STUDIES ON SEQUENTIAL POST-MORTEM CHANGES OF ISOLATED CHICKEN EYE

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Thesis submitted in partial fulfilment of the requirement for the degree of

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DECLARATION

I hereby declare that this thesis, entitled **'STUDIES ON SEQUENTIAL POST-MORTEM CHANGES OF ISOLATED CHICKEN EYE'** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other university or society.

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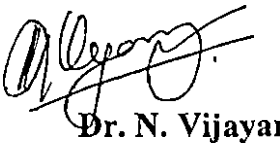
Certified that this thesis, entitled **'STUDIES ON SEQUENTIAL POST-MORTEM CHANGES OF ISOLATED CHICKEN EYE'** is a record of research work done independently by **Arya Aravind**, under my guidance and supervision and it has not previously formed the basis for the award of any degree, diploma, associateship, or fellowship to her.



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Introduction

1. INTRODUCTION

The use of cosmetics, synthetic detergents and agrochemicals are increasing day by day. To rule out any adverse reactions in human beings, these are tested in experimental animals through different routes before being released into the market.

Experimental animals like rabbits, mice, cats, monkeys and invertebrates are used for various experimentation tests as in Draize test. This test attempts to measure the harmfulness of chemicals to human by observing the damage they cause to the eyes and skin of animals. All these tests lead to unreasonable cruelty to the animals. In Draize test for eye irritancy, solutions of products are applied directly into the animal's eyes, which can cause intense burning, itching and pain. Clips are placed on the eyelids to hold them open during the test period, which lasts for several days and to keep the animals from blinking away the solution. The animals are placed in restraining stocks that hold their heads in place, which prevents them from moving throughout the test period. In addition to causing terrible pain, the test compounds often leave the animal's eyes ulcerated and bleeding.

The Botox animal test uses laboratory mice to determine how much Botulinum toxin is required to kill 50 per cent of the animals. The Botox animal test assesses the effects of Botox on the animal over a period of several days whereby the animals experience severe muscle paralysis. Eventually death occurs due to muscle failure and an inability to breath.

Another test employed in guinea pigs is the skin sensitization test. Here test substance is applied on the surface of skin or injected onto their shaved skin. In the Guinea Pig Maximization Test, a chemical adjuvant is injected with the test substance to boost the immune reaction. In the Buehler test, no adjuvant is used but the test is less sensitive. In both of these skin sensitization tests, multiple doses are

applied in order to cause an allergic reaction. Dermal penetration or skin absorption tests analyze the movement of a chemical through the skin and into the blood stream. In these tests, rats are most often used. After the test chemical is administered, the rats are killed and the amount of test substance absorbed is estimated.

All these tests lead to unreasonable cruelty to the animals. It causes pain and discomfort to the animals involved. Once the tests are completed, these animals are killed so that their internal organs can be examined.

These tests are often cruel and unscientific violating the animal ethics. Due to mounting pressure from animal rights activists and consumers, there is a call for developing animal free testing alternatives.

Many alternatives to animal testing experiments are being developed. The use of slaughter animals such as the cow, the pig and the chicken as possible eye donor for the enucleated eye test (EET) were examined. From these candidates, the chicken appeared to be the most suitable and highly accurate in the assessment of eye irritation potential without the use of laboratory animals (Prinsen and Koeter, 1993).

Prinsen (1996) assessed the eye irritation/corrosion potential of test materials using chicken enucleated eye test (CEET) and identified non irritating/severely irritating compounds. Statistical analysis of the CEET and the rabbit in vivo scores showed high linear correlations between the critical values of both tests and confirmed the relevance of this assay with respect to ocular effects.

Due to the development of the broiler industry, broiler chicken is being slaughtered in large numbers. The decapitated heads of chicken serves as a valuable source of chicken eyes, which can be used for ex vivo testing including histopathological examination. For this the eyes should be collected fresh and have

to be fixed in appropriate fixative. So far no standardization has been done on the fixatives to be used for eyes. Slight autolytic change may reflect upon the result and leads to false interpretation. Hence it is essential to evaluate and record the structural changes of the eye associated with autolysis for better interpretation.

Therefore the present study is planned for finding out an ideal fixative and also studying the sequential postmortem changes of the different segments of the eye at varying time intervals before and after fixation. It will provide a better understanding of the ocular changes which can be used for ocular tests and thereby avoiding the live animal experiments.

The main objectives of the study include:

(1) Sequential postmortem changes of the different components of the eye.

(2) To choose the ideal fixative, for the eye.

(3) To choose the appropriate special staining techniques.

Review of Literature

2. REVIEW OF LITERATURE

2.1 NORMAL STRUCTURE OF THE EYE IN GENERAL

According to King and Mc Lelland (1975) in domestic fowl, 'flat' eyeball is seen since the intermediate region is a flat disc and is almost parallel with the surface of the body. Prakash and Arora (1998) reported that in pigeons the eyeball is partly concave.

Berger (2005) observed that the owl's eyes are more like cylinders or tubes, long from front to back, shorter from side to side.

Textbook of Veterinary Histology (Dellmann and Eurell, 1998) described the structure of the eye as follows. The eye consists of three tunics. The three tunics are 1) Tunica fibrosa 2) Tunica vasculosa and 3) Tunica interna.

The corneoscleral coat or the fibrous tunic forms a capsule enclosing and protecting the other components of the eye. The cornea covers the anterior one sixth of the eye and is continuous with the fibrous sclera posteriorly. Cornea consists of five layers. The outer layer consists of stratified squamous, non keratinized corneal epithelium. Next layer is the Bowman's membrane, upon which the corneal epithelium rests. The corneal stroma (substantia propria) forms most of the thickness of the cornea. Flattened fibrocytes, referred to as keratocytes, are located between the layers of collagen fibres of corneal stroma. Next layer is the Descemet's membrane. The inner most layer is called the corneal endothelium consisting of cuboidal cells. Blood vessels are not normally found in the cornea and the cells are not pigmented. The sclera consists of dense, irregular connective tissue, making it opaque. A characteristic feature of avian sclera is the presence of overlapping scleral ossicles anteriorly.

The vascular middle coat of the eye, comprises of the choroid, ciliary body and the iris. The choroid consists of loose connective tissue, which houses a

dense network of blood vessels, connective tissue cells and melanocytes. It supplies the retina with nutrients. The ciliary body is an inward extension of the choroid at the level of the lens. Ciliary processes are short extensions of the ciliary body towards the lens. The ciliary body continues posteriorly until it merges with the retina at the ora serrata. The layers of the ciliary body include a stroma and an epithelium. The stroma is divided into two layers, the outer ciliary muscle, which alters the shape of the lens in accommodation and an inner vascular region extending into the ciliary process. The epithelium of the ciliary body is double layered. The superficial, non-pigmented epithelial layer secretes aqueous humour, which passes into the anterior chamber of the eye. The deeper layer is pigmented. The iris extends over the anterior surface of the lens from the anterior border of the ciliary body. The iris is the colored portion of the eye. It divides the front portion of the eye into two chambers – the anterior and posterior chambers. The opening in the middle of the iris is called the "pupil," which appears as the dark center of the eye. The iris either dilates or constricts the pupil to regulate the amount of light entering the eye. It is accomplished by the presence of sphincter and dilator muscles of the iris.

The nervous tunic is a layer of photoreceptor cells called the retina. A roughly circular opening exists, where the optic nerve exit, called the optic disc. Often the optic disc is called the blind spot, because there are no photoreceptor cells there, so no images can actually be perceived at that position. The retina consists of retinal pigment epithelium, layers of rods and cones, external limiting membrane, outer nuclear layer outer plexiform layer, inner nuclear layer, inner plexiform layer, ganglion cell layer, nerve fiber layer and internal limiting membrane. The retina of the chicken is avascular.

The pecten or pecten oculi is a comb-like structure of blood vessels in the eye of birds. It is non-sensory and is a pigmented structure that projects into the vitreous body from the point where the optic nerve enters the eyeball. The pecten is believed to nourish the retina and control the pH of the vitreous body and acts as an occular shade against sun's glare.

2.2 FIBROUS TUNIC

2.2.1. Corneal Architecture in Birds

According to King and Mc Lelland (1975), the cornea in the domestic fowl was approximately 450 μm thick and played an important role in refracting light.

Waring *et al.* (1982) reported that the endothelium's main function is to control corneal hydration and providing nutrition.

Lucio and Smith (1984) studied architecture of corneal stroma in adult chicken and reported that it consisted of two systems of lamellae, a superficial or subepithelial system formed by a fan like spreading lamellae disposed in a spiral fashion and a deep system formed by an orthogonal gridwork of lamellae.

Tuft and Coster (1990) reported that the endothelium is a monolayer of cells on the posterior corneal surface that transports water from the stroma into the anterior chamber.

Perez-Torres *et al.* (2002) observed under electron microscopy that Langerhans cell-like dendritic cells were present in the cornea of the chicken (*Gallus gallus*) except that they were deficient in Langerhans cell granules.

Pigatto *et al.* (2005) studied the morphology of the corneal endothelial surface by performing morphometric analysis of the normal corneal endothelial cells of Megallanic penguin (*Spheniscus magellanicus*) using scanning electron microscopy and reported that the cell borders were clearly evident and cell size and shape were uniform.

Boote *et al.* (2008) employed wide angle X-ray diffraction to assess collagen organization in nine month-old chicken corneas. They found that the central two-four mm corneal region featured a preponderance of fibrils directed along the superior-inferior and nasal-temporal orthogonal meridians and more

peripherally the orientation of fibrils altered in favour of a predominantly, tangential arrangement. The chicken cornea appeared to be circumscribed by an annulus of fibrils that extended into the limbus.

According to Venktesan *et al.* (2008), the cornea was the second thickest structure in the eye ball and its thickness remained increased upto 20 weeks of age in layers and eight weeks in broilers.

2.2.2. Corneal Architecture in Other Species

Muller *et al.* (1997) concluded that the nerve bundles in the sub basal plexus of the human cornea formed a regular dense meshwork with equal density over a large central and central-peripheral area.

By transmission electron microscopy and freeze etching techniques, Meek and Fullwood (2001) revealed that the corneal stroma in humans contained high concentration of collagen fibrils which showed cross bridge structures between the fibrils.

Mathew *et al.* (2008) investigated the fine structure of the interface between the anterior limiting lamina and anterior stromal fibrils of the human cornea and revealed at least three different types of fibrillar arrangements.

2.2.3. Avian Sclera

King and Mc Lelland (1975) reported that the wall of sclera nearest to the cornea was modified into a ring of small, roughly quadrilateral overlapping bones, the scleral ossicles. The number of ossicles varied in different species of birds and in domestic fowl there were fourteen to fifteen ossicles.

Canavese *et al.* (1994) studied the scleral bone of Japanese quail (*Coturnix coturnix japonica)* and concluded that when the ossicle number decreased; single ossicle area was increased, retaining a constant scleral bony ring area.

A remarkable feature of avian sclera was the presence of a ring of overlapping scleral ossicles anteriorly and a cup-shaped layer of hyaline cartilage, the scleral cartilage, posteriorly. The latter terminated internal to the scleral ossicles (Bacha and Bacha, 2000).

Venkatesan *et al.* (2008) reported that the thinnest structure of the eyeball of chicken was the sclera and there was a sudden change in the thickness of the cornea and sclera at corneo-scleral junction.

2.3 VASCULAR TUNIC

2.3.1 Choroid in Birds

Junghans *et al.* (1997) compared the ultrastructure of chicken and rabbit outer choroids and concluded that chick had large lymphatic sinusoids, whereas rabbit only had a system of large vacuoles contained within the processes of individual fibroblasts.

Stefano and Mugnaini (1997a) reported that the avian choroid was a highly vascularised muscular sheath that might be endowed with degree of motility and elasticity higher than those of the mammalian choroid. On electron microscopic examination they observed fenestration of choriocapillaries which faced towards the retina.

Stefano and Mugnaini (1997b) studied the fine structure of the choroidal coat of the avian eye and concluded that the lacunae of the avian choroids represented a system of posterior short lymphatic vessels, which drained intraocular fluids directly into the eye's venous system and that the villous structures were the sites of communication between lacunae and veins.

Fitzgerald *et al.* (2001) carried out functional and morphological assessment of age-related changes in the choroids and outer retina of pigeon and opined that choroidal blood flow and choroidal vascularity declined significantly with age in pigeons, also the acuity and photoreceptor abundance.

Venkatesan *et al.* (2008) studied the age and sex related histometry of the eyeball in chicken and observed that the thickness of the cornea, sclera, choroid, iris, lens capsule and the retina increased upto the sexual maturity and maintained in layers. In broilers the values increased upto eight weeks of age.

2.3.2. Choroid in Other Species

Guymer *et al.* (2004) examined the cytoarchitecture of choroidal capillary endothelial cells of human donor eyes and monkey and hamster eyes by transmission electron microscopy. They found that the cellular processes of choroidal capillary endothelial cells penetrated their basal laminae and concluded that these processes served to stabilize choroidal endothelial cells physically thereby playing an important structural role in the maintenance of potency of the choriocapillaries.

2.3.3. Ciliary Body

Ricardo *et al.* (1983) opined that in chickens as in mammals, tight junction between the non-pigmented cells of the ciliary epithelium represented the structural equivalent of the blood aqueous barrier.

Murphy *et al.* (1995) studied the anatomy of the ciliary region of the chicken eye and concluded that the ciliary musculature of the chicken eye was composed of two major muscle groups with fibres arranged in five different forms. The anatomy of the ciliary muscle was consistent with the proposed functions of altering corneal curvature for corneal accommodation and moving the ciliary body anteriorly as a part of the lenticular accommodative mechanism.

Uehara *et al.* (1996) investigated hyalocytes in the pecten oculi and ciliary body of adult chicken and reported that the hyalocytes were mainly found on the pleats of the pectin oculi and on the ciliary body.

Pardue and Sivak (1997) described the functional anatomy of the ciliary muscle in four avian species and concluded that the majority of ciliary muscle

fibres in chickens, pigeons and kestrels were in the anterior muscle fibre group which indicated corneal accommodation. In the ciliary muscle of the hooded merganser, the majority of fibres were in the internal and posterior muscle fibre groups, indicating that lenticular accommodation was the predominant form of accommodation.

