximately 50 percent (Cook et al., 2013). If this continues, rainfall and other forms of precipitation would get more heavier, the frequency would increase and it would hence, become unpredictable.

The biodiversity of Earth may fail to sustain with all the abrupt changes in the environment. Many species are not accustomed to the severe weather conditions and long seasons, or the changes in the chemical makeup of the surrounding. In the Arctic region, the melting of permafrost is releasing carbon and mercury which is not only causing global warming but also has toxic effects on plants and animals (du Pontavice et al., 2020). When permafrost melts, it releases carbon dioxide and more-potent methane, making climate change worse. The change in sea levels and currents is a result of the melting of the freshwater. The gases erupting from the volcanic activity can also be absorbed by the water, thus changing the chemical composition, making it unsuitable for certain life forms.

The water temperature is rising rapidly which is damaging the growth of zooplanktons which are the main source of aquatic life. It is very well known to us how warming up of Earth's atmosphere is affecting the melting ice caps at polar regions, and how warming ocean waters have melted a substantial segment of ice in the Southern Ocean, the influence is such that a breakdown of a far larger mass of ice may be unavoidable. This melting of ice and glaciers adds more water to the oceans and as well as depleting one of the major habitats on which many animal species are dependent on (Welch, 2015). Each species has a particular self defence mechanism and the inherent capability to fight disease. With the changing climatic conditions and landscape, certain species are losing their ability to fight off the disease. They are becoming more vulnerable to disease and epidemic, which can lead to their eventual extinction (Sutherland et al., 2019)

Natural habitats, such as bogs and meadows, are also being converted into agricultural land or housing areas. Habitat loss is a main threat for 85% of species that are listed as "threatened" or "endangered". About half of the earth's forests are gone, and they are still being removed much faster than they can regrow (Pecl et al.,2017). Invasive species are a group of organisms, plants that are not native to the ecosystem and may alter it thereby, having a negative impact. Due to the introduction of invasive species by humans, the competition for survival has increased which will eventually lead to killing of the natural species (Kumar Rai et al., 2020). In certain regions of the United States, Asian carp, a fast-growing, aggressive, and adaptable fish are outcompeting the native fish species for food and habitat.

Coral reefs are referred to as the "rainforests of the sea" which form the part of diverse ecosystems of the ocean. Coral reefs occupy less than 1% of the ocean floor, and are home to more than 10% of all marine species like Crustaceans, Seaweeds, several species of bacteria, etc. and over 4000 species of fishes. With a global economic value of $375 billion per year, coral reefs provide food and resources for more than 500 million people. Corals which occupy less than 1% of the ocean floor are now endangered due to overfishing, usage of improper fishing techniques, coastal development, pollution, etc (Harvey et al., 2018). Eventually this results in increase in the CO_2 content and warming up of the water bodies. About 75% of the world's coral reefs are at risk from local and global impacts. About a quarter of them have already been damaged and are beyond repair. It is speculated that if the situation continues to be the same then 90 % of coral reefs will be in danger by 2030 and nearly all of them would be endangered by 2050 (Vanwonterghem et al., 2020).

Amphibians are often referred to as 'canaries of coal mines' and approximately 7,000 known species are dependent on clean fresh water and damp habitats. Some of these species are now considered to be vulnerable to habitat loss generally due to deforestation, changes in water or soil quality and the possible influence of climate change. Since amphibians are sensitive and are dependent on both terrestrial and aquatic habitats; they serve as signalling systems, thus acting as indicators of the wellbeing of the environment. Unfortunately, they are declining at an alarming rate. The recent figures from the IUCN Red List of Threatened Species show that there are just about as many species of amphibians categorized as threatened as those of threatened birds and mammals put together, with an estimated 40% of amphibian species in danger of extinction. The reptiles too are declining due to climatic changes and habitat loss and trading. Studies estimate that 19% of the world's reptile population are threatened to be extinct (Pabijan et al., 2020).

At present, many precious species are endangered and several are on the verge of extinction. In the year 2018, the last male northern white rhino became extinct. Australia in the year 2019-2020 experienced its worst bushfires which estimates that about a billion animals including bats and insects are in total loss

of their habitat, which will make these animals endangered in the near future.

If we fail to conserve the ecosystem and its rich biodiversity the consequences are going to be gravely damaging and devastating. Conservation of endangered species and ecosystems is important to humans, since a well-balanced ecosystem results in a healthier and pure environment, giving us clean air to breathe, a healthy water system to support the divine marine life and arable land for agricultural purposes. By saving endangered species, we are helping ourselves. Our ancestors left us with an incredible natural world and an endless number of amazing species and now it's our duty to do the same for our future generation. By conserving wildlife, we're ensuring that our future generations can enjoy the natural world and its incredible species. While natural disturbances occasionally happen, human disturbances put a constant pressure on the ecosystems and dramatically create an impact on the species around. The minute the ecosystem begins adjusting to one stress, another appears. Many ecosystems that we depend on are not given enough time to adapt to the new conditions. The natural cycle of disturbances, growth, die down of the plants and forest and then regrowth again, cannot function properly because too many disturbances put pressure on the ecosystem (Batáry et al., 2015; Burgess, 2019).

Conservationists mainly focus on ecosystems with higher numbers of species like the ones in rainforest and other hotspots of biodiversity. We as the inhabitants of our planet can follow a "Discipline with a Deadline" to make it a better place to live in. With a fact that not all are well versed with the concepts of ecosystems and their interactions with the environment as that of the experts in the field yet a few steps with a positive approach can do a lot more to save the environment. Volunteering at non-governmental organizations (NGO) and wildlife refuges, spreading awareness for saving wildlife through social media, simple lifestyle changes like eating less meat, usage of products which are cruelty free, beach clean-ups, avoiding the use of one-time usable plastic can serve the purpose and would make a big difference in the years to come (May,2011).

Earth right now is the middle of its sixth major animal and plant extinction, the sixth to happen in the last 500 million years (Bellard et al., 2012). Based on several studies many scientists believe that it might be too late to stop the melting of ice caps in Antarctica causing sea levels to rise by 16 feet, inundating regions that are home to hundreds of millions of people.

It's time for us to understand and realize that we are going to reap the benefits of what we sow. Human society cannot function without biodiversity (Isbell et al., 2017). Every single animal on the planet is an important component that helps maintain balance in our ecosystem in different ways. They keep our planet in the order it should be. The exploitation of species will end up in a tragedy we no longer can fix. This is a calling to each and every one of us to make a difference, not only because animals are getting extinct, but also because Earth needs us. The tiniest effort from each human being will make a difference, and eventually, nature will pay us in ways we won't be able to believe (Lewis, 2006). What may still be possible, however, is for humans to control just when that might happen.

"The fact is that no species has ever had such wholesome control over everything on earth, living or dead, as we now have. That lies upon us, whether we like it or not, an awesome responsibility. In our hands now lies not only our own future, but that of all other living creatures with whom we share the earth." - David Attenborough.

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Review article

Exploring a link between obesity and diabetes through autophagy

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Abstract

Obesity poses a severe threat to human health, involving increased prevalence of cancer, diabetes mellitus, hypertension, inflammation and other chronic diseases. Current therapies of obesity-associated disorders and disease concentrate mainly on calorie intake, but the efficacy of this approach is limited. A better understanding of pathophysiology of obesity is necessary for managing obesity and its complications. Recent studies have provided mechanisms underlying the cause of obesity mainly emphasizing on energy imbalance and neurohormonal dysregulation. These processes are tightly regulated by a process called autophagy. Diabetes mellitus is characterized by insulin resistance and failure of beta cells that produce insulin. Obesity is closely related to metabolic disturbances in adipose tissues. For this reason, it is considered as the primary site for initiation and worsening obesity and Type 2 Diabetes Mellitus. There is an emerging interest in the role of autophagy, a conserved homeostatic process for cellular quality control, which plays a major role in the cellular homeostasis and organ function by selectively clearing cells of potentially toxic proteins, lipids and organelles. This review will provide insight into the current understanding of autophagy, its regulation, and its role in obesity and obesity-associated diabetes.

Introduction

Over the last century, obesity has emerged as one of the leading global health concerns due to lifestyle, environmental, and social changes. Technological advancements and sedentary life aid in building up fats in our body. Obesity has tripled since 1975. Overall, about 13% population of the world was obese in 2016. It has increased among adults from 4-7% in 1980 to 8.5% in 2014. The prevalence of obesity among children and adolescents has also risen significantly from 4% in 1975 to 18% in 2016 (World Health Organization, 2020).

Obesity or overweight is defined as an abnormal or excessive accumulation of fat. So how do we determine whether we are overweight or not? A BMI or Body Mass Index is a measure of obesity, a person's weight in Kilogram is divided by the square of his heights in meters. BMI greater than or equal to 25 is considered as Overweight, whereas, BMI greater or equal to 30 is referred as Obese. Another way to measure is waist size. Obesity is one of the crucial elements, possessing severe threat for human health for chronic diseases such as cardiovascular diseases,

cancer, diabetes, liver and kidney diseases, psychological disorders as well.

Diabetes mellitus (DM) is a serious, chronic disease caused by the absence of insulin secretion due to progressive/ marked inability of β-Langerhans islet cells of the pancreas to produce insulin or by the defect in the uptake of insulin in peripheral tissue (American Diabetes Association, 2020). The global prevalence of diabetes has doubled from 4.7% to 8.5% since 1980 in the adult population. More than 87% of adults with diabetes are overweight or obese (Centers for Disease Control and Prevention, 2020; World Health Organization, 2020).

Autophagy, a type of cell death, plays an important role in the development of obesity that may be related to insulin sensitivity and β-cell function. It might be a cause or effect of obesity that might create a vicious cycle of worsening obesity and associated complications (Zhang et al., 2018). Current therapies mainly aim to suppress caloric intake. The study of autophagy in glucose metabolism is still in its preliminary stage. Therefore, it is still worth further exploration of deeper mechanisms linking autophagy, obesity, and diabetes. Hence targeting autophagy in obesity might create and develop a new cure. Eventually, preventing and treating obesity will help us to prevent diabetes.

What is Diabetes mellitus?

Clinically, there are two types of diabetes- Type 1 and Type 2. Type 1 is also known as insulin-dependent or childhood-onset. It is caused due to damage to the β-cells of the pancreas. This damage leads to an inability in the production of sufficient amounts of insulin required for the body. On the other hand, Type 2 is called non-insulin-dependent or adult-onset. It results from low insulin secretion or peripheral insulin resistance. The incubation period for type 2 diabetes typically lasts more than 10-15 years, hence diagnosed after ages where complications have already risen. Insulin level might be high in those patients (a condition called hyperinsulinemia); although it might not be enough to normalize the glucose level. As a result, the glucose level in the blood is always higher than normal. This condition is called hyperglycaemia (Kharroubi& Darwish, 2015).

Role of Pancreas

As an endocrine organ, the pancreas plays a very essential role, concerning the regulation of blood glucose level. It secrets a variety of hormones such as insulin, glucagon, somatostatin. Especially insulin and glucagon signal cells to normalize the blood glucose level.

Both hormones work in the opposite manner. Insulin is secreted by β Langerhans islet cells whereas glucagon is secreted by α cells of the pancreas. Insulin is secreted when glucose in the blood is high. It signals the uptake of excess glucose into cells like adipose tissues, skeletal muscles, hepatocytes in the liver. Then glucose molecules are used in various manners such as energy production by the process of glycolysis or stored in the form of glycogen by the process of glycogenesis (Al-Goblan et al., 2014). Glucagon is secreted, when the glucose level is low. It is used to elevate glucose by breaking down glycogen stores in the liver, by the process called glycolysis.

It is very important to keep the glucose level within range. For normal adults without diabetes, the blood glucose level before a meal is supposed to lie between 80 to 130mg/dl. While the glucose level after a meal within 1-2 hrs, is to be below 180mg/dl. 8hr post fasting blood glucose more than 126mg/dl and 2-hr post-meal blood glucose more than 200mg/dl is a clear-cut indication of diabetes (Center of Disease Control and Prevention, 2019).

Progression of Obesity to Diabetes:

I. Development of Obesity

The decline of physical activity owing to transportation facilities, changes in diet, sedentary work ultimately brings on energy imbalances. Humans consume nutrients such as carbohydrates, proteins, fats, vitamins required for normal growth, and maintenance of the body. Obesity occurs when the intake of energy exceeds

consumption. It gets stored in the form of fats and glycogen. The major stored form of energy is fats- triglycerides, which accumulate in adipocytes under the skin or around organs. Development of obesity depends not only on the balance between food intake and energy expenditure but also on the amount of white adipose tissue to that of brown. White adipose tissue is an energy reservoir while brown adipose tissue is mostly involved in energy expenditure (Gomez-Hernández et al., 2016).

II. Role of adipocyte tissues

Fat defined as adipose tissue, is a complex organ playing an important role in energy homeostasis. Changes in adipocyte activity and nutrient handling are the core of obesity and many metabolic diseases. They are highly active secretory cells, functioning far beyond metabolism. These secretory factors contribute to immunity, inflammations, vasculogenesis, matrix remodelling (Sun et al., 2011).

Adipose tissue is remarkably flexible in terms of energy storage and its release. Responding to hormonal and energetic cues, it provides energy, by reducing lipid stores and releasing fatty acids during low energy state. In contrast, esterification, lipid uptake, and storage allow the expansion of adipose tissue. It is a useful adaptive response to overnutrition to prevent lipotoxicity and ectopic fat accumulation (Rutkowski et al., 2015). Insulin signalling performs a major role in adipose tissue metabolism. In a fed state, high circulating insulin binds to its receptors on adipocytes, activating signal cascade, thereby transporting GLUT4 transporter from cytosol to the cell membrane of adipocytes. As a result, there is a great influx of glucose into the cell.

The determining factor of metabolic health is the ability of Subcutaneous Adipose Tissue (SAT) which are located under the skin, to store excess fat rather than allowing them to deposit in ectopic depots including the heart, liver, muscle which leads to complication of obesity. The development of obesity includes both hypertrophy (an increase in the size of adipocytes) and hyperplasia (adipogenesis) of fat cells. Once adipocyte reaches maximum size, no more storage of lipid occurs and new cells from mesenchymal stem cells start to differentiate into preadipocytes. If adipocyte exhausts its ability of storage, they become lipolytic causing an elevated level of free fatty acids in plasma.

Recent studies proved that elevated level of free fatty acid causes insulin resistance in the liver and muscle by activation of pro-inflammatory factor NFκB, PKCθ, mitochondrial dysfunction, increased oxidative stress and ER stress, and also decreased PGC1α/β activation (Boden et al., 2005; Baker et al.,2011; Griffin et al.,1999; Ragheb et al., 2009; Burgos-Morón et al., 2019; Goodpaster & Coen, 2014).

Consumption of a diet that is rich in saturated fatty acids has a profound effect on insulin resistance (Kopp, 2019; Merat et al., 1999); whereas diets rich in mono or polyunsaturated fatty acids have less effect or even improve insulin sensitivity (Imamura et al., 2016). Saturated FFA also activates inflammation by indirectly secreting several cytokines such as TNF-α, IL-1β, IL-6, and interacting with toll-like receptors (Volpe & Nogueira-Machado, 2013; Tripathy et al., 2003).

III. Progression to Insulin resistance

Obesity is a critical risk factor for Type 2 diabetes (T2D). By 2025, more than 300 million people are expected to have T2D as a problem of obesity (NCD risk factor collaboration, 2016). The rising number of cases in children and adults with diabetes is related to widespread obesity. The primary cause of T2D is obesity driven insulin resistance in white adipose tissue (WAT), liver, skeletal muscles.

Insulin resistance is a condition in which normal doses of insulin does not produce sufficient normal biological response. In this condition, tissue sensitivity to insulin decreases, and compensatory insulin secretion by β-cell increases (Kusminski et al., 2016; Leitner et al, 2017). This failure is a result of impaired insulin signalling

pathway, causing hyperinsulinemia and hyperglycemia.

Obesity linked with insulin resistance increases the functional demand per beta cell which amplifies the burden and accelerates β-cell dysfunction. Free fatty acids have a specific effect on insulin secretion depending on the type of fatty acid and its exposure. Short term exposure of β cells to saturated fatty acids is good enough for insulin secretion. On the other hand, long-term exposure increases basal level secretion and blunted response towards glucose (Opara et al., 1994; Paolisso et al., 1995; Chen et al.,1994). Butler et al have reported that β cell mass is decreased by 40% and 65% in lean and obese people with T2D respectively (Butler et al., 2003). Chronically exposure to an elevated level of glucose, free fatty acids, cytokines, oxidative stress has a deleterious effect on β cells leading towards pathways of cell death, resulting eventually in the reduction of cell mass (Zhou & Grill, 1994; Lupi et al., 2002; Ebato et al.,2008).

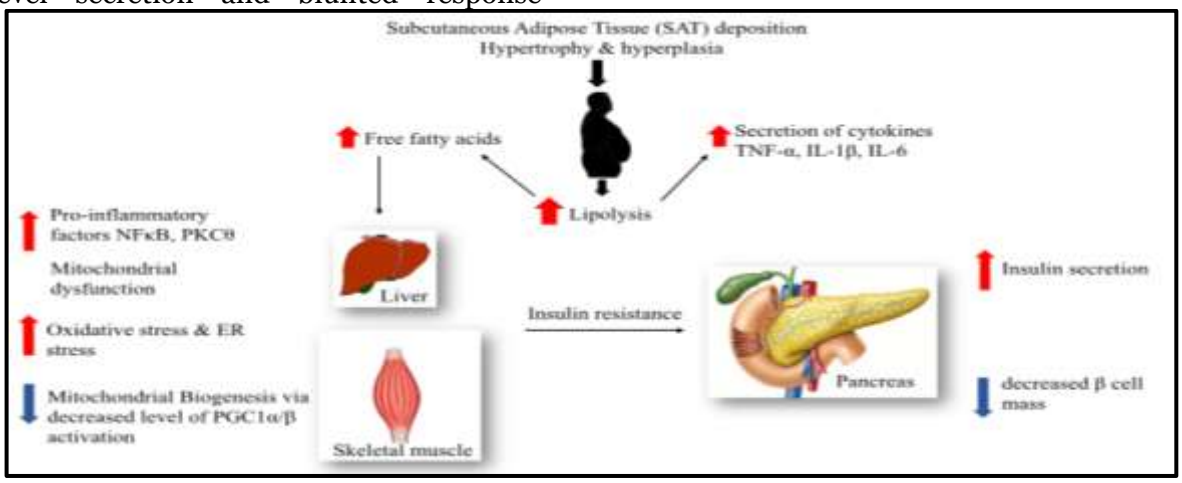

Fig.1: Schematic showing development & progression to insulin resistance

Cell Death: Autophagy

Cell death is a very crucial process involved in a variety of biological processes such as development, homeostasis, immune regulation. Its imbalance is connected with many pathological conditions. It is classified under three domains- apoptosis, autophagy, and necrosis.

Out of three, autophagy has a dual role directing towards - survival or death of cells. Autophagy is a Greek term meaning self-eating. It is a process in which cellular organelles are degraded into the lytic compartment (lysosome) of cells and is reused for cell survival in the case of nutrient deprivation. Its elevated level promotes cell death by removing dysfunctional organelles during cellular stress (Menikdiwela et al., 2020). The pathway starts with the formation and expansion of an isolated membrane that engulfs an organelle or protein. This structure is called the 'autophagosome.' This structure then fuses with lysosomes where components are degraded by hydrolytic enzymes. This process is tightly regulated by autophagy-related proteins called ATG proteins, such as the activating complex UNC-51-like kinase (ULK1)/ATG1, the Beclin /PI3K (VPS34) complex, two transmembrane proteins (ATG9 and VMPL), two ubiquitin-like conjugation systems (ATG12-ATG5 and ATG8/LC3), and other proteins that mediate the fusion between the phagosome and the lysosome (Bhattacharya et al., 2018).

I. Role in insulin secretion

Autophagy and insulin secretion are both concerned with nutrient status. Hence, it is suggested that they might be related. Because Type 2 diabetes is critically dependent on the metabolism of nutrients, insulin action, and its regulation. Dysregulated autophagy may play a role in

the pathogenesis of Type 2 diabetes. Many studies have proven that autophagy is involved in diabetes by controlling hormone action and organelle function (Kasuga, 2006; Karpe et al., 2011).

Autophagy was first observed in glucagon perfused rat liver (Ashford & Porter, 1962). The first evidence of the role of autophagy in β cell functioning was shown in 1984 (Orci et al.,1984). During nutrient surplus, insulin inhibits autophagy by two mechanisms - one by activating mTORC1 resulting in inhibition of ULK1 (Ganley et al., 2009; Mizushima & Komatsu, 2011) and second by inhibiting transcription factor FoxO3 for Atg gene expression (Mammucari et al., 2007). On the other hand, glucagon activates autophagy specifically in rat hepatocytes (Ashford & Porter, 1962; Deter & De Duve; 1967).

II. Function in β-cells of the pancreas

Autophagy is mandatory for the normal architecture and function of β cells. Even in normal conditions, these cells are always subjected to oxidative and ER stress. Counteracting with numerous stressors through different mechanisms, it plays a vital role in maintaining its normal function.

Once insulin is synthesized it is packed into secretory granules called B-granules, ready for secretion by exocytosis. Intracellular insulin level is regulated by the lysosomal degradation of secretory granules, a special form of autophagy named 'Crinophagy' (Orci et al., 1984). It depends on the glucose level. When the level of glucose is low, there is no need for insulin. Hence it has to be degraded. Elevated activity of crinophagy causes fall off intracellular insulin level. However, when glucose level rises, insulin degradation stops and is directed towards its secretion (Keller et al., 1983; Schworer & Mortimore, 1979). During hyperglycaemia, ER cannot maintain its protein folding ability, resulting in ER stress. Normal mitochondrial metabolism is central in glucose-induced insulin secretion. Insulin secretion is dependent on the intracellular ATP/ADP ratio (Maechler & Wollheim, 2001). Hence mitochondrial dysfunction especially in the respiratory chain results in defective insulin secretion. A specialized form of autophagy, 'Mitophagy' aids to clear out defective mitochondria. It helps in keeping a healthy mitochondrial population in β-cells (Twig et al., 2008).

Intending to uncover the role of autophagy in the pancreas, several studies have focused on β-cell-specific knockout of the Atg-7 genes in rat pancreas. These knockout mice clearly exhibit hallmarks of type-2 diabetes such as defective insulin tolerance and its secretion, decreased β-cell mass due to extensive cell death by apoptosis, reduced β-cell proliferation (Jung et al., 2008; Jung & Lee, 2010; Ebato et al., 2008). Also, a human β-cell study on diabetes showed more dead β-cells than in the control, massive vacuole overload suggesting altered autophagy, dense volume of vacuole & autophagosomes. Exposure of non-diabetic islets to free fatty acids leads to a marked increase in vacuole accumulation with enhanced β-cell death (Masini et al., 2009). Skeletal muscle accounts for 80% of insulin for glucose utilization in humans (Baron et al., 1998). Autophagy in myocytes plays a role in glucose utilization and the development of insulin resistance. Physical exercise induces autophagy in mouse muscle cells by activating the AMPK pathway (Dagon et al., 2015). Defective autophagy is causal in hepatic insulin sensitivity and glucose metabolism in obesity. In the liver of obese mice, there is an overload of lipid which is eventually causing suppression of autophagy by downregulating Atg-7 (Yang et al., 2010).

III. Role in adipocytes

Apart from providing energy in nutrient deprivation, it is involved in cell modelling and differentiation. Adipocyte differentiation is highly dependent on autophagy (Choi et al., 2016; Zhang et al., 2009). Studies showed that Atg-7 knockout mice exhibited drastically reduced adipogenesis despite diet and severe morphological abnormalities such as decreased cell size, increased cytoplasmic volume with higher mitochondrial population, and a high percentage of multilocular lipid droplets

(Zhang et al., 2009). After a high-fat diet, mice adipocytes underwent metabolic changes before inflammation, by loss of mitochondrial biogenesis due to the downregulation of proteins involved in the biogenesis process (Cummins et al., 2014).

Brown adipocyte activity is reduced in obese and diabetic patients (Lee et al., 2010). Mitophagy plays a very essential role in the browning of white adipose tissue. Inhibiting mitophagy and activating the mitochondrial biogenesis can increase the mitochondrial population resulting in the browning of white adipose tissue thus preventing human health from nutrition. But mitophagy seems much more controversial as the basal level is very important to clear out dysfunctional mitochondria from accumulating ROS; however hyperactive mitophagy can convert brown adipocyte or beige fat (brown in white adipocytes) to white by a process called 'whitening' or 'reverse browning' (Cummins et al., 2014; Altshuler-Keylin et al., 2016; Gospodarska et al., 2015; Liu et al., 2016; Ferhat et al., 2019).

There is convincing evidence from animal models that improving the function of brown adipocytes or browning of white adipocytes could be an effective way to treat obesity and its associated conditions such as type 2 diabetes (Scheele & Nielsen, 2017).

Conclusion

Autophagy is essential for balancing sources of energy at the time of development as well as during nutrient stress. Housekeeping functions performed by autophagy includes degradation of defective proteins and organelles, removal of intracellular pathogens. Such functions are fundamental for autophagy mediated protection against aging, cancer, neurodegenerative diseases and infections.

Given the clear role of autophagy in developing and regulating obesity, diabetes, and its complications, the question arises whether the modulation of autophagy might offer a therapeutic approach. However, alterations in autophagy seen in diabetes and obesity are complex. They are known to vary from cell to cell and are not yet fully understood. Targeting altered autophagy will significantly benefit for the treatment of type-2 diabetes or even the progression of obesity to type-2 diabetes. However, the development of therapies needs much more understanding of the role of autophagy in obesity and diabetes.

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Review article

Epigenetics and Autoimmunity

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Abstract

Epigenetic modifications bring about changes in the functioning of genes without modifying the DNA sequence. Very broadly, they act as an additional layer of information and bridge the gap between the genotype and phenotype. Several epigenetic modifications have been identified and elucidated, this includes DNA methylation, histone modifications and the role of non-coding mRNAs as well. For a long time, it was seen that some monozygotic twins are discordant for certain diseases such as cancer and many autoimmune diseases. This indicates that epigenetics may be playing a role in the same. This review looks at the epigenetic modifications involved in situations where there is a breakdown of immunological tolerance- autoimmunity. Some epigenetic mechanisms underlying certain autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis, have been examined. The plausible effect of certain environmental stimuli and lifestyle factors on epigenetic mechanisms leading to autoimmunity have also been considered. A hallmark feature of epigenetic modification is its reversibility. This implies that a greater understanding of how epigenetic processes shape immunological tolerance (or the lack thereof) could possibly open new avenues for better diagnostic techniques and therapeutics to prevent the progression of autoimmune diseases.

Introduction

The term 'epigenetics' was introduced by Waddington in 1942, when he used the word to refer to changes in the phenotype without any changes in the genotype. Epigenetics can be broadly considered as an added layer of information which occurs alongside the genetic information, that is, an alteration of gene activity without a change in the DNA sequence. A good example of this is the cells in our body. All the cells of a particular individual have the same genotype, but different cell types perform distinct functions and also look different. This is due to differential gene expression, which is partly because of various epigenetic marks applied to the DNA. These epigenetic marks can either be mitotically heritable or could be de novo (or laid down newly). Many epigenetic marks are also reversible. Currently, epigenetics is defined as the "study of mitotically heritable changes in gene expression that occur without changes in the DNA sequence".

Over the years, many epigenetic modifications have been identified including DNA methylation, histone modifications (acetylation, methylation, phosphorylation, citrullination, sumoylation, etc.), and non-coding RNAs.

Some of these epigenetic modifications are described briefly below-

DNA Methylation

DNA methylation is the most widely studied epigenetic modification. DNA methylation can either be de novo or it can be carried forward during DNA replication. It occurs almost exclusively at CpG dinucleotides in mammals (often found in promoter regions) and this results in a change in the activity of a particular gene. DNA methylation is laid down by methyltransferases or DNMTs. De novo methylation is done usually by DNMT3A and DNMT3B. During the process of replication, DNMT1 is an important enzyme which recognizes hemi-methylated DNA and subsequently, methylates the daughter strand to maintain the pattern of DNA methylation (Goldberg et al., 2007). Generally, it is the intergenic and repetitive elements which are usually methylated. Initially it was thought that methylated CpG regions directly blocked transcription sites, thus causing transcriptional repression. However, now it is known that the methylated DNA is bound by certain proteins like MeCP1 and MeCP2. These proteins have methylated-DNA binding domains and transcriptional repression domains which can further recruit the nucleosome remodeling and histone deacetylase protein- NuRD, which eventually causes chromatin condensation and gene silencing (Feng & Zhang, 2001; Greer & Mccombe, 2012).

Histone Modifications

Histone modification refers to the chemical modifications of histones (highly conserved proteins). Modifications usually occur on the N-terminal tails which jut out from the nucleosome (Greer & Mccombe, 2012). The most widely studied histone modifications are histone acetylation and methylation. Histone acetylation is correlated with gene activity. This process is carried out by two enzymes- histone acetyltransferases and histone deacetyltransferases. Histone acetyltransferases work by catalyzing the transfer of an acetyl group from acetyl-CoA to the lysine residues on histones. Histone acetylation is thought to control gene expression in two ways. One explanation is that since lysines on the histones have positively charged side chains, acetylation neutralizes this positive change causing reduced attraction of the histones with the negatively charged DNA. This may change the structure of the nucleosomes. Acetylated histone sites may also act as docking sites for other proteins (such as the bromodomain proteins) which open up the chromatin (Verdone et al., 2005). On the other hand, histone deacetylases (HDACs) reduce the expression of a particular gene. As their name suggests, they do this by removing acetyl groups from the histones, which would close the chromatin and thus, repress gene expression (Yan et al., 2015). Unlike histone acetylation, histone methylation can correlate with gene activity or inactivity. Histone methyltransferases use S-adenosyl methionine as a donor to add the methyl group to the basic histones- lysine, arginine, and histidine. Methylated histone tails are recognized by proteins with methyl-binding domains such as the plant homeodomain or PHD fingers, ankyrin repeats, and so on (Greer & Shi, 2012).

Non-coding RNAs

Non-coding RNAs (ncRNAs) interact with the mRNA or DNA post-transcriptionally to mediate certain processes. Two important types of ncRNAs are long non-coding RNAs (lncRNAs) and microRNAs (miRNAs). It is classified as long and short, and miRNA is one of the short ncRNAs.

There is a lot of excitement surrounding miRNA research since miRNA dysregulation can lead to several disorders and diseases ranging from autoimmune diseases to cardiovascular diseases and they could also act as biomarkers for certain diseases. Hence, miRNAs would be the ncRNA in focus for this review. miRNAs block the translation of mRNA into protein by binding to the 3' UTR, reducing the stability of the mRNA, or simply by acting as a transcriptional

inhibitor on the target gene (Abdellatif, 2012; Dwivedi et al., 2019).

It is very evident from the above descriptions that epigenetic modifications would have quite a prominent role to play in shaping the health and disease of an individual. Epigenetic modifications have been implicated in a range of diseases and disorders- cancer, schizophrenia, Rett syndrome, and of course, autoimmune diseases. An autoimmune disease arises when the immune system loses its ability to distinguish between self and non-self and starts attacking cells or tissues or organs which belong to one's own body. They can be organ-specific where only one organ or tissue is affected or systemic wherein multiple organs or tissues are attacked.

An autoimmune disease occurs when there is a breakdown of immunological tolerance, not allowing the immune system to recognize between self and non-self. The process of gaining immunological tolerance involves several checkpoints which adaptive immune cells have to undergo as they mature and a failure in any of these checkpoints could lead to the disastrous consequence of autoimmunity, highlighted in diseases such as multiple sclerosis (Schwartz, 2012).

Monozygotic (MZ) twins show variable degrees of concordance with regard to diseases such as autoimmune diseases. Twin studies have been helpful in the study of genes that make one susceptible to autoimmune diseases. However, when the levels of discordance of the incidence of autoimmune diseases between MZ twins were studied they revealed factors other than purely genetic factors. Therefore, since monozygotic twins are genetically similar, such studies may provide valuable information regarding the epigenetic factors involved in the pathogenesis and progress of autoimmune diseases and complications associated with them (Javierre et al., 2010).

