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Effect of molecular crowding on native Cytochrome C: A Time-Dependent study

Neha Kausar Ansari, Imtiaz Ahmad, Gufran Ahmed Siddiqui, and Aabgeena Naeem *

Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202002, Uttar Pradesh, India

** Corresponding author at: Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202002, Uttar Pradesh, India.*
e-mail: anaeem.bc@amu.ac.in (A. Naeem).

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ABSTRACT

The interior of the cell is crowded with various types of macromolecules that can effectively interact with proteins and alter their native conformation, consequently resulting in protein aggregation. Protein aggregation has been linked to various pathological conditions such as Alzheimer's and Parkinson's disease. In this study, we analyze the effect of macromolecular crowding on the native structure of Cytochrome C using polyethylene glycol of different molecular weights (PEG 4000 and PEG 6000) at a constant concentration of 200 mg/mL. Time-dependent conformational alterations were analyzed over a 32-hour incubation period at room temperature using turbidity, thioflavin T fluorescence (ThT), Soret absorption and fluorescence microscopy. The notable increase in turbidity at 350 nm suggested crowder-induced aggregation. Increased ThT fluorescence further confirmed the formation of amyloid-like fibrillar assemblies in the presence of PEG. Furthermore, the kinetic analysis revealed a nucleation-dependent mechanism of cytochrome C aggregation, specified by an initial lag phase of 8 hours, followed by a rapid growth phase, and finally a saturation phase at 32 hours, marking the presence of mature fibril-like structures. The red shift of 4 and 9 nm in the presence of PEG 4000 and PEG 6000, with increased Soret absorbance, confirmed the exposure of the heme group to the solvent as a result of structural distortions. Fluorescence microscopy confirms the formation of fibrillar assemblies by direct visualization, with a more pronounced fibrillation in the presence of PEG 6000. Altogether, these results exhibit that macromolecular crowding alters the native structure of cytochrome C and drives the protein toward fibril formation, suggesting a stronger aggregation-promoting effect of higher molecular weight crowders along with increased incubation time. Therefore, this study emphasizes the importance of the size of the crowding agent and the time of incubation in promoting the conformational perturbations of globular proteins, providing insights into protein aggregation in the crowded microenvironment of the cell.

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1. Introduction

The space inside the cell is crowded heavily due to various available macromolecules such as proteins, nucleic acids, carbohydrates, lipids, *etc*. All these molecules in combination contribute to the crowded intracellular environment [1]. A substantial fraction (10-40%) of the intracellular space is occupied by these macromolecules, which confines the volume available to other macromolecules present [2,3]. The concentration of biomolecules reaches up to 400 g/L, leading to a crowded intracellular environment known as macromolecular crowding [4]. No single molecular species is present at an extremely high concentration, but various macromolecules and cell organelles physically occupy a significant proportion of the volume within the cell [5]. Macromolecular crowding plays a significant role in affecting the native structure of proteins, thus increasing the rate and extent of protein aggregation and fibril formation [6-8]. A variety of different molecules have been used as potential macromolecular crowding agents: proteins (hemoglobin and bovine serum albumin), polysaccharides (Dextran) and synthetic polymers such as polyethylene glycol (PEG) or Ficoll, to study aggregation under *in vitro* conditions [9-11]. The crowding agents are chemically inert and tend to provide natural crowded environments [12]. The addition of inert macromolecules (crowding agents) enhances interaction, increases the reaction rate, or shifts the equilibrium to the association.

Cytochrome C (Cyt-C) is a small globular protein that serves as an electron carrier in the inner mitochondrial membrane, thus being crucial for respiration [13]. It is a highly conserved protein consisting of around 104 amino acid residues in a single chain and has been found to play a significant role in apoptosis. It consists of both α-helices and β-sheets. It has been used as a model as it is evolutionarily conserved, small in size, and has a significant fraction of α-helix to study α-to-β transformation [14]. It possesses a heme prosthetic group, which allows it to be studied through a variety of spectroscopic techniques.

