3
Trehalose Promotes Clearance of Proteotoxic Aggregation of Neurodegenerative Disease-Associated Aberrant Proteins

3.1 RESULTS

In cells, the PQC system regularly discriminates against proteins that require active folding or failure of folding (Buchberger et al., 2010); attempts of abnormal proteins lead to their proteolytic degradation (Gregersen et al., 2006). Different mechanisms of PQC transport misfolded proteins for their clearance; UPS is majorly accountable for intracellular protein degradation (Lilienbaum, 2013). It has been shown that the over-accumulation of abnormal proteins can obstruct the Proteasome assembly and consecutively impair their respective proteolytic functions (Thibaudeau et al., 2018). How we can restore the proteasome functions under such conditions and avoid proteotoxic insult is a significant critical challenge.

3.1.1 Exogenous Treatment of Trehalose Induces Proteasome Levels and Accelerates Bona Fide Abnormal Protein Degradation

In our current finding, we try to address this question; in our preliminary experiments, A549 cells were exposed to Trehalose. Post-treatment collected cells were used for immunoblotting using antibodies against 20S and GAPDH, as shown in Figure 3.1 (a). Our result demonstrates that using Trehalose can induce endogenous levels of proteasome.

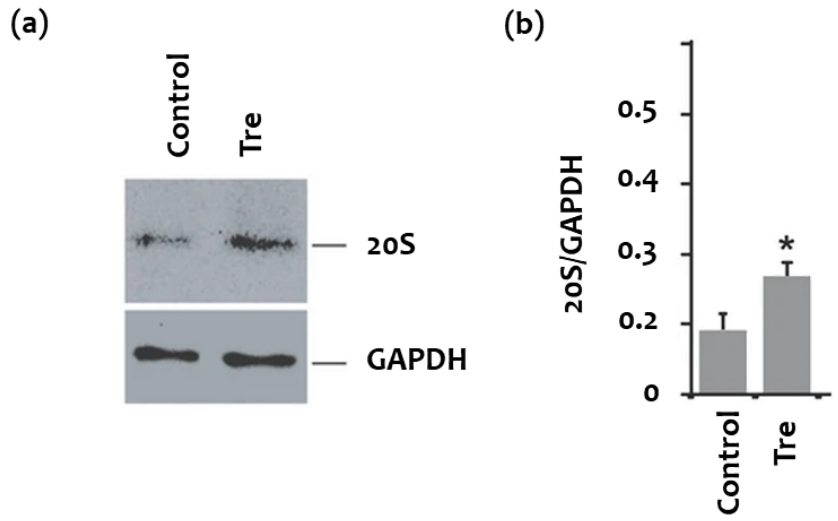

Figure 3.1: Effect of trehalose on endogenous levels of 20S proteasome: (a) A549 cells were subjected to trehalose (1.5 mM) and control (distilled water). Treated cells were then gathered for immunoblotting experiments with anti-GAPDH and anti-20S antibodies. GAPDH was utilized for normalizing the protein amount in each immunoblotting sample. (b) The intensity of immunoblotting bands was quantified and plotted for treatment dependent samples utilizing ImageJ analysis application with *$p < 0.05$ (compared with control) demonstrating statistical significance.

To confirm this result, we further treated cells with trehalose in a time-dependent manner, and cell lysates were used for immunoblotting, as shown in Figure 3.2 (a) Further

detailed quantitative analysis of immunoblotting results via image analysis using ImageJ software indicates that trehalose can elevate the endogenous levels of proteasome (Figure 3.1 (b) and Figure 3.2 (b)).

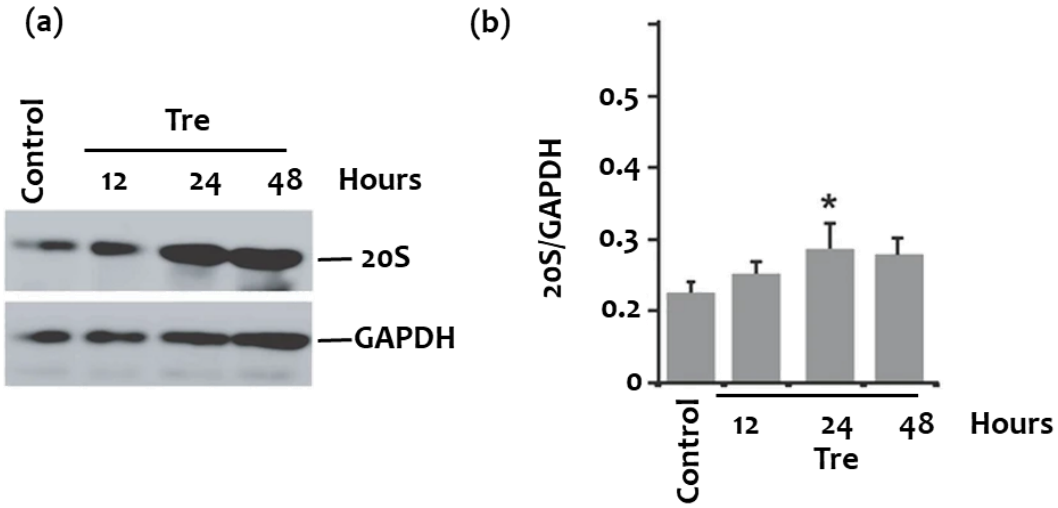

Figure 3.2: Effect of Trehalose on endogenous levels of 20S proteasome in time-dependent manner: (a) A549 cells were subjected to trehalose (1.5 mM) and control (distilled water) for different time periods. Treated cells were then gathered for immunoblotting experiments with anti-GAPDH and anti-20S antibodies. GAPDH was utilized for normalizing the protein amount in each immunoblotting sample. (b) The intensity of immunoblotting bands was quantified and plotted for time-dependent samples utilizing ImageJ analysis application with $*p < 0.05$ (compared with control) demonstrating statistical significance.

The exogenous induction of proteasome levels after the treatment of trehalose engendered us to check its effect on heat-denatured luciferase misfolded protein. Cells were transfected transiently with luciferase (firefly) plasmid, and the same group of samples was treated in the presence or absence of trehalose. These cells were shifted to 43 °C for 30 min for the heat-shock treatment and, after heat treatment, returned to recovery at 37 °C for 2 h. After recovery, we performed immunofluorescence as represented in Figure 3.3 (a); treatment of trehalose elevated clearing of heat-denatured luciferase protein. In another group of experiments, similar sets of samples described above were used to analyze luciferase activity in presence of established proteasome and autophagy inhibitors like MG132 and chloroquine and stressors like DMSO; as shown in Figure 3.3 (b), our findings indicate that trehalose treatment elevates the clearance of heat-denatured misfolded inclusions.

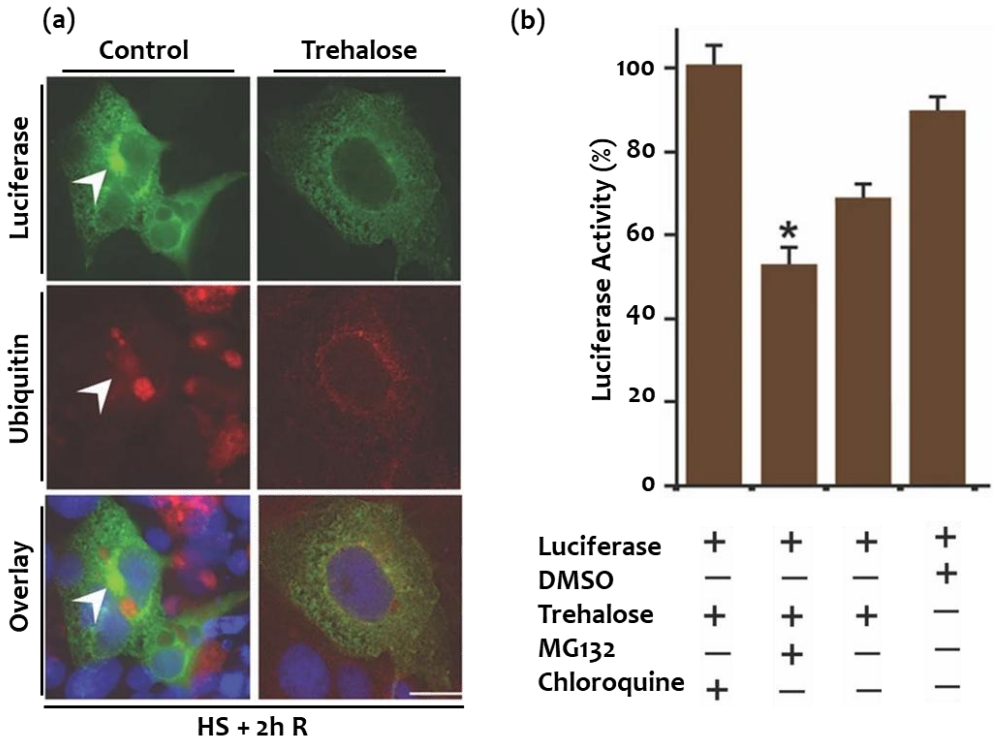

Figure 3.3: Trehalose induces degradation of heat denatured luciferase: (a) The COS-7 cells transfected transiently with a construct expressing firefly luciferase. They were exposed to trehalose (1.5 mM) post-transfection and control for 48 h. After the treatment, the cells were subjected to 43 °C for 30 min and again placed back to 37 °C for 2 h for recovery. Afterwards, cells were processed for immunofluorescence study utilizing antibodies against luciferase (green) and ubiquitin (red), and DAPI staining was done to localize nucleus. Arrowheads show colocalization of luciferase's peripheral nuclear aggregates positive for ubiquitin staining. (b) As explained above, few groups of cells were administered MG132 (10 μM), DMSO, and chloroquine (20 μM) for 12 h and then were collected for luciferase activity assay. Three experiments were performed independently and in triplicates; data depicted in graph as mean ± SD. *$p < 0.05$ compared with the control. *Scale bar 20 μm.*

Clearance of heat-denatured luciferase protein also gives us an important clue that the use of trehalose can target the elimination of d1EGFP, a model substrate of proteasome, containing several PEST sequence regions at its carboxyl terminus. Cells were transfected (transient) with pd1EGFP; a few sets of cells were given trehalose and MG132. Cells form plenty of d1EGFP inclusions in the presence of MG132 (Figure 3.4 (a)), but the treatment of trehalose (Figure 3.4 (b)) induces the destabilization of d1EGFP intracellular inclusions. Our current findings suggest that trehalose treatment can induce the cellular levels of proteasome, which may consequently promote the degradation of abnormal aggregate-prone proteins.

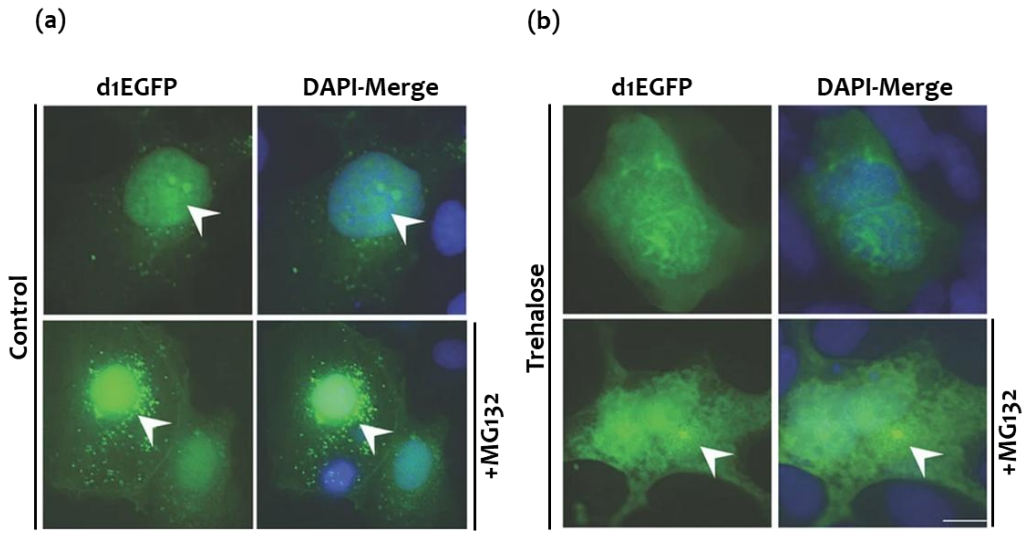

Figure 3.4: Trehalose promotes clearance of d1EGFP a proteasomal substrate in cells: COS-7 cells transfected transiently with pd1EGFP. Post-transfection, they were subjected to (a) control and (b) trehalose (1.5 mM) (H) for 48 h; some cells were exposed with MG132 (10 µM) for 12 h. Counterstaining with DAPI (blue) was done to localize the nucleus. Cells were analyzed under an epi-fluorescence microscope, as depicted in micrographs. The arrowheads indicate the perinuclear aggregates of misfolded proteins. *Scale bar 20 µm.*

3.1.2 Trehalose Induces Proteasome Functions Linked with PQC Components

Cells consume energy in terms of ATP molecules and promote abnormal polypeptide degradation to avoid unwanted interactions with other functional proteins (Goldberg, 2003;Ciechanover and Kwon, 2015). Over-accumulation of large proteinaceous inclusions can block the proteasome activity, adversely affecting overall cellular health and fitness (Thibaudeau et al., 2018). Improving the effective degradation of unwanted proteins is a major challenge. Our previous results imply that the Trehalose treatment can elevate the cell's intrinsic levels of proteasome, which promotes clearance of bona fide proteasomal substrate d1EGFP protein. These results prompted us to check direct effect of Trehalose on proteasome activities. We treated cells with various compounds (like Aspirin, Lactacystin, and MG132 as proteasomal inhibitors and control) along with Trehalose. We measured both chymotrypsin-like (Figure 3.5 (a)) and post-glutamyl peptide hydrolysing (PGPH)-like activities (Figure 3.5 (b)).

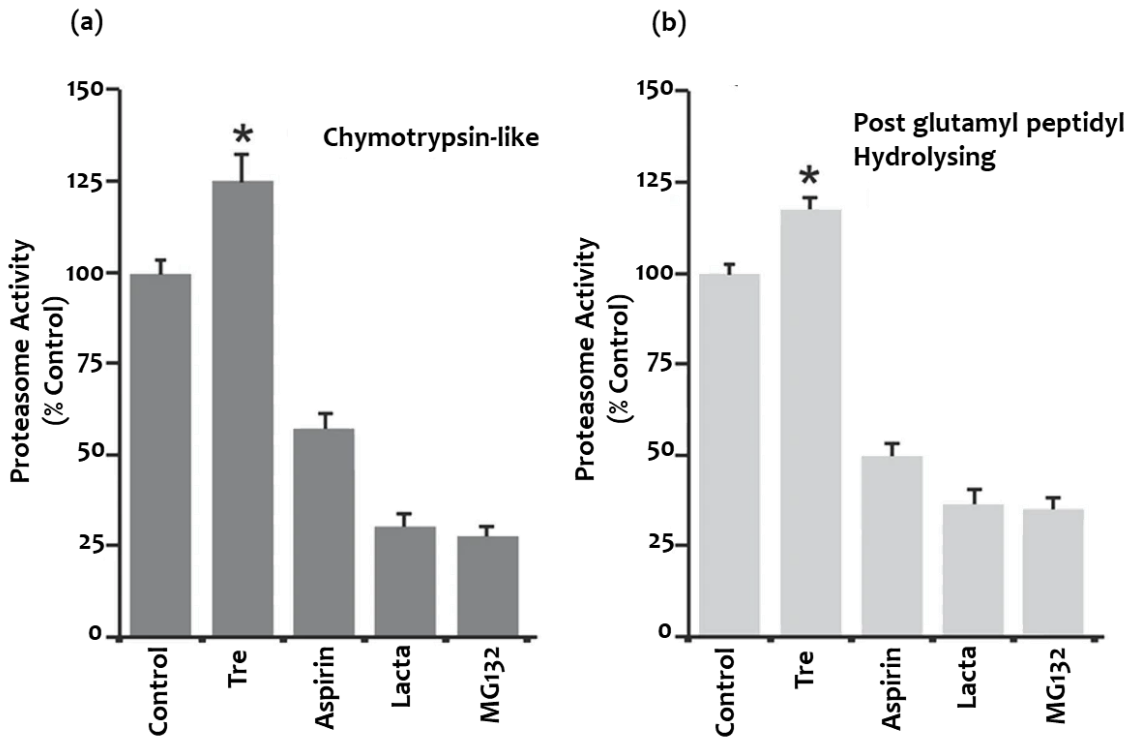

Figure 3.5: Trehalose exposure can enhance the proteasome functions: A549 cells in 6-well plates were subjected to various proteasomal inhibitors aspirin (2.5 mM), MG132 (10 μM), and lactacystin (10 μM) for 12 h along with trehalose (1.5 mM) for 48 h. Afterwards, cells were harvested to perform proteasome (a) chymotrypsin and (b) PGPH-like activity assays. All data are depicted as mean ± SD of three experiments in triplicate performed independently. *$p < 0.05$ when compared to control.

This observation was interesting, Trehalose induces proteasome activities, and now it is also significant for us to check whether the treatment of Trehalose influences normal cellular health in an adverse manner or not. As expected, we noticed that the treatment of Trehalose does not generate any adverse effect on cellular health used in both time (Figure 3.6) and concentration (Figure 3.7) dependent manner.