Studies of epigenetic modifications in autoimmune disease can provide significant clues regarding the development and progression of autoimmune diseases and this review is an attempt to understand the same.

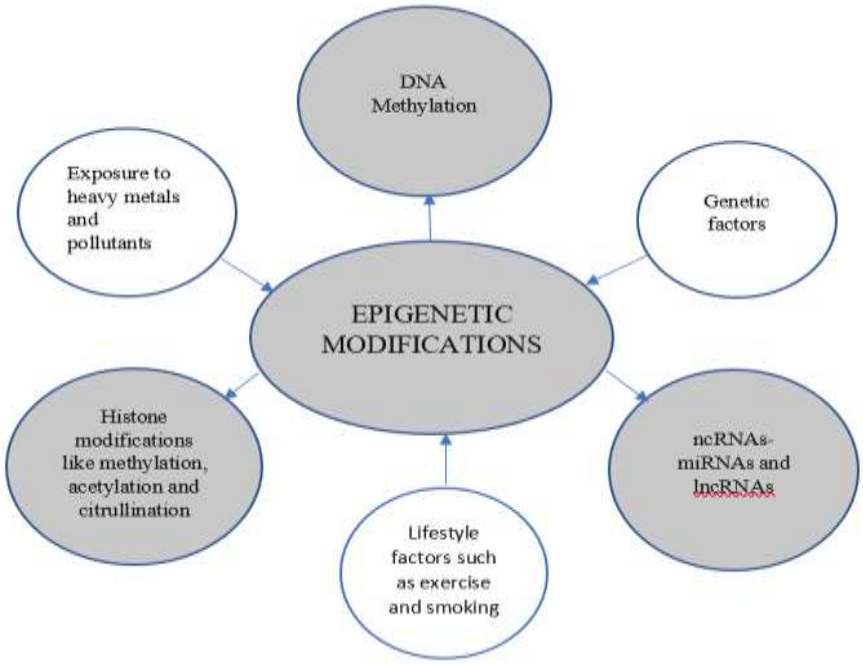

Fig. 1: An overview of epigenetic modifications & their causes

Epigenetic T cell regulation and autoimmunity.

Dysregulation of certain T cell reactions because of deviant epigenetic regulations may cause certain autoimmune disorders or may accelerate their progression. The role of certain proteins like Aire and FOXP3 as well as that of miRNAs has been well established in epigenetic regulation of T cells in autoimmunity. The two major types of T cells are cytotoxic and helper T cells. Cytotoxic T cells usually express the CD8 membrane glycoprotein on its surface and kill cells which are damaged or infected whereas helper T cells usually express the CD4 membrane glycoprotein and 'help' in the activation of B cells. Regulatory T cells (Tregs) are a type of CD4+ T cells which play a role in modulating immune tolerance preventing autoimmune disease.

T cell tolerance to auto-antigens occurs in thymus by the Aire protein

This is controlled by the Aire (autoimmune regulator) protein. The Aire protein binds to H3 methylated histone via its PHD fingers. For establishing central tolerance, it is of utmost importance that the Aire protein binds to the hypomethylated H3K4 in the PHD. The D299 residue is important for the interaction of the Aire protein with chromatin and this residue is blocked by the heavy methylation of H3K4. In a study that was carried out in transgenic mice expressing the Aire D299A mutation in thymic epithelial cells, it was seen that the mutation in the PHD finger of the Aire protein caused a reduced binding to the methylated histone H3, eventually causing autoimmunity to develop because of a weakening in the transcription of the Aire (Koh et al., 2010).

The FOXP3 transcription factor

Forkhead box P3 or FOXP3 is a transcription factor which is involved in immune responses and it is expressed in Treg cells. These cells apart from their numerous other functions, also regulate autoimmunity. FOXP3 associates with a wide range of proteins to control gene expression including histone acetyltransferases or histone deacetylases. They epigenetically control transcriptional activity. This may be via FOXP3 itself (directly) or with FOXP3 associating with proteins to form complexes (indirectly) like those with histone acetyltransferases and histone deacetylase. In mice, it was seen that when FOXP3 failed to associate with these proteins, the regulatory T cells were adversely impacted. It is seen that a loss in the control of epigenetic modifications controlled by FOXP3 may cause a quicker development of autoimmunity (Bettini et al., 2012).

miRNA

miRNA levels may play an important role during the development of T cell tolerance too. In normal cells of the immune system, it is seen that the levels of miRNAs are regulated very strictly. However, variations in the same have been seen in cases of breakdowns in immune tolerance. Inhibition of two important proteins (PTEN and Bim) by miRNA has been implicated in the pathophysiology of autoimmunity. The PTEN (phosphatase and tensin homolog) protein is an inhibitor of a cell cycle regulating pathway called the P13K pathway. A mutation in the PTEN gene can increase the risk of autoimmunity (as well as cancer) in an individual. Similar to PTEN, the pro-apoptotic protein called the Bim protein also plays an important role in autoimmunity and cancer. A cluster of miRNAs- the miR-17-92 cluster which produces mature miRNAs (via transcription) targets the mRNAs of the PTEN and Bim proteins. It was seen in mice that when the miRNAs transcribed from this cluster were overexpressed, there was a downregulation in the expression of these two proteins leading to the development of autoimmunity in the mice (Xiao et al., 2008).

Epigenetic studies of autoimmune diseases

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease. In a study, it was found that there was a global hypomethylation in the T cell DNA of RA patients as compared to healthy controls. However, since the patients were taking

medicines for their condition, a clear connection could not be established between a specific feature of the disease and T cell hypomethylation (Richardson et al., 1990).

The inflammation seen in RA patients is caused by a cytokine called TNF-α. When miRNA expression profile analysis was carried out on RA patients, it was found that the expression of miR-146a was high in peripheral blood T cells and synovial fluid T cells with high levels of TNF-α as compared to that of healthy controls (Li et al., 2010). This means that an overexpression of miRNA-146a may have a key role in the joint inflammation seen in RA patients and this could potentially be a new avenue for therapy.

A very high-risk factor for RA is the HLA-DR1 & HLA-DR4 genes. Smoking when a risk gene for RA is present causes alterations in the DNA methylation profile which implies that epigenetic changes are bridging the environmental conditions with the genetic component which may trigger RA (Meng et al., 2017).

When the synovial fibroblasts obtained from the synovial tissues from patients with osteoarthritis and RA were compared, it was found that the acetylation of histone H3 was significantly higher in RA synovial fibroblasts, thus causing an increase in the level of the interleukin (IL)-6 mRNA (Wada et al., 2014). It is known that IL-6 is known to be pro-inflammatory and plays a heavy role in the pathogenesis of RA. IL-6 inhibitors such as Tocilizumab have already been used to treat RA (Tanaka et al., 2010). Targeting aberrations in histone acetylation patterns (such as that which causes an increase in IL-6) can possibly help treat RA (Wada et al., 2014).

Certain systemic lupus erythematosus (SLE) susceptibility genes like STAT4 or MECP2 are associated with DNA methylation. When SLE discordant twins were screened for their DNA methylation profiles certain differences were revealed. It is interesting to note that the methylation in the SLE twin did not show a significant methylation change when the DNA methylation profile was compared to that of unrelated controls who had SLE. This may mean that indeed it may be differences in the DNA methylation that cause different phenotypes in SLE discordant twins (Javierre et al., 2010). Abnormal histone modification patterns have also been found in the CD4+ T cells of patients with SLE. It was found that there was global hypomethylation of histone H3K9 in the CD4+ T cells of patients with both active as well as inactive SLE as compared to controls. The same study also found that there was increased H3/H4 acetylation in patients with active and inactive SLE and a lower extent of H3 acetylation was correlated with higher disease activity (Hu et al., 2008).

In systemic lupus erythematosus (SLE) patients it was seen that the levels of certain miRNAs were noticeably dysregulated- nine of them were upregulated and seven downregulated as compared to healthy controls (Pauley et al., 2009).

A study by Cheng and colleagues in 2017 attempted to determine the role of miR-451a in SLE. The levels of miR-451a were higher in SLE mice as compared to wildtype (WT) mice. Interestingly, in miR-451a knockout SLE mice, it was seen that there was a suppression in the levels of autoantibodies when compared to the SLE mice. The level of the interferon regulatory factor (IRF)-8, which is thought to play a role in SLE, is low in SLE mice compared to the WT mice. However, when the levels of miR-451a had decreased, there was an improvement in the IRF-8 levels. In conclusion, the study showed that miR-451a “negatively modulated” the expression of IRF-8. These findings can potentially help establish a new therapeutic target for SLE (Cheng et al., 2017).

Multiple Sclerosis (MS) is a neurodegenerative autoimmune disease. Studies have revealed that the probability of monozygotic twins having MS is only 26% (Van Den Elsen et al., 2014) indicating that there may be a significant epigenetic component(s) playing a role in the pathogenesis of this disease. Peptidyl

arginine deiminase type-2 (PAD2) is an enzyme widely found in the brain. The PAD2 enzyme causes the citrullination of myelin basic protein (MBP). Research has shown that the promoter region of the CpG islands of PAD2 is hypomethylated effectively meaning that the transcription factors will have access to the promoter region and allow transcription which in turn, will lead to an increase in PAD2 expression. This causes an increase in the citrullination of MBP, something which is observed in the normal-appearing white matter of individuals suffering from MS (Miyazaki & Niino, 2015). A small study indicated that seven plasma miRNAs may also play a critical role in MS. One particular miRNA- miR-648 is notably overexpressed compared to the controls. miR-648 regulates the expression of the MOB protein and the NR2C2 steroid nuclear receptor which play important roles in the pathogenesis of MS and the production of inflammatory cytokines in MS-affected individuals, respectively. However, it is to be noted that this study was conducted with a small number of participants from the same ethnic group, therefore further research is necessary to confirm these findings (Siege et al., 2012).

Autoimmune thyroid diseases (AITD) which include Graves' disease (GD) and Hashimoto thyroiditis are regulated by B and T cells. It has long been thought that epigenetic factors play key roles in the pathogenesis of those diseases including the loss of immune tolerance causing antibodies to attack the thyroid tissues. Studies of patients with AITD have revealed global DNA hypomethylation which may cause an increased expression of certain genes in immune cells resulting in the production of autoantibodies. In GD patients, it was also observed that there was a lower level of acetylation of the histone H4 in peripheral blood mononuclear cells along with several other aberrant histone modifications whose effects have not been fully elucidated (Wang et al., 2017). In the PBMCs of GD patients, it was found that there was a global reduction in the acetylation of histone H4 along with an increase in the mRNA levels of histone deacetylase (HDAC)1 and HDAC2 (Yan et al., 2015).

Epigenetic B cell regulation & autoimmunity

There is a lot of evidence pointing to the fact that B cells are epigenetically regulated. For example, it is known that an important gene involved in memory B cell formation in humans, the CD27 gene, is regulated by histone modifications- the levels of histone methylation are higher in active memory B cells. Dysregulation in the epigenetic processes in B cells may lead to or aid in the progression of certain autoimmune diseases. In the B cells of SLE patients, lower methylation levels of the LINE-1 gene have been reported as well as decreased levels of miR-30a. Higher levels of miR-30a are associated with lower levels of Lyn, which is a negative regulator to maintain tolerance to self-antigens. Similarly, epigenetic dysregulations are also seen in patients with RA. B cells from the synovium have increased levels of miR-155 which have an impact on B cell regulation (Wu et al., 2018). However, there is a significant dearth in literature regarding epigenetic process in B cells regulating autoimmunity and further research is required in this field to establish conclusive findings.

Environmental stimuli and lifestyle may trigger autoimmunity

It is a well-known fact that (autoimmune) disease may be caused in genetically susceptible individuals by environmental triggers via epigenetic modifications.

Autoimmunity has also been reported in individuals who have been exposed to heavy metals like arsenic, cadmium, nickel, mercury, and lead. This is due to differences in the levels of epigenetic factors like miRNAs, DNA methylation, and histone modifications (Greer & Mccombe, 2012).

Arsenic exposure, for example, has been linked with DNA hypomethylation or hypermethylation. Inorganic arsenic is detoxified in the body by methylation. S-adenosyl methionine (SAM) is a methyl donor for DNA methyltransferases (DNMTs) that plays an important role in DNA methylation and is used for the detoxification process when inorganic

arsenic enters the human body. Prolonged exposure to arsenic causes a decrease in the SAM and this in turn decreases the DNMT activities eventually causing global DNA hypomethylation. In stark contrast, it was seen that prolonged exposure to cadmium caused global DNA hypermethylation and increased DNMT activities. Such effects of heavy metals may cause a breakdown in normal immune function and contribute to the development of autoimmunity (Greer &Mccombe, 2012; Hou et al., 2012).

Exercise may also play a role in autoimmunity. In a study to investigate the epigenetic modifications that take place during exercise, (McGee et al., 2009) made subjects exercise for 60 minutes. Immediately afterward, when muscle samples were analyzed, it was seen that there was a 64% increase H3K36 acetylation (H3K36 acetylation may control transcriptional elongation). Another study has revealed that exercise results in epigenetic modifications like DNA hypomethylation in certain genes of the skeletal muscles (Barrès et al., 2012), and thus, such modifications may have an effect on various organs and systems in the body including the immune system impacting autoimmunity. However, most of these studies are small and further research is vital to understanding the further implications of exercise on autoimmunity.

It is very clear that integrating genetic data and epigenetic factors could unravel the causes as well as the potential treatments for a range of autoimmune diseases. The key to doing so would be further investigating aspects that are not fully understood regarding epigenetic processes impacting autoimmunity. The chief advantage of focusing on epigenetic marks for the treatment of autoimmune diseases is due to the fact that many epigenetic marks are reversible. Reversing aberrant modifications characterizing certain autoimmune diseases may be valuable in delaying its onset or treating it.

It is evident that monozygotic twin studies can be utilized to study epigenetic differences such as differences in the DNA methylation and miRNA profile (and the factors causing them) significant to the onset and progression of autoimmunity in the absence of genetic differences. Dysregulation in the miRNA levels such as that of miR-146a in RA, miR-451a in SLE, and miR-648 in MS could act as biomarkers, notably because they are present in body fluids such as the synovial fluid and plasma. This means that they could efficiently be used in the screening, diagnosis, and prognosis of autoimmune disorders. Other epigenetic marks like histone acetylation marks or DNA methylation marks can be used as therapeutic targets to alleviate symptoms associated with autoimmune diseases or possibly even cure it. Since proteins like the Aire protein and FOXP3 transcription factor have been demonstrated to play significant roles in the development of autoimmunity, further understanding regarding the roles they play in epigenetic processes is needed.

Most of the studies pertaining to epigenetic processes in autoimmune diseases have been made with regard to DNA methylation and histone acetylation. Although these epigenetic marks are undoubtedly important, it is necessary to study others too, and their roles in autoimmunity to obtain a full picture. It is also vital that human studies be conducted with a larger number of subjects belonging to different age groups and ethnicities to get a more precise understanding of how epigenetic modifications may vary with these factors. The determination of the proportion of individuals who acquire aberrant epigenetic marks due to a certain lifestyle or because they live in a particular location has to be studied for maximum efficacy in creating therapeutics, especially if those individuals are genetically predisposed to autoimmune diseases.

Overall, significant breakthroughs have been made, however, future research is critical to tap the emerging potential for diagnosis and remediation of autoimmune diseases.

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Review article

Emergence of The New Superbug - *Candida auris:* A mini review

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Abstract

Candida auris is a multidrug-resistant pathogen that is mainly found in healthcare facilities. The first case of infection caused by *C. auris* was reported in Japan in 2009. After that, it was isolated in many countries like South Korea, India, Pakistan, Kuwait, Israel, Oman, United States, Canada, Norway, Germany, Venezuela, South Africa, Columbia, Europe, and Spain. Pathogenesis due to Candida is due to germination, adherence, biofilm formation, or production of proteinase and phospholipase. The clumps formed by *C. auris* are very difficult to break in vitro. *C. auris* shows drug resistance due to mutation in the gene responsible for a drug target, overexpression of efflux pumps, etc. *C. auris* can be isolated on Chromagar on which it shows pink to beige color colonies. Salt Sabouraud Dulcitol broth is used for the enrichment of *C. auris*. Antifungal susceptibility test shows that a combination of micafungin and voriconazole can be used to treat infection caused by this fungus. *C. auris* was also found to be susceptible to 5-flucytosine. It was observed that amphotericin B and chlorhexidine can be used against sessile cells and planktonic cells. To avoid transmission of *C. auris* iodine-based cleaners as well as chlorhexidine can be used as they show a killing effect on *C. auris*. Hydrogen peroxide and chlorine-based disinfectants can also be used.

Introduction

The first case of *Candida auris* was reported in Japan in 2009. It was isolated from the ear canal of a 70-year-old Japanese woman (Satoh et al, 2009). However, the earliest strain of *C. auris* dates back to 1996 and was reported from South Korea (Centers for Disease Control and Prevention, 2019). It was subsequently isolated from many patients in multiple countries. Cases of *C. auris* have now been reported globally from South Korea, India, Pakistan, Kuwait, Israel, Oman, United States, Canada, Norway, Germany, Venezuela, South Africa, Columbia, Europe, and Spain (Jeffery-Smith et al., 2017).

In the last 10 years, many cases of *C. auris* have been reported in more than 35 countries in five continents including Asia, Africa, Europe, South America, and North America. Due to colonization, survival, and

persistence of *C. auris* on surfaces, hospitals contribute largely to the outbreak (Zhu et al., 2020).

A study has been conducted with isolates from 1400 candidemia cases over 27 ICU across India. It was observed that around 5.3 % i.e. 74 patients in 19 out of 27 ICU acquired candidemia due to *C. auris* (Rudramurthy et al., 2017).

It is important to study this organism because it is a global threat due to its multidrug resistance. Around 30 to 60 percent of people die due to the *C. auris* infection (Centers for Disease Control and Prevention, 2019). This pathogen is mainly found in health care facilities. *C. auris* is resistant due to mutation in the gene responsible for a drug target, overexpression of efflux pumps, etc. It shows reduced susceptibility towards many antifungals, hence combinations of these agents have to be used. MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Technique of Flight Mass Spectroscopy) is used for identification of *Candida spps,* molecular techniques like PCR assay which are specific for *C. auris* are also used.

Detection and Identification

Salt Sabouraud Dulcitol broth procedure is used for isolation of *C. auris*. Dulcitol is used as the main carbon source to decrease the growth of *C. glabrata* and *C. parapsilosis*. The swab is taken from the infected site and immersed in liquid amines, vortexed, inoculated in Salt Sabouraud Dulcitol broth and incubated at 40°C at 250 rpm for 5 days. The suspension is then streaked on Chromagar (Kordalewska & Perlin 2019).

When isolated on chromogenic agar *C. auris* forms pink to beige colonies. The optimum temperature is 42°C but it can also grow at a higher temperature. When supplemented with 0.01% cycloheximide no growth is observed. Oval or elongated yeast cells are observed on staining. Unlike fungi, no hyphal or pseudohyphal forms are present (Jeffery-Smith et al., 2017). Pathogenesis due to *Candida* is due to germination, adherence, biofilm formation, or production of proteinase and phospholipase. The clumps formed by *C. auris* are very difficult to break in vitro (Spivak & Hanson 2018). The pathogen can form biofilm on various equipment in hospitals like intensive care settings, catheters, etc. *C. auris* can be misidentified as some other *Candida* species. Hence phenotypic and biochemical methods like API 20C, Phoenix (BD), MALDI-TOF MS (Matrix-Assisted Laser Desorption Ionization Technique Of Flight Mass Spectroscopy), PCR assay which is specific for *C. auris* are used. In Venezuela and India AFLP (Amplified Fragment Length Polymorphism) has been used to demonstrate *C. auris* (Jeffery-Smith et al., 2017).

Treatment

C. auris shows reduced susceptibility to azoles, polyenes, and echinocandins. One of the isolates from India was observed to be resistant to fluconazole. Reduced susceptibility to triazole antifungal drugs, voriconazole, posaconazole, itraconazole, and isavuconazole have also been reported. Amphotericin B shows variable activity against *C. auris*. Due to the resistance to these drugs, echinocandins are used as first-line therapy (Kordalewska & Perlin 2019).

Combination of micafungin and voriconazole gave a more promising result than the combination of azole and echinocandins. *C. auris was* found to be susceptible to 5-flucytosine. It was also observed that amphotericin B and chlorhexidine can be used against sessile cells and planktonic cells (Jeffery-Smith et al., 2017).

Infection, prevention and control

C.auris is transmitted usually in hospitals, intensive care units, from patients to the environment or workers from surfaces like medical equipment, sinks, etc. To avoid transmission iodine-based cleaners as well as chlorhexidine can be used as they have a killing effect on *C. auris*. Hydrogen peroxide and chlorine-based disinfectants can also be used (Spivak & Hanson 2018).

Conclusion

Many cases are still being reported globally due to diseases caused by *C. auris*. It has become drug-resistant due to mutations in its molecular mechanisms. As mentioned by CDC, *C. auris* is a serious global health threat because it is multidrug-resistant, difficult to identify and prone to misidentification as it is similar to other *Candida* species and it has caused an outbreak in many health care facilities (Centers for Disease Control and Prevention, 2019). Hence to overcome this, diagnostic methods need to be improved, more combinations of antifungal therapies need to be tried and PCR assays for accurate identification of *C. auris* need to be used.

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Review article

Bacteriocins - A safer alternative to harsh food preservation techniques: A mini review

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Abstract

Food provides nutritional support, an important aspect required for the growth of an organism. Hence, spoilage of food is a common occurrence. To avoid this, it is important to develop methods that increase the shelf life of the food product without causing any changes in the nutritional value or chemical nature of food. Current methods of food preservation either affect the quality of food, have adverse effects on human health or are expensive. Thus, the need for safer alternatives is increasing day by day. Bacteriocin are antimicrobial peptides produced by an organism to inhibit the growth of organisms that may or may not be closely related. It does not affect the food quality or human health and therefore, a safer alternative. Hence, it is important to explore the different bacteriocins and their effectiveness in food preservation.

Food preservation is an important aspect of the food industry. Intense preservation techniques can lead to damage to the food quality. Therefore, safer methods of food preservation is the focus of research in this area.

Food preservation is a method of maintaining foods such that their properties or nature are kept unchanged so as to provide maximum benefit to the consumer. In general, every step of handling, processing, storage, and distribution affects the characteristics of food, and this can be desirable or undesirable (Rahman, 2007). Many types of microbiological spoilage are preventable to a great degree by a wide variety of preservation techniques. Most act by preventing or inhibiting microbial growth. Other techniques act by inactivating microorganisms (e.g., pasteurisation, sterilization, etc.). Additional techniques limit the access of microorganisms to merchandise (e.g., sterile processing and packaging). New and rising preservation techniques that are returning into use or are under development include those that act by inactivation (e.g., ultrahigh-pressure, electroporation, etc.).

An additional trend is towards the utilization of procedures that deliver merchandise that is less heavily preserved, has better quality, and is more natural, freer from additives, and nutritionally superior. Less severe preservation procedures are

being developed that use preservative factors in combination with others, to deliver products with less damage and better quality (hurdle technologies); (a) new strategies of heating that are better controlled and thus deliver milder heat to products; (b) cook-chill combos that deliver longer high-quality shelf lives; (c) changed atmosphere packaging to retain quality longer; and (d) use of antimicrobial systems that are natural. Several of the prevailing and upcoming preservation techniques act by harnessing the homeostatic mechanisms that organisms have evolved in order to survive extreme environmental stresses (Gould, 1996).

Bacteriocins are ribosomally-synthesized peptides with antimicrobial activity, made by completely different groups of bacteria. Many LAB bacteriocins offer potential applications in food preservation, and their use within the food business will facilitate cutback of the addition of chemical preservatives and also the intensity of heat treatments, leading to foods that are more naturally preserved and richer in organoleptic and nutritional properties. This will be a viable alternative to satisfy the increasing demands for safe, fresh-tasting, ready-to-eat, minimally-processed foods and additionally to develop "novel" food merchandise (e.g., less acidic, or lower salt content). Broad-spectrum bacteriocins may be used more specifically to selectively inhibit certain high-risk bacteria in foods like *Listeria monocytogenes* while leaving harmless microbiota untouched. Bacteriocins may be added to foods as food preservatives, additives, or maybe made in situ by bacteriocinogenic starters, adjuncts, or protecting cultures. Immobilized bacteriocins also can find applications in the development of bioactive food packaging. In recent years, the application of bacteriocins as a part of hurdle technology has gained great attention. Many bacteriocins show synergistic effects together with chemical preservatives, natural phenolic compounds, and also with other antimicrobial proteins. The effectiveness of bacteriocins is commonly determined by environmental factors like pH, temperature, food composition, and structure, as well as food microbiota (Gálvez et al., 2007). Bacteriocins are proteinaceous agents that are quickly digestible by proteases within the human gastrointestinal tract resulting in their safe use as natural preservatives in foods. Their production may well be thought-about as a bonus for food and feed producers since, in adequate amounts, these peptides will kill or inhibit pathogenic bacteria that compete for identical status (Saavedra et al., 2004). Most analysis has centered on antimicrobial agents made by lactic acid bacteria and several bacteriocins of different Gram-positive bacteria have been isolated and documented. One area of interest is to manage the expansion of undesirable microorganisms, e.g. *C. botulinum* and *L. monocytogenes* (Deegan et al., 2006). The ability to produce antimicrobial peptides specifically bacteriocins is wide-spread among a range of Gram-positive bacteria such as *Staphylococcus*, *Clostridium*, and *Bacillus* spp. These substances are directed against competitive microorganisms and thereby generate a selective advantage for their producers (Tagg, 1992). Though the utilization of those bacteriocins is precluded from foods because the producer strain is a pathogen, recent developments in biotechnology have made the transfer of genes encoding for bacteriocin production from such bacteria to food-grade microorganisms, if possible (Heng et al., 2007).

Bacillus species are ubiquitous in distribution within the atmosphere and are often found as commensals, transient organisms within the duct systems of mammals, insects, invertebrates, etc., in addition to being present in soil, clays, rocks, food, etc. (Abriouel et al., 2011). Members of the genus *Bacillus* are rod-shaped, Gram-positive, aerobic, endospore-forming bacteria characterised by the production of the enzyme catalase. Bacillus species are phenotypically and genotypically

heterogeneous (McCormick et al., 1999; Nicholson, 2002) and consequently, they exhibit numerous physiological properties such as the ability to degrade many different substrates derived from plant and animal sources (Priest, 1993). Recent studies have disclosed that Bacillus will suppress infection against *E. coli 070: K80*, *Salmonella enterica*, *Clostridium perfringens*, and *Citrobacter rodentium* (Slepecky & Hemphill, 2006). The expansion of two viable food pathogens viz. *L. monocytogenes* and *S. aureus* may be suppressed by a newly isolated bacteriocin from *Bacillus mycoides* (Lutz et al., 2006). Similarly, paenibacillin (bacteriocin from Paenibacillus sp.), was found to be active against several bacteria including Bacillus spp., *Clostridium sporogenes*, *Lactobacillus* spp., *Listeria* spp., and *S. aureus* (Permpoonpattana et al., 2012). This proves that bacteriocins secreted from different bacteria behave differently and have their specific inhibition spectra (Sharma et al., 2008). Several bacteriocins made by Bacilli inhibit Gram-positive however not Gram-negative bacteria (He et al., 2007) The presence of Bacillus species in food does not imply spoilage or food poisoning, and some species or strains are even employed in human and animal foods, for example, Bacillus strains that are employed in Natto, an East Asian fermented food production (Sharma et al., 2011). Furthermore, specific *B. subtilis* strains are used as a starter culture for fermenting soybeans into the traditional West African flavouring Dawa Dawa (Cherif et al., 2003) for fermenting African mesquite seeds in the production of the Nigerian food flavouring okpehe (Kiuchi & Hosoi, 2003). Toxin-producing *B. cereus* strains occur in okpehe, and the use of a subtilisin-producing starter culture was envisaged to boost the protection of this product (Terlabie et al., 2006; Oguntoyinbo et al., 2007). Bacteriocins from Bacillus species supply a much broader spectrum of potential applications compared with existing laboratory bacteriocins (Oguntoyinbo et al., 2010). The process of bacteriocin purification can be made cost effective by using techniques like immunoaffinity chromatography (Suárez et al., 1997).

Conclusion

The use of bacteriocin may be a safer alternative than the harsh techniques used to preserve food. However, if a purified bacteriocin is used as a food preservative, the substance must be approved as GRAS (Generally Regarded as Safe).

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Review article

Azo Dyes: Microbial Degradation and Mechanisms

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Abstract

Azo dyes due to their high utility in industries have garnered a lot of attention regarding their effect on the environment. These dyes are now known to be carcinogenic and mutagenic and their high toxicity and resilience to degradation have become a cause for concern. Existing physio-chemical processes for degradation of azo dyes are unable to effectively remove them from effluents. They are also not economically feasible or environmentally sustainable. Microbial decolourization and degradation of azo dyes in varied environmental stressors has gained attention as a bioremediation strategy as these are inexpensive, eco-friendly, and can be applied to a wide range of such dyes. Microbial enzymes such as laccase and azoreductase, are cost-efficient, easy to harvest, easily downstream processable, and effortlessly mobilizable. Therefore, this review aims at discussing microbial-based decolourization of certain azo dyes and understanding their mechanisms.

1. Introduction

Azo dyes are the largest group of synthetic dyes used in various industries like food, pharmaceutical, cosmetic and textile industries. Release of azo dyes through effluents is one of the major causes of water pollution. Their "colour" comes from the azo functional group. Their complex aromatic structures make them difficult to biodegrade (Fu &Viraraghavan, 2001). Therefore conventional treatment methods such as photochemical or activated sludge are inefficient and are unable to degrade the dye completely (Singh et al., 2014). Various microorganisms can mutate or develop into resistant strains under stressful environments of pH variation, salt toxicity, and other inorganic contaminants. As a result of the primary or secondary metabolic activities, a wide variety of microorganisms including bacteria, fungi, algae, and yeast are capable of decolorizing different pollutants, comprising dyes (Manu & Chaudhari, 2002) under different anaerobic or aerobic conditions. Bacterial enzymes are known to degrade or break down various toxic chemicals into less potent forms. (Zimmermann et al., 1982). In anaerobic condition, the azo bond undergoes cleavage to generate aromatic amines and it was mineralized by nonspecific enzymes through ring cleavage under aerobic condition. Therefore, coupled anaerobic treatment followed by aerobic treatment can be an efficient degradation method of azodyes (Ali et al., 2011). Major microbial enzymes that carry out degradation include

azoreductases, laccases, and peroxidases. These enzymes are known to achieve degradation via oxidation reductions reaction under aerobic as well as anaerobic conditions. Azoreductases are found in bacteria, while laccases and peroxidases are common enzymes used by fungi to carry out degradation. However, before proceeding to industrial application, the nature of the end products must be studied to check for toxicity.

2. Toxicity of azo dyes:

Azo Dyes are known to be toxic to aquatic life with LD_{50} values as low as 1 mg/L (Clarke &Anliker, 1980). Besides, the dye also blocks the penetration of light and impacts algal photosynthesis (Chung & Stevens, 1993). Toxicity of azo dyes was studied in various microorganisms and it was observed that azo dyes can cause inhibition of luminescence in *Vibrio fischeri*, growth inhibition in the microalga*Elenastrumcapricornutum*, and variety of effects on the viability, growth, grazing, and morphometry of the ciliate *Tetrahymena pyriformis* (Novotný et al., 2006). Bioaccumulation of the azo-dye is observed through the food chain (Weisburger, 2002). Accumulation of these toxic chemicals is found in certain tissues and their fish gills (Vargas et al., 2009). The Sudan I dye (Solvent yellow 14) inside an animal or human body is enzymatically transformed in the intestinal into carcinogenic aromatic amines (Piątkowska et al., 2018). Tartrazine and carmoisine were evaluated for their toxicity in rats by oral administration. Deterioration of liver and kidneys observed and oxidative stress markers were increased (Amin et al., 2010). Formation of micronuclei in RBCs of fishes was observed under the exposure of chlorotriazine reactive azo red 120 (Al-Sabti, 2000). Intermediate metabolites of Direct Red 28 stimulated DNA degradation and apoptosis (Bafana et al., 2009). Germ cell reduction in mice was observed during exposure to Congo red (Gray &Ostby, 1993).

