Reduction of Protein Aggregation in Huntington's Disease by Recombinant DNA Technology Using Chaperone Protein HspB5

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ABSTRACT

Huntington's disease (HD) is a neurodegenerative disorder linked with a mutation in the HTT gene, encoding toxic mutant forms of the Huntingtin protein. In this study, the focus was on using chaperone proteins, which play a pivotal role in the organisation of misfolded proteins, were used to target this problem in HD. The HspB5 chaperone protein was selected to be used in the study. In order to be used in protein characterisation, the data for the GFP and HspB5 protein genes was obtained, and cDNA was prepared for the PCR stage and appropriate primer designs were made accordingly. Following the PCR stage, the pYES2 vector selected in the study was then combined with the gene by cutting and ligation. Recombinant DNA was then transfected into *S. cerevisiae* cells. The prepared *S. cerevisiae* cells were tested with URA3 and GFP and a cell colony was formed with positive cells that carried the vector, selected from the tests. Cell lysis was then performed, and proteins were purified. The protein purification process was checked by SDS Page and Western Blot methods. To test synthetic HspB5 chaperone proteins, appropriate cell culture media were established with the HT22 mouse cell line. Both control and test cultures were incubated for a sufficient time and ThT staining, SDS Page, Western Blot and Confocal microscopy methods were applied to evaluate the anticipated aggregate reduction. This study provides a promising basis for biotechnological approaches targeting protein aggregation in the treatment of HD.

Keywords: Huntington's disease, Chaperone, Recombinant DNA, S. cerevisiae, HspB5, URA3, GFP

Introduction/Aim of the Study

Huntington's disease is an autosomal dominantly inherited neurodegenerative disorder. It is the most common monogenic neurological disorder in the world. 10.6-13.7 individuals per 100,000 suffer from Huntington's disease [1]. In this disease, psychological disorders, chorea-type movement disorders, and a decline in cognitive and motor coordination will be observed [2]. While 15-26 CAG triple nucleotide group repeats are observed in the 1st exon of the HTT gene located on chromosome 4p16.3 in a healthy individual, this number increases to 50 in severe Huntington's disease patients. This abnormal repetition leads to the formation of a mutant, dysfunctional Huntingtin protein. In the Huntingtin protein of a healthy individual, a polyQ structure containing 23 glutamines and 3144 amino acids is expected. This mutant protein contains an abnormally long polyQ (polyglutamine) sequence and misfolds. The toxic structure of the protein formed in this way causes dysfunctions and neuron deaths. In the later stages of the disease, severe tissue loss is observed, especially in the caudate/putamen duo [1].

Chaperones prevent the accumulation of misfolded proteins and direct them to the folding process for their destruction or reconstitution [3]. These proteins, also called ‘heat shock proteins’ (HSP) or ‘stress proteins’, are activated in cellular stress situations such as high temperature, pH change, oxygen deficiency/excess, starvation and the presence of heavy metals [4]. In this way, they play an important role in protecting the organism against stress [5]. Due to their functions, heat shock proteins (HSPs) come to the forefront together with RNAi-based therapies for the treatment of Huntington's disease, for which there is still no definite treatment method [6].

This study aims to prevent protein aggregation, which is the secondary cause of the disease, with the HspB5 chaperone, one of the heat shock proteins (HSP). In this study, it is aimed to synthetically produce HspB5 chaperone protein by recombinant DNA technology. In order to minimize the incompatibility of the HspB5 chaperone protein to be produced when transferred to individuals suffering from the disease, the cells selected for production should have common characteristics with eukaryotic cells. *S. cerevisiae* cells, which are frequently used in industrial biotechnology and have common features with eukaryotic cells, were used in this study because they can be easily modified in the laboratory environment such as E. coli and have high efficiency. To prevent aggregation, HspB5 chaperones produced synthetically using *S. cerevisiae* will be administered to patients. Accordingly, it is aimed to minimise the symptoms of Huntington's disease by slowing down

the loss of neurons in individuals. The tests of the mentioned chaperone protein will be carried out in a culture containing a mouse cell line (HT22) with transfected Huntington's disease for biosafety and cost reasons.

