INTRODUCTION

The past decade has seen tremendous development in the wireless telecommunications industry with the introduction of cellular phones (cell phones / mobile phones). They have revolutionized the telecom industry by making the telecommunication faster, economical and more convenient. With the introduction of new applications and multifunctional features in the mobile hand set, the telecom industry is wooing both young and old generation and the number of cell phone users are increasing day by day. Gadgets like tablets, smartphones and the like are multiplying at a rate five times faster than global human population.

According to the latest input from digital analysts in the GSMA Intelligence (Group Speciale Mobile Association), the number of active mobile devices has crossed 7.22 billion marks which has crossed the global human population that is placed between 7.19 and 7.22 billion (US Census Bureau). In the history of mankind, the mobile phone stands as the fastest growing man-made phenomenon ever discovered. With the rise in the number of cell phone users, the telecom industry is installing more and more base stations for better network coverage and signal transmission. These wireless telecommunication devices operate with the help of radio frequency (RF) fields just like television, radio, radar, microwave oven, etc.

The radio frequency fields are a part of the electromagnetic spectrum, which consists of low frequency electromagnetic fields, high frequency electromagnetic fields and visible light (FCC., 1999, ARPANSA., 2011) (Figure 1). Electromagnetic radiation consists of waves of electric and magnetic energy moving together in space (Cleveland et al., 1999). Electromagnetic field is the area where these waves are found. The distance covered by one cycle of the wave is known as wavelength (λ) and is measured in meter (m). The number of waves passing in one second at a given point is known as frequency (f) and its unit of expression is known as hertz (Hz). One hertz means one wave per second.

The high frequency electromagnetic fields are also known as radio frequency fields, since they are used for broadcasting and telecommunications.
Their frequency ranges from 9 KHz – 300 GHz and are found in the environment as a natural high frequency field or man-made electromagnetic fields.

The natural sources for electromagnetic environment include

- Electrical discharges in the earth’s atmosphere (Schumann Resonances from tropical thunderstorms, lightning and blackbody radiation.)
- Radiation from sun (solar flares, solar winds.) and

Though naturally occurring, these radiations in higher doses are hazardous to health, causing gene mutations leading to malignancies. However, most of these radiations are prevented from entering the earth’s atmosphere by the Ozone layer and the magnetic fields of the earth (Van Allen radiation belts).

The man-made fields are produced either intentionally or unintentionally as the by-products of the use of electrical devices and systems. These include
• Devices used for telecommunications and broadcasting (FM, television, mobile and wireless communications technologies, satellite uplinks.)
• Devices used for medical applications (diathermy, hyperthermia, MRI, CT scans, RF ablation, RF telemetry.)
• Devices used for industrial and domestic applications (dielectric heating, induction heating, microwave ovens, electronic article surveillance, radio frequency identification.) and
• Devices used for safety applications and navigation (radar, air traffic control, marine radar.).

In addition to the above said applications, new technologies are emerging day by day that join the bandwagon of man-made electromagnetic fields. These include wireless LANs (wireless local area network), Bluetooth, DECT (digital enhanced cordless telecommunication, UWB technology (ultra-wide band technology) and wireless power transmission (WPT) from solar power satellites (SPS). Among all these devices that emit man-made electromagnetic fields, the cellular mobile phone industry has undergone tremendous growth and development since its inception in Europe in early 1980s.

Introduced in early 1980s, the analogue cellular radio systems formed the first generation (1G) mobile telephone. 1G mobile technology was designed to operate in the frequency range of 450 MHz or 800/900 MHz and to provide mainly voice services.

By early 1990s, second generation (2G) mobile communication systems were introduced that used digital technology for its operation. 2G refers to GSM (global system for mobile communications) system that uses TDMA (Time Division Multiple Access) technology where each user is on for 0.58 milliseconds and again comes back periodically at a frequency of 217 Hz. The remaining 7/8 of the time is used for other users. They function in the frequency range of 900-1800 MHz’s. GSM phones are mainly used in Europe, parts of Asia and America. Another version of 2G system that uses CDMA (Code Division Multiple Access) technology was also developed in North America. Unlike the TDMA technique, where the user is on for only 0.58 milliseconds, the CDMA technique allows the users to be on simultaneously, but is separated by codes. The 2G system provides voice services, sending and receiving of text messages and also carries various data. But with the increasing popularity of internet and
personal computers, a GPRS (General Packet Radio Services) was introduced into 2G systems. The GPRS can support data up to 140.8 Kbit/s.

To cater to the need for higher data rates, third generation (3G) system was introduced in late 2000 which operates in the frequency between 1900- 2200 MHz. 3G system provides voice services, texting, email access, internet browsing, video telephony, high speed data access, music downloads and video games. It can support higher data rates ranging from 384 Kbits/s – 2 Mbits/s. The 3G systems are also known as UMTS in Europe and CDMA- 2000 in North America.

Recently, the fourth generation (4G) system was introduced in a few selected cities in India. The 4G cell phone operates in the frequency of 2300 MHz and can support data rates of 100 Mbits/s – 1 G bits/s. The fifth generation (5G) may emerge very soon!!

When we speak into a cell phone, the voice goes through a transmitter that encodes the sound into a sine wave. A sine wave is a continuously fluctuating wave and the transmitter sends the signal to the antenna, which transmits the signal out into space in all directions thus creating an electromagnetic field (Fathi, E et al., 2014). As these electromagnetic fields build-up and collapse, an electromagnetic radiation is created.

The increasing scientific evidence of various health hazards on exposure of radiofrequency radiation (RFR) emitted from both the cell phone and base stations have caused significant media attention and public discussion in recent years. It is alarming to note the increased usage of cell phone among children, who have more time to use mobile phones than today’s older generation who started using the mobile phones only a decade ago. The children are exposed to a lifetime of potentially harmful RF radiation. In this regard, usage of cell phones among pregnant ladies is also of equal concern.

Researches involving these two groups are very less due to various ethical issues. Again, the mechanism of interaction of RF fields with developing tissues of children and fetuses may be different from that of adults due to their smaller physical size and variation in tissue electromagnetic properties. The present study may provide an insight into the basic mechanisms by which RF fields interact with developing tissues in an embryo. However, the direct extrapolation of the outcome of the present study to human population may be limited due to
differences in species, volume and size, life span, functional and anatomical organization of tissues.