3. Degradation of azo dyes by physiochemical methods

Textile waste water treatment by physical processes for degradation of azo dyes. Various physicochemical processes such as anion-exchange resin, flotation, electro floatation, electro-chemical destruction, irradiation, ozonation, activated carbon, chemical coagulation, chemical oxidation, and adsorption have been found to be expensive, have limited versatility, prone to interference by other wastewater constituents, and/or generate waste product that pose additional serious disposal problem. (Banat et al., 1996), Ion exchange resins were applied to decolorize the textile wastewater and to reduce its COD (Karcher et al., 2001). Sodium hypochlorite decolorizes azo dyes by chemical oxidation which generates aromatic amines; often carcinogenic and induce toxicity (Anliker, 1979). Azo dyes (such as Reactive yellow 2, Orange P, and Red Px) can be effectively adsorbed by carbon-based sorbents. However, carbon sorbents are expensive and there is a large requirement for complete degradation. This becomes economically unfeasible and therefore cannot be applied on a large industrial scale (Forgacs et al., 2004). Ozonation can effectively decompose azodyes, however a high amount of dissolved oxygen is required and the risk of ozone toxicity to living things also makes it hard to work with (Forgacs et al., 2004). In advanced oxidation processes (AOP) (photochemical and photocatalytic), oxidizing agents such as O_3 and H_2O_2 or heterogeneous photocatalysts are used with catalysts, such as TiO_2, ZnO_2, Mn and Fe, in the presence or absence of an irradiation source which generates (OH) radicals for the destruction of hazardous dye pollutants (Forgacs et al., 2004).

4. Degradation of azo dyes by microorganisms:

Physical and chemical methods of degradation of azo dyes generate various toxic by-products and metabolites. Therefore, the need of the hour is to explore

bioremediation for the treatment of industrial effluents. Microorganisms are known to adapt and acclimatize to environmental stress such as osmotic pressure, pH variations, and temperature variation. As a result of the primary or secondary metabolic activities, a wide variety of microorganisms including bacteria, fungi, algae, and yeast are capable of decolorizing different pollutants, comprising dyes under different anaerobic or aerobic conditions (Manu & Chaudhari, 2002).

4.1. Degradation of azo dyes by bacteria:

In the early 70s, *Bacillus subtilis* was used to degrade azo dye, (Horitsu et al., 1977) which led to further experimentation of azo dye degradation using bacteria spp. Anaerobic degradation of azo dye appears to be effective due to higher decolourization however anaerobic reduction by slicing of –N=N– bonds results in toxic, carcinogen, and lethal by-products such as aromatic amines (Sarkar et al., 2017). Novacron Super Black G (0.2mg/ml) was decolorized by *Alcaligenes faecalis AZ26* to 65% in 48 hours, however, the strain was not able to decolorize Novacron Turquoise HGN in 48 hours (Hossen et al., 2019). Methyl red is used as the sole carbon source for growing *Staphylococcus aureus* to study its degradation under aerobic conditions with decolourization observed was 66.07% (Abioye et al., 2015). *Lysinibacillus spp.* have also been studied for the degradation of azo dyes. For example, 90.6% decolourization of Remazol Yellow RR by *Lysinibacillussphaericus* (Srinivasan &Sadasivam, 2018). *Lysinibacillus fusiformis W1B6* was studied for degradation of Methyl Red, Congo Red, and Methyl Orange. It exhibited maximum potency towards degradation of Methyl Red at 94% (Sari &Simarani, 2019). These studies indicate that *Lysinibacillus fusiformis W1B6* is able to decolorize Methyl Red better than *S. aureus*. Another species of Staphylococcus, *Staphylococcus hominis RMLRT03* was studied against Acid Orange decolorization. (Singh et al., 2014). *Pseudomonas entomophila BS1* isolated from the Ganges River was able to decolorize Reactive Black Dye 5 in just about 120 hours. (Khan & Malik, 2015). It has been reported that the bacteria capable of carrying out decolourization of colorants aerobically are difficult to isolate, specifically, those involving sulfonated azo dyes (Lin et al., 2010; McMullan et al., 2001; Pearce et al., 2003). Study conducted using GC-MS examination, proved that *Staphylococcus lentus* 1 M produces several end products enzymatically by acting on azo colorants (Ajaz et al., 2018). The metabolites produced are significant in various pathways; for example like the utilization of 4-guanidinobutyric acid as a substrate in the metabolism of amino acid. Amino acid catabolism leads to the production of pyruvate, which leads to formation of acetyl coenzyme A, which in turn undergoes TCA cycle initiating generation of reduced molecules. Similarly, spectrum of fatty acids and aldehydes can be produced by transformation of phthalate derivative, an end product. These molecules yield manufacture of $NADH_2$ and $FADH_2$, either directly or indirectly through β-oxidation reactions of fatty acids (Sarkar et al., 2017).

4.2. Degradation of azo dyes by fungi:

Decolourization of azo dyes has been demonstrated by various fungal organisms such as *Pleurotusostreatus, Pichia sp., Penicillium sp., and Candida tropicalis* (Gou et al., 2009; Kalmiş et al., 2008; Tan et al., 2013). The white rot fungi such as P*hanerochaetechrysosporium*has been extensively researched and its use has been reported in the treatment of textile effluents. White-rot fungi produces lignin peroxidase, manganese peroxidase, and laccase that degrades many aromatic compounds due to their nonspecific enzyme systems (Forgacs et al., 2004). Degradation by *Penicillium chrysogenum*of Direct Yellow 86, Direct Black 22 and Direct Blue 200 were studied. *P. chrysogenum* was able

to completely decolorize Direct Yellow 86 and Direct Black 22 and could decolorize Direct Blue up to 87.5% in 12 days (Durruty et al., 2015). *Trichoderma tomentosum* was able to decolorize 52% of Acid Red 3R in 24 hours and 97.3% in 48 hours (Si et al., 2013). 90% decolorization was observed when Scarlet Red RR was degraded by *Peyronellaeaprosopidis* and maximum decolorization was obtained at pH 6.0(Bankole et al., 2018). Direct Red 81 was decolorized to 94.929% in 120 hours by *Aspergillus clavatus* (Shareef et al., 2018). At pH of 5.8, Malachite green gets decolorized by *Aspergillus flavus strain F10* to 98% (Barapatre et al., 2017).

5. Mechanisms of enzymatic degradation of bacteria:

Azo dyes are known to be highly stable in nature and their xenobiotic properties make them recalcitrants. Therefore, conventional treatment methods such as light, chemicals, or activated sludge are inefficient and are unable to degrade the dye completely (Singh et al., 2014). Enzymatic bioremediation of these compounds is known to be promising and almost complete decolourization has been reported in waste waters. Major microbial enzymes that carry out degradation include azoreductases, laccases, and peroxidases. These enzymes are known to achieve degradation via oxidation reductions reaction under aerobic or anaerobic conditions. Azoreductases are found in bacteria, while laccases and peroxidases

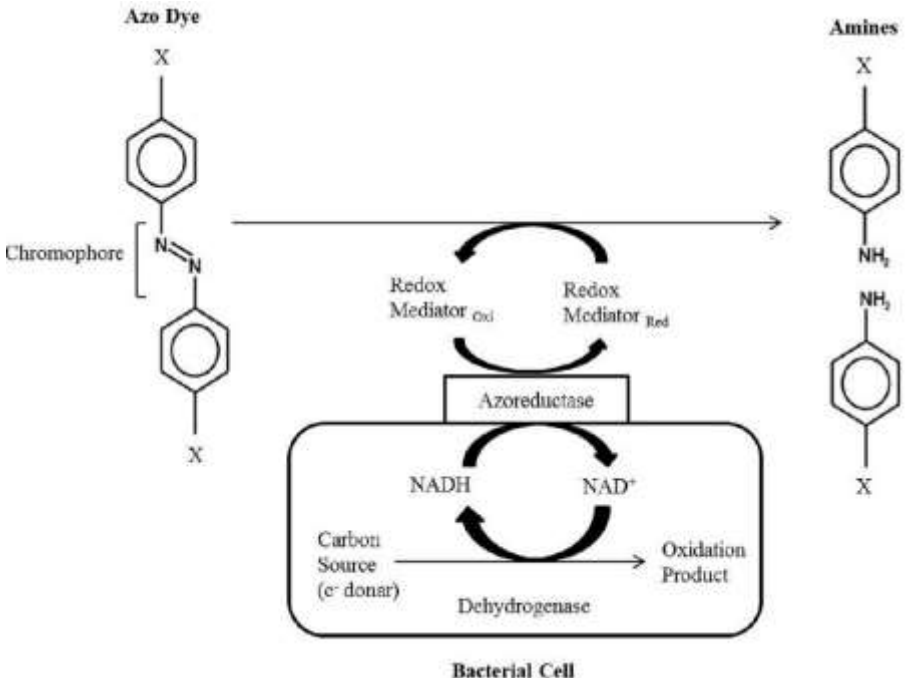

Fig. 1: General mechanism of degradation of azo dye by azo reductases (R. L. Singh et al., 2015).

Table 1: Azo dye degradation demonstrated by microorganisms

Azo Dye Degradation by Bacteria

Strain	Source of Microbe	Dye in Study	Conc of dye	Experimental Setup	Results	References
Lysinibacillus sphaericus	MTCC - 9523	Remazol Yellow RR	0.1mg/ml	Decolorization was studied for 48 hours	90.6% decolorization - 48 hours	Srinivasan & Sadasivam, 2018
Lysinibacillus fusiformis WIB6	Textile effluent - Malaysia	Congo Red, Methyl red, Methyl Orange	0.1mg/ml	Decolorization was studied for 4 hours	Time (hr)/ DYE: Methyl Red 94.00%, Congo Red 58%, Methyl Orange 10% (4hr)	Sari & Simarani, 2019
Staphylococcus hominis RMLRT03	Textile effluent - Tanda, Uttar Pradesh, India	Acid orange	(0.1-0.6)mg/ml	Decolorization was studied at 12 hours interval for 60 hours	Concentration/ Time: 0.1mg/ml 91.31%, 0.2mg/ml 80%, 0.3mg/ml 60%, 0.4mg/ml 41%, 0.5mg/ml 31%, 0.6mg/ml 18% (60hrs)	R. Singh, Singh, & Singh, 2014
Pseudomonas entomophila BS1	Ganges river bank, Kanpur, India	Reactive Black Dye 5	0.5mg/ml	Decolorization was studied at 1 day intervals for 5 days.	Day->/ Concentration: Day 1 20%, Day 2 62%, Day 3 83%, Day 4 85%, Day 5 87% (0.5mg/ml)	Khan & Malik, 2015
Alishewanella Spp CBL2	Industrial waste water, Lahore, Pakistan	Sumifex tourqi blue	100ul/ml 200ul/ml	Decolorization was studied at 6 day intervals for 24 days.	Day->/ Concentration: Day 1, Day 2, Day 3, Day 4, Day 5, Day 6; 100ul/ml 15% 25% 35% 55% 70% 78%; 200ul/ml 17% 26% 40% 60% 73% 83%	Ajaz, Elahi, & Rehman, 2018
Staphylococcus aureus	Dump site, Kuntukwo village, Niger state, Nigeria	Methyl Red	0.08mg/ml, , 0.04mg/ml, 0.026mg/ml	Decolorization was studied at 3 day intervals for 12 days.	Day->/ Concentration: Day 3, Day 6, Day 9, Day 12; 0.02mg/ml 59.04% 60.02% 61.73% 66.07%; 0.04mg/ml 52% 54.08% 59.07% 60.04%; 0.08mg/ml 47.11% 48.07% 50.17% 51.04%	Abioye, Iroegu, & Aransiola, 2015
Alcaligenes faecalis AZ26 *Bacillus spp* *Bacillus cereus*	Textile Effluents - Chittatong, Bangladesh	Novacron Super Black G	0.2mg/ml	Decolorization was studied at 24 hour interval for 96 hours	Time (hr)/ Organism: 24hr, 48hr, 72hr, 96hr; *Alcaligenes faecalis AZ26* 63% 63% 83% 89%; *Bacillus spp* 55% 75% 83% 88%; *Bacillus cereus* 85% 87% 90% 89%	Hossen et al., 2019

Azo Dye Degradation by Fungi

Strain	Source of Microbe	Dye in Study	Conc of dye	Experimental Setup	Results	References
Aspergillus clavatus	Texile Zone, Micado, Cairo, Egypt	Direct Red 81	0.1 mg/ml	Decolorization was observed after 120 hours	94.929% decolorization - 120 hours	A. Al Shareef, S.I. Affifi, A. Ramadan, & R. Sakr, 2018
Peyronellaea prosopidis	Sunflag Nigeria Limited, Surulere, Lagos State, Nigeria	Scarlet Red RR	0.01 mg/ml	Decolorization was studied at 24 hour intervals for a period of 120 hours	Day/ Concentration: Day 1 32%, Day 2 46%, Day 3 62%, Day 4 72%, Day 5 90% (0.01mg/ml)	Bankole, Adekunle, Obidi, Chandanshive, & Govindwar, 2018
Aspergillus flavus strain F10	Chandigarh, India	Malachite Green	100 mg/ml, 250 mg/ml, 500 mg/ml, 750 mg/ml, 1000 mg/ml	Decolorization was studied at 24 hour intervals for a period of 192 hours	Day/ Concentration: Day 2, Day 4, Day 6, Day 8; 100 mg/ml 10% 92% 96% 98%; 250 mg/ml 8% 35% 91% 98%; 500 mg/ml 1% 15% 47% 98%; 750 mg/ml 1% 5% 20% 45%; 1000 mg/ml 1% 3% 4% 13%	Barapatre, Aadil, & Jha, 2017

are common enzymes used by fungi to carry out degradation. The mechanism of azo dye degradation includes the cleavage of the azo bond (N=N) followed by other processes like oxidative cleavage, desulfonation, deamination, demethylation, and dihydroxylation which are subject to the type of functional groups present on the azo dye (Gumiero et al., 2010). Enzymes like laccase are highly dependent on the redox

potential of the substrate (Legerská et al., 2016). Some of these mechanisms are discussed below.

5.1. Azo reductases:

The enzyme azoreductase (Singh et al., 2015) can degrade azo dyes into colourless aromatic amines. It is a reducing enzyme and by means of a reductive cleavage mechanism can break down azo dyes using FADH and NADH as the electron donors (Solís et al., 2012). The general mechanism of azoreductase in azo dye degradation is demonstrated in figure 1.

Azoreductases from anaerobic microorganisms are highly oxygen-sensitive, while azoreductases from aerobic microorganisms are usually oxygen insensitive. Based on the oxygen tolerance, azoreductases were classified in two major groups, oxygen-sensitive, and oxygen-insensitive azoreductases. Oxygen tolerant azo reductases do not require Flavin based molecules (Misal & Gawai, 2018). Under aerobic and anaerobic conditions, aromatic amines are produced which are broken down further into simpler amines by enzyme systems such as monooxygenases, dioxygenases, and hydrolases (Hu, 2001; Idaka et al., 1987; Russ et al., 2000). Even though azo-reductases obtained from aerobic and anaerobic strains differ in properties which include molecular weight, optimum conditions of pH and temperature, and thermal stability, most of them share structural homology (Bryant & DeLuca, 1991; Morrison et al., 2012). On the basis of structure, azo dyes are classified to be homodimeric or monomeric in nature. Flavin free azo reductases are known to be monomeric and NADPH dependent azoreductase is found to have a tetrameric form (Chen et al., 2005; Liu et al., 2007; Nakanishi et al., 2001). An FMN-dependent azoreductase isolated and extracted from *Enterococcus faecalis* contains two functional dimers each bound to one another. It was further determined that most FMN-dependent azo reductases were known to be homodimers in structure (Liu et al., 2008; Liu et al., 2007).

Enzymatic cleavage of the azo bond (–N=N–) results in release of four electrons. The two electrons are transferred to the azo dye at every stage which in turn gets reduced. This reduction leads to decolorization of the dye giving an amine. This amine is aerobically broken down into simpler products (Pandey et al., 2007).

Anaerobic degradation differs in a manner, wherein a redox mediator (eg. anthraquinone sulfonate) is used as an electron shuttle which are redox mediators that carry out transfer of electrons by oxidation-reduction reactions (Watanabe et al., 2009). Glucose serves as a carbon source and electron donor that when metabolized helps in maintaining anaerobic conditions and provides intermediates such as NADPH (Lima et al., 2014). This mechanism is of significance since the enzyme system is oxygen-sensitive thereby making the anaerobic degradation more efficient than aerobic (Russ et al., 2000; Singh et al., 2015). The mechanism of anaerobic azo dye degradation is shown in figure 2.

5.2. Laccases:

Laccases are one of the most widely studied enzymes and are known to be found abundantly in nature. They belong to the multicopper oxidase family containing 1,4-benzenediol: oxygen oxidoreductases. The composition of the enzyme is also known to contain a 15% to 30% carbohydrate moiety having molecular weight of 60–90 kDa (Gianfreda et al., 1999; Couto & Herrera, 2006). The polymeric enzyme is known to contain four copper atoms (type1 Cu T1, type 2 Cu T2, type 3 CuT3α & CuT3β) of copper subunits, wherein Cu T2 and Cu T3 associate forming a trinuclear copper cluster (Shraddha et al., 2011). The efficiency of laccase to carry out degradation is well known due to its capability to degrade phenolics and azo compounds (Sarkar et al., 2017).

The action of laccases on the substrates depends on its redox potential. Therefore, laccase are characterized on the basis of their redox potentials as low, medium, and high (Legerská et al., 2016). Laccases found in bacteria have a significantly lower redox potential than the fungal laccases (Mate & Alcalde, 2015), because of which bacterial laccases are unable to oxidize azo bonds as efficiently as fungal laccases. For eg, enzymatic oxidation of sudan orange G by bacterial laccase results in the production of oligomers due to the inability of the enzyme to cleave the azo bond (Pereira et al., 2009). Mechanism of the azo dye by laccases is mediated by production of non-specific free radicals (Telke et al., 2009). The Copper complexes play a major role in this process. The process of degradation begins by asymmetric cleavage of the azo bond by the enzyme. This is followed by other processes like oxidative cleavage, desulfonation, deamination, demethylation, and dihydroxylation which are subject to the types of functional groups present on the dye structure (Adnan et al., 2015; Telke et al., 2009, 2011; Yang et al., 2015; Zheng et al., 2016). Degradation of mono azo dyes such methyl orange is carried out by fungal laccases which results in the formation of hydroxybenzene sulphonic acid and dimethyl phenyldiazine which are colourless end products. A carbocation is first formed which is highly electron deficient. Nucleophilic attack by groups such as $-SO_3$ results in the asymmetric cleavage of the azo bond (Telke et al., 2010). This mechanism is shown in figure 3. For the degradation of bis azo dyes such as congo red, electrons are required (Nam & Renganathan, 2000). The shuffling of electrons occurs via the copper centres present in the enzyme (Zheng et al., 2016). The dye is cleaved asymmetrically and the products formed are further broken down via processes such as oxidative cleavage, desulfonation, and deamination ultimately leading to the formation of napthylamine, and other derivatives (Shleev et al., 2005). This mechanism is depicted in figure 4. A study conducted by (Si et al., 2013) showed further degradation resulting in products with diminished toxicity. To increase the efficiency of degradation on non-phenolic substrates, mediators are used which are high active cation radicals (Shraddha et al., 2011). Examples of mediators include -hydroxy benzotriazole (HOBT), N-hydroxyphthalimide (NHPI), 2, 2 - azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), and 3 hydroxyanthranilic acid (Bourbonnais, Paice, Reid, Lanthier, & Yaguchi, 1995; Gochev & Krastanov, 2007).

5.2. Peroxidases:

Peroxidases are part of the hemeprotein family which are known to mediate reactions when subjected to hydrogen peroxide (Durán et al., 2002). Peroxidases show three substrate binding sites which are denoted as heme d and c edges and an exposed tryptophan residue (Gumiero et al., 2010). Peroxidases are widely used in vitro and represent studies that are feasible in nature due to their simplicity and ease of system; minimal requirements of compounds; low cost and stable enzyme; and short treatment periods (Mielgo et al., 2003). The heme peroxidase family can be further classified as (a) non animal peroxidase (b) animal peroxidase (c) catalase (d) haloperoxidase (e) di-heme cytochrome-c peroxidase and (f) DyP-type peroxidase families. The classification is carried out on the basis of organism, primary structure, and substrate (Gumiero et al., 2010).

Fungi are known to produce peroxidases such as lignin peroxidases (Lip) and manganese peroxidases (Mnp) (Kuwahara et al., 1984; Tien & Kirk, 1984). Most of the mnps can carry out degradation of many sulfonephthalein (SP) dyes like bromophenol blue, thymol blue (Shrivastava et al., 2005). Mechanism of degradation of sulfonated azo dyes begins with the oxidation of the manganese ions (Mn2þ) to Mn3þ, which is then chelated with organic acids. The chelated Mn3þ diffuses readily from the active site of the enzyme which can further oxidize to the secondary substrates (Mester & Field, 1998)

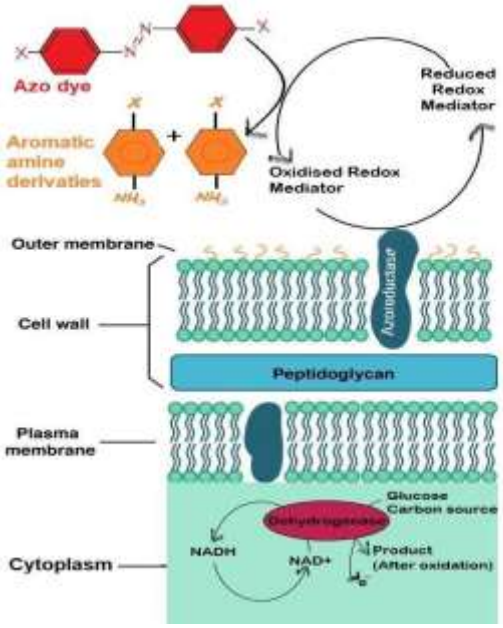

Fig. 2: Degradation of azo dyes by azo reductase anaerobically using redox mediator (Adapted from Joshni & Subramaniam, 2011; Keck et al., 1997)

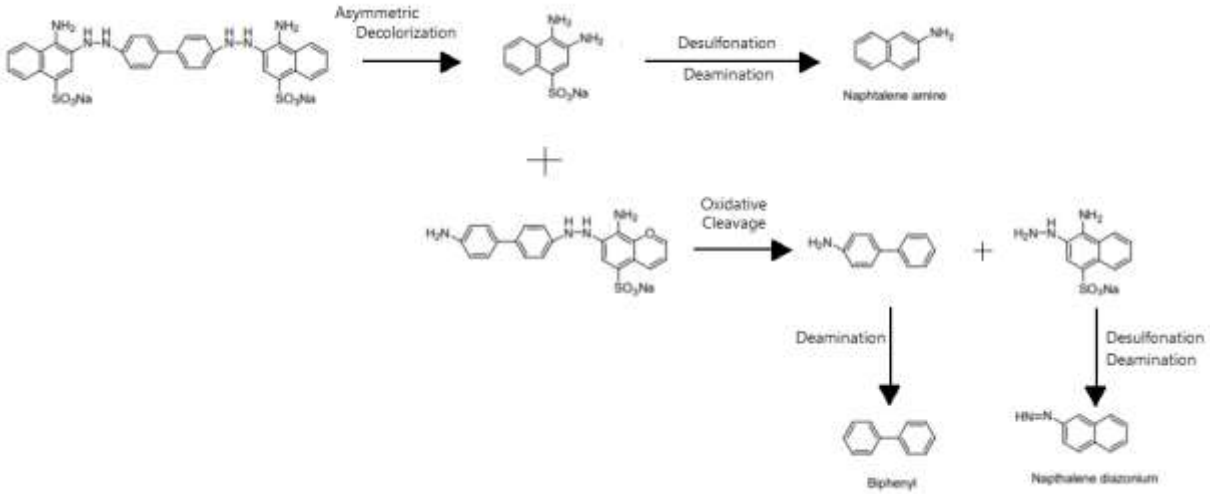

Fig. 3: Degradation of methyl orange by Degradation mechanism of mono azo Methyl Orange by laccase obtained from Aspergillus ochraceus (Adapted from Telke et al., 2010)

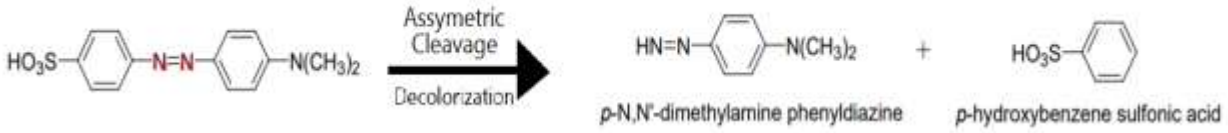

Fig. 4: Degradation mechanism of Congo Red by purified laccases from Trametes pubescens (Adapted from Nam & Renganathan, 2000).

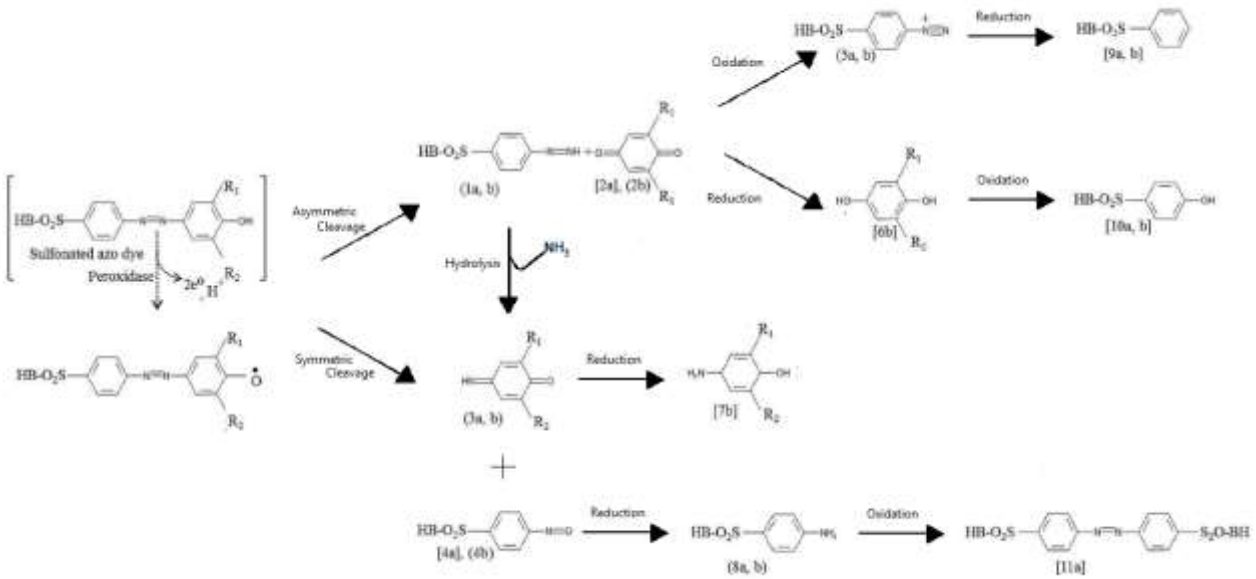

Fig. 5: Proposed pathway for peroxidase catalyzed degradation of sulfonated azo dyes (Adapted from McMullan et al., 2001).Substitution pattern (as in I), R1=R2=O and B=O; substitution pattern (as in II), R1=H, R2=OCH, and B=NH. [2a] 2,6 dimethyl-1,4-benzoquinone, [4a] 4 nitrosobenzenesulfonic acid, [6b] 2 methoxyhydroquinone, [7b] 2-methoxy-4 aminophenol, [8a] sulfanilic acid, [8b] sulfanilamide, [9a] 4-hydroxybenznesulfonic acid, [9b] 4-hydroxybenzenesulfonamide, [10a] benzenesulfonic acid, [10b] benzenesulfonamide, [11a] azobenzene-4,40 disulfonic acid, [12] ammonia.

Conclusions:

Azo dyes are the most commonly used synthetic dyes in pharmaceuticals, food, and textile industry due to the ease of manipulation for desired colour, higher affinity to mordant, and less wear out. The property of it being stable is what makes it a threat. It cannot be easily broken down and cannot be left untreated. Azo dyes are toxic to aquatic life, can move up through the food chain by bioaccumulation, and lead to various health problems as azo dyes have been found to be carcinogenic and mutagenic. The physical and chemical methods of degradation often lead to large sludge formation and some are not economically feasible to be applied to a larger scale. Therefore, bioremediation is an option to explore. Microorganisms have the ability through their enzymatic processes to effectively degrade azo dyes that are eco-friendly. Microbes can acclimatize in harsh environments easily. This becomes an important factor as industrial effluents can induce pH, temperature, salt, and organic stress. Therefore, it is important to know which microbe has the better potential to degrade which dye and also to understand the factors and conditions that manipulate its activity. However, before an industrial application, one needs to determine the end products produced from the degradation, assess its toxicity, and also account for its activity under different environmental stressors.

In case of azo-reductase anaerobic degradation is more efficient compared to aerobic since the enzyme system is oxygen sensitive. Redox potential plays a crucial role in action of laccases, higher the potential greater the ability of the enzyme to degrade the dye via oxidation reactions. Hence, fungi oxidize the azo-bonds more efficiently compared to bacteria as they have higher redox potential. Microorganisms further have the ability to break down the products formed by decolourisation which can be toxic and sometimes even carcinogenic. These products are shuffled via the TCA cycles which in turn produces energy for the microbe. Redox mediators are compounds that increase the range of substrates and conditions for the enzymes which in turn accelerates the rate of degradation and its effectiveness. Thus azo dye degradation via microbial cultures is found to be an effective, economic and a simpler method when compared to physiochemical method, and bioremediation of waste waters can be carried out considering other parameters such as toxicity of products.

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Review article

Nest building in birds: A behavioral and neurobiological perspective

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Abstract

Nest building is no small feat for birds. It is also no surprise that nest building behavior involves complex planning and cognition. Since nest building usually precedes mating and egg-laying, the young ones have not seen their parents build a nest. Yet, in adulthood they manage to accomplish this mammoth task which leads us to ask some intriguing questions. Does nest building stem from instinctive behavior or does it have to be learned by trial and error/watching peers? Which brain areas/neurological pathways are involved in nest building? In this narrative review, we summarize the current research on these topics and attempt to bring forward the behavioral and neuroanatomical aspects of nest building in birds.

A glimpse into the world of birds' nests

Birds are natural engineers. It is truly remarkable that a bird accomplishes the task of nest building with his/her feet and beak. The study of birds' nests (caliology) is indeed a fascinating subject of research. There is a great deal of variation in size, complexity, shape, composition, and technique used for nest building in different species. Number of nests built and location/altitude also vary in different species. Birds intelligently incorporate many changesin the form of colour and texture to ward off predators (usually green plant material and feathers) (Clark, 1991; Dubiec et al., 2013). Nest building is usually performed during the spring/summer due to the aptness of weather and availability of food. It is either the males or females who build nests or both the partners, depending upon the species. An important aspect of nest building is its integral link to reproductive success in most birds. While the primary goal of nest building is to ensure a safe home to lay eggs and subsequently an insulated environment for incubating them and nurturing the young ones; a secondary, albeit, an important goal is sexual signalling. Accordingly, the design and sturdiness of the nest determine the success of the courtship. For example, in weaverbirds (genus: *Ploceidae*) it is the male who builds the nest which is not only elaborate but also intricate and time-consuming. He then waits for a female, and

if no female approves of his nest, he needs to start all over (Collias & Victoria, 1978).

While most birds build their own nests, some birds lay their eggs in nests made by other birds, either belonging to the same species i.e., intraspecific (for example, some duck species) or belonging to another species i.e., interspecific (for example, cuckoos and cowbirds). This trait is called 'brood parasitism'. Their young ones are usually reared by foster parents as their eggs resemble their own (egg mimicry). However, if the host foster parents happen to notice, they strive to prevent the brood parasites from laying their eggs. Brood parasitism, though a feat of laziness, enables the parasites to avoid investing energy into building nests and caring for the young while focusing on more important tasks such as foraging and reproduction (Davies, 2000).

