CELL VIABILITY IN X-RAY IRRADIATED HUMAN LENS EPITHELIAL CELL (HLEC) CULTURE BY USING TRYPTAN BLUE EXCLUSION TEST

Rajesh Dehankar¹, Shivani Bapat², D. D. Ksheersagar³, V. Paikrao⁴

¹Associate Professor, Department of Anatomy, NKP Salve Institute of Medical Sciences & Research Centre, Digdoh Hills, Hingna Road, Nagpur.
²Student, Department of Anatomy, NKP Salve Institute of Medical Sciences & Research Centre, Digdoh Hills, Hingna Road, Nagpur.
³Professor & HOD, Department of Anatomy, NKP Salve Institute of Medical Sciences & Research Centre, Digdoh Hills, Hingna Road, Nagpur.
⁴Technician, NKP Salve Institute of Medical Sciences & Research Centre, Digdoh Hills, Hingna Road, Nagpur.

ABSTRACT

INTRODUCTION

Cataract is one of major cause of blindness, 44% cases of blindness is due to cataract only. In developing countries such as India cataract is more common and it manifest earlier in life than the most developed countries of the world. Patients receiving therapeutic radiation may have developed cataracts as well as radiation retinopathy and optic nerve damage. In this study we are aimed to compare effects of X-ray irradiation on cell viability of HLEC (Human lens epithelial cell) culture by using trypan blue dye exclusion test against the unexposed control HLEC culture. Total 20 cataract patients from Dept. of Ophthalmology of Lata Mangeshkar Hospital, Nagpur were included in this study. The capsulorhexis sample from cataract surgery taken for study from eye OT. The capsulorhexis sample without any x-ray exposure taken as control. For culture of HLEC’s RPMI 1640 medium containing 10% fetal calf serum used. The HLEC cultures were X-ray irradiated with increasing count of skull limit parameters of range 150kV, 250mAs and 50mA for 5sec using GE® 650 M.A. X-ray machine. The Trypan blue dye exclusion test was used to analyze cell viability. The slides were analyzed using Olympus® BX51 Research microscope with oil immersion and 100 were observed per sample. The cell viability was calculated using Epi info® (version 6.0), as subject means with standard deviation. Statistical significance will be defined at P≤0.001. Out of 100 cases the 12 were female and 8 were male. Mean age of both male and female patients were 62.45±3.18(SD). A positive linear correlation was found (r:0.819, p<0.001) between the No. of X-Ray exposure and death cells per 100 observed LECs.

KEYWORDS

Human Lens Epithelial Cells, Trypan Blue.

HOW TO CITE THIS ARTICLE: Dehankar R, Bapat S, Ksheersagar DD et al. Cell viability in X-ray irradiated human lens epithelial cell (HLEC) culture by using trypan blue exclusion test. J Evid Based Med Healthc 2015; 2(60), 9003-08, DOI: 10.18410/jebmh/2015/1277

INTRODUCTION:

Cataract is one of the major causes of blindness. 44% cases of blindness are due to cataract only. In developing countries such as India cataract is more common and it manifests earlier in life than the most developed countries of the world.(1)

The cataract caused by ionising radiation as X-ray, different from that caused by age.(2) The age related cataracts are most commonly found in the nuclear region and cortical cataracts are commonly found in diabetic patients while the ionising radiation is generally although not exclusively associated with posterior sub capsular and sometimes cortical opacities.(3) The posterior sub capsular opacification was thought to be a characteristic of the radiation damage to the lens; although more recent data suggest that radiation induced opacities can be found in the lens cortex as well. The various studies have found that nuclear cataracts are not associated with radiation exposure. Although much work has been carried out in this area, the exact mechanisms of radiation cataractogenesis are still not fully understood.(4)

The effects of radiation on living organisms are varied and depend on dosage as well as species and tissue specific sensitivities. With regard to the eye, most ultraviolet radiation is absorbed by the cornea and conjunctiva. However, ionising radiation, because of its better penetration power, can reach all parts of the eye. The lens seems to be particularly sensitive because of effects on the lens epithelium.(5) Cataracts have been diagnosed in worker who have been routinely exposed to radiation and in atomic bomb survivors. Patients receiving therapeutic radiation have developed cataracts as well as radiation retinopathy and optic nerve damage. (6) There have been few reports of the effects of diagnostic X-Ray exposure on the lens, although Storrs and Byrd(7) have suggested that repeated exposure to computerised axial tomography (CAT) scans of...
the head delivers a dose of radiation to the lens that may have cataractogenic potential.