Materials and Methods

1. Primer Design

In this study, a single cDNA construct encoding the Hspb5 chaperone protein and the GFP protein will be obtained and PCR will be applied for amplification. A plasmid will be used for the PCR application. A plasmid is a suitable carrier for expressing foreign genes in an organism. pYES2 will be selected as the target vector as it is commonly used for gene expression in yeast. It contains features such as the GAL1 promoter, Ura3 gene, ampicillin resistance, and multiple cloning sites (MCS). For these reasons, pYES2 is chosen as the target vector. Forward primer (5'GGATCCATGAGTAAAGGAGAAG3') and Reverse primer (5'GAATTCTGACTGTTGCTGAATG3') will be designed using the Primer-BLAST program. The forward primer will be 22 bases in length, with a melting temperature of 62°C and a GC content of 45%. The reverse primer will also be 22 bases in length, with a melting temperature of 60°C and a GC content of 42%. The amplification product length will be targeted at 1442 base pairs [7][8][9][10][11].

2. PCR

A 20 µL total reaction mixture will be prepared for the PCR reaction. This mixture will include 0.2 µM forward primer (10 µM), 0.2 µM reverse primer (10 µM), 1 µL cDNA(bought), 0.25 mM dNTPs, 1× LA Taq buffer, and 2.5 U LA Taq. The PCR program will be optimized as follows: initial denaturation at 95°C for 2 minutes, followed by 25–30 cycles of denaturation at 98°C for 20 seconds, annealing at 60°C for 120 seconds, and elongation at 72°C for 120 seconds. A final elongation step will be carried out at 72°C for 7 minutes. To evaluate the accuracy of the amplification products, the PCR products will be run on a 2–3% agarose gel (metaphor) electrophoresis at 120V for 45–60 minutes. The gel will be stained with ethidium bromide at a concentration of 0.5 µg/mL and visualized under autoradiography. The correctness of the primer design will be demonstrated by obtaining bands of the target size. These products will be purified and their concentration measured before being transferred to the yeast expression system for recombinant protein production. PCR products will be gel purified using the Qiagen MiniElute reaction cleanup kit as specified by the manufacturer [11][12][13][14][15][16][17][18].

3. Cloning of PCR Product into Vector

3.1 Cutting and Ligation

The cDNA product consisting of GFP and HspB5 genes obtained by PCR will be cut using appropriate restriction enzymes before cloning into the target vector. For the cutting process, the restriction recognition regions located within the multiple cloning region (MCS) on the PCR product and vector DNA will be used. In this project, EcoRI and BamHI enzymes were selected for double enzyme cutting. EcoRI and BamHI restriction enzymes will be selected for the cutting process because they cut only from one region on the selected vector and are among the non-cutter enzymes of the desired gene sequence to be added to the vector. The primer designs used in the PCR process will also be made according to these selected enzymes.

3.1.1 Cutting Protocol

The cutting process will be performed on PCR products and expression vectors using EcoRI and BamHI restriction enzymes. Each reaction tube will include 2 µL of EcoRI (10 U/µL) and 2 µL of BamHI (10 U/µL). Additionally, 2.75X volume of 10X reaction buffer will be added, and the total volume will be adjusted to 1.25X with dH2O. The reaction will be incubated at 37°C for 1 hour in a total volume of 50 µL. This protocol will also be applied to the expression vector intended for cloning. After digestion with EcoRI and BamHI enzymes, alkaline phosphatase will be used to remove phosphate groups from the vector. This step prevents self-ligation of the vector and increases cloning efficiency. The same protocol, including digestion with EcoRI and BamHI and subsequent treatment with alkaline phosphatase, will be applied to the expression vector. Removing the phosphate group ensures that the vector does not self-ligate, thereby enhancing the efficiency of the cloning process [15].

3.1.2 Ligation Protocol

The sheared vector DNA (50 ng, 4 kb) and PCR product will be used in the ligation reaction at a ratio of 2.2 µL and 2.3 µL, respectively. 1 µL of T4 DNA Ligase and 2 µL of 10X ligation buffer will be added to the mixture. The reaction volume will be completed to 20 µL with nuclease-free water.

The temperature and time for the ligation process will vary depending on the type of DNA end used. For cohesive ends, the reaction will be incubated at 16°C overnight or at room temperature for 10 minutes. For ends containing blunt ends or single base overhangs, incubation will be done at 16°C overnight or at room

temperature for 2 hours. Alternatively, when using a high concentration of T4 DNA Ligase, blunt end ligation will be completed in 10 minutes. After ligation, T4 DNA Ligase will be inactivated by heating at 65°C for 10 minutes. After the reaction mixture is cooled on ice, 1-5 µL of ligation product will be used for transformation of competent cells. After ligation is complete, the cutting will be confirmed successfully by agarose gel electrophoresis (1% [15], 90-150V [20], 30-60 minutes [21]). The cut PCR product will be purified using the QIAquick gel extraction kit (QIAGEN) and the DNA concentration will be measured with a NanoVue spectrophotometer [22][19].