Nest building: A behavioral perspective

Behaviour can be broadly thought of as either innate or learned behaviour. Just as the names suggest, innate behaviour refers to an instinct that is genetically hardwired and predictable, for instance, the act of a baby bird feeding/gaping or the act of egg rolling in new mothers (fixed action pattern); whereas, learned behaviour can be learnt or acquired over the course of life, for instance, bird songs and nest building in birds have strong learned components. Until about 150 years ago, it was largely believed that bird nesting is a purely innate behaviour as nests built by the same species showed obvious similarities. In 1868, Alfred Russel Wallace was the first to suggest that nesting behaviour in birds may be dependent on experiences. In 1964, Colias and Colias, also showed that instinct alone is insufficient for nest building (Colias & Colias., 1964). They reported that male 'village weaver' (*Ploceuscucullatus*) birds built loose and untidy nests for the first time compared with the tightly woven neat nests constructed by older and more experienced birds. In fact, not only nest building but most behaviours stem from an interplay between genetic and experiential components. An intriguing question that may be posed - to what extent do each of these contribute to the behaviour of nest construction in birds?

Low repeatability/heritability in nest building: Bird nesting has a component of 'repeatability' in the same species. This is true as the same bird will not experiment with strikingly different types of nests. Repeatability is also a measure of 'heritability'/'innateness' of nest construction; hence, scientists used this simple rationalization to measure the extent of repeatability over time. Studies on nest dimensions (length, breadth, height) in male weaverbirds showed low repeatability (R=0.21 for southern masked weavers and R=0.07 for village weavers) (Walsh et al., 2010). Further, these birds usually construct nests in two phases – initial attachment phase and ring phase. The same group reported another study showing that their actions performed during these two phases were variable, yet, slightly more repeatable in the ring phase (Walsh et al., 2011). A recent report of a study conducted over a period of 10 years and over 1000 nests belonging to the wild bird population called blue tits (*Cyanistes caeruleus*) also revealed low heritability (12-13%) with regards to nest size and nest composition (Ja¨rvinen et al., 2017). An apparent exception to this is the canary bird (*Serinuscanaria*) which seems to display a high instinctive component in nest construction (Hindes and Matthews, 1958).

Evidence of learning as an important part of nest building

Ways of learning: If learning happens during the early period of life (also called the critical period), it is referred to as 'imprinting'. This was first shown by Konrad Lorenz in 1935 wherein he demonstrated that some species of geese formed an attachment to the first moving object they observed. So, imprinting is a form of learning from early cues that are picked up

during this initial period (for birds, this ranges from a few days to a few months). The other types of learning are 'associative learning' and 'social learning'. The former refers to learning by experimenting by oneself and making associations while learning from one's own mistakes, and the latter means learning from seniors or peers or anybody who is more experienced or is a part of a respectable social environment. While only few studies have addressed the role of imprinting, many have actually provided evidence of associative and social learning in bird nesting, of which, we provide a few examples below.

Imprinting and Early-life experiences: Imprinting during early life influences the preferences in the adult years in certain aspects/choices. Early on, Sargent studied the role of imprinting in bird nesting using elegant experiments (Sargent, 1965). Sargent reared zebrafinches (*Taeniopygia guttata*) and he sought to understand whether the birds' preferences were based on the nests from which they fledged from as young ones based on three aspects, namely, colour of the nesting material, structure of the nest (open/closed), and location (in the cage or in an extension) (Sargent, 1965). He found no evidence of imprinting in terms of color and structure, although imprinting was seen in terms of location (Sargent, 1965). Also, this effect was seen to decrease during their second nesting (Sargent, 1965). In addition, some of these findings were confirmed recently in a similar study (Muth & Healy, 2012).

Associative learning: The selection of nesting site is an important contributor to the overall success of breeding. This decision is based on the bird's own past experiences (Haas, 1998). Marking and recapture studies of birds have shown that those who have a successful past nesting and parenting experience are more likely to return to the same nesting site next season and those who have an unsuccessful attempt are more likely to change the location entirely (Hoover, 2003). Birds that felt threatened by predators changed their location from the previous year and moved to areas with dense vegetation (Eggers, 2006). Birds may also change/improve the structure, provide a wall that separates the front room in the nest while the chicks are reared in the back room to hinder the accessibility, if at risk or threatened by a predator (Stanback et al., 2013).

Social learning: A bird's choice of location is influenced by other conspecifics, especially the first-time builders look up to the decisions of the more experienced individuals, also as trial and error can be a costly affair in terms of time and effort. The birds that were unsuccessful due to predation were more likely to copy their successful peers nesting location the next time (Boulinier et al., 2008). Birds also carefully track the success/failure of their neighbors' attempts at nesting and breeding by virtue of the clutch size, in order to decide whether to copy their location or not (Seppänen et al., 2011). Remarkably, newbies would rather copy the choices (regarding nesting site and material) from seniors who are familiar to them rather than from strangers (Guillette et al., 2016).

Besides instinctive and acquired behaviors, birds also seem to put their own touch into their individual nests which later becomes fixed called 'signature style of nest'. For example, male weaverbirds display specific weave patterns in computer aided imaging technology enabling recognition of an individual nest builder (Bailey et al., 2015).

Nest building: A neurobiological perspective

A bird's brain has to give instructions to execute a sequence of tasks right from selecting the site to the choice of materials to the actual process of building the nest. These stages involve decisions that require higher order thinking followed by motor skills. As stated earlier, many species of birds build nests biparentally and this involves an additional layer of cognition

owing to the alliance and co-operation. The neurobiology of bird nesting is somewhat an under-researched area; however, a few recent studies have somewhat been able to demystify this subject. Behavioural scientists generally use techniques like 'functional Magnetic Resonance Imaging' to determine which areas of the brain are activated while the subject performs the actions. This is obviously difficult to achieve with birds. Hence, alternative means have been adopted to understand the neurobiology of nesting.

Recent work on zebrafinches (*Taeniopygia guttata*) by Healy and colleagues reveal interesting insights into the neurobiology of nest building. This species was chosen as it readily builds nests in captivity and displays majority of the learning behaviours seen in nest building. They investigated the expression patterns of 'immediate early genes' (genes that are activated i.e. transcribed and translated in certain groups of neurons immediately after performing a task) to study which areas of the brain are involved in nest building. An important 'immediate early gene' used in this type of research is c-fos; hence, detecting c-fos protein in a particular brain area indirectly indicates that the area was active during the task. In the studies reported by Healy and colleagues, zebrafinches were allowed to build nests for 90 minutes after which the patterns of c-fos protein in various brain regions were compared to the non nested birds. It was found that the anterior motor pathway, mesotocinergic, vasotocinergic, and dopaminergic reward pathways were all activated during different time points in the next building process (Hall et al., 2014; Hall et al., 2015). (Mesotocin and vasotocin are pituitary neuropeptides homologous to oxytocin and vasopressin in mammalian brains).

Regarding neuroanatomy, studies have conducted a cross-species comparison in the cerebellum with respect to the type of nests built. It was suggested that species that do not use tools like sticks etc. have simple cerebellar architecture (less cerebellar foliation) than those who use tools (Iwaniuk et al., 2009). This makes sense since the cerebellum is largely responsible for motor coordination but also points towards its possible cognitive role. In parallel with this, an increase in folds was seen in the cerebellum of the birds as per the complexity of the nest built, wherein, species that construct cup-shaped nests had the most foliation followed by those who construct platform nests followed by those who do not construct nests had the least foliation (Hall et al., 2013).

Conclusion

Birds' brains are quite high up in the evolutionary ladder (just before mammalian brains) and can shed light on important relationships between brain and behaviour. It has become increasingly apparent that nest building may have a low innate component while several recent studies have highlighted the importance of learning (imprinting, associative and social) in the nest building behavior of birds. An amalgamation of behavioral aspects with the neuroanatomical understanding will provide deeper insights into the neurobiology behind the incredible task of nest building in birds.

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Review article

Role of metals in neurodegenerative diseases

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Abstract

Neurodegenerative diseases are characterized by the loss of vulnerable neurons in a progressive manner. Abnormalities in folding of proteins and their structures play an important role in the pathology of these diseases. Alzheimer's disease is very common among the elderly and is associated with memory decline and cognitive deficits. It is characterised by the accumulation of the Aβ 40 and 42 peptides in the neurons and blood vessels of the brain. Parkinson's disease is caused by the mutations of the parkin genes and is characterized mainly by the degeneration of the nigrostriatal dopaminergic neurons as well as that of other neuronal networks. This article emphasizes on the importance of metal ion homeostasis in our nervous system. Various proteins and their receptors are involved in maintenance of homeostasis, and disruption of the function of any one of them can be extremely harmful and contribute to neurodegeneration. In order to deal with these effects, metal chelation therapy could be a way out. A combination of several chemicals may be one of the solutions for dealing with neurodegenerative diseases in the near future.

Introduction

Neurons are the basic unit of our nervous system. Once formed, they do not regenerate. Hence any sort of damage to them is a matter of huge concern. The term Neurodegeneration, implies degradation or loss of function in neural cells, tissues or organs. With increasing stress in our lives, neurodegenerative disorders (NDs) have become very common, with the elderly being the most vulnerable. Research suggests that in the past century, population in the industrialised countries majorly consists of people belonging to the age of 65 and beyond. This means that the number of people at the risk of suffering from neurodegenerative diseases will also increase in the near future. This prediction is a growing concern because it would increase the emotional, social and financial burden on the patients, caregivers and the society as a whole. Degradation in the quality of the environment surrounding us, exposes us to various harmful substances, which in general increases our susceptibility towards such disorders. Metals are a component of environmental pollutants, often causing neuronal damage, apart from triggering general physiological and metabolic dysfunctions. In response to this scenario, the study of various neurodegenerative diseases in terms of their etiology, ways to prevent them and various

drugs and therapeutics for their treatment is extremely essential.

An overview of neurodegenerative diseases:

Alzheimer's disease:

Alzheimer's Disease (AD) is the most common, progressive neurodegenerative disease particularly among the elderly and is phenotypically associated with memory decline and cognitive deficits (Maslow, 2008). It is characterized by the accumulation of Aβ40 and Aβ42 peptides due to the abnormal processing of the amyloid precursor protein (APP) by the β- and γ-secretases and the formation of the neurofibrillary tangles (NFTs) due to hyperphosphorylation of tau proteins associated with microtubules in neurons. Amyloid precursor protein (APP), Presenilin 1(PSEN 1) and Presenilin 2 (PSEN 2) are the genes associated with autosomal dominant AD. APP gene is present in chromosome 21 and is a type I integral membrane protein. It is expressed in a variety of tissues and is concentrated at the synapses. It is known to play a role in neural plasticity and synapse formation. PSEN1 gene is located on chromosome 14 and is a polytopic membrane protein and it forms the catalytic core of the γ-secretase complex. Defects in this gene causes the most severe form of AD. PSEN 2 is a part of chromosome 1. It is also a part of the γ-secretase complex. Increase in the ratio of Aβ42 to Aβ40 has been reported as a result of mutation in PSEN2. The APOE gene, present on Chromosome 19 plays an important role in sporadic AD. It shows isoform mediated toxicity due to *APOE* ε4. This isoform mediates amyloid aggregation and tau hyperphosphorylation (Bekris et al., 2010). Eosinophilic Hirano bodies, granulovacuolar degeneration (GVD) and cerebral amyloid angiopathy (CAA) are also frequently observed, ultimately leading to synaptic loss and neuronal death (Serrano-Pozo et al., 2011).

Parkinson's Disease:

Parkinson's Disease (PD) is the second most common neurodegenerative disease, manifesting as tremor, rigidity, and bradykinesia with postural instability (Kouli*et al*., 2018). Autosomal dominant PD is caused by mutation in *SNCA* (*PARK*1), and *LRRK2* (*PARK8*) genes. Mutations in *Parkin* (*PARK2*), *PINK1* (*PARK6*), *DJ-1* (*PARK7*), and *ATP13A2* (*PARK9*) are responsible for autosomal recessive PD (Klein & Westenberger., 2012). Pathologically, it presents as degeneration of the nigrostriatal dopaminergic neurons. The most distinctive macroscopic morphological characteristic of PD is the loss of dark pigmented area observed in the substantia nigra pars compacta (SNpc) and locus coeruleus in the transverse sections of the brain stem (Dickson, 2012). Microscopic pathological features of PD involve formation and axonal deposition of the Lewy neurites (Spillantini et al., 1997).

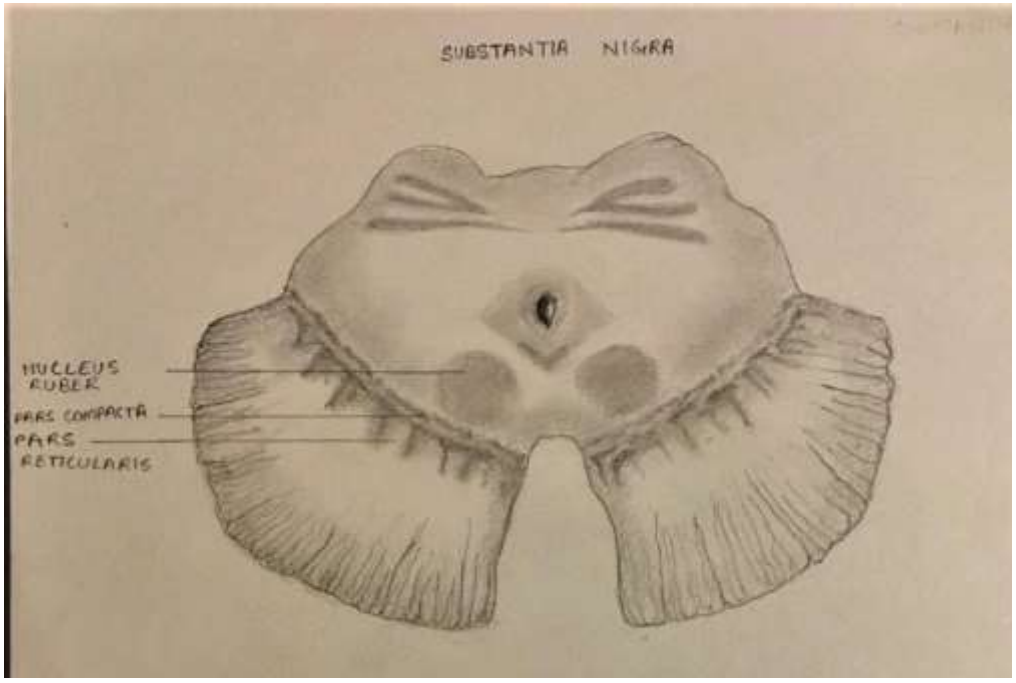

Fig.1: The substantia nigra. The loss of the dark pigmented area in this region is characteristic in case of Parkinsons disease. Image credits: Sanjana Krishnakumar

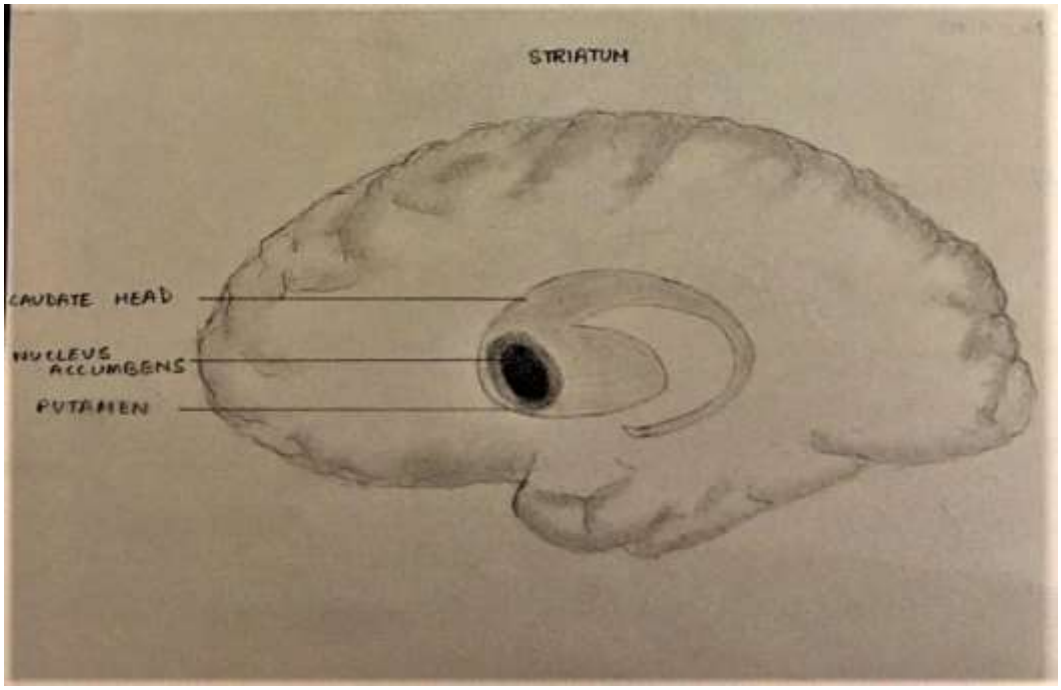

Fig. 2: The striatum and the cortex undergo maximum neurodegeneration in Huntington's disease. Image credits: Sanjana Krishnakumar

Huntington's disease:

Huntington's disease (HD) is a neurodegenerative disease that is inherited as a dominant genetic disease due to an unstable expansion of a CAG repeats within the coding region of the IT15 gene on the short arm of chromosome 4, which encodes the Huntingtin (Htt) protein. Mutation in this gene leads to the formation of extended series of glutamines (polyQ>36) near its amino terminus (Strong et al., 1993). Symptoms of HD include chorea, cognitive disturbances, mood swings, depression, motor abnormalities and epileptic seizures. These conditions alleviate with time, ultimately leading to death. Its neuropathology includes loss of neurons in the striatum and the cortex (Vonsattel & DiFiglia, 1998).

METAL TOXICITY:

Normal aging is accompanied with a dysregulation in metal homeostasis. The rate of this is accelerated in case of various neuropathological conditions. It has been suggested by various experimental evidence that exposure to toxicants present in the environment can increase the risk of developing neuronal damage in many neurodegenerative disorders (Rivera-Mancia *et al.,* 2010). For example, the levels of heavy metals in both terrestrial and aquatic environments are increasing due to anthropogenic activities like mining, use of insecticides and fertilisers, etc. Natural causes like volcanic eruption, metal erosion, etc. also contribute to accumulation of heavy metals in the environment. Exposure to them is known to be extremely harmful to living organisms (Briffa et al*.,* 2020). Accumulation of metals in the nervous systems of patients suffering from NDs has also been observed. This indicates that metals have a role in a range of neurobiological processes, regulation of synaptic transmission being one of them.

Iron

Disruption in metal ion homeostasis is common with age, but its rate has been identified to be accelerated in case of various neurodegenerative diseases. Iron is harmful for our system primarily in the form of iron oxide released from industrial processes, including iron ore mining, steel processing, welding, and pyrite production (Kornberg *et al.,* 2017). The involvement of iron in AD is proved by its increased levels in the brains of the patients. It is also involved in Multiple Sclerosis causing demyelination of neurons. The accumulation of ferrous iron in the oligodendrocytes causes its degeneration, and its further removal into the extracellular space increases the toxicity. In case of PD, increased levels of reactive Fe(II) lead to auto-oxidation of dopamine, releasing hydrogen peroxide. Excessive levels of iron in the SN leads to its progressive degeneration (Yantiri & Anderson, 1999).

Copper

Exposure to high doses of copper occurs mostly by consumption of copper contaminated food or beverages. This may cause mucosal ulcers and bleeding in the gastrointestinal tract and adversely affect the liver, CNS and the cardiovascular system. Abnormality in copper ion homeostasis contributes to neurodegenerative diseases namely Menkes and Wilson's disease. Around 95% of copper found in the human body is present in ceruloplasmin. If the genetic loss of ceruloplasmin is inherited, it leads to progressive neurodegeneration in the retina and the basal ganglia. Mutations leading to the gain of function of the copper enzyme, Superoxide dismutase present in the cytoplasm results in degeneration of motor neurons in case of Amyotrophic Lateral Sclerosis. Recent evidences suggest that copper plays a role in this process as well as in neuronal damage in Aβ and prion mediated encephalopathies (Waggoner *et al.*, 2000).

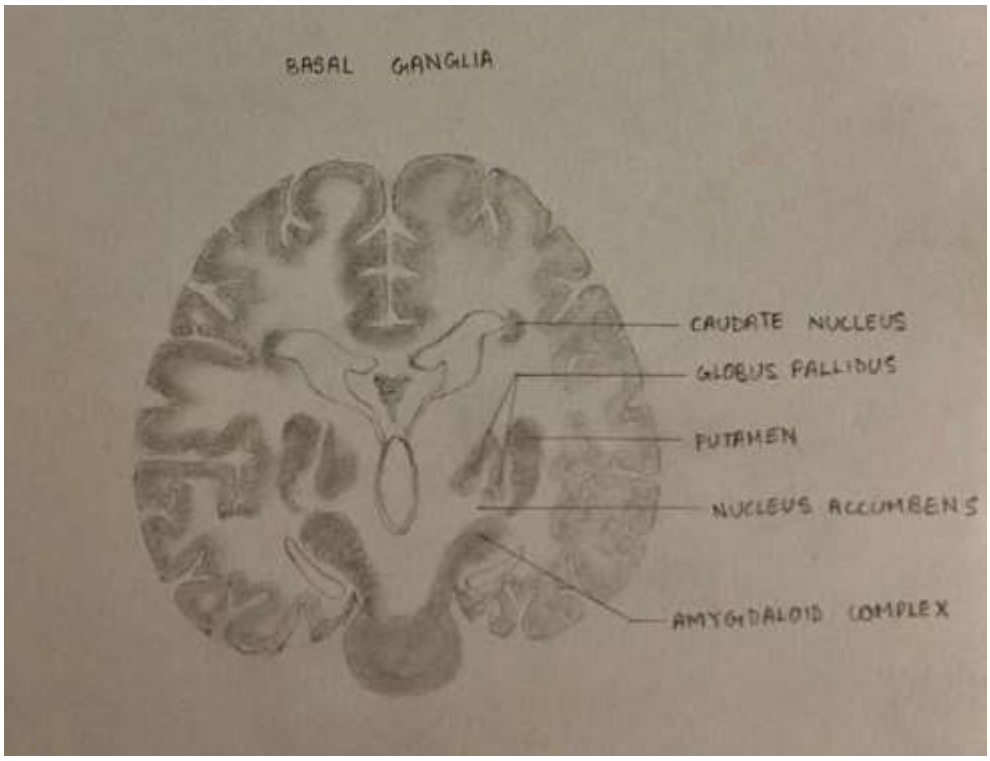

Fig. 3: The basal ganglia progressively degenerates if there is deficiency of copper. Image credits: Sanjana Krishnakumar

Zinc

Excess of zinc leads to an increased risk of vulnerability towards neurological disorders. It also leads to disruption of the process of neurogenesis and promotes neuronal apoptosis, leading to memory deficits. Alteration of zinc homeostasis also increases the risk of depression, AD, aging and a range of other neurological disorders. In normal conditions, cellular homeostasis of zinc is maintained with the help of coordinated regulation effected by membranous zinc transporters (ZnT and Zip) and metallothioneins (MT), involved in its uptake, excretion and intracellular storage/trafficking (Szewczyk, 2013). Air in the industrial areas and jobs such as zinc mining, smelting, welding and manufacture of zinc containing alloys like brass, bronze etc. are the source of exposure to high levels of zinc.

Calcium

Although calcium is not a metal, it is important to briefly include it in our discussion here. Neuronal calcium signalling is affected due to aging. Inability of the Ca^{2+} buffering capacity of the cytosol of the neurons increases the susceptibility of aged neurons towards AD. Hence, the neurons expressing low levels of Ca^{2+} binding proteins are extensively damaged. Calpain activation in case of excessive Ca^{2+} levels leads to the cleavage of proteins important for the normal functioning of the neurons, leading to neuronal dysfunction and apoptosis. Oversaturation by Ca^{2+} causes oxidative stress, thereby damaging the mitochondrial DNA, can be the probable cause of damaged mitochondria observed in case of AD (Bezprozvanny, 2010).

Manganese

Intoxication due to Mn (manganese) causes manganism, initially characterised by psychological symptoms which include hallucinations, psychoses and a myriad of behavioural disturbances. This might happen due to consumption of contaminated food (e.g. grains, beans,

soybean) and oral formulations and also by exposure to natural or man-made manganese contamination via water, air or soil (O'Neal & Zheng., 2015). Motor defects are observed in the later stages which include gait dysfunction with a propensity to fall backward, postural instability, bradykinesia, rigidity, micrographia, mask-like facial expression and speech disturbances (Olanow, 2004). Manganism also affects the cortex and hypothalamus of the CNS. Mn can increase the expression of α-synuclein, which then increases the formation of neurofibrils. These fibrils lead to formation of Lewy bodies and Lewy neurites found in PD. Mn plays a role in the dopaminergic death by overexpression of α-synuclein, which then activates the transcription factor NF-κB, the kinase p38 MAPK, and apoptotic signalling cascades. It has also been linked to mitochondrial impairment and oxidative stress. Altered activity of Mn dependent enzymes such as arginase, glutamine synthetase, pyruvate decarboxylase, and Mn superoxide dismutase 2 (SOD2) has been observed in the brains of humans suffering from HD and also animal models of it. Mn is also known to increase the intracellular levels of PrP (the protein involved in bovine spongiform/"mad cow" disease) which induces PrP misfolding and proteinase resistance (Brown et al., 2000) at micromolar dosages at physiological pH. Animals and humans suffering from Prion's disease also have high levels of Mn in CNS and blood. In a study, chronic exposure of macaques to Mn induced an upregulation of amyloid-like protein 1 and accumulation of diffuse amyloid-beta plaques in the frontal cortex (Banta & Markesbery, 1977).

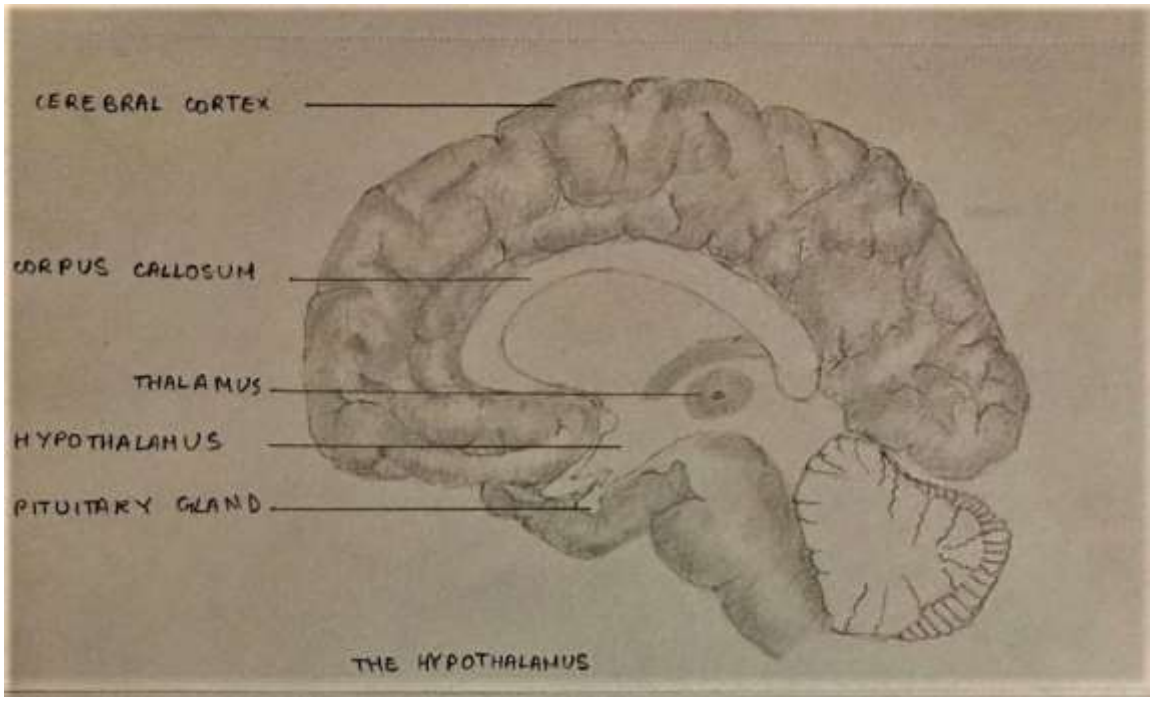

Fig.4: The cortex and the hypothalamus are adversely affected in case of manganism. Image credits: Sanjana Krishnakumar

Aluminium

Aluminium is well known for its neurotoxic effects and occupations such as aluminium welding, powder manufacture, etc. are the main source of its exposure (Klotz et al., 2017). It is involved in pathogenesis of AD by contributing in abnormal Aβ aggregation, tau hyperphosphorylation, OS and cellular dysfunction. It can, by itself, lead to ROS accumulation and work together with other metal ions to increase the oxidative stress of the surrounding environment of the neurons. It is also known to be co-localized with other ions in the Aβ plaques. The etiological role of Al in PD was established by studying its ability to increase oxidative stress in the brains of AD mice. It contributes to the increase in the levels of lipid peroxidation in the substantia nigra of these brains and adversely affects the antioxidant activity of various enzymes like SOD (superoxide dismutases), GPX (Glutathione peroxidases) and CAT (catalase).

Heavy metals:

There are various metals like lead, mercury, cadmium etc., which are extremely toxic for the biological system. Exposure to these metals at any point in life has harmful effects on the body.

Lead

Lead has a primary role in degenerative brain diseases as it can modify gene regulation in exposed individuals. Lead toxicity is an occupational hazard and jobs like production of gasoline, pottery, lead based paintings etc. are the primary sources of exposure to the metal (Wani et al., 2015). In an experiment, exposure to lead caused disruption in regulation and expression of a number of genes involved in development of neurons and in the response of neural and glial cells to stressors like metals and pathogens. Many of these genes involved include serine/threonine protein phosphatases (implicated in tau pathology), AβPP and the beta-secretase enzyme (implicated in amyloid-β pathology). Lead appears to alter gene expression primarily by decreasing DNA methyl transferase activity in affected cells (Reuben, 2018).

Cadmium

Exposure of the foetus to Cadmium (Cd) in the neonatal developmental state makes the CNS extremely vulnerable to it. This is primarily because Cd in fully grown organisms cannot reach the brain due to a well-developed blood brain barrier (BBB), which is not fully developed in young organisms. Cd affects the CNS by increasing damage due to oxidative stress, disrupting metal homeostasis and inducing neurotoxicity by reducing neuronal differentiation and axonogenesis, leading to neuronal cell death (Wang & Du, 2013). Exposure to cadmium occurs via certain food (e.g. cereals, pulses, potatoes) substances and majorly through smoking as tobacco plants take up a lot of it from the environment.

Mercury

Mercury is a global pollutant. It bioaccumulates through the aquatic system and is hazardous when exposed through contaminated water and food (Bose-O'Reilly *et al.,* 2010). Mercury in its vapour form is extremely harmful because it is inhaled by the lungs and 80% of it is absorbed. Its uncharged monoatomic form can easily cross the BBB and lipid bilayers of cells and their organelles. Research suggests presence of higher levels of Hg in the brains of AD patients. Extremely small amounts of mercury results in changes in the nerve cells that are characteristic of AD. Presence of both organic and inorganic forms of Hg induces biochemical changes in tubulin structures that are found in case of AD (Carocci et al., 2014).

Table 1: A summary of role of metals in neurodegeneration.