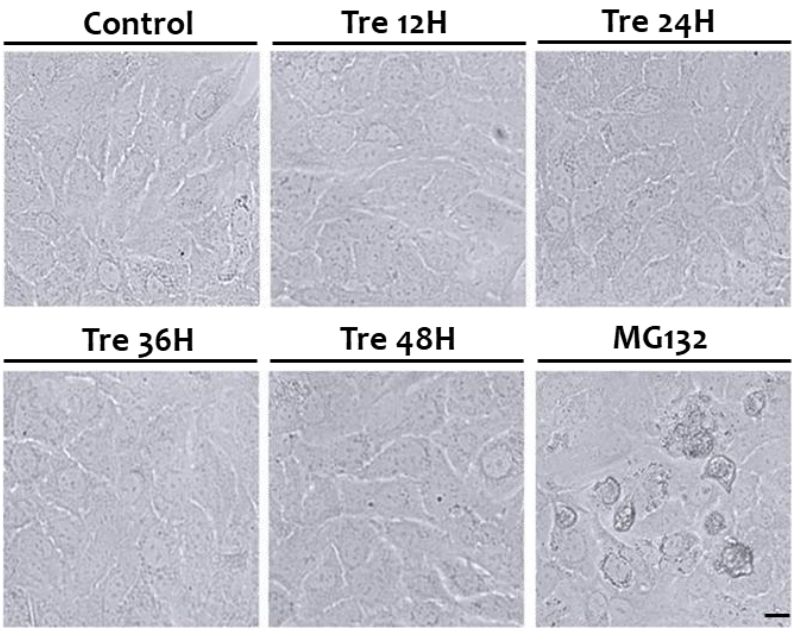

Figure 3.6: Effect of trehalose in time-dependent manner on morphology of cells as compared to MG132: A549 cells were grown and exposed to trehalose at different time intervals (1.5 mM) and MG132 (10 µM) for 12 h. Afterwards, bright-field images were taken, as depicted in micrographs. *Scale bar: 20 µm.*

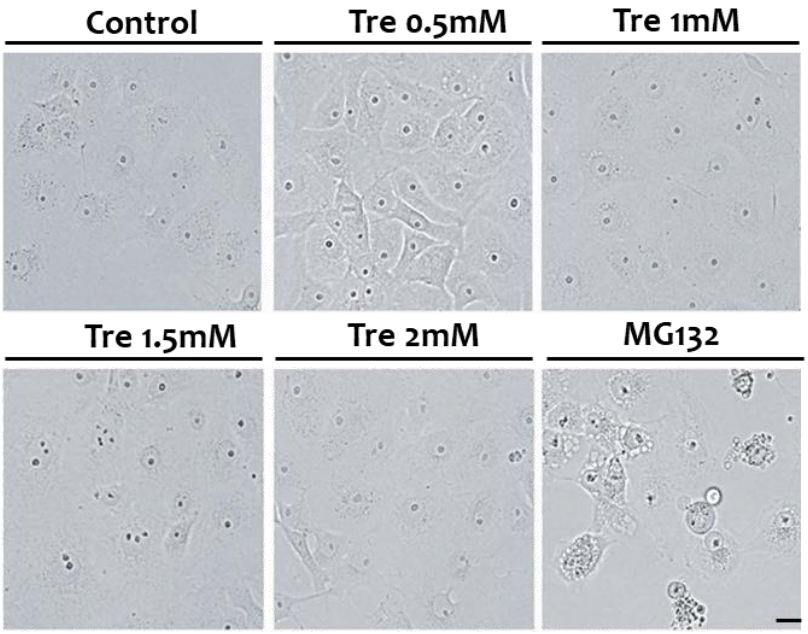

Figure 3.7: Effect of trehalose in concentration-dependent manner on morphology of cells as compared to MG132: A549 cells were treated with trehalose at different concentrations (48 h) and MG132 (10 µM) for 12 h. Afterwards, bright-field images were taken, as depicted in micrographs. *Scale bar: 20 µm.*

Next, we examined trehalose effect on a few critical PQC players. Trehalose-treated cells were processed for immunofluorescence analysis deploying anti-20S, anti-ubiquitin, and anti-p62/SQSTM1 antibodies (Figure 3.8). Our results suggest that trehalose most likely retains a potential to modulate the endogenous levels of PQC molecules, which may be moreover helpful in removing the misfolded proteins from the cells.

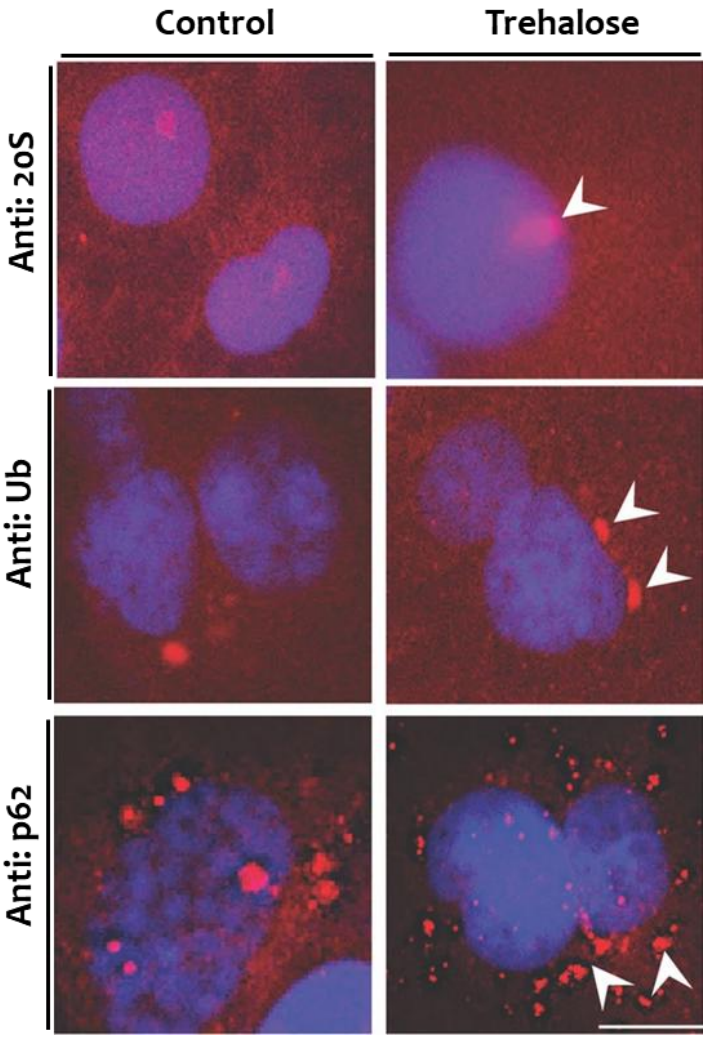

Figure 3.8: Trehalose induces the levels of different PQC elements: A549 cells were cultured in 2-well chambered slides and were subjected to trehalose (1.5 mM) for 48 h and with control (distilled water). Afterwards, immunofluorescence analysis was done, via labeling with antibodies anti-p62, anti-20S proteasome, and anti-ubiquitin. The nucleus is stained with DAPI for localization. *Scale bar: 20 μm.*

As shown in our previous results, trehalose can increase the levels of proteasome and also may affect proteasome activities. Most likely, trehalose may possibly interact with proteasome subunits. It is important for us to explore this question; therefore, we executed a thorough docking analysis. To identify the high-probability interaction sites, we have performed blind docking, as depicted in Figures 3.9, 3.10 and 3.11. The outcome obtained from docking analysis implies that energy at the binding sites of proteasome 20S subunits β1, β2, and β5 are -5.7, -5.9, and -6.1 kcal/mol, respectively, and the molecules are interacting with forming polar interactions. Our current findings suggest that trehalose treatment retains the possible potential to activate proteasome activities without inflicting any cellular insults. The above results also establish a better understanding and correlation of trehalose treatment against misfolded protein aggregation, most likely targeting proteasome activities underlying the PQC mechanism.

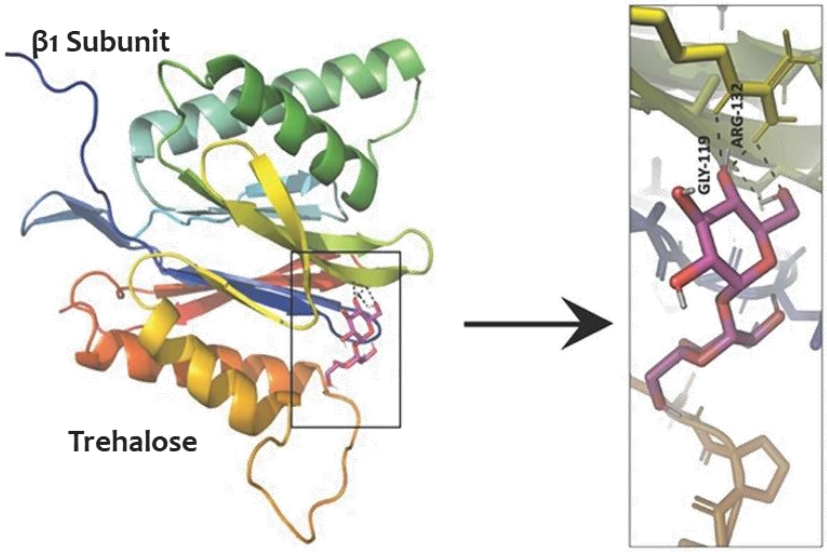

Figure 3.9: *In silico* molecular docking with trehalose and β1 subunit of 20S proteasome: To validate the assumption of possible interaction between catalytic subunits of proteasome 20S and the small ligand trehalose, *in silico* molecular docking was done using AutoDock Vina (version 1_1_2). In given figure, a generalized view of resulted (cartoon) models is represented where trehalose is interacting with proteasome 20S subunit β1.

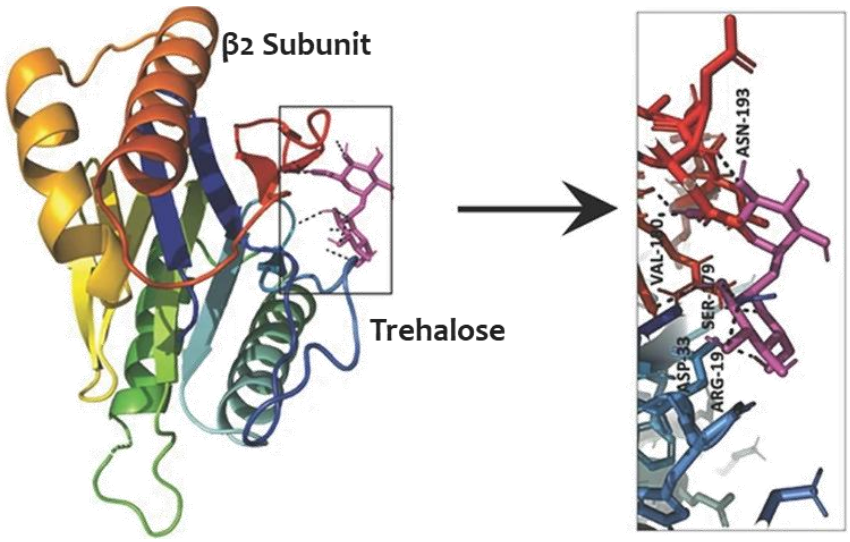

Figure 3.10: *In silico* docking with trehalose and β2 subunit of 20S proteasome: In given figure, a generalized view of resulted (cartoon) models is represented where trehalose is interacting with proteasome 20S subunit β2.

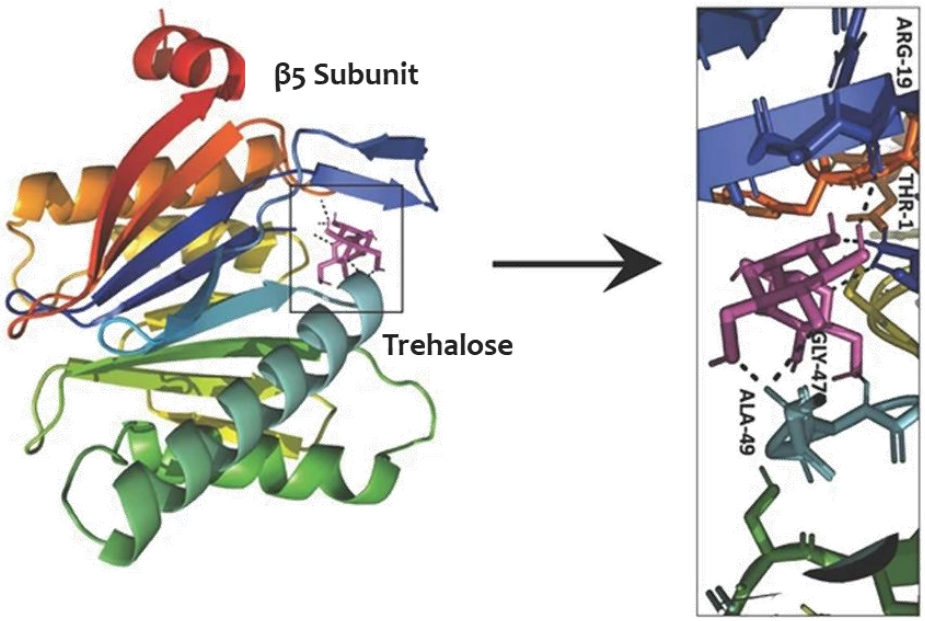

Figure 3.11: Molecular docking with trehalose and β5 catalytic subunit of 20S proteasome: In given figure, a generalized view of resulted (cartoon) models is represented where trehalose is interacting with proteasome 20S subunit β5.

3.1.3 Trehalose Can Interact with Proteasome and Affect the Stability of Proteasome Substrate

Further, we investigated the resulted models from molecular docking of β1, β2, and β5 subunits of proteasome with Trehalose which are represented as surface model as shown in Figure 3.12 (a), 3.13 (a) and 3.14 (a) respectively. The results of molecular docking were analyzed further with LigPlot+ where 3D coordinates were taken and a 2D representation was done for detailed investigation. The two-dimensional result representation shows hydrogen bonding and other possible hydrophobic interactions of β1, β2, and β5 subunits of proteasome with ligand as shown in Figure 3.12 (b), 3.13 (b) and 3.14 (b) respectively.

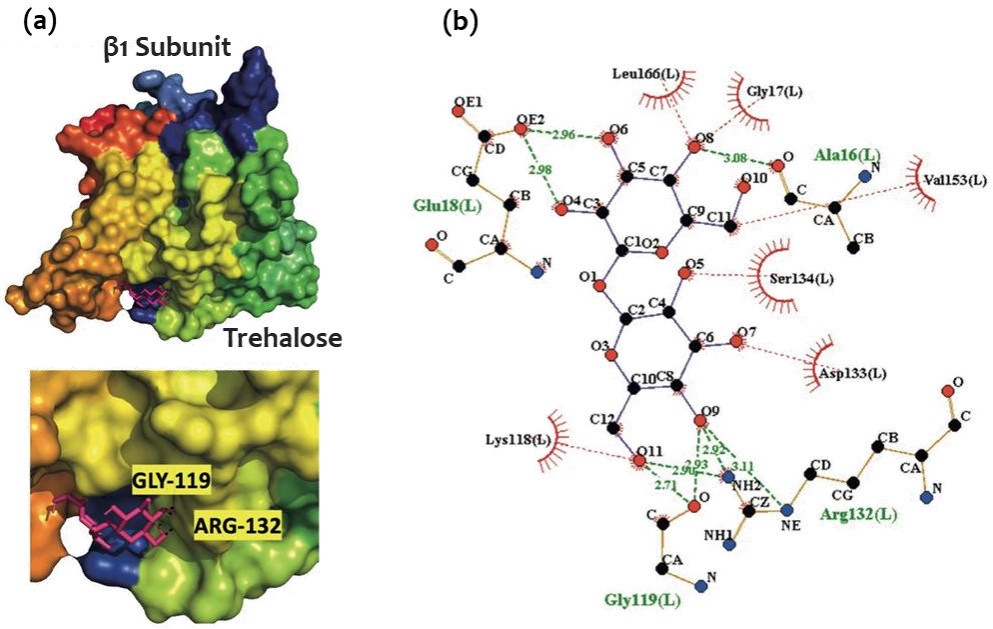

Figure 3.12: Possible Trehalose interaction with proteasome β1 subunit subunits and its surface and 2D representation: (a) Analysis of *in silico* molecular docking result. Images here represent the interaction of protein–ligand where proteins β1 subunits of proteasome are represented as surface models. (b) The images shown represents the docking results in 2D representation where the ligand–protein interactions are depicted. The dotted line in green shows the hydrogen bond and in red shows hydrophobic interactions. The nonbonded contact of residues made with ligand is depicted with spoked arc wheel.

