Project Report

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FECD (Fuch's Endothelial Corneal Dystrophy) | Glioma

The following paragraphs gives a brief understanding of my project work under the categories 'Understanding the genetic gameplay behind the disorder - Fuch's endothelial corneal dystrophy (FECD) and 'Evaluation of Dihydro-artemisinin as anticancer drugs in rat brain model'

Evaluation of Dihydro-artemisinin as anticancer drugs in rat brain model : . Cancer cells are characterised with abnormal cell growth and lack of specificity. Glioma, or brain cancer is a type of brain tumour that grows from glial cells.Glial cells support nerve cells with energy and nutrients and help maintain the blood-brain barrier.Recent studies have reported that artemisinin is effective against cancers and that it has positive effects on the immune system. Using rat (SD strain) as model,C6 cell line was derived from a rat glial tumour induced by N-nitrosomethylurea.Dihydroartemisinin(DHA) ,metabolised from artemisinin has been found to inhibit proliferation to induce apoptosis of rat glioma C6 cells.

Understanding the genetic basis of late-onset Fuch's endothelial corneal dystrophy : . The aim of my project was to understand the genetic gameplay behind an eye disorder prevalent widely among middle aged individuals, known as Fuch's Endothelial Corneal Dystrophy(FECD).The aim was to genetically assess part of intronic sequences of a transcript of chromosome 18 and telomere portion of chromosome 13 where several single nucleotide polymorphism and unusual trinucleotide repetitions as a result of mutation, either epigenetic or hereditary has been found to cause decreased expression of the aforementioned transcript .This is critical as the result is deficiency of a protein, called ZEB1.This protein succesively regulates expression of RNA transcripts responsible for synthesis of certain collagen fibres, expresssed by the epidermis of corneal endothecium and whose function is to maintain the structure of the Descemet's membrane of cornea.Thinning of Descemet's membrane results in edema, the last phase of FECD,causing chronic blindness. Though symptomatic cure is possible through usage of drugs, the only permanent cure of FECD is corneal transplantataion. Further goals are investigation of TCF8 Genome as well as the reason for its oxidative degenration.

Lab Protocols:

The following protocols were followed :

Genomic DNA Isolation (Using Column Method) : .

- Take 200 micro-litre of blood
- Add 20 micro-litre of protease and incubate for 10 mins.
- Add 200 micro-litre of Al buffer and vortex for 15 sec.
- Incubate at 56 °C for 10 mins(water bath).
- Briefly centrifuge(short spin) to remove anything attached to the lid.
- Add 200 micro-litre of EtOh (absolute) and vortex for 15 sec.
- Brief spin.
- Add to column and spin at 6,000g/10,000rpm for 3 mins.
- Add 500 micro-litre of AW1 and spin at 6,000g for 1 minute.
- Take a fresh collecting tube.
- Add 500 micro-litre of AW2, keep for incubation (5 mins.) and spin at 14,000rpm for 3 mins.

Significance Statement

Evaluation of dihydro-artemisinin as anti-cancer drug : The objective of the experiment was to study the effect of dihydroartemisinin on rat brain tumour model and check for its regression. Dihydroartemisinin is highly specific towards oncogenic cells and reacts less with normal tissue cells, hence avoiding any infliction of side-effects apart from the killing of malignant tumour cells.The experiment has extensive potential in the treatment of glioma in higher mammals including humans, proving to be a very efficient and specific treatment procedure.

Genetic understanding of FECD : The late-onset FECD is classified as a dystrophy of type 2 with insufficient genomic data to account for the disorder.Along with symptomatic effects of FECD like edema and corneal pains ,the intronic variation in the TBF4 gene and decreased expression of the protein result in a very severe phenotype of mental retardation, seizures, and episodic hyperventilation. The most prevalent clinical procedure of treating this disease is penetrating keratoplasty or Descemet's stripping endothelial keratoplasty, both of which involves corneal transplantation using surgical methods and is thus associated with chronic pains, high maintainence and surgical costs and restriction vision . The possibility of gene therapy at the fetal stage or gene regualtion at the later stage may provide an highly effective treatment to this disease at minimal cost. Gene therapy is also accompanied with little or no side effects as only a particular portion of the genome (approx. 100 bases) will be artificially mutated to restore normal function of the RNA transcript. Thus the project has social as well as institutional relevance.

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- New collecting tube is taken, dry spin at 14,000rpm for 3 mins.Then air dry for 10-15 mins.
- Place the column in a 1.5 ml tube, add 200 micro-litre AE buffer and incubate for 5 mins.
- Spin at 6,000g/8,000rpm for 1 min.
- Quantitate and store at -20°C.
- Precautions :
  - Rinse the disposable tips in bleach before disposing.
  - If blood touches the pipette, erase the blood away with a tissue soaked in ethanol.
  - Before disposing the collecting tube, dispose the contents of the tube in a beaker containing bleach and absorb the remaining liquid inside the tube using a tissue paper.
  - Air dry should allow sufficient time for the ethanol to escape.
  - After adding each buffer leave for 5 mins.
- Keys : A. AW1- Wash Buffer 1 B. AW2- Wash Buffer 2 C. Al- Lysate Buffer D. AE- Elution Buffer
- Centrifuge Temperature = 15°- 25°C

Polymerase Chain Reaction (PCR) : .