Tedesco *et al.* (2005) studied the architecture of the ciliary muscle of domestic fowl (*Gallus domesticus)* and described the existence of three main muscular groups defined as anterior, posterior and intermediary. The anterior and posterior muscular groups were inserted in the sclera, around the Schlemm's canal wall and ciliary process stroma. The vitreal intermediary muscle had fibers inserted in Schlemm's canal wall and ciliary process stroma.

2.3.4. Avian Iris

In fowl the colour of the iris was dependent on breed and age and ranged from grey or greyish-yellow through light yellow, golden yellow to orange red or brownish-red. This variation in colour of the iris resulted from the variation in the amount and colour of the fat which was enclosed in follicles (Seiferle, 1977).

Scapolo *et al.* (1988) determined the distribution and typology of fibres in the muscular system of the iris in chicken (*Gallus gallus)* and reported that the sphincter muscle in proximity to the ciliary margin was composed predominantly of slow fibres and in the dilator muscle, the oblique system was uniquely composed of fast oxidative fibres.

Danilov and Ishmeeva (1991) studied the iris musculature tissue in chick embryos and in chicken and reported that the leading contractile tissue of the iris was the striated muscular tissue and was formed as a cellular-simplastic system with its own cambium- myosatellitocytes.

Bortolotti *et al.* (2003) compared the iris colour of American kestrels with age, sex and exposure to polychlorinated biphenyls (PCBs) and opined that, old

birds had all brown irides, whereas those of the older birds were red-brown. Age and PCB exposure consistently had an effect on colour, while sex was significant only for red.

Tu *et al.* (2004) studied the non-visual photoreception in the chick iris and reported that the chick iris was most sensitive to short wave length light, demonstrating an action spectrum consistent with cytochrome rather than with opsin pigments. Also the photosensitivity of the iris was not affected by retinoid depletion or inhibition of visual phototransduction.

2.3.5. Iris of Other Species

Sun *et al.* (2006) suggested that the iris pigment epithelium derived cells had retinal stem/progenitor properties and neurogenic potential without gene transfer, thereby providing a novel potential source for both basic stem cell biology and therapeutic application for retinal diseases.

Mac Neil *et al.* (2007) opined that a population of cells derived from the adult iris, pars plana and ciliary body of the pig, had progenitor properties and neurogenic potential, thereby providing novel sources of donor cells for transplantation studies.

2.4. NERVOUS TUNIC

2.4.1. Retinal Structure in Birds

Braekevelt (1984) investigated the retinal pigment epithelial (RPE) layer in the night hawk and reported that it was composed of a single layer of low cuboidal cells joined laterally by tight junctions.

Braekevelt (1990) studied the fine structure of retinal pigment epithelium (RPE) of the mallard duck (*Anas platyrhyncos*) by light and electron microscopy and concluded that in this species the RPE consisted of a single layer of cuboidal cells which displayed numerous deep basal infoldings and extensive apical

processes which enclosed photoreceptor outer segments laterally joined by tight junctions. Melanosomes were plentiful only within the apical processes of the RPE cells.

Cuadros *et al.* (1991) described the existence of specialized phagocytic cells in the region of retinal neuroepithelium undergoing intense cell death during early differentiation of the avian embryo retina.

The light and electron microscopical study of the fine structure of the retinal epithelium was conducted by Braekevelt and Thorlakson (1993). They reported a single layer of cuboidal cells joined laterally by tight junctions. Melanosomes were located almost exclusively within the apical processes indicating retinomotor movements.

Braekevelt and Richardson (1996) studied the fine structure of retinal epithelium in the galah and reported that it consisted of a single layer of low cuboidal cells joined basally by a series of zonulae occludentes. The melanosome of the RPE were almost exclusively located within the apical processes indicating retinomotor movement of this pigment. Similar observations were also made in barred owl (*Strix varia*) by Braekevelt *et al.* (1996).

Braekevelt (1998) concluded that the retinal pigment epithelium consisted of a single layer of cuboidal cells joined basally by a series of tight junction in emu. The melanosomes of the RPE were almost exclusively located within the apical processes of these cells.

Imagawa *et al.* (1999) studied quantitatively and morphologically the optic nerve fibre layer of the chicken retina and suggested that the dorsal area in the retina had thin nerve fibre layer (NFL) and contained largest number of nerve fibres, which were mainly thin and unmyelinated. The ventral area had a thick NFL and contained relatively small number of nerve fibres, many of which were myelinated. The nasal band had the thickest NFL and contained as many nerve

fibres as the dorsal area, with the temporal band showing a high ratio of myelinated fibres.

An investigation was undertaken to study the histochemical nature of the eyeball of the layer and broiler chicken by Venkatesan *et al.* (2008) and reported that periodic- Acid Schiff positive areas were the Descemet's membrane of the cornea, internal limiting membrane of ciliary body, outer segments of the rods, cones, aqueous humour, vitreous body, pecten, hyalocytes and central part of the lens in all the age groups under study.

2.4.2. Retina in Other Species

Burns *et al.* (1984) analysed the retinal pigment epithelium of aging human eyes using ultrastructural and morphometric techniques and lipofuscin, melanin and complex granules (melanolipofuschin and melanolysosomes) were recorded in cells from macular, equatorial and peripheral retinal specimens.

Davila *et al.* (1987) investigated the cytoarchitecture of intraocular portion of the optic nerve in the turtle (*Mauremys caspica)* and reported that the glial cells in the intraocular portion were disposed among the axons, either scattered or forming columns, and bordering the nervous parenchyma. Astroglia formed the limiting glia that separated the optic nerve from the vitreous body, retina, choroid and sclera.

Frenkel *et al.* (2005) carried out experiments on the histological measurement of retinal nerve fibre layer (RNFL) thickness and reported that the RNFL thickness rapidly diminished with increasing distance from the disc margin and at different location the ratio of axons to supportive tissue varied significantly.

Sasoh *et al.* (2006) investigated the changes in localization of amino acids in the detached cat retina and concluded that excess intracellular glutamate,

aspartate and glutamine in photoreceptor cells might cause a part of neuronal death after retinal detachment.

2.5. PECTEN

2.5.1. Fine Structure

Dieterich *et al.* (1973) reported that in chicken the pecten oculi capillaries formed an extensive anostomatic network and their endothelial cells had apical as well as basal, longitudinally oriented microfolds. They concluded that the pecten plays an important role in the nourishment of the retina and vitreous body.

Braekevelt (1991) reported that the pecten oculi of the Red-Tailed Hawk was of pleated type and joined apically by a heavy pigmented bridge of tissue and held pecten in a fan like shape and was indicative of a heavy involvement in the transport of materials to the avascular avian retina.

Bacha and Bacha (2000) discussed that the pecten in chicken was draped by a covering membrane, which was thought to be continuous with the inner limiting membrane of retina.

Kiama *et al.* (2006) suggested that the pecten oculi of ostrich as vaned type, consisting of a vertical primary lamella and laterally located secondary lamellae with tertiary lamellae.

2.5.2. Functions

Crozier and Wolf (1944) studied the modification of the flicker response contour and the significance of the avian pecten and suggested that the avian pecten might be to increase the sensory effect of small moving images.

2.6. REFRACTIVE MEDIA

2.6.1. Lens

Glasser *et al.* (1995) studied the mechanism of lenticular accommodation in chicken and proposed that in the chick eye, lenticular accommodation was induced primarily by contraction of muscle fibers at the peripheral edge of the iris.

Bagchi *et al.* (2001) carried out experiments in chicken lens and revealed the presence of heat shock proteins HSP-four0, HSP-seven0 and HSC.seven0 in all areas of both adult and embryonic chicken lens.

2.7. POSTMORTEM CHANGES

2.7.1. Fibrous Tunic

Suzutani *et al.* (1978) investigated the relationship between the postmortem interval and the turbidity of the cornea and concluded that slight variations of the turbidity of the cornea of the cadavers were observed only within 12 hours after death.

Grierson (1981) investigated the postmortem changes in the baboon outflow system and found that the meshwork cells closest to the chamber angle were particularly susceptible to post mortem advance. Post mortem vacuoles developed in the endothelium of Schlemm's canal and by light microscopy these structures could be mistaken for giant vacuoles.

Slettedal *et al.* (2008) examined the changes in the endothelium of donor cornea with extended post mortem time and concluded that the cornea showed increasing endothelial cell damage with increasing postmortem time. After five-seven days postmortem, most cells were structurally damaged.

2.7.2. Vascular Tunic

Prasad *et al.* (2003) suggested that there was a linear increase in vitreous potassium level with rise of postmortem interval.

Abraham *et al.* (2008) conducted experiments on postmortem iris color change in the eyes of *Sus scrofa* and reported that all isolated blue eyes in the experiment, at room temperature and higher, changed to brown/black within 48hours.

Felby *et al.* (2008) analysed the postmortem distribution of ketone bodies between blood, vitreous humor, spinal fluid and urine and reported that the ketone bodies in spinal cord showed best correlation to blood, followed by vitreous humor and last urine. In vitreous humor, the dependence was mainly due to protein bindings of acetoacetate and beta-hydroxybutycate in blood and the difference in dry matter between blood and vitreous humor.

2.7.3. Nervous Tunic

Schmidt and Berson (1980) investigated the postmortem metabolic capability of photoreceptor cells in human and rat retina and found that retinas of postmortem human donor eyes retained the high affinity mechanism for uptake of three H-taurine, the capacity to synthesize rhodopin from 14 C – aminoacids and the ability to incorporate inorganic 32 p – phosphate into rhodopsin with exposure to light for four to 4½ hours. These processes declined at a rate of about 16 per cent to 19 per cent per hour between two and 4½ hours after death. Parallel studies with rats showed that all these processes declined linearly at rates of 8 per cent to 12 per cent per hour.

Wakakura and Ishikawa (1982) carried out ultrastructural studies on centrifugal fibres in the feline retina by performing unilateral optic nerve transaction at six mm behind the eye ball via soft palate and reported that at 40 hours the degenerative changes were observed in the neuronal processes in the

outer part of inner plexiform layer and were thought to represent degeneration of the centrifugal fibers.

Napper and Kalloniatis (1999) studied the neurochemical changes following postmortem ischemia in the cat retina and opined that the postmortem ischemia in mammalian retina predominantly resulted in a loss of glutamate and gamma-aminobutyric acid (GABA) from neurons and accumulation of these amino acids, within Muller cells. This accumulation might occur as a result of increased release of these neurotransmitters from neurons and decreased degradation.

Chen *et al.* (2007) conducted image analysis for degradation of DNA in retinal nuclei of rat after death and concluded that retinal nuclear DNA was degraded gradually and had a good correlation with postmortem interval.

2.8. FIXATIVE FOR EYES

2.8.1. Neutral Buffered Formalin

Margo and Lee (1994) reported that the whole eyes fixed in 10 per cent neutral buffered formalin demonstrated a variety of artifacts including separation of the neurosensory retina from the retinal pigment epithelium and postulated that the osmolarity of the fixative caused contraction of the internal compartments of the eye leading to several artifactual changes commonly observed in routine histological sections.

Janardhan *et al.* (2001) conducted a study to standardize the procedure for preparation of eyeball of rats and mice for histopathological evaluation in toxicological studies and opined that when 10 per cent neutral buffered formalin was used for fixation the lens could not be sectioned intact along with other structures. Also artifactual changes were observed in retina, cornea and sclera which might probably be because of mechanical damage, caused by hardened lenses, during sectioning.

Barash and Shepardo (2002) compared the fetal cat eyes fixed in neutral buffered formalin and Bouin's and Lillie's fluids and reported that the number of mitotic figures at one or six hour postmortem was significantly higher in Bouin's and Lillie's fixed material than that fixed in formalin. Excessive shrinkage and fragmentation of formalin fixed lenses were also observed.

2.8.2. Gluteraldehyde Formaldehyde Fixative

Margo and Lee (1994) concluded that the immersion fixation of whole eyes for 36 hours (or longer) with a mixture of one per cent buffered formaldehyde and 1.25 per cent gluteraldehyde reduces tissue distortion without compromising cellular preservation.

Doughty *et al.* (1997) compared the shrinkage and distortion of the rabbit corneal endothelial cell mosaic caused by a high osmolarity gluteraldehyde-formaldehyde fixative and gluteraldehyde and concluded that with the gluteraldehyde-formaldehyde fixative, transmission electron microscopy (TEM) revealed gross shrinkage and distortion of the cytoplasm and organelles, while the regions of the cell-cell junctions were not attenuated but included dilated extracellular space. With the gluteraldehyde fixative, TEM also revealed shrinkage but the cytoplasm was less compact than with the high osmolarity fixative.

2.8.3. Other Fixatives

Izami *et al.* (2000) compared the rat retinal fixation techniques using chemical fixation and microwave irradiation and concluded that the retina of chemically fixed whole eyes showed neuronal swelling similar to excitotoxic ischemic damage suggesting that conventional immersion methods provided poor whole eye fixation. Also the neuronal degeneration observed with conventional immersion fixation was not found in retina of whole eyes fixed with 20 sec of microwave irradiation.

Janardhan *et al.* (2001) conducted studies for preparation of eyeballs of rats and mice for histopathological examination and opined that Davidson's fluid proved to be a good fixative, but prolonged fixation in the same, hardened the lenses.

Latendresse *et al.* (2002) studied the preservation of ocular histomorphology by Davidson's fluid (DF) and modified Davidson's fluid (mDF). For histological examination of eye, apposition and preservation of rods and cones and nuclear layers of the retina was slightly inferior with mDF compared to DF.

Materials and Methods

3. MATERIALS AND METHODS

3.1. SAMPLE COLLECTION

Chicken eyes were obtained from a local slaughter house. Eyes were collected from apparently healthy broiler chickens slaughtered, which were approximately eight weeks old, either male or female. The weight of the birds ranged from 2.5 to 3 kg. The eyes collected were preserved in clean petri dishes till the time of fixation.