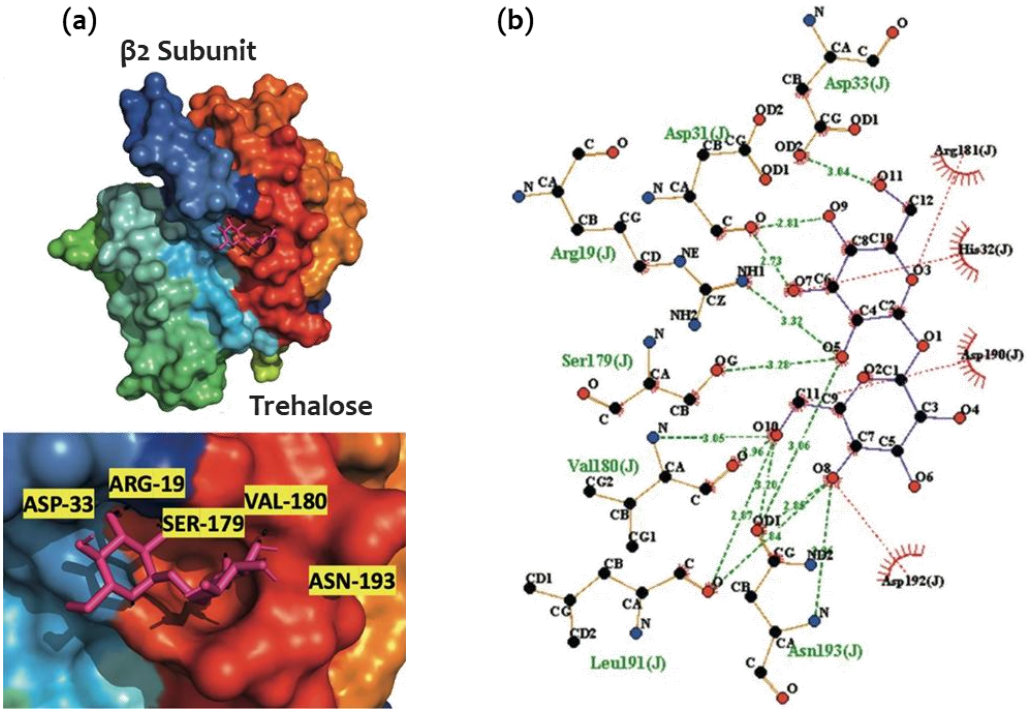

Figure 3.13: Probable Trehalose interaction with proteasome β2 subunit subunits and its surface and 2D representation: (a) analysis of *in silico* molecular docking result. Images here represent the interaction of protein–ligand where proteins β2 subunit of proteasome are represented as surface models. (b) The images shown represents the docking results in 2D representation where the ligand–protein interactions are depicted. The dotted line in green shows the hydrogen bond and in red shows hydrophobic interactions. The nonbonded contact of residues made with ligand is depicted with spoked arc wheel.

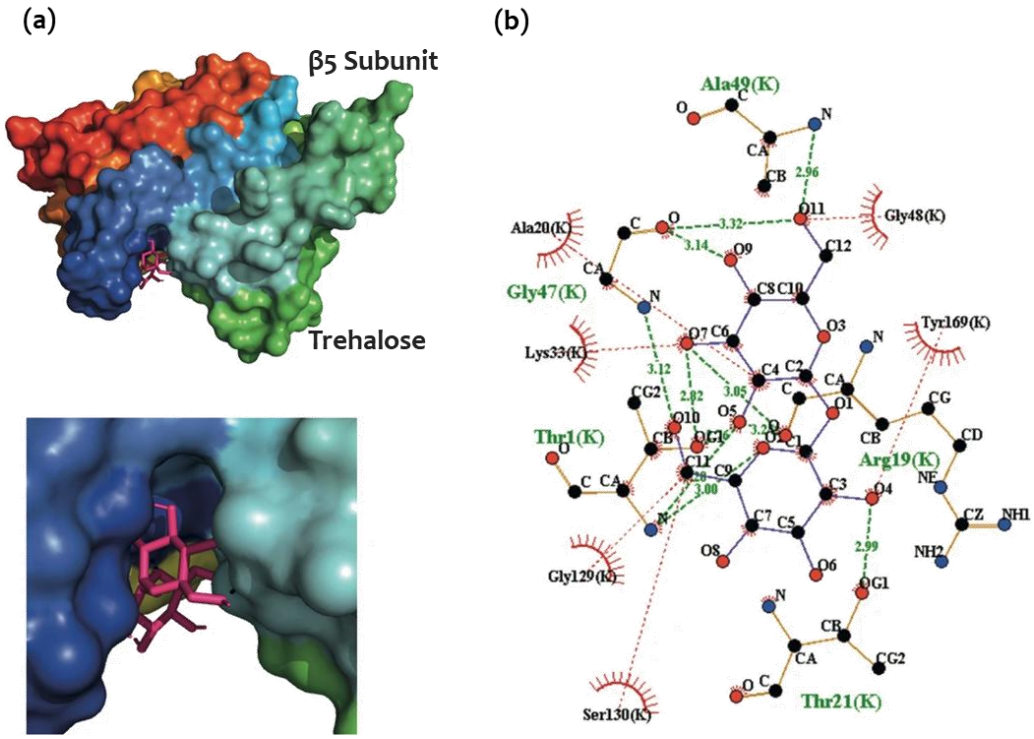

Figure 3.14: Probable Trehalose interaction with proteasome β5 subunit subunits and its surface and 2D representation: (a) analysis of *in silico* molecular docking result. Images here represent the interaction of protein–ligand where proteins β5 subunit of proteasome are represented as surface models. (b) The images shown represents the docking results in 2D representation where the ligand–protein interactions are depicted. The dotted line in green shows the hydrogen bond and in red shows hydrophobic interactions. The nonbonded contact of residues made with ligand is depicted with spoked arc wheel.

Next, we have performed cycloheximide chase analysis where the cells were transfected transiently with the construct of pd1EGFP, coding for d1EGFP, which is a model substrate of proteasome. After the transfection, the cells were exposed to control or trehalose (1.5 mM). Afterwards, cells were examined upon cycloheximide treatment at various time intervals, and collected as shown in Figure 3.15. The collected samples were then processed for immunoblotting experimentation with anti-GFP antibody (Figure 3.15 (a)) with corresponding ponceau staining. We also observed effect of trehalose on the induced rate of clearance of d1EGFP, and few sets of samples were analyzed under the fluorescence microscope (Figure 3.15 (b)).

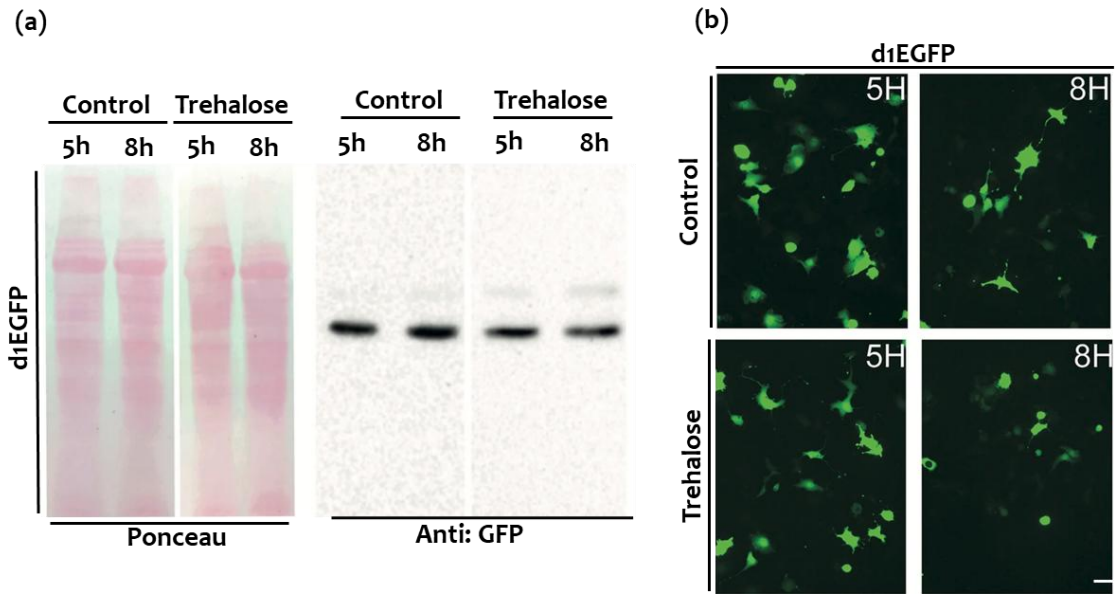

Figure 3.15: Trehalose treatment effect on d1EGFP upon cycloheximide treatment: Cells were transfected transiently with pd1EGFP plasmid construct. Subsequently post-transfection, these cells were subjected to trehalose (1.5 mM) and then were chased with cycloheximide (10 µM). Afterwards, the treated cells were collected for immunoblotting utilizing anti-GFP antibodies (a) with corresponding ponceau staining. Similar sets of treated cells were also analyzed and imaged under the fluorescence microscope (b). *Scale bar: 20 µm.*

3.1.4 Trehalose Exposure Induces Intracellular Proteasome Activities

Trehalose is a non-reducing disaccharide; it can alleviate cytotoxicity in neurodegenerative disease models (Emanuele, 2014); however, the detailed mechanism by which Trehalose induces the degradation of abnormal proteins is still unclear. Previous findings suggest that Trehalose can prevent protein aggregation by preserving proteasome Activity (Li et al., 2017). Based on our preliminary results and the above findings, it was essential for us to address the ability of Trehalose to act upon the Proteasome activities. We designed an experiment where we investigated the Trehalose effect on proteasome activities in both time- and concentration-dependent manner. As represented in Figure 3.16 and 3.17, cells were exposed to various concentrations and time intervals; post-treatment, cell lysates were processed for both the measurement of chymotrypsin-like (Figure 3.16 (a) and 3.17 (a)) and PGPH-like (Figure 3.16 (b) and 3.17 (b)) activities; in the similar experiments as a control, MG132 (known Proteasome inhibitor) treatment was done to a few sets of cells.

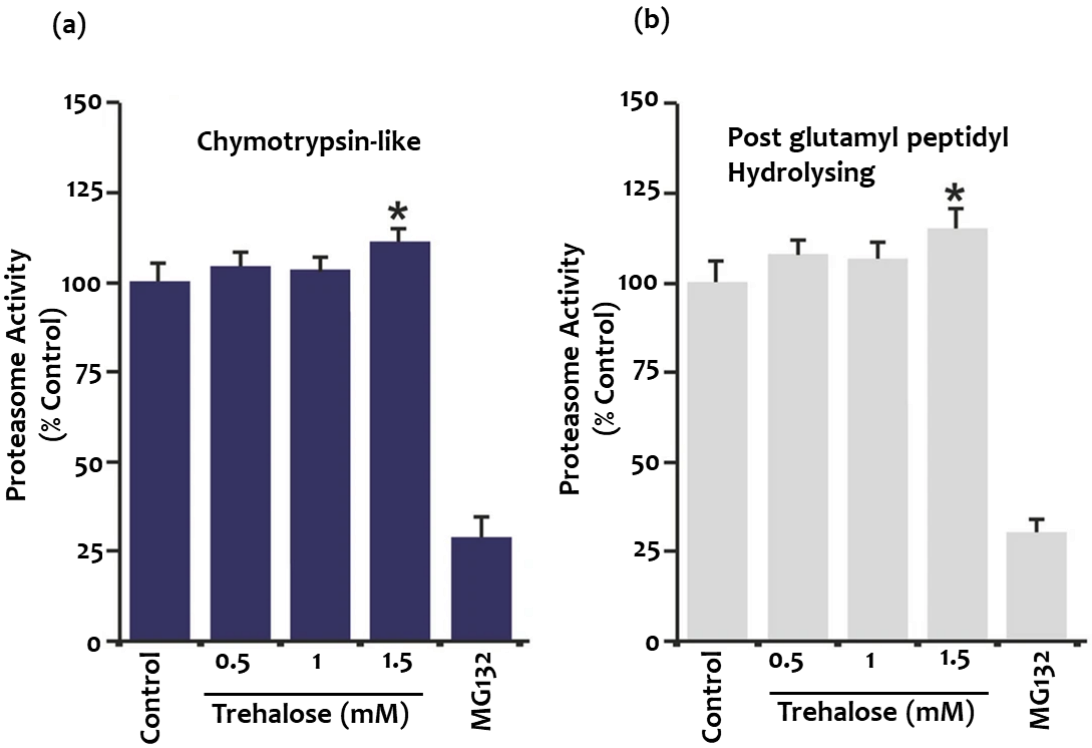

Figure 3.16: Treatment with trehalose enhances intracellular proteasome activity: A549 cells were subjected to various concentrations of trehalose for 36 h and 10 µM MG132 for 12 h (a, b). Afterwards, cells were harvested to perform proteasome (a) chymotrypsin and (b) PGPH-like activity assays. All data are shown as mean ± SD of triplicate experimentation executed independently three times. *p < 0.05 when compared to the control.

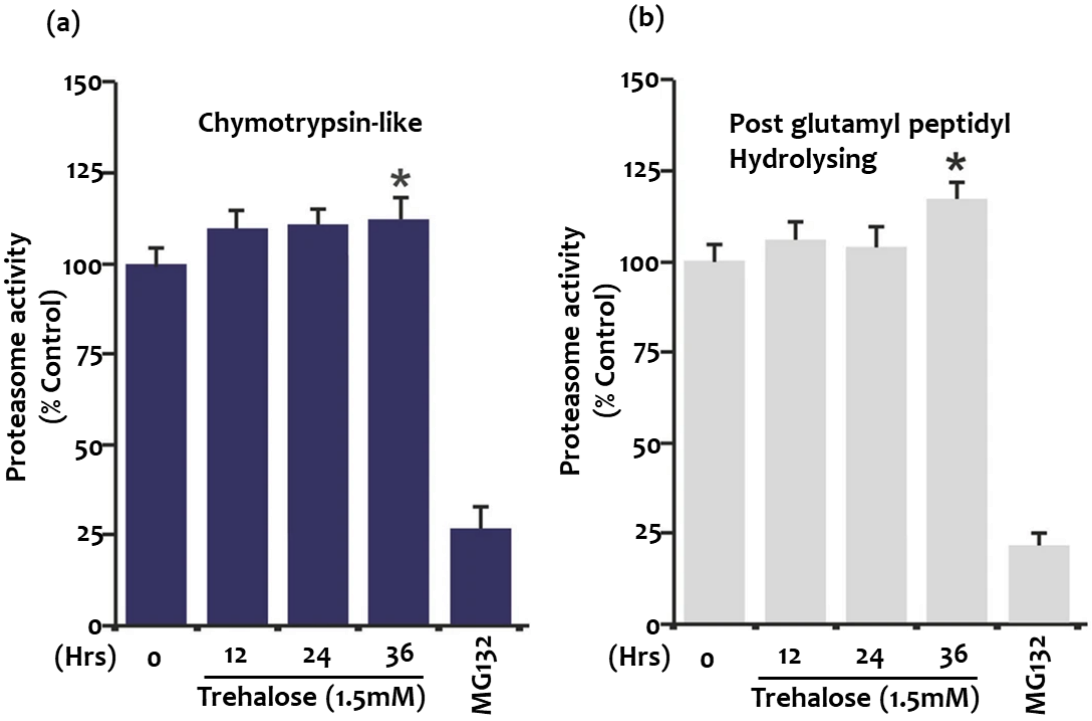

Figure 3.17: Effect of time-dependent treatment of trehalose on proteasome proteolytic activities: A549 cells treated with a 1.5 mM concentration of trehalose for various intervals of time. Post-treatment, the cells were processed to perform proteasome activity (a) chymotrypsin and (b) PGPH-like activity assay. All data are shown as mean ± SD of triplicate experimentation executed independently three times. *p < 0.05 compared to control.

Our results suggest that exposure of Trehalose in both concentrations (Figure 3.16) and time-dependent manner (Figure 3.17) effectively induces chymotrypsin-like and PGPH-like proteasomal activities. Earlier reports suggest that misfolded protein aggregation can initiate proteostasis disturbance due to the over accumulation of unwanted proteinaceous inclusions together (Rajan et al., 2001). Effective removal of these aggregated proteins is a critical challenge for cells. Failure in the clearance mechanism could result in series of lethal cellular insults and consequently cause neuron death. Our current results suggest a possible promising therapeutic strategy against abnormal protein accumulation; the use of Trehalose most likely elevates proteasome activities, which possibly can be helpful in the degradation of aberrant proteinaceous aggregates.

3.1.5 Trehalose Suppressed the Intracellular Aggregation of Bona Fide Abnormal Proteins

How can cells overcome the deleterious challenge of unwanted protein accumulation during their lifespan? The abnormalities in PQC lead to the failure of abnormal protein degradation and impact multiple cellular pathways (Morimoto and Cuervo, 2014). Exogenous support for PQC activation can be an effective strategy against the problem of misfolded protein overaccumulation. To explore this idea, we executed a detailed immunofluorescence analysis as represented in Figures 3.18-3.21; COS-7 cells in 2-well chambered slides were transfected transiently with wild-type (GFP-wtCAT) and mutant (GFP-Δ9CAT) constructs. Cells were also subjected to Trehalose, and some similar sets were also treated with MG132, as shown in Figures 3.19 and 3.21. Before performing the detailed immunofluorescence staining, we confirmed expression of wild-type (GFP-wtCAT) and mutant (GFP-Δ9CAT) plasmids under a fluorescence microscope. We observed the formation of significant GFP-Δ9CAT abnormal inclusions. We examined that expression of the GFP-wtCAT construct (Figure 3.18) does not form apparent amorphous aggregates, which were noticed after the addition of MG132 and treatment of Trehalose to reduce the aggregation phenomenon (Figure 3.19).