There is need to investigate the effects of X-ray irradiation on the eyes, though there is vast literature on the effects of X-ray irradiation on body tissues and tissue cultured cells in vitro and in vivo, these literature lack the work on lens epithelial cells (LEC) of humans, such an information would describe the actual relation between the X-ray irradiation and viability in human lens epithelial cells (HLEC).

The word ‘cataract’ dates from the middle ages and has been derived from the Greek word ‘katarrates’ which means ‘waterfall’.

ANATOMY OF EYE: Chaurasia, 2013(9) and Khurana, 2012(9) explain the Anatomy of eye as under.

The eyelids are covered externally by the skin and internally by conjunctiva which is reflected over the globe of the eye. The lacrimal glands which are compound racemose glands are situated at the upper angle of the orbit. The globe of the eye is composed of 3 layers: The Cornea Sclera, Choroid Iris, and Retina. The eyeball is the organ of sight. It is almost spherical in shape and has a diameter of 2.5cm. It is made up of three concentric coats. The outer or fibrous coat comprises the sclera and cornea. The middle or vascular coat also called the uveal tract consists of choroid, the ciliary body, and the iris. The inner or nervous coat is the retina.

Outer Coat
Sclera: The sclera is opaque and forms the posterior five sixths of the eyeball. It is composed of dense fibrous tissue which is firm and maintains the shape of the eyeball. It is thickest behind, near the entrance of the optic nerve, and thinnest about 6mm behind the sclerocorneal junction where the recti muscles are inserted. However, it is weakest at the entrance of the optic nerve. Because of its sieve like appearance, this region is called the lamina cribrosa. The outer surface of the sclera is white and smooth; it is covered by Tenon’s capsule. Its anterior part is covered by conjunctiva through which it can be seen as the white of the eye. The inner surface is brown and grooved for the ciliary nerves and vessels. It is separated from the choroid by the perichoroidal space which contains a delicate cellular tissue, termed the suprachoroidal lamina or lamina fusca of the sclera.

The sclera is a continuous anteriorly with the cornea at the sclerocorneal junction or limbus. The deep part of the limbus contains a circular canal, known as the sinus venosus sclera or the canal of Schlemm. The aqueous humour drains into the anterior sclera or ciliary veins through this sinus. The sclera is fused posteriorly with the dural sheath of the optic nerve. It provides insertion to the extrinsic muscles of the eyeball: the recti in front of the equator, and the oblique muscles behind the equator.

The Sclera is pierced by a number of structures:
1. The optic nerve pierces it a little inferomedial to the posterior pole of the eyeball.
2. The ciliary nerves and arteries pierce it around the entrance of the optic nerve.
3. The anterior ciliary arteries derived from muscular arteries to the recti pierce it near the limbus.
4. Four venae vorticosae or the choroid veins pass out through the sclera just behind the equator.

The sclera is almost avascular. However, the loose conjunctiva tissue between the conjunctiva and sclera called as the episclera is vascular.

Cornea: The cornea is transparent. It replaces the sclera over the anterior one sixth of the eyeball. Its junction with the sclera is called the sclerocorneal junction or limbus. The cornea is more convex than the sclera, but the curvature diminishes with the age. It is separated from the iris by a space called the anterior chamber of the eye. The cornea is avascular and is nourished by lymph which circulates in the numerous corneal spaces. It is supplied by branches of the ophthalmic nerve and the short ciliary nerve. Pain is the only sensation aroused from the cornea.

Middle Coat
Choroid: It is a thin pigmented layer which separates the posterior part of the sclera from the retina. Anteriorly, it ends at the ora serrata by merging with the ciliary body. Posteriorly, it is perforated by the optic nerve to which it is firmly attached. Its outer surface is separated from the sclera by the suprachoroidal lamina which is traversed by the ciliary vessels and nerves. Its attachments to the sclera is loose, so that it can be easily stripped. The inner surface is firmly united to the retina.