3.2. GROSS EXAMINATION

The eyes were examined for any physical deformities, colour change, abnormal growth etc. (fig. 1)

3.3. FIXING AND PROCESSING

The eyes collected were fixed in 10 per cent neutral buffered formalin and gluteraldehyde – formaldehyde fixative (Menocal *et al.,* 1980). For excellent fixation of the inner layers, fixing fluid was injected into the globe using syringe attached with a needle of 21 gauges. The fixative was injected midway between the edge of the cornea and the equator avoiding the anterior part of retina since it is detached easily. Six eyes each were fixed at 0 hour, ½ hour, 1 hour, 1 ½ hours, 2 hours, 4 hours and 12 hours after the collection. Eyes were processed after a minimum of three weeks fixation time.

3.4. PROCEDURE

The eyes were sectioned by making a clean cut through the midline of the eye but slightly to one side of it, so that the origin of the optic nerve and the pupil are together in one of the pieces. (Fig. 2) The tissues were processed in high melting paraffin as per the procedure adopted by Disbrey and Rack (1970).

3.4.1. Steps for Processing Procedure:

Washing in running tap water - over night

Dehydration

1. 50per cent alcohol - 1 hour
2. 70per cent alcohol - 1 hour
3. 80per cent alcohol - 1 hour
4. 90per cent alcohol - 1 hour
5. Absolute Alcohol I - 1 hour
6. Absolute Alcohol II - 1 hour
7. Absolute Alcohol III - 1 hour

Clearing

1. Cedar wood oil - over night
2. Benzene - 10 minutes

Impregnation

1. Paraffin Wax I - 15 minutes
2. Wax II - 45 minutes
3. Wax III - 45 minutes
4. Embed in fresh wax

Paraffin sections of 4 to 5 µm thickness were cut and stained for histological examination.

3.5. STAINING

The sections were stained with Hematoxylin and Eosin (H&E) as per the procedure adopted by Luna (1968).

The sections were also stained with special stains like Masson's Trichrome method and Periodic Acid – Schiff's staining.

1. Masson's trichrome method (Luna, 1968).

Weigert's iron hematoxylin solution

Solution A

Hematoxylin crystals	1 gm
Alcohol, 95 per cent	100 ml

Solution B

Ferric chloride, 29per cent aqueous	4 ml
Distilled water	95 ml
Hydrochloric acid, concentrated	1 ml

Working solution

Equal parts of solution A and B

Biebrich Scarlet- acid fuchsin solution

Biebrich scarlet, aqueous 1per cent	90 ml
Acid fuchsin, aqueous 1per cent	10 ml
Glacial acetic acid	1 ml

Phosphomyolybdic-phosphotungstic acid solution

Phosphomolybdic acid	5 gm
Phosphotungstic acid	5 gm
Distilled water	200 ml

Aniline blue solution

Aniline blue	2.5 gm
Glacial acetic acid	2 ml
Distilled water	100 ml

1per cent glacial acetic and solution

Glacial acetic acid	1 ml
Distilled water	100 ml

Staining procedure

1. Deparaffinize and hydrate to distilled water.
2. Mordant in Bouin's solution for one hour at 56°C.
3. Cool and wash in running water until yellow color disappears.
4. Rinse in distilled water.
5. Weigert's iron hematoxylin solution for 10 minutes. Wash in running water for 10 minutes.
6. Rinse in distilled water.
7. Biebrich scarlet- acid fuchsin solution for two minutes. Save solution.
8. Rinse in distilled water.
9. Phosphomolybdic-phosphotungstic acid solution for 10 to 15 minutes.
10. Aniline blue solution for five minutes.
11. Rinse in distilled water.
12. Glacial acetic solution for three to five minutes.
13. Dehydrate in 95per cent alcohol, absolute alcohol and clear in xylene, two changes each.
14. Mount with DPX mount.

2. PAS Staining (Bancroft and Gamble, 1996).

a. Per iodic acid solution

Per iodic acid	1 gm
Distilled water	100 cm^3

b. Schiff's reagent

Dissolved one gram basic fuchsin in 200 cm^3 of boiling distilled water, removed the flask of water from the Bunsen just before adding the basic fuchsin.

Allowed the solution to cool to 50°C and added two gram potassium metabisulphite with mixing. Allowed to cool to room temperature then added two cubic centimeters concentrated hydrochloric acid, mixed and allowed to stand

overnight at dark. Added 0.2 gm activated charcoal and was shaken for one to two minutes. Filtered through No.1 Whatmann paper when the solution should be either clear or a pale yellow colour. Stored in a dark container at 4°C.

Method

1. Dewax sections and bring to distilled water.
2. Treat with Per iodic acid for two minutes.
3. Wash well with several changes of distilled water.
4. Cover with Schiff's solution for eight minutes.
5. Wash in running tap water for 5-10 minutes.
6. Stain nuclei with Harris' haematoxylin, differentiating as appropriate in acid alcohol and bluing as usual.
7. Wash in water.
8. Rinse in absolute alcohol.
9. Clear in xylene and mount in DPX mount.

The stained sections were examined critically to evaluate the histological features of the sequential post-mortem changes in the fibrous, vascular and nervous tunics of the eyes. The suitability of the fixative was also assessed based on the sectionability and the artifacts of the tissue.

Results

4. RESULTS

4.1 GROSS MORPHOLOGY OF EYES

The chicken eyes were in the form of an ellipsoid with greater diameter through the equator than across the poles. The colour of the iris was greyish yellow. Retinal detachment of the eyes was observed from zero hour onwards. (Fig. 3)

The fibrous tunic at different time intervals viz, half an hour, one hour, one and a half hour, two hours, four hours and twelve hours showed a dried up appearance with shrinkage of corneoscleral capsule. The colour of the iris remained greyish yellow, but the intensity was reduced gradually at the fourth hour postmortem.

4.2 FIBROUS TUNIC

Fibrous tunic consisted of the opaque, white sclera and transparent avascular cornea.

4.2.1 Histology

4.2.1.1 Cornea

Cornea consisted of five layers. From outside inwards it showed (1) a multilayered, anterior epithelium, (2) Bowman's membrane, (3) substantia propria or corneal stroma which consisted of collagen fibres arranged in parallel bundles, (4) Descemet's membrane and (5) single layered posterior epithelium or corneal endothelium (fig. 4, 5 & 6). The cornea was continuous with the sclera at the corneoscleral junction or the limbus. The multilayered anterior epithelium consisted of a single layer of basal cells, two to three layers of polyhedral cells and two to three layers of non-keratinised squamous cells. Basal layer of cells was columnar and

the most superficial cells were flattened. The multilayered anterior epithelium gradually changed to conjunctival epithelium at the limbus. Bowman's membrane was more apparent in histologic preparation. Stroma occupied 90% of the corneal thickness and was made up of collagen fibres arranged in parallel with the corneal surface. Fibrocyte (keratocyte) was the predominant cell type of the corneal stroma. Keratocytes showed very little cytoplasm with ill defined borders. Descemet's membrane was less distinct. The posterior epithelium consisted of a single layer of cells. The posterior epithelial layer continued with the anterior surface of the iris across the iridocorneal angle. Cornea was devoid of any blood vessels, except at the region of the limbus. At the corneoscleral junction there was a sudden decrease in the thickness of the sclera.

4.2.1.2 Sclera

Sclera mainly consisted of dense network of collagen and elastic fibres. Ventral to the posterior pole it showed a small cribriform area through which passed the fibres of the optic nerve. At the corneoscleral junction, the regular arrangement of the collagen fibres of corneal stroma merged with the interwoven fibres of the sclera. Overlapping scleral ossicles anteriorly was a cup shaped layer of hyaline cartilage, the scleral cartilage (fig. 7 & 8). The scleral ossicles strengthened the eyeball and provided attachment for the striated muscles of accommodation (fig. 9). A large scleral venous plexus was evident at the corneoscleral junction. The pectinate ligament joined the sclera to the iris and to the ciliary body at the corneoscleral junction.

4.2.2 Effects of Delayed Fixation

There were only mild to moderate changes in the histological structure of the components of the fibrous tunic at half an hour, one hour, one and a half hours and

two hours. The intensity of the changes aggravated as the postmortem time was prolonged.

The changes included condensation of the nucleus of multilayered anterior epithelium of the cornea, leaving a halo around the nucleus from one hour. But the most appreciable changes were the erosion or the exfoliation of the cells of multilayered anterior epithelium after four hours postmortem (fig. 10). The cytoplasm also revealed vacuolar and degenerative changes. The corneoconjunctival epithelium mainly consisted of the columnar type of cells which showed degenerative changes. Seperation of the fibres of corneal stroma observed from one hour onwards. (fig. 11). Lysis and detachment of corneal endothelium were seen from half an hour onwards(fig.12 & 18).

The Bowman's membrane was not much affected by the delayed fixation. The collagen fibres which were thickely packed at zero hour fixing showed separation of the fibres from one hour. The keratocyte did not reveal any characteristic postmortem changes on delayed fixation. Postmortem changes did not affect the Descemet's membrane.

Focal areas of lysis and detachment of the endothelium was prominent in the eyes fixed after one and a half to two hours and more Lysis and complete peeling of corneal endothelium was noticed at twelve hours post-mortem (fig. 13). Condensation of the nucleus of multilayered anterior epithelium of the cornea leaving a halo around the nucleus was observed from one hour (fig. 14). The interepithelial junctions of multilayered anterior epithelium and corneal endothelium also showed tendency of detachment from one hour (fig. 15). The endothelial layer showed swelling of the cells with protrusion into the anterior chamber from one hour (fig.16). . The epithelium in the corneoscleral junction or limbus contained a centrally placed round nucleus with moderate cytoplasm. The cytoplasm revealed

swelling, but the nucleus was not found to be affected by the delayed fixation upto four hours (fig.17).

The dense network of collagen and elastic fibres in the sclera did not reveal any appreciable histological changes due to delayed fixation. But the collagen fibres at the corneoscleral junction showed moderate separation from two hours suggestive of autolytic changes. The Canal of Schlemm was dilated along with the swelling of the endothelial cells but without any contents inside from half an hour onwards (fig. 19). The Bowman's membrane was not much affected by the delayed fixation. (fig. 20). Desquamation of cells of corneo-conjunctival epithelium and separation of collagen fibres was observed at twelve hours (fig. 21).

4.3 VASCULAR TUNIC

Vascular tunic consisted of choroid, ciliary body and iris, in posterio-anterior sequence. Choroid lined the sclera from the optic nerve almost to the limbus, the ciliary body followed as a thickened zone opposite to the limbus and iris projected into the cavity of the eyeball posterior to the cornea.

4.3.1 Histology

4.3.1.1 Choroid

The choroid consisted of dense network of blood vessels and heavily pigmented connective tissue. The external surface of the choroid was connected to sclera; the internal surface was adjacent and intimately attached to the pigmented epithelium of the retina. The vascular layer of the choroid showed many blood vessels and large spaces embedded in loose connective tissue. No tapetum lucidum was present in the chicken.

4.3.1.2 Ciliary body

The choroid was thickened towards the limbus to form the ciliary body. The ciliary body consisted of ciliary epithelium, a vascular layer and the ciliary muscles. The ciliary body was covered by two layers of cuboidal epithelial cells, outer pigmented epithelial layer and inner non-pigmented epithelial layer.

4.3.1.3 Iris

The iris consisted of an anterior epithelial layer which blended with the posterior epithelium of the cornea, a middle layer of connective tissue stroma consisting of sphincter and dilator muscles of the iris and the posterior layer of the iris consisting of the pigmented epithelium, which was extended as the pigmented layer of the retina.

4.3.2 Effects of Delayed Fixation

The presence of intact erythrocytes was noticed in the blood vessels at zero hour (fig. 22). The lysis was mild to moderate from half an hour to two hours after which the lysis of the erythrocytes was prominent. The vascular endothelium was swollen at two hours (fig. 23). The presence of lysed erythrocytes was noticed in the blood vessels at twelve hours (fig. 24).

The pigmented connective tissue showed varying degrees of dispersion of the melanin pigments as the delayed fixation advanced. The cellular borders were indistinct compared to the zero hour fixing.

The ciliary body showed an intact epithelium, but the epithelial arrangement was disturbed in the eyes fixed after two hours and later. The swelling of the cytoplasm was more evident in the non-pigmented cuboidal epithelial layer. The blood vessels in the region of the ciliary body showed thickened arterial muscles

with intact erythrocytes inside. The epithelium of the iris was seen detached and fragmented in a number of eyes examined.

4.4 NERVOUS TUNIC

Nervous tunic mainly consisted of the retina.

4.4.1 Histology

The sensory portion of the retina consisted of (1) retinal pigment epithelium (2) layers of rods and cones (3) external limiting membrane (4) outer nuclear layer (5) outer plexiform layer (6) inner nuclear layer (7) inner plexiform layer (8) ganglion cell layer (9) nerve fiber layer and (10) internal limiting membrane. The retina was devoid of any blood vessels. The cells of pigmented epithelium were tall and narrow. They were firmly bound to the choroid layer and showed melanin pigment. Light sensitive portions of the retina were the layers of rods and cones. Outer nuclear layer contained rod and cone cell bodies and nuclei. Cone nuclei were ovoid and limited to a single row. Rod nuclei were rounded and distributed in several layers. Outer plexiform layer was made up of axons of rod and cone cells and dendrites of bipolar cells. Inner nuclear layer contained nuclei of bipolar neurons and associated neurons. Inner plexiform layer showed processes of amacrine cells, axons of bipolar cells and dendrites of ganglion cells. Nerve fiber layer consisted of non-myelinated axons of ganglion cells. At the optic disc, optic nerve left the eye. Bundles of fibres of the optic nerve passed through perforations of the sclera.

4.4.2 Effects of Delayed Fixation on Retina

Retinal detachment was noticed in most of the eyes fixed from zero hour to four hours later. The layers of the detached retina were intact and continuous at the zero hour to one and a half hours of fixation. But the fragmentation of the retinal layers was more frequent in the eyes fixed later than one and a half hours. The

nucleus of the epithelial cells including retinal pigmented epithelium, layers of rods and cones and outer nuclear layer showed varying degrees of pyknotic changes in the eyes fixed after two hours. The changes were more prominent in the ganglion cells which showed condensation of the nuclei along with vacuolar lytic changes of the cytoplasm at two hours.

4.5 PECTEN

Pecten was observed as a highly vascular pleated membrane protruding into the cavity of the vitreous humor (fig. 25). Apically it was modified into bridge of pecten (fig. 26). Numerous melanocytes and moderate number of hyalocytes were interspersed throughout the reticular membraneous framework. Large numbers of capillaries lined by thick endothelial cells with plump nuclei were evident in the meshwork of pecten. The pecten was draped by a covering membrane.