The present study has been designed to evaluate the possible effects of chronic exposure to RFR emitted from 2G and 3G mobile phones on the developing tissues of the chick embryo.
SCOPE AND PLAN

SCOPE OF THE STUDY:

The Cell phone once considered as a status symbol in early 90s, has now become an integral part of everyone’s life. They are used extensively by all age groups, including the children, elderly and pregnant women. A rapid growth of cell phone industry and the introduction of higher version of generation phone in a competitive market to attract all age groups and the major concern of controversial scientific reports on the health hazards on long term exposure to radiofrequency radiation, has prompted us to design this study. The scope of present study is to create awareness among the users about the possible ill effects of chronic exposure of RFR emitted from 2G and 3G cell phone during prenatal growth and differentiation of various biological tissues.

PLAN OF THE STUDY:

- To expose chick embryos to RFR radiation emitted from 2G and 3G cell phone at regular intervals for a period of 12 days (organogenesis period).
- Since solid organs absorb more RFR, brain, liver, kidneys and eyes are chosen for the present study.
- Gross morphological features and structural changes on brain, liver, kidney and eyes of chick embryo with and without exposure to RFR are studied and compared.
- The possible alterations in the level of enzymatic antioxidants (SOD & GPx) due to oxidative stress are analyzed in brain, liver, and eyes of the chick embryo.
- The extent of DNA damage, if any, is assessed using the alkaline comet assay technique in brain, liver, and eyes of the chick embryo.
All living organisms are exposed to two different forces on the earth – gravitational force and electromagnetic force (Delgado, J.M.R., 1985, Balmori, A., 2009). Man-made electromagnetic fields are also added to these forces. Thus we have interfered too much with nature causing unintended and undesirable negative impacts on the natural environment and living beings (Orendacova, J., 2007). When in operation, the cell phones emit a pulsed radio frequency electromagnetic field (RF - EMF) that is absorbed into the user’s body, especially in the head region as the handsets are held against the ear (Mary, H.D et al., 2014b).

**Specific Absorption Rate (SAR) and Power Density (PD)**

The Variables used in the measurement of RF radiations are power density (PD) and specific absorption rate (SAR). Power density is defined as the amount of power per unit area in a radiated microwave field and is usually expressed in Watts per meter squared (W/m\(^2\)). SAR is the term used to describe the absorption of RF-EMF radiation in the body. It is defined as the rate of energy that is actually absorbed by a unit of tissue and is measured in watts per kilogram (W/Kg) of tissue. The SAR is averaged over the whole body (whole body exposure) or over a small volume of tissue (1 gm. or 10gm) (local body exposure). This absorption depends on multifactor like frequency and intensity of transmission, the duration of exposure, the number of exposures, the distance from the radiation source, the shape and size of exposed organism, the water and mineral content of the organism (Philips, J.L., 2009, Sivani, S et al., 2011).

**Exposure standards for cell phone and cell phone tower in India**

According to International Commission on Non-ionizing Radiation Protection (ICNIRP., 1998) guidelines, the general public exposure limit to RF fields from mobile handsets is set at 4.5 W/m\(^2\) (power density) for 900 MHz and 9 W/m\(^2\) for 1800 MHz (Sivani, S et al., 2011). The safety limit for average whole body SAR is set at 0.08 W/kg, SAR for the head and trunk is 2 W/kg and for limbs it is 4 W/kg. Our Indian Telecommunications department (DoT) has set safety limits of exposure to radio frequency energy produced by mobile handsets for general public at 1.6 W/kg with effect from September 1, 2012 (The Hindu., 2012). Exposure levels
below this limit may not produce any thermal stress and could be tolerated by the body through various heat dissipating mechanisms without any possible damage (Sivani, S et al., 2011)

**EFFECT OF RFR ON BIOLOGICAL SYSTEMS**

The interaction of RF radiation from cell phone on biological tissues has been studied and is reported to cause thermal and non-thermal stress in the body (De la Hoz, A et al., 2005, Challis, L.J et al., 2005, Glaser, R., 2005, Foster, K.R et al 2007)

**Thermal effects**

Thermal effects are produced due to an increase in core temperature in the tissues during RFR exposure (Lin, C et al., 2007b, Fathi, E et al., 2014). This depends on SAR and power density of emitted EMF. The increased temperature of the body is then reduced by various heat dissipating mechanisms in three ways (Hamada, A.J., et al., 2011):

- Heat conduction to other tissues
- Convection through blood perfusion
- Radiation to the surroundings

Exposure to RF radiations may result in an imbalance between heat generation and heat dissipation resulting in thermal effects. This could adversely affect the functioning of biological systems (Deepinder, F et al., 2007, Habash, R.W.Y., 2008).

**Non-thermal effects**

Non-thermal effects are the interactions of RF- EMF with biological tissues of the body without causing any visible increase in body temperature. Research on the role of non-thermal effects causing oxidative stress are gaining momentum and various scientific reports are cited on this aspect (Blackman, C.F et al., 1982, Bernat, R et al., 1985, Lew, V.L et al., 1988, Ha, B.Y., 2001, Glaser, R.,2005, Ozuguner, F et al., 2006, Foster, K.R et al 2007, Li, H.W et al., 2007, Balci, M et al., 2007, Yu ,Y et al., 2008, Bormusov, E et al., 2008, Yu, Y et al., 2010, Jelodar, G et al., 2012). The various mechanisms by which non-thermal effects exert a damaging effect on a tissue are by,
- changes in ionic transport (Neil, C., 2000, Rao, V.S et al., 2008)

**RFR & OXIDATIVE STRESS**

Oxidative stress (OS) is a condition associated with increased generation of reactive oxygen species (ROS) induced by oxygen and oxygen-derived oxidants (Sikka, S.C., 2001). ROS are highly reactive oxidizing agents belonging to the class of free radicals (Aitken, 1984). A free radical is defined as —any atom or molecule that possesses one or more unpaired electrons (Warren, J.S et al., 1987, Halliwell, B et al., 1999). They are the byproducts of normal cellular metabolism and they play a dual role as both deleterious and beneficial to living organisms (Valko, M et al., 2006). Beneficial effects of ROS include its physiological roles in cellular responses to anoxia and in the function of a number of cellular signaling systems. The harmful effect of free radicals causing potential damage to biological tissues is termed as oxidative stress (Kovacic, P et al., 2001, Valko, M et al., 2001).

Oxidative stress includes,

- Oxidation of polyunsaturated fatty acids (PUFA) in lipids (Siems, W.G et al 1995, Juutilainen, J et al., 2009)
- Interference with mitochondrial oxidative phosphorylation causing ATP depletion (Anjana, V.Y et al., 2009)
- Initiation and promotion of carcinogenesis. (Juutilainen, J et al., 2009)
The ROS include superoxide anion radical (O$_2^\cdot$), hydrogen peroxide (H$_2$O$_2$), peroxyl (ROO$^\cdot$) radicals, and very reactive hydroxyl radicals (OH$^\cdot$). (Venkatasamy, M et al., 2013).