Ciliary Body: It is a thickened part of the uveal tract lying just posterior to the corneal limbus. It is continuous anteriorly with the iris and posteriorly with the choroid. It suspends the lens and helps it in accommodation for near vision. The ciliary body is triangular in cross section. It is thick in front and thin behind. The sclera surface of this body contains the ciliary muscle. The posterior part of the vitreous surface is smooth and black. The anterior part is ridged anteriorly to form about 70 ciliary processes. The central ends of the processes are free and rounded.

Iris: It is the anterior part of the uveal tract. It forms a circular curtain with an opening in the centre, called the pupil. By adjusting the size of the pupil, it controls the amount of the light entering the eye, and thus behaves like an adjustable diaphragm. It is the continuation of the choroid which extends in front of the lens. It is similar in structure to the choroid but contain pigment cells.

Inner Coat
Retina: It is a part of central nervous system and corresponds in extent to the choroid which it lines internally.
The retina is composed of a number of layers of cells and their synaptic processes are of three types:

1. External photoreceptor cells (rods and cones).
2. Intermediate relay layer of bipolar cells.
3. Internal layer of ganglion cells with their axons running into the central nervous system.

The central fovea is a specially differentiated spot in the retina posteriorly which consists only of photosensitive cones but is devoid of photoreceptor rods and blood vessels. Macula lutea or yellow spot surrounds the central fovea and though not as sensitive as central fovea, it is more than the other parts of the retina. At the optic disc, the fibres of the nerve fibre layer of the retina pass into the optic nerve.

**Anterior Chamber:** It is the space filled with the aqueous humour, and is bounded by the cornea in front and the iris behind, with anterior surface of the lens exposed in the pupil.

**Posterior Chamber:** Posterior chamber containing the aqueous humour is the triangular space between the back of the iris, the anterior surface of the lens and the ciliary body forming its apex at the papillary margin.

**Vitreous Chamber:** It is the large space behind the lens containing gelatinous material, the vitreous humour. The main function of the eye is visual acuity which depends upon a transparent focusing system comprised by the cornea, lens, transparent media consisting of aqueous and vitreous humours, and a normal retinal and neural conduction system. The cornea and lens receive their nutrient demands from the aqueous humour produced by the ciliary processes. The intraocular pressure is normally 15 to 20 mmHg and depends upon the rate of aqueous production and on the resistance in the outflow system.

**Anatomy of Lens:** The lens is a transparent biconvex structure which is placed between the anterior and posterior segments of the eye. It is circular in outline and has a diameter of 1 cm. Its refractive index is 1.39 and total dioptric power is about 18D. The accommodative power of the lens varies with age, being 14 to 16D (at birth), 7 to 8D (at 25 yrs. of age) and 1 to 2D (at 50 yrs. of age). The central points of the interior and posterior surfaces are called the anterior and posterior poles. The line connecting the poles constitutes the axis of the lens, while the marginal circumferences termed the equator. The chief advantage of the lens is that it can vary its dioptric power. It contributes about 15 dioptries to the total of 58 dioptric power of the eye. The posterior surface of the lens is more convex than the anterior. The anterior surface is kept flattened by the tension of the suspensory ligament. When the ligament is relaxed by contraction of the ciliary muscle, the anterior surface becomes more convex due to elasticity of the lens substance. The lens is enclosed in a transparent, structure less elastic capsule which is thickest anteriorly near the circumference. Deep to the capsule, the anterior surface of the lens is covered by a capsular epithelium. At the centre of the anterior surface, the epithelium is made up of a single layer of cubical cells, but at the periphery, the cells elongate to produce the fibres of the lens. The fibres are concentrically arranged to form the lens substance. The centre (nucleus) of the lens is firm, whereas the periphery (corpus) is soft and is made up of more recently formed fibres.

The suspensory ligament of the lens retains the lens in position and its tension keeps the anterior surface of the lens flattened. The ligament is made up of a series of fibres which are attached peripherally to the ciliary processes, to the furrows between the ciliary processes, and to the ora serrata. Centrally, the fibres are attached to the lens, mostly in front, and a few behind the equator.

The crystalline lens is a transparent structure playing main role in the focusing mechanism for vision. Its physiological aspect includes; Lens transparency, Metabolic activities of the lens and Accommodation.