4. Transfer to Yeast Cells

The Lithium Acetate (LiAc) transformation protocol is a commonly used method for introducing plasmid DNA into yeast cells. This method enhances the permeability of the cell membrane, allowing plasmid DNA to enter the cells. After the transformation process, the yeast cells are grown on a selective medium to evaluate the expression of the target gene. [24]

4.1 Preparation of Yeast Culture

Selected yeast strain: Saccharomyces cerevisiae BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) mutant strain will be used. This strain will not grow in a uracil-deficient medium, and therefore cells containing vectors carrying the URA3 gene will be able to grow selectively [23]. In the culture preparation stage, a single colony is inoculated in 5 mL of liquid medium. 2x YPAD (Yeast Extract Peptone Dextrose) will be used for the medium. The culture will be grown at 30°C, 200 rpm, shaking for 12 hours. In addition, 50 ml of double-density 2x YPAD medium and 250 ml culture flask will be placed in the incubator and warmed up [24][27]. At the cell density stage, approximately 8-10 hours later, the optical density of the cells (OD600) is measured. To measure this value, 10 µl of cell suspension will be mixed with 1.0 ml of water and added to a spectrophotometer cuvette and the measurement will be made. This will indicate the optimal growth of cells in the log phase. The OD600 value of the suspension with a density of 1x10^6 cells/ml will be expected to be between 1.0 and 1.6. Then, 50 ml of double-density YPAD medium, which was previously heated, will be transferred to a 250 ml culture flask. Then, 2.5 x 10^8 cells are added and the cell density is adjusted to 5 x 10^6 cells/ml and the culture will be incubated at 30°C at 200 rpm [24][25]. At the harvesting stage, when the cell density is 2 x 10^7, the cell culture will be centrifuged at 3000-4000 rpm for 5 minutes and the supernatant will be discarded. The cell pellet will be washed with sterile 25 ml dH2O and then washed again with 1 ml dH2O [24][25][26]. At the resuspension stage, water will be added to the obtained cells and the total volume will be adjusted to 1.0 ml. The mixture will be vortexed vigorously and resuspended. For each transformation process, 100 µL samples containing approximately 108 cells will be taken and these samples will be transferred to 1.5 ml microcentrifuge tubes. The cells will be centrifuged at maximum speed for 30 seconds.

4.2 Transformation

To prepare the transformation mixture, the specified components will be taken in the specified proportions and mixed. For the mixture: 240 µL PEG 3350 (50% (w/v)), 36 µL LiAc (1.0 M), 50 µL boiled and denatured single-stranded carrier DNA (sonicated fish DNA, 2.0 mg/mL), 34 µL plasmid DNA, and the necessary amount of water will be added, and all components will be mixed homogeneously to prepare a mixture suitable for transformation. For the transformation mixture step for a single reaction, 360 µL of the transformation mixture will be added to each transformation tube, and then the cells will be vigorously vortexed to resuspend. During the heat shock step, the tubes will be incubated in a 42°C water bath for 15 minutes. This step will allow the plasmid DNA to pass through the weakened cell membrane into the cell. After the heat shock, the tubes will be immediately placed on ice and quickly cooled to prevent cell damage. Afterward, the cells will be centrifuged at maximum speed for 30 seconds, and the supernatant will be discarded. The cell pellet will be resuspended in sterile dH2O [24][25][27].

5. Verification of Recombinant Vectors

The selective marker URA3 gene in the vector will be used to control the presence of the vectors transferred to the cells. The GFP reporter gene, which can work with the GAL1 promoter and is inserted into the MCS, will be used to monitor the proper placement of the desired gene in the vector and protein expression. While cells carrying vectors containing the URA3 gene will be able to continue to grow in an environment lacking uracil, the growth of cells not containing the vector will be prevented. This process will take place in an environment containing SD-URA and glucose. Then, the selected cells will be placed in an environment containing SD-URA and galactose to induce GAL1. In this way, the expression of GFP and HSPB5 will be ensured. After the cells have been incubated for a sufficient time, GFP expression will be confirmed by fluorescence microscopy analysis [28][29]. During the selection phase in an environment lacking uracil, no cells other than cells carrying the pYES2 vector will grow. Glucose will be

used as a carbohydrate source in the environment for this test. SD-URA medium will only allow the growth of URA3-positive cells. For this purpose, an SD-URA medium containing glucose with appropriate agar will be prepared. For preparation, 1.7 g/L amino acid-free YNB (Yeast Nitrogen Base), 20 g/L glucose, 5 g/L ammonium sulphate and 20 g/L agar will be mixed and 1 L of distilled deionized water will be added. A 100 µL volume of cell-containing suspension will be taken from the cell pellets prepared in the previous step with a sterile pipette. The suspension taken with the pipette will be carefully dropped onto the centre of the agar medium. Then, the loop will be sterilized with 70% ethanol and dried by burning in a flame. The suspension dropped onto the medium will be spread evenly on the medium surface with the help of a sterilized loop. During the process, it will be ensured that the cells are evenly distributed on the agar surface. The mixture will be sterilized in an autoclave at 120 °C for 20 minutes. After sterilization, the plates will be incubated at 30 °C for 48 hours [27][30][31]. As a result, only cells carrying the vector will be able to grow in this process, and the growth of cells not containing the vector will be inhibited. In the visual screening test stage with GFP, gene expression will be proven by observation under a microscope to verify and visualize the expression of GFP in yeast cells. SD-URA medium with the same content will be used in this test, except for the carbohydrate source. Galactose will be added for the activation of the GAL1 promoter for this test. Thanks to galactose, the GFP gene is induced under the control of the GAL1 promoter. Positive colonies carrying the vector that will grow in the medium in the previous stage will be transferred to the SD-URA cell culture medium containing galactose. Cells in SD-URA medium containing galactose will be incubated for 48 hours at 30°C. Cell growth is monitored by measuring OD600. 10 µL of cell suspension (prepared in PBS) is dropped onto the slide and gently covered with a coverslip. The GFP filter of the microscope will be set so that the excitation wavelength is 488 nm and the emission wavelength is 509 nm. Then the light intensity and camera settings will be optimized. The objective of the microscope will be adjusted appropriately for imaging and the microscope will be switched to fluorescence mode. In this mode, the GFP signal will be observed. Several images from different areas will be taken to evaluate the status of the cells. If the cells emit a green fluorescent signal, these cells will be understood to be GFP positive [32][33][34].

6. Protein Expression and Induction

In the initial stage with a single colony, one of the positive colonies growing in a selective medium with SD-URA will be taken in a sterile manner. (A single colony will be inoculated in a medium containing 5 mL of YPD (Yeast Peptone Dextrose) and ampicillin (50 µg/mL) [35]. The culture will be grown at 30°C, 225 rpm, and shaken overnight. This step ensures that the cells healthily pass to the log phase. In the expansion stage of the culture, 1-10% of the overnight culture will be transferred to a new tube [35][36]. The cells will be grown in 10 mL of YPD + ampicillin (20 µg/mL) medium and the OD600 value will be brought to 0.6-0.8. This is the stage where the cells reach the log phase.

7. Confirmation of Protein Expression

7.1 Cell Lysis

In the Cell Culture stage, the yeast culture is grown to 50 mL after induction. The culture is centrifuged at 5,000 rpm for 5 minutes at 4°C to pellet the cells and the supernatant is discarded [36]. In the washing stage, the resulting pellet will be washed with 1X PBS and resuspended. The cells are centrifuged at 15,000 rpm for 15 minutes at 4°C and the supernatant is discarded [37]. In the Lysis Buffer preparation stage, the Lysis buffer will be prepared with components containing 50 mM Tris-Cl (pH 8.0), 1 mM imidazole, 100 mM NaCl, and 0.1 mM EDTA [38]. These components will be mixed in appropriate proportions to form the buffer. In the Lysis Process stage, the cell pellet is resuspended in the prepared lysis buffer (1 mL) and then the cells will be lysed with an ultrasonicator. Sonication will be performed on ice in 3 sets of 1 minute each. The lysate will then be centrifuged at 8,000 rpm at 4°C for 20 minutes to remove insoluble cell debris.