- Composition -
  - NFW - 15 micro-litre
  - DMSO - 2.5 micro-litre
  - Taq Buffer - 2.5 micro-litre
  - dNTPs - 2 micro-litre
  - Forward Primer - 0.5 micro-litre
  - Reverse Primer - 0.5 micro-litre
  - Taq Polymerase - 0.3 micro-litre
  - Template DNA - 2 micro-litre
  - Total volume of solution - 25.3 micro-litre
- Temperature setup-
  - 96°C-10 sec
  - 50°C-5 sec
  - 60°C-4 min
  - Return to step 1 (24 cycles)

Gel Casting : .

- Composition -
  - 1x TAE Buffer
  - 1 percent Agarose
  - 7 micro-litre Ethidium Bromide
- Procedure -
  - Take 300 ml of Buffer solution and add 24gm of Agarose powder to it (0.8 percent).
  - Microwave for 3 mins.
  - Cool under tap water.
  - Add 7 micro-litre of ethidium bromide.
  - Place the combs and cast from one side of the chamber. Allow to solidify for 30mins.
  - Stain the DNA and pipette on the wells. Electrophorize for 20 mins at 300V.
  - Observe under UV Ray. Cut portions where DNA is located.

DNA Elution : .

- Take a colourless tube. Add 3 volume of QG Buffer to 1 volume of gel.
- Vortex and then incubate at 52°C for 15 mins(until the gel is dissolved).
- Check the colour of the mixture (yellow). If the colour is orange or violet, add 10 micro-litre of NaCH3COOH, pH-5.0 and mix.
- Add the volume to spin column and centrifuge for 1min at 6,000 rpm, until all the samples have passed through column.
- Discard flow through.
- Add 500 micro-litre PE buffer for washing. Keep for 2-5mins after addition of buffer PE and then centrifuge at 13,000rpm for 1 min. Repeat the step again.
- Then do a dry spin to remove residual wash buffer (13,000rpm for 10 mins.)
- Place the column into a clean 1.5ml tube.
- To elute DNA, add 20 micro-litre elution buffer (EB) to the centre of the membrane. Stand for 5 min and then centrifuge for 1 min at 13,000rpm.
- Store the eluted product for further use at -20°C.

Sequencing PCR : .

- Composition -
  - DB - 1.75 micro-litre
  - RR- 0.3 micro-litre
  - Primer- 2 micro-litre
  - Template- 5.95 micro-litre
  - Total volume - 10 micro-litre
- Temperature setup -
  - Same as before

Cleanup : .

- Composition -
  - Master Mix 1 -
    - EDTA - 2 micro-litre
    - Milli-Q - 10 micro-litre
  - Master Mix 2 -
    - NaOAc - 2 micro-litre
    - EtOH - 50 micro-litre
- Procedure -
  - Add 12 micro-litre MM1 to new tubes (0.5 ml tubes)
  - Add PCR products to it and mix
  - Add 52 micro-litre MM2 to it and mix
  - Wait for 20 mins (Occasionally tap at 20 min interval)
  - Centrifuge at 12,000g for 20 mins
  - Discard supernatent gently
  - Add 250 micro-litre of 70 percent EtOH to each tube
  - Centrifuge at 12,000g for 15 mins
  - Discard the supernatent gently and keep the volume of EtOH to minimum
  - Keep for drying overnight
  - Add Hi-Di (10 micro-litre) and incubate for 10 mins
  - Then do sequencing (Sanger sequencing)

Loading Dye : .

- Composition -
  - 10mM Tris Hcl (pH 7.6) - 0.5 ml
  - 0.03 percent BPB - 15 mg
  - 60 percent Glycerol - 30 ml
  - 60mM EDTA - 6 ml
  - NFW - 13.5 ml
- Procedure -
  - Add Tris Hcl,EDTA and NFW
  - Add 0.03 percent BPB
  - Slowly add glycerol and mix
  - Store overnight

50x TAE Buffer solution : .

- Weigh out 242 gm of Tris base
- Prepare 100 ml of 0.5M EDTA, by dissolving 14.62 gm of pure solid EDTA in distilled water (100 ml).Add 6ml of NaOH for better dissolution
- Measure 57.1 ml of Glacial acetic acid, add it to a beaker containing 242gm of Tris and 100 ml EDTA.
- Add water and make the volume upto 1000 ml. Stir the solution to ensure complete dissolution of Tris buffer (the solution should turn colourless).(pH 8.5)
- Add NaOH (100 ml) to ensure complete dissolution.

10x TBS Buffer solution : .