**Lens Transparency:** Factors that play significant role in maintaining outstanding clarity and transparency of lenses are;

1. Avascularity.
2. Tightly packed nature of lens cells.
3. Narrow lens fibre membranes.
4. Loss of organelles.
5. The arrangement of lens proteins.
6. Semi permeable character of lens capsule.
7. Pump mechanism of lens fibre membranes that regulate the electrolyte and water balance of the lens, maintaining relative dehydration and,
8. Auto oxidation and high concentration of reduced glutathione in the lens maintains the lens proteins in a reduced state and ensures the integrity of the cell membrane pump.

**Classification of Cataract:**

The classification of eye lens opacities classification system III.

**A. Etiological Classification:**

1. Congenital and developmental cataract.
2. Acquired cataract:
   1. Senile cataract.
   2. Traumatic cataract.
   3. Complicated cataract.
   4. Metabolic cataract.
   5. Electric cataract.
   6. Radiational cataract.
   7. Toxic cataract.
      i. Corticosteroid induced cataract.
      ii. Miotics induced cataract.
      iii. Copper (in chalcosis) and iron (in siderosis) induced cataract.
   8. Cataract associated with skin diseases (dermatogenic cataract).
   9. Cataract associated with skin diseases.
Sterilisation of glassware was carried out by the standard sterilisation of lab ware and culture room. Cleaning and sterilisation of glassware was carried out by the standard sterilisation of lab ware and culture room. The type of cataract was noted as per the standard sterilisation of lab ware and culture room.

AIMS & OBJECTIVES:
In this study we aimed at:
1. Standardising the process of human lens epithelial cells (HLEC) culture.
2. Performing the HLEC culture and standardisation of trypan blue exclusion test.
3. Comparing the effects of x-ray irradiation on HLEC Culture by using trypan blue exclusion test against the unexposed control HLEC culture.
4. Analysing the result using the advanced statistical analysis tools.
5. Giving the significant relation between x-ray irradiation on HLEC Culture and cell viability, if any.

MATERIALS & METHODS:
Patient selection: This study comprised cataract patients of all ages, who had undergone phacoemulsification. The informed consent was obtained from the participants. The patients lived in with the surroundings of contact of ionising radiation as x-ray was excluded from study.

Sample Collection: The circular pieces of human anterior lens capsulorhexis sample was collected post operatively in sterile normal saline from cataract patients from the eye OT of institute’s hospital. The type of cataract was noted as per lens opacities classification (LOC) system III. (10)

Tissue Culture of Human Lens Epithelial Cell(HLEC): Sterilisation of lab ware and culture room. Cleaning and sterilisation of glassware was carried out by the standard protocol using appropriate disinfectants followed by autoclaving of glassware at 15 lbs for 20mins. Inoculation room was fumigated with 5% formaldehyde spray in the culture room. The UV light was switched on for half an hour, before preparing the culture media. RPMI 1640 media used to culture the HLECs.

Human Lens Epithelial Cell (HLEC) Culture: Cell culture was carried out as per Goyal et al 2010 (11) to acclimatize the HLEC Cells, a single rhexis was placed in 1ml of RPMI medium containing 10% foetal calf serum in a single well of a 24 well plate and was incubated for 1 hour 30 minutes on appropriate CO₂ pressure at 37°C.

X-Ray Irradiated Human Lens Epithelial Cell (HLEC) Culture: The culture plate with human lens epithelial cell (HLEC) culture was x-ray irradiated using GE Healthcare Automatic 630MA x-ray machine. For irradiation the increasing count of 630 milli ampere/sec dose of x-ray was used. The control was without any x-ray irradiation.

The Trypan Blue Exclusion Test: The trypan blue exclusion test was used to ensure cell viability. In this test, a few drops of trypan blue was added on a rhexis, placed on a glass slide and then microscopically examined to determine whether cells take up or exclude the dye. A viable cell had a clear cytoplasm whereas a nonviable cell had a blue cytoplasm. (12)

Slide Preparation: The rhexis was removed from the trypan blue dye and was washed twice in PBS (phosphate buffer solution). Then rhexis was immediately mounted on a drop of PBS, on clean dry grease free slide. The slides were analysed using Olympus Research microscope with oil immersion and 1,000 cells were observed per sample. Only nucleated cells that were separated without overlapping or folds were analysed. Micronuclei (MN) were counted when the structures had a regular border and were located inside the cytoplasm.