METAL	ROLE IN NEURODEGENERATION
IRON	Ferrous ions lead to rise in production of reactive oxygen species (ROS), resulting in an increase in the rate of neuronal death. Involved in hyper-phosphorylation of tau
COPPER	Deficiency promotes progressive neurodegeneration of retina and basal ganglia. Excessive Cu causes degeneration of the motor neurons.
ZINC	Deficiency of zinc disrupts the process of neurogenesis and promotes neuronal apoptosis
CALCIUM	Low levels damage neurons. Excessive Ca leads to: Neuronal dysfunction and apoptosis Oxidative stress, thereby damaging mitochondrial DNA
ALUMINIUM	Affects the antioxidant activity of enzymes by increasing lipid peroxidation in the substantia nigra and accumulation of ROS Involved in Aβ aggregation and tau hyperphosphorylation
LEAD	Causes disruption in regulation and expression of a number of genes involved in development of neurons and in the response of neural and glial cells to stressors like metals and pathogens.
CADMIUM	Reduces neuronal differentiation and angiogenesis. Affects the CNS by increasing the ROS levels.
MERCURY	Biochemically changes the structures of tubulins present within neurons.
MANGANESE	Affects cortex and hypothalamus of the CNS Promotes the formation of neurofibrils by increasing the expression of α-synuclein

IRON METABOLISM:

The molecules involved in iron metabolism and homeostasis include transcriptional levels of mRNAs, iron regulatory proteins (IRPs), Tf (Transferrin), TfR1(Transferrin receptor protein1), ferritin, FPN1 (Ferroportin1), DMT1 (Divalent Metal Transporter 1), etc. (Crielaard et al., 2017). Iron regulatory elements (IREs) are special amino acid sequences present in the mRNAs of these proteins. Iron regulates the transcription of iron-related proteins by controlling the binding of IRPs to IRE (Zhou & Tan, 2017). Hepcidin is an anti-bacterial peptide which plays a significant

role in iron homeostasis regulating both its input and output in the cell. APP can interact with FPN1 to regulate the efflux of iron. Tau mediated iron homeostasis is APP dependent (Tuo *et al.*, 2017) as it transports the APP produced to the surface of the cell, thereby promoting iron output (Li et al., 2015). Overproduction and accumulation of Aβ42 and its alloforms {Aβ (1-42)} is linked to abnormal iron metabolism as they can interact with both ferric and ferrous forms of iron. The ferroxidase activity of APP can prevent the

FPN1-mediated iron loading to Tf resulting in iron retention in the neurons and further causing neuronal death. Several studies have shown that ferritin containing microglia are present in the Alzheimer brains, thus suggesting a role of ferritin in the disruption of the iron homeostasis in them. Magnetite (iron oxide), present in the senile plaques is an indication of anomaly in iron redox chemistry.

Interaction between the peptides is enhanced by ferrous ions, thereby causing the amyloid monomers to form oligomers and fibrils (Boopathi & Kolandaivel, 2016; Tahirbegi et al., 2016). High iron levels can affect the amyloidogenic and non-amyloidogenic pathway of APP processing by modulating the α-secretase cleavage activity of APP. Furin expression is inhibited by the presence of excessive iron. This results in activation of β secretase, thereby promoting the production of Aβ from the amyloid pathway. (Ward *et al.*, 2014). Fe2+ and Fe3+ interact with APP and Aβ to promote aggregation of Aβ into fibrous forms (Ha et al., 2007). Fe3+ bound to Aβ is easily reduced to Fe2+. Ferrous ions lead to an increase in production of reactive oxygen species (ROS), which promote the cleavage of Aβ monomer by β secretase resulting in an increase in the rate of neuronal death (Liu *et al.*, 2018). Iron is involved in the hyperphosphorylation of tau protein through the activation of the cyclin dependent kinase (CDK5)/P25 complex and glycogen synthasekinase-3β (GSK-3β). Fe3+ can reduce the release of superoxide radicals by the mitochondria (Kudin et al., 2004). Reduction in levels of Fe3+ leads to a rise in the production of these radicals, which react with nitric oxide (NO) to form per- nitrate. In case of AD, nitration of tau plays a role in destabilization of tau, tau entanglement and formation of senile plaques (SPs).

Iron chelation is the most straight forward and promising method of targeting iron accumulation and redistributing its systemic availability. This approach is very important keeping in mind the toxicity of iron overload Chelation therapy involves the use of metal chelating agents which bind to metals to form a stable compound. It is aimed at balancing the rate of iron accumulation in the body. It is extremely essential to avoid excessive iron chelation. Another challenge is adherence to regular therapy. If not followed properly, it can have damaging effects on the body (Porter et al., 2014).

METAL ION CHELATORS:

Clioquinol

Clioquinol is a metal ion chelator that can cross the BBB. Administration of clioquinol to animal models of AD showed a decrease in the amyloid deposition and improvement in memory (Cherny*et al.*, 2001; Grossi*et al.*, 2009). The high binding affinity of CQ to zinc, iron and copper allows competitive synthesis of these metals from the amyloid plaques, thereby preventing their accumulation. Reduction in the size of SPs was observed on CQ administration to 15-month-old APP Tg2576AD mice (Cherny et al., 2001).

Deferoxamine

Deferoxamine (DFO), used to deal with iron overdose, is non-toxic and is known to inhibit the toxic effects of ROS, the formation of β-sheets of Aβ1-42, translation of APP mRNA, expression of the protein

and secretion of Aβ peptides. It can dissolve amyloid plaques and influence the formation of tau protein. (House *et al.*, 2004). Deferiprone can very efficiently bind to iron, thereby chelating almost all the iron in the body leaving it unavailable for any sort of further ROS production. Deferiprone is more effective if it is administered orally (Liu *et al.*, 2018).

α-lipoic acid

α-lipoic acid (LA) can bind with Cu2+, Zn2+, Pb2+, Hg2+,and Fe3+ forming metal complexes (Ou et al., 1995). In vivo study demonstrated that feeding LA for two weeks in aged mice showed a reduction in iron accumulation in the age related cortical neurons (Suh *et al.*, 2005). LA can efficiently resolubilize Aβ and reduce deposition of amyloid plaques in the brains of AD patients. Interestingly, in another study it was observed that in LA treated mice, memory retention was significantly improved and changes in the levels of soluble and insoluble Aβ in the brain were not observed (Quinn *et al.*, 2007). It also alleviates oxidative stress, inflammation and ferroptosis in the brains of transgenic mice (Zhang et al., 2018).

Lactoferrin

Lactoferrin (LF) is a non heme iron binding protein which is present in various body fluids like milk, saliva and urine. Each molecule of LF can reversibly bind with two molecules of metal ions. The vascular endothelial cells of the BBB contain LF receptors which allows the exogenous LF to enter the cells easily. Injection of lactoferrin-DFO conjugates in the peritoneal cavity have shown to decrease the levels of Aβ plaques and improve memory in AD rats (Kamaliniа*et al.*, 2013). It can also regulate immunity and cell autophagy by these mechanisms. All these studies establish the role of LF as an important drug in the treatment of AD.

However, it should be noted that, in case of iron overload, DFO, deferasirox and deferiprone are being used as a first line chelator, but there are many studies which show that there are a various side effects including neuronal hearing loss, allergic reactions and liver and renal damage (Borgna-Pignatti & Marsella, 2015).

CONCLUSION

Metal homeostasis is extremely important for the normal functioning of our system. Various proteins and their receptors are involved in it, and disruption of the function of any one of them can be extremely harmful. A number of factors like living conditions, occupation and diet also influence the type and concentrations of metal exposure. This makes them vulnerable to neurological disorders, especially young kids and the elderly. Hence, an approach to easily monitor the homeostasis of various metal ions and a way to restore it becomes essential.
To use ion-chelating drugs without hesitation to treat neurodegenerative diseases, further research and development of iron chelation therapy is definitely required. However, the chelators synthesized naturally by our biological system which include α-lipoic acid and lactoferrin may prove less detrimental. A combination of these chemicals, which can deal with the various mechanisms of disruption of iron homeostasis, might be one of the solutions to neurodegenerative diseases in the near future.

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Review article

Diagnostic methods for Alzheimer's Disease

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Abstract

Alzheimer's Disease is a type of disorder mainly affecting memory, thinking and behaviour. It is a common cause of dementia mainly occurring in individuals of age 65 years and above. This article explains Sporadic and Familial Alzheimer's Disease and discusses the causes, symptoms, and diagnosis, including physical, biochemical, molecular and genetic tests for early detection of the ailment. It also describes treatments and medications used to help improve memory and cognitive impairment.

Introduction

Alzheimer's disease is a neurodegenerative ailment and causes loss of neurons mainly in the cortex. There are toxic changes in the brain of a person suffering from AD. These changes might occur years before the disease is detected in the person. It is a common cause of dementia in individuals of age 65 years and above. (Crawford &Loprinzi., 2019).

Symptoms

The symptoms of Alzheimer's are often slow and progress over years. The rate of this progress depends on the individual. The symptoms include memory loss, forgetfulness, misplacing items, delusions, mood swings, loss of senses, loss of weight, loss of speech, and no work is done properly without assistance (Lanctotet al., 2017).

Causes

The thoughts, feelings, and memories of a person are all a result of signals that pass-through nerve cells in the brain. Neurons constantly communicate with the help of ions that travel down axons, causing the release of chemicals between two neurons. Other cells such as microglia and astrocytes clear debris and help in keeping neurons healthy. The cause of Alzheimer's disease is not completely known but research shows that pathological changes include plaques and tangles in the neuronal cells in the brain.

The cell membranes of neurons have the Amyloid Precursor Proteins (APP). APP is usually broken down by enzymes, such as α-secretase and γ-secretase. The part broken by these enzymes is soluble. But in Alzheimer's disease, β-secretase teams up with γ-secretase, the part broken is not soluble and it forms a monomer called amyloid-beta (Kang et al.,1987). This monomer formed is sticky and binds to other monomers outside neurons to form clumps of monomers, the beta-amyloid plaques. These plaques can get in between neurons and disrupt signalling between neurons. They can also trigger an immune response and cause inflammation. They can also deposit around blood vessels in the brain in a condition called amyloid angiopathy, which weakens the walls of

blood vessels, and increase their rupture, leading to blood loss (Hiltunen et al., 2009).

The tangles are found inside the neurons, which are held together by the cytoskeleton, made up of microtubules. These structures ship nutrients and molecules to different areas within the cell. A special protein, tau, makes sure that these tracks made up of microtubules do not break. Amyloid plaque build-up leads to activation of kinase, an enzyme that transfers phosphate groups to tau protein. This leads to the hyperphosphorylation of tau protein; therefore, it stops supporting microtubules and bonds with other tau proteins and leads to neurofibrillary tangles (Braak&Braak, 1991). As a result, the neurons cannot signal properly, sometimes leading to cell death. As neurons die the brain shrinks.

Table 1: Sporadic and Familial Alzheimer's Disease

Sporadic	Familial
It's a late-onset disease. The rate of occurrence is 90-95%.	It's an early-onset disease. The rate of occurrence is 5-10%.
It is caused by several genetic and environmental risk factors working simultaneously.	It is caused by several genetic mutations. It can also be caused by Down's syndrome/Trisomy 21.
The risk factors are age and certain genes. AGE: 1% of people with the age of 60-65 years 50% of people over the age of 85 years. GENE: Risk in e4 allele of apolipoprotein E gene Risk is increased when a person inherits one e4 allele and even more if two e4 alleles.	AGE: Its onset has generally been observed after the age of 40 years. GENE: It is because of mutation in PSEN-1 or PSEN-2 genes in chromosome 14 or chromosome 1 respectively. Presenilin 1 and 2 both are protein subunits of γ-secretase. Down's syndrome is because of an extra 21st chromosome. The gene responsible for producing APP is located on chromosome 21, makes people with Down's syndrome more vulnerable to this form of AD as they have one extra gene (i.e. APP). Therefore, the more APP the more plaques.
Apolipoprotein E helps in breaking down beta-amyloid but e4 allele is less effective than other alleles, like the APOE-e2 allele.	Mutations in these lead to the production of γ-secretase instead of β-secretase. Hence γ secretase chops APP increasing the length of beta-amyloid molecules forms more plaques.

Diagnosis

The clinical diagnosis of a patient with Alzheimer's includes the medical history of the patient, the medications taken, the family history, a medical neurological examination, mental status examination, CT or MRI and PET scan, laboratory tests include thyroid-stimulating hormone (TSH), B12. It would have been much better if we could detect Alzheimer's in a person way before the mental status deteriorates. The research on this is still ongoing but there are some methods for this:
They are as follows:

- Brain imaging:
  Initially, a definitive diagnosis of Alzheimer's was only possible by post-mortem observations of the brain. Brain imaging is an advanced research technique to detect Alzheimer's. It includes techniques like structural imaging, functional imaging, and molecular imaging.

  In structural imaging, we can know about the structure and volume of the brain. As neurons die the brain shrinks, i.e. the gyri get narrowed, and sulci get wider. It includes techniques like Computer Tomography (CT) scan and Magnetic Resonance Imagining (MRI). In functional imaging, we can know about brain cell activity in some regions i.e. how well the brain uses glucose (sugar) and oxygen. It includes techniques like Positron Emission Tomography (PET) and Functional MRI (fMRI) (Reiman & Jagust, 2012). Molecular imaging uses radioactive tracers to detect cellular and chemical changes in the brain. In molecular imaging, we can know about the beta-amyloid and tangles formed. Functional PET or single photon emission compound tomography can analyze brain function (Chu, 2012). PET scan is used to measure the brain metabolic energy and brain scans tell us about the bilateral hypometabolism of superior and posterior lobes. In addition to hypometabolism of temporoparietal areas, there is bilateral frontal lobe hypometabolism present in advanced Alzheimer's (Chu, 2012). 99mTc hexamethyl propylene amine oxime is a single emission photon compound which helps in studying cerebral perfusion. PET scan is more sensitive and specific than single-photon emission compound.

  Amyloid PET brain scans can detect Aβ deposits in the brain of an individual with AD. Pittsburgh compound B (PiB) PET brain scans are a considerably best-reported technique for AD patients (Chu, 2012). If a PiB PET scan is positive it confirms AD in patients, in case if an elderly person does not have dementia but the test for PiB PET scan is positive then it suggests that the individual has high risks of cognitive decline and AD development. Brain scans like PET scan and MRI help in better studying of neuropathological and neuronal degeneration information.

- Cerebrospinal Fluid biomarkers:
  Cerebrospinal fluid surrounds the brain and spinal cord providing protection. CSF also supplies nutrients and chemicals that help keep brain cells healthy. CSF biomarkers, amyloid- β(Aβ42), total tau(t-tau) and phosphorylated tau(p-tau) are very important for AD (Blennow et al., 2018). These biomarkers are mostly for early-onset Alzheimer's. These biomarkers help in studying the cognitive decline pathophysiology in aging. The CSF (cerebrospinal fluid) levels of amyloid-beta proteins and tau proteins will be higher in a person suffering from Alzheimer's and that can be known through a lumbar puncture or spinal tap. It has been observed that amyloid beta-protein levels are low and that of tau proteins are high (Bucchave et al., 2012).
- Blood and Urine Tests:
  They detect the level of tau and beta-amyloid proteins in the blood or urine of the person. Blood tests very accurately predict Alzheimer's. For many years, the diagnosis of Alzheimer's is done using these biomarkers, but these are all useful for detection at a

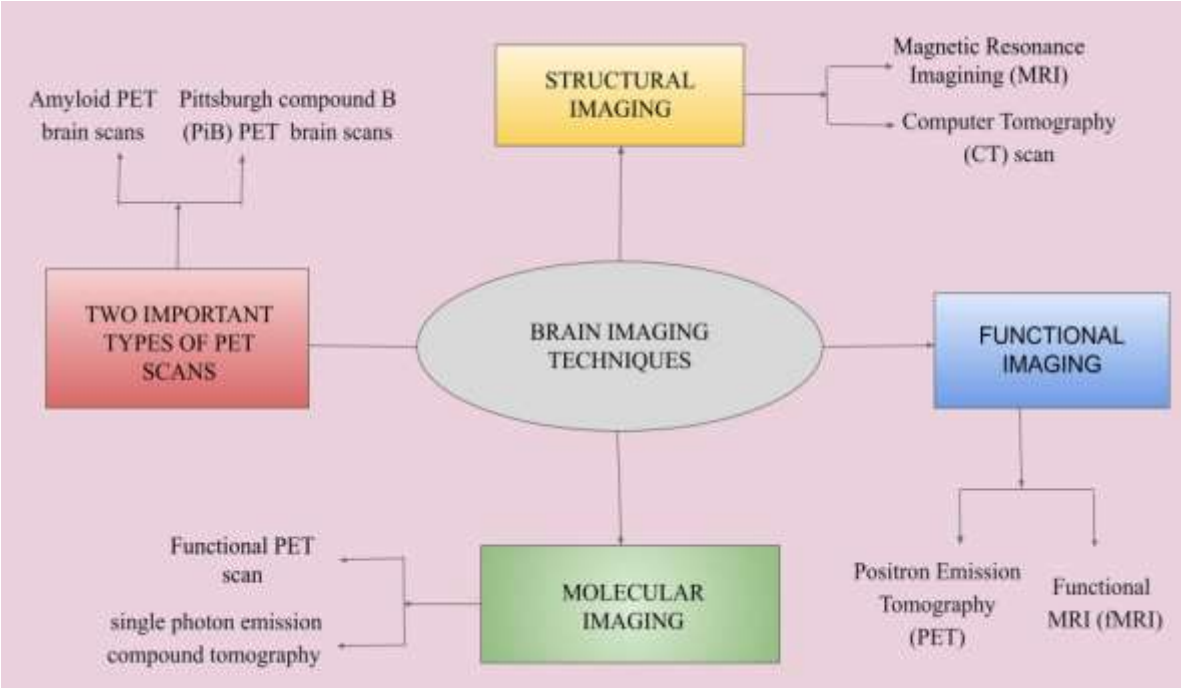

Fig. 1: Techniques used for brain imaging.

later stage. A new study, published on 28 July 2020 in the Journal of American Medical Association (JAMA) and also presented at Alzheimer's Association International Conference, states that a new blood test for plaques and tangles has a remarkable promise in person with or without Alzheimer's and it can be detected as early as 20 years before the cognitive impairment. According to the study, the measurements of phospho-tau protein217 (p-tau217) can act as better indicators for both plaques and tangles than p-tau 181 and other blood tests (Palmqvist et al., 2020).

The study involved 1402 participants, who were divided into three cross-sectional cohorts. Cohort 1 was an Arizona based neuropathology cohort, which included 34 individuals suffering from AD and 47 participants without it. Cohort 2 was the Swedish Bio FINDER, which consisted of 301 cognitively unimpaired participants, 178 patients who were clinically diagnosed with mild cognitive impairment, 121 patients suffering from AD-related dementia, and 99 individuals with other neurodegenerative diseases. Cohort 3 was a Colombian autosomal dominant AD causing mutations which included 365 participants carrying a mutation in PSEN1 E280A and 257 participants who were non-carriers of the mutation (Palmqvist et al., 2020). In cohort 1, neuropathological AD was defined from non- AD by the analysis of P-tau217 in the antemortem (i.e. the participants had already provided blood in the last years of their life) plasma with the help of a ROC curve, using the area under the curve. An accuracy of 89% was obtained in the plasma p-tau217 report between Arizona Brain donors with and without the neuropathological diagnosis of Alzheimer's (intermediate or high i.e. containing plaques as well as tangles that have spread to temporal lobe memory areas), also 98% accuracy was obtained between with and without a diagnosis of Alzheimer's (high), there were higher p-tau217 measurements with an individual having higher brain tangles counts and amyloid plaques. In Swedish Bio FINDER, the participants were with clinical diagnoses of Alzheimer's and some other neurodegenerative diseases and they obtained 96% accuracy. 93% accuracy

was obtained between those with and without an abnormal tau PET scan. In Colombia, they obtained the result between mutation carriers and non-carriers 20 years before their estimated age of MCI. (Palmqvist et al., 2020).

It is said that urine detects Alzheimer's earlier than CSF and blood testing as it obtains changes with AD (Zhang et al., 2017). Urine AD7c-NTP (Neuronal Thread Protein) is found to be a more sensitive and specific test for AD (Zhang et al., 2017). It is too small to be excreted in urine and is stable in AD patients.

- Genetic testing:
  Genetic testing for APOE-4 and other rare genes are available but these tests are not generally recommended because there is no treatment available for AD as they are dangerous and highly costly. Genetic counselling should be done (Atkins &Panegyres, 2011). The test for APOE-4 is mainly for individuals at higher risk of developing Alzheimer's. Carrying these genes does not exactly indicate that a person will develop Alzheimer's. If there is strong autosomal dominant history or early onset AD, genetic testing should be recommended by the clinician. (Goldman et al, 2018). Genetic testing will definitely clarify the diagnosis. Earlier, before NGS (next generation sequencing) Technology, based on family history single gene testing or disease specific sequencing was done. NGS is an advanced technology which allows to analyse many genes. (Goldman et al., 2018).
- Mild Cognitive impairment:
  It is a memory disorder dealing with just memory loss of a person. This can be detected by performing a mini-mental status examination. Individuals with MCI can develop Alzheimer's (Korolev, 2014).
  MCI in AD requires cognitive and functional tests and cannot be diagnosed by a normal laboratory test. PET scan and CSF will help in differentiating dementia-like symptoms but molecular and structural causes should also be kept in mind. (Hane et al, 2017). A positive Aβ biomarker i.e. an increase in the level of CSF Aβ biomarker and Pittsburgh compound b in PET scan and a high level of neuronal impairment can indicate MCI is due to AD. (Hane et al., 2017).

Treatment:

Although there is no perfect cure for AD, there are some medications which have small benefits, and proper care of an individual should be taken. There is no such treatment yet which can halt this disease (Bhushan et al., 2018).
These medications can help with memory and cognitive impairment (Joe et al., 2019). There are two types of these medications:

1. Cholinesterase Inhibitors:
These drugs boost levels of cell-to-cell communication and improve neuropsychiatric symptoms. Commonly prescribed cholinesterase inhibitors are Donepezil, Galantamine, and Rivastigmine (Wilkinson et al., 2004). There are some side effects of these medications such as nausea, loss of appetite, sleep disturbance, etc.

2. Memantine:
This works in brain cell communication and slows down the progress of symptoms. It is sometimes used in combination with cholinesterase inhibitors (Matsunaga et al., 2018). Side effects of this are dizziness and confusion.
Other medications like antidepressants can help in controlling the behavioural symptoms.

Summary:

Alzheimer's disease is caused due to plaques and tangles formed in neurons resulting in the disruption of signalling. This leads to blood loss due to the weakening of walls of the blood vessels. Sporadic AD is caused due to the e4 allele of the apolipoprotein E gene whereas Familial AD is because of mutation in PSEN-1/PSEN-2 genes in chromosome 14 or chromosome 1 and also due to Down's syndrome. The methods used for diagnosis are Brain Imaging, Cerebrospinal Fluid biomarkers, Blood and Urine Tests, Genetic

testing, Mild Cognitive impairment. An article published on 28 July 2020, in the Journal of American Medical Association (JAMA) which was also presented at the Alzheimer's Association International Conference, stated that a new blood test for plaques and tangles have shown remarkable promise in person with or without Alzheimer's, by measuring the levels of phospho-tau protein217 (p-tau217). There is no proper treatment for Alzheimer's disease but medications like Cholinesterase Inhibitors, Memantine can help with memory and cognitive impairment. Hence, further enhancement of the method of measuring phospho-tau protein217 in the blood can be an extremely promising method of early detection and cure of Alzheimer's disease.

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Review article

Getting high on social media *versus* Getting high on exercise: A mini review

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Abstract

There is a slow but gradual shift of population interest towards the shining media networking world. This shift has brought about a decrease in physical activity, thus increasing the occurrences of disease and disorder. Fads like 'Dopamine detox' indicate that there is some awareness regarding the issue of addiction. This mini-review gives a broad overview of the issue at hand and it also addresses the importance of pleasure derived from exercise.

We use social media as a mirror to evaluate our self-worth. Perception of ourselves is deeply influenced by what others think about us. We invest a great deal of time in establishing ourselves online and making our presence felt. Coupled with our packed schedule, this often discourages us to move around and we tend to remain sedentary for extended periods of time. Of late, less active lifestyles have caused a surge in cases of obesity. A study conducted by WHO states that approximately 2.8 million deaths worldwide have core cause due to obesity (WHO, 2017). However, it is a need of the hour to network with people, especially employers and peers. It becomes our sole responsibility to ensure we don't get addicted to the multitude of social networking sites in the process of securing a job or gaining information.

Social Networking websites attract us and keep us hitched by utilizing the prime concern of how others perceive our character. Comparisons and estimations about our reputation are known to be performed by the Nucleus Accumbens (Korn et al., 2012). It is a member of the basal ganglia and is often associated with the Mesolimbic pathway. The pathway plays a role in motivational and reward-seeking behavior (Adinoff, 2004). It doesn't come as a surprise when this pathway from the Ventral Tegmental Area to the Nucleus of Accumbens is claimed to be one of the centers responsible for addictions (Neasta et al., 2011).

But exercise also gives us a high! Another pathway associated with the basal ganglia gets activated when we undergo aerobic stress, the Nigrostriatal pathway. It also consists of dopaminergic neurons, as in the

Mesolimbic pathway (Playford & Brooks, 1992). Research on patients suffering from Parkinson's has suggested that they display abnormalities in the Nigrostriatal pathway which explains its contribution to motor control. It has also been proven that treatment of the same first manifests as improvement in mood and subsequently of ataxia (Willner, 1995).

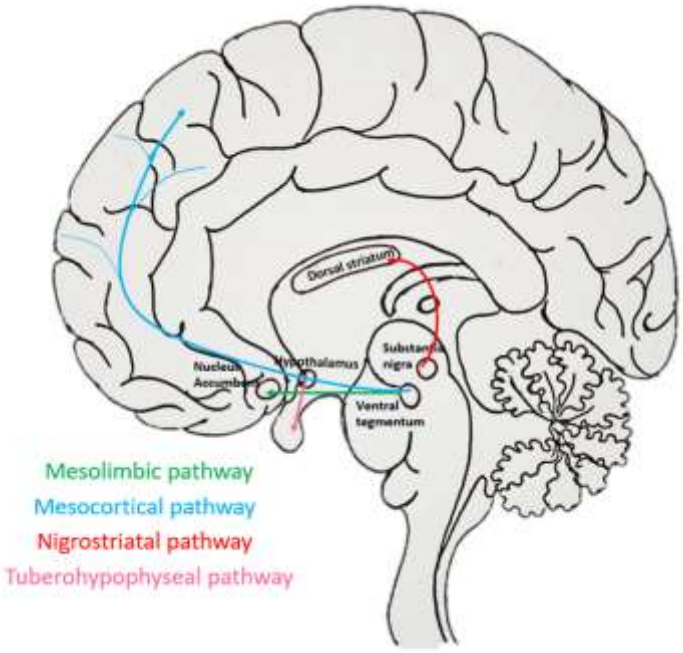

Fig.1: The four dopaminergic pathways

This implies that although a different pathway plays a role in the core function of reward and movement, the activation of dopaminergic neurons generates feelings of ecstasy post successful completion of each (Glimcher, 2011).

However, when we use media apps, we underestimate the time spent on them. This leads to gross justification to spend more time. This phenomenon is known as 'time distortion' (Lin et al., 2015). Users who display addictive symptoms feel anxious when they are unable to use the applications. The cravings become so uncontrollable that the brain's impulsive system springs into action. In the process, the inhibitory mechanisms fail to exercise any control and instant gratification becomes the need of the hour (Noël et al., 2013). Therefore, they often retort to avail themselves of a few more minutes of usage for instant gratification (He et al., 2017).

Addictive behaviors corresponding to exercise, have seldom been reported. The cases reported frequently involve adolescents suffering from body dysmorphic disorders such as anorexia and pro-athletes (Lichtenstein & Hinze, 2020). More research is required to elaborate on this niche. However, it has been proven that BDNF (Brain-Derived Neurotrophic Factor) increases in individuals who do chronic or even acute exercises (Knaepen et al., 2010). BDNF plays a role in enhancing neuroplasticity. Tests on subjects exhibited a significant improvement in cognitive functions (Ferris et al., 2007).

We have been told that aerobic exercises increase blood flow to all extremities as well as the brain and it is good for health. Adding to that, research says that regular cardio exercises can lead to an increase in the gray

matter volume in the brain (Killgore et al., 2013). This holds true even for the adult and old aged population. (Wittfeld et al., 2020). An increase in gray matter volume is associated with improved executive functions such as memory and emotional control (Erickson et al., 2014). Social media, unfortunately, is known to reduce gray matter volume in the Amygdala, thus sprouting addiction-like symptoms (He et al., 2017). Social activity becomes so addictive, statistics state that users unconsciously use it even during mono-focus preferred tasks such as driving (Turel& Bechara, 2016).

Euphoria gained by extreme social networking is thrown off balance by the vices associated with it. On the other hand, along with euphoria, exercise also provides long term physical and mental benefits. So, instead of performing a 'Dopamine detox' which consists of staying away from all pleasurable activities (McCarthy, 2020), we could stay away from excessive doses of those. It involves reducing screen time, allotting time for focused networking, dedicated exercise time, and so on. This should be considered as a wakeup call to prevent brain shrinkage and make rational choices of how much involvement is a boon and when does it turn into a bane.

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Review article

Mental Health during COVID-19: A Mini Review

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Abstract

While COVID-19 has led to severe physical illness and death of millions of individuals, it has also led to an unpredicted rise in anxiety, mood disorders, and depression globally. Apart from the outcomes of the disease itself, patients have also suffered from mental health issues. Healthcare workers, too, have experienced anxiety and worry while working at the frontlines. Spending an increased amount of time in safe home environments, though protective against infection, leads to increased aggression and violence in some households. Students and teachers have also faced stress and anxiety with new forms of digital learning and uncertainty about the future. This mini review focuses on effects of the pandemic on the aspect of mental health among people world over.

On the 11th of March 2020, the World Health Organization declared that the Coronavirus outbreak caused by Sars-CoV-2 that had originated in Wuhan, China, was a pandemic. As of 7th February 2021, there have been over 23 lakh COVID related deaths worldwide (Worldometers.info, 2020). A definitive cure has not yet been developed and the distribution of some preventive vaccines have only just begun while other vaccine candidates are still in phase three clinical trials. Hence, governments across the globe have enforced physical distancing, isolation, and quarantine to help break the chain of transmission of the virus. Though such measures help reduce the chances of contracting COVID-19 infection, it could also lead to unexpected outcomes such as mood disorders, higher anxiety levels, and depression amongst isolated individuals. Such mental health issues need to be seriously addressed as they have been shown to interfere with the individual's wellbeing. Moreover, diverse strata of society have been affected to varying degrees by these mental health issues.

One of the prominent groups of people suffering from mental health issues due to COVID-19 are the patients themselves. Numerous studies across the world have confirmed the negative psychological impact that the COVID-19 outbreak has had on patients. A Spanish study reported that the major contributors to the reduced

mental wellbeing of the patients were the discrimination that they faced due to their COVID-19 positive status and the subsequent loneliness that followed (Gonzalez-Sanguino et al. 2020). Individuals experiencing mild symptoms of COVID-19 too can display symptoms of anxiety. When socially isolated, the anxiety itself, and not the viral infection, may lead to death through suicide (Epstein et al. 2020). The degree of care that patients receive in physical isolation may be reduced, which has also been repetitively seen through past epidemics (Abad et al. 2010). These factors may slow down or interfere with the patient's recovery.

Another important group that needs to be addressed with respect to mental health during COVID-19 is that of doctors, nurses, and healthcare workers. Factors that contribute to their adverse mental health symptoms include being at constant risk of infection, worrying about lack of safety equipment, and constantly being around the plight of patients (Rajkumar, 2020). The mental wellbeing of healthcare professionals is essential to ensure continued patient care and sustenance of health care systems.

While quarantining and isolating at home can keep one safe, vulnerable individuals such as women can face ill effects in hostile home environments. Aggression and agitation caused due to the quarantine measures may lead to acts of domestic violence (Mazza *et al.* 2020). Social isolation can lead to an increase in mood disorders and higher anxiety levels. People living with Intellectual Disability may be adversely affected as they may not be able to cope with the sudden change in personal hygiene measures and everyday routines. This puts them at a higher risk for changes in their behaviour and mental health conditions (Courtenay & Perera, 2020).

Students, as well as teachers, have experienced a dramatic change in the learning process in the form of online education, while many final year exams have been cancelled or postponed. Such measures can be immensely stressful for students. A study conducted in China found that graduating and final year students in quarantine showed more signs of Post-Traumatic Stress Disorder (PTSD) and depression as compared to students studying in other years and this may be due to uncertainty regarding the future, in finding a job or pursuing higher education (Tang *et al.* 2020). This is of particular concern, as suffering from PTSD and depression can cause problems with retention of jobs, productivity, completion of a higher degree, etc., affecting the future of students (Beck et al., 2011).

Many other ill effects of COVID-19 may have gone unnoticed. It is essential for countries all over the world to assess the mental health of their individuals and take the necessary actions required to ensure their wellbeing.