Postmortem changes in the pecten were appreciated by the lysed erythrocytes in the capillaries along with the vacuolar degeneration and lytic changes affecting the melanocytes and the hyalocytes. The eosinophilic staining cytoplasm of the hyalocyte was prominent in the pecten of the eyes fixed upto one hour, but the intensity of eosinophilic staining was less in the eyes fixed after one hour or later.

4.6 REFRACTIVE MEDIA

4.6.1 Lens

Lens was covered by a lens capsule. Within the capsule, lens was divided into annular pad and the lens body (fig. 27). Annular pad formed an outer ring around the equator of the lens body. The nucleus of the cells of annular pad was prominent in the eyes fixed at zero to one hour.

The section of the lens frequently revealed artefacts, distorsion and fragmentation of the lens. The distorsion and fragmentation also affected the attachment of the iris with the lens.

4.7 COMPARISON OF FIXATIVES

The eyes that were fixed in gluteraldehyde formaldehyde (GF) fixative gave good sections when compared with neutral buffered formalin (NBF) fixative. The sections gave good structural details in GF fixative. Also there was detachment of retina from 0 hour onwards when fixed in both the fixatives. The lens of the eyes showed extensive fragmentation and artefacts when fixed in NBF fixative.

4.8 SPECIAL STAINING

4.8.1 Periodic-Acid Schiff's (PAS) Staining

The Descemet's and Bowman's membranes of the cornea, the layer of rods and cones of retina, capsule of the lens and the pecten showed positive Periodic-Acid Schiff's (PAS) reaction positive indicated by deep magenta staining (fig. 28, 29 & 30). The intensity of the magenta staining was reduced in the eyes fixed after one and a half hour and later.

4.8.2 Masson's Trichrome Staining

Collagen fibres were stained deep blue in colour. The cytoplasm of the anterior epithelial and endothelial layer and muscle fibres were stained red and the nucleus black in colour. Lens was stained red in colour. This staining gave a better understanding of the different layers of retina (fig. 31).

4.9 HISTOMETRY OF THE EYEBALL

The histometrical observations of different parts of the eyeball viz., cornea, sclera and retina were recorded and analysed. The observations were analysed when fixed in both neutral buffered formalin and gluteraldehyde formaldehyde fixative (Table 1) using paired t-test. Also the parameters in different time intervals were compared with zero hour for both the fixatives (Table 2 and Table 3) using t-test. Using t-test, significant difference was observed in the thickness of cornea at 120 minutes when compared with zero hour in neutral buffered formalin fixative. The thickness of cornea showed significant difference at 30 minutes, 60 minutes, 90 minutes and 120 minutes when compared with zero hour and also the thickness of retina showed significant difference at 30 minutes, 60 minutes, 90 minutes and 120 minutes in gluteraldehyde-formaldehyde fixative. Sclera also showed significant difference at 120 minutes compared to zero hour in GF fixative.

Table. 1 Micrometrical parameters of the layers of the eye in neutral buffered formalin (NBF) fixative and gluteraldehyde formaldehyde (GF) fixative (µm)

Time intervals	Layers of the eye	NBF Mean ± SE	GF Mean ± SE
0 hour	Cornea	290.25 ± 9.70 S	328.50 ± 10.26 S
	Sclera	201.25 ± 3.02 NS	213.75 ± 5.42 NS
	Retina	164.25 ± 13.23 NS	184.50 ± 5.69 NS
30 min	Cornea	312.75 ± 9.49 NS	306.00 ± 6.67 NS
	Sclera	217.25 ± 8.11 S	202.50 ± 3.49 S
	Retina	141.75 ± 4.61 S	164.25 ± 5.42 S
60 min	Cornea	303.75 ± 3.02 S	265.5 ± 5.69 S
	Sclera	209.25 ± 3.02 S	195.75 ± 3.02 S
	Retina	148.50 ± 4.93 NS	146.25 ± 5.42 NS
90 min	Cornea	276.75 ± 4.61 NS	285.75 ± 5.42 NS
	Sclera	200.25 ± 4.15 NS	200.25 ± 4.15 NS
	Retina	146.25 ± 4.15 NS	148.50 ± 4.93 NS
120 min	Cornea	252.00 ± 4.50 S	229.50 ± 7.79 S
	Sclera	202.50 ± 3.49 NS	198.00 ± 4.50 NS
	Retina	148.50 ± 4.93 NS	148.50 ± 3.49 NS

S – Significant difference exists for the layer of eye when fixed in NBF and GF fixative for that particular time interval

NS – No significant difference

Table. 2 Comparison of the layers of eyes at different time intervals with zero hour fixation in neutral buffered formalin (NBF) fixative

Layers of eye at different time intervals	Probability (P)
Cornea 0 – Cornea 30	0.205 ^{NS}
Cornea 0 – Cornea 60	0.175 ^{NS}
Cornea 0 – Cornea 90	0.275 ^{NS}
Cornea 0 – Cornea 120	0.023 ^{S}
Sclera 0 – Sclera 30	0.235 ^{NS}
Sclera 0 – Sclera 60	1.000 ^{NS}
Sclera 0 – Sclera 90	0.102 ^{NS}
Sclera 0 – Sclera 120	0.296 ^{NS}
Retina 0 – Retina 30	0.224 ^{NS}
Retina 0 – Retina 60	0.180 ^{NS}
Retina 0 – Retina 90	0.158 ^{NS}
Retina 0 – Retina 120	0.220 ^{NS}

S – Significant difference exists for the layer of eye for that time interval compared to zero hour

NS – No significant difference exists

Table. 3 Comparison of the layers of eye at different time intervals with zero hour fixation in Gluteraldehyde-formaldehyde (GF)

Layers of eye at different time intervals	Probability
Cornea 0 – Cornea 30	0.004 S
Cornea 0 – Cornea 60	0.004 S
Cornea 0 – Cornea 90	0.010 S
Cornea 0 – Cornea 120	0.001 S
Sclera 0 – Sclera 30	0.224 NS
Sclera 0 – Sclera 60	0.082 NS
Sclera 0 – Sclera 90	0.076 NS
Sclera 0 – Sclera 120	0.013 S
Retina 0 – Retina 30	0.007 S
Retina 0 – Retina 60	0.000 S
Retina 0 – Retina 90	0.005 S
Retina 0 – Retina 120	0.000 S

S – Significant difference exists for the layer of eye for that time interval compared to zero hour

NS – No significant difference exists

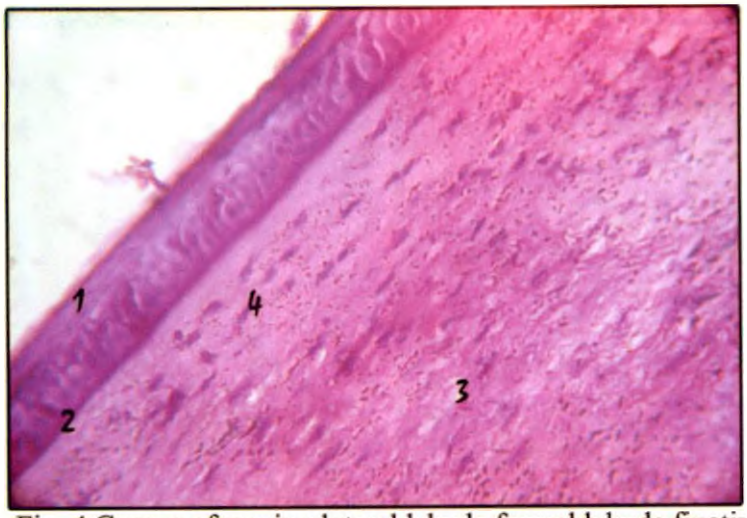
Fig. 1 Whole eye

1 Optic nerve

Fig. 2 Section of eye showing optic nerve

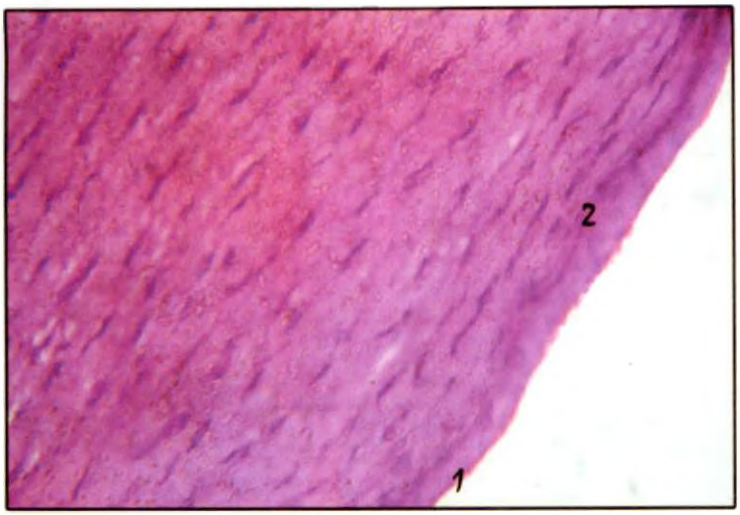
1 Lens
2 Retinal detachment

Fig. 3 Section of eye showing retinal detachment



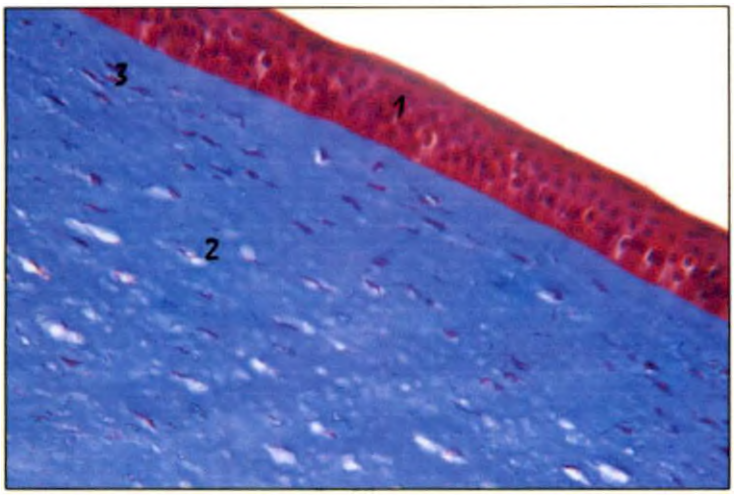
1 Anterior epithelium
2 Bowman's membrane
3 Corneal stroma
4 Fibrocyte

Fig. 4 Cornea of eye in gluteraldehyde formaldehyde fixative (GF) at 0 hr-H & E x 400



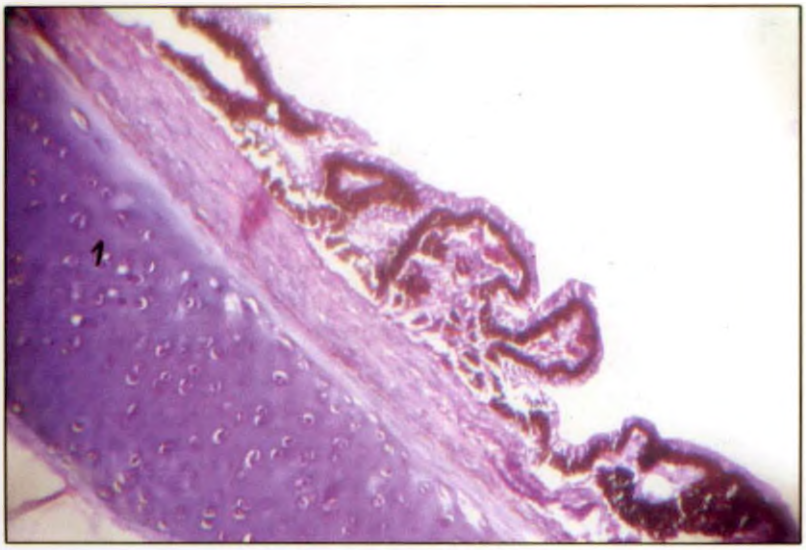
1 Corneal endothelium
2 Descemet' s membrane

Fig. 5 Cornea of eye in gluteraldehyde formaldehyde fixative (GF) at 0 hr-H & E x 400



1 Anterior epithelium
2 corneal stroma
3 fibrocyte

Fig. 6 Cornea of eye in gluteraldehyde formaldehyde fixative (GF) at 0 hr using Masson's trichrome stain-H & E x 400



1. Scleral cartilage

Fig. 7 Sclera of the eye in GF fixative at 0 hr-H & E x100

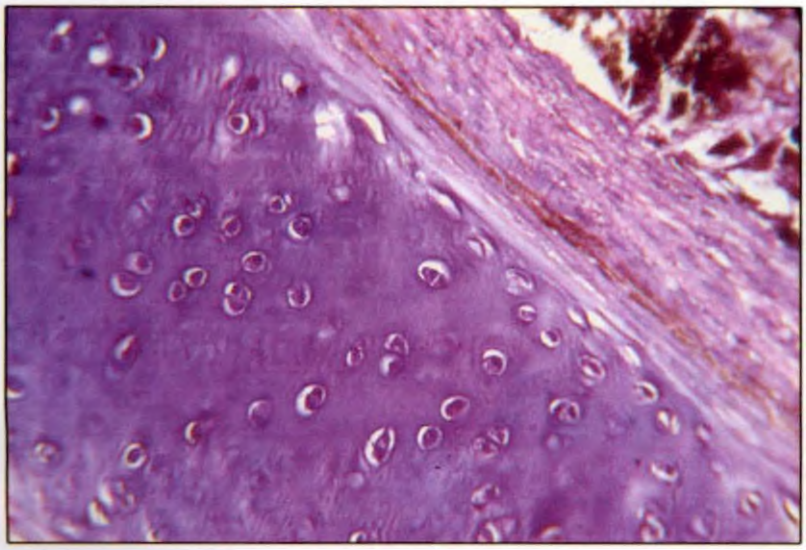
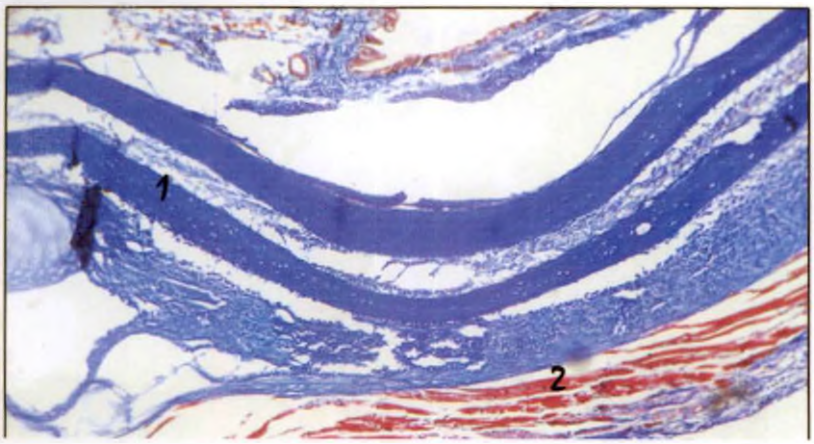