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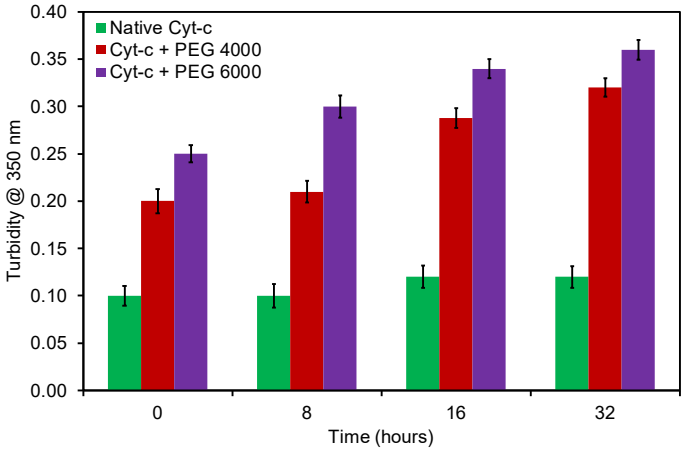


Figure 1. Turbidity assessment at 350 nm of Cyt-C in the presence and absence of 200 mg/mL PEG 4000 and PEG 6000 at different time intervals (0-32 hours).

This study aims to analyze how the macromolecular crowding affects the native structure and stability of Cyt-C, mimicking the cellular crowded environment under *in vitro* conditions. The objective of the study is to explore the time-dependent effects of polyethylene glycol, particularly PEG 4000 and PEG 6000, on the conformation of the globular protein, Cyt-C, by incubating it with and without PEG 4000 and PEG 6000 for different time intervals (0-32 hours) at room temperature. The macromolecular crowder was kept constant, *i.e.*, 200 mg/mL.

The macromolecular crowding is a fundamental aspect of the intracellular environment, where high concentrations of biomolecules can crucially alter protein folding, stability, and dynamics compared to diluted in vitro conditions. Therefore, it is important to understand how Cyt-C behaves in a crowded environment, as it plays a central role in the electron transport system and programmed cell death. Macromolecular crowding-induced conformational alterations could potentially influence its structure, facilitating its aggregation, consequently hindering its biological function. By incubating Cyt-C with PEG 4000 and PEG 6000 as inert crowding agents at a fixed concentration, this study provides information on the possible effects of crowder size on conformational behavior over time. This finding will help narrow the gap between *in vitro* biochemical studies and *in vivo* cellular conditions and the potential role of crowding in protein dysfunction.

2. Experimental

Bovine heart cytochrome C (Cyt-C), sodium phosphate monobasic, and sodium phosphate dibasic were purchased from the Sisco Research Laboratory (Mumbai, India). Polyethylene glycol 4000 (PEG 4000), polyethylene glycol 6000 (PEG 6000), and thioflavin T were bought from Sigma Chemicals Co. (St. Louis, MO, USA).

2.1. Preparation of samples

The Cyt-C stock solution (10 mg/mL) was prepared in a 20 mM sodium phosphate buffer of pH = 7.4. The protein was dialyzed overnight against the same buffer, and the concentration was determined using the molar extinction coefficient of 106,100 M^{-1} cm^{-1} at 410 nm on a Shimadzu UV-1900 spectrophotometer [15]. The working solution of Cyt-C (3 mg/mL) was prepared by diluting the stock solution with sodium phosphate buffer, pH = 7.4. The stock solutions of PEG 4000 and PEG 6000 were also prepared in sodium phosphate buffer. Cyt-C samples were incubated for different time intervals, *i.e.*, 0, 8, 16, and 32 hours in the presence and absence of 200 mg/mL PEG 4000 and PEG 6000 at room temperature. All experiments were carried out three times separately, and the samples were analyzed for aggregate formation using various biophysical assays.

2.2. Turbidity analysis measurements

Proteins, when they lose their native functional conformation, exhibit turbidity. The turbidity of protein formulations increases with an increase in particle size, concentration, and insoluble aggregates. It is used as an indicator for the detection of protein aggregates and fibrils. An increase in absorbance at 350 nm would indicate the presence of aggregates. Absorption was recorded on a Shimadzu UV-1900 spectrophotometer. The path length of the cuvette is 10 mm. The turbidity of Cyt-C in the absence and presence of PEG was determined by monitoring the change in absorbance at 350 nm.