**Superoxide anion radical**

Superoxide anions are produced by the addition of one electron to the oxygen molecule

$$\text{O}_2 + \text{electron (e-$^-$)} \rightarrow \text{O}_2^\cdot.$$  

They are produced mainly due to mitochondrial respiration within the cells (Cadenas, E et al., 1998) and are implicated in the pathophysiology of various diseases (Kovacic, P et al., 2005, Valko, M et al., 2004). These anions are then converted into hydrogen peroxide (H$_2$O$_2$) by an enzymatic antioxidant, superoxide dismutase (SOD) (Cadenas, E., 2004).

**Hydrogen peroxide (H$_2$O$_2$)**

Hydrogen peroxides are produced either by peroxisomes present in the cells or by dismutation of superoxide anion by SOD (Valko, M et al., 2004). Hydrogen peroxide thus formed reacts either with superoxide anion (Haber-Weiss reaction), or interacts with free iron to form highly reactive hydroxyl radicals (OH$^\cdot$) (Fenton reaction).

$$\text{O}_2^\cdot + \text{H}_2\text{O}_2 \rightarrow 2\text{O}_2^- + \text{HO} + \text{HO}^\cdot \quad \text{(Haber-Weiss reaction)}$$

$$\text{Fe}^{++} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{+++} + \text{HO} + \text{HO}^\cdot \quad \text{(Fenton reaction)}$$

$$\text{Fe}^{+++} + \text{H}_2\text{O}_2 \rightarrow \text{OOH} + \text{H}^+$$

H$_2$O$_2$ is highly stable and can easily diffuse through the plasma and nuclear membranes, thus contributing to DNA adduct formation.

**Hydroxyl radicals (HO$^\cdot$)**

The hydroxyl radical is a highly reactive neutral form of the hydroxide ion. It is considered as a very dangerous radical with a very short in-vivo half-life of approximately 10−9 sec. (Pastor, N et al., 2000). They produce the covalent cross-linking of a variety of biological molecules as well as the propagation of other free radicals through more complex reactions leading to cell injury and cell death.
Peroxyl radicals (ROO•)

They are another group of oxidative radicals present in the cells and the simplest peroxyl radical is HOO•, which is the protonated form of superoxide (O2•−) and is usually termed either as hydroperoxyl radical or perhydroxyl radical. (De Grey, 2002).

Antioxidant enzymes

The ROS are eliminated by various antioxidant enzymes that provide a defense mechanism for all organisms. Antioxidants can safely interact with free radical and can terminate the chain reaction before vital molecules are damaged (Fang, Y.Z et al., 2002). The antioxidants scavenge free radicals directly, or interfere with the generation of free radical-mediated events, and inhibit cellular injury (Sun, Y 1990). Antioxidants are of two types – enzymatic and non-enzymatic antioxidants. The main enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), and Glutathione peroxidase (GPx). Non-enzymatic antioxidants include Glutathione (GSH), Ascorbic acid (Vitamin C), Vitamin E and lipid peroxidase.

SOD converts superoxide anion radical to H2O2, thus preventing the cellular injury and the H2O2 in turn is metabolized by Catalase (CAT) and Glutathione peroxidase (GPx). The homeostasis of a cell depends on the delicate balance in the production of ROS and its removal by various antioxidant mechanisms present in the cell. Failure of antioxidants to detoxify the ROS and imbalance in the production of ROS will lead to oxidative stress causing enzyme inactivation, protein, DNA and lipid degradation (Di Giulio et al., 1995).

Effect of RFR on antioxidant activity - Animal model

The effects of RFR on antioxidant activities of various tissues in different animal models are contradictory. Dasdag, S et al., 2009 reported an increase in antioxidant capacity, catalase enzyme activity and alterations on apoptosis of glial cells in rat brain on exposing the rats to 900 MHz radiation. The repeated exposures of New Zealand rabbit’s eyes to microwaves for 10, 20 and 30 days (5 minutes per day) at power densities of 5 and 10mW/cm² resulted in decreased glutathione concentrations in the cortex and the core of the lens and increased enzymatic activity of carboxypeptidase A and aminopeptidase (Bernat, R., 1985). A long term exposure of albino Wistar rats to RFR from 900 MHz mobile phones resulted in decreased concentration of anti-
oxidant enzyme glutathione peroxidase, catalase and SOD (Ozuguner, F et al., 2006). The exposure of adult rabbits to 1800 MHz mobile phone radiation resulted in structural changes in the testes and decreased antioxidant enzymes SOD, GPx and catalase (Salama, N et al., 2010). The exposure of albino Wistar rats to RFR of 900 MHz and SAR of 1.2 W/kg for four weeks (10 minutes four times a day) resulted in an increased MDA level showing decreased antioxidant activity in the cells. The increased MDA level was reduced by the addition of non-enzymatic antioxidant Vitamin C. The study proved the role of antioxidants in reducing the oxidative stress (Balci, M et al., 2007, Jelodar, G et al., 2012, Motawi, T.K et al., 2014). In-vitro exposure of rat lymphocytes treated with iron ions to 930 MHz electromagnetic fields showed an increased ROS by means of the Fenton reaction (Zmyslony, M et al., 2004). Oxidative stress was reported in the rat kidneys exposed to mobile phone radiation (Oktem, F., 2005).

**Effect of RFR on antioxidant activity – Human beings**

In vitro exposure of human erythrocytes to RFR caused decreased SOD and GPx activity (Moustafa, Y.M et al., 2001). Exposure of MDA-MB-231 breast cancer cell line to 900 MHz radiation from mobile phone resulted in increased apoptosis and oxidative stress (Kahya, M.C et al., 2014). Increased free radical activity in the cells (epithelial, endothelial, epidermal, cardiac muscle cells, fibroblasts etc.) on exposure to EMF resulted in both apoptotic and necrotic changes causing cell death (Singh, N et al., 1994, Simonian , N.A et al 1996, Blumenthal, N.C et al., 1997, Ismael, S.J et al., 1998, Phillips, J.L et al., 1997, Simko, M et al., 1998, Lai, H et al., 2004,). The exposure of human oropharyngeal epidermoid carcinoma KB cells to 1950 MHz GSM fields at SAR of 3.6 W/ kg for three hours resulted in increased apoptosis (Caraglia, M et al., 2005). In vitro exposure of human CCRF- CEM T- lymphoblastic leukemia cells to 900 MHz CW with SAR of 1 mW/kg for 48 hours resulted in increased apoptosis (Marinelli, F et al., 2004 a, b).