Interestingly, the expression of the mutant (GFP-Δ9CAT) construct formed plenty of abnormal intracellular inclusions, and the addition of Trehalose appears helpful in the degradation of these inclusions (Figure 3.20). To examine and clarify the effect of Trehalose under similar conditions, a few sets of samples were subjected to MG132 and observed the abolishment of Trehalose impact on cellular proteinaceous inclusions (Figure 3.21). In the conclusion of the above experiment, our findings indicate that using Trehalose can improve degradation of bona fide mutant misfolded proteins, most likely through the proteasome pathway. Because the addition of MG132 (proteasome inhibitor) reverses the impact of Trehalose against over-accumulation of mutant proteins, the addition of MG132 consequently leads to the accumulation of mutant bona fide misfolded inclusions.

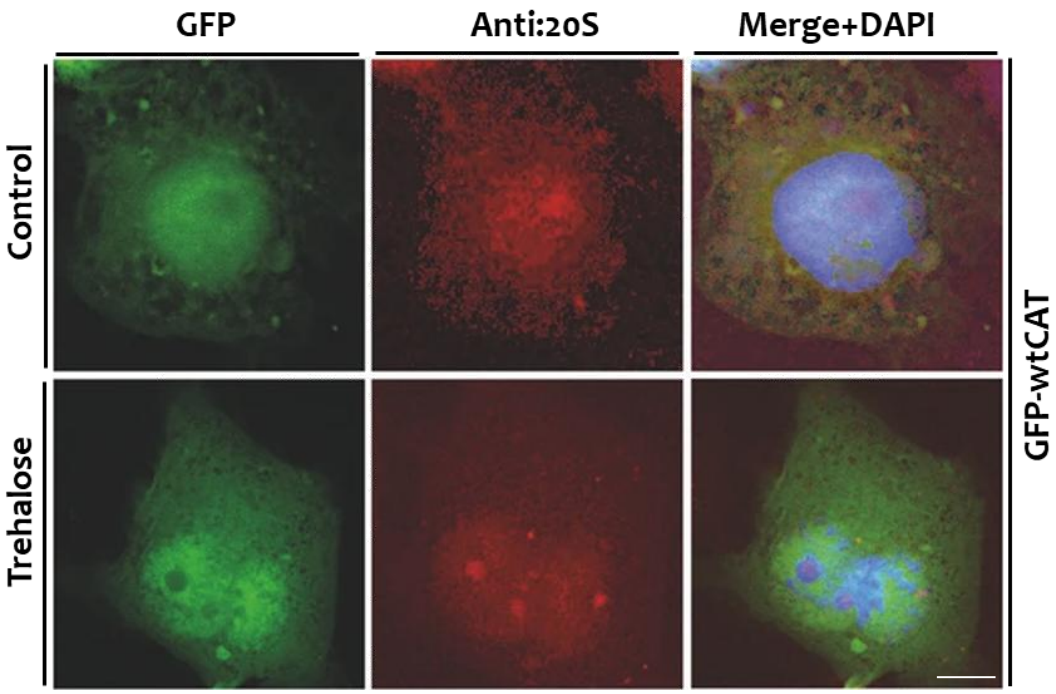

Figure 3.18: Trehalose treatment effect on wild-type CAT protein: COS-7 cells were cultured in 2-well chambered slides and were transfected transiently with GFP-tagged wtCAT constructs. Post-transfection, cells were subjected to control and trehalose (1.5 mM) for 48 h; post-treatment, immunofluorescence staining with antibodies against 20S was done. The secondary antibodies used were Texas red-conjugated (red). Furthermore, counterstaining with DAPI (blue) was done to localize the nucleus. Cells were analyzed with epi-fluorescence microscopy. *Scale bar 20 µm.*

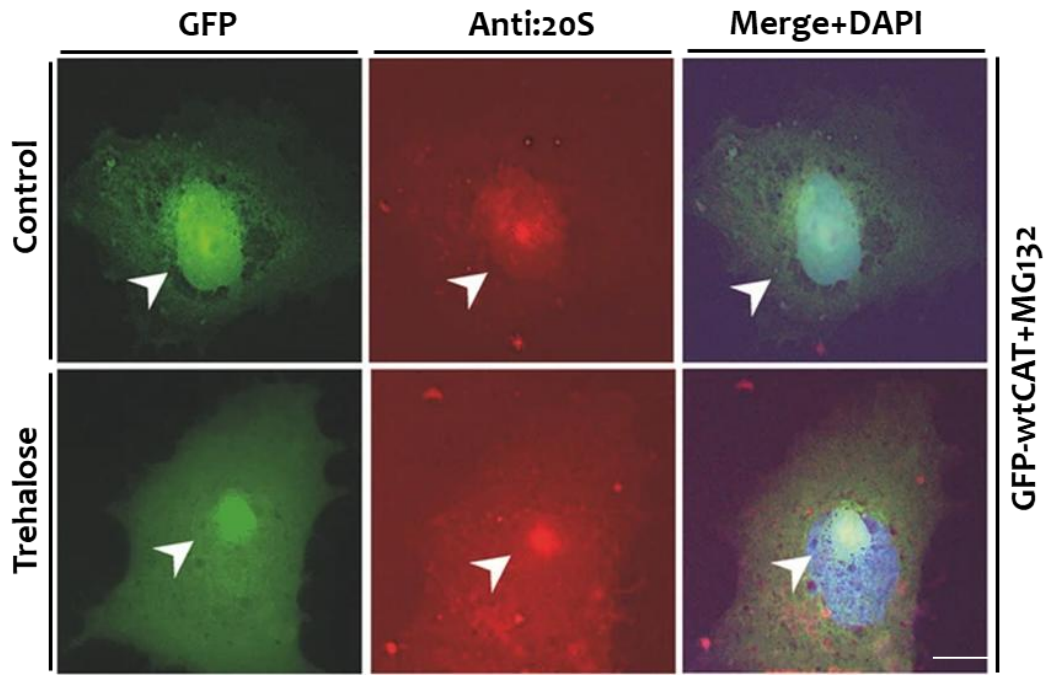

Figure 3.19: Trehalose treatment effect on wild-type CAT in presence of MG132: COS-7 cells were cultured in 2-well chambered slides and were transfected transiently with GFP-tagged wtCAT constructs. Post-transfection, cells were subjected to control and trehalose (1.5 mM) for 48 h and were also subjected to MG132 (10 µM) for 12 h. Post-treatment, immunofluorescence staining with antibodies against 20S was done. The secondary antibodies used were Texas red-conjugated (red). Furthermore, counterstaining with DAPI (blue) was done to localize the nucleus. Cells were analyzed with fluorescence microscopy. Arrowheads indicate cytoplasmic perinuclear aggregates of GFP-wtCAT misfolded proteins colocalizing with 20S proteasome. *Scale bar 20 µm.*

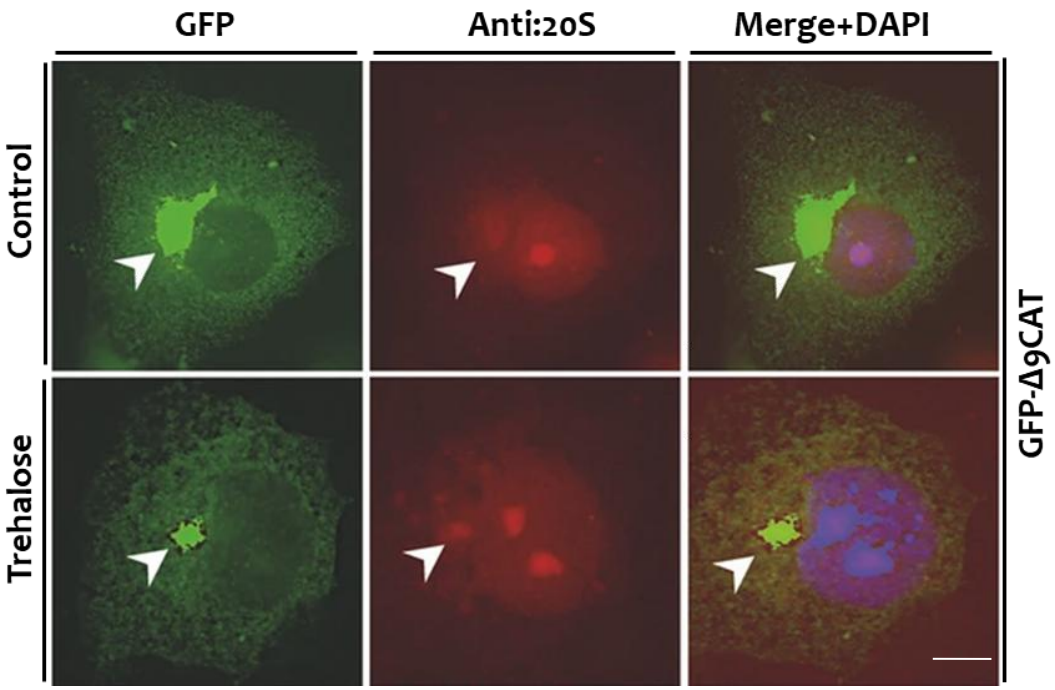

Figure 3.20: Trehalose treatment enhances the clearance of perinuclear aggregates of Δ9CAT from the cytoplasm: COS-7 cells were transfected transiently with GFP-tagged Δ9CAT constructs. Post-transfection, cells were subjected to control and trehalose (1.5 mM) for 48 h. Post-treatment, immunofluorescence examination with antibodies against 20S was done. The secondary antibodies used were Texas red-conjugated (red). Furthermore, counterstaining with DAPI (blue) was done to localize the nucleus. Arrowheads indicate cytoplasmic perinuclear aggregates of GFP-Δ9CAT misfolded proteins colocalizing with 20S proteasome. *Scale bar 20 μm.*

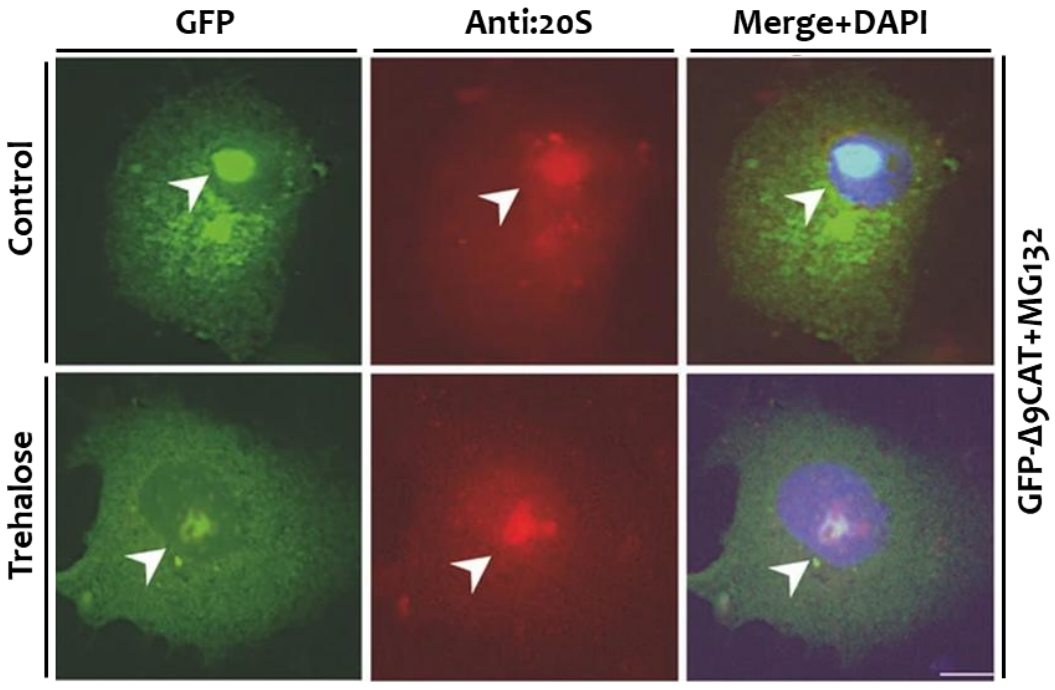

Figure 3.21: Trehalose-induced clearance of Δ9CAT protein aggregates is reverted by MG132: COS-7 cells were transfected transiently with GFP-tagged Δ9CAT constructs. Post-transfection, cells were subjected to control and trehalose (1.5 mM) for 48 h; cells were also subjected to MG132 (10 μM) for 12 h. Post-treatment, immunofluorescence examination with antibodies against 20S was done. The secondary antibodies used were Texas red-conjugated (red). Furthermore, counterstaining with DAPI (blue) was done to localize the nucleus. Arrowheads indicate cytoplasmic perinuclear aggregates of GFP-Δ9CAT misfolded proteins colocalizing with 20S proteasome. *Scale bar 20 μm.*

3.1.6 Treatment of Trehalose Induces Degradation of Mutant Superoxide Dismutase (SOD1) Proteins

Cells retain several mechanisms to maintain proteostasis and intracellular protein folding (Chhangani and Mishra, 2013a). It has been observed that disturbance of cellular proteostasis leads to several complexities, such as proteotoxic effects, and regularly threatens the survival of cells (Nakamura and Lipton, 2009). In this study, we have noted that the treatment of Trehalose enhances the removal of misfolded bona fide proteins; these results prompt us to cross-check the effect of Trehalose on different proteins linked with neurodegenerative diseases. To analyze mutant SOD1 protein that the treatment of Trehalose can degrade, we transfected cells transiently with wild-type (SOD1-WT) and mutant SOD1-G37R (GFP-tagged) expression constructs; few cell sets were co-treated in presence/absence of Trehalose and MG132 (Figure 3.22-3.25).

Post-treatment, the cells were used for immunocytochemistry studies utilizing anti-20S antibodies. As represented in Figure 3.22, wild-type (SOD1-WT) does not form apparent misfolded inclusions. After the addition of MG132, few inclusions of wild-type (SOD1-WT) were observed; addition of Trehalose promotes their solubilization, as shown in Figure 3.23. Expression of mutant SOD1-G37R construct form misfolded aggresomes-like structures, which were effectively degraded by the treatment of Trehalose, as shown in Figure 3.24. The addition of MG132 in a few samples of the experiment mentioned above reverted the effect of Trehalose (Figure 3.25), which suggests that the treatment of Trehalose promotes the clearance of misfolded mutant SOD1 inclusions most probably via the Proteasome system. Together, our results suggest a new possible insight that governs the clearance of mutant proteins with the help of small molecules treatment, such as Trehalose.

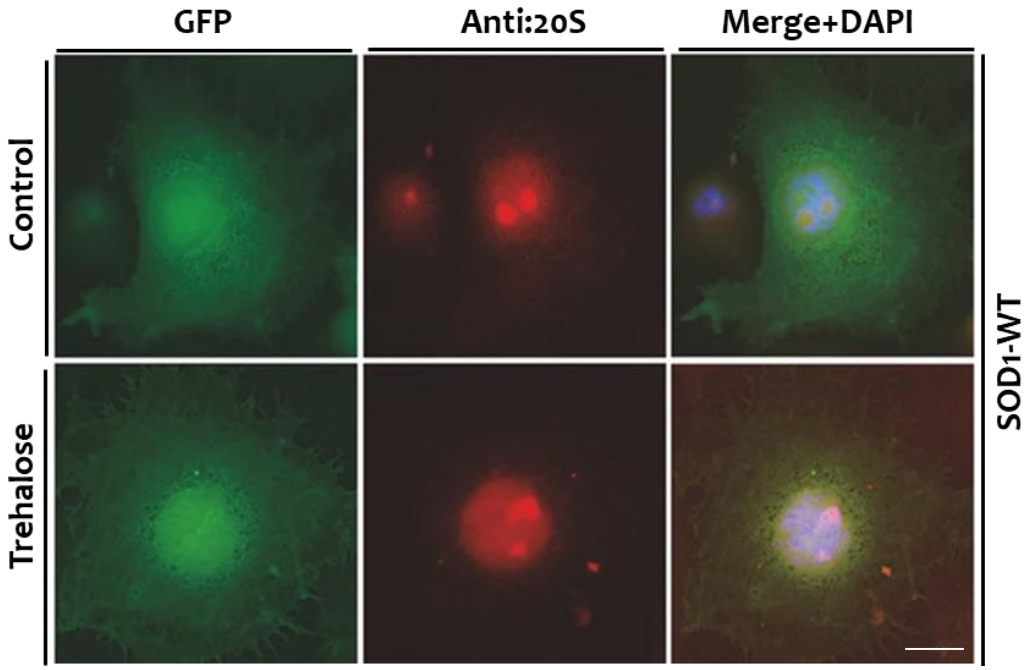

Figure 3.22: Effect of trehalose on wt-SOD1 protein: for experimentation, COS-7 cells were grown and transfected transiently with GFP-tagged non-pathogenic SOD1-WT for 24 h and afterwards exposed to control and trehalose (1.5 mM) for 48 h. Post-treatment immunocytochemistry was executed by utilizing anti-20S antibody. A TR-conjugated secondary antibody was employed to stain 20S proteasome (red) for the analysis. Further DAPI (blue) staining was done to confirm the localization of the nucleus, and cells were analyzed with epifluorescence microscopy. *Scale bar 20 µm.*