Statistical Analysis: Statistical analysis was carried out using the Epi Info® statistical package, after breaking the code. The cell viability was calculated as subject means with the standard deviation. Statistical significance was defined at P≤0.05.

RESULT:
Out of 20 cases the 12 were female and 8 were male. Mean age of both male and female patients were 62.45±3.18(SD). A positive linear correlation was found (r: 0.819, p<0.001) (Graph 1) between the No. of X-ray exposure and death cells per 100 observed LECs (Fig. 1).
Fig. 1: Trypan blue dye exclusion test. (a) Controls LECs without any x-ray exposure; (b) X-Ray exposed LECs

Graph 1: Observed Cell Death

Table 1: X-Ray Exposed LECs with X-Ray Exposure
DISCUSSION: Cataract is the multifactorial disease. Various causative factors are behind the development of cataract. Though the age related cataract is more prevalent the other factors also play an important role in the pathogenesis of cataract. Beside this cataract is more common in dry and hot area where sun (UV) exposure is more than prolong cloudy cover. The cataract caused by ionising radiation as x-ray, different from that caused by age. A lot of researchers worked on the pathogenesis of cataract but most of them were concentrated on the age related cataract and some on non-ionising radiation. Therefore it was significant to know the comparative effects of X-Ray irradiation on HLEC against the unexposed control HLEC Culture. From the proposed research we have achieved the viability of the HLEC in the X-Ray radiation and determined the minimum count through X-Ray exposure.

Apart from the scientific research we had interaction with the patients, the knowledge of research designing and the exposure to the scientific protocols and biostatistics analysis. We collect data regarding alcohol consumption of cataract patients of Vidarbha region, our data also support the all research report. We also found risk of cataract more in alcohol drinker.

CONCLUSION: From this research it can be concluded that the repeated exposure of skull X-ray dose is might be lethal to the LECs and it may have cataractogenic potential, as these cells are precursor of eye lens.

REFERENCES:
1. Leibowitz H, Krueger D, Maunder C, Milton R, Mohandas M, Kahn A, Nickerson R, Pool J, colton T, Ganley J, Loewenstein J, and Dawber T. (1980): the Framingham eye study monograph. Surv Ophthalmol
2. Chatterjee A, Milton R and Thyle S. (1982): prevalence and etiology of cataract in Punjab. Brit J Ophthalmol 19.
3. International atomic energy agency, radiation and cataract: staff protection, radiation protection of patients, Vienna international centre, Austria.
4. Ainsbury E, Bouffler S, Dorr W, Graw J, Muirhead J, Edwards A, Cooper C, radiation cataractogenesis: a review of recent studies, Radiat Res 172.
5. Kleiman N, Worgul B, lens. In: tasman w and jaeger ea, editors. Duane’s clinical ophthalmology, Philadelphia: Lippincott & co. (1994).
6. Klein BEK, Klein R, Linton KLP and Franke T. Diagnostic xray exposure and lens opacities: the beaver dam eye study. Am J Pub Health. 1993, 83.
7. Storns BB, Byrd S. Radiation exposure to the ocular lens during CT scanning. Pediatr Neurosci 1984.
8. Chaurasia BD, Human Anatomy, CBS publishers & Distributors Pvt.Ltd.vol. 3 Edn. 6, 2013. Pg. 288-294. ISBN:978-81-239-2332
9. Khurana AK, Comprehensive ophthalmology, New Age International Publishers, Edn. 5th, 2012. ISBN: 978-81-224-3331-9
10. Chylack LT Jr., Wolfe JK, Singer DM, Leske MC, Bullimore MA, Bailey II, Friend J, McCarthy D, Wu SY. The lens opacities classification system III. The longitudinal study of cataract study group Arch Ophthalmol 1993.
11. Goyal M M, Vishwajeet Pinkaro, Mittal Rashi, Sune Pradeep. A potential correlation between systemic oxidative stress and intracellular ambiance of the lens epithelia in patients with cataract. J Clin Diagno Res 2010.
12. Nanavaty MA, Johar K, Sivasankaran M, Vasavada A, Praveen M, Zetterstrom C. Effect of trypan blue staining on the density and viability of the lens epithelial cells in white cataract. J cataract refract surg 2006.