2.3. Thioflavin T-binding assay

Thioflavin T (ThT) is a fluorescent molecule that preferentially binds to protein fibrils. ThT was added to the aliquots in the absence and presence of PEG 4000 and PEG 6000 at 16 hours. Furthermore, the fibrillation kinetics of Cyt-C in the absence and presence of PEG 4000 and PEG 6000 were monitored by measuring the fluorescence of the samples at different time intervals, *i.e.*, 0, 8, 16, and 32 hours. Protein samples were excited at a wavelength of 440 nm and emission was recorded in the range 460-600 nm using a Shimadzu RF 5301 spectrofluorometer. After adding the dye, the aliquots were kept in the dark for about 30 minutes at room temperature. The protein to dye used in the experiment was 1:3.

2.4. Soret absorbance spectroscopy

Soret absorbance studies were performed to analyze the conformational state of the heme protein. The absorbance was recorded in the range between 350 and 550 nm on a Shimadzu UV-1900 spectrophotometer, using a 1 cm path-length cell.

2.5. Fluorescence microscopy

ThT was added to Cyt-C aliquots with and without different crowding agents, *i.e.*, 200 mg/mL PEG 4000 and PEG 6000, incubated for 16 hours, and then incubated in the dark for 30 minutes at room temperature, washed efficiently and viewed under a fluorescence microscope having a 20× oil immersion objective.

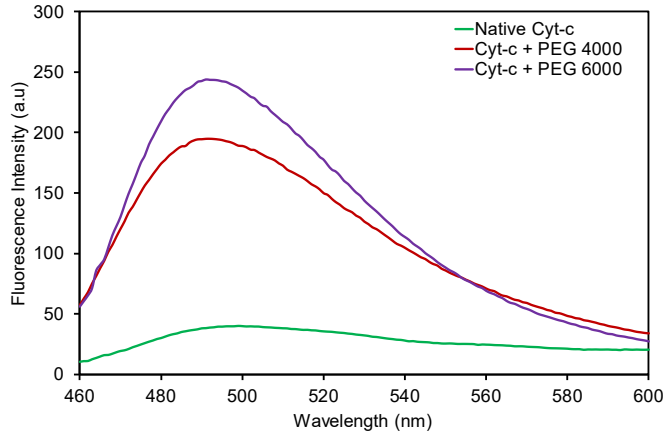


Figure 2. Cyt-C thioflavin T fluorescence in the presence and absence of 200 mg/mL PEG 4000 and PEG 6000, incubated for 16 hours.

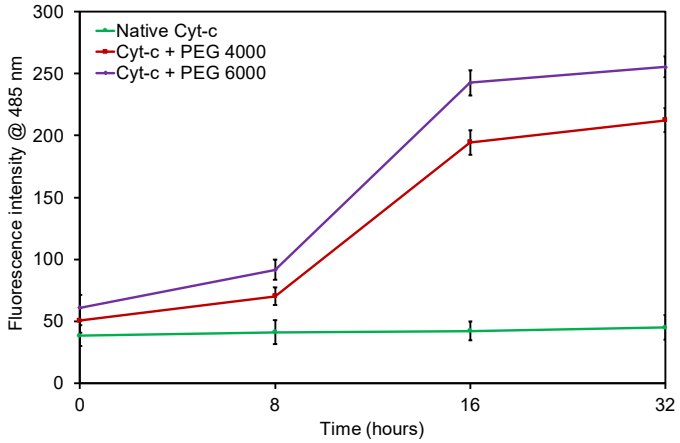


Figure 3. Cyt-C fibrillation kinetics in the presence and absence of 200 mg/mL PEG 4000 and PEG 6000 at different time intervals (0-32 hours) using thioflavin T.

3. Results

3.1. Turbidity assay

Turbidity measurements at 350 nm were used to assess the effect of macromolecular crowding on Cyt-C protein. The assay was performed for Cyt-C incubated in the absence and presence of 200 mg/mL of crowding agents PEG 4000 and PEG 6000 for different time intervals. It is clear from Figure 1 that native Cyt-C is minimal and was found to be the same throughout the incubation time, suggesting that the protein does not show any aggregation in the absence of any macromolecule. However, when PEG 4000 and PEG 6000 were added to the Cyt-C solution, the turbidity of the solutions increased markedly with increasing incubation time, and this can be attributed to the formation of crowder-induced Cyt-C aggregates. Furthermore, PEG 6000 showed more turbidity than PEG 4000 at 32 h, indicating that the heavier the crowding agent, the more prominent the results will be. Similar results have been obtained from our laboratory when hemoglobin was incubated with PEG 4000, PEG 6000, and dextran 70 [16]. However, the results of the turbidity assay were further validated by the thioflavin T assay.