Fig. 8 Sclera of the eye in GF fixative at 0 hr-H & E x400



1. Scleral ossicles
2. ciliary muscles

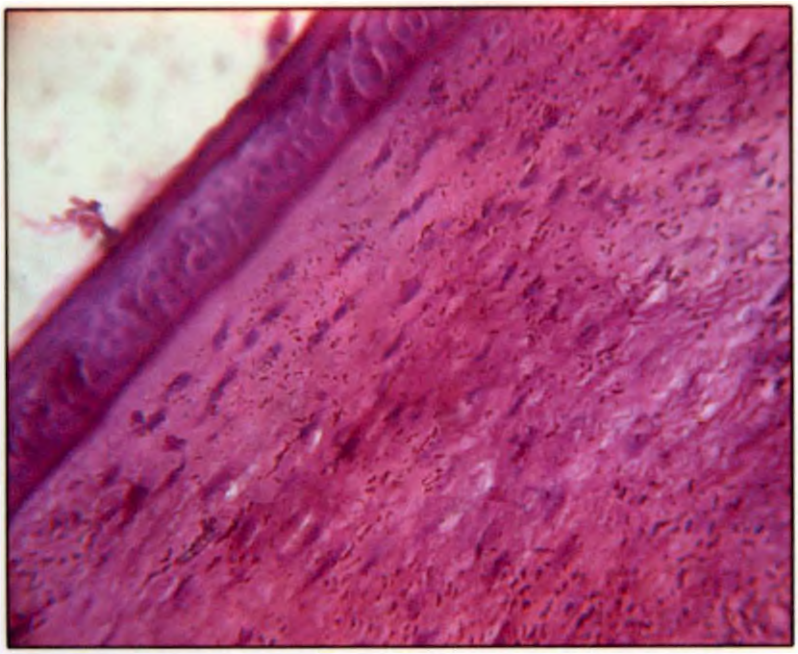


Fig. 10 Exfoliation of epithelium of cornea at 4 hrs- H & E x400

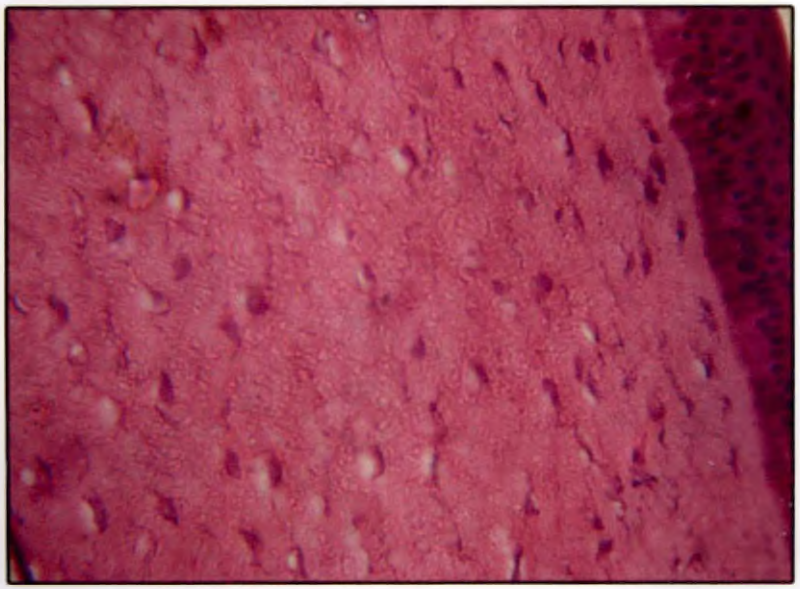


Fig. 11 Seperation of collagen fibres at 1 hr- H & E x400

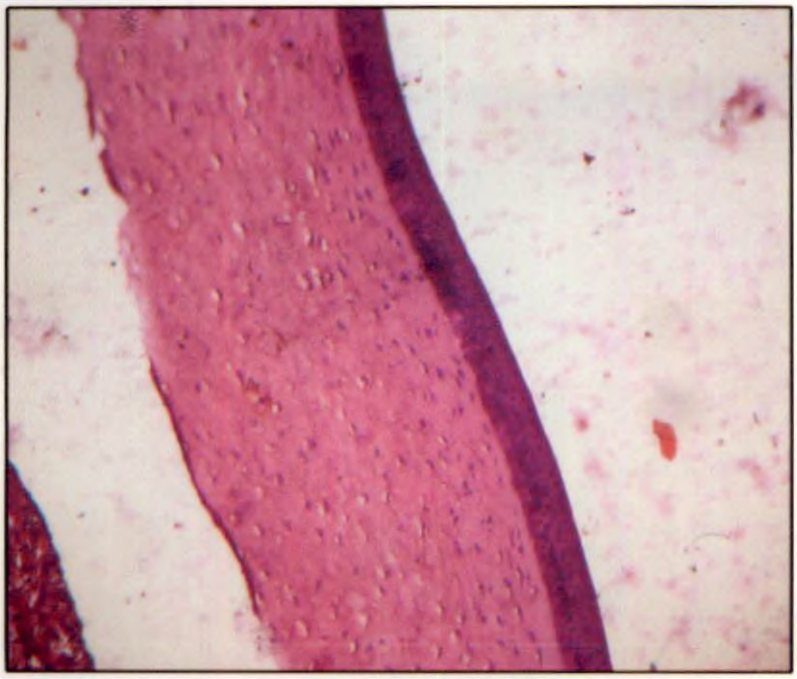


Fig. 12 Lysis and detachment of corneal endothelium at 12 hrs- H & E x100

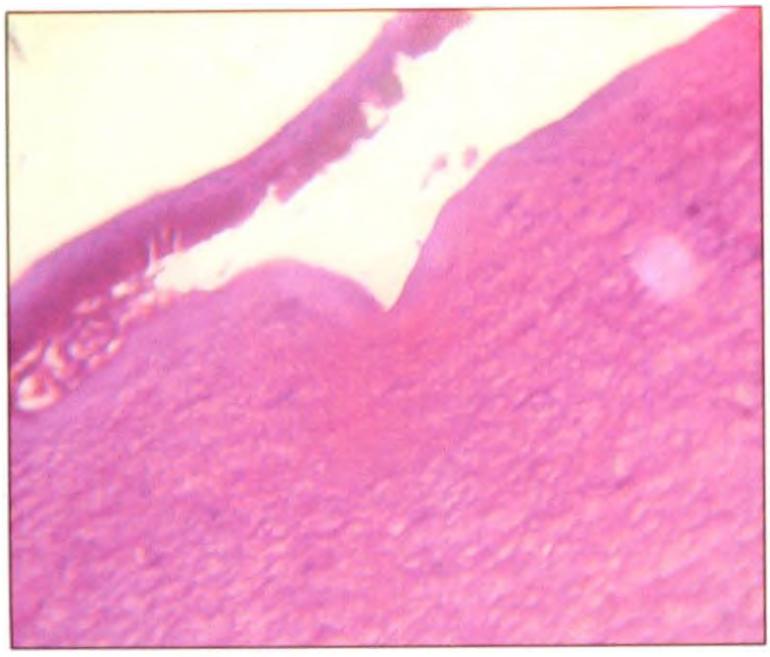


Fig. 13 Complete peeling off the corneal endothelium at 12 hrs- H & E x400

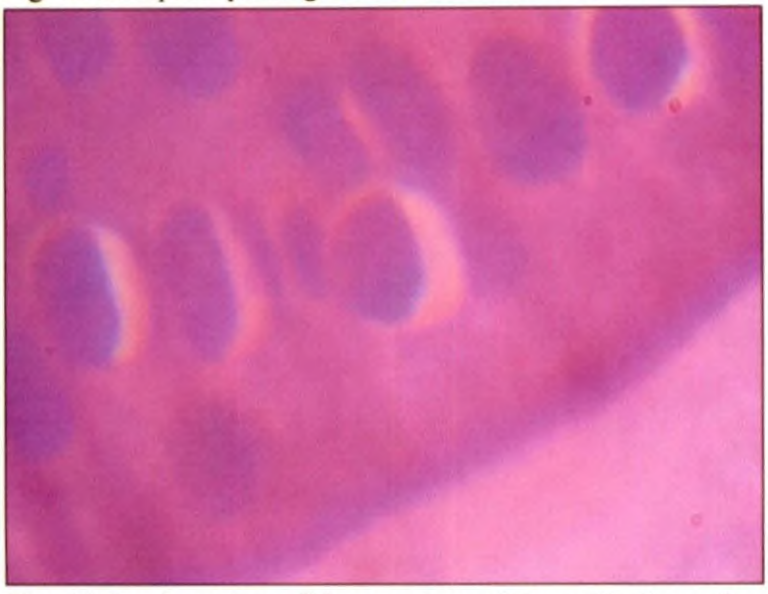
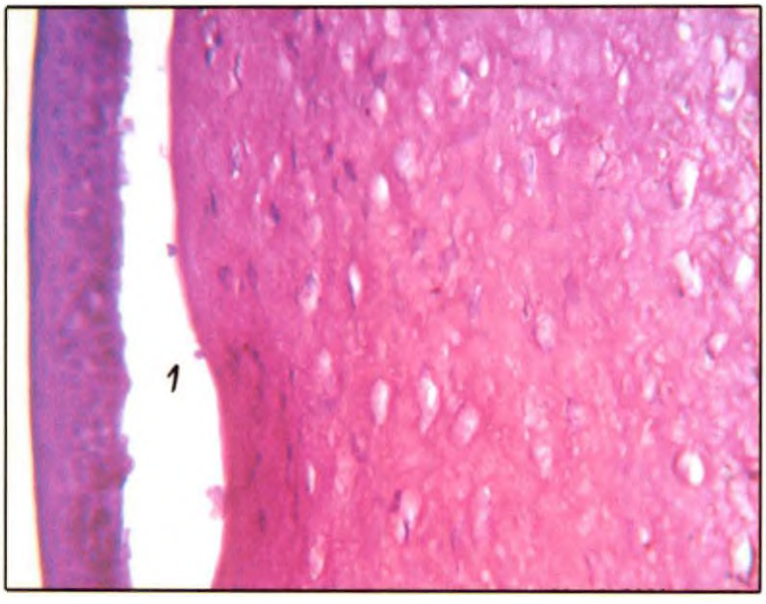


Fig. 14 Condensation of the neucleus of the epithelium of cornea leaving a halo around the neucleus at 1hr- H & E x1000



1. Detachment of corneal epithelium

Fig. 15 Detachment of corneal epithelium and seperation of collagen fibres at 1 hr- H & E x400

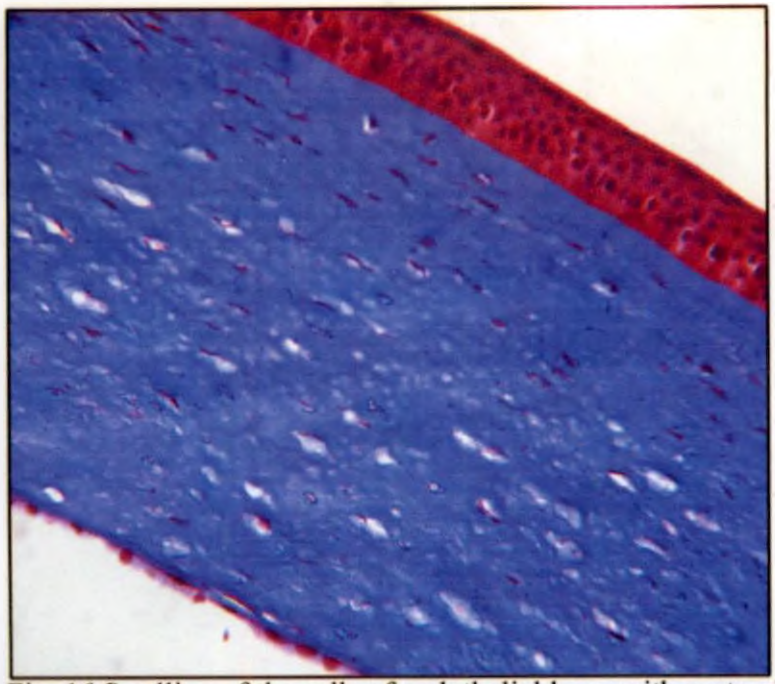


Fig. 16 Swelling of the cells of endothelial layer with protrusion into the anterior chamber at 1 hr x400

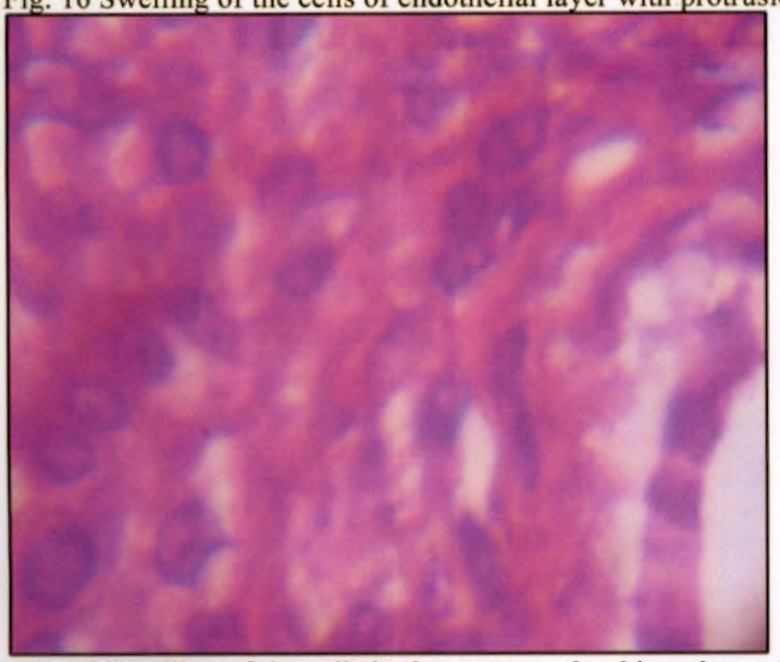


Fig. 17 Swelling of the cells in the corneo-scleral junction at 2 hrs- H & E x1000