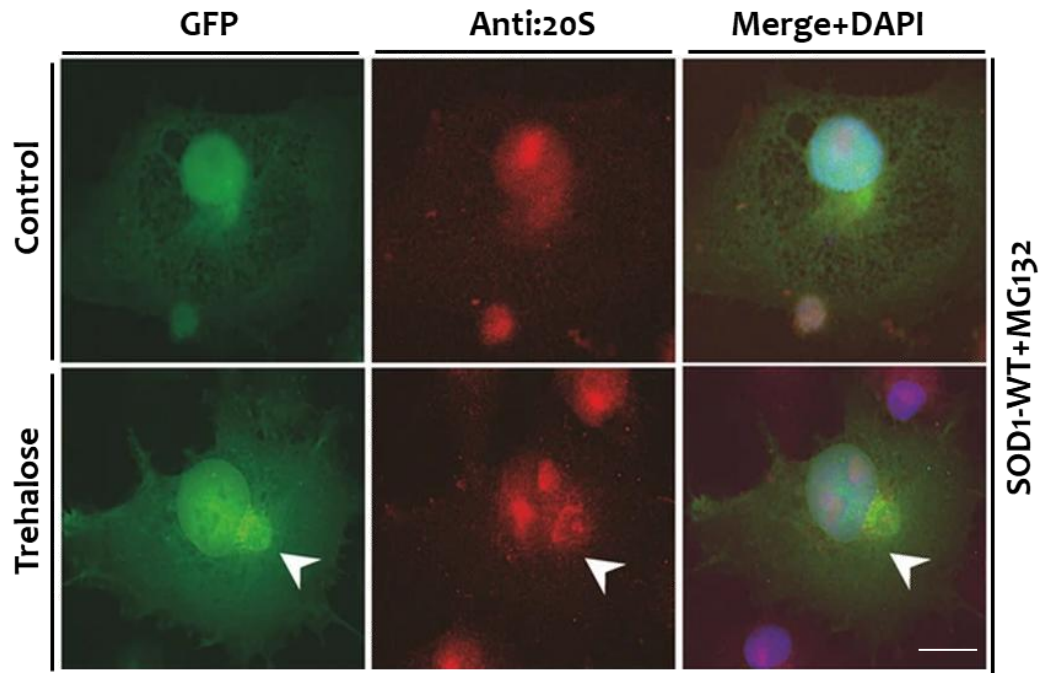

Figure 3.23: Effect of trehalose on wt-SOD1 in presence of MG132: COS-7 cells were grown and transfected transiently with GFP-tagged non-pathogenic SOD1-WT for 24 h and afterwards exposed to control and trehalose (1.5 mM) for 48 h; cells were also subjected to MG132 (10 µM) for 12 h. Post-treatment immunocytochemistry was executed by utilizing anti-20S antibody. A TR-conjugated secondary antibody was employed to stain 20S proteasome (red) for the analysis. Further DAPI (blue) staining was done to confirm the localization of the nucleus, and cells were analyzed with a fluorescence microscope. The arrowheads point to the formed aggregates and the colocalization of 20S proteasome with the aggregates of SOD1 proteins. *Scale bar 20 µm.*

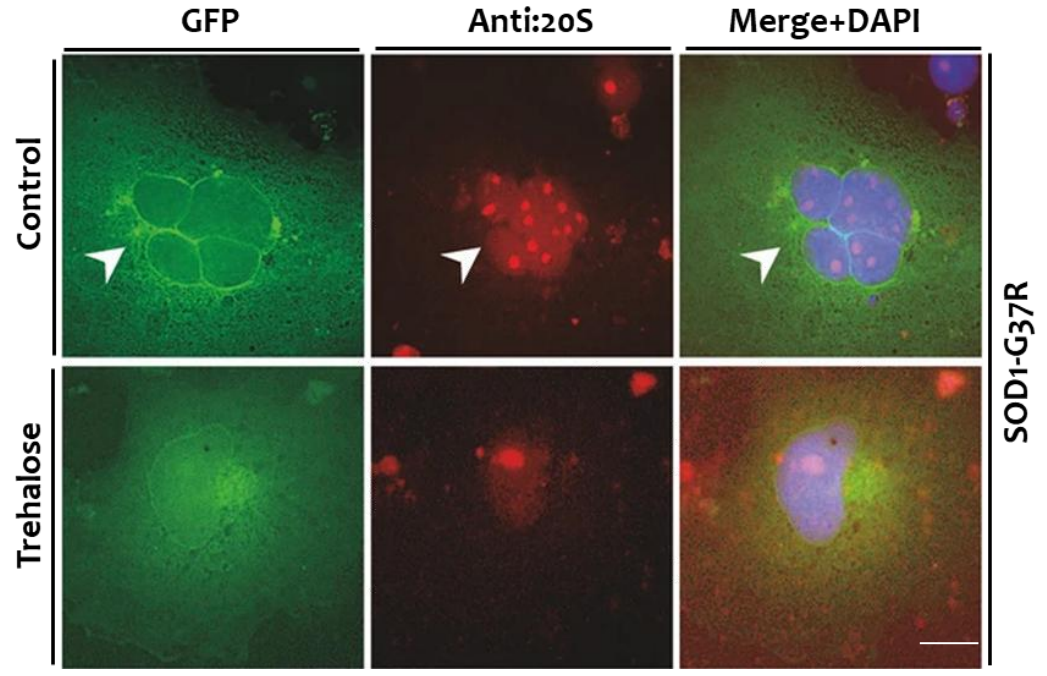

Figure 3.24: Trehalose suppresses accumulation of ALS-linked SOD1 mutant protein: COS-7 cells were grown and transfected transiently with GFP-tagged pathogenic mutant SOD1-G37R for 24 h and afterwards exposed to control and trehalose (1.5 mM) for 48 h. Post-treatment immunocytochemistry was executed by utilizing anti-20S antibody. A Texas-Red-conjugated secondary antibody was employed to stain 20S proteasome (red) for the analysis. Further DAPI (blue) staining was done to confirm the localization of the nucleus, and cells were analyzed with a fluorescence microscope. The arrowheads point to the formed aggregates and the colocalization of 20S proteasome with the aggregates of SOD1 proteins. *Scale bar 20 µm.*

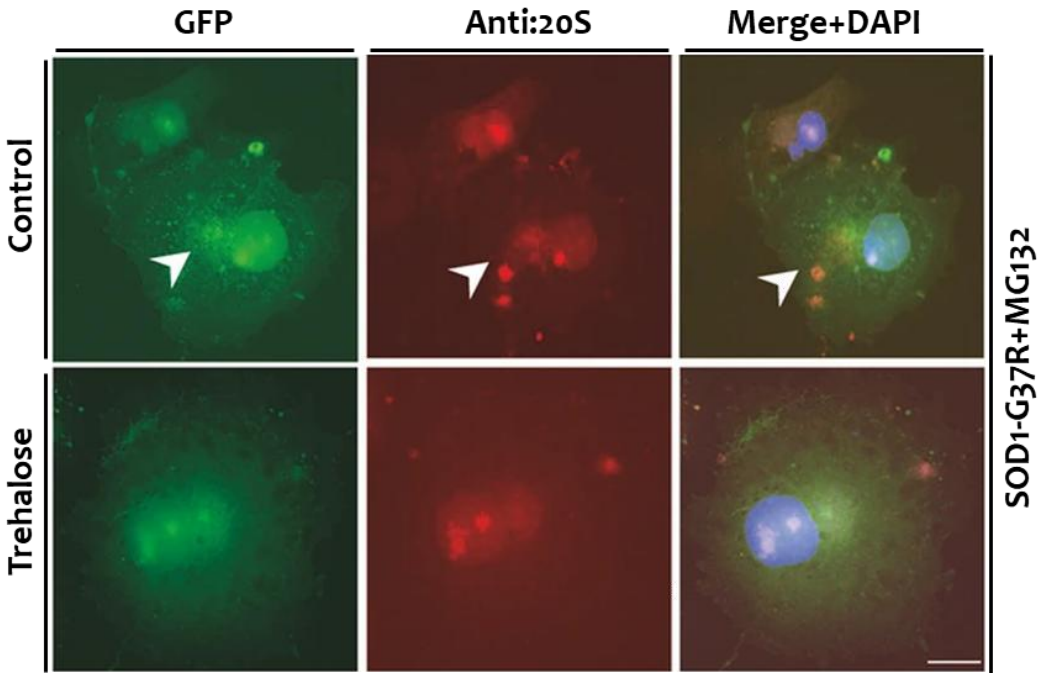

Figure 3.25: When subjected to cells, trehalose can enhance the clearance of mutant SOD1-G37R aggregates: for experimentation, COS-7 cells were grown and transfected transiently with GFP-tagged pathogenic mutant SOD1-G37R for 24 h and afterwards exposed to control and trehalose (1.5 mM) for 48 h; cells were also subjected to MG132 (10 μM) for 12 h. Post-treatment immunocytochemistry was executed by utilizing anti-20S antibody. A TR-conjugated secondary antibody was employed to stain 20S proteasome (red) for the analysis. Further DAPI (blue) staining was done to confirm the localization of the nucleus, and cells were analyzed with a fluorescence microscope. The arrowheads point to the formed aggregates and the colocalization of 20S proteasome with the aggregates of SOD1 proteins. *Scale bar 20 μm.*

3.1.7 Trehalose Promotes the Degradation of Spinocerebellar Ataxia Type 3 Expanded Polyglutamine Protein

Previous findings suggest that CAG trinucleotide repeat expansion in certain genes is involved in various neurodegenerative diseases (Lieberman et al., 2019). Proteasome-mediated degradation facilitates the removal of polyglutamine-expanded abnormal inclusions from the cells (Michalik and Van Broeckhoven, 2004). We observed that using Trehalose promotes the proteasome-dependent clearance of ALS-associated mutant SOD1 protein. To determine whether Trehalose treatment can also improve the clearance of abnormal spinocerebellar ataxia type 3 expanded polyglutamine protein, we tested the influence of Trehalose exposure on the propensity of aggregation of ataxia type 3 expanded polyglutamine protein. COS-7 cells were transfected transiently with both normal repeat ataxin-3(Q28) and polyglutamine expanded repeat containing ataxin-3(Q84) GFP-tagged constructs and then were also treated with or without Trehalose as shown in Figures 3.26-3.29. A few similar sets of experiments were also exposed with MG132.

We observed that expression of expanded polyglutamine repeat containing ataxin-3 (Q84) plasmid produces more abnormal inclusions (Figure 3.28) as compared to normal repeat ataxin-3(Q28) (Figure 3.26). Adding MG132, induces the aggregation propensity of both normal repeat ataxin-3(Q28) and polyglutamine-expanded repeat containing ataxin-3(Q84), which were reverted, when cells were exposed to Trehalose as shown in Figure 3.27 and Figure 3.29. Together, this strongly suggests Trehalose induces proteasome-dependent proteolytic function. Such elevated activities of the Proteasome likely contributed to the adequate clearance of intracellular over-accumulated expanded polyglutamine repeat containing ataxin-3(Q84).

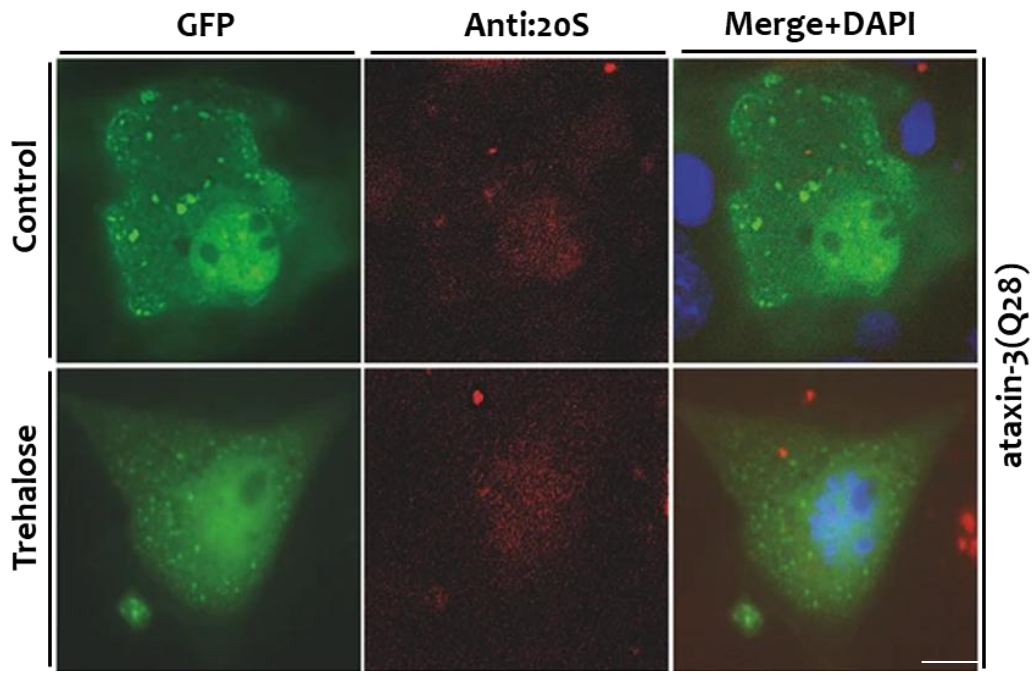

Figure 3.26: Effect of trehalose treatment on ataxin-3 (Q28) polyglutamine protein: COS-7 cells were grown and transfected transiently with GFP-tagged non-pathogenic ataxin-3(Q28) for 24 h and afterwards treated with control and trehalose (1.5 mM) for 48 h. Post-treatment immunocytochemistry was executed by staining with 20S antibody. A secondary antibody (Texas red-conjugated) was utilized to stain 20S proteasome (red) for the analysis. Further, DAPI (blue) staining was done to confirm the localization of the nucleus. Cells were studied under the fluorescence microscope. *Scale bar 20 µm.*

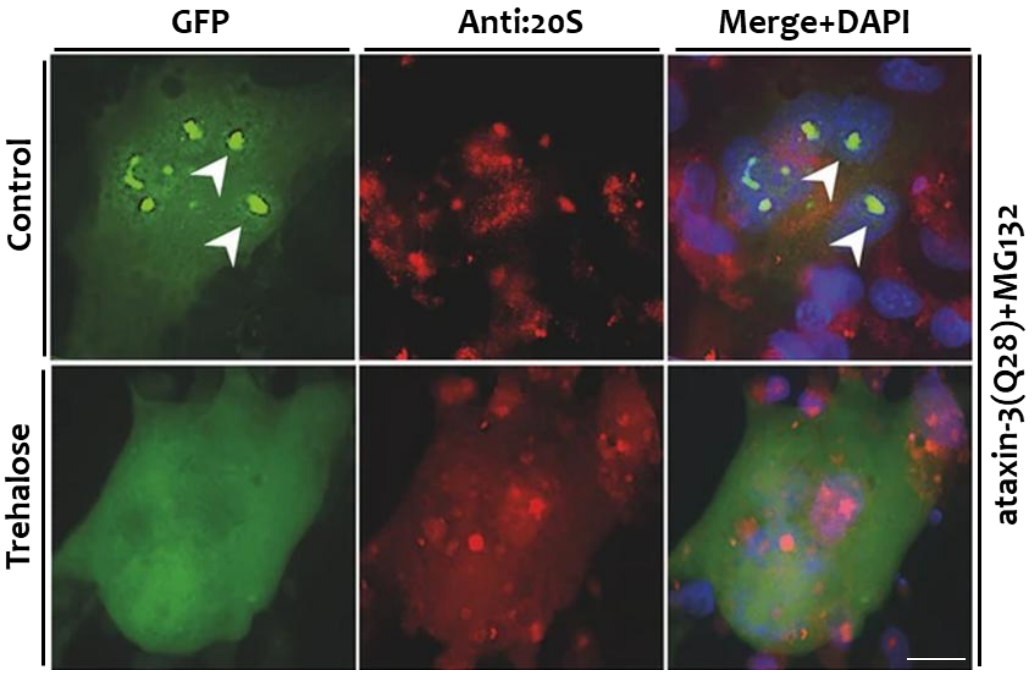

Figure 3.27: Trehalose treatment to cells with ataxin-3 (Q28) in presence of MG132: COS-7 cells were grown and transfected transiently with GFP-tagged non-pathogenic ataxin-3(Q28); afterwards control and trehalose (1.5 mM) for 48 h and MG132 (10 µM) for 12 h treatment was done. Post-treatment immunocytochemistry was executed by staining with 20S antibody. A secondary antibody (Texas red-conjugated) was utilized to stain 20S proteasome (red) for the analysis. Further, staining of DAPI (blue) was done to confirm the localization of the nucleus and imaging was done under the fluorescence microscope. The arrowheads point to the formed aggregates and the colocalization of 20S proteasome with the cytoplasmic aggregates of ataxin-3 proteins. *Scale bar 20 µm.*