3.2. Thioflavin T assay

Thioflavin T is a benzothiazole dye that usually binds and forms a highly fluorescent complex with amyloid and amyloid-like fibrils, thereby intercalating between the cross-β sheets of the fibrils [17]. Figure 2 shows ThT fluorescence measurements of Cyt-C alone and in the presence of different crowding agents, PEG 4000 and PEG 6000. ThT shows an insignificant binding to native Cyt-C compared to Cyt-C aggregates, as the dye does not bind to native proteins [18]. However, Cyt-C in the presence of the crowding agent PEG 4000 exhibits an increased intensity, indicating that the native conformation of the protein has been altered to a fibrillar-like structure, which consequently leads to the formation of Cyt-C aggregates [19]. The fluorescence intensity corresponding to Cyt-C in the presence of the crowding agent PEG 6000 shows a further increase in the ThT intensity, resulting in the formation of protein aggregates [19]. The increase in ThT intensity was due to the fact that the Cyt-C aggregates retained a binding site where the dye molecules got locked sterically. In addition, the kinetics of Cyt -c fibrillation were also monitored at different time intervals, as shown in Figure 3. At the initial hour of incubation, Cyt-C showed a lag phase of 8 hours when native Cyt-C monomers were present, which is followed by an exponential growth phase (nucleation) for 16 hours, leading to the formation of Cyt-C aggregates by the elongation and propagation of misfolded proteins, and finally culminating in a stationary phase at 32 hours where the matured fibrils were present. This type of curve is attributed to the fact that Cyt-C follows nucleation-dependent kinetics in the presence of PEG 4000 and PEG 6000 [20]. Although it has the same kinetic mechanism for both crowding agents, the only difference is that the crowder with a higher molecular weight causes more aggregation of the Cyt-C model protein Cyt-C [21].

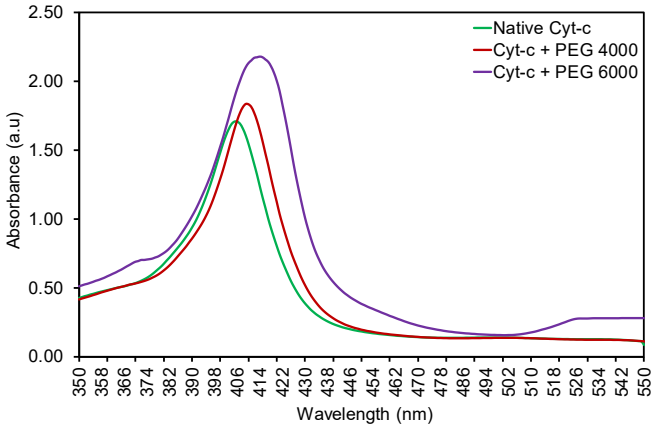
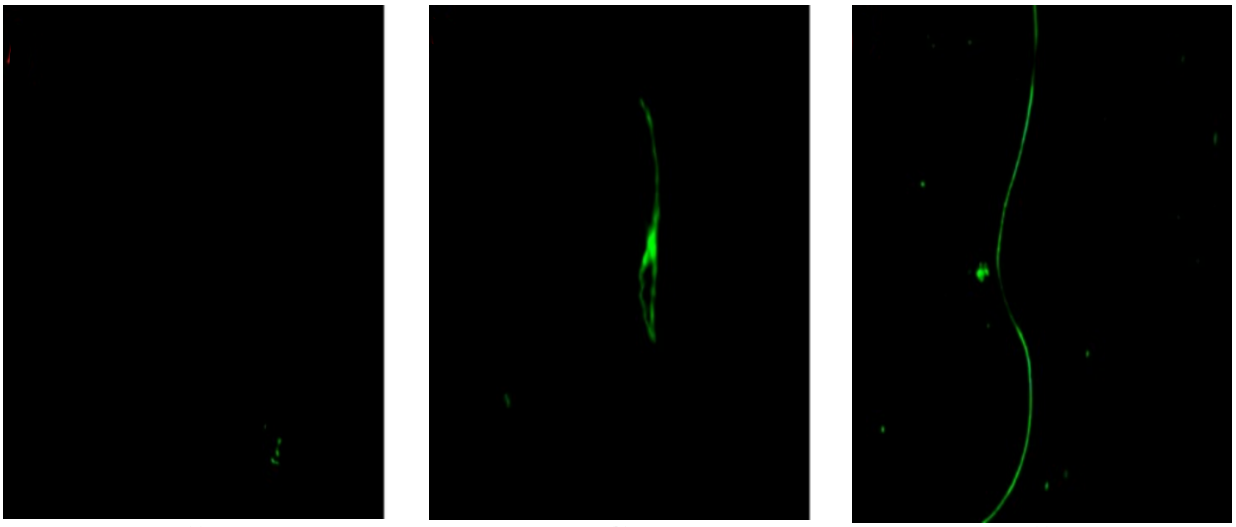