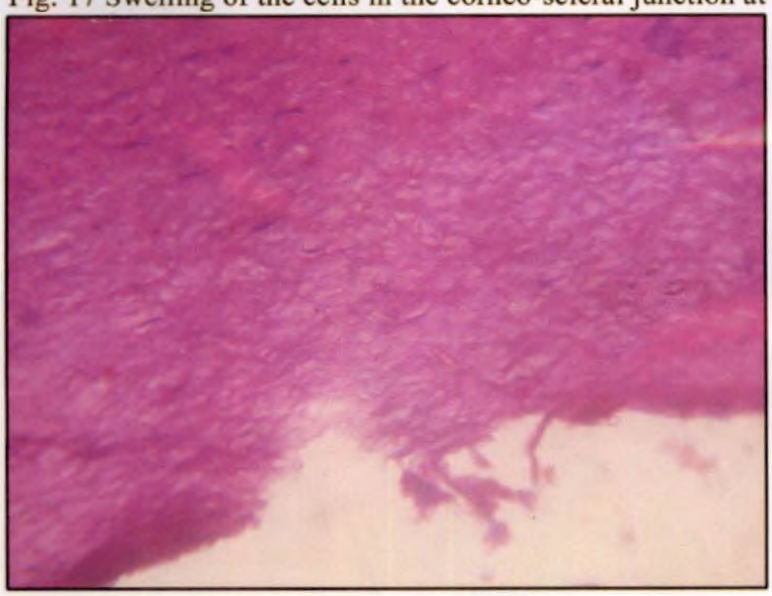


Fig. 18 Lysis of the corneal endothelium at 12 hrs- H & E x400

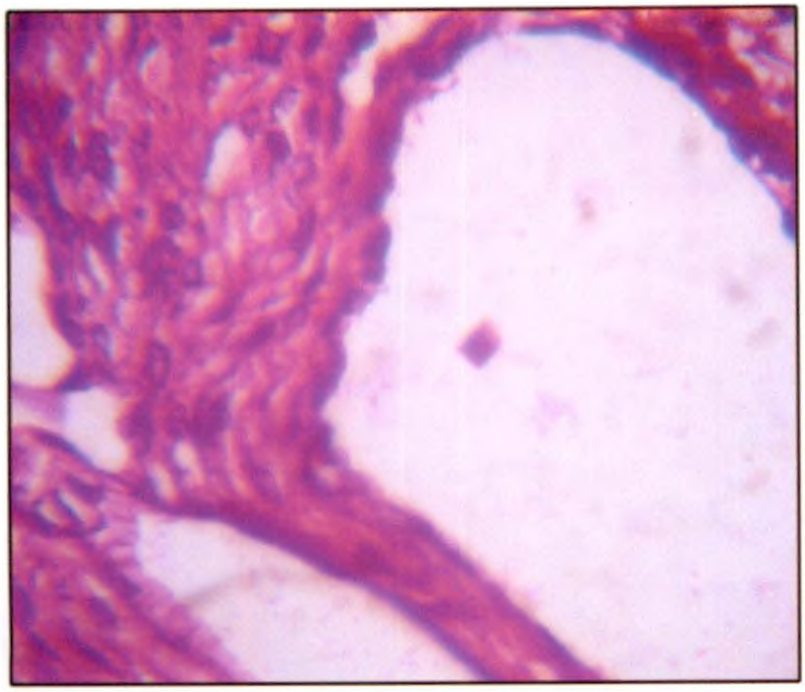
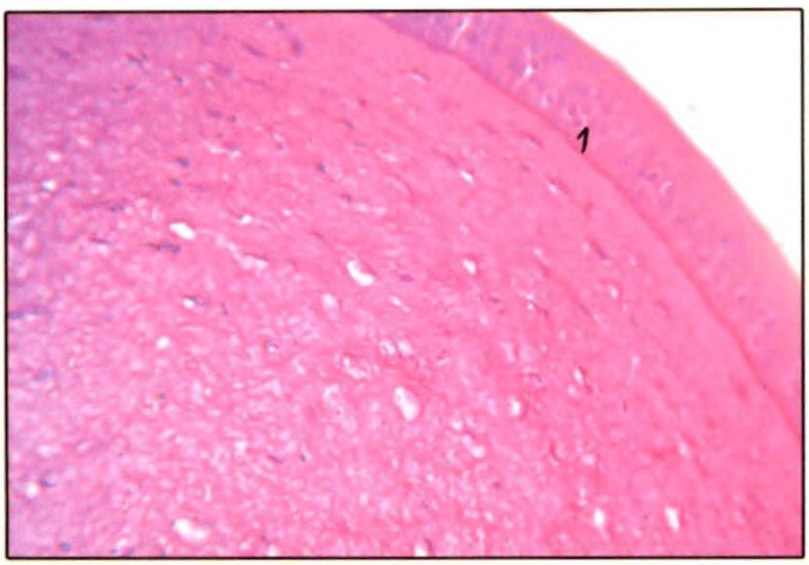


Fig. 19 Dilation of Canal of Schlemm at half an hr-H & E x1000



1. Bowmans' membrane

Fig. 20 Bowmans' membrane on periodic-Acid schiffs'(PAS) staining at 1 hr-H & E x400

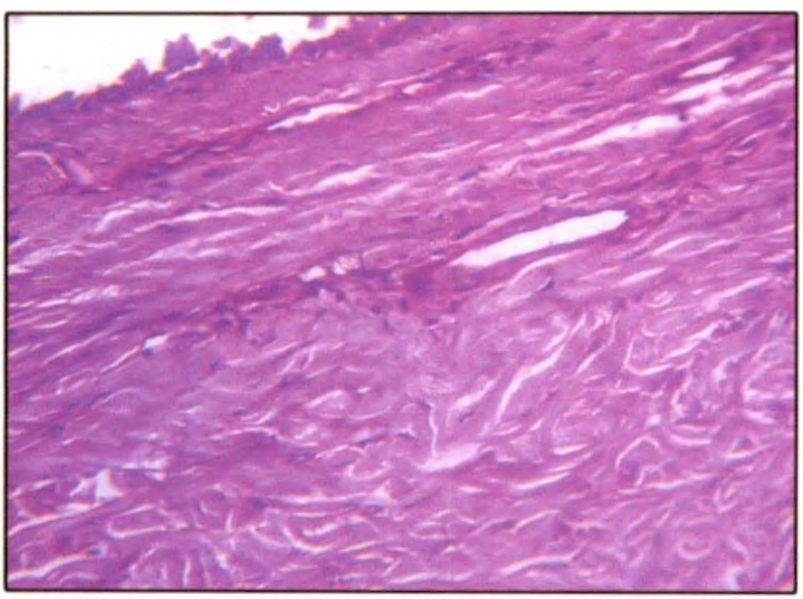


Fig. 21 Desquamation of the cells of corneo- conjunctival epithelium and seperation of the collagen fibres at 12 hrs-H & E x400

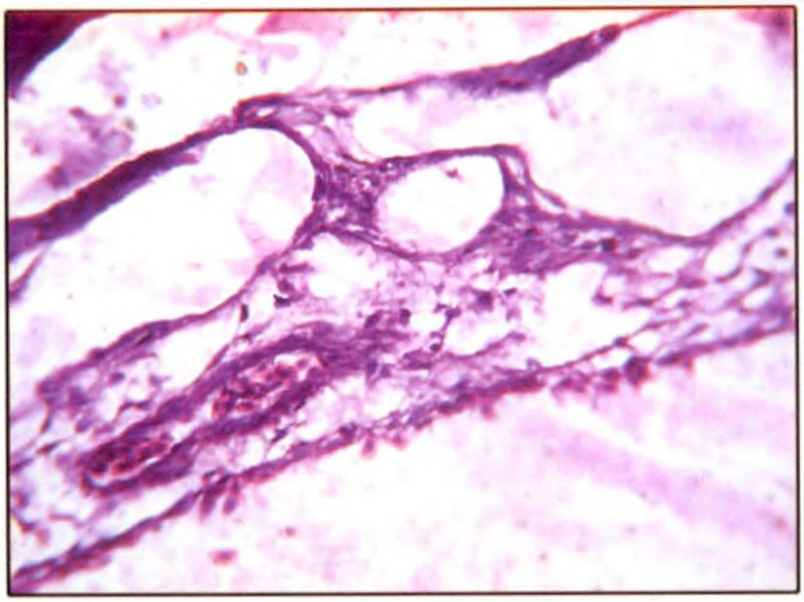


Fig. 22 Intact RBCs in the capillaries of choroid at 0 hr-H & E x400

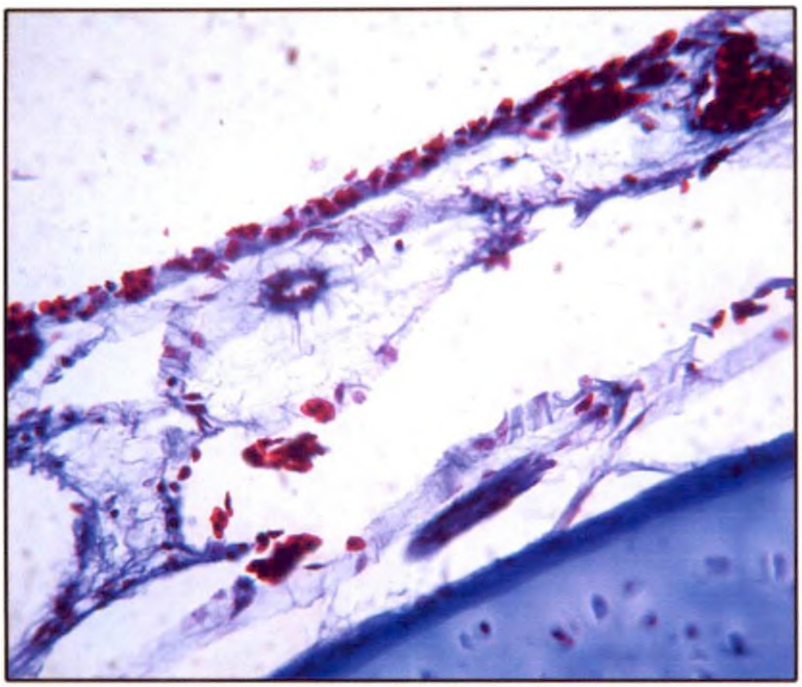


Fig. 23 Lysis and swelling of the vascular endothelium of choroid at 2 hrs x400

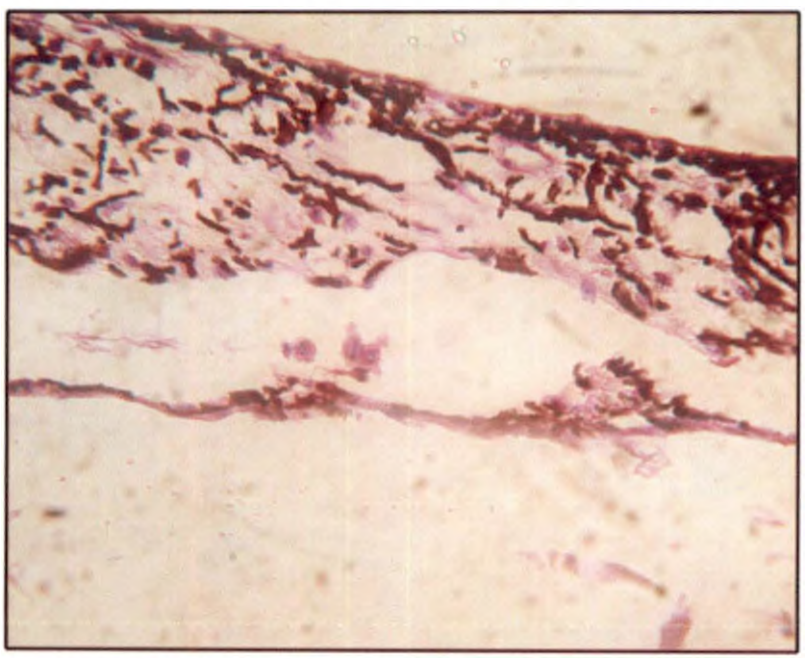
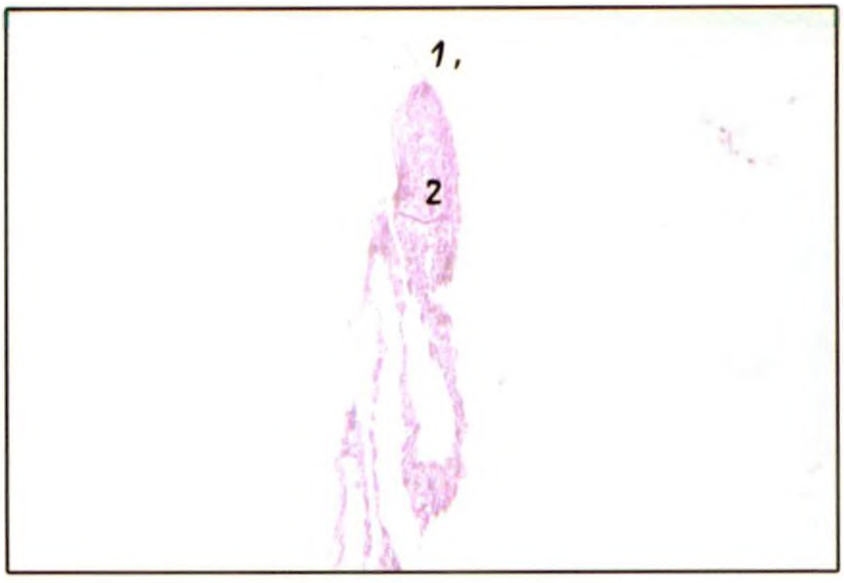