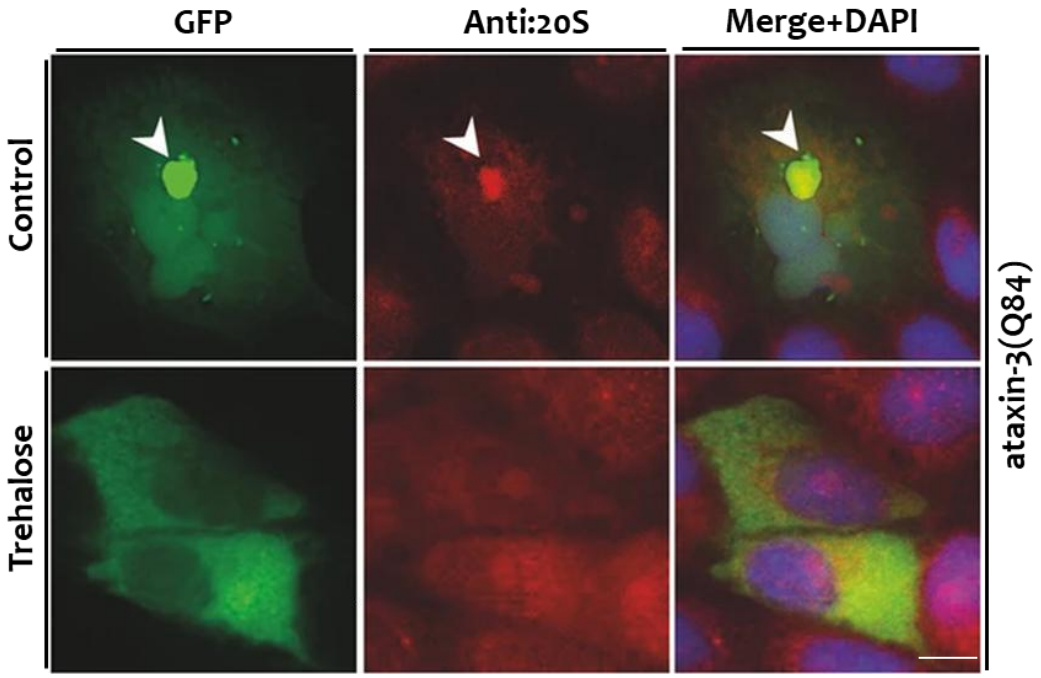

Figure 3.28: Treatment with trehalose clears the polyglutamine aggregates of ataxin-3: for experimentation, COS-7 cells were grown and transfected transiently with GFP-tagged pathogenic ataxin-3(Q84) and afterwards control and trehalose (1.5 mM) treated for 48 h. Post-treatment immunocytochemistry was executed by staining with 20S antibody. A secondary antibody (Texas red-conjugated) was utilized to stain 20S proteasome (red) for the analysis. Further, DAPI (blue) staining was done to confirm the localization of nucleus. The arrowheads point to the formed aggregates and the colocalization of 20S proteasome with the cytoplasmic aggregates of ataxin-3 proteins. *Scale bar 20 µm.*

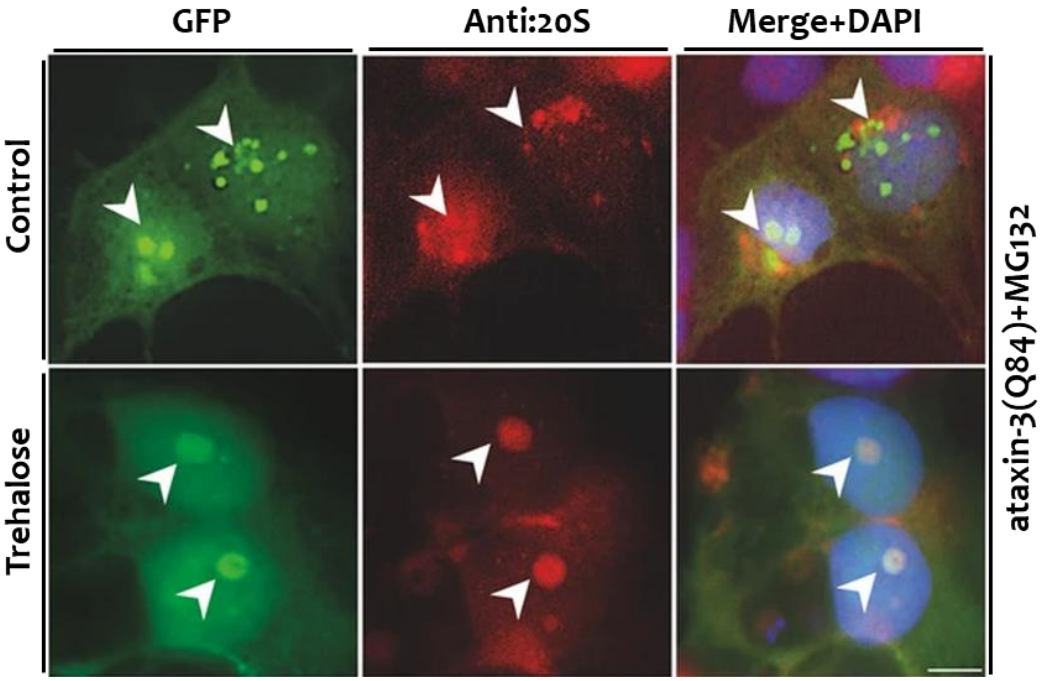

Figure 3.29: Anti-aggregation effect of trehalose on ataxin-3 (Q84) is reduced by MG132: COS-7 cells were grown and transfected transiently with GFP-tagged pathogenic ataxin-3(Q84) and afterwards control and trehalose (1.5 mM) for 48 h and MG132 (10 µM) for 12 h treatment was done. Post-treatment immunocytochemistry was executed by staining with 20S antibody. A secondary antibody (Texas red-conjugated) was utilized to stain 20S proteasome (red) for the analysis. Further, staining of DAPI (blue) was done to confirm the localization of nucleus and imaging under the fluorescence microscope was done. The arrowheads point to the formed aggregates and the colocalization of 20S proteasome with the cytoplasmic aggregates of ataxin-3 proteins. *Scale bar 20 µm.*

3.1.8 Improved Proteasomal Degradation of Polyglutamine-Expanded Huntingtin Proteins in the Presence of Trehalose

Neuronal degenerative disorders are characterized by the existence of abnormal proteinaceous inclusions (Ross and Poirier, 2004). The presence of such inclusions indicates the failure of their removal due to the possible defects in the PQC mechanism, which most likely depleted their functions and consequently led to the massive accumulation of misfolded proteins (Ross and Poirier, 2004). We initially monitored the effect of Trehalose on bona fide abnormal or misfolded proteins and various neurodegenerative diseases-associated proteins. Next, we needed to examine the effect of Trehalose on Huntington's Disease (HD) associated expanded polyglutamine repeats; therefore, we transfected cells transiently with both normal (EGFP-HD23Q) and expanded (EGFP-HD74Q) polyglutamine repeat containing constructs as shown in Figure 3.30-3.33. We analyzed that expression of expanded (EGFP-HD74Q) polyglutamine repeat forms numerous abnormal inclusions (Figure 3.32) as compared to normal (EGFP-HD23Q) polyglutamine repeat expression (Figure 3.30).

Our results also suggest that exposure to MG132 induced the aggregation propensity of both normal (EGFP-HD23Q) polyglutamine (Figure 3.31) and expanded (EGFP-HD74Q) polyglutamine repeat-containing proteins (Figure 3.33). Reduced aggregation of expanded polyglutamine repeats was observed after adding Trehalose, as shown in Figure 3.32. Together, these observations are congruent with earlier results and suggest Trehalose treatment is linked to Proteasomal dependent malfolded proteins degradation and most probably Trehalose induced the function of Proteasome for better removal of abnormal proteins from the crowded pool of protein in the cells.

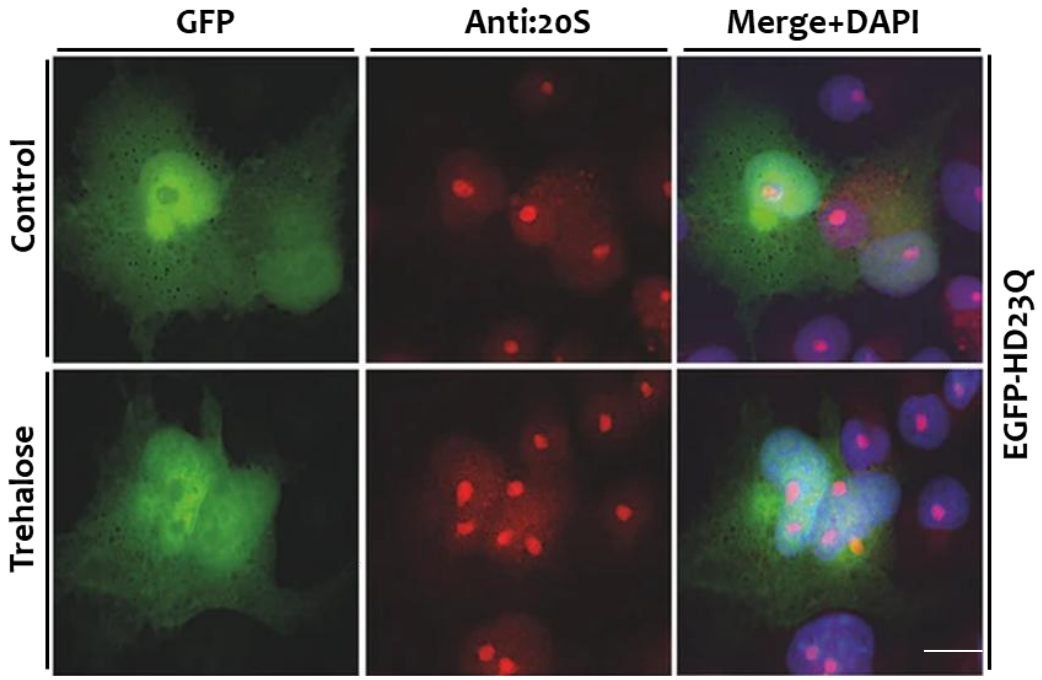

Figure 3.30: Effect of trehalose on EGFP-HD23Q: COS-7 cells cultured in 2-well chambered slides were transfected (transient) with EGFP-HD23Q plasmids. Post-transfection cells were exposed to control (distilled water) and trehalose (1.5 mM) for 48 h. Post-treatment, immunocytochemistry analysis with antibodies against 20S was done. Secondary antibodies used were Texas red-conjugated (red); furthermore, counterstaining with DAPI (blue) was done to localize the nucleus. Cells were analyzed with fluorescence microscope. *Scale bar 20 μm.*

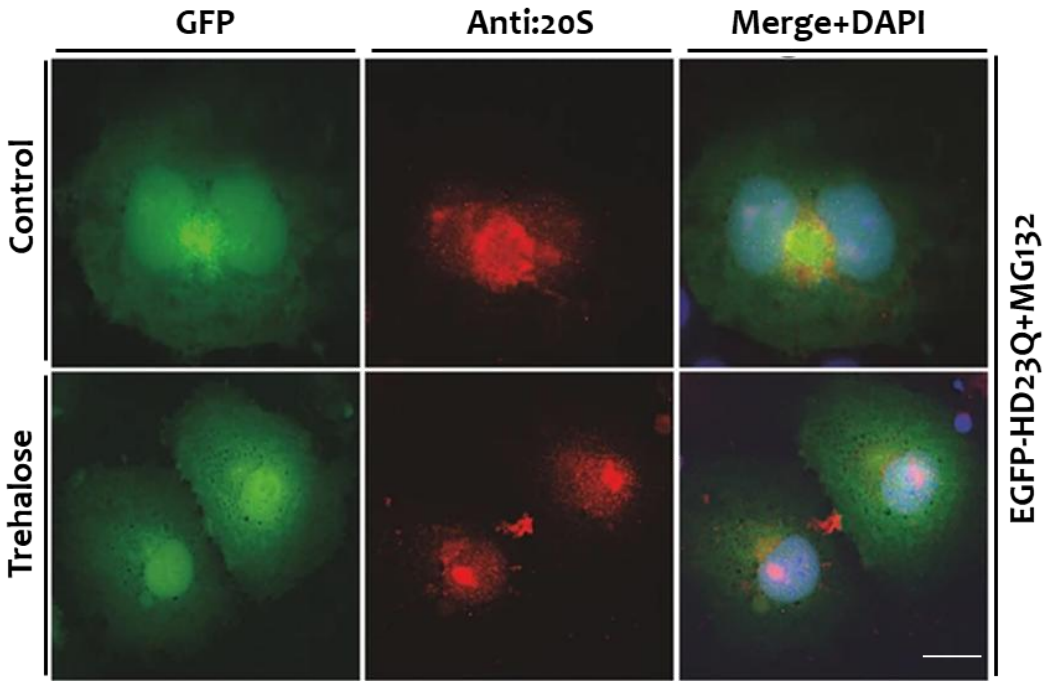

Figure 3.31: Effect of trehalose on EGFP-HD23Q in presence of MG132: COS-7 cells cultured in 2-well chambered slides were transfected (transient) with EGFP-HD23Q plasmids. Post-transfection cells were exposed to control (distilled water) and trehalose (1.5 mM) for 48 h; cells were also subjected to MG132 (10 µM) for 12 h. Post-treatment, immunocytochemistry analysis with antibodies against 20S was done. Secondary antibodies used were Texas red-conjugated (red); furthermore, counterstaining with DAPI (blue) was done to localize the nucleus. Cells were analyzed with fluorescence microscope. *Scale bar 20 µm*

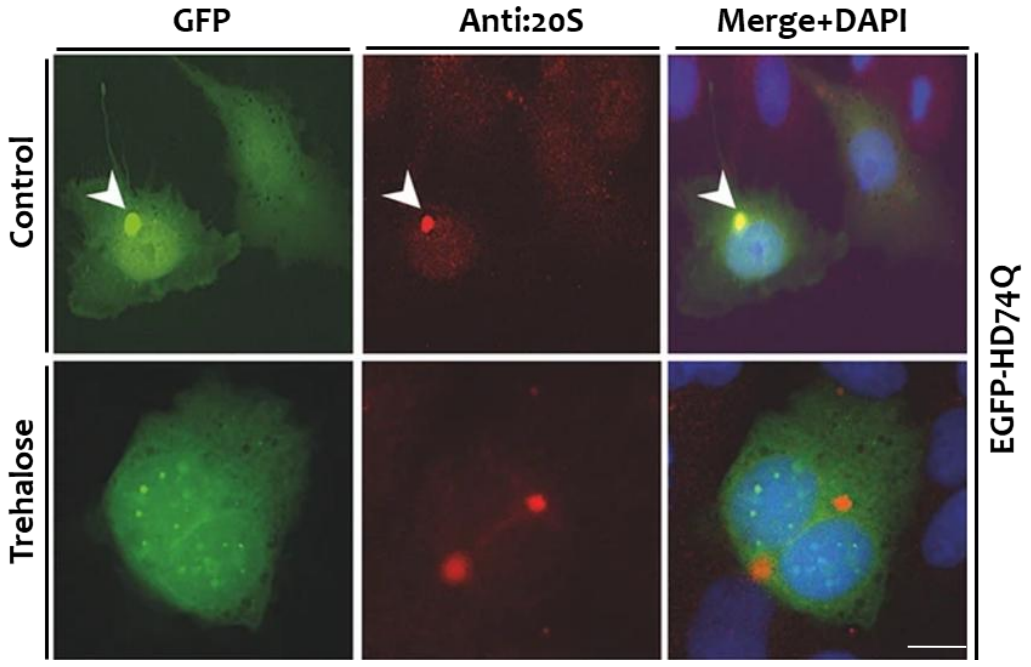

Figure 3.32: Ameliorating effect of trehalose on expanded polyglutamine aggregates of HD74Q: COS-7 cells cultured in 2-well chambered slides were transfected (transient) with EGFP-HD74Q plasmids. Post-transfection cells were exposed to control (distilled water) and trehalose (1.5 mM) for 48 h. Next, immunocytochemistry analysis with antibodies against 20S was done. Secondary antibodies used were Texas red-conjugated (red); furthermore, counterstaining with DAPI (blue) was done to localize the nucleus. Cells were analyzed with the fluorescence microscope. The arrowheads indicate the expanded polyglutamine aggregates of HD74Q misfolded proteins colocalizing with 20S proteasome. *Scale bar 20 µm.*

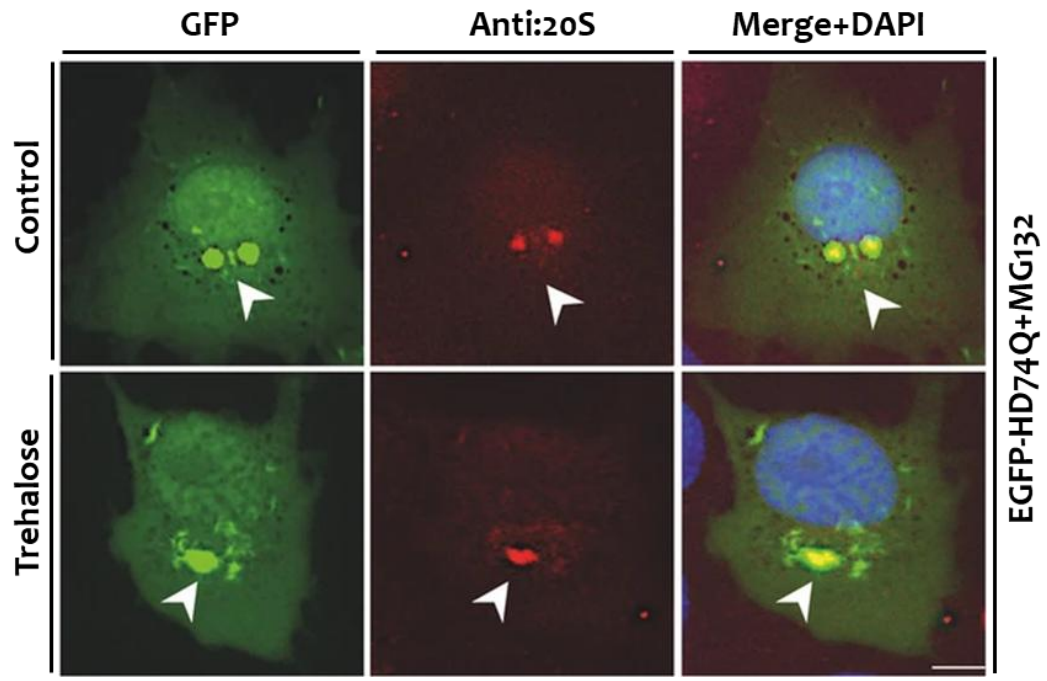

Figure 3.33: MG132 diminished the effect of trehalose induced clearance of Huntington disease associated aggresomes: COS-7 cells cultured in 2-well chambered slides were transfected (transient) with EGFP-HD74Q plasmids. Post-transfection cells were exposed to control (distilled water) and trehalose (1.5 mM) for 48 h; cells were also subjected to MG132 (10 µM) for 12 h. Next, immunocytochemistry analysis with antibodies against 20S was done. Secondary antibodies used were Texas red-conjugated (red); furthermore, counterstaining with DAPI (blue) was done to localize the nucleus. Cells were analyzed with the fluorescence microscope. The arrowheads indicate the expanded polyglutamine aggregates of HD74Q misfolded proteins colocalizing with 20S proteasome. *Scale bar 20 µm.*

3.1.9 Trehalose Reduces the Misfolding of Proteins And Increases Cell Viability In High-Stress Environments

We observed exposure of Trehalose to cell elevates the proteasome levels endogenously and induces the proteasomal activities. Consequently, as a further step, we investigated direct effect of Trehalose on tendency of misfolded proteins for aggregating. Following that, we carried out a dot-blot experiment with cell lysates expressing a wide variety of aberrant proteins. Our results imply that utilising Trehalose enhances the solubility of defective proteins, as demonstrated in Figure 3.35, and exposure to Trehalose encourages the clearance of aberrant proteins, as shown in Figure 3.34.