Figure 4. Cyt-C soret absorption spectra in the presence and absence of 200 mg/mL PEG 4000 and PEG 6000 incubated for 16 hours.



(a) (b) (c)

Figure 5. Fluorescence microscopy of: native Cyt-C (A) with 200 mg/mL PEG 4000 (B) and with 200 mg/mL PEG 6000 (C) incubated for 16 hours.

Different proteins showed the same degree of linearity in ThT fluorescence emission under different conditions [16,22]. Therefore, it can be inferred from the results that the native structure of Cyt-C has been altered to fibrillar assemblies in a macromolecular crowded environment induced by PEG 4000 and PEG 6000 [23]. However, not only the type of crowding agent, but also the time of incubation, play a consequential role in the crowding-induced alteration in which the structural transition of globular protein occurs [4].

3.3. Soret absorbance studies

Heme-containing proteins show the characteristic absorption spectrum in the range of 400-450 nm [24]. Figure 4 shows the absorption spectra of Cyt-C in the absence and presence of 200 mg/mL PEG 4000 and PEG 6000. Native Cyt-C showed a peak at 405 nm, illustrating that the protein molecule retained its heme group and structural integrity [23]. As Cyt-C was supplemented with PEG 4000, the spectrum showed a red shift of 4 nm with an increase in absorbance, while with PEG 6000 a red shift of 9 nm was observed with an additional increase in absorbance [21]. The most likely reason for this is that, in the presence of a crowding agent, the micro-environment around the heme moiety loosens and becomes more exposed to the solvent [25]. The greater the exposure of the heme moiety, the higher the absorbance and the higher the red shift. The PEG 6000 has more absorbance and red shift than the PEG 4000 sample, suggesting that large crowder molecules cause more protein aggregation at the same concentration [26]. The Soret results are in accordance with the thioflavin T and turbidity results obtained [16].

3.4. Fluorescence microscopy

Fluorescence microscopic images of Cyt-C incubated with PEG 4000 and PEG 6000 were captured using a fluorescence microscope. Images of native Cyt-C and Cyt-C incubated with PEG 4000; PEG 6000 are shown in Figure 5. In Figure 5, panel A corresponds to native Cyt-C and does not show the presence of any aggregate [27]. As the protein was incubated with PEG 4000 and PEG 6000 (panels B and C, respectively) and showed fibril formation [27]. The fibril formation was more pronounced under the influence of PEG 6000 than PEG 4000. The results of microscopy further authenticate the results obtained in the ThT measurements.

4. Discussions

In reality, living cells are heavily crowded with various types of macromolecules that occupy up to 40% of the total volume of the cell [28], making it difficult for the other molecules to function properly [29]. Natural and artificial

crowder molecules can be used to mimic the crowding environment under *in vitro* conditions. The crowding agents of different natures exhibited different effects on various proteins. For example, the macromolecular crowder, polyethylene glycol 2000, induces α-lactalbumin aggregation [30]. Ficoll 70 efficiently disrupts the native conformation of myoglobin, leading to its unfolding [31]. Consequently, dextran 70 converts the recombinant PrPC (cellular prion protein) to the neurotoxic β-oligomers [32]. From the above studies, it is confirmed that macromolecular crowding influences the native structure of proteins, leading to their aggregation and fibril formation [33].