**Stress proteins and stress response**

Moreover, reports are available on the alteration of expression of heat shock proteins (HSP) on RF exposure. The heat shock proteins are activated when there is cellular stress and it can potentially harm a cell. Various physical and chemical stimuli are reported to induce cellular stress and RFR is also included in this list. The exposure of Wistar rats to 3G cell phone
radiation caused a transient increase in phosphorylation of HSP 27, HSP 70 and P38 mitogen–activated protein kinase (P38MAPK) which leads to mitochondrial dysfunction resulting in apoptotic cell death (Kesari, K. K et al., 2014). In vitro exposure of human lens epithelial cells (HLEC) to RFR of 1.8 GHz frequency at SAR of 3W/kg for 0 -30 minutes caused an increase in HSP-70 and HSP-27 protein expression (Lixia, S et al., 2006). The exposure of HLEC’s to 1800-MHz GSM-like radiation for two hours with SAR of 1.0, 2.0, or 3.5 W/kg resulted in up-regulation of HSP-70 (Li, H.W et al., 2007, Yu, Y et al., 2008). An increase in extracellular signal regulated kinase (ERK) and HSP 70 was observed in human lens epithelial cells on exposure to 1800 MHz fields with SAR in 2, 3 and 4 w/kg. The effect was seen five minutes after exposure and reached its peak at thirty minutes and lasted for two hours (Yu, Y et al., 2008). Increased levels of reactive oxygen species (ROS) were observed in human lens epithelial cells on exposure to microwave radiation of 1800 MHz for two hours at SAR of 2, 3 and 4 w/kg (Yao, K et al., 2008). Human endothelial cells also showed increased levels of stress proteins on exposure to mobile phone radiation (Leszczynski ,D et al., 2002). The increased stress protein levels with exposure to RFR in the cells clearly demonstrate that they consider the radiation as a potential threat for their survival (Blank, M et al., 2009).

Contradictory reports

However, controversial reports are also available stating exposure of chick embryos to RF radiations for 4 days resulted in 27% decrease in HSP (Di Carlo., 2002). No significant change was observed in antioxidant activities in Wistar albino rats exposed to 3G mobile phone radiations (Dogan, M et al., 2012, Demirel, S et al., 2012). No significant change in apoptosis was observed in human peripheral blood mononucleated cells on both continuous and intermittent exposure (5 min on / 30 min off) to 900-1800 MHz GSM (Capri, M et al., 2004 a, b). In vitro exposure of human K562 cells to 1800 MHz GSM signals with SAR of 2 W/kg for forty five minutes showed no significant change in free radical production (Lantow, M et al., 2006)

RFR AND DNA DAMAGE

DNA is continually damaged by various endogenous factors (free radicals) and exogenous factors (UV, ionizing and nonionizing radiation, chemicals and so on). The damaged DNA is usually repaired by DNA repair enzymes (Philips, J.L., 2009). Any imbalance in DNA
damage and its repair mechanisms or mistakes during repair may result in accumulation of damaged DNA resulting in

- Ageing of the cell (Hart, R.W et al., 1974, Liber, M.R., 1998, Li, H et al., 2008) or

The most common types of DNA damage are DNA strand breaks and DNA cross links (Philips, J.L., 2009). The DNA strand breaks are of two types – Single strand breaks (SSB) and double strand breaks (DSB). DSBs are more lethal. Researchers have postulated different mechanisms by which an EMF interacts with DNA producing damages. Since the energy associated with RF fields is very low \((1.24 \times 10^{-5} \text{ ev})\), it cannot produce any direct breakage of chemical bonds within the molecules. However, RF radiation emitted per cell phone enhances the activity of free radicals in the cells by Fenton reaction. These free radicals are known to produce damaging effects on macromolecules such as DNA, proteins and membrane lipids (Lai, H, et al., 2004, Okten, F et al., 2005, Ozguner, F et al., 2006, Yariktas, M et al., 2005). Another possible mechanism is that, RF radiation is known to produce alterations in the structure of proteins (Bohr, H et al., 2000,. Chiabrera, A et al., 2000, George, D. F, et al, 2008). Thus, structural alteration in DNA repair proteins might have caused changes in its function, leading to DNA damage. (Philips, J.L., 2009)

**Effect of RFR on DNA - Animal model**

Various reports are available on the deleterious effect of RFR on DNA molecules. The exposure of mice to 2450 MHz microwaves for two hours per day at a power density of 1mw/cm\(^2\) for 120, 150 and 200 days resulted in increased DNA strand breaks and rearrangement of DNA segments in testis and brain (Sarkar, S et al., 1994). The exposure of rat brain cells to a 2450 MHz RFR at whole body specific absorption rate (SAR) between 0.6 – 1.2 w/kg for 2 hours resulted in an increased SSB and DSB but the effects were blocked by antioxidants (Lai, H et al., 1995, 1996, 1997, 2005). Their study suggested the role of free radicals in producing DNA strand breaks. Exposing Chinese hamster lung cells to 1800 MHz field at SAR of 3w/kg resulted in DNA damage after 24 hours of exposure (Zhang, D.Y et al., 2006). The acute
exposure of mouse embryonic stem cells to 1.7 GHz fields resulted in DSB (Nikolova, T et al., 2005)

**Contradictory reports in animal studies**

However, some studies have shown contradictory results in DNA damage on exposure to RFR fields. No significant DNA damage was observed in murine C3H10T1/2 fibroblasts after 2 hours of exposure to 847.74 and 835.02 MHz fields (Li, L et al., 2001). The exposure of Molt-4 cells to CDMA, FDMA, IDEN or TDMA modulated RFR for 24 hours did not show any significant change in DNA damage (Hook, G.J et al., 2004). Lagaroye, I et al., 2004a, 2004b reported no significant change in DNA strand breaks, protein–DNA cross links, DNA–DNA cross links in rat brain cells on exposure to 2450 MHz RFR. No significant DNA strand breaks was observed in the cells of rat on long term exposure (two hours/day, five days/week for two years) to 900 MHz GSM microwaves at 0.3 and 0.9 w/kg (Verschaeve, L et al., 2006). No significant double strand breaks was reported in rat brain on exposure to 915 MHz GSM mobile signals (Belyaev, I.Y et al., 2006). Aitken, R.J et al., 2005, reported no significant change in DNA strand breaks from the spermatozoa of mouse caudal epididymis on exposing the mice to 900 MHz GSM signals at SAR of 90mW/kg (two hours/day for seven days).