Fig. 24 Lysed RBCs in the capillaries of choroid at 12 hrs-H & E x400



1. Covering membrane
2. Bridge of pecten

Fig.25 Pecten of eye at 0 hr-H & E x100

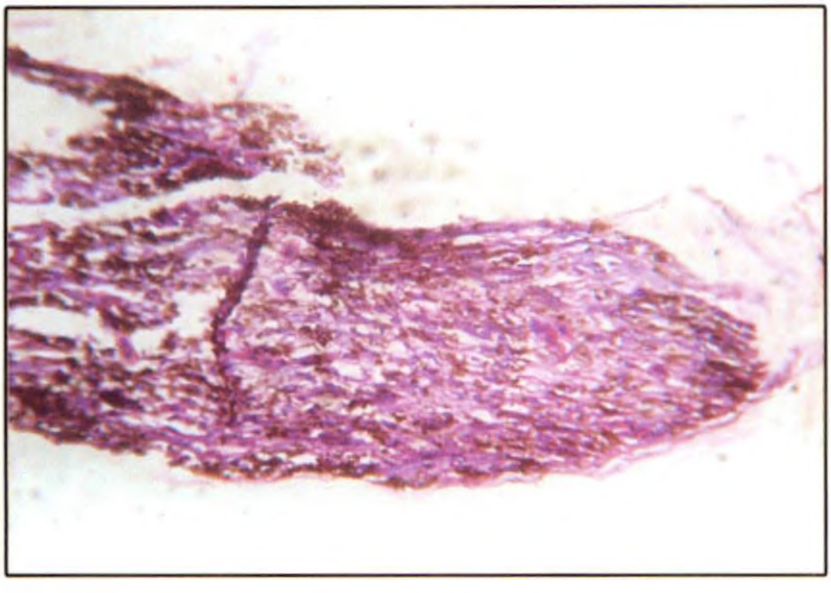
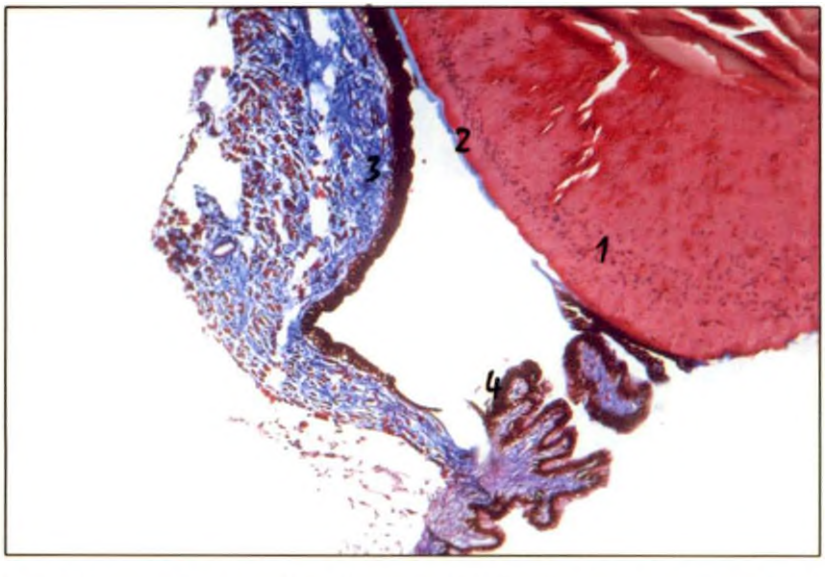
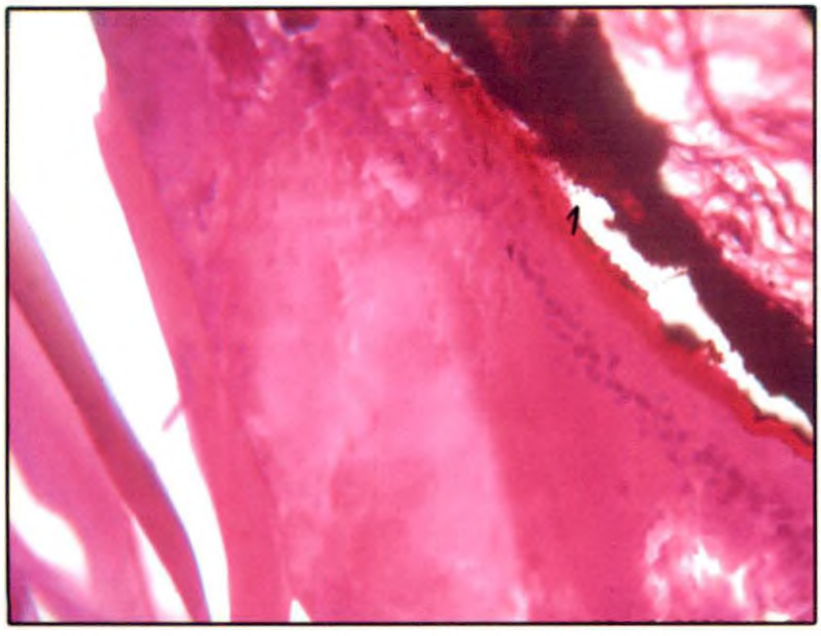


Fig. 26 Bridge of pecten at 1 hr-H & E x400



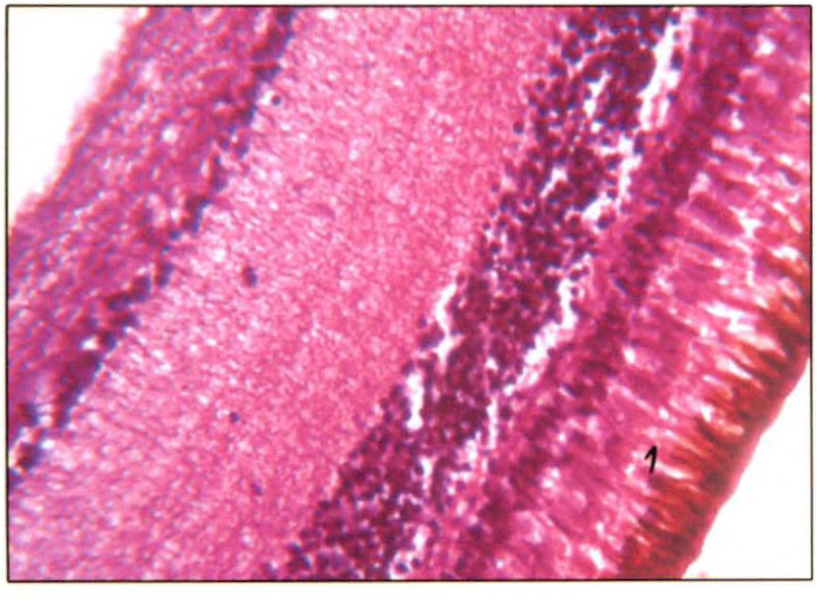
1. Annular pad
2. Lens capsule
3. Iris
4. Ciliary process

Fig. 27 Lens of eyes using Massons' trichrome stain at 0 hr-x100



1. Lens capsule

Fig. 28 Lens of eye using PAS staining at 0 hr x400



1. Layer of rods and cones

Fig. 29 Retina on PAS staining at 0 hr x400

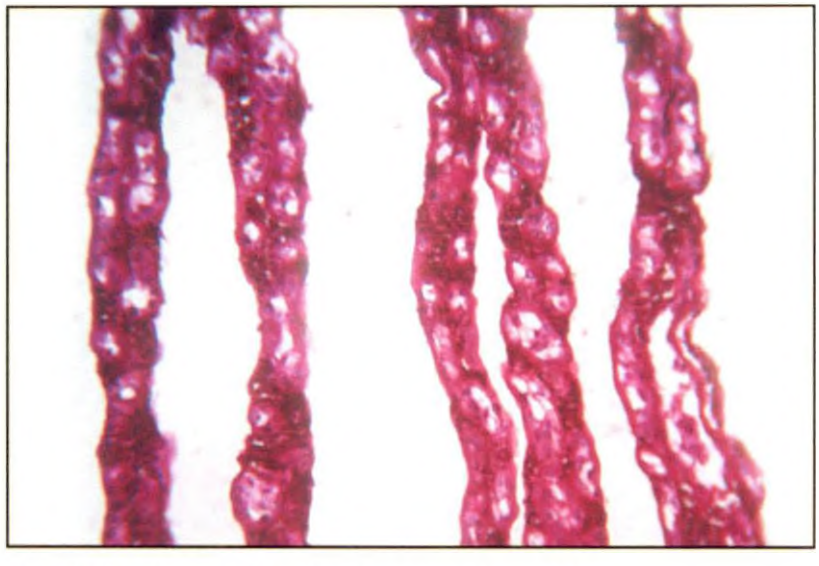
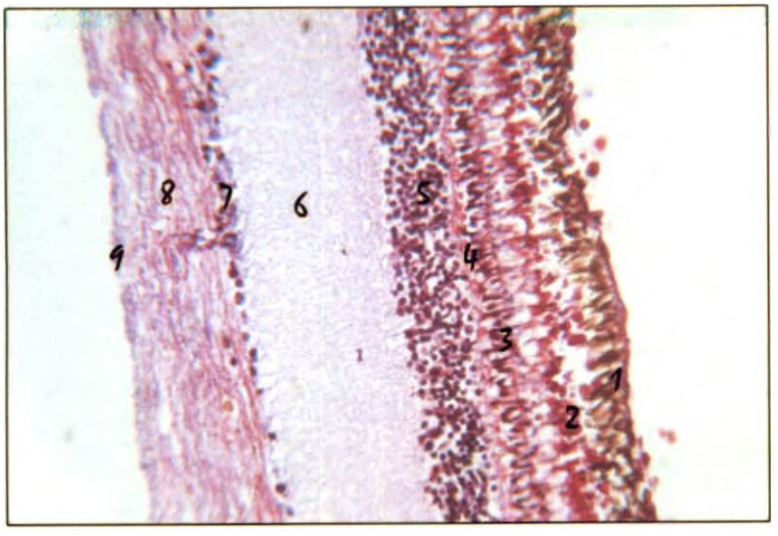


Fig. 30 Pecten on PAS staining at one hr x400



1 Retinal pigment epithelium
2. Layer of rods and cones
3. Outer nuclear layer
4. Outer plexiform layer
5.Inner nuclear layer
6. Inner plexiform layer
7. Ganglion cell layer
8. Nerve fibre layer
9. Internal limiting membrane

Fig. 31 Retina on using Massons' trichrome staining at one hr x400

Discussion

5. DISCUSSION

The experiment was designed to study the sequential postmortem changes in the isolated chicken eyes. The effect of the delayed fixing time on the histological structure of the eye can be made use of as basic information in the experimental studies using isolated chicken eye as a model in toxicological evaluations.

5.1 MORPHOLOGY OF THE EYES

The eyes of the chicken were in the form of ellipsoid with a greater diameter through the equator than across the poles. This is in accordance with Seiferle (1977). According to King and Mc Lelland (1975) in domestic fowl, 'flat' eyeball was seen since the intermediate region was a flat disc and was almost parallel with the surface of the body. Berger (2005) observed that the owl's eyes were more like cylinders or tubes, long from front to back and shorter from side to side. Prakash and Arora (1998) reported that in pigeons the eyeball was partly concave. The eyes of the chicken collected were devoid of any tumors or clots. The colour of the iris was greyish yellow, but reduced in intensity as fixing time was delayed. The decrease in colour may be due to the depletion of fat enclosed in the follicles as a result of the postmortem changes as suggested by Seiferle (1977) who opined that the variation in colour of iris may result from the amount and colour of the fat.

5.2 FIBROUS TUNIC

5.2.1 Cornea

Cornea consisted of five layers. From outside inwards it showed (a) a multilayered anterior epithelium (b) Bowman's membrane (c) corneal stroma (d) Descemet's membrane and (e) corneal endothelium. Tuft and Coster (1990) reported that the endothelium was a monolayer of cells on the posterior corneal surface that transports water from the stroma into the anterior chamber. Waring *et*

al. (1982) reported that the function of endothelium is to control corneal hydration and providing nutrition. Cornea met with sclera at the limbus. A sudden decrease in the thickness of the cornea and the sclera at the corneoscleral junction was observed in this study which is in agreement with Venkatesan *et al.* (2008). The Descemet's membrane and Bowman's membrane showed Periodic-Acid Schiff's (PAS) reaction positive as reported by Venkatesan *et al.* (2008). But the PAS reaction became less intense as the fixing time was delayed which might be due to the depletion of the glycogen.

Separation of multilayered anterior epithelium from the corneal stroma was observed in the eyes fixed after two hours. This observation is significant since the separation of the superficial layers cannot be considered as criteria for the evaluation of the effect of any toxicological agents. The corneal endothelium showed disintegration and swelling of the cells. This was more pronounced in trichrome staining which shows the advantage of this stain over Hematoxylin and Eosin (H&E) stain. There was dilatation of scleral venous plexus at the corneoscleral junction. Also the lining endothelial cells of the scleral venous plexus were swollen. Shrinkage of the nucleus of anterior epithelium with formation of a halo and vacuolation of the cytoplasm was observed in this study. There was separation of the collagen fibres in the stroma and formation of spaces in between them. Also the densely arranged collagen fibres turned into loosely packed stroma. The epithelial cells in the limbus showed degenerative changes. Grierson (1981) reported that the meshwork cells closest to the chamber angle were particularly susceptible to postmortem advance.

The post-mortem changes of cornea included condensation of the nucleus of epithelium of the cornea, leaving a halo around the nucleus. This change may be an apoptotic change which needs to be studied. The interepithelial adhesions also showed tendancy of detachment. But the most appreciable changes were the erosion or the exfoliation of the epithelium after four hours postmortem. The cytoplasm also revealed vacuolar and degenerative changes. The corneoconjunctival epithelium also showed the degenerative changes. The

collagen fibres which were thickly packed at zero hour fixing showed separation of the fibres as the fixing time was delayed.