In this study, an artificial crowding environment that mimics a cellular system was created *in vitro* using synthetic inert crowding agents PEG 4000 and PEG 6000. The Cyt-C model protein was incubated with 200 mg/mL of PEG 4000 and PEG 6000 to induce aggregation. The increased absorbance at 350 nm as a result of the turbid protein solution correlates with the larger particle size as an indicator of protein aggregation [34,35]. It is a preliminary experimental approach to confirm aggregation. In addition, to further validate the formation of fibrillar Cyt-C aggregates in the presence of PEG 4000 and PEG 6000, thioflavin T fluorescence measurements were carried out. The increase in fluorescence of the samples corresponding to PEG 4000 and PEG 6000 supported the fact that conformational alterations drive Cyt-C to form a fibrillar structure [36]. From the fibril formation kinetic analysis, it was found that the model protein follows a nucleation-dependent mechanism in the presence of both crowder molecules [37]. Since PEG 6000 is larger than PEG 4000, for the same concentration, PEG 6000 showed enhanced fluorescence attributed to the increased aggregation rate [8]. The reason for this may be that larger crowder molecules have a substantial effect on the propensity for aggregation as a result of increased excluded volume [38]. Soret band absorption gives an idea about the integrity of the heme group in native proteins. The increase in absorbance with the red shift in the Cyt-C spectra suggested the loosening and exposure of the heme groups to the microenvironment as a result of fibril formation [39]. PEG molecules are generally hydrophilic, but due to the presence of ethylene groups, they show some hydrophobicity, and this may be the reason for inducing protein aggregation mainly through hydrophobic interactions [40]. Fluorescence microscopic images also authenticate the formation of Cyt-C fibrils in the presence of PEG 4000 and PEG 6000. In addition to mimicking cell-like *in vivo* conditions, it is important to assess the role of various crowding molecules in the native conformation of proteins [41]. However, in living systems, macromolecular congestion alters processes like the protein folding mechanism and changes in the activity of enzymes, *etc*. Therefore, it is of the hour to gain better insights into how macromolecular crowding induces protein aggregation to cope with actual situations.

5. Conclusions

We have demonstrated that the macromolecular crowding alters the native structure of Cyt c in a time-dependent manner and promotes aggregation. From our results, it can be concluded that the crowding agents PEG 4000 and PEG 6000 behave similarly to Cyt c and show a similar pattern and kinetics of protein aggregation. These findings can be related to various age-related protein conformational diseases, including neurodegenerative disorders, in which macromolecular crowding plays an important role as a result of a lack of water hydration and shrinkage of cells. Furthermore, research should be carried out to test the antiamyloidogenic potential of small molecules, either natural or synthetic, to prevent protein aggregation in a crowded environment.

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Disclosure statement

Conflict of interest: The authors declare that they have no conflict of interest.
Ethics approval: All ethical guidelines have been followed.
Availability of data and material: All data supporting the findings of this study are available in the paper.

CRediT authorship contribution statement

Conceived and designed the experiments: Neha Kausar Ansari, Imtiaz Ahmad, Gufran Ahmed Siddiqui and Aabgeena Naeem. Performed the experiments: Neha Kausar Ansari, Imtiaz Ahmad, Gufran Ahmed Siddiqui and Aabgeena Naeem. Analysed the data: Neha Kausar Ansari, Imtiaz Ahmad, Gufran Ahmed Siddiqui and Aabgeena Naeem; Formal Analysis: Neha Kausar Ansari, Imtiaz Ahmad, Gufran Ahmed Siddiqui and Aabgeena Naeem; Investigation: Neha Kausar Ansari, Imtiaz Ahmad, Gufran Ahmed Siddiqui; Resources: Aabgeena Naeem; Data Curation: Neha Kausar Ansari, Imtiaz Ahmad, Gufran Ahmed Siddiqui; Writing - Original Draft: Neha Kausar Ansari, Imtiaz Ahmad, Gufran Ahmed Siddiqui; Writing - Review and Editing: Aabgeena Naeem; Visualization: Neha Kausar Ansari, Imtiaz Ahmad, Gufran Ahmed Siddiqui; Supervision: Aabgeena Naeem.

ORCID and Email

Neha Kausar Ansari
nehakausar786@gmail.com
https://orcid.org/0009-0002-1287-2618
Imtiaz Ahmad
imtiaz8ahmad@gmail.com
https://orcid.org/0009-0003-0462-6369
Gufran Ahmad Siddiqui
gufran786indian@gmail.com
https://orcid.org/0000-0002-6980-5574
Aabgeena Naeem
aabgeenanaim@gmail.com
anaeem.bc@amu.ac.in
https://orcid.org/0000-0003-3562-780X

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