**Effect of RFR on DNA – Human studies**

An increased DNA damage and SSBs was reported on human lens epithelial cells (HLEC) on exposing to 1800 MHz at SAR of 3 and 4 W/Kg and the DNA damage observed was irreversible on exposing the cells to the fields at 4 W/Kg (Lixia, S et al., 2006, Sun, L.X et al., 2006). However, on exposure of human lens epithelial cells to microwaves of 1800 MHz, showed repairable DNA damage, especially SSB (Lixia, S et al., 2006, Yao, K et al., 2008). Exposing the human fibroblasts and rat granulose cells to 1800 MHz RFR with SAR of 1.2 or 2 w/kg for 4, 16 and 24 hours continuously or intermittently (5 min on / 10 min off) resulted in both SSB and DSB in both cell types (Diem, E et al., 2005). Their study also showed that the intermittent exposure caused more damage than continuous exposure. Gandhi, G et al., 2005, reported increased DNA strand breaks and micro nucleation in lymphocytes obtained from cell phone users. An increased DNA SSB was observed in human hair root cells on short term exposure to 900 MHz RFR from a cell phone. The SSB was predominantly seen in the hair root
cells located around the ear which is used for phone calls (Çam, S.T et al., 2012). The exposure to GSM signals caused double strand breaks (DSB) in human lymphocyte culture (Markova, E et al., 2005).

**Contradictory reports on human studies**

The exposure of in vitro human lymphocytes to 2450 MHz at 2.135 w/kg for two hours showed absence of DNA damage (Vijayalaxmi, B.Z et al., 2000). The exposure of human leukocyte culture to RFR emitted from various forms of cell phone signals for 3 or 24 hours at an average SAR of 1.0 -10.0 w/kg did not show any significant DNA damage (Tice, R.R et al., 2002). Two hour exposure of human leukocytes to 900 MHz GSM signal at 0.3 and 1 w/kg didn't produce significant DNA damage. (Zeni, O et al., 2005)

**EFFECT OF RFR ON HUMAN BEINGS**

The exposure to continuous RFR poses greater risk to the children, the elderly, the frail and pregnant women (Cherry N., 2001). There are many scientific reports confirming the association of exposure to electromagnetic fields from the base stations and cell phones causing depressive symptoms, headache, dizziness, memory changes, tremors, and sleep disturbances. (Hocking, B., 1998, Hocking, B., 2000, Santini, R, et al., 2002, Oberfeld, G., 2004, Hutter, H.P et al., 2006, Abdel – Rassoul, G et al., 2007., Kundi, M, et al., 2009)

**Cognitive functions**

Acute exposure to 2G and 3G cell phone radiation affected human cognitive functions (Leung, S et al., 2011). A decrease in reaction time was observed in volunteers on exposure to GSM mobile phone signals in the frequency range of 902- 915 MHz for 30 minutes to one hour (Preece, A.W et al., 1999, Koivisto, M et al., 2000a, 2000b). Decreased reaction time was also observed in adolescents on exposure to 1900 MHz GSM phone for 25 minutes (Lee, T.M et al., 2003). The exposure to 945 MHz GSM, 1840 MHz GSM (2G) and 2140 MHz UMTS (3G) base station radiation to electromagnetic hypersensitive (EHS) individuals and normal controls showed a significant reduction in overall wellbeing score and cognitive performance in both groups, especially on exposure to UMTS radiation (Kundi, M et al., 2009). The exposure to UMTS signals also caused increased anxiety symptoms, somatic symptoms, inadequacy
symptoms, and hostility symptoms in the sensitive individuals, whereas in the control group only inadequacy symptoms were increased after UMTS exposure. (Zwamborn, A.P.M et al., 2003, Regel, S.J et al., 2006, Eltiti, S et al., 2007, Kundi, M et al., 2009)

**Cancer risk**

A number of scientific reports are available on increased risk of cancer associated with cell phone use. An increased risk of astrocytoma (Hardell, L, et al., 2003), acoustic neuroma (Lonn, S, et al., 2004, Hardell, L, et al., 2006 a) and glioma (Schüz, J et al., 2006) have been reported in cell phone users after ten years of utilization. There were studies which also showed an increased cancer incidence within five years of exposure to radiations from base stations (Wolf, R et al., 2004, Eger, H et al., 2004). The incidence of malignant brain tumors was found to be significantly higher in the age group of 20-29 with the highest risk for those who started using cell phone below 20 years of age (Hardell, L et al., 2004, 2006 a, 2006 b). Based on the increasing evidence of cancer risk in cell phone users, the WHO has included mobile phone radiation on the IARC scale as **group 2B**- possibly carcinogenic (WHO, 2011).

**Reproductive system**

A retrospective study conducted on 304 men showed that frequent use of cell phone for more than two years resulted in decreased sperm motility and increased percentage of abnormal sperm (Wdowiak, A et al., 2007). Another study conducted on 371 men showed increased cell phone use resulted in decreased sperm motility and 30% lower sperm count ((Fejes, I., 2005). The exposure to semen samples to RFR signals at SAR of 1.46 W/kg for 60 minutes resulted in decreased sperm motility and viability. The study also showed increased levels of ROS proving that oxidative stress produced due to RFR exposure in human spermatozoa leads to decreased motility and viability (Agarwal, A et al., 2008).

**Contradictory reports**

Controversial reports are also available showing no health risk on exposure to RFR radiations from cell phone and base stations on human beings. No significant change in wellbeing was found on exposure and no exposure to UMTS (3G) radiation at a frequency of 2167 MHz from the base station. The study was conducted on 300 employees working on a
building where the UMTS antenna was deployed (Heinrich, S., 2007). A study on acute and chronic onset of headache, neurological symptoms, cardiovascular symptoms, concentration problems, sleeping disorders and fatigue on exposure to GSM (900 MHz), GSM (1800 MHz), UMTS, DECT and WLAN (2.45 GHz) showed no significant change in the study population (Thomas, S et al., 2008). The in vitro exposure of semen samples to RFR showed no significant increase in ROS level (Falzone, N et al., 2010). No effect on memory was observed on exposure of healthy volunteers (20-24 years) to 902 MHz GSM phone operating at 0.25 W for 65 minutes (Haarala, C et al., 2004). No effect on cognitive function of children in the age group of 10-14 years were observed on exposure to 902 MHz GSM mobile phone radiation (Preece, A.W et al., 2005, Haarala, C et al., 2005). A case control study by the United States of America (USA) and five European countries showed no increased risk of brain tumors in chronic cell phone users as compared with people who never used or rarely used cell phone (Lahkola, A et al., 2008, Hardell, L et al., 2008, Ahlbom, A, et al., 2009). A series of study by IARC also showed no increased risk of brain tumors in people who used cell phone for more than 10 years (IARC., 2002).