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Review article

SARS – CoV2: Structure, life cycle, and specialized bioinformatic databases

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Abstract

Early in the year 2020, our world was taken by an unpleasant surprise – the COVID-19 pandemic by causative agent SARS-CoV2 (Severe Acute Respiratory Syndrome -associated coronavirus 2). Not surprisingly, there was a global burst of biological research and information on the novel coronavirus; hence, there was a need for specialized databases focusing on SARS-CoV2. This review commences with a brief description on SARS-CoV2 structure and life cycle, followed by specialized bioinformatic databases in a nutshell. These databases are of immense help to scientists conducting research on diagnostics, therapeutics and vaccines. They are freely available online repositories made especially as platforms for researchers to gain access to biological information related to SARS-CoV2.

Severe Acute Respiratory Syndrome -associated coronavirus 2 (SARS-CoV2).

At the start of the new year people all over the world were alarmed by an emerging virus, the novel coronavirus, and in January 2020, WHO declared this to be a pandemic. Those who contracted this virus were noted to show symptoms like fever, dry cough, fatigue and shortness of breath. The rapid spread of this virus, since then, started posing major public health and governance challenges.

Coronaviruses (CoVs) belong to the family Coronaviridae. These are enveloped viruses that consist of extraordinarily large single stranded RNA genomes ranging from 26 to 32 kilobases in length (Su et al., 2016). CoVs were regarded as pathogens that cause mild diseases in the immunocompetent individuals until the emergence of coronavirus causing severe acute respiratory syndrome (SARS-CoV) in 2002 (Fouchier et al., 2003). Currently, there are 7 known coronavirus species that can cause diseases in humans. 229E, NL63, OC43 AND HKU1 viruses cause mild diseases, whereas, the remaining three viruses i.e. SARS CoV, Middle East Respiratory Syndrome (MERS-CoV) and SARS-CoV2 can cause severe illness.

SARS-CoV2 is an enveloped, non-segmented, positive sense RNA virus. It is included in the sarbecovirus, ortho corona virinae subfamily which is highly

distributed in humans and other mammals (Huang et al., 2020; Ul Qamar et al., 2020). It has a diameter of about 65-125 nm and it consists of single RNA strands. Crown-like spikes are seen on the outer surface. SARS-CoV2 has four main structural proteins which are a) spike (S) glycoprotein, b) nucleocapsid (N) protein, c) membrane (M) glycoprotein, d) small envelope (E) glycoprotein and several other accessory proteins (Jiang et al., 2020).

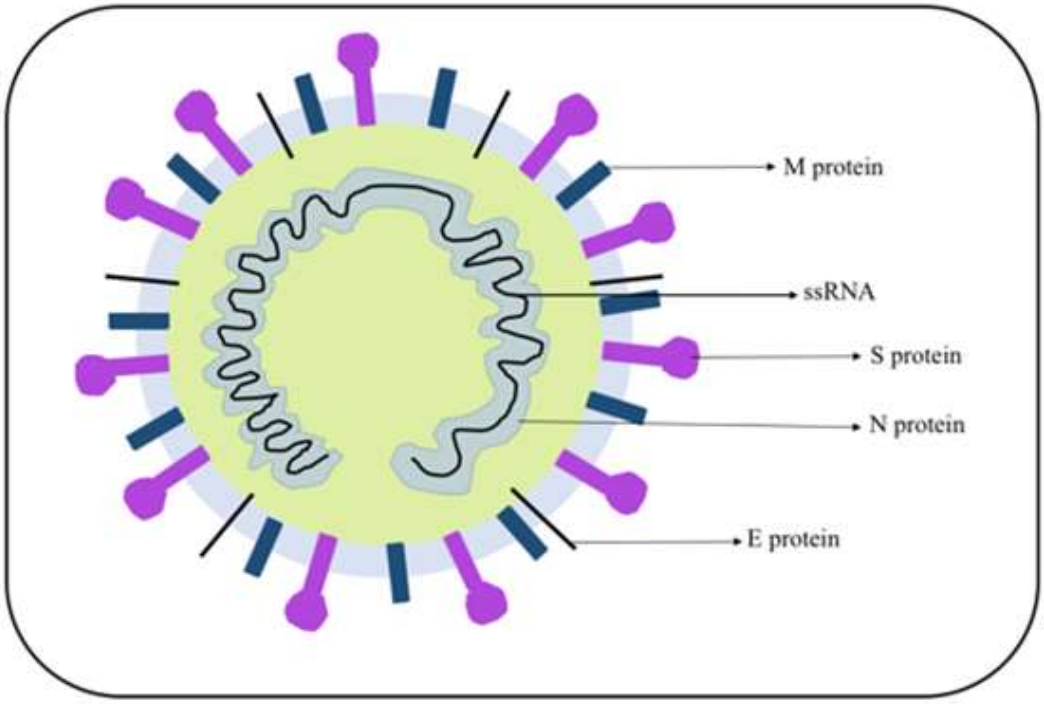

Fig. 1: 2-D representation of SARS CoV-2. SARS-CoV-2 has four main structural proteins including spike (S) glycoprotein, small envelope (E) glycoprotein, membrane (M) glycoprotein, and nucleocapsid (N) protein, and also several accessory proteins. S protein facilitates binding of the virus to host cells by attraction with angiotensin-converting enzyme 2 (ACE2).

S protein is responsible for forming homotrimers that protrude from the viral surface and hence facilitate binding of viruses to the host cells. This binding is made possible by the attraction of the virus surface to the angiotensin-converting enzyme 2 (ACE2) which is expressed in lower respiratory tract cells. This glycoprotein is cleaved by the host cell furin-like protease into 2 sub units namely S1 and S2 (Fehr and Pearlman, 2015).

N protein, known as the nucleocapsid protein, is the structural part of CoV and is present in the ER-golgi region of the virus which is bound to ssRNA of the virus. Since the protein is bound to the nucleic acid material of the virus, the protein is involved in processes related to the viral genome like the viral replication cycle, etc. (Schoeman and Fielding, 2019).

M protein, or membrane protein, plays a role in determining the shape of the envelope of the virus. This protein can bind to any other structural protein and the binding helps in the stabilization of N proteins which in turn promotes the viral assembly (Schoeman and Fielding, 2019).

E protein, or envelope protein, is the smallest protein of this virus that plays a role in the production and maturation of the virus (Schoeman and Fielding, 2019).

Entry and Life Cycle of SARS-CoV-2

The animal source of SARS-CoV2 responsible for the COVID-19 pandemic, has not yet been confirmed by scientists, although it is believed that it originated from the bat species (Wu et al., 2020; Zhou et al., 2020). Various organs of humans, like kidneys, lungs, etc., express angiotensin-converting enzyme 2 (ACE2) receptors which in a healthy human, aids in modulating the activities of angiotensin II (ANG II) protein that increases blood pressure and inflammation, increasing damage to blood vessel linings and various types of tissue injury. According to Sungnak and colleagues, nasal epithelial cells, specifically goblet, or, secretory cells and ciliated cells, display the highest ACE2 expression throughout the respiratory tract in humans (Sungnak et al., 2020). The S protein of the virus binds to the ACE2 receptors of the host cells. The entry and binding is then followed by the fusion of the viral membrane and the host cell (Walls et al., 2020). The binding of the virus with ACE2 receptors now promotes binding to a protease that is present on the cell membranes of the host cells, the type II transmembrane serine protease (TMPRSS2) which then activates the receptor-attached spike-like, S proteins of the virus (Rabi et al., 2020). The virus now enters the host cell, thus releasing its genetic material, ssRNA which leads to translation and further production of the virus inside the host cell.

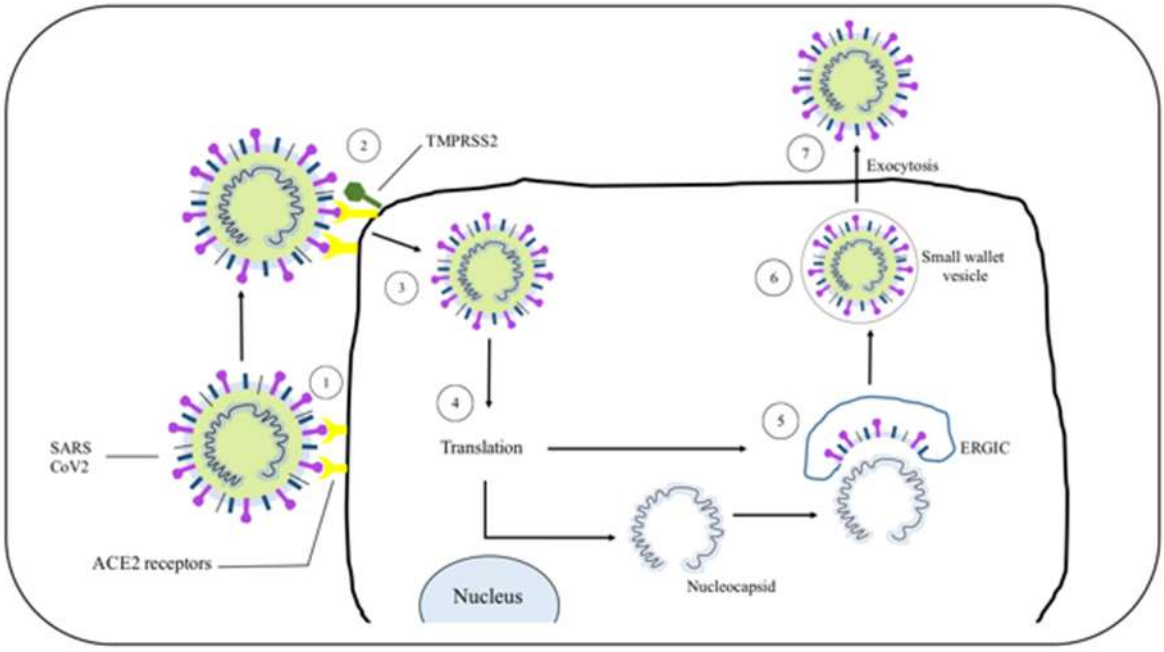

Fig. 2: Entry and life cycle of SARS CoV-2. 1.Spike proteins on the surface of the virus bind to angiotensin-converting enzyme 2 (ACE-2) receptors on the surface of the target cell; 2.The type II transmembrane serine protease (TMPRSS2) binds to and cleaves the ACE-2 receptor. In this process, the spike protein is activated; 3. Cleaved ACE-2 and activated spike protein facilitate viral entry; 4. Viral entry leads to the translation of the viral nucleic acid material; 5. The intracellular cargo ERGIC (ER-Golgi intermediate compartment) aids in the assembly of viral proteins inside the envelope; 6. ERGIC finally mediates vesicle formation. 7. Finally, the viral cells exit host cells via vesicle mediated exocytosis.

Bioinformatics

There was a time when biology only meant observing the external properties of living specimens. Then, came along tools like microscopes that aided in the better understanding of these living specimens. Along the way, major discoveries in the field of technology made us realise the interdisciplinary nature of all the branches of science and hence the need to utilize them simultaneously in order to get the complete story. With the desire to attain complete biological data of various organisms, the use of bioinformatics started becoming very common. A huge demand for analysis and interpretation of the data generated by the human genome project and various sequencing projects in other organisms is hence being managed by the evolving science of bioinformatics. Bioinformatics, basically, is an interdisciplinary field which makes use of mathematics, biology and computer science (Bayat, 2002). It is the application of tools of computation and analysis to capture and interpret biological data.

With an unprecedented wealth of biological data being generated, bioinformatics is essential for management of data. The fundamental activity in bioinformatics, using the basic tool i.e. computer software programs and the internet, is to search the plethora of recorded databases, of all the biological entities, across the web to match, create and thus obtain information about the desired biological entity. The basic bioinformatics databases can help anyone, from clinicians to molecular biologists, to freely gain access to biological data. Generally, complex software programs are used by bioinformaticians for storing, organzing/sorting, retrieving,
analysing, and predicting biological data (Bayat, 2002). The evolution of bioinformatics has been a global venture, leading to generation of computer networks that allows easy access to biological data and lead to development of software programs for effortless analysis.

Specialized bioinformatic databases for SARS-CoV2

Bioinformatic databases are online repositories of biological information with the purpose to store, organize and enable ease of access for researchers for further analyses. A good database is usually manually curated, well annotated and regularly updated with current information. In contrast to general bioinformatic databases that harbour gene or protein sequence/ structural/ functional/ bibliographic information from all species, specialized databases are a conglomerate of existing knowledge on a particular species which may be a model organism or a disease-causing pathogen. With regard to SARS-CoV2, early on in the year 2020, the primary step was to sequence the genome of SARS-CoV2, which was done, very soon (Wan et al., 2020; Zhou et al., 2020). In no time, there were reports on sequence alignments and phylogenetic trees, mutations, primers for PCR (polymerase chain reaction), protein structures, gene/protein expression arrays in response to the virus, pathways affected, protein interactomes, important proteins targeted by miRNA, immune-informatics, chemi-informatics, virtual screening of drugs, drug repurposing, so on and so forth. Hence, there was a need to make specialized databases for SARS-CoV2 to store, organize and make this mammoth of information available on easily retrievable platforms. Some of these databases are also linked to other popular databases like NCBI, PubMed, PubChem, KEGG etc. Recent specialized databases on SARS CoV2 that are freely available on the internet have been enlisted below and also described briefly -.

- CoronaVR (http://bioinfo.imtech.res.in/manojk/coronavr/) is categorized into 4 main sections: Genomes (consisting of genomic information including phylogenetic analysis, codons, protein structure in Protein Database etc), Epitopes (potential epitope-based vaccine candidates), Therapeutics (various RNA based therapeutics) and Primers (for diagnosis) (Gupta et al., 2020).

- DockCoV2 (https://covirus.cc/drugs/) is database for predicting the binding affinity of FDA-approved and Taiwan National Health Insurance (NHI) drugs (total 3,109 drugs) with the seven important proteins belonging to SARS-CoV2, namely, spike protein, 3C-like protease (3CLpro), RNA-dependent RNA polymerase (RdRp), Papain-like protease (PLpro), nucleocapsid (N) protein, human angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease family member II (TMPRSS2). The results are presented in three categories: Docking structure, Ligand information, (including PubChem links and KEGG pathways) and Experimental data (including Gene Set Enrichment Assay data) (Chen et al., 2021).

- GESS (Global Evaluation of SARS-CoV2 Sequences; https://wan-bioinfo.shinyapps.io/GESS/) is a database that harbours thousands of SARS-CoV-2 complete genome sequences by GISAID initiative. Although it does not include phylogenetic analyses, it provides the user information in 5 categories: Genome position, Genome region, Country/Area, Co-occurrence, SNV birth query (Fang et al., 2021).

- CORDITE (CORona Drug InTEractions database; https://cordite.mathematik.uni-marburg.de) ports data from PubMed, MedRxiv, BioRxiv, ChemRxiv, ClinicalTrials.gov. on SARS-CoV2 and provides information on drug interactions based on experimental, clinical and *in silico* predictions in the following categories: Interactions, Targets, Drugs, Publications and Clinical Trials (Martin et al., 2020).

- COV2ID (http://covid.portugene.com/cgi-bin/COVid_home.cgi) is a database focused on oligonucleotides of SARS-CoV2 besides also having the reference genome, alignments, genome variation and also protocols by WHO and CDC (Carneiro et al., 2020).

- H2V (http://www.zhounan.org/h2v) is a database for the knowledge about human genes and proteins influenced by SARS-CoV-2 (SARS2), SARS-CoV (SARS1), and MERS-CoV. It consists of DEGs (differentially expressed human genes after virus infection), PPIs (Human-virus protein-protein interactions), DEPs (Differentially expressed human proteins after virus infection), DPPs (Differentially phosphorylated human proteins after virus infection), DTPs (Differentially translated human proteins after virus infection), DUPs (Differentially ubiquitinated human proteins after virus infection), SAPs (Human proteins in association with disease severity).

- CoronaVIR (https://webs.iiitd.edu.in/raghava/coronavir/index.html) is a database for genomic annotation (whole genome, protein and nucleotide sequences), therapeutics (drug designing), molecular diagnostics (primers), comparative genomic analysis and tools while also providing useful links to worldwide trends and recent bibliography on SARS-CoV2.

- Other important and useful specialized databases on viruses include those which were made prior to the pandemic such as ViPR (Virus Pathogen Resource; https://www.viprbrc.org/brc/home.spg?decorator=vipr) and GISAID (Global initiative on sharing all influenza data; https://www.gisaid.org/). Apart from these, one of the most popular databases - NCBI (National Centre for Biological Information) provides a mammoth of information related on SARS-CoV2 for researchers via 'NCBI SARS-CoV2 Resources' (https://www.ncbi.nlm.nih.gov/sars-cov-2/) and 'LitCovid' (https://www.ncbi.nlm.nih.gov/research/coronavirus/).

Conclusion

The SARS-CoV2 pandemic around the world is a reminder of the problems of zoonotic diseases for public health and economy. There is a huge requirement for vaccines and new drug compounds against

the SARS-CoV2 virus. For this, computational methods offer a fast and cost-efficient approach to support laboratory research. Specialized bioinformatic databases on SARS CoV2 are important platforms which store and manage information and serve to enable researchers worldwide to gain easy access and analyse this information, and thereby, expedite the process of ending this deadly pandemic.

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Review article

Pregnancy and COVID-19: What are the risks?

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Abstract

As the novel Coronavirus is increasing globally, there is a lot of concern raised about pregnant women who are one of the most vulnerable members of our society. The novel Coronavirus is contagious and spreads primarily through droplet transmission. Symptoms of COVID-19 may include cough, fever, loss of taste and smell. COVID-19 uses ACE-2 as the major receptor to gain access to the cell. Data collected from other types of similar virus infections like Severe Acute Respiratory Syndrome (SARS) and Middle Eastern Respiratory Syndrome (MERS) there is an indication that the risk to the mother increases moderately during the last trimester of pregnancy. Similar to pregnancies with SARS and MERS infection, there is a lack of data regarding the intrauterine transmission of SARS-CoV-2 from COVID-19 positive mothers to their fetuses. Even though there are different antiviral treatments available, Lopinavir-Ritonavir has been the preferred drug regimen as it is known to be safe during pregnancy. The Indian Council of Medical Research (ICMR) has issued safety guidelines to be followed for COVID-19 positive pregnant women during delivery and breastfeeding. In this review, we have explored the current scientific literature available on the impact of COVID-19 on pregnant women and the child. It is still uncertain whether pregnant women are more susceptible to Coronavirus than other groups. More scientific data is required to gain greater insight into the risk posed by COVID-19 to pregnant women and the child.

Introduction

The novel Coronavirus (COVID-19) has caused an outbreak of a contagious disease that culminates in respiratory illness (Munster et al., 2020). Coronavirus usually spread through droplets of saliva discharged from the nose when an infected person coughs or sneezes (Xu et al., 2020). Majority of people infected with Coronavirus experience mild to moderate respiratory illness and manyrecover without special treatment. Older people and people with a compromised immune system are likely to develop serious illness. At present, there are multiple vaccines developed for this novel Coronavirus (World Health Organization, 2020). However, it will be a considerable period before a majority of the world population is vaccinated. Coronaviruses are enveloped, positive-sense single-stranded viruses, and they belong to the family Coronaviridae (Li et

al., 2020). The Coronaviruses (CoV) are classified into four genera, α,β,γ, and δ(CoV). Amongst these, α-CoV and β-CoV are found to infect mammals, while γ-CoV and δ-CoV tend to infect birds. Many research groups have identified that SARS-CoV-2 belongs to β-Coronavirus, with a highly identical genome to bat Coronavirus, pointing to bat as the natural host. The novel Coronavirus consists of the same receptor, angiotensin-converting enzyme II (ACE2) as that for SARS-CoV (Guo et al., 2020). Human transmission of Coronavirus infection worldwide has been shown to occur rapidly (Riou & Althaus, 2020).

Symptoms of COVID-19 may include fever, dry cough tiredness, and less commonly, body ache, sore throat, diarrhoea, headache, loss of taste and smell. Serious cases could cause Acute Respiratory Distress Syndrome (ARDS) and could be fatal. It can take from 2 to 14 days for the symptoms to exhibit from the time of infection (Cascella et al., 2020). Since angiotensin-converting enzyme II (ACE2) is known to be the major receptor through which Coronavirus gains access into the body cells, it leads to angiotensin II accumulation in human cells (Patel & Verma, 2020). Excess accumulation of angiotensin II may lead to acute lung injury and vessel dysfunction such as vascular permeability, vasoconstriction, and abnormal myocardial remodeling. (Bouaziz et al., 2020).

Effects of Coronavirus on pregnant women

As the novel Coronavirus is spreading globally, there is a lot of concern about pregnant women who test positive for Coronavirus. Pregnant women with symptoms of fever, cough, myalgia, sore throat, or shortness of breath have been advised to seek timely medical consultation and help. (Chen et al., 2020). Women with a travel history to endemic areas and those with a clinical suspicion of infection are recommended isolation and investigations. (Liang & Acharya, 2020). Much time into the pandemic, things are still unclear about how the Coronavirus affects pregnant women and newborn babies. Data collected from other types of Coronavirus (SARS, MERS) infection indicates that the risk to the mother increases moderately during the last trimester of pregnancy (Yue et al., 2020). For COVID-19, there is no evidence to show that pregnant women are more severely affected than other healthy adults. If they are affected, then this might be due to their altered immunity during pregnancy (Chen et al., 2020). According to a recent study from China, 38 pregnant women who tested positive recovered successfully (Schwartz, 2020).

However, there are contradictory reports which suggest that COVID-19 positive women can suffer from miscarriage, respiratory distress, and preterm delivery but newborn children are not affected (Panahi et al.,2020). According to a recent study conducted at Northwestern University, COVID-19 can potentially cause some abnormalities in the placenta of pregnant women leading to adverse pregnancy outcomes including villous edema and a retroplacental hematoma (Shanes et al., 2020). A study of 441 pregnant women with COVID-19 from 16 different countries shows that 96% had pneumonia. The study also recorded 9 maternal deaths, 6 stillbirths, and 4 neonatal deaths. This analysis also found that pregnant women across countries, who were infected presented similar symptoms with fever being the most common (56%) followed by cough (43%) and muscle ache (19%). Around (18%) also experienced dyspnea (Debroy, 2020).

Effects of Coronavirus on newborn babies

Babies born to pregnant women who were positive for COVID-19 may or may not acquire the disease. Similar to pregnancies with SARS and MERS infection, there is a lack of data regarding the intrauterine transmission of SARS-CoV-2 from COVID-19 positive mothers to their fetuses. In Cheluvamba hospital at Mysuru, India, two pregnant infected women gave birth to healthy babies through a cesarean section (Star of Mysore, 2020). Nair Hospital in Mumbai has successfully ensured the safe delivery

of 300 newborns, where the mothers had tested positive (Times Now News, 2020).

However, a case study in London showed that there is a transmission from mother to child (Govind et al., 2020). It is still unclear whether the baby contracts the viral infection in utero or shortly after birth. On the other hand, two cases of neonatal COVID-19 infection from a hospital in Wuhan have been confirmed, both appear to be infected postnatally (Zeng et al., 2020).

According to the latest research, there is no evidence of COVID-19 transmission in the neonates or placentas in 31 documented deliveries where the mother was COVID-19 positive. In the above study, two mothers died due to respiratory complications after delivery. Based on limited data, there is no evidence showing the intrauterine transmission of COVID-19 from mothers to their fetuses (Liang & Acharya, 2020).

Report of the Center for Disease Control and Prevention (CDC)

The Centers for Disease Control and Prevention (CDC) report for June showed that 10,537 pregnant women have tested positive for COVID-19. The report also compares the medical data between pregnant and non-pregnant COVID-19 positive women and shows that mortality rates for both infected pregnant and non-pregnant women are the same but pregnant women are more likely to be admitted to the ICU or need a ventilator (Center for Disease Control and Prevention, 2020).

Hence, it is likely that there is no transmission of Coronavirus from infected mothers to the fetus during pregnancy. The newborns may contract the Coronavirus post-delivery. However, if the precautions for cleanliness and hygiene are not taken, there is a chance of negative newborn babies turning positive.

Antiviral Treatment

The antiviral treatment that has been used to treat COVID-19 in China is also recommended for pregnant patients suffering from COVID-19. Recent studies revealed that the drugs like Remdesivir and Chloroquine are used for the treatment of COVID-19. It is seen that Remdesivir is a drug that inhibits the SARS-CoV-2 replication in vitro and Chloroquine shows a broad spectrum for antiviral and immunomodulating activities (Colson et al., 2020). However, Lopinavir-Ritonavir has been the preferred drug regimen as it is known to be safe in pregnancy. The recommended dose is 2 capsules of Lopinavir and Ritonavir orally together with nebulized alpha-interferon inhalation twice a day. Ribavirin which is used in conjunction with Lopinavir and Ritonavir are to be avoided as it is teratogenic and can be harmful to pregnant women as it leads to miscarriages and limb defects in fetuses when consumed (Sinclair et al., 2017; Dong et al., 2020)

Precautions to be taken before and after delivery

Guidelines are provided by the ICMR for deliveries where the mother is infected. Intrapartum care should be provided by monitoring temperature, respiratory rate, and oxygen saturation. Postnatal care should be provided by arranging separate rooms for the newborn. Breastfeeding should be facilitated using a breast pump. Hand hygiene should be practiced by the mother before breastfeeding the child. The expressed breast milk should be fed to the newborn by a healthy caregiver (Indian Council of Medical Research, 2020; Okunade et al., 2020).

Conclusion

According to the literature review conducted it can be concluded that there is no clear evidence that pregnant women are more likely to develop more complications due to COVID-19 infection. The initial scientific data conveys that SARS-CoV-2 infection during pregnancy is not associated with an increased risk of spontaneous abortion. There is no evidence of vertical transmission of SARS-CoV-2 infection when the infection manifests in the third trimester of pregnancy (Alzamora et al., 2020). On the

other hand, there is data suggesting that there is an increased risk of preterm births. More than ten months have passed into this pandemic and still, the data is not enough to derive a conclusion.

The Government of India has included pregnant women in the list of people at moderate risk (clinically vulnerable) as a precaution. Pregnant women should follow social distancing, refrain from unnecessary travel, avoid crowds, public transport, and most importantly practice and maintain good personal and social hygiene and stay away from anyone who has symptoms suggestive of Coronavirus (Indian Council of Medical Research, 2020).

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Review article

COVID-19 and its current diagnostic techniques

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Abstract

The SARS-CoV-2 virus is the causative agent of COVID-19 which has spread all across the globe at an unprecedented rate giving rise to a pandemic. The rapid infection rate of the virus demanded the need for accurate and sophisticated techniques for the detection of the virus. Currently, the commercially available tests for COVID-19 fall under two categories: molecular assays and immunological assays. Both categories of tests are carried out in order to detect and confirm the presence of the virus. This review explains the current diagnostic techniques used for the identification of COVID-19 along with their advantages and disadvantages. It also talks about the future prospects of existing and new techniques.

Introduction

The Coronavirus is a single-stranded RNA virus which was discovered in the late 1960s. The virus gets its name from the Latin word 'coronam' which means a crown, owing to a crown-like image seen under the electron microscope. The virus has spike-like protein projections on its surface. Coronaviruses are known to infect a wide range of animals such as, mammals, humans, birds, and rodents. Once transmitted to a new host, the virus has the capability to adjust to the new host owing to its high rates of recombination and mutation (Huang et al., 2018). Novel Coronavirus or SARS-CoV-2 is zoonotic in nature. The virus was passed from bats to humans. It is believed that the virus originated from a wholesale seafood market in the city of Wuhan in the province of Hubei (China) towards the end of November 2019. Exotic animals like marmots, birds, rabbits, bats, and snakes, were being traded illegally in this market. It is thought that the first people to be infected were the stallholders from the seafood market who were in direct contact with the animals. Thereafter, the virus went on infecting more people and the number of cases increased exponentially in Wuhan. Within no time the virus spread to nearby cities and the number of cases soared across 34 regions in Mainland China. The Coronavirus disease or COVID-19 was declared an international public health emergency on 30th January 2020 (World Health Organization, 2020).

SARS -CoV-2 is observed to have a faster rate of transmission than other coronaviruses in the past, such as SARS and MERS (Middle East Respiratory Syndrome). No specific drug or vaccine was available back then. Social distancing, facial masks, appropriate hygiene, and sanitizing measures seemed like the only way to curb the mass transmission of the virus. Hence, most countries in the world resorted to a complete national lockdown in order to get control over the situation. However, this has resulted in significant disruption of the living and working conditions of people worldwide. A massive drift from people's normal lives is seen to be detrimental, as this pandemic has also affected people mentally. This had given rise to many questions regarding public health, wellbeing, and resilience, at an international level. COVID-19 may have long-lasting rippling effects. Nations were facing challenges in dealing with the virus. The availability of accurate and rapid testing procedures was an urgent necessity. Laboratories, universities, and companies around the world have joined the common quest for developing critically needed test kits.

Early diagnosis could be key to preventing the spread of the disease. A major challenge to curbing the spread of COVID-19 is the inability to identify asymptomatic cases.It is seen that more than 30% of confirmed cases are asymptomatic, and the high false-negative rate (FNR) of a single assay calls for the development of novel diagnostic techniques, effective approaches, sampling from different locations, and finally detection (Yuan et al., 2020). The use of accurate, convenient, and rapid testing for widespread deployment can help in eliminating the silent spread of COVID-19 by asymptomatic viral carriers.

As COVID-19 has a wide range of clinical manifestations, ranging from mild flu-like symptoms to more severe conditions, it is very necessary for us to have techniques that help in efficient testing and detection during the early stages of infection. This will help in identifying COVID-19 from those patients with other illnesses having overlapping symptoms. It will be beneficial as it will avoid unnecessary quarantine and treatment of negative patients and will help in reducing the load on healthcare sectors. Early diagnosis will help physicians provide immediate intervention and treatment for patients who are at a higher risk for developing more serious complications due to COVID-19 illness.

Nature of Coronavirus

Coronavirus or SARS-CoV-2 is a single-stranded RNA virus. The virus is named SARS-CoV-2 because of its genetic similarity to the Severe Acute Respiratory Syndrome Coronavirus-1 (SARS-CoV-1) discovered in 2003. SARS-CoV-2 RNA genome is 30 kilobases in size and virus particlesare80–120 nm in diameter. It is the seventh Coronavirus known to cause infections in humans.

The genome of the virus is seen to include two-thirds of RNA which are responsible for encoding viral polymerase RNA-dependent RNA polymerase (RdRp). It is also responsible for the synthesis of RNA materials, and two large non-structural polyproteins that are not involved in host response modulation, open reading frames (ORF1a-ORF1b). The rest of the one-third of the genome is seen to encode for four structural proteins which are: spike (S), envelope (E), membrane (M), nucleocapsid (N), as well as other helper proteins (Sahin et al., 2020).

SARS-CoV-2 and SARS-CoV are seen to utilize Angiotensin-converting enzyme II (ACE2) as a receptor to enter a cell (Zhou et al., 2020). Once SARS-CoV-2 enters the host, the S protein recognizes and binds to ACE2 which is on the host cell membrane. The binding of the virus to the ACE2 receptor leads to the fusion of the virus membrane with the host cell membrane. This is followed by the entry of the virion or its RNA genome into the cell. The viral

antigen is then presented by the Antigen Presenting Cells (APCs), which eventually stimulates humoral and cellular immunity. The primary humoral immune response is typically the production of IgG, IgM, and IgA antibodies. During an infection IgM is secreted as the first line of defense, while IgG is secreted later. IgG provides long-time immunity and imparts immunological memory. (Yuan et al., 2020).