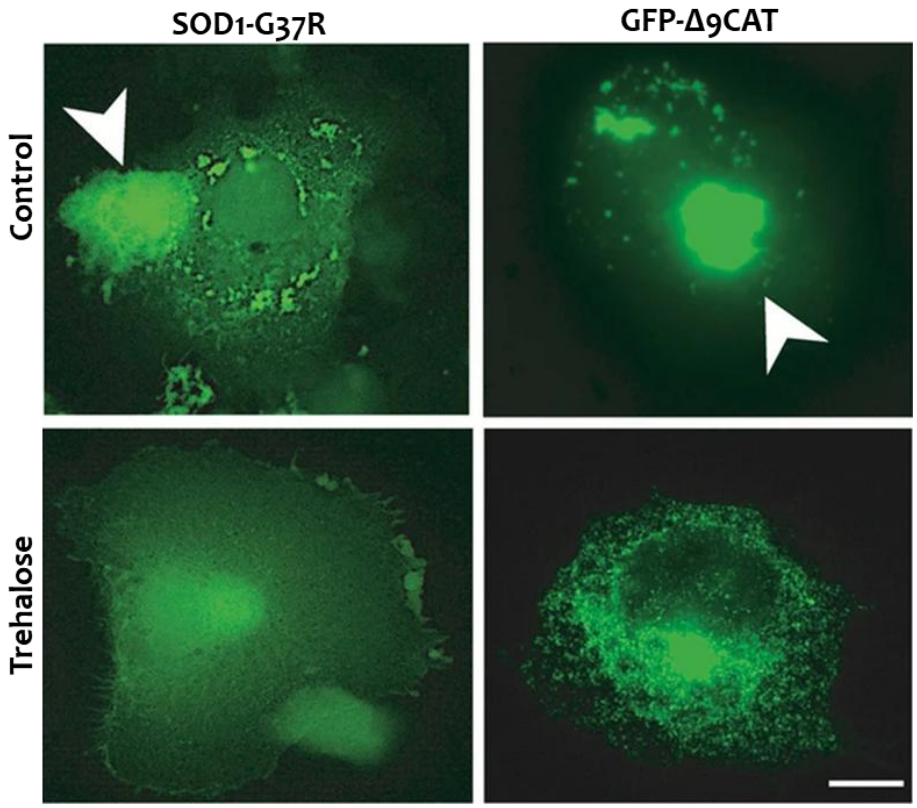

Figure 3.34: Trehalose efficiently provides cytoprotection against proteotoxic inclusions: Cells were transfected transiently with constructs of SOD1-G37R and GFP-Δ9CAT. Few sets of cells were treated with or without trehalose (1.5 mM). Cells were analyzed with fluorescence microscope, and the arrowheads shows aggregates of misfolded protein. *Scale bar 20 μm.*

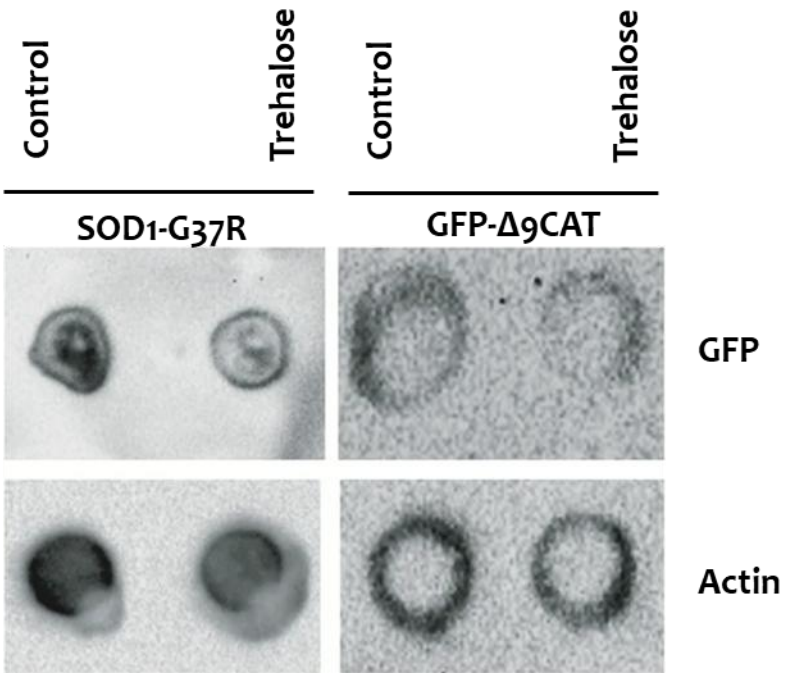

Figure 3.35: Trehalose efficiently promotes solubilization of aberrant proteins: Cells were transfected transiently with constructs of SOD1-G37R and GFP-Δ9CAT, respectively. Few sets of cells were either treated or not with trehalose (1.5 mM). Next, cells were collected, and their extracts were analysed with dot blot via antibodies anti-GFP and anti-β-actin.

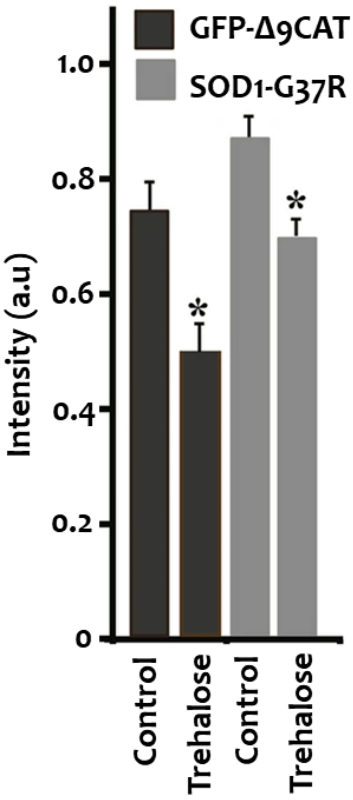

Figure 3.36: Trehalose efficiently enhances clearance of aberrant proteins aggregates: The quantification of the dot blot analysis shown in Figure 3.35 is represented here. NIH image analysis software used the densitometric method to analyze the accumulated protein inclusions. *p < 0.05 when compared to the control.

Previous research suggested that the production of aberrant proteins may lead to cellular stress and affect cells survival. Under circumstances that generate oxidative and ER stress, we investigated the impact that Trehalose has on cells survival. Following the treatment of the cells with H_2O_2 and Tunicamycin, some of the cell samples were transfected with Hsp70 or CHIP constructs. According to the findings that we obtained, treatment with Trehalose seems to provide cytoprotection against cellular stress conditions. Furthermore, the combined impact of Trehalose and the exogenous molecular chaperones expression (Hsp70 and CHIP) appears to be more favourable for the cells in terms of their survival, as shown in Figure 3.37. During the aforementioned tests, we also verified the exogenous expression of CHIP and Hsp70, as shown in Figure 3.38. In general, our findings suggest that combining PQC components with small compounds such as Trehalose may increase the survival of cells under stressful situations. This is most likely because the heightened functions of PQC and the proteasome-based clearance of aberrant proteins promote cell survival.

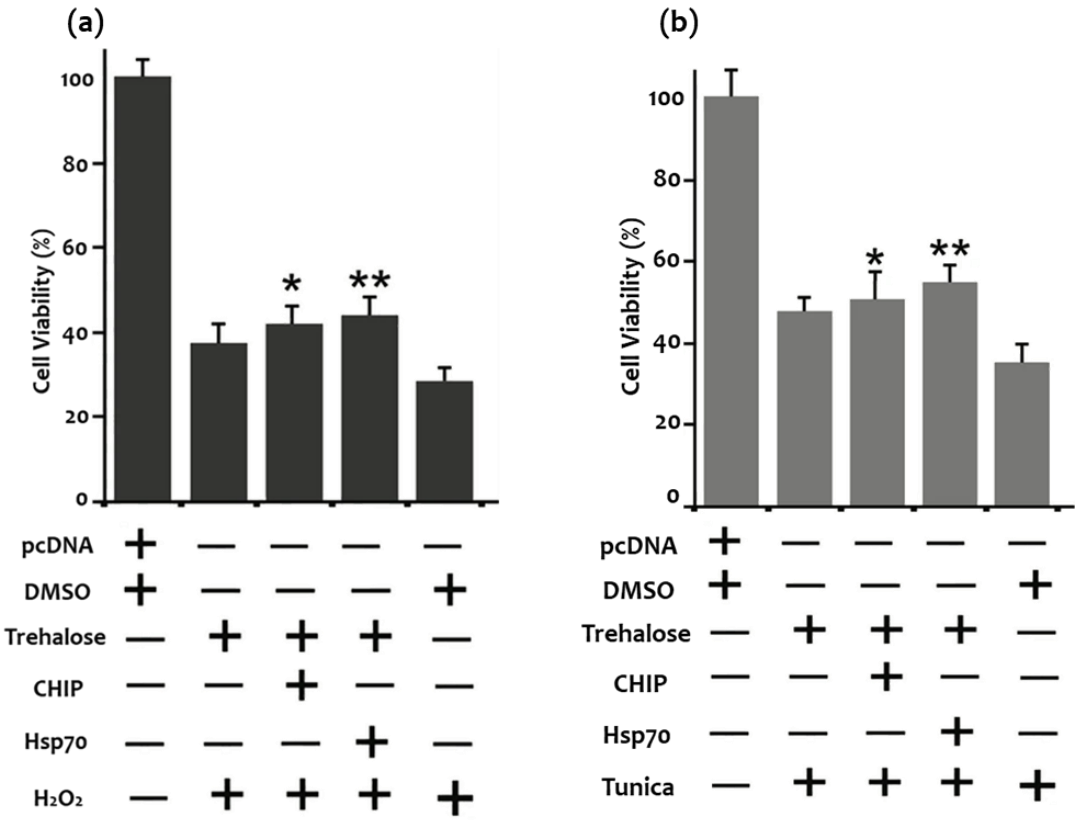

Figure 3.37: Trehalose with chaperones may increase the survival of cells under stressful situations: COS-7 cells were treated with various drugs for different time intervals like MG132 (10 µM) for 10 h; some sets of cells were exposed with or without trehalose (1.5 mM) for 24 h. Few sets of cells were transfected with plasmid constructs of Hsp70 or CHIP. Afterwards, stressors tunicamycin (10 µg/ml) for 12 h (b) and H2O2 (0.2 mM) for 6 h (a) were subjected to some samples. Post-treatment, the cell samples were subjected to MTT assay and cell viability was measured. The results are the mean ± SD of three individual studies, with each experiment being done three times. *p < 0.05 as compared with control.

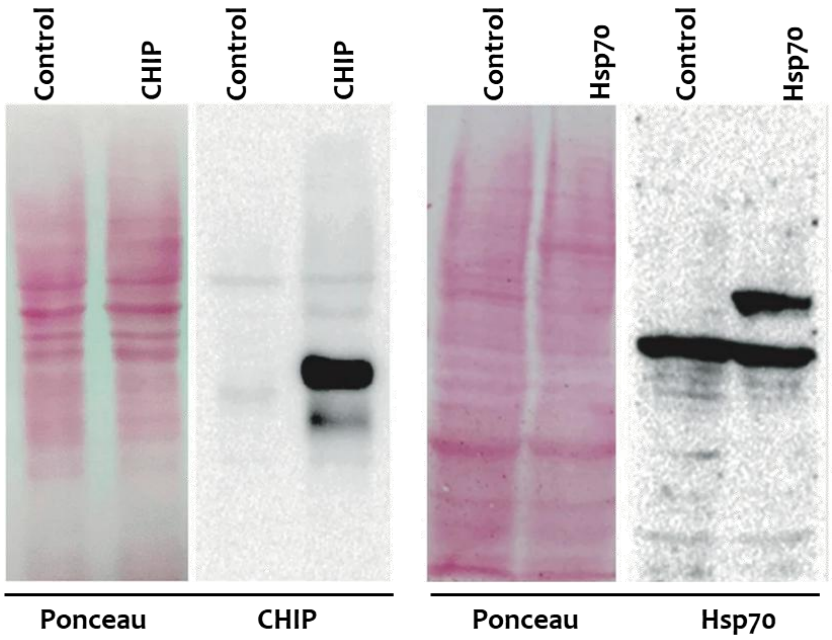

Figure 3.38: Confirmation of exogenous expression of CHIP and Hsp70: the COS-7 cells were transfected transiently with Hsp70 or CHIP constructs and were collected afterwards for immunoblotting and probed using anti-CHIP and anti-Hsp70 with respective ponceau staining.

3.1.10 Trehalose Reduces the Aggregation Load of Abnormal Proteins and Protects the Cells Against Stress Conditions

Previous findings suggest that the elevation of intracellular proteasome functions protects the cells against various cellular insults, e.g., oxidative stress (H_2O_2), proteotoxic stress (MG132), and ER stress (Thapsigargin and BME) (Njomen and Tepe, 2019a). To determine whether treatment of Trehalose protects the cells under stress situations, we examined Trehalose effect on cellular health under different stress conditions. As depicted in Figure 3.39, cells were exposed to varying conditions of stress, and a few sets of samples were also treated with Trehalose. Based on bright-field images, morphological changes can be observed. We noticed Trehalose was sensitive against various cellular insults and provided cryoprotection during stress inflicted by different stress-inducing agents. We found that the treatment of Trehalose under concentration (Figure 3.40 (a)) and time-dependent (Figure 3.40 (b)) fashion did not adversely affect the treated cells viability. These results suggest Proteasome modulation cytoprotective effect by Trehalose without any adverse effects on cells. We previously showed that treatment of Trehalose improves the degradation of neurodegenerative disease-linked proteins.