EFFECT OF RFR ON ANIMAL MODELS

Mortality rate

Significant increase in the mortality rate was observed in chick embryos on exposure to RFR emitted from 2G cell phone (Bastide, M et al, 2001, Grigov’ev Iug, 2003, Batellier, F., 2008, Ingole, I.V et al., 2006a, Zareen, N et al., 2009b, Fatima Al Qudsi et al., 2012, Lotfi, A et al., 2012). The mortality rate increased to 75% in chick embryos exposed to GSM mobile radiation and the control embryos showed 16% of mortality rate (Grigov’ev Iug, 2003). The continuous exposure of chick embryos to 900 MHz at a power density of 2 W/kg for 21 days increased the mortality rate to 52-54% in comparison with control group that showed a mortality rate of 15% (Youbicier, B.J et al., 1998). The exposure of chick embryos to 900 MHz radiation increased the mortality rate up to 4.5% in the first four days of incubation, 1% mortality rate in day 5-7, less than 1% on day 7-14 and 6.1% on day 18-21 (Batellier, F., 2008). The exposure of chick embryos to different doses of 900 MHz GSM radiation for 10 days and 15 days resulted in increased mortality rate. (Zareen, N et al., 2009b). Intermittent exposure to 900 MHz RFR from a cell phone with SAR of 0.37 W/kg for a short duration and long duration showed an increased
mortality rate ranging from 11.11% to 22.22% and 11.11% to 77.78% respectively. Maximum casualty was observed in the embryos kept near the antennae (Ingole, I.V et al., 2006a). The continuous exposure to 428 MHz RFR at a power density of 5.5 mW/cm² with a SAR of 3.1 to 47.1 mW/kg for 20 days resulted in increased mortality rate of 60% in the exposed group as compared with 16% of mortality rate in the control group (Saito, K et al., 1991). All the studies showed the correlation of lethality with duration of exposure.

**Teratogenicity**

Various researchers have reported teratogenicity of RFR on developing chick embryos (Saito, K et al., 1991, Farrell, J.M et al., 1997, Lahijani, M.L et al., 2007, Lahijani, M.L et al., 2011, Fatima Al Qudsi et al., 2012). The exposure of chick embryos to 900-1800 MHz RFR from cell phone for 14 days resulted in congenital malformations - Subcutaneous hemorrhage, anophthalmia, head abnormalities and abdominal hernia (Fatima Al Qudsi et al, 2012). On exposing white leghorn chick embryos to 50-60 MHz EMF for 24 hours before incubation resulted in hemorrhage in various tissues, sinusoidal denaturation, increased lymphoid tissue, spina bifida, anophthalmia, monophthalmia, microphthalmia, growth retardation, brain malformation and increased apoptotic cells (Lahijani, M.L et al., 2007, Lahijani, M.L et al., 2011, Mary, H.D et al., 2014b ). The exposure of chick embryos to pulsed magnetic fields resulted in neural tube defects (Farrell, J.M et al., 1997). On exposing chick embryos to EMF resulted in blood brain barrier permeability, increased cellular apoptosis and torn blood vessels (Kalantari, S et al., 2014).

**Growth and development**

Controversial reports are cited on the role of RFR on the growth and development of chick embryos. The chick embryos exposure to 0.07 T magnetic field intensity increased the mortality rate and low weight of hatching chicken (Veterany, L et al., 2001). Retarded growth was observed in chick embryos on exposure to 50 Hz for 24 hours before incubation (Lahijani, M.L et al, 2007). The exposure of chick embryos to 900-1800 MHz increased the growth parameters (the CR length, body length and beak length) and also increased the eye development (eye weight, eye diameter, eye area, eye perimeter) on the tenth day of incubation and a decrease in these parameters was observed on further exposure (Fatima Al Qudsi et al., 2012). Zareen, N
et al., 2009b reported the effect of duration of exposure on growth parameters of chick embryos. They reported increased body growth on long term exposure and decreased growth on short term exposure to 900-1800 MHz radiation. Exposure of chick embryos to 900 MHz signals at different duration and power density showed an increased number of somite pair than normal control group and increased mortality rate on long term exposure with high power density (Jyoti et al., 2014). Decreased fetal weight and crown-rump length was observed in intrauterine exposure of rat and mouse animal models to RFR ranging from 27.12 MHz to 2450 MHz (Lary, J.M, et al., 1982, Marcickiewicz, J et al., 1986., O’Conner et al., 1999., Amer, F.I et al., 2013).

**Structural changes in tissues**

RFR emitted from a cell phone produced both thermal and non-thermal effects. The eyes and testes are the two organs that have poor heat dissipating mechanism, making them vulnerable to the thermal effects of RFR (Habash, R.W.Y., 2008, Hamada, A.J et al., 2011). However, plenty of scientific reports are available on the non-thermal effect of RFR on the eye of various animal models. The retina of chick embryos showed enhanced development up to 10th day of incubation and prolonged exposure till 14th day showed impaired retinal growth and malformed brain on exposing them to RFR of 900 - 1800 MHz for 15 minutes four times a day (Fatima Al Qudsi et al., 2012). However, Zareen, N et al., 2009a reported impaired growth of retina in chick embryos till 10th day of incubation and further exposure up to 15 days showed growth enhancement and hyperpigmentation of retinal pigment epithelium (RPE) on exposing the embryos to RFR emitted from 1800 MHz GSM mobile phone for 15 minutes twice a day. Increased pigmentation of RPE due to RFR exposure was also reported by Jeffrey, G et al., 1994, Ilia, M et al., 2000, Agar, N et al., 2005. According to them, the increased pigmentation could be due to RFR inducing melanogenesis in RPE. Exposure of rats to electromagnetic waves of 50-60 Hz for 4 weeks resulted in increased retinal thickness (Khaki, A.A et al., 2011). In another study, the pregnant CD-1 mice were exposed to RFR from 900-1800 MHz for 2 hours per day from 7th – 14th day of gestation and the retina of exposed fetuses on the 18th day showed increased vacuolations in the cytoplasm of outer and inner nuclear layers, pyknotic nuclei and DNA fragmentation as compared with control group (Amer, F.I et al., 2013).