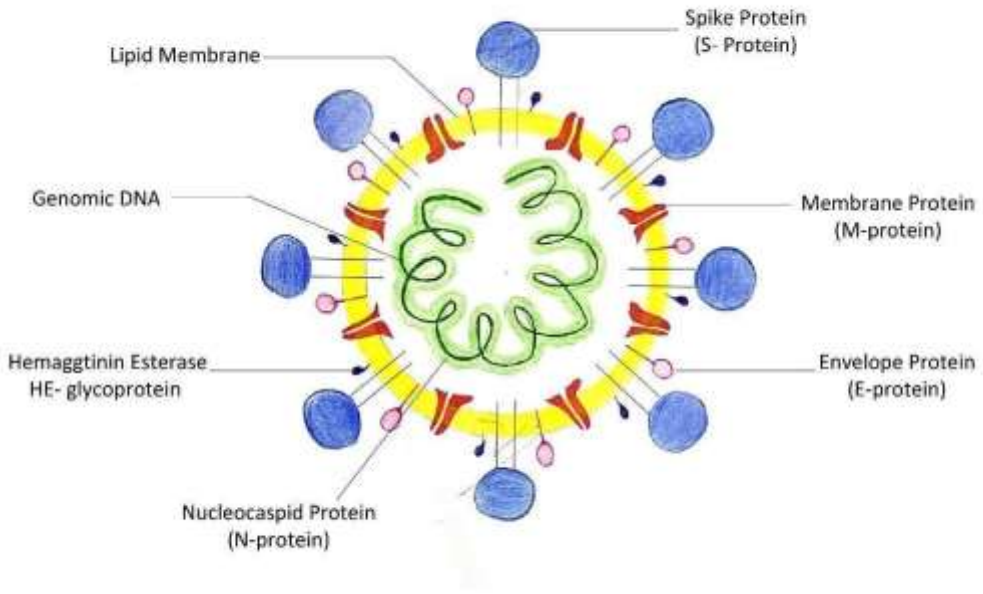

Fig.1: Schematic of the structure of SARS-CoV-2

Effect of the virus on the body

Once Coronavirus infects an individual, it is known to give rise to flu-like symptoms. Following are the most common symptoms of COVID-19: fever, cough, shortness of breath, myalgia, fatigue, loss of sense of smell and taste, decreased leukocyte count, and ground-glass opacities. Along with these symptoms, patients might experience headaches, hemoptysis (coughing up blood or blood in sputum), abdominal pain, diarrhoea, and the production of sputum (Harapan et al., 2020). SARS-CoV-2 is seen to affect lymphocyte count which resulted in depletion of CD4 and CD8 cells (Rodriguez-Morales et al., 2020). It also obstructs the interferon signalling pathways, which in turn lead to higher respiratory virus load, positive viremia, and eventually, poor prognosis. Acute respiratory problems, kidney failure, Hypoxemia, organ damage, acute respiratory distress syndrome (ARDS), arrhythmia, shock, acute cardiac injury, and cytokine storm have acted as complications and have caused the death of many patients (Harapan et al., 2020). The incubation period for SARS-CoV-2 is 1-14 days with no noticeable symptoms.

Moreover, mutations are known to aid in faster transmission of viruses from animals to humans and then from humans to humans. Patients with COVID-19 were seen to have a similar pattern of viral load change to those with influenza but were different from that of SARS and MERS. In SARS and MERS viral load was known to

reach its maximum value in about 10 days, after the appearance of symptoms. In SARS CoV-2, high viral loads were seen in the upper respiratory tract and were reported in the early days, that is from the onset of symptoms.

Asymptomatic cases seem to pose a serious risk as they remain unidentified until symptoms show and yet are capable of spreading the disease in the meantime. Moreover, most of the symptoms of COVID-19 are similar to those of normal influenza and cold making it difficult to distinguish from the later cases. Hence, more sensitive diagnostic techniques for COVID-19 were a pressing priority. Early and accurate diagnosis of infected individuals will help prevent the extensive spread of this deadly disease. Detection in the early stages of COVID-19 will help patients get the appropriate cure on time, before developing serious complications which can be a hindrance to treatment and recovery.

Current detection techniques for COVID-19

Currently, the commercially available tests for COVID-19 fall under two categories. The 1st category is known to include molecular assays for the detection of SARS-CoV-2 viral RNA using polymerase chain reaction (PCR)-based techniques or nucleic acid hybridization-related strategies. The 2nd category includes serological and immunological assays that mainly rely on detecting the viral antigenic proteins or antibodies produced by individuals as a result of exposure to the virus. The widely used molecular test is RT-PCR while the commonly used serological test is Rapid Antigen Test (RAT). It is important to know that the detection of infection by both methods are important and they need to complement each other. The determination of the viral protein antigensor RNA helps in detecting the virus in its active stage, while serological assays help in identifying people whose immune system has already developed antibodies against the infection. These individuals could be potential convalescent plasma donors (Carter et al., 2020).

Amplification techniques

1. Reverse transcription-polymerase chain reaction

RT-PCR is currently considered to be the gold standard for the identification of the SARS-CoV-2 virus. PCR methods are based on the amplification of genes and their RNA transcripts isolated from biological samples. DNA polymerase enzyme, extracted DNA samples, primers, and deoxynucleoside triphosphates are the essential components that are included in a PCR test kit. Reverse transcription PCR (RT-PCR) is a type of PCR method that uses the enzyme reverse transcriptase to convert RNA molecules to cDNA molecules. Then cDNA acts as a template sequence for the PCR reaction.

RT-PCR begins with the conversion of viral genomic RNA into DNA by RNA-dependent DNA polymerase (reverse transcriptase). This reaction depends on short DNA sequence primers designed to specifically recognize complementary sequences on the RNA viral genome. The entire genetic sequence of SARS-CoV-2 was uploaded to the Global Initiative on Sharing All Influenza Data (GISAID) platform on January 10, 2020, so that this information could be used to develop diagnostic kits (Carter et al., 2020).

The short DNA primers along with reverse transcriptase help in generating a short complementary DNA (cDNA) of the viral RNA. In real-time RT-PCR, the amplification of DNA is known to be monitored in real-time as the PCR reaction advances. This is brought about using a fluorescent dye or a sequence-specific DNA probe labelled with a fluorescent molecule and a quencher molecule, like in the case of TaqMan assays. An automated system is known to repeat the amplification process for about 40 cycles. This is done until the viral

cDNA can be detected, which is usually by a fluorescent or an electrical signal (Carter et al., 2020).

Currently, RT-PCR testing for COVID-19 is conducted using samples collected from the upper respiratory system with the help of swabs. Additionally, a few studies have been conducted wherein serum, stool, or ocular secretions were used as samples for testing. (Xia et al., 2020). Although RT-PCR is currently the widely used method for the detection of SARS-CoV-2, it has a disadvantage as it requires expensive laboratory instrumentation. There is also a need for highly skilled laboratory personnel, and it might even take days to generate results. Owing to this, laboratories are working towards improving the efficiency and timeliness of the RT-PCR technologies. Various other techniques are being developed and assessed for their accurate and rapid detection of SARS-CoV-2 virus.

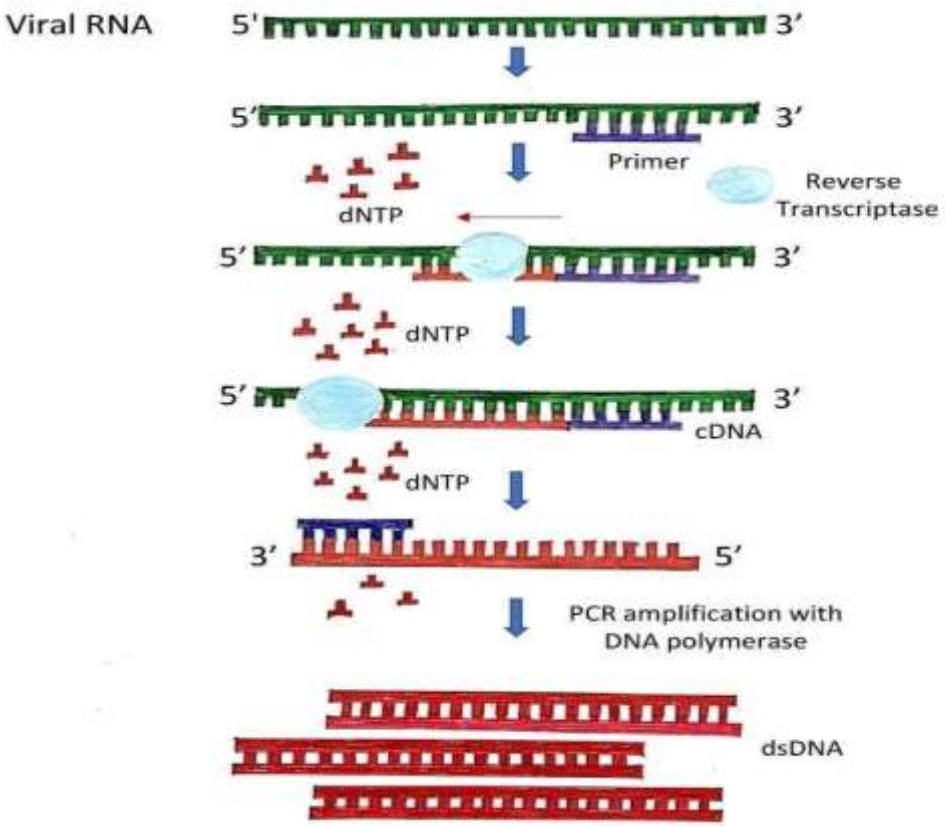

Fig. 2: Reverse transcription-polymerase chain reaction (RT-PCR)

2. Isothermal nucleic acid amplification

It is a technique that is being used to amplify nucleic acids at a constant temperature by avoiding the complex requirement of regular PCR that needs multiple changing temperatures in each cycle (Zhao et al., 2015). Various isothermal nucleic acid amplification techniques had been developed for the detection of SARS-CoV which are mentioned below.

(a) Loop-mediated isothermal amplification (LAMP)
RT-LAMP is carried out in one step at 63°C within 30 min to detect SARS-CoV-2. In this method, three gene amplifications are combined to detect SARS-CoV-2. The amplification product in the LAMP method is then detected by measuring the turbidity of the solution or

the fluorescence of an intercalating dye. Also, unpurified samples can directly be used in LAMP.

(b) Rolling circle amplification (RCA)

RCA is also a promising isothermal amplification technique that has received appreciable attention in recent years because of its sensitivity and capability of amplifying up to 10^9 fold within 90 min. Circle-to-circle amplification and isothermal nucleic acid quantification methods were coupled with an optomagnetic chip for sensitive (0.4 f M) detection of the SARS-CoV-2 virus (Zhao et al., 2014).

(c) Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a family of nucleic acid sequences found in prokaryotic organisms, like bacteria. CRISPR uses bacterial enzymes such as Cas12 and Cas13 that are programmed to cut certain viral RNA sequences which are then followed by isothermal amplification (Broughton et al., 2020).

Indian scientists employed the CRISPR-Cas 9 technology to develop a rapid, accurate and a cost-effective test called 'Feluda.' Feluda stands for FNCAS9 Editor-Limited Uniform Detection Assay. The test is capable of detecting low quantities of the genetic material of the virus based on very minute differences in their RNA sequences. Efforts are being taken in making Feluda more point of care, simpler and more deployable (Pacha, 2020).

3. Nucleic Acid Hybridization Using Microarray

This technique was used for the rapid high-throughput detection of SARS-CoV nucleic acids. It relies on the generation of cDNA from viral RNA using reverse transcription and subsequent labelling of cDNA with specific probes (Carter et al., 2020).

Table 1. Summary of the current molecular tests being used for SARS-CoV-2 detection

Molecular tests				
Technique	**Principle**	**Advantages**	**Disadvantages**	**Time taken**
Reverse transcription-polymerase chain reaction (RT-PCR)	Viral RNA is initially transcribed into complementary DNA (cDNA) by reverse transcriptase. The cDNA is then used a template for PCR reaction	Highly accurate and reliable	Sample generation takes about 24 hrs, Expensive, Requires qualified clinical laboratory personnel, Quality of the RNA extracted from the swabs might affect results	24 hours
Isothermal nucleic acid amplification	Amplifies nucleic acids at a constant temperature thus, eliminating the			

	complex requirement of temperature change			
(a) Loop-mediated isothermal amplification (LAMP)	The target sequence is amplified at a constant temperature of 60–65 °C using 2- 3 sets of primers and a polymerase with high strand displacement activity in addition to a replication activity	Rapid and cost-effective, Doesn't require expensive reagents or instruments	Limited to only one sample per run, Primer designing is complicated due to the requirement of large number of primers, can give false positive results	30 minutes
(b) Rolling circle amplification (RCA)	It involves amplification of short DNA or RNA primer to long single stranded DNA or RNA using a circular DNA template and special DNA or RNA polymerases	Uses minimal agents, avoids false positive results, can be handled by minimally trained personnel	Requires an additional ligase step, since it needs to amplify circular DNA templates, circularization of the DNA template might be low-yielding if folding is not optimal	Technique has not been deployed for detection of SARS-CoV-2 at this point.
(c) Clustered Regularly Interspaced Short Palindromic Repeats	Techniques utilizes enzymes such as Cas12 or Cas13 from the bacterial immune system, along with RNAs to direct enzyme binding to specific target areas on pathogenic DNA or RNA sequences	Fast and robust, allows the determinati-nation of the of the path of the infection the virus takes without having to sequence it, Low-cost	Dependent on the collateral activity of Cas enzymes and their readout is qualitative	Less than 1 hr

Nucleic Acid Hybridization Using Microarray	Generation of cDNA from viral RNA using reverse transcription and subsequent labelling of cDNA with specific probes	Capability of starting with either DNA or RNA, requires small sample volume	High chances of obtaining false-positive results	15 min

Immunological assays

Immunoassays are based on the detection or quantitation of antigen/antibody interactions. They give us data about the dynamics of infections and earlier exposures. Antibodies or immunoglobulins are entities that are produced by the immune system to defend the host against foreign agents. IgG is the most applicable antibody among that of IgA, IgD, IgE, IgG, and IgM in immunoassay techniques. Infections lead to the production of IgM as the first line of defence and IgG is generated in the next stage and persists as immunological memory. IgG may be used to suggest the presence of post-infection immunity. Hence, serological assays involve the testing of blood serum/ plasma/saliva/ sputum/ other biological fluids for the presence of IgM and IgG antibodies. Many sophisticated immunoassays like Enzyme-Linked Immunosorbent Assay (ELISA), Lateral Flow Immunoassay, Neutralization Assay, Luminescent Immunoassay help in diagnosing SARS-CoV-2.

1. Lateral Flow Immunoassay

It is a qualitative (positive or negative) chromatographic assay. It is small, portable, and is used at the point-of-care. This test is considered to be a kind of rapid diagnostic test (RDT) as the result is obtained in 10–30 min. Fluid samples are applied to a substrate material that allows the sample to flow past a band of immobilized viral antigen. If the virus is present, anti-CoV antibodies get collected at the band, where, along with co-collected tracer antibodies, a colour is developed which gives the results. Rapid antigen tests allow for a more direct assessment of ongoing infection (Carter et al., 2020).

Rapid Antigen Test is based on membrane-based lateral flow immunoassay. It allows the detection of viral antigens and is used complementary to molecular genetic assays. This test relies on specific monoclonal antibodies to provide a means for the capture of viral antigens from an analytical sample. Results are obtained in 15 minutes.

2. Enzyme-Linked Immunosorbent Assay (ELISA)

It is a microwell, plate-based assay designed to detect and quantify substances like peptides, proteins, antibodies, and hormones. The test can be qualitative or quantitative. It generally takes 1-5 hours to generate the result. For the detection of SARS-CoV-2, the plate wells are coated with a viral protein. If the virus is present, antiviral antibodies in the patient samples will bind to the viral protein on the plate. Then the bound antibody-protein complex can be detected with an additional tracer antibody to produce a colorimetric or fluorescent-based readout.

3. Neutralization Assay

In this technique, patient samples of whole blood, serum, or plasma are diluted. Then they are added at decreasing concentrations to the cell cultures. If neutralizing antibodies are present, their levels can then be measured by determining the threshold at which they can prevent viral replication in the

infected cell cultures (Postnikova et al., 2019).

Table 2. Summary of the current immunological tests being used for SARS-CoV-2 detection

Technique	Principle	Advantages	Disadvantages	Time taken
Lateral Flow Immunoassay (Rapid Antigen Test)	Antibodies against SARS-CoV-2 present in the sample will attach to chemicals in the device. These antibodies are captured at the test and control lines	Rapid results with high sensitivity and specificity, Easy to read, Useful in detecting asymptomatic cases	Response is based on the individual's immune system and hence timing of when the test is conducted matters. If the test is conducted too early, false-negative result will be obtained simply because the immune response wasn't mounted then	15 minutes
Enzyme-Linked Immunosorbent Assay (ELISA)	Plate-based immunoassay uses enzymes linked to antibodies that can attach to the molecule that is being tested for, and cause a colour change that can be measured by a specialised machine	Cheaper and Point-of-care, can detect previous exposure to infections, Large number of people can be tested	ELISA testing for SARS-CoV-2 COVID-19 has not been well established yet	1-5 hrs
Neutralization Assay	Serological test used to detect the presence and magnitude of antibodies that prevent infectivity of a virus	Useful in vaccine efficacy during clinical trials and vaccination	Results take long time to be generated	3-5 days

Novel prospects for Covid-19 detection: Biosensor Test

Biosensors are bioanalytical devices that are known to combine the selective features of a biomolecule with the sensitivity of a physicochemical transducer. It is based on converting the specific interaction of biomolecules into a measurable readout via optical, electrical, enzymatic, and other methods. Surface plasmon resonance (SPR) is a technique that is seen to measure the interference with incident light at a solid boundary due to local disturbances such as the adsorption of antibody or antigen. An SPR-based biosensor has been developed for the diagnosis of SARS using coronaviral surface antigen (SCVme) anchored onto a gold substrate (Park et al., 2009). A biosensor that detects the novel SARS coronavirus has been developed. It makes use of a cell-based immunosensor that couples capture of the virus with signal amplification to provide a result in 3–5 minutes.

Conclusion

The recent months have witnessed rapid advancement in technologies for the detection of the virus that has led to a global pandemic. Molecular tests and serological assays go hand in hand when it comes to the detection and identification of the SARS-CoV-2 virus. Even though RT-PCR is the dominant technique for the detection of viral RNA, other techniques like nucleic acid assays including isothermal amplification assays, hybridization microarray assays, amplicon-based metagenomics sequencing, and the cutting-edge CRISPR-related technologies might soon be used for mass testing, owing to their fast rate of advancement. Rapid Antigen Tests, although not being as sensitive as RT-PCR have been widely used for mass screening. Rapid antigen test kits are cheaper and the results are obtained in 30 minutes making it a very effective strategy to test maximum people in high-risk areas. This helps in locating super spreaders and hence curbing the further spread of the virus. Development of Ultrarapid test kits and point-of-care tests are in progress as these tests will help in rapid detection and will eliminate the necessity of expensive laboratory setup. Serological tests conducted along with molecular tests do not only confirm the detection of the virus,they also give us crucial information about the course of the immune response as well as the durability of immunity in both symptomatic and asymptomatic individuals. Despite the odds, significant progress has been made in the diagnostic techniques of SARS-CoV-2, and advancements in these techniques still continue. These sophisticated approaches will help us be better prepared for such situations in the future.

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Review article

Nobel Prizes in Science 2020

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Abstract

The Nobel prizes were instituted to recognize discoveries and contributions that benefit mankind. The long tradition of awarding the Nobel Prizes started over a century ago in 1901. Although it was initially for research in the field of Chemistry, it soon came to encompass other areas like Physics, Physiology or Medicine, Economic Science and Peace and Literature. It is the most prestigious award in the fields and carries a citation and cash component of USD 350,000. The main value of the award is the recognition of usually almost a lifetime of dedicated work in the area of research.

In this article, we bring to you the latest awards in the fields of Physiology or Medicine, Chemistry, and Physics to give a glimpse of the work that received recognition.

I.NOBEL PRIZE IN PHYSIOLOGY OR MEDICINE 2020

The Nobel Prize for Physiology or Medicine is awarded by the Nobel Foundation for excellent work and discoveries in the field of Life sciences and Medicine. Even when the world is going through a pandemic, the tradition of awarding the Nobel Prizes continues. It is the right time to celebrate those that have worked hard for the advancement of mankind. This year's Nobel Prize for Physiology or Medicine was awarded to Harvey J. Alter, Michael Houghton and Charles M. Rice for the discovery of Hepatitis C Virus. It was discovered in 1989. (The Nobel Prize in Physiology or Medicine 2020, 2020)

THE PATH TO THE DISCOVERY OF HEPATITIS C VIRUS

Hepatitis was first described by the physician Hippocrates and he called it the"epidemic jaundice". Now, we know that hepatitis can be caused by more than one virus and they affect the liver thus decreasing its function and integrity. Hepatitis can be classified into infectious, autoimmune, ischemic, metabolic, genetic or toxic. Of these, infectious hepatitis are caused by five types of viruses (both RNA and DNA). Infectious hepatitis is the most common type reported all over the world.

Before the viruses were discovered, infectious hepatitis was divided into two types: A and B based on the route of transmission. (MacCallum, 1947). Since then, we have discovered and studied in detail the different types of infectious hepatitis. Two different RNA viruses are responsible for "epidemic hepatitis. The first one belongs to the family *Picornaviridae*and is called Hepatitis A or HAV. The second one belongs to the family *Hepeviridae* and is called Hepatitis E or HEV. These are mainly transmitted through contaminated food and water. They have a very short incubation time and result in an acute illness. Life-long immunity follows recovery. Three different types of viruses cause "serum hepatitis". The DNA virus of *Hepadna* family known as the Hepatitis B virus or HBV with/without RNA virus belonging to the *Deltaviridae* family known as the Hepatitis D virus or HDV or RNA virus of *Flaviviridae* family known as the Hepatitis C virus or HCV. "Serum hepatitis" spreads through contaminated body fluids and has a long incubation period. The individuals infected by this type can spread the virus during the incubation period. The infection can become chronic leading to liver cancer.

Hepatitis B virus or HBV was discovered by geneticist Baruch Blumberg. He observed an unusual reaction between the serum of a multiple transfused patient and that of an Australian aborigine. He was under the impression that he had discovered a new lipoprotein. He was able to prove that the serum from the multiple transfused patients contained a new antigen. (Au Antigen). (Blumberg et al., 1967). He observed that an individual formed antibodies against this antigen and that the serum of all individuals that were affected by post-transfusion contained the same antigen. (Bayer et al., 1968). This resulted in the development of vaccines and tests to diagnose HBV. Until its discovery, serum hepatitis was a health threat and the risk of transmission to patients undergoing surgery was very high. (Chalmers et al., 1971). Baruch Blumberg received the 1976 Nobel Prize for Medicine and Physiology for his discovery of HBV. Even after its discovery and the development of vaccines and tests, individuals that underwent blood transfusions were still affected by chronic hepatitis.

At the start of his career, Harvey J Alter had collaborated with Baruch Blumberg and contributed to the discovery of the Au antigen. Post HBV discovery, a lot of research groups started looking into the HBV infection in blood donors and the development of the infection after transfusion. (Alter et al., 1972; Gocke, 1972; Grady et al., 1972). It was observed that even after removing the HBV antibody positive blood, 80% of the recipients still developed hepatitis. This new form of hepatitis or non-B hepatitis was very common. This non-B hepatitis had a shorter incubation period compared to HBV hepatitis and the former showed milder symptoms during the acute period compared to the latter. (Gocke, 1972). One of Alter's patients developed the non-B hepatitis first and then later developed HBV hepatitis. (Alter et al., 1972). From this he concluded that two different viruses were responsible for the two types of hepatitis. The assumption that the non-B hepatitis could be caused by some antigen of HAV were abandoned after the discovery of HAV. Following the discovery of immune – electron microscopy techniques by Stephen Feinstone and Robert H. Purcell, Alter collaborated with them and examined the serum samples of individuals with non-B hepatitis. They concluded that non-B hepatitis was not caused by HAV or any other known virus. (Alter et al., 1975; Feinstone et al., 1975). The name "non-A, non-B hepatitis" or NANBH was coined shortly after. It was very clear that NANBH was responsible for several post transfusion hepatitis cases but the circumstances were particularly dangerous as the infected individuals presented themselves with no clinical symptoms. There was little to no progress in the identification of the agent

causing NANBH in the following years. Health workers, patients in need of blood transfusions and intravenous drug users were in serious risk. Alter and colleagues succeeded in developing a primate model of infection. They showed that the disease could be transmitted to chimpanzees through blood transfusions. (Alter et al., 1978; Tabor et al., 1978; Hollinger et al., 1978). This helped them to study the infection better. Alter and Purcell identified that this virus contained essential lipids and that it had a diameter of around 30-60nm. (Feinstone et al., 1983; He et al., 1987).

Even after this important breakthrough, the identity of NANBH was still unclear. Michael Houghton started his search for NANBH in 1982 by using cDNA library isolated from the infected chimpanzees. After a couple of unsuccessful approaches, Houghton with the help of Qui-Lim Choo and George Kuo decided to try a novel immune screening approach. They created a cDNA library from the RNA obtained from the plasma of NANBH infected chimpanzees and then transferred it into bacteria using an efficient lambda bacteriophage. They then examined the serum of a patient that had a sudden onset of NANBH for expression of viral antigens. After screening one billion bacterial colonies by this technique, they isolated one colony which did not have chimpanzee or human DNA sequences. (Choo et al., 1989). This sequence was named clone 5-1-1. It hybridised to an RNA of about 10,000 nucleotides. The RNA encoded an open reading frame and showed homology with the genome of other known viruses. Proteins were translated from the RNA strand which showed that the virus contained a positive strand RNA genome. After obtaining the above-mentioned information, it was easy to classify and name the virus. The virus was named Hepatitis C virus or HCV and it became a part of the *Flaviviridae* family. After the identification of HCV, Houghton developed an immunoassay which helped in the detection of HCV specific antibodies and also helped to identify such antibodies in the blood of the donor (Kuo et al., 1989). Alter and Houghton were able to link NANBH and HCV infection. But further proof was needed as the transmission of the infection through contaminated blood did not exclude the role of essential cofactors. In order to demonstrate causality, the virus that could reproduce the same infection had to be isolated from the blood of the infected host. The initial step towards achieving this was taken by a group of Kunitada Shimotohno from the National Cancer Center Research Institute in Tokyo and Charles Rice from Washington University in St. Louis, found in close succession a conserved, non-coding region at the 3'end of the RNA genome of HCV virus which they thought could play an important role in replication (Kolykhalov et al., 1996; Tanaka et al., 1995). Rice made viral genomes containing the above mentioned 3'region and then injected them into the liver of chimpanzees and observed the blood for newly made viruses but failed to find any. With the knowledge that RNA virus replication is error prone and that viral mutations carry inactivating mutations; Rice developed a new set of RNA genomes with both the 3'region and a consensus sequence that would exclude potential inactivating mutations. He then injected this new set into the liver of chimpanzees and this time he was able to observe newly formed viruses. The animals developed the infection with the clinical signs of hepatitis and the virus was found in their blood for a couple of months (Kolykhalov et al, 1997). Hence, Rice proved that HCV alone can cause hepatitis.

This discovery led to the development of antiviral drugs against hepatitis. The clones made by Rice did not replicate properly within cell lines which hindered the study and testing of the virus. This drawback was overcome by Ralph Bartenschlager from the University of Heidelberg in Germany. He created the first HCV sub-genomic clones that replicated in transfected hepatoma cell lines (Lohmann et al., 1999). Following this,

sub genomic replicons were produced that upon transfection into hepatoma cells secreted viral particles that were infectious (Wakita et al., 2005). The second drawback was the narrow hot spectrum of HCV. It only affected humans and chimpanzees. The unavailability of smaller hosts made it difficult to assess the pathological and immunological profile of the infection and also testing of pre-clinical drugs. This was overcome when T and B cell deficient with SCID were grafted with human hepatocytes. (Mercer et al., 2001). The availability of *in vitro* systems that replicated as well as smaller models accelerated the development of antiviral drugs. The generation of drugs that targeted RNA dependent RNA polymerase NS5B, for instance sofosbuvir (Bhatia et al., 2014) and regulatory replicon protein NS5A, for instance ledipasvir was a major breakthrough.

II. CRISPR- Cas9: A pathway for Hope and revolution in Genome Editing and Designer DNA

The dawn of October 7th 2020, brought with it the announcement of the new recipients of the Nobel Prize in Chemistry- Jennifer Doudna and Emmanuelle Charpentier, the first all-female duo to have won the prize for Chemistry. This gave young girls all over the world new **hope** that they are in fact capable of having their dreams manifested and realized perhaps in any field that evoked a keen interest within them. “Ain’t no mountain high enough….”

Undoubtedly, the system and its adapted versions have revolutionized genome editing, making it far simpler, efficient and effective to make edits in genomes. Consequently, a PubMed search of “CRISPR Cas9” revealed how research in the field has increased over the years in various fields (Fig.1.). The tool has been used for research in genome engineering, cancer therapeutics, neurodegenerative diseases and even as a diagnostic tool for research in COVID-19.

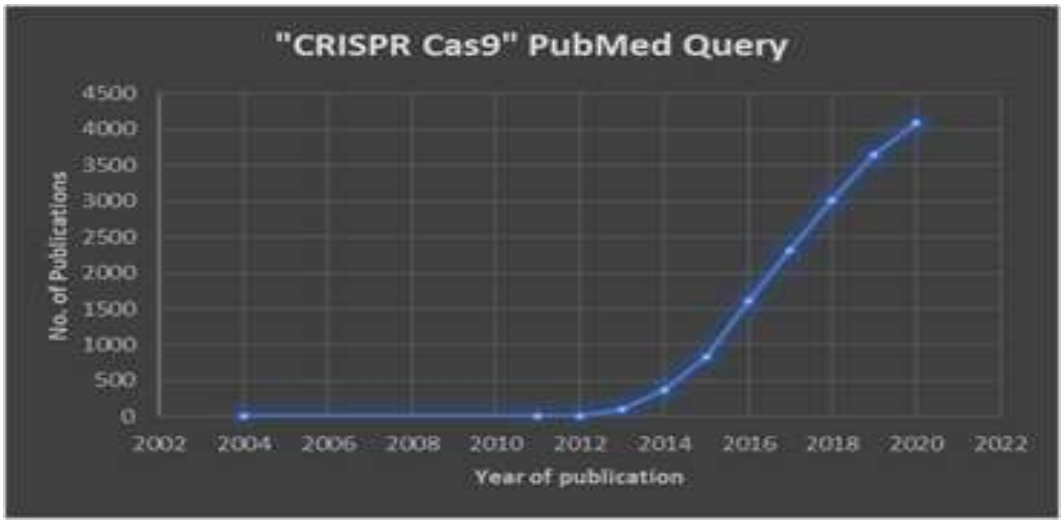

Fig. 1: The search "CRISPR Cas9" on the PubMed search tool provided the number of articles that contained the terms.

The information listed above provokes the question 'what exactly is CRISPR-Cas9?' The term CRISPR stands for 'Clustered Regularly Interspaced Short Palindromic Sequences'. The term was coined in 2002 (Jansen et al., 2002). From the discovery of CRISPR in *E. coli* 1987 (Ishino et al., 1987), to the discovery of their function as an adaptive immune system in bacteria and archaea against invading viruses and plasmids (Barrangou et al., 2007), there have been a lot of small contributions by key players pronouncing many assumptions of the system's function and target of action (Lander, 2016).

The CRISPR/Cas system constitutes the Cas genes which include operons and CRISPR arrays composed of the target sequences, fragments of genomes of foreign, invading pathogens (a.k.a. spacers) distributed with identical palindromic repeats (Wiedenheft et al., 2012). CRISPR system is essentially divided into three classes, with five subclasses (Makarova et al., 2015). The class II system functions with the help of a single protein effector system like Cas9 (Cas: CRISPR associated) and a transactivating crRNA (tracrRNA) (Lander, 2016; Deltcheva et al., 2011).

The adaptive immune system studied in the bacteria, *Streptococcus pyogenes*, functions in three steps: the i) adaptive phase which includes the addition of fragments of foreign DNA of the invading pathogen (protospacers) to the existing CRISPR array of the host bacteria proximally, the ii) expression and interference phases which include the transcription of the repeat-spacer sequence into precursor crRNA. The action of enzymes on it leads to formation of short crRNAs that are complementary to the target sequence within the genome of the pathogen. The silencing of foreign sequences and subsequent degradation is achieved by the crRNA with the help of the Cas9 protein and the tracrRNA.