7.2 Protein Purification

7.2.1 Ni-NTA Column Preparation

In the equilibration stage, Ni-NTA (Nickel-Nitrilotriacetic Acid) resin will be prepared as 3 mL. The resin will be washed 3 times with an equilibration buffer containing 10 mM imidazole. The column will be filled with buffer and brought to equilibrium conditions [39].

7.2.2 Protein Binding

In the Application of Lysate to the Column stage, the supernatant will be loaded onto the prepared Ni-NTA column. The lysate will be passed through the column by gravity or low-speed centrifugation. The column will be incubated for 1 hour for the protein to bind to the resin. In the washing stage, the column is washed with a wash buffer containing 10 mM low concentration imidazole to remove non-specifically bound proteins. The washing process will be repeated

4 times, each with 50 mL. In the elution stage, an elution buffer containing 10 mM high-concentration imidazole will be added to the column. The elution fractions will be collected as 5.0 mL each, making a total of 50 mL fractions. In the Elution Control stage, 2 µL of sample will be taken from each fraction. The presence of the target protein in the fractions will be checked using SDS-PAGE [39]. In the dialysis stage, protein purification will be performed to remove high concentrations of imidazole or other small molecules in the buffer. The pure protein sample will be made more stable and passed into a buffer suitable for the experimental environment. The protein will be dialyzed in a suitable buffer to reduce the high imidazole concentration in the elution buffer. The buffer will be changed 3 times while the dialysis time is 4-to 6 hours [40].

7.2.3 Protein Concentration Measurement

The purified protein will be concentrated with Amicon filters or ultrafiltration systems. The Bradford Method will be used for concentration calculation. The Coomassie Brilliant Blue G-250 dye used in the method changes colour by binding to basic and aromatic amino acids of proteins. Thanks to this colour change, the protein concentration will be analysed by measuring it with a spectrophotometer at a wavelength of 595 nm. To prepare the Bradford indicator, 0.1 g of Coomassie Brilliant Blue G-250 dye will be dissolved in 50 ml of ethanol at 95% concentration. Then, 100 ml of phosphoric acid at 85% concentration will be added to the solution. Then, dH2O will be added so that the total volume of the solution will be 1 L. The solution will be filtered with Whatman-1 filter paper or a similar paper to remove particles. Standard protein solutions with concentrations of 0, 20, 40, 60, 80, and 100 mg/L from BSA are prepared with dH2O. 0.1 mL of standard protein solutions with different concentrations will be added to each tube and 5 mL of Bradford solution will be added to it. The tubes will be kept at room temperature for 5–10 minutes. Optical density (OD) will be read at 595 nm with the help of a spectrophotometer. Blank measurement will be made with 0.1 mL of distilled water and 5 mL of Bradford solution. Blank measurement value will be subtracted from other absorbances. Measured absorbance values (y-axis) will be plotted on the graph against known protein concentrations (x-axis). The accuracy of the curve will be checked. r^2 (correlation coefficient) value should be 0.98 or above, slope should be 2 and around. According to the data obtained, the equation of the curve because of regression analysis will be y=0.0125x. 0.1 mL of protein samples to be measured in concentration will be taken and placed in clean tubes. 5 mL of Bradford solution will be added to each tube and vortexed. The procedure described previously will be applied in the same way. Absorbance values will be measured at 595 nm. The measured absorbance values of protein samples to be measured in concentration will be placed in the standard curve equation. The results will be multiplied by the dilution factor to determine the final concentration [41][42]. The purified protein will be loaded onto the SDS-PAGE gel and the target Hspb5 protein will be confirmed by Western Blot. The band of the target protein will be detected on the membrane using Hspb5 antibodies.

8. SDS-PAGE

In the preparation of the protein sample for Sds-Page; the purified protein will be prepared as 20-30 µg.[43] Loading buffer will be added and denatured at 95°C for 5 minutes. Loading buffer will be prepared using 250 mM Tris-HCl (pH 6.8), 10% SDS, 40% glycerol, 0.02% bromophenol blue, and 5% beta-mercaptoethanol or 1 mM DTT. After this process is completed, the denaturation process will be started [15]. Since the molecular weight of the Hspb5 protein is in the range of 20-24 kDa, SDS-PAGE (10-12% polyacrylamide) gel will be prepared. When preparing the gel, a 10% polyacrylamide solution will be created as follows: 30% acrylamide, 1 M Tris-HCl (pH 8.8), 10% SDS, 10% APS (ammonium persulfate), and TEMED will be used. After preparing the gel, it will be ensured that it is fully polymerized. For the loading process of the protein samples, 15 µL of protein samples will be loaded into each well. Electrophoresis for the Hspb5 protein will be performed at 150 V for 60 to 120 minutes and running will be ensured. After completing the SDS-PAGE, the proteins will be transferred to the membrane for Western Blot analysis [47][49].

9. Western Blot Analysis

In the Western blot transfer step, proteins will be transferred to the PVDF membrane at 100 V for 1-2 hours at 4°C. The buffer used for the transfer process will be prepared to contain 25 mM Tris, 192 mM glycine and 20% methanol. The wet transfer method will be applied at this stage [45][46][15][49]. In the blocking process, the membrane will be blocked in 5% milk solution for 1 hour. The blocking buffer will be prepared using 1x TBS (Tris-Salt-Water), 0.1% Tween-20 and 5% non-fat milk.[44][49] For primary antibody incubation, rabbit anti-HSPB5 (αB-crystallin) antibodies will be prepared at a concentration of 1:1000-1:2000 and incubated overnight at 4°C. After this process, the membrane will be washed 3 times with TBS-T for 10 minutes each. The wash buffer will be prepared using 1x TBS and 0.1% Tween-20 [45][46][49]. In the secondary

antibody incubation, the HRP-conjugated secondary antibody (anti-rabbit IgG-HRP) will be incubated at room temperature for 1 hour. The secondary antibody solution will contain 1x TBS, 0.1% Tween-20 and 1% BSA. After the secondary antibody incubation, the membrane will be washed again in TBS-T buffer. The wash buffer will be prepared using 1x TBS and 0.1% Tween-20 [44][45][47]. The buffer will be washed again in TBS-T buffer for the last time after the secondary antibody incubation. The wash buffer will be prepared using 1x TBS and 0.1% Tween-20. The membrane will be treated with HRP substrate solution (Enhanced Chemiluminescence - ECL) and visualized with a chemiluminescence imaging device. The dimensions of the protein bands will be recorded. The location of the bands will be determined according to the Hspb5 weight (20-24 kDa) and the results will be analyzed. The analysis results of the purified protein will be compared with the analysis results of the Hspb5 protein. At this stage, the properties of the purified protein will be expected to have the properties of Hspb5 [44][47].

10. In Vitro Cell Models – Testing in Huntington's Disease Cell Models

10.1 Cell Culture and In Vitro Cell Modeling

The mouse cell line HT22 will be used to prepare the cell culture. The cells will be cultured for 24 hours with the addition of 10% FBS to DMEM/F12 (1:1) medium and 1% penicillin-streptomycin against bacterial contamination. Then, 10 µM Retinoic Acid (RA) will be added and the cells will be incubated for 6 days in an environment where the serum ratio has been reduced to 2%. This incubation will be carried out at 37°C, in a 5% CO_2 atmosphere and in a humidity-controlled incubator. The cell culture medium will be changed every 2-3 days. This change was made to ensure optimal growth and healthy proliferation of the cells. The resulting cell culture will be transfected with the mHTT plasmid to have Huntington's disease. In the transfection step, 2 µg of mHTT plasmid pAAV-hSyn-Htt171-66Q-myc-WPRE [53] and 6 µL of transfection reagent Lipofectamine 3000 will be mixed in 100 µL of Opti-MEM and left at room temperature for 10 minutes. This mixture will be added to the cell culture medium. The cells will be incubated with the transfection reagent at 5% CO_2, 37°C for 6 hours. After these procedures, a fresh growth medium will be added. The mentioned culturing and transfection process will be repeated in a different medium. One of the obtained media will be used as the control group and the other as the experimental group [50][51][52][53][54].

10.1.2 Addition of Chaperone to In Vitro Cell Model

The experimental group, which contains mHTT-expressing cell cultures added to fresh growth medium after transfection, will be supplemented with previously obtained synthetic HspB5 chaperone proteins at a concentration of 10 µg/mL and PBS. The control group, which underwent the same procedure, will be supplemented with only PBS. The cells will be incubated at 5% CO_2 and 37°C for 48 h after HspB5 addition, and then mHTT aggregation will be analyzed by Western blot [55][56].