On exposing the cultured bovine lens to RF radiation of 1.1 GHz and power of 2.2 mw for eight days caused histopathological changes in lens epithelial cells (LECs) leading to the
formation of smaller cells with pyknotic nuclei and the lens also showed an increase in adolase enzyme activities (Bormusov, E et al., 2008). In another experiment, the optical function of cultured bovine lens was impaired on exposure to RF radiation of 1.1 GHz for two weeks. But on withdrawing the radiation the optical function was reversed. However, the microscopic changes in the form of bubbles near the sutures of lens fibres and granulations in the cells persisted even after removal of radiation (Dovrat, A et al., 2005). On exposing white New Zealand rabbit eyes to RFR of 2.45 GHz continuous waves ranging from 12 hours to 123 days resulted in the structural changes in LEC in the form of granular degeneration of the lens epithelial cells at the equator, the appearance of large spherical or ovoid —balloon cells— and distortion of lens fibres. The distortion of lens fibres also resulted in the aberrant arrangement of lens nuclei leading to irregular —lens bowl— arrangement of nuclei and formation of aberrant posterior lens epithelial layer (Carpenter, R.L., 1979). The exposure of cultured rabbit lens epithelial cells (LEC) to 2450 MHz microwave radiation and power density of 0.10, 0.25, 0.50, 1.00, 2.00 mW/cm$^2$ for 8 hours decreased the proliferation of lens epithelial cells. Exposure also caused distorted arrangement of LEC's, cell shrinkage and inhibited the DNA synthesis (Wang, K.J et al., 2003).

Exposure to RFR of 900 MHz with a power of 2 watts and SAR of 0.37 W/kg caused structural changes in the developing kidneys of the chick embryo. The changes reported were in the pattern of increased vacuolations in cells, disrupted basement membrane and brush border of PCT and DCT and clumped glomerulus (Ingole, I.V et al., 2006b).

**Contradictory reports**

No change in testicular structure and sperm count was reported in Sprague-Dawley rats exposed to 890-915 MHz pulsed GSM fields with SAR OF 0.5 W/kg for 20 minutes/day for a period of 1 month (Dasdag, S et al., 2003). On exposure to 900 MHz pulsed GSM with SAR of 4W/kg for 1 hr/day from day-1 gestation to day-19 gestation in mice showed no effect on blood-brain barrier permeability (Finnie et al., 2006a). Under similar exposure conditions, day-1 to day-7, neonatal mice showed no effect on blood-brain barrier permeability (Finnie, J.W et al., 2006b). An exposure of 2.45 GHz at a power density of 430W/m$^2$ for 4 hours showed no abnormalities in the corneal epithelium, lens, retina and vitreous body of monkeys (Kamimura, Y et al., 1994). No histopathological changes in cornea, iris and lens was reported from anaesthetized monkey and rabbit eye to pulsed 60 GHz RFR with power density of 100 W/m$^2$ for
4-8 hours for 5 consecutive days (Kues, H.A et al., 1992). The exposure to 1.25 GHz pulsed RFR with SAR of 20 W/kg for 4 hours/day for 9 days resulted in no histopathological changes in eye of conscious monkeys (Lu, S.T et al., 2000). No structural malformation was reported in chick embryos after exposure to continuous wave of 915 MHz with a power density ranging from 200-800 mW (Krueger, W.F et al., 1975).

The cell phone has become an integral part of our day to day life. Nowadays, they are not just used as devices for communication, but also serve the function of camera, video-recorder, hand-held computer and the like. The growing demand for cell phone usage has led to the installment of more and more base stations / cell towers for effective coverage and communication. These cell towers are kept haphazardly on top of commercial buildings, near schools and colleges, hospitals and also in residential areas (Shivani, S et al., 2012). The general public is not only exposed to radiations from a cell phone, but also to continuous radiations from the cell towers (Kundi, M et al., 2009).

The various inconclusive controversial scientific reports and the rapid proliferation of cell phone industry going for a higher version of Generation cell phones and their possible health impacts on the public has prompted me to undertake this research study.
AIM AND OBJECTIVES

AIM

The present study has been designed to evaluate the damage caused by radiofrequency radiation emitted from 2G and 3G cell phones in developing chick embryos from the 5th day up to the 12th day.

OBJECTIVES OF THE STUDY:

- To study the prenatal effects of RFR exposure from 2G and 3G cell phone on developing and differentiating tissues of the chick embryo.
- To identify the gross congenital anomalies occurred due to RFR exposure from 2G and 3G cell phone.
- To establish the severity of structural damages caused by RFR exposure on developing solid organs like brain, liver, kidneys and eyes of the chick embryo.
- To analyze the gross morphology and microstructural changes caused by RFR exposure.
- To analyze structural changes in quantitative terms using stereological and histomorphometric data to prove the potential damage caused by RFR exposure in comparison with the non-exposed group.
- To evaluate the oxidative stress caused by RFR exposure by analyzing the level of enzymatic antioxidants SOD and GPx.
- To assess the DNA damage in the form of double strand breaks (DSB) using the alkaline comet assay.
- To compare the effects of radio frequency radiation emitted from 2G and 3G cell phones on the chick embryo tissue.
- To compare, to evaluate, to identify and to conclude the range of frequency emitted from 2G and 3G cell phone that imparts maximum damage on the developing tissues of the chick embryo.
MATERIALS AND METHODS

Organogenesis period is regarded as the most critical phase in the dynamic process of development of an embryo. The external and internal environmental insult during this period could result in an adverse outcome (Mary, H. D et al., 2014b). The teratogenicity of radiation emitted from 2G and 3G cell phones were assessed by analyzing the histopathological changes at structural level, the alterations in antioxidant levels and the extent of DNA damage in various organs of developing chick embryo and then compared with the findings of unexposed and sham controlled embryos. All the procedures were followed as per Ethical Guidelines for care and use of experimental animals approved by Institutional Animal Ethics Committee (IAEC). The designed methodology was as follows:

**Animal Model - Gallus domesticus**

The fertilized hen eggs are chosen because of easy availability, they are relatively easy to maintain and handle, growth and development is faster and easy to monitor. Moreover, the risk of changes in the internal environment of mother interfering with the fetal development does not arise. The chick eggs have been extensively used to find the teratogen effect of various drugs, chemicals, insecticides, pesticides and also for the culture of viruses. Hence the developing chick embryo was used as the experimental animal model for the present study.