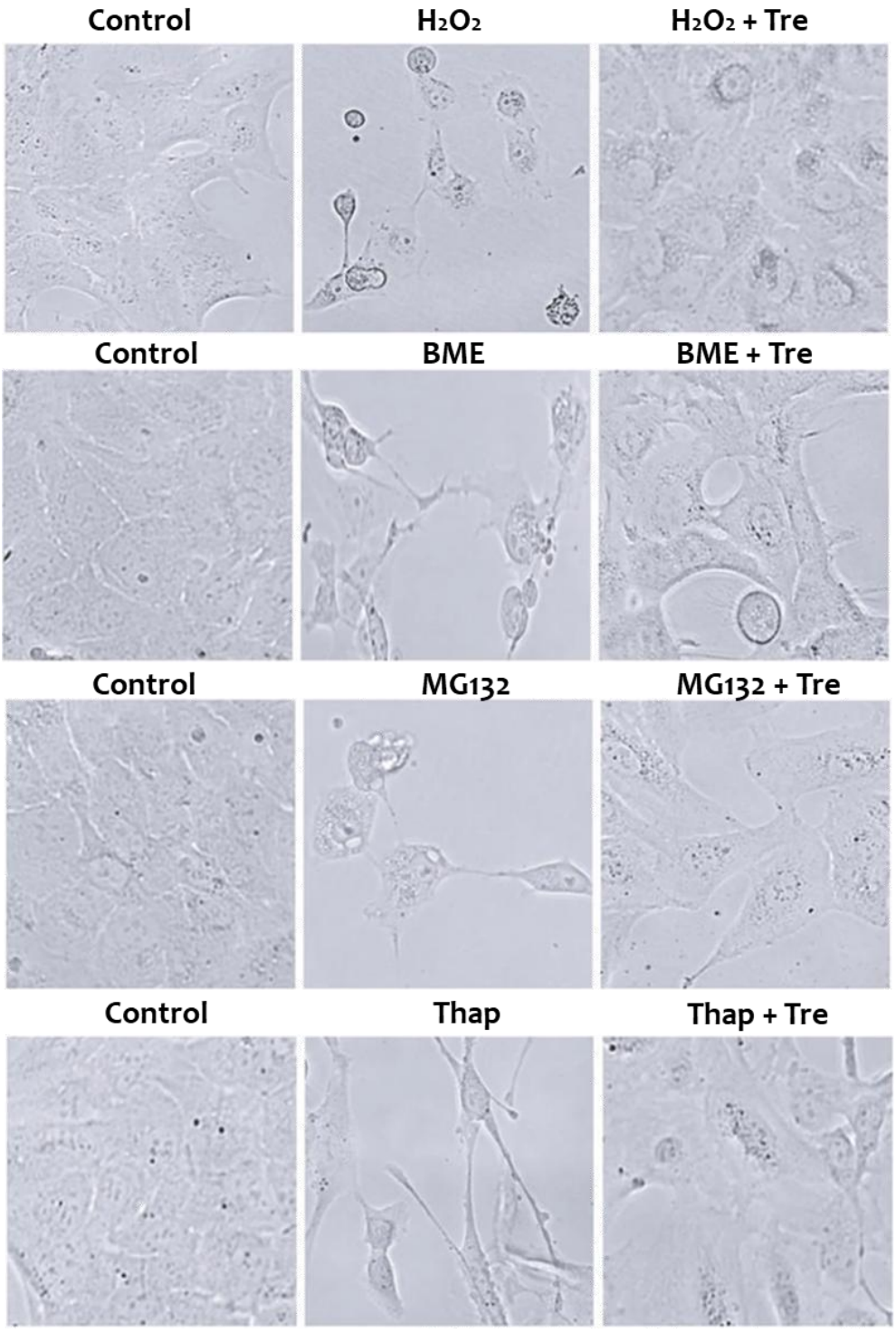

Figure 3.39: Trehalose alleviates the stress-induced cytotoxicity: A549 cells were cultured on 6-well and subjected to different stresses 5 mM BME for 3 h, 10 µM MG132 for 12 h, 5 µg/ml thapsigargin for 8 h, and 0.2 mM H2O2 for 3 h with/without 1.5 mM trehalose (48 h). Post-treatment, bright-field imaging was done.

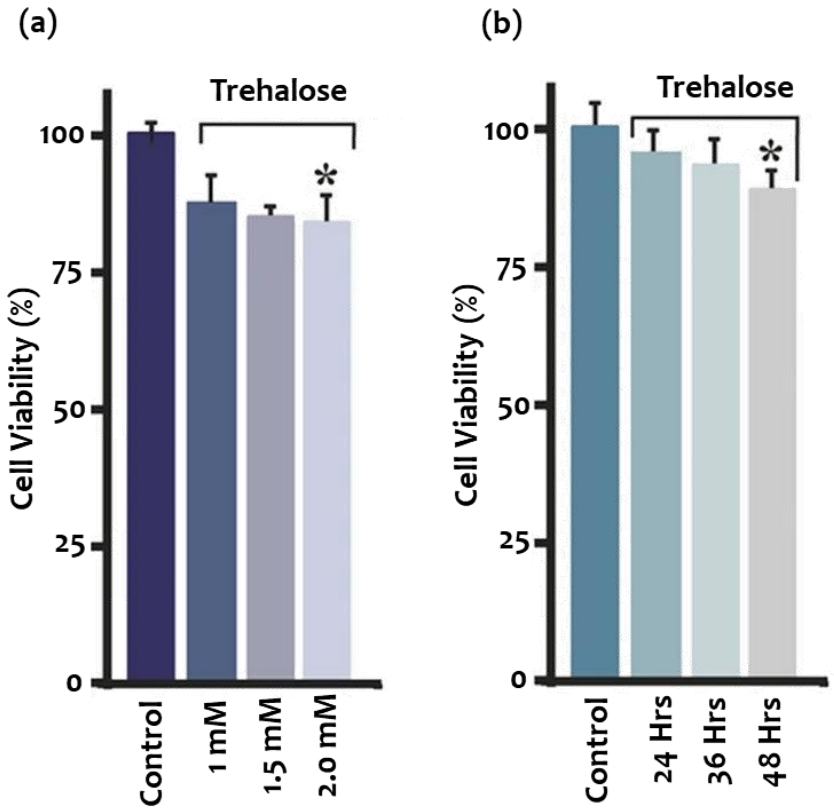

Figure 3.40: Trehalose effect on cellular viability: To measure the viability of cells when exposed to trehalose at different concentrations (48 h) (a) and at various time intervals (1.5 mM) (b), MTT assay was performed. Values represented are means ± SD from three experiments executed independently and in triplicate. *$p < 0.05$ as compared with control.

To see whether the exposure of Trehalose lowers the overall aggregation propensity of various misfolded proteins, we performed a detailed quantitative aggregation analysis, as shown in Figures 3.41 and 3.42. Upon incubation with Trehalose, the aggregation of misfolded proteins (ataxin-3 Q84 (Figure 3.42 (a)); SOD1-G37R (Figure 3.42 (b)) ; GFP- Δ9CAT (Figure 3.42 (c)) were dramatically reduced. The above findings strongly indicate that the treatment of Trehalose most likely enhances endogenous Proteasome functions and improves cellular proteolytic activities, which clears the load of abnormal protein accumulation. Together with all the results, our current findings suggest that Trehalose provides cytoprotection from various cellular damages caused by different proteotoxic insults.

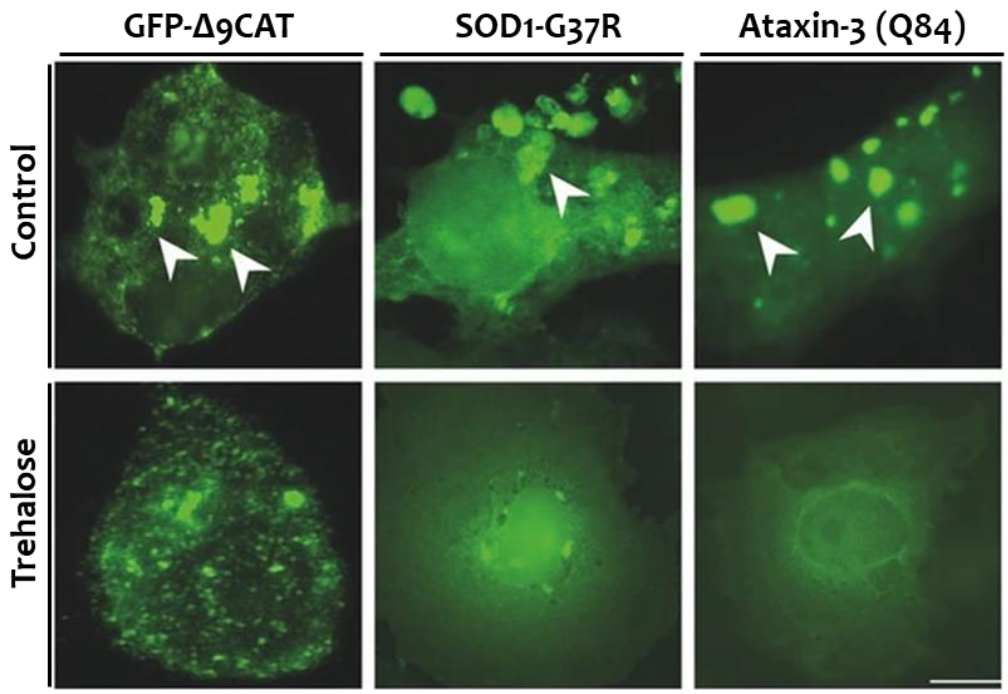

Figure 3.41: Trehalose exposure can reduce aberrant protein aggregation: COS-7 cells in 2-well chambered slides and transfected with aggregation-prone GFP-tagged mutant protein plasmids ataxin-3(Q84), Δ9CAT, and SOD1-G37R and aggregation were monitored in absence/presence of trehalose (1.5 mM; 48 h) by epifluorescence microscopy. *Scale bar: 20 µm.*

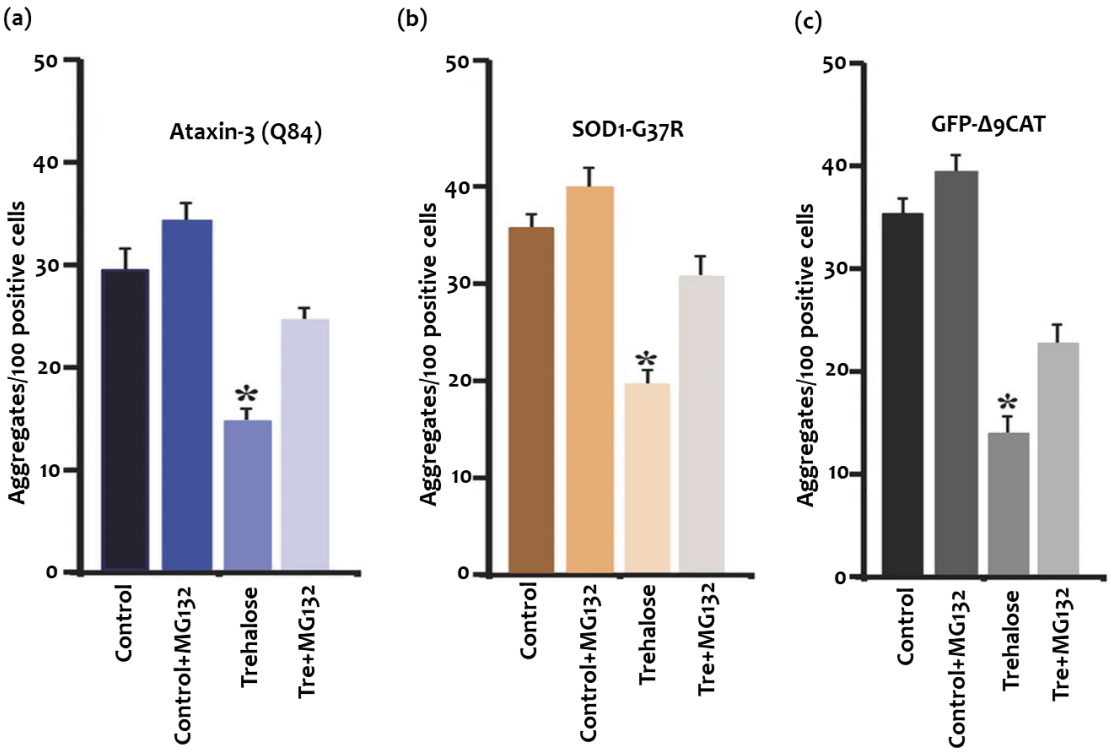

Figure 3.42: Treatment of Trehalose reduces aggregation count of aberrant proteins: Quantification was done for aggregates of different misfolded proteins in cells that were transfected with respective plasmids and subjected to trehalose (1.5 mM) and MG132 (10 µM) (a-c). The values presented are mean ± SD of datasets from three experiments independently and in triplicate. *$p < 0.05$ compared with the control.

3.2 DISCUSSION

Recent findings suggest that identifying proteasome activators is a key approach and retains therapeutic potential for ameliorating neurodegeneration. An earlier finding explored the screening of more than 2000 compounds provided ten compounds, which increased the proteasome activity and model substrates degradation (Leestemaker et al., 2017). Still, it is challenging to find an efficient proteasome activator that can improve the clearance of abnormal proteins without affecting overall cell health and fitness. Our preliminary results indicate treatment of Trehalose induces endogenous levels of proteasome even in a different time-dependent manner. Next, we observed that using Trehalose induces the removal of heat-denatured luciferase and suppresses the aggregation of d1EGFP, a specific proteasome model substrate protein. To further validate the effect of Trehalose on other modeled misfolded proteins, we use GFP-Δ9CAT and observe that Trehalose based strategy synchronously improves the degradation of misfolded inclusions of GFP-Δ9CAT from the cytoplasm and MG132 addition prevented their degradation.

However, the removal of abnormal aggresomes of neurodegeneration-linked proteins is a critical change after their intracellular formation. In an interesting finding, peptide proteasome-activating peptide 1 (PAP1) was shown to increase the chymotrypsin-like proteasomal catalytic activity, which also suppressed the protein aggregation in amyotrophic lateral sclerosis cellular model (Dal Vechio et al., 2014). Similarly, in our present finding, we noticed that the use of Trehalose prevented the intracellular accumulation of ALS disease-associated mutant SOD1-G37R protein, and the effect of Trehalose was reverted when MG132 was added, which suggests that most likely Trehalose induces the clearance of mutant SOD1-G37R through proteasome-based degradation. Another study indicates that the use of oleuropein, a natural compound constituent from *Olea europea* leaf extract, demonstrates enhancement of proteasome activities, most likely through proteasome conformational modulation (Katsiki et al., 2007).

Here, we observe that the use of Trehalose induces Proteasome both PGPH-like and chymotrypsin-like activities; it was also important to confirm the same effect of Trehalose in concentration and time -dependent fashions. In another detailed experiment, we noticed that exposure of Trehalose elevates both chymotrypsin-like and PGPH-like proteasomal activities in dose and time-dependent fashion. In similar sets of experiments, we also noticed that the use of Trehalose does not affect cellular fitness and viability even when treatment at different concentrations and time periods of Trehalose was done. Earlier, several studies suggested the significance of proteasomal activation as a promising therapeutic application (Trader et al., 2017;Njomen et al., 2018). Small compounds mediated stimulation of proteasomal functions also supports the removal of damaged proteins via PQC-dependent proteolytic mechanisms (Medina et al., 2011). It is expected that the efficient degradation of abnormal proteins by activated proteasomes can alleviate proteotoxic insults. We observed in our detailed immunofluorescence analysis experiment that the Trehalose exposure also promotes the endogenous levels of various PQC mechanism-associated molecules such as ubiquitin, p62 (SQSTM1) as well as proteasome as observed in previous experiments.

Earlier findings indicate that the use of Trehalose inhibited expanded polyglutamine protein aggregation and improved cellular survival of the HD cellular model. The beneficiary impact of Trehalose is also demonstrated in transgenic HD mice; oral administration of Trehalose lowered aggregation of expanded polyglutamine proteins in HD mice (Tanaka et al., 2004). In our present report, we noticed that exposure of Trehalose reduced the proteotoxic aggregation of both ataxin-3(Q84) and EGFP-HD74Q expanded polyglutamine proteins. Importantly, our observations provide a framework that the use of MG132 reverts Trehalose's abnormal protein aggregation solubility enhancing effect. A previous study also supports our findings that both bicalutamide and Trehalose ameliorate motor neuronal degeneration

associated with Spinal and Bulbar Muscular Atrophy (SBMA) pathomechanisms in mice (Galbiati et al., 2023).

Next, it was essential for us to observe the impact of Trehalose on cellular health and fitness under different stress conditions. The results indicate increased cellular survival and decreased proteotoxic insults in the presence of Trehalose under the existence of various cellular stress conditions. Reduced aggregation propensity of different neurodegenerative disease-linked proteins in the presence of Trehalose also recommends that using small compounds could open a new therapeutic strategy against protein conformational diseases. Overall, the results demonstrate that using Trehalose induces endogenous proteasome levels and promotes the clearance of modelled misfolded (heat-denatured luciferase and Δ9CAT) proteins. We also examined the Trehalose meditated concentration and time-dependent elevation of chymotrypsin-like and PGPH-like activities of proteasome. Next, we determine that using Trehalose enhanced the removal of abnormal proteins linked with neurodegenerative diseases (SOD1-G37R and expanded polyglutamine aggregates of HD74Q and ataxin-3). Current findings suggest that Trehalose reduces the cytotoxic insults of aberrant proteins and alleviates stress-induced cell death. Our result highlights a cytoprotective function of Trehalose against the aggregation of abnormal proteins.

3.3 CONCLUDING REMARKS

Cells ensure the timely removal of unwanted proteins and avoid the disturbance of proteostasis via the PQC mechanism (Jayaraj et al., 2020). Therefore, finding the compounds that can trigger the Proteasome functions and elevate the removal of misfolded proteins is essential. Here, we also observed that the use of Trehalose improves the cytosolic removal of abnormal proteinaceous inclusions. Proteasome machinery targets the selective removal of intracellular proteins that are defective, misfolded or senescent in nature (Tanaka, 2009;Mishra et al., 2018). Previous findings suggest that over-accumulation of aberrant proteins blocks the Proteasome functions and leads to the progression of protein conformational disorders (Soto, 2001;Deriziotis et al., 2011;Andre and Tabrizi, 2012). Improving the clearance of the overburden of abnormal proteins from the cells is a critical challenge. Elevating the PQC mechanism through small molecules can be a therapeutic action plan for promoting degradation of non-native proteins by activated UPS. Identifying and characterizing such small molecules is another challenge because their treatment should not adversely affect cellular fitness and viability.

In our present report, we noted that using Trehalose elevates endogenous levels of proteasome and promotes the function of proteasome machinery. These observations suggest that Trehalose elevates the degradation of both model and NDDs-linked proteins without affecting cellular fitness and viability. The present study suggests a possible small compounds-based molecular strategy that needs detailed research in the near future for therapeutic interventions of neurodegenerating conditions. Our current study is also in line with the earlier findings. It indicates that a detailed understanding of these critical molecules can help design an appropriate and better possible therapeutic strategy based on upcoming emerging knowledge.

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