10.1.3 Aggregation Test

Two types of aggregate tests will be applied to the mHTT cell line. Thioflavin T staining will be performed as the first aggregate test to obtain rapid results. The control group and the experimental group will be stained with Thioflavin T, and after staining, the presence and distribution of protein aggregates within the cell will be analyzed with a confocal microscope. In the analysis phase, the aggregate reduction rate between the two media will be determined quantitatively. In the second aggregate test to be conducted, synthetically produced HspB5 will be added to the cell culture and incubated for 48 hours, then washed with PBS and separated with trypsin, then centrifuged and suspended. The cells will be lysed with protein extraction buffer (RIPA buffer + protease inhibitors) for aggregate analysis and centrifuged at 12,000 rpm for 5 minutes to obtain the supernatant. The cell suspension obtained after centrifugation will be centrifuged at 100,000 x g for 1 hour to separate the aggregates, and the supernatant obtained will contain pelleted aggregates when taken as the soluble protein fraction. The aggregate fraction will be solubilized with a 1X lysis sample buffer and heated at 98°C for 5 minutes. Both fractions (soluble and aggregate) will be separated by SDS-PAGE and transferred to the PVDF membrane and incubated with an anti-mHTT primary antibody for mHTT detection. Anti-mouse will be used as the second antibody and immunoreactivity will be measured with a chemiluminescence detection kit. The results will be examined by comparing the mHTT distribution in the soluble and aggregate fractions, and the effect of HspB5 on aggregate formation will be evaluated [55][57][58][59][60].

10.2 Intracellular Analysis and Immunofluorescence Microscopy

In the preparation of cells stage, cells will be seeded on glass slides with 8 chambers, 100,000-200,000 cells per chamber [61]. Cells will be fixed with 4% PFA solution for 10 minutes to stabilize their morphology and protein structures [62]. In the

permeabilization stage, cells will be permeabilized by incubating in 0.1% Triton X-100 solution for 5 minutes so that antibodies can reach target proteins [63]. In the blocking stage, cells will be blocked by incubating with 1% BSA or 5% powdered milk solution for 30 minutes at room temperature to prevent non-specific binding and ensure that antibodies bind only to target proteins [64]. In the primary antibody application stage, Anti-Hspb5 or anti-His antibody will be diluted 1:1000 according to the manufacturer's recommendation [65]. Diluted primary antibody will be added to the cells and incubated overnight at 4°C. In the washing step, the cells will be washed 3 times with PBS. Each wash will last 5 minutes [66]. In the secondary antibody application step, fluorescently conjugated secondary antibodies such as Alexa Fluor or FITC will be diluted 1:1000 [67]. Diluted secondary antibodies will be added to the cells and incubated for 1 hour at room temperature. In the imaging step, the cells will be stained with the nuclear dye DAPI and visualized under a confocal microscope. Hspb5 localization will be analyzed within the cell [68].

Conclusion

In conclusion, this study represents an innovative approach to targeting protein aggregation, which is the most significant secondary cause of the disease, since research on inhibition of HTT gene mutation, the primary cause of HD, is still ongoing. In this direction, the production of the HspB5 chaperone protein for use in cells has been achieved by the application of recombinant DNA technology. As an experimental model, *S. cerevisiae* yeast cells, which have similar characteristics to the cells in which the disease is observed, were preferred to avoid incompatibility when applied to sick individuals. The disease was modelled in the appropriate cell line, synthetically produced HspB5 was applied and its effect on misfolded mutant Huntington's proteins produced because of the mutations in the HTT gene was observed. Since this study used many biotechnological methods such as synthetic production of HspB5 protein, gene sequence cloning, plasmid vector transfer and gene expression, it provides a promising basis for future biotechnological approaches targeting protein aggregation in the treatment of HD.

Biosafety

Our project aligns with biosafety and biosecurity regulations. Genetically modified Saccharomyces cerevisiae which can be used for protein production is a safe model organism with low risk of release into the environment. Experiments involving low-risk organisms can be conducted in a Biosafety Level 1 (BSL-1) laboratory. The genetic materials to be used should be produced and screened in accordance with IGSC standards. Personal protective equipment (PPE), including lab coats, gloves, and eye protection, should be always worn during experimental procedures. Separate areas should be designated for handling pre- and post-PCR products to prevent contamination. In order to avoid contamination in the studies, all materials must be sterilized, biological waste must be destroyed by autoclaving, and experiments that will be carried out should be only in closed laboratory environments. No human or animal samples are needed to be used, and all processes must be developed under ethical principles and national and international safety standards.

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