**Procurement and selection of eggs**

Fresh fertile hen eggs (Gallus domesticus) were procured from Rajiv Gandhi college of Veterinary and Animal Sciences, Puducherry after obtaining permission from the animal ethical committee. The eggs were weighed and eggs having approximately similar weight (65 - 70gms ± 5gms) were selected for incubation in one particular batch. The eggs were initially candled to identify the healthy viable one and to discard the defective one. Selected healthy eggs were washed and then kept in the standard digital egg incubator.
Testing and standardization of the incubator

The following were the specifications of the incubator used:

- **Brand** – Standard digital incubator (Technico, India)
- **Capacity** – 50 eggs.
- **Temperature range** – ambient to 70 °C ± 0.5 °C with thermostat (Figure 2)

![Figure 2: Egg Incubator used for the study.](image)

The efficacy of the standard digital incubator to maintain the 38°C temperature was tested before starting our work. The humidity of the incubator was maintained at 50-55% by keeping a tray full of water. The water was replaced every alternate day and maintained at the same level to maintain the same percentage of humidity throughout the incubation period. The eggs were incubated in three batches of 8 eggs each and the embryos were terminated from 5<sup>th</sup> day up to 12<sup>th</sup> day. The gross morphological features and growth parameters (CR length, volume and
weight) of the embryos of all the three batches were compared for each day (5th day – 12th day). The results showed perfect efficacy of the incubator with embryos showing normal development.

**Procedure for incubation of eggs**

The eggs were incubated in an egg tray in the horizontal array of 6 x 2. A total of 19 batches of 12 eggs each (19 x 12 = 228 eggs) were incubated. The temperature of the incubator was maintained at 37±0.5°C with 50-55% of humidity and adequate ventilation. The eggs were kept with their broad end up. The eggs were rotated manually twice a day along the longitudinal and vertical axis. The eggs were routinely checked with a Candler for the viability of embryos. The dead embryo was removed immediately from the incubator and the viable embryos were allowed to develop further.

**Egg grouping**

The embryos were divided into four groups.

Group A – Exposed to 2G cell phone radiation.

Group B – Exposed to 3G cell phone radiation.

Group C – Sham exposed group (incubated with cell phone kept in null status).

Group D – Control group (incubated without cell phone).

**EXPERIMENTAL DESIGN:**

**INCUBATION OF CONTROL GROUP (GROUP C AND GROUP D)**

The first 4 batches (48 eggs) was grouped as control (Group –D) and the eggs were incubated in a normal procedure without any external factors interfering with the developmental process. Next 6 batches (72 eggs) were treated as sham exposed group (Group-C). They were incubated along with a popular brand cell phone with the SAR of 0.310 watts/kilogram. The cell phone was hung above at a constant distance of 5 cm separating the egg and the phone was put up in null status (switched off mode) (Plate 1A). Six embryos per day were sacrificed from 5th day to 12th day. Morphological and structural features of both Group D and C were analyzed
and found to be similar. Hence we have considered the sham exposed group (Group C) as the control group for comparison with experimental group.

**INCUBATION OF EXPERIMENTAL GROUP (GROUP A AND GROUP B)**

**Source of radiation:**

A popular brand cell phone hand set and a service provider were selected for our study with following specifications.

1. Central frequency - 900-1800 MHz - 2G module
   - 1900 – 2100 MHz - 3G module
2. Power - 2 Watts
3. Specific Absorption Rate (SAR) - 0.310 watts/kilogram.

The same brand hand set operating on 2G and 3G and same service provider were used for both experiment exposures.

**Dosimetry and experiment procedure:**

Different researchers have followed different methodology regarding the duration and frequency of exposure. In the present study, the embryos were exposed for 72 minutes duration over a 12 hour period (4.30am - 4.30pm) followed by 12 hours of exposure-free period in a day. In the first experimental group A, 72 eggs in 6 batches were incubated with a 2G cell phone kept in silent operative mode with the head phone plugged in (switched on) (Plate 1B). This arrangement ensured that the cell phone got activated automatically each time it received a call. For exposure activation, the cell phone was rung from another cell phone and the first exposure was initiated at the 12th hour of incubation at 4.30 AM for 3 minutes duration period. Thereafter, on every half an hour lapse, the cell phone was rung for duration of 3 minutes each till 4.30 PM. This was repeated regularly on subsequent days up to 12th day of incubation. In the second experimental group B, 72 eggs in 6 batches were incubated with a 3G cellphone setup (video call facility) and same standardized procedure of same duration and incubation period for 2G were followed.
PLATE 1: Experiment set up.

A. Experiment set up for control group embryos (Group – C). A popular brand mobile hand set (red arrow) was hung 5 cm above the fertilized eggs and was kept in null status (switched off mode).

B. Experiment set up for Experimental group embryos (Group – A and Group - B). A popular brand mobile hand set (red arrow) was hung 5 cm above the fertilized eggs and was kept in switched on mode (head phones plugged in). A Radio frequency meter (blue arrow) was kept inside the incubator to measure the intensity of radiation.
The internal reflection of frequency waves from the walls of the incubator was prevented by keeping absorptive materials and the intensity of radio frequency waves was measured using radiofrequency meter (RF meter, Less EMF Inc, USA) (Plate 1B). Six embryos per day were terminated from 5\textsuperscript{th} day to 12\textsuperscript{th} day. The total duration of RFR exposure from 5\textsuperscript{th} – 12\textsuperscript{th} day is given in (Table1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Age in days</th>
<th>Number of embryos sacrificed</th>
<th>Duration of Exposure/day</th>
<th>Total duration of exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (exposed to 2G cell phone radiation) and B (exposed to 3G cell phone radiation)</td>
<td>5</td>
<td>6</td>
<td>72 min/day</td>
<td>360 minutes</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6</td>
<td>72 min/day</td>
<td>432 minutes</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>6</td>
<td>72 min/day</td>
<td>504 minutes</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>6</td>
<td>72 min/day</td>
<td>576 minutes</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>6</td>
<td>72 min/day</td>
<td>648 minutes</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6</td>
<td>72 min/day</td>
<td>720 minutes</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>6</td>
<td>72 min/day</td>
<td>792 minutes</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>6</td>
<td>72 min/day</td>
<td>864 minutes</td>
</tr>
</tbody>
</table>

1. GROSS MORPHOLOGICAL STUDY:
   HARVESTING OF EMBRYOS

   The embryos were sacrificed from 5\textsuperscript{th} day onwards and were collected with extreme care and caution in the following manner.

   1. The broader end of the egg shell was cracked by a light stroke with the blunt end of a small plane forceps.
2. The shell was removed in piecemeal with the forceps and the opening was enlarged gradually, thus exposing the vascular layer. (Plate 2-A and B).

3. The yolk was poured out from the egg as much as possible to prevent the embryo getting drowned in the yolk.

4. The following observations were made at this stage:
   - **Condition of vasculosa**: Whether the blood vessels were looking bright red and healthy or appearing brownish and unhealthy indicating moribund state of embