MOLECULAR CHAPERONES AS A THERAPEUTIC APPROACH TO HUNTINGTON'S DISEASE

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We suggest you to cite this article as:

Kennedy, N. 2019. Molecular chaperones as a therapeutic approach to Huntington's disease. *Junior Scientific Researcher*, Vol V, No. 1, pp. 17-31.

ABSTRACT

Neuronal cell death in subpopulations of brains affected by Huntington's disease has been theorised to be due to cell cytotoxicity. The cause of this cytotoxicity, however, is not yet known. One mutant allele of the Huntingtin gene is sufficient to cause the disorder through the synthesis of the Huntingtin protein. Despite the ubiquitous expression of the HTT gene, expansion of the polyglutamine (polyQ) tract in HTT leads to progressive cell death in affected brains, predominantly in the basal ganglia. The polyQ of the protein expressed when this mutant gene is translated has shown to increase the chance of protein aggregates forming. In the Centre for Genomic Regulation, the transformation and study of S.cerevisiae cells have demonstrated a positive correlation between the number of polyQ repeats and the number of protein aggregates that develop within cells. This was evident in the Western Blot and confocal microscopic images. An extra protein band in the Western Blot indicated protein aggregation in the well with the highest number of CAG repeats, implying that with more repeats, the higher the propensity for aggregation. The growth observed in these cells was slower, indicating the inextricable link between aggregation and cytotoxicity within cells. The detrimental effect of protein aggregation is known, therefore the methods that will be proposed will consist of preventing or reducing protein aggregation within cells. HTT is a functional protein, essential to brain development and neuronal survival and thus, by targeting only the aggregates, the functionality of the protein is not affected. Harnessing the essential role of molecular chaperones in promoting the refolding of proteins within cells will form the main body in discussing a therapeutic approach to Huntington's disease; by studying the aggregation propensity in different concentrations of a molecular chaperone, the effectiveness of the molecule can be investigated. Following the results, a discussion on how effectively this method combats aggregation will follow. This discussion depends on the cytotoxicity that remains in the cells studied.

Keywords*: aggregates; cytotoxicity; heat shock response; huntingtin; molecular chaperones; polyglutamine*
JEL Classification*: I1, I10*

INTRODUCTION: HUNTINGTON'S DISEASE AS A POLYGLUTAMINE DISEASE

Huntington's disease is one of the most devastating and disabling disorders as it results from progressive neurodegeneration, depriving the person of their motor skills and the overall cognitive ability of their brain. This lethal disorder arises from a mutation of the Huntingtin protein (HTT). The genotype for the protein is affected by a phenomenon known as trinucleotide repeat

expansion which results in the excess of CAG repeats, a codon that is present within the genome. During the synthesis of mutant HTT, the expanded CAG repeats within the gene sequence are translated into a series of uninterrupted glutamine amino acids forming what is known as a polyglutamine tract (polyQ), exceeding the pathological threshold for polypeptide lengths. This is when the cells cannot physically manage the expanded length of the polypeptide and so cannot efficiently promote protein folding - which is dependent on the cooperativity inherent in a folding/unfolding reaction (Malhotra, 2016).

The expansion of the polyQ has been proven to be related to the development of 9 different neurodegenerative disorders, including Huntington's disease. The only similarity within these disorders is the sequence in the CAG tract. Polyglutamine diseases are therefore neurodegenerative disorders resulting from a CAG expansion leading to a large polyglutamine tract. The propensity to aggregate is due to the physicochemical properties of the polyQ tract itself. Protein aggregation is the biological phenomenon in which misfolded proteins accumulate and clump together and is often correlated with disease. Amyloids are aggregates of proteins that become folded into a shape that allows many copies of that protein to stick together, forming fibrils that are referred to as fibrillar aggregates (Drombosky, 2018).

The risk of trinucleotide repeat expansion occurring is increased by dynamic mutations; this is where the probability of expression of a mutant phenotype is a function of the number of copies of the mutation. Therefore, as the number of CAG repeats increase, the earlier the onset of disease and the more severe the symptoms present to be. This has been a well-discussed concept before and it has been theorised that there is a critical threshold length of the CAG repeat required for disease, and further expansion beyond this threshold is correlated with age of onset and symptom severity (Adegbuyiro et al., 2017).

Mutations that append additional CAG codons become more likely with each successive generation meaning that the progeny are more likely to express the disease than the individual. In previous experiments, it has been discovered that 'at lengths over 40 repeats, a single allelic dose of the HD mutation is sufficient to cause HD within a typical human lifespan and to determine age at motor onset' (Lee et al., 2012) meaning that the risk of the disease expressing itself dramatically increases over a fairly constant number of CAG repeats. Applying the concept that the probability of disease expression increases as the number of repeats increase, it can be explained why individuals that have had Huntington's disease in their family for a longer period of time show an earlier onset of the disease and faster disease progression (Lee et al.)

How the mutant HTT elicits cytotoxicity

It becomes very understandable that mutations in a key protein needed to ensure proper neuronal development can also be the cause of a neurodegenerative disease that deprives the brain of neurons and the person of full motor control. The central dogma is that DNA is transcripted into RNA and RNA is translated into protein, as a simplified explanation of protein synthesis. The central dogma lays at the crux of Huntington's disease.

There is no cure for Huntington's disease because it is still not known whether the HTT protein aggregates arising from the misfolding of the proteins in the polyQ are the cause of the disease or a consequence of it. Therefore, it is not known what to target. In order to discuss a therapeutic approach towards neurodegenerative diseases, we must first understand how protein aggregates can elicit cytotoxicity within cells. Cytotoxicity is the quality of a substance being toxic to cells. We see the effects of toxicity when the rate of growth in cells is slower or they

divide more slowly. There is strong evidence to suggest that aggregation results from the expansion of the polyQ, but it is not known whether this aggregation causes the toxicity responsible for neuronal cell death in subpopulations of the brains affected by the disorder, and therefore the symptoms of Huntington's disease. Targeting the aggregates is a well justified approach to potentially alleviating the symptoms of the disease as symptoms usually arise from increased levels of toxicity in the surrounding environment. The cells' natural response to the increased aggregation is the degrade the protein, which causes a influx in cellular toxicity (San, 2017). This pro-survival pathway is induced under the conditions of cellular stress which acts to maintain proteostasis, the regulation of proteins.

The functionality of molecular chaperones

Ordered protein in the cellular matrix is only possible under the supervision of specialised proteins, collectively referred as molecular chaperones. Molecular chaperones are a highly conserved group of proteins, that have the ability to stabilise proteins when they are partially folded during processes such as translation in protein synthesis, transport across organelle membranes, folding and degradation and also facilitating the refolding of damaged proteins after cells have been exposed to stress. The function of molecular chaperones is implemented mainly in the recovery/adaptation phase after cells have been exposed to stress so that aggregated proteins can engage in chaperone-assisted disassembly (San, 2017).

The natural cellular response to combat the negative effects on proteins caused by stressors such as increased temperatures, oxidative stress, and heavy metals is to increase the number of molecular chaperones; this is known as the heat shock response. The environment in which cells affected by Huntington's disease is one that imitates cells under stress. Therefore, an intervention that involves adding an increased concentration of molecular chaperones to the cells can be considered as a synthetic version of the heat shock response, as explored by San (2017).

Many years of effort have uncovered some of the mechanisms employed by molecular chaperones, which can be broadly divided into three basic types: (1) passive blockage of aggregation, (2) direct facilitation of protein folding and (3) structural disassembly and disaggregation (Lin, 2004). The structural disassembly and disaggregation of protein structures is the least well understood of the mechanisms, yet this is the function of molecular chaperones that could potentially be used to treat the symptoms of Huntington's disease and therefore justifies why they need to be studied in more depth.

Aggregation is the consequence of abnormally folded proteins and therefore, if the production of the misfolded proteins exceeds the cell's degradative capacity, the polypeptides are able to form insoluble intracellular aggregates. When this threshold is reached, the molecular chaperones present within the cell cannot effectively promote refolding of proteins and so the rate of protein aggregation cannot be controlled. At this point, the addition of a higher concentration of molecular chaperones may be productive in controlling the rate of protein aggregation.

Protein aggregates have been referred to as the hallmark of neurodegenerative diseases (Smith, 2015) and as they have been proven to overwhelm the protective capacity of the molecular chaperone machinery, the root problem within the oxidative stress and chaos can be targeted.

S.cerevisiae as the model organism

The validity of the chosen model organism determines how transferable the conducted research is to human cells affected by Huntington's disease. *S.cerevisiae* show high similarity to human animal cells; the sequence comparisons of the classical genetic, biochemical approaches have shown that there is symmetry in many of the major biological processes between the two species.

In molecular biology, transformation is the genetic alteration of a cell resulting from the direct uptake and incorporation of exogenous genetic material from its surroundings through the cell membrane. Another intrinsic property of yeast cells is that a plasmid inserted into a few cells can be horizontally transferred to other cells, meaning that the plasmid need not be inserted into every cell. Also, through vertical transfer, yeast cells divide by budding and thus the plasmid in the parent yeast cell will be transferred into the daughter cells. Similarly, the plasmid does not need to be inserted into many yeast cells as it will be propagated through cell division. Evidently, *S.cerevisiae* is ideal for genetic manipulation and show an array of molecular and genetic tools that have been developed because of the ability to transform cells. Containing an inserted plasmid, the yeast cells can execute the normal functions that the synthesised protein would normally perform. The mechanisms of this can be observed to elucidate the underlying causes of cellular dysfunction (Konstanze, 2010) which contributes to the defect in these processes, such as disaggregation and protein refolding after cells are exposed to stress.

Although yeast cells are also relatively inexpensive to maintain, simple to culture and have a fast generation time of 1-2 hours, it is not possible to model cellular processes involve complex interaction between multiple specialised cells such as synaptic transmission and inflammation. This means that the process of disaggregation in human cells may not be performed to the same complexity in yeast cells, as they are unicellular and lack the same level of coordination between multiple differentiated cells; the observations made from yeast cells are not transferable to human cells because of the disparity between the complexity of specific cellular pathways, crucially protein disaggregation (Bilinski, 2017).

OBJECTIVES

The main aim of this project proposal is to investigate how the concentration of molecular chaperones affects the protein aggregation that develops in transformed yeast cells, containing the HTT gene. The method described will be a theoretical approach of the subject and will explore the proposed relationship between aggregation rate and the efficiency of the synthetic heat shock response that this study will endeavour to instigate in the yeast samples.

Different organisms organise their protein disaggregation mechanisms in different ways. Bacteria, many unicellular eukaryotes and plants use a bi-chaperone network composed of an Hsp70 system coupled with an Hsp100 system (Rampelt et al., 2012). The model organism used in our experiments was a sample of *S.cerevisiae* cells, which also employ this bi-chaperone network.

In order to assess the amount of aggregation remaining within cells after a determined concentration of molecular chaperones have been added (and therefore after the process of disaggregation has been engaged), the toxicity of the solution of aggregates and molecular chaperones can be measured. There is reasonable evidence to suggest that increased aggregation leads to higher levels of toxicity within cells (Doran et al., 2012) and therefore we would expect to see a relationship between the concentration of the molecular chaperones (and thus the effectiveness of disaggregation in the sample of yeast cells) and the toxicity of the solution after

a controlled amount of time. Previous studies, where the model organism used was *D.melanogaster*, have indicated that 'the overexpression of Hsp70 and Hsp40 chaperones acts synergistically to rescue neurodegeneration' (Landles, 2004) in the organism and has been shown to alter the biochemical properties of fibrillar aggregates. Interestingly, it seems that only specific subclasses of molecular chaperones affect polyQ aggregation and toxicity and therefore a different couple of molecular chaperone systems would dominate the disaggregation process in *S.cerevisiae.*

Following the results of the experiment, the concentration of the molecular chaperone that most effectively disables aggregation can be identified and a proposal into how this molecular chaperone could dissolve aggregates in yeast cells, and potentially human cells, can be undertaken.

HYPOTHESIS

The alternate hypothesis is that there will be lower toxicity in the sample of yeast cells with a controlled number of CAG repeats when an increased concentration of a molecular chaperone has been added. The chaperone-assisted disassembly of aggregates is expected to be more efficient after adding a higher concentration of molecular chaperones to cells exposed to stress. Therefore, the toxicity in these cells should decrease. It is expected that the growth of cells to be slowest in the control that is to be included without an addition of molecular chaperones; there are no molecular chaperones for disaggregation to occur, so the level of toxicity increases and rate of cell division decreases.

The null hypothesis is that there will be no variation in the growth of yeast cells between samples containing different concentrations of molecular chaperones. This hypothesis ensures that researchers do not become biased towards their research and particularly, the outcome of it. Until data from the growth assay is collected and analysed, the null hypothesis is maintained. This means that there is will be no difference when a comparison is done through data collection and analysis. The threshold that must be breached in order for the null hypothesis to be rejected will depend on a statistically significant difference in growth of the yeast cells in the different samples; therefore, the alternate hypothesis can be considered.

PREVIOUS WORK: CENTRE FOR GENOMIC REGULATION

To investigate the effect of the number of CAG repeats within the HTT protein, the proteins were classified into size order. Therefore, we used three different strains of DNA containing: 25 repeats (25Q), 46 repeats (46Q) and 103 repeats (103Q). We transformed *S.cerevisiae* cells by inserting a plasmid containing one strain of the DNA into each sample of yeast cells that were pre-grown. A plasmid is a small DNA molecule within a cell that is physically separated from chromosomal DNA that already exists in the cell and can replicate independently. As yeast cells divide by budding, the plasmid in the parent yeast cell will be transferred into the daughter cells; therefore, the plasmid doesn't need to be inserted into many yeast cells as it will be propagated through cell division.

The Western Blot was one test used to evaluate the amount of aggregation in cells with different repetitions of the CAG codon in the plasmid inserted. Gel electrophoresis was used in this technique, which separates protein according to its size and charge. The general trend in the

Western Blot was that the longer the polyQ chain (the higher the number of repeats), the higher up in the gel it will be. The 25Q was the lowest band and the 103Q was the highest. Interestingly, at the top of the 103Q column there was a band indicating protein aggregation, where the protein was too large of a fragment to travel further down the gel. What was even more intriguing was that we also saw an extra observable protein band in the 103Q column, below the HTT band, which may have explained cellular degradation. It could have indicated protein degradation during the manipulation of the experiment, but this is unlikely since this was not observed in the other samples. If the band did indicate protein degradation, this explains why the band is lower than the HTT band as smaller parts of the protein can travel further down the gel. Observations from the growth assays of the *S.cerevisiae* cells implied that as the number of repetitions increases, the division rate of the cells decreases. Degradation usually occurs due to increased toxicity within cells however, our results alone were not sufficient to conclude that aggregation can elicit cytotoxicity within cells as our data was qualitative. According to further research from the *Neurology* Journal, it has been proven that the number of repetitions does indeed affect the growth of the cells (Lee et al., 2012). If the cells had been left for more days to mature and grow, we would have seen results as clear as these ones.

Investigating the mechanism of disaggregation within cells presents to be a great technical challenge that requires focused analysis into the population distribution of aggregates. In order to overcome this challenge, we used a green fluorescent tag protein to identify aggregates in the confocal microscope images as they appeared as bright green spots within the yeast cells. We assessed that this was a qualitative form of assessing the amount of aggregation that formed in yeast cells containing plasmids of different strains of DNA. It was easily identifiable that the yeast cells containing the 103Q plasmid had the most aggregates and the cells with the 25Q plasmid had very little if any, aggregation. The observations from the images corroborated the conclusion reached by the Western blot - where most aggregation formed in the 103Q column. Although the results from the Western Blot can be quantified by the intensity of each band, we only relied on observations from the gel. Hence, both the microscopy and the Western Blot were qualitative so the conclusions that could be drawn were limited. Also, the degradation that was assumed in the 103Q could not be explained by doing these two tests as the toxicity of the cells was not measured.

METHODS

The following method described can be employed to future studies which engage in the study of transformed yeast cells. Plasmids inserted may vary with objectives of different studies. In this study, plasmids containing 103 polyQ repeats are used to ensure substantial aggregation in the samples, in order to observe the effect of additional molecular chaperones in the heat shock response.

Defining the system:

The purpose of the experiment will be to observe the effectiveness of molecular chaperones in managing aggregation in a sample of *S.cerevisiae* cells. The natural cellular response to aggregation is the mechanism of degrading the protein to prevent further aggregation. The intervention of introducing a known concentration of molecular chaperones that contains the bi-chaperone network, consisting of an Hsp70 system coupled with an Hsp100 system, will affect the recovery of yeast cells after they are exposed to stress.

The independent variable will be the concentration of the molecular chaperone solution that is added, and all other variables will be controlled. A control containing no molecular chaperones confirms that no other external factors are affecting the aggregation in the sample, and therefore the toxicity of the overall solution.

Transformation of yeast cells

Transformation involves inserting the HTT gene into a plasmid with 103Q repeats and appending a promoter to the gene to create an inducible system. The promoter, which is galactose in this case, named GAL1_promoter. The most important components of Plasmid P426 103Q GAL are the 103Q repeats of the HTT gene, URA3, URA3_promoter, HTT gene, GAL1_promoter, Ampicillin, AmpR_promoter, T7 _promoter and the CYC1_terminator.

Reagents and Materials:

-Selective plates
-YTB
-YTB-PEG
-Salmon Sperm 10mg/mL
-Plasmid DNA
-DMSO
-Glass beads

Equipment:

-Spectrophotometer
-Incubator at 30°C
-Thermoshakers
-Centrifuge for large tubes (15ml)
-Centrifuge for small tubes (1,5ml)

Thermoshaker is set at 95°C and salmon sperm is heated to 95°C for at least 10 minutes. Thermoshaker is then set at 42°C for heat shock transformation.

Procedure:

1. Pour 50ml of cells in a 50ml Falcon tube.
2. Centrifuge at 700g for 2 minutes.
3. Discard the supernatant.
4. Wash/resuspend the pellet in 6ml YTB (1ml for each transformation, producing 6 samples) and transfer each 1ml to an Eppendorf tube.
5. Centrifuge at 500g for 2 minutes.
6. Discard the supernatant.
7. Resuspend the pellet in 72ul YTB.
8. Add 8ul of salmon sperm to cells.
9. Add 1ul 103Q plasmid DNA.
10. Add 500ul YTB-PEG.
11. Vortex briefly.
12. Incubate for 30 minutes in rotation at room temperature.
13. Add 60ul of sterile DMSO.
14. Vortex briefly.
15. Heat shock for 15 minutes at 42°C.
16. Centrifuge at 500g for 2 minutes.
17. Discard the supernatant and resuspend the pellet in 300ul of sterile water.
18. Plate the 300ul of cells onto each plate (without uracil, also referred to as -ura) and spread with glass beads.
19. Incubate the plates at 30°C. Observe colonies after 2-3 days.

20. Streak the colonies in fresh -ura plates. As there is only one strain of DNA used, the colonies need not be isolated in the -ura plates.
21. Incubate plates at 30°C ON.

(*CRG Courses*, *2018*)

Growth curve

Reagents and Materials:
-Non-inducing medium (raffinose)
-Inducing liquid medium (galactose)
-Centrifugation medium
-20 MM sterile glass tubes
-96 wells plate
-Multi-pipette

Equipment:
-Plate reader - TECAN

Procedure:

1. Grow cells from the streak in 4ml non-inducing media. Incubate at 30°C shaking ON.
2. After 24 hours, measure the optical density (OD) and take the volume of cells to obtain an OD=0.2
3. Grow cells in 3ml at 30°C shaking for 4-6 hours.
4. Measure OD and take the volume of cells to obtain an OD=0.2 in 1.5ml.
5. Add a few drops of centrifugation medium and then centrifuge the tubes at 500g for 2 minutes. Following this, discard the supernatant.
6. Add 1.5ml of inducing medium (galactose, also referred to as +gal) to each of the 6 tubes.
7. Pipette 100ul of each sample into 6 wells of a 96 well plate, performing technical replicates of the same sample to test variability in the testing protocol itself.
8. Fill the upper and bottom wells of the plate with medium to avoid evaporation.
9. Allow yeast cells to culture for at least 24 hours, until sufficient aggregation has occurred within the cells.

(*CRG Courses*, *2018*)

Intervention of molecular chaperones:

Assuming the solution containing the bi-chaperone network, consisting of an Hsp70 system coupled with an Hsp100 system has already been obtained, a serial dilution should be performed in order to produce the six different concentrations with even intervals to be added to each sample. Include a control with no molecular chaperones, and therefore a concentration of 0ug/0uL molecular chaperone solution to test if anything externally affects growth of the yeast cells.

The concentrations used should be determined by the natural existing concentration of the bi-chaperone network in *S.cerevisiae* so that it can be ensured that an increased concentration is added. After yeast cells have been cultured for at least 48 hours, to ensure that aggregates have formed (this period of time should be increased if the cells have not been exposed to sufficient stress), the molecular chaperones should be added. First, 240ul of the solution should be pipetted into 1 well of a 96 well plate. The interval between each concentration should be so that every well will be 5 times more diluted compared to the previous one. Add 240ul of each concentration of molecular chaperones to each of the six yeast samples in the first 96 wells plate. Finally, place the plate in a plate reader, TECAN, to measure OD values for 72 hours.

This machine measured the optical density (OD) of each sample every 10 min for 48 h in our previous experiments. In this experiment, to increase the accuracy of the growth curve

produced from the results, the populations of yeast should be monitored for a longer time to observe potential secondary effects of the molecular chaperones added.

INTERPRETING RESULTS

After saving the results into an Excel file, the analysis can be proceeded using bioinformatics software. The growth measured is during the exponential phase of the curve; therefore, the slope is calculated and the slopes of each can be compared and condensed into a boxplot. This statistical representation of data is effective in presenting the spread of the data, including relevant percentiles that can be specified. It also allows for a suitable and visual comparison between the growth of cells in each sample in an empirical way. A designated space in the different boxplots should represent the whole spectrum of results taken by the TECAN machine. The box in the middle represents the results taken by the 25th to 75th percentiles, which includes the majority of the sample. The curved lines represent a Gauss curve/distribution of the results.

Analysis of results:

The sample with the fastest exponential phase contains cells with fastest growth. According to the theory pursued in the hypotheses, this is the sample with lowest toxicity present in cells. The concentration of molecular chaperone solution used in the sample therefore is defined to have the best performance in the growth assay and as the concentration at which disaggregation occurs most efficiently.

FURTHER CONSIDERATION INTO THE METHODS

Measuring toxicity

Cytotoxicity itself cannot be observed, only the effects of it can be measured in order to quantify it. The toxicity remaining in the cells will be measured using a growth assay, monitoring the growth of the yeast cells containing aggregates after a certain concentration of molecular chaperones have been added to the sample.

In the growth assay performed at the Centre for Genomic Regulation, it was predicted from the results that if the yeast cells had been left for longer, we would have observed all the phases of the population growth of yeast cells. Therefore, the toxicity should be measured after a sufficient amount of time so that the change in the rate of growth of yeast cells is more definitive. The phases of growth should be at different rates with samples containing different concentrations of molecular chaperones, according to the alternate hypothesis.

Monitoring growth: Growth curve for yeast

Growth curves represent a change in the population over a period; in biology, they are measured by recording the Optical Density (OD) of each sample and plotting it on the Y-axis of the graph against time on the X-axis. The optical density, also known as ‘Absorbance’, is a logarithmic ratio of the intensity of light entering the yeast cell suspension to the intensity passing through it. A spectrophotometer is used to find the OD at a specific wavelength. The results are in the form of absorbance and have no units (Desjardins, 2009).

We can use the spectrophotometer to obtain results for absorbance through the implementation of Beer’s Law. Beer's Law is that the absorbance, through a known length, is

directly proportional to the concentration of the solution. In other words, as long as we know how far the light travelled through the sample, then we can determine the concentration of the solution based on the absorbance. Since we know how long the cuvette is (typically 1 cm), we can determine the concentration.

The yeast growth curve is divided into three major phases: lag, logarithmic, and stationary phase. During lag phase, cells are acclimating to the environment and are growing in size but are not dividing. Later, in the logarithmic phase, the cells are actively dividing, leading to cell doubling. Stationary phase is reached once available nutrients are depleted and cell division slows down. The population therefore remains constant. The stationary phase is followed by the decline phase and this happens as resources become scarce and the gradient of the curve becomes negative as cells start to die.

Protocol for Growth Curve:

In order to do a growth assay comparison, we will use the exponential/logarithmic phase rate for each group of replicates. This is because it is expected that as the toxicity level rises, the exponential growth rate declines.

Data is extracted from the exponential growth phase since this phase is when changes in the actual growth of cultures is appreciated. As there is no significant difference between the slope of any of the phases except the exponential phase, we use this value to represent the rate of growth of yeast cells in each sample.

Significance of the growth assay

The growth assay should be used to monitor the growth of the yeast cells with the same number of CAG repeats (103Q) after each solution (each with different concentrations) of molecular chaperones has been added. The protocol follows the prediction that the solution of yeast cells with a higher concentration of molecular chaperones will have the highest absorbance due to the levels of toxicity being lower and yeast cells being able to divide more frequently and rapidly.

Due to the variation amongst the samples of yeast cells at the start of the experiment in terms of the concentration of cells and the protein aggregates within the cells, the growth assay should be taken as relative to each sample. The increase in the population as a percentage of the initial population size should be measured to increase the accuracy of the growth assays because there will be variance in each starting population in terms of its size; this is enabled by the plate reader used.

EXPECTED RESULTS

According to the hypotheses, it is expected that the sample with the highest concentration of molecular chaperones will contain yeast cells with the lowest toxicity and therefore the fastest growth rate - which will be apparent in the growth curve of the yeast cells. There should be slowest growth in the control, in other words, the gradient of the logarithmic phase of the growth assay from the control should be smaller.

DISCUSSION AND EVALUATION

Evaluation of the Method:

There are features of the method that are desirable and are in accordance with the objectives of observing transformed yeast cells. For example, inducible promoters are extremely useful as they can be regulated by a positive or negative control; the system implemented in the method is a positive inducible system as the promoter will be inactive if the activator protein cannot bind. After an inducer (galactose) binds to the activator protein, the activator protein can bind to the promoter, turning it ON and initiating transcription of HTT.

A high number of CAG repeats is used to increase the propensity for aggregation so they are more likely to form. This is desirable as the maximum effect of molecular chaperones can be observed. Therefore, it is useful to use 103Q repeats as aggregates form more quickly; the rate of aggregation is much higher than the rate of disaggregation.

However, even after identifying that there is fastest growth of yeast cells in the solution containing the highest concentration of molecular chaperones, the quality and productivity of the yeast cells produced cannot be guaranteed. In the growth assay, we assume that the produced yeast cells have the same rate of cell division as the original cells containing the aggregates. If the increased concentration of molecular chaperones proves to have a detrimental effect despite increasing the growth rate of the yeast cells, this approach would need to be adapted as the slower growth in cells with higher toxicity is not the only issue that arises due to aggregation.

The Molecular Chaperone Approach

The nature of Huntington's disease means that the symptoms become more severe with successive generations, resulting in an earlier onset of the disease and faster disease progression in the progeny. The problem with treating the symptoms of a genetic disorder is that the root cause of the problem is not addressed, which lies in the number of CAG repeats and the expansion of the polyglutamine tract during the synthesis of the Huntingtin protein. The main argument against altering the genotype of the protein is that this will invariably affect the synthesis of the protein in its ubiquitous expression. Huntingtin is a functional protein and therefore, by changing the genotype, there will be direct repercussions on the phenotype. By targeting the aggregates, we are able to observe the effect of lower cell toxicity and how this leads to faster cell division. As a hopeful future development, we can investigate how the increased cell growth potentially enabled by the addition of molecular chaperones can combat the cell death caused by neuro-degeneration.

ETHICAL CONSIDERATIONS

Treating the symptoms expressed by people suffering from this disease only addresses short term consequences of the disease; targeting the aggregates does not, at this stage, alleviate the symptoms or prevent the rapid progression of the disease. This also means that the progeny of a patient with Huntington's has an unreduced chance of developing the disease as the genome remains unchanged. The sustainability of the proposed therapeutic approach must be considered as the potential benefits of this approach should ideally outweigh the potential risks and costs if it were to reach a clinical trial.

This approach must be rigorously tested and challenged before the intervention of the most suitable concentration of molecular chaperones can be brought to a clinical trial. This should hold great importance in the future considerations and developments of this therapeutic approach. Potentially treating the symptoms of the disease is a very distant perspective and even if it is brought to clinical trial, there is no guarantee that the molecular chaperones that once mitigated cell toxicity in a controlled lab environment or in animal tissue will behave in the same way when under stress in humans. This is due to the absence of specific cellular pathways and proteins in

S.cerevisiae that are involved in the disease pathogenesis as well as the evolution of pathways and proteins to fulfil different functions in the cell (JoVE database, 2019).

Ultimately, this means that the conclusions drawn about the dependence of yeast cells on molecular chaperones will differ for that of human cells. Since the endeavour into imitating the heat shock response in cells in a controlled laboratory environment is a feat in itself, implementing this method into human cells is an ethical and immensely technical challenge. The fundamental differences within the model organism are always a limiting factor that must be considered when applying the theory to multicellular organisms, especially humans affected by the disorder.

Clinical trials are essential in increasing understanding of human biology as the path to confirming whether a new drug or treatment is safe or effective, for example, involves testing it on patient volunteers. Only when there is a favourable risk-benefit ratio, a study can be considered to be ethical. There has always been a bone of contention over whether subjecting patient volunteers to potential harm for the benefit of others is just and equitable. In order to avoid the exploitation of volunteers, it is critical to uphold the principle of respect for individual autonomy (Muthuswamy, 2013). A clinical trial appertaining to the proposed therapeutic approach must be similarly treated with respect and sensitivity as the potential benefits of an intervention could transform the current concepts associated with molecular chaperones. Inappropriate infringements on the free actions of competent individuals would upset the dynamic of the endeavour of clinical research, damaging the reputation and integrity of the study.

Predictive testing

Huntington's disease is characterised by midlife onset which consists of cognitive, physical, and emotional deterioration. The principle of autonomy has traditionally guided genetic testing in previous years. However, severe psychological consequences such as depression, anxiety, survival guilt, and suicide have ‘complicated the ethical issue of providing a presymptomatic yet definitive diagnosis for an incurable disease genetic presymptomatic testing for Huntington's disease’ (Coustasse et al., 2009). The inherent personal battle to know or not to know the results of a predictive test for this genetic disorder changes the behaviour and suicidal ideation in preclinical Huntington’s disease, creating a very complex and sensitive responsibility that must be adopted by healthcare professionals. Duties of upholding autonomy, justice are beneficence are employed when strict protocols are followed, and extensive counselling and vigilance are provided to the patient. Ultimately, it is the individual’s choice whether they want to be tested. It is the responsibility of healthcare to ensure the psychological wellbeing of the patients; whether this includes promoting the predictive test in the interests of equipping the individual with awareness, preparation and support is an ethical challenge to be discussed. Perhaps, the most driving factor to being tested is the choice of having children and unwittingly subjecting them to a life of progressive deterioration. In the cruellest, most unfortunate circumstance, testing might, in fact, be too late. Predictive testing, the condemnable truth, is essentially a burden that the individual carries with them, alone. It is their inherited right to choose whether they want the boon or the punishment of knowledge.

CONCLUSIONS

Before drawing conclusions from the experiments, it is important to confirm whether the results are valid and repeatable, and even reproducible if another method is used and the same

results are reached. After repeating this experiment to prove that it is achievable to obtain repeatable results and a reliable trend of decreasing toxicity in samples of yeast cells containing higher concentrations of molecular chaperones to a certain extent, the hypothesis can be reflected on, followed by an evaluation as to whether the results reached are well justified and reflect the expected results.

In the case that the expected results are replicated in the actual results from the experiment, then the null hypothesis can be rejected, and it becomes very interesting when the alternate hypothesis can start to be accepted. This means that the toxicity of cells and the surrounding environment inside the sample is mitigated by the intervention of a specific concentration of the molecular chaperone systems in the bi-chaperone network. A faster growth is interpreted in this sample from the growth assay, which validates the lower toxicity of the environment because as emphasised before, the toxicity is quantified by the growth rate of the cells. The significance of this conclusion emphasises that it is possible to intervene in the natural cellular heat shock response and combat the intrinsic quality of cells that increases their cytotoxicity.

FUTURE PERSPECTIVES

A higher concentration of molecular chaperones is expected to be more effective in managing the protein aggregation in the yeast cells. After obtaining valid and reliable results, the first thing that should be investigated is the mechanism behind the chaperone network in managing aggregation, compared to the performance of networks of lower concentrations in different samples.

Another important consideration of this approach is to observe the secondary effects of adding increased concentrations of molecular chaperones into cells. This is very important as the detrimental effect caused by molecular chaperones could amplify the cellular chaos that is already present when cells undergo stress and therefore should be avoided through further study into the events that occur immediately after adding the most effective concentration of molecular chaperones. The concentrations used in this project proposal are only suitable for yeast cells in a controlled lab environment. Therefore, they would need to be scaled to human cells and trained to perform as efficiently in a multicellular environment, requiring further investigation beyond the confines of a synthetically grown culture.

After evaluating if the approach is safe within yeast cells, in that the benefits of molecular chaperones outweigh the risks, we should investigate if human cells respond to their own molecular chaperones or if it would be suitable to use yeast molecular chaperones and treat the intervention as exogenous.

Only after meticulous consideration and investigation of the above, the theory as to how molecular chaperones should be injected to target only neuronal cells can be developed.

Further developments:

These developments rely on the assumption that it is possible to obtain a suitable concentration of functioning molecular chaperones, that human cells respond to, to promote disaggregation within cells affected by Huntington's disease in humans. This should be done without presenting any detrimental secondary effects in the cells that cannot be reversed or targeted by another approach.

The use of molecular chaperones is useful in managing readily occurring aggregation and treating symptoms in the short term. However, the expression of the mutated HTT is not addressed in this approach. As previously justified, the expression of HTT cannot be moderated as Huntingtin is a functional protein and is necessary at specific levels in certain parts of the brain for neuronal survival. Aggregation is normal within cells and excess aggregates are well managed by molecular chaperones in a controlled environment such as in a lab with samples contained in test tubes. However, further research and development of this proposal are required in order to ascertain how well molecular chaperones can perform under stress - such as that of cells in patients with Huntington's disease. An observation made in a lab cannot be assumed to have similar results within human cells; thus the disparity between the model organism, *S.cerevisiae*, and human cells must be considered when predicting possible outcomes and physiological reactions to increased concentration of molecular chaperones in cells under stress.

The therapeutic approach of adding an increased concentration of molecular chaperones to cells that have been exposed to stress closely resembles the natural cellular heat shock response and is intended to be implemented when the rate of aggregation exceeds the rate of protein refolding and disaggregation, decreasing the cytotoxicity within cells. The aggregation can be effectively managed in this method, but the probability of mutation remains a function of the number of copies within the gene and therefore, the onset and the initial severity of the symptoms that arise from Huntington's disease aren't mitigated. The overriding effect of the genotype in Huntington's disease condemns an individual with a 50% of expressing the disease. Therapeutic approaches merely combat the symptoms, let alone the root cause that orchestrates the progression of neurodegeneration - a mutation in an essential protein. The challenging dilemma that Huntingtin is vital for neuronal survival and protein development and yet a single mutation leads to the progression of such a debilitating disorder is a sensitive and ever-present antagonist in the field of neurology that is one to be reckoned with.

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Acknowledgements

The study of S.cerevisiae cells at the Centre of Genomic Regulation was supervised by M.N. Lorenzo Gotor and A. Alcaine. The methods used were a replication of the method used at the CRG, for previous experiments in which the background information and prior research were collected.