- Composition -
  - 20mM tris base - 12.1 gm
  - 150mM sodium chloride - 43.8 gm
- Add the above items to distilled water and make the volume to 500ml.

Column washing : .

- Add 500 micro-litre of TE buffer to each tube
- Incubate for 2 hrs
- Centrifuge at 13,000 rpm for 1 min.
- Discard the liquid
- Do dry centrifuge at 13,000 rpm for 5 mins

Food preparation for Drosophila : .

- Composition -
  - Agar-3 grams
  - Maize powder-17 grams
  - Sugar-15 grams
  - Yeast powder-6 grams
  - Nipagin-1 gram
  - Ethanol-2ml
  - Distilled water
- Procedure -
  - At first in a big beaker(1 liter) take 100ml of water and mix 3 grams of agar thoroughly. Then boil it in the oven for 1 to 2 minutes.
  - Then in another small beaker take 100 ml of water and mix the maize powder and sugar in the specified quantity. Then put this mixture in the agar solution.Then wash the small beaker with 50ml of water and pour the water in the big beaker containing the agar, maize and sugar solution. Mix it properly and boil for 1-2 minutes.
  - Then take 60 ml of water in the small beaker and put 6 grams of yeast powder in it.Mix it properly using a stirrer and pour the mixture into the big beaker containing all the mixtures. Then wash it properly with 50 ml of water and put the water in the big beaker. Mix this properly and boil it for 1-2 minutes.
  - In another tube take 1 gram of Nipagin and mix it with 2 ml of ethanol.
  - Put this nipagin mixture into the big beaker containing the food mixture when it comes to room temperature and stir it properly.
  - If you want to mix any drug then you can add this at this time after mixing nepagin

- If you want to test that whether the fly has eaten the drug formulated food or not then you can add nilkon blue at this time to the drug formulated food.
- Then pour the food into vials (5 ml each). The food will be solidify after some times
- You can use it immediately or can store and use in future.

C6 cell line injection in subcutaneous rat tissue : .

- Model organism and drug used -
  - Sprague Dawley Rats (SD Rats) -
    - Outbred multipurpose breed of rat
    - Advantage is its calmness and ease of handling
    - Lifespan of 3.5 years
    - Female adult body weighs 250-300gms. Male adult body weighs 450-520gms.
    - Sprague Dawley rats produce secretions from their eyes when stressed that contain a pigment which when dry, has the appearance of dried blood. These "tears" glow fluorescently under UV light
  - C6 Cell line -
    - C6 cell line is derived from a rat glial tumour induced by N- nitrosomethylurea.
    - The cells produce S-100 protein which is found in the neural tissue of vertebrates and has been found in brain tumours.
  - Dihydro-artemisinin (DHA) -
    - Artemisinin is effective against cancers and that it has positive effects on the immune system. Every artemisinin compound is metabolized to dihydroartemisinin (DHA) by replacing the C-10 lactone group of artemisinin with hemiacetal.
    - The artemisinin molecule contains an endoperoxide bridge that reacts with a ferrous iron atom to form free radicals .The free radicals generated by the artemisinin and its derivatives induce macromolecular damage and cell death
    - The cytotoxic effects of artemisinin are greater against cancer cells than normal cells because cancer cells take up more iron, which is an essential nutrient for cellular growth
- Methodologies -
  - Culturing C6 cell line in vivo. seeding effective dosage in rat for tumour growth
  - Inhibition of C6 cell line growth by DHA will be determined using MTT calorimetric assay(Dosage kinetics)
  - Subcutaneous implantation of C6 glioma cells to induce tumor in SD rat.
  - To determine whether dihydro-artemisinin could suppress tumor growth, different dosages are orally administered to the rat with appropriate controls after a certain stage of tumour growth(100 ,500 and 1000 cubic cm).
  - Analysis of tumour regression by measurement using vernier callipers.

Observations :

- DNA extraction and purification data -
  - Sample Used : FN 215 (control sample) -
    - Date - 18.05.2018
    - Nano-Drop reading - 36.4 ng/microlitre
    - 260/280 ratio - 1.65
    - 260/230 ratio - 0.1
  - Sample Used : FN 377 (control sample) -
    - Date - 22.05.2018
    - Nano-Drop reading - 4.8 ng/microlitre
    - 260/280 ratio - 2.42
    - 260/230 ratio - 4.65
  - Sample Used : FN 378 (control sample) -
    - Date - 22.05.2018
    - Nano-Drop reading - 5.0 ng/microlitre
    - 260/280 ratio - 2.06
    - 260/230 ratio - 4.50
  - Sample Used : FN 385 (control sample) -
    - Date - 28.05.2018
    - Nano-Drop reading - 21.8 ng/microlitre
    - 260/280 ratio - 1.83
    - 260/230 ratio - 1.86
  - Sample Used : FN 386 (control sample) -
    - Date - 28.05.2018
    - Nano-Drop reading - 14.3 ng/microlitre
    - 260/280 ratio - 1.83
    - 260/230 ratio - 1.95

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