The tracrRNA contains a complementary sequence to the precursor crRNA (pre-crRNA) and initiates the processing of pre-crRNA by (ds) RNA-specific ribonuclease RNase III, with the help of the Cas9 protein (Jinek et al., 2012). The Cas9 protein consists of two endonuclease domains: the HNH and the RuvC endonucleases. The former is responsible for cleaving the complementary DNA strand while the latter is responsible for cleaving the non-complementary strand (Jinek et al., 2012). The tracrRNA is responsible for pre-crRNA processing and DNA cleavage of invading pathogens as part of the Cas9tracrRNA-crRNA complex. The recognition of the protospacer sequence is mediated by the "protospacer adjacent motif" or PAM. The PAM is situated one base-pair upstream to the protospacer sequence in the pathogen. It usually consists of an NGG sequence containing two G:C base-pairs. The PAM makes it easier for the Cas9-tracrRNA-crRNA complex to trace, recognize and bind to the protospacer sequence whilst scanning through the entire genome of the pathogen. The cleavage takes place 3 base-pairs upstream to the PAM sequence (Jinek et al., 2012).

Notably, the tracrRNA was jointly discovered by Emmanuelle Charpentier and Jörg Vogel. Charpentier was working on the regulatory role of RNAs in microbes when she met and collaborated with Vogel (Lander, 2016).

Charpentier met Jennifer Doudna in March of 2011, whilst she was in Puerto Rico for a lecture on tracrRNA at American Society for Microbiology. At the time, Doudna was working on deciphering the structures of the constituents of the more complex CRISPR I system in *E. coli* (Lander, 2016).

The two formed an alliance and worked on the CRISPR system in *S. pyogenes* in vitro with purified pathogen DNA that could be degraded by customized crRNA molecules within the Cas9-tracrRNAcrRNA complex. They discovered the strand specificity of the Cas9 endonuclease domains (HNH and RuvC) and the working of sgRNA (single-guide RNA) (Lander, 2016).

The sgRNA is a pruned version of the tracrRNA:crRNA complex. The tracrRNA:crRNA complex is 42 nucleotides long with base-pairing observed between 22 nucleotides from the 3' end of the crRNA and the 5' end of the tracrRNA. Two versions of the chimeric RNA were created with a hairpin loop structure between the positions where the base-pairing between the two occurred. The longer chimeric RNA was found to function efficiently. Furthermore, the efficacy of five additional pruned tracrRNA:crRNA complex versions was checked against a plasmid in vitro (Jinek et al., 2012).

Their entire work has established a frontier for a new era of "**designer DNA**". However, Doudna explains there being a need for scientists to use the technology with caution and keep ethical issues in mind (Doudna & Charpentier, 2014). The use of CRISPR to create twins with edited genomes has already sparked a major controversy and the debate for essential therapy v/s enhancement therapy seems imperative now more than ever.

Nevertheless, the quest for science will continue always. "My wish is that this will provide a positive message to the young girls who would like to follow the path of science, and to show them that women in science can also have an impact through the research they are performing." ~Emmanuelle Charpentier on receiving the news of her win (Rincon, 2020). The thought is destined to inspire a vision among young girls.

III. THE SUPERMASSIVE OBJECT AT THE CENTRE OF OUR GALAXY: BLACK HOLE

The Royal Swedish Academy of Sciences, Sweden has awarded the Nobel Prize in Physics 2020 to three laureates this year. The first laureate this year is Roger Penrose for his discovery based on "Black hole formation is a robust prediction of the general theory of relativity." Reinhard Genzel and Andrea Ghez have been jointly awarded the prize for their work on "The discovery of a supermassive compact object at the center of our galaxy" (The Nobel Prize in Physics 2020, 2020).

Roger Penrose proved that the general theory of relativity leads to black holes formation, by using mathematical methodology in the proof. A black hole is a region of spacetime, wherein the gravity is very strong. It can capture anything and no particle can escape from it (The Nobel Prize in Physics 2020, 2020).

Reinhard Genzel and Andrea Ghez have studied a region, 'Sagittarius A', located at the center of our galaxy, the Milky Way. The measurements of the groups led by these two scientists have found an extremely heavy and invisible object that forces the stars to swirl around. According to the current theory of gravity, this object is a supermassive black hole, which makes these stars swing around at great distorted speeds, at the center of our galaxy.

Black holes are known to hide a 'singularity'. A singularity is a boundary, at which all the known laws of nature cease to exist. The question regarding the existence of black holes was brought back in 1963, with the discovery of 'quasars', which are known to be the brightest objects in the Universe (Genzel et al., 2010). Penrose had an idea that he called as "trapped surfaces", which was a mathematical tool he used to describe a black hole. Penrose, with the help of trapped surfaces, was able to prove that a black hole always hides a 'singularity', which is a boundary where time and space are known to end. Even though the black hole cannot be seen, it is possible to establish its

properties, by observing how its colossal gravity is able to direct the motions of the surrounding stars (The Nobel Prize in Physics 2020, 2020).

Genzel and Ghez used the orbits of the stars as guides, and were able to show the presence of an invisible supermassive object at the center of the Milky Way. Genzel and his team used the VLT ((Very Large Telescope) facility on Paranal mountain for imaging. Ghez and his team used the Keck Observatory, in Hawaiian mountain of Mauna Kea. Telescopes that were used were equipped with an extra thin mirror, for compensating for the air's turbulence, and thereby correcting the blur images. They have tracked around thirty bright stars in the multitude. They were able to map the entire orbit of a star, called S2, which is known to complete an orbit of the center of galaxy in around 16 years. Over the years, using better adaptive optics, the image resolution has improved by at least a thousandfold. Evidence has shown that a supermassive black hole is hiding in Sagittarius A, and is estimated to weigh about 4 million solar masses, and is packed into a region of the size equivalent to our solar system (Abuter et al, 2018)

Penrose has shown that black holes are formed, as a direct consequence of the general theory of relativity. However, the theory ceases to apply in the infinitely strong gravity at the 'singularity' of the black holes. Intensive research is being conducted in theoretical physics, for creation of a new theory of quantum gravity. The need of the hour is uniting theory of relativity and quantum mechanics, which meet inside the black holes (Genzel et al., 2010). The pioneering work done by Genzel and Ghez, has provided the framework for testing the general theory of relativity, and thereby have provided insightful clues. This year's discoveries have provided the basic framework for future research pertaining to compact and supermassive objects, in the domain of their inner structural organization. This research has led to questions on how to test the theory of gravity under extreme conditions in the immediate vicinity of a black hole.

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Review article

Women in Science: A Crack in the Glass Ceiling

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Abstract

Women have made crucial contributions to the field of Science since time immemorial. But for a large part of history, their scientific accomplishments have remained either largely concealed or understated. The present review summarizes the history of women in Science, and their imperative discoveries and achievements, unfolding from ancient times and culminating in the present-day 21st Century. In addition, it highlights the difficulties faced by women in their strife for scientific inclusivity. It concludes by spotlighting a few recent changes in society in the form of attitudes and policies and emphasizes the need to acknowledge women's triumphs in the field of science.

"I hadn't been aware that there were doors closed to me until I started knocking on them."
-Gertrude B. Elion

Gertrude B. Elion was a 20th Century Nobel Prize-winning chemist who was denied a job because the authorities thought a woman in the laboratory would be a "distracting influence". Now women in the STEM fields may not find the same doors closed for them, but there are plenty still left to be knocked upon.

To realize where women stand in the current scientific setting it is essential to know their journey through history.

A Brief History of Women in Science

A quick internet search about the "first woman scientist" will direct you to the enigmatic name of *Merit-Ptah,* a purported ancient Egyptian doctor dated back to 2700s BC. The name was popularized by the second wave of feminism which tagged her as the "First Female Physician". However, the existence of Merit-Ptah is dubious due to a prominent lack of historical evidence corroborating her place in the Old Kingdom. But the legend of Merit-Ptah was inspired by the true account of Peseshet, who was named the "Overseer of Woman Physicians" (Kwiecinski, 2020). The egalitarian Egyptian civilization also saw the advent of genius women such as Queen Hatshepsut (1458 BC), who was passionate about botany and encouraged botanical excursions through her trade routes making Egypt the torchbearer of phytomedicine (Bernardi, 2016).

In the 4th Century BC, Hypatia of Alexandria (a part of Egypt under the rule of the Roman Empire,) emerged as a scientifically gifted woman who taught math, astronomy, and philosophy. Considered the best mind in mathematics

and astronomy in her time, she is also credited as the commentator of pioneering works in mathematics such as Apollonius of Perga's Conics (Geometry) and Diophantus of Alexandria's Arithmetic (Number Theory) (Richeson, 1940). TapputiBelatekallim, a 13th Century BC perfume-maker in Mesopotamia, Ancient Babylonia is recorded as the world's first chemist. She was the inventor of the swan-neck still used till this day to distill cognac (Kass-Simon et al., 1993). The world's first known woman astronomer was perhaps EnHedu'anna, a 23rd Century BCE Akkadian priestess of Ur (modern-day Iraq). She was tasked with keeping a written account of astronomical calculations and observations, a formidable duty since writing had been recently developed. She was also the creator of calendars still used to date religious holidays (Howard, 2017). In Greece, women came to the forefront of the intellectual community as mathematicians like Agnodike, and physicians such as Algonike.

We remember these names with pride, but the truth is the occurrence of women scientists (they were known as astronomers, physicians, mathematicians, philosophers, and priestesses) in these ancient civilizations wasn't an anomaly. It is only in retrospect we attribute importance to this phenomenon as we consider progress to be a linear event. In reality, these ancient civilizations had achieved a level of egalitarianism that has long since been lost with the advent of the dark ages.

As the last lights of the Ancient Civilizations fizzled into the darkness, the number of women scientists dimmed along with them (Mark, 2019). The sole way a woman could take autonomy into her own hands during the Dark Ages was by entering the nunnery. One such nun, Hildegaard of Bingen was one of the most prominent women of science during the medieval period. She wrote treatises on medicine and natural history which are often referred to as the most comprehensive scientific works of the Middle Ages. Although the Renaissance is considered a period of intellectual loftiness, it was a time during which women's participation in science dwindled. The outbreak of the bubonic plague, the ongoing Hundred Years' War, and the infamous Salem Witch Trials were all reasons responsible for the decrease in scientific pursuit during the 14th and 15th Centuries (Herzenberg, 1990).

Nonetheless, amidst this time the Islamic State flourished with achievements and discoveries in arts, sciences, architecture, and technology. This came to be known as The Golden Age of Islam. The government at the time heavily patronized scholars, and their compensation can be compared to that of professional athletes of current times (Falagas et al., 2006). Islam did not impose any restrictions on female education and numerous Islamic women were scientifically successful. One such woman of science was Fatima al-Fihri, who inherited her father's assets and used them to establish the Qarawiyin Mosque in 859, making it presumably the first University in the world. It garnered students from all across the world and produced scholars in various fields such as astronomy, languages, sciences, and Islamic Studies. It was responsible for popularizing Arabic numerals throughout Europe (Siddiqi, 2018).

The 17th Century saw the Age of Reason paving the way for scientific discoveries and progress (Bristow, 2010). A turning point for science during this era was the establishment of various prestigious scientific institutions such as The Royal Academy of Sciences, London in 1660, The French Academy of Sciences, Paris in 1666 and Akademie der Wissenschaften, Berlin in 1700. But it would be a couple of centuries before women found themselves to be members of these conventions (Schiebinger, 1987). Throughout the world, fallacious ideas about women's corruptness, frailty, intellectual inferiority, and irrationality persisted. Women continued to be denied autonomy and legal rights. Even so, women subsisted their scientific endeavors, rising above the tide of discrimination and suppression.

Margaret Cavendish (1623-1673) is the most prominent name that comes up when discussing the limited scene of women scientists of the 17th Century. Cavendish was a natural philosopher, and her version of naturalism is expounded by scholars even to this day. She wrote multiple treatises on complex subjects such as the true nature of perception, intelligent cause, the limits of words, ideas, and language, and even on the mechanisms of transfer of motion between bodies. She was highly involved and influential in the topics defining the intellectual life of 17th Century philosophers, being on par with her more well-known male counterparts the likes of Thomas Hobbes and David Hume (James, 1999).

In the 18th Century, the participation of women in science was still a rare privilege reserved for those belonging to high society. Out of all the astronomers working in Germany in the 1700s, 14% of them were women. Maria Winkelmann (1670-1720), a German astronomer, was the first woman astronomer to ever discover a comet, though the credit for it was given to her husband, the Royal Astronomer at the time. Winkelmann petitioned to become a member of the Berlin Academy of Sciences as an assistant astronomer but was denied admission due to her sex which set the tone of exclusivity these institutions desired to maintain against women. It would be two centuries before the first woman scientist would be inducted into the academy (Schiebinger, 1992). Laura Bassi (1711-1778) was an 18th Century Italian physicist who became the first female member of the University of Bologna at the young age of twenty. She was heavily inspired by Newtonian physics, and publicly defended her thesis to achieve her degree of doctor of philosophy. Bassi became the first woman to become a professor in Europe, and in 1776 became the first female Chair of Physics in the university of Bologna (Findlen, 1993). The first woman to have her dissertation published by the French Academy of Sciences was the French mathematician and physicist Emile du Chatelet. She was also responsible for the first and only French translation of Newton's pioneering work, Philosophiae Principia Mathematica (Reichenberger, 2018).

Previously, *scientists* used to be called "cultivators of science". It was in 1834 that William Whewell coined the term "Scientist" to refer to Mary Somerville, a Scottish polymath with the sobriquet of "Queen of Science"; when he was reviewing one of her works. In 1835, Mary Somerville, and Caroline Herschel, a German astronomer, were the first elected female members of the Royal Astronomical Society (Patterson, 1969). Mary Somerville's tutelage Ada Lovelace was an English mathematician who translated an article on Charles Babbage's Analytical Engine. The notes she added to her translation was later used to construct the first-ever computer. Hence, she's often credited as the first computer programmer (Callaghan et al., 2020). Astronomer Maria Mitchell is considered to be America's first female scientist of note who went on to become the director of Vassar's Observatory in 1865 (Kohlstedt, 1978).

In the 19th Century India too witnessed the emergence of its women scientists to the foreground. Kadambini Ganguly and Anandibai Joshi were the first female physicians to hail not only from India but the entire British Empire (Karlekar, 2012). Dr. Rupa Bai Furdoonji gained acclaim for becoming the first female anesthetist in the world. She also obtained a medical degree from Johns Hopkins University, Baltimore (Ala et al., 2010). These were the first few women to ever receive a degree in India. Mary PoonenLukosewas an obstetrician, gynecologist, and the first woman Surgeon General of India (Mohindra, 2015).

The Nobel Prizes were established at the dawn of the 20th Century, in 1901. Marie Curie became the first woman awardee in 1903, and the first to be awarded the Nobel Prize twice and in two separate fields in 1911. Curie was still not inducted into the French Academy of Sciences in the very year she received her second

Nobel Prize. Instead of recognizing her scientific prowess the academy instead chose to "respect the immutable tradition against the election of women" (Schiebinger, 1987). It wouldn't be until almost seventy years later, in 1979, when the gates of the French Academy of Sciences would open for women, with Yvonne ChoquetBruhat becoming the Academy's first female member. Bruhat, a brilliant mathematician and physicist, went on to become the President of the International Committee of General Relativity and Gravity, from 1980 to 1983 (Jones & Hawkins, 2015).

In 1902, Inventor Hertha Ayrton was denied fellowship to the Royal Society on the grounds of being married (a criterion exclusive for women). She was the first woman to present her paper, "The Origin and Growth of Ripple Marks", before the Royal Society in 1904. Ayrton also received the prestigious Hughes Medal in 1906 for her contributions to the understanding of electric arcs. Since its conception in 1902, only two women have been awarded the Hughes Medal (Mather, 1923). The consequent Sex Disqualification (Removal) Act of 1919 ruling no person should be discriminated against on the grounds of sex and marriage from being admitted to any society facilitated the entry of women into the Royal Academy (UK Government Legal Department, 2019). This revolutionary act led to the induction of Kathleen Lonsdale, crystallographer and discoverer of the flat structure of benzene, and Marjory Stephenson, prolific biochemist, as the first women to be members of The Royal Society in 1945 (Jones & Hawkins, 2015).

A total of eleven women received the Nobel Prize in the 20th Century, the most prominent of them being Barbara McClintock (discoverer of transposons, also known as "jumping genes"), Gerty Cori (who discovered the catalytic conversion of glycogen, known as Cori Cycle), Dorothy Hodgkin (pioneered X-ray Crystallography leading to the discovery of molecular structures) (Modgil et al., 2018). But there were many more female scientists whose accomplishments were buried under the privileged positions their male counterparts held. The term "The Matilda Effect" was coined by Historian of Science Margaret Rossiter to define the lack of prominence of women scientists in history. The name came from the 19th Century suffragette Matilda Josely Gage who elaborated on this ignorance of the scientific community towards the innovations of women in her paper "Woman as an Inventor (1893)" (Rossiter, 1993).

Dr. Lise Meitner, a prominent nuclear physicist and the first woman to be inducted into the Berlin Academy of Science discovered nuclear fission but never received credit for it. Instead, her colleague of thirty years, Otto Hahn took the accolades for the discovery and even received the Nobel Prize in 1944 (Modgil et al., 2018). African-American Chemist Alice Augusta Ball pioneered the treatment for leprosy but died at the young age of twenty-four before her discoveries could attain publication. The treatment, known as the "Dean Method" was wrongfully named after the University President Dr. Arthur Dean, who was Ball's colleague and took credit for her work (Nortman, 2020). Esther Lederberg's crucial invention of the Replica Plating technique and her discovery of the lambda phage set the stage for her husband Joshua Lederberg's Nobel Prize (1958) winning work of transmission of bacterial genetic material. However, Esther never received her deserved recognition (Loeb, 2018). Jocelyn Bell Burnell too was denied her rightful ovation when her discovery of radio pulsars forever changing the field of astrophysics was accredited to her thesis supervisor Antony Hewish, who won a Nobel Prize for the same in 1974, even though Burnell was a co-author in the very article in Nature that led to his nomination (Modgil et al., 2018).

Rosalind Franklin, however, remains the most notable victim of the Matilda Effect. A deft x-ray crystallographer, she was responsible for discovering the molecular structure of DNA. Her vision and understanding of molecular science even

led her to make scientific predictions about the helical structure of DNA and the manner of storage of genetic traits. The now-famous picture of the DNA molecule she isolated, known as Photo 51, was obtained by Francis Crick and James Watson without her knowledge and used as evidence to put forth their claims about the DNA structure that ultimately won them their Nobel Prize four years after Franklin died in 1958 at the age of thirty-seven (Modgil et al., 2018).

At the advent of the 1900s, India experienced a spurt in the education of girls. Prominent freedom fighters and social activists advocated for the education and emancipation of women. The years to come saw some of the first Indian women achieving higher education and degrees in science. Dr. Janaki Ammal, a prolific botanist, holds the prestige of being the first woman to attain a Ph.D. in Botany in the US in 1931. She created the crossbreed sweet-sugarcane hybrid enabling India to become self-sustained in its sugarcane production (Subramanian, 2007). Dr. Asima Chatterjee, an organic chemist, carries the title of the first woman to be awarded a Ph.D. from an Indian University (The University of Calcutta) in 1944. She was the first female recipient of the Shanti Swarup Bhatnagar Award in Chemical Science; and the first woman to become the General President of the Indian Science Congress Association (INSA, 2007). Anna Mani was a physicist from Kerala who worked under C. V. Raman as an undergraduate. Even though she had years of research experience she was denied a Ph.D. on the basis that she did not have a Master's degree (this was at a time when Ph.D.'s were conferred based on research experience as well). She went on to study at Imperial College, London, and was named the "Weather Woman of India" (Sur, 2001). Dr. Kamala Sohonie was an Indian biochemist, who became the first woman to receive a Ph.D. from a British University (Cambridge University), in 1939. Before her Ph.D., the vivacious scientist had applied to the Indian Institute of Science (IISc) for a research fellowship under C. V. Raman. At the time, Raman was against women entering his University and turned down Sohonie's application. But not one to give up easily, Sohonie, a devout Gandhian, performed Satyagraha in front of Raman's office until he conceded and allowed Sohonie's entry into the Institute under the condition that she would have to be on a year's probation. After her Master's she was invited to Cambridge University to continue her research. Here she discovered the presence of cytochrome-c in all plant tissues which earned her a Ph.D. It was her success that convinced Raman to open the doors of IISc to women. Sohonie also went on to become the first female Director of the Institute of Science, Bombay (Mitra, 2016).

It has been only but twenty years into the 21st century, and women have made leaps and bounds in the field of science. There have been 12 women who have won the Nobel Prize in sciences in this century, 2018 being the first year women received the prize both in Chemistry and Physics and again in 2020. In 2006, Frances E. Allen became the first female recipient of the Turing Award, the Nobel equivalent in Computer science (Finkelstein, 2007). Maryam Mirzakhami won the Fields Award in 2014, which is the Nobel equivalent in Mathematics, and became the first and only woman to receive it to date (Rafi, 2017). In 2019, ChandrimaShahaholds the title of the first woman president of the Indian National Science Academy (Basu, 2019).

The second wave of feminism had pushed and campaigned for the equality and education of women causing more women to pursue higher schooling and degrees, serving to bridge the gender gap in various scientific fields. Nevertheless, there is still a gross underrepresentation of women in STEM fields. According to the UNESCO Institute for Statistics (UIS), women accounted for less than 30% of researchers in the world. In India, less than 15% of total researchers are women, one of the lowest percentages of women in science in the world. Myanmar ranks the highest in women representation in science with more than 80% of women in the research field. Countries such as Azerbaijan,

Thailand, and Georgia are a select few wherein there is a higher percentage of female researchers in comparison to males. On the other hand, developed countries including North America and Western Europe report around 30% of female researchers (UNESCO Institute for Statistics, 2019).
The ongoing pandemic is a stark reminder of the gender gap omnipresent in all organizations of our society as well. The global health workforce is predominantly composed of women (above 70%) despite which only about 25% of women are appointed to significant decision-making positions (Daalen et al., 2020).

The Gender Bias Prevalent in STEM Fields

The gender-bias in science can be traced back to Aristotle's argument about women being "weaker" and "colder" which deprived them of the heat required to "purify one's soul". In the late 18th and early 19th centuries, craniologists tried to falsely equate cranium volume with intelligence. Anatomists believed larger cranium size of men signified their greater intelligence. The mid-19th century brought in social Darwinists who used evolutionary biology to incorrectly argue that women have stunted mental abilities. Even in the 1920s and 1930s "hormonal research" was being used in hopes of proving women as being the "inferior sex" (Fausto-Sterling, 1992).

All of these dubious claims are heralded under the term "Biological Determinism". This school of thought holds men to be more adept at scientific endeavors because they made up the major chunk of neolithic hunters in the prehistoric African Savanna, and had hence evolved "better brains" with higher spatial intelligence, which was equated to the higher performance of boys in mathematics. Ruth Hubbard, the first woman to attain tenure in biology at Harvard University, challenged this claim of "innate gendered behavior" in her essay "Have only men evolved?" where she put forth the point that in all these gendered studies, none of the scientists took into account other variables such as the environment, society, and culture each gender grew up and developed in. She went on to elaborate on the "political nature" of humankind, which states that people belonging to different races, classes, and genders aren't exposed to equal opportunities and resources, which influence their consequent behaviors (Benderly, 2016).

The term "neurosexism" is given to the distorted practice of claiming the existence of innate differences between male and female brains. These thoughts pigeonhole women into specific roles, which leads to the propagation of "stereotype threat" which is the widespread anxiety felt by people who feel suffocated and helpless by the constraints of the stereotypes they are perceived by. The effect of this kind of anxiety serves to enhance the hindrance of women towards entering STEM fields. This hindrance and the consequent under-representation of women is seen to be more apparent in fields such as computer science, mathematics, and engineering, as opposed to social sciences or life sciences (Rippon, 2016; Fausto-Sterling, 1992).

This chasm in gender representation is counterintuitively seen to be more in countries with impressively high gender-equalities. This phenomenon is known as the "Educational-Gender-Equality Paradox". Finland is a classic example of this paradox, being one of the countries excelling in gender-equality (with a Gender Inequality Index (GII) of eight), but also having one of the largest gender gaps in collegiate STEM degrees, with less than 25% of STEM graduates being women. Women from more gender-equal countries are less likely to pursue science, even when showing high performance and high self-efficacy in the subject when compared to women from less gender-equal countries. One hypothesis states that since less gender-equal societies tend to have less financially secure economies, this pushes women into STEM careers due to their high pay-off (Stoet& Geary, 2018).

On the other hand, the discrepancy noticed between the positive performance

and attitude shown by girls in school towards STEM subjects, and the gender statistics of STEM careers in developed countries could be a reflection of the deep-seated societal biases about the sexual differentiation of the brain. These biases are known as "Gender Schemas" and are non-conscious beliefs about characteristics that are distinctly "male" or "female". Most studies about gender and brain are carried out taking these beliefs at face value and do not strive to uncover their origin and pervasiveness. This helps create a more strongly polarized society that categorizes men and women into gender roles, which in turn influences their behaviors (Stoet& Geary, 2018; Valian, 2005).

To counter these biases, neuroimaging experiments have been conducted which depict substantial variability to be prevalent in brain scans of all genders. In a study conducted using the brain scans of 169 females and 112 males, only 6% of brains were observed to be consistent with the prevalent gender norms (i.e. males being instrumental, and task-oriented; and females being communal, nurturing, and expressive). These results proved to be highly reproducible in much larger cohorts. In this particular study, the human brain was noticed to be non-dimorphic, with vast overlaps between "male" and "female" brains. This further drives home the point that gender differences aren't due to biological determinism and instead may be more attributable to environmental factors. Most human beings are deemed to possess a "mosaic" of different personality traits, attitudes, interests, etc., (Joel et al., 2015).

Reforms and Restitutions

Currently, the difficulties faced by women in science include the "masculine culture" of certain fields such as those of mathematics, engineering, and computer science, the harassment they face on account of their gender, and the perennial wage gap (Somerville & Gruber, 2020).

The creation of a gender-imbalanced atmosphere causes women to feel alienated, which in turn becomes the basis of their hesitation to pursue STEM fields. When such young, impressionable women are presented with a plethora of women scientists who have beat all odds and made it to the top of their fields (such as women Nobel Laureates) it becomes a source of inspiration and confidence, which help them feel like they "belong" in science too. To keep a track of such biases specifically in the field of neuroscience, the website BiasWatchNeuro rates the level of equality in gender representation in various seminars and conferences (Somerville & Gruber, 2020).

Harassment is a grave area of concern in the mission to make science more accessible to women and often leads to many talented women being compelled to leave the field. In a 2018 report on sexual harassment by the National Academies of Sciences, Engineering, and Medicine (NASEM), 20 to 50% of women reported having faced harassment from faculty members and colleagues (National Academies of Sciences, Engineering, and Medicine, 2018).

A 2017 Annual Census by the US National Science Foundation calculated a wage gap of eighteen thousand dollars between prospective salaries of men and women who have recently obtained their Ph.D. (National Science Foundation, 2018). The origin of the wage gap, especially in scientific fields, can be attributed to the age-old tradition of marginalizing the efforts of women scientists and their contribution towards the progress of science. This peripheralization of women was termed "Little Lady Syndrome" by sociologist Barbara Reskin. Women in science have historically always been referred to as "assistants" or "support personnel", which automatically relegated them to a rank lower than that of male scientists, and hence put their work at a lower monetary value (Reskin, 1993).

Recent attempts to remedy the gender bias in Science

There have, however, been numerous schemes and policies targeted towards helping women flourish in STEM fields. Women Scientist Schemes (WOS) is a set of such policies inaugurated by the Department of Science and Technology (DST) in 2002-2003. This scheme is particularly intended for facilitating the entry of women scientists belonging to the age-group of 27-57 years who have had to take a "break in career" back into the mainstream profession. There are three policies (WOS A, B, and C) under the banner of WOS which provides grants to women scientists, and are categorized based on basic research in applied or engineering fields, technological research for societal benefit, and self-employment (Ministry of Science and Technology, 2020). In 2020, DST drafted a new Science, Technology, and Innovation Policy mandating equal representation of women in science named Gender Advancement through Transforming Institutions (GATI). The program works by requiring the participating institutions to analyze their data on gender equity (which includes gender gap, the wage gap, reports of harassment, and unequal representation) and systematically address any issues that may arise. The institutions are consequently accredited by bronze, silver, or gold standards (Ministry of Science and Technology, 2020).

The women scientists mentioned here do not even form the tip of the iceberg that is the contribution of women in the progress of science and technology. Here, their achievements and trials are mentioned fleetingly, owing to the constraints of a journal article. These women were passionate, fiery, and zestful about the pursuit of science, standing tall and bold in the face of prejudice and humiliation. Every one of these women deserves the recognition and accolades they have been denied in their lifetime. It is up to us to write their names back into the history, present, and future of science, where they truly belong.

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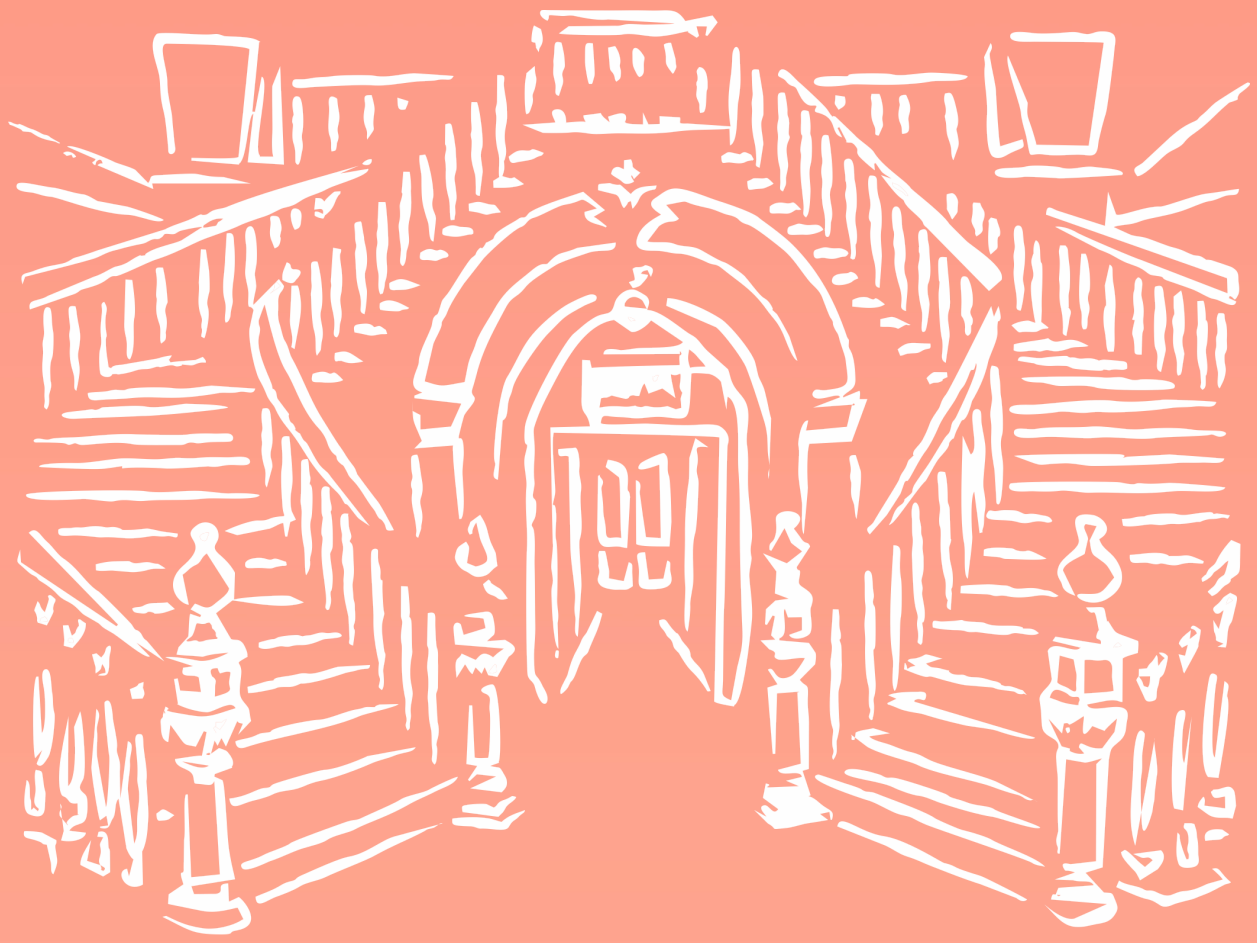

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