The endothelial layer showed swelling and protrusion of the cells into the anterior chamber. Focal areas of lysis and detachment of the endothelium was prominent in the eyes fixed after one and a half to two hours and more. The collagen fibres at the corneoscleral junction showed moderate separation suggestive of autolytic changes. The Canal of Schlemm was dilated from half an hour. But the postmortem vacuoles reported by Grierson (1981) in the endothelium could not be observed in this study.

Complete peeling of the corneal epithelium and endothelial disintegration was observed at 12 hours of time interval. This is in agreement with Slettedal *et al.* (2008) who reported that the cornea showed increasing endothelial cell damage with increasing postmortem time.

5.2.2 Sclera

Sclera consisted of dense network of collagen and elastic fibres and cartilages. A remarkable feature of the sclera was the presence of overlapping scleral ossicles anteriorly and a cup shaped layer of hyaline cartilage, the scleral cartilage, posteriorly. This is in accordance with Bacha and Bacha (2000). King and Mc Lelland (1975) reported that the scleral ossicle strengthened the eyeball and provide attachment for the muscles of accommodation and there were 14 to 15 ossicles in domestic fowl. No postmortem changes could be detected in the scleral cartilage and ossicles for the four hours time interval.

5.3 VASCULAR TUNIC

5.3.1 Choroid

The presence of lysed or intact erythrocytes was noticed in the blood vessels in different fixation intervals. The lysis was mild to moderate from half an hour to two hours after which the lysis of the RBCs was prominent. The

vascular endothelium was swollen. There were no changes in the musculature of the vessels. The pigmented connective tissue showed varying degrees of dispersion of the melanin pigments as the postmortem changes advanced. The cellular borders were indistinct compared to the zero hour fixing. The changes observed may be useful to interpret changes in the study of ocular pathology.

5. 3. 2 Ciliary Body

The ciliary body consisted of ciliary epithelium, a vascular layer and the ciliary muscles. Tedesco *et al.* (2005) studied the architecture of the ciliary muscle of domestic fowl (*Gallus domesticus*) and described the existence of three main muscle groups defined as anterior, posterior and intermediary. The swelling of the cytoplasm was more evident in the non-pigmented cuboidal epithelial layer. These microscopic changes may lead to the increased potassium level as described by Prasad *et al.* (2003) and the ketone bodies as described by Felby *et al.* (2008). The blood vessels in the region of the ciliary body showed thickened arterial muscles with intact RBCs inside.

5.3.3 Iris

The colour of the iris of chicken eyes collected was grayish yellow at zero hour fixation. The colour was less intense as the fixation time delayed. This may be due to the depletion of fat as reported by Seiferle (1977). The results of the study could not reveal any color change of iris as described by Abraham *et al.* (2008) who observed colour change in the iris of *Sus scrofa* and reported that all isolated blue eyes in the experiment, at room temperature and higher changed to beam/black within 48 hours of postmortem.

The iris was suspended between the cornea and the lens which divided the space between the lens and the cornea into anterior and posterior chamber. The epithelium of the iris was observed detached and fragmented in a number of eyes examined. At 12 hours of time interval there was seperation of the connective tissue stroma of iris. The detachment and fragmentation of the iris can be

associated with the detachment of the retina, since the pigmented epithelium of the iris is extended as the pigmented layer of the retina.

5.4 NERVOUS TUNIC

5.4.1 Retina

Retina consisted of the following layers (1) retinal pigment epithelium (2) layer of rods and cones (3) external limiting membrane (4) outer nuclear layer (5) outer plexiform layer (6) inner nuclear layer (7) inner plexiform layer (8) ganglion cell layer (9) nerve fiber layer and (10) internal limiting membrane. The layer of rods and cones showed periodic-acid Schiff's reaction positive. But the intensity of the reaction was reduced as the fixation was delayed. This may be attributed to the depletion of the glycogen content. This is in accordance with Venkatesan *et al.* (2008). The retina of the chicken was avascular as reported by Bacha and Bacha (2000).

Detachment of retina was seen from zero hour of fixation in neutral buffered formalin (NBF) fixative and gluteraldehyde formaldehyde (GF) fixative. The fragmentation of the retinal layers was more frequent in the eyes fixed later than one and a half hours. The nucleus of the epithelial cells including retinal pigment epithelium, layers of rods and cones and outer nuclear layer showed varying degrees of pyknotic changes in the eyes fixed after two hours. The changes were more prominent in the ganglion cells which showed condensation of the nuclei along with vacuolar lytic changes of the cytoplasm. The detachment of the retina cannot be considered as a postmortem change. Other changes like fragmentation and the changes in the retinal pigment epithelium and ganglion cells are indicative of the postmortem changes.

The changes reported in the ganglion cells, retinal pigment epithelium and outer nuclear layer of the rods and cones could be correlated with the results of Schmidt and Berson (1980) that the postmortem metabolic capacity of photoreceptor cells in human and rat retina showed decline of 16 per cent to 19

per cent per hour between two and four and a half hours after death. It also coincides with the observation of Chen *et al.* (2007) that retinal nuclear DNA was degraded with postmortem interval.

5.5 PECTEN

Pecten is a thin, highly vascular, pleated membrane that protruded into the cavity of vitreous humor. Apically it was modified into bridge of pecten and draped by a covering membrane which is in agreement with Bacha and Bacha (2000) who described that this covering membrane was thought to be continuous with the inner limiting membrane of retina.

Postmortem changes in the pecten were appreciated by the lysed RBCs in the capillaries along with the vacuolar degeneration and lytic changes affecting the melanocytes and the hyalocytes. The eosinophilic staining cytoplasm of the hyalocyte was prominent in the pecten of the eyes fixed upto one hour, but the intensity of eosinophilic staining was less in the eyes fixed after one hour or later. Since the pecten plays an important role in the nourishment of retina and vitreous body, the understanding of the changes in pecten is significant in the study of the pathology of the retina.

5.6 REFRACTIVE MEDIA

5.6.1 Lens

Lens was covered by a lens capsule. Lens capsule showed a positive reaction for periodic-Acid Schiff's reaction positive. Within capsule, lens was divided into annular pad and lens body as reported by Bacha and Bacha (2000). There was fragmentation of the lens fixed in NBF fixative. Janardhan *et al.* (2001) reported that when ten per cent NBF was used for fixation of eye balls of rats and mice, the lens could not be sectioned intact along with other structures and he also reported that the brittle nature of the lens caused fragmentation and artifactual changes.

5.7 COMPARISON OF FIXATIVES

The eyes that were fixed in GF fixative gave good section when compared with NBF fixative. Also there was detachment of retina from zero hour onwards when fixed in both the fixative. The lens of eyes showed extensive fragmentation when fixed in NBF fixative. Margo and Lee (1994) reported that the whole eyes fixed in ten per cent NBF demonstrated separation of retina from retinal pigment epithelium and also several artifactual changes.

The excessive shrinkage and fragmentation of formalin fixed lens supports the findings of Barash and Shepardo (2002).

5.7 HISTOMETRY OF THE EYEBALL

Using t-test, significant difference was observed in the thickness of cornea at 120 minutes when compared with zero hour in neutral buffered formalin fixative. The thickness of cornea showed significant difference at 30 minutes, 60 minutes, 90 minutes and 120 minutes when compared with zero hour and also the thickness of retina showed significant difference at 30 minutes, 60 minutes, 90 minutes and 120 minutes in gluteraldehyde-formaldehyde fixative. Sclera also showed significant difference at 120 minutes compared to zero hour in GF fixative. No literatures were available for further discussion of this topic.

Summary

6. SUMMARY

Experimental animals are used to test the toxicity and adverse reactions of agents like cosmetics, synthetic detergents and agrochemicals. This leads to extreme pain and discomfort for these animals. Alternatives to the animal experimentation studies are needed to avoid the cruelty to the animals. Among these alternatives, isolated chicken eye (ICE) test gained more importance due to the high linear correlation between the results of the in vitro and in vivo tests. In order to study histological changes that happen in the isolated chicken eye on application of cosmetics and chemicals, the normal post-mortem changes in the eye should be known to prevent false interpretation.

The present study focuses mainly on the sequential post-mortem changes that happen in the isolated chicken eye (ICE) upto twelve hours of time interval after enucleation. Six eyes each were fixed at zero hour, half an hour, one hour, one and a half hours, two hours, four hours and twelve hours at ten per cent neutral buffered formalin and gluteraldehyde-formaldehyde fixative. A clean cut was made near the midline of the fixed eye slightly to one side of it, so that the origin of the optic nerve and pupil are included in the sections. The tissues were processed in high melting paraffin.

The eyeball consisted of three concentric tunics. The outer fibrous tunic consisted of the rostral transparent cornea and the caudal opaque sclera. The middle vascular pigmented tunic consisted of the iris, ciliary body and choroid. The inner nervous tunic consisted of the retina.

Cornea was avascular and consisted of five layers. From outward to inward it showed the stratified squamous non-keratinized corneal epithelium, Bowman's membrane, corneal stroma, Descemet's membrane and corneal endothelium. The anterior epithelium gradually changed to conjunctival epithelium at the limbus. Stroma formed 90% of the corneal thickness, which

consisted of collagen fibres. Between the lamellae of cornea, there were freely branching fibrocytes.

Sclera consisted of dense network of collagen and elastic fibres. There was presence of overlapping scleral ossicles anteriorly and a cup shaped layer of hyaline cartilage, the scleral cartilage, posteriorly. A large scleral venous plexus (Canal of Schlemm) lay at the corneoscleral junction.

The post-mortem changes of the fibrous tunic included condensation of the nucleus of the epithelium of the cornea, leaving a halo around the nucleus. There was tendency of detachment for the interepithelial adhesion. After four hours there was exfoliation of the epithelium. The cytoplasm also revealed vacuolar and granular degenerative changes. There was separation of the collagen fibres of corneal stroma which were thickly packed at zero hour time fixing. The corneal endothelium showed swelling, lysis and detachment of the endothelium. Sclera did not reveal any appreciable histological changes.

The choroid consisted of dense network of blood vessels and heavily pigmented connective tissue. The ciliary body consisted of ciliary epithelium, a vascular layer and the ciliary muscles. The ciliary body was covered by two layers of cuboidal epithelial cells, outer pigmented epithelial layer and inner non-pigmented epithelial layer. The smallest part of the vascular tunic was the iris.

In the vascular tunic, the presence of lysed erythrocytes was prominent after two hours in the choroid. The vascular endothelium was swollen. There were varying degrees of dispersion of melanin pigment. The swelling of the cytoplasm of the ciliary body was more evident in the non-pigmented epithelial layer. The epithelium of the iris showed detachment and fragmentation.

In the nervous tunic, the important change noticed was the retinal detachment in eyes fixed from zero hour. The nucleus of the epithelial cells including the retinal pigmented epithelium, layers of rods and cones and outer nuclear layer showed varying degree of pyknotic changes in the eyes after two

hours. The changes were prominent in the ganglion cells which showed condensation of the nuclei along with vacuolar and lytic changes of the cytoplasm.

Pecten was a highly vascular pleated membrane protruding into the cavity of the vitreous humor. Post-mortem changes of pecten were appreciated by the lysed erythrocytes in the capillaries along with the vacuolar degeneration and lytic changes affecting the melanocytes and hyalocytes.

Lens was covered by a lens capsule. Within capsule, lens was divided into annular pad and the lens body. Annular pad consisted of radially arranged lens fibres with peripheral nuclei. Fragmentation of lens was extensive in eyes fixed in ten per cent NBF fixative.

PAS positive areas were detected in the Descemet's membrane and Bowman's membrane of cornea, lens capsule, pecten and layer of rods and cones of retina.

Masson's trichrome staining helped to differentiate the different tissue components like collagen fibres and muscles of the avian eye. GF fixative proved to be better fixative than ten per cent NBF since the cellular details were more evident when eyes were fixed in the former. There was extensive fragmentation of the lens in ten per cent NBF fixative. The histometry of the eyeball showed a significant decrease in thickness of cornea at 120 minutes when compared with zero hour in neutral buffered formalin fixative.

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STUDIES ON SEQUENTIAL POST-MORTEM CHANGES OF ISOLATED CHICKEN EYE

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ABSTRACT

The present investigation was undertaken to study the sequential postmortem changes of different components of chicken eye and to choose an ideal fixative.

Six eyes each were fixed at zero hour, half hour, one hour, one and a half hours, two hours, four hours and twelve hours in ten per cent neutral buffered formalin and gluteraldehyde fixative. Eyes were fixed for a minimum of three weeks. A clean cut was made near to the midline of the fixed eye slightly to one side of it, so that the origin of the optic nerve and pupil were included in the sections.

The eye consisted of three tunics; tunica fibrosa consisting of cornea and sclera, tunica vasculosa with choroid, ciliary body and iris and tunica interna consisted of retina.

The postmortem changes were mostly appreciated in the cornea, which included condensation of the nucleus of the epithelium of cornea, interepithelial detachment and exfoliation of epithelium. The cytoplasm revealed vacuolar and degenerative changes and there was separation of collagen fibres of corneal stroma. The corneal endothelium showed swelling, lysis and detachment.

The presence of lysed erythrocytes was prominent after two hours in the choroid. As the time interval advanced, there were varying degrees of dispersion of melanin pigment. The swelling of the cytoplasm of the ciliary body was more evident in the non- pigmented epithelial layer. The epithelium of the iris showed detachment and fragmentation.

Retinal detachment was noticed from zero hour of fixation in GF fixative and ten per cent NBF fixative. The changes were prominent in the ganglion cells which showed condensation of the nuclei along with vacuolar and lytic changes

of the cytoplasm. Postmortem changes of pecten were appreciated by the lysed erythrocytes in the capillaries along with the vacuolar degeneration and lytic changes affecting melanocytes and hyalocytes.

PAS positive areas were detected in the Descemet's membrane and Bowman's membrane of cornea, lens capsule, pecten and layer of rods and cones of retina. Masson's trichrome staining proved to be useful to appreciate the changes in the collagen fibres, muscles and epithelium. The histometry of the eyeball showed a significant decrease in thickness of cornea at 120 minutes when compared with zero hour in neutral buffered formalin fixative. From the study it can be concluded that GF fixative is a better fixative for eye than 10 per cent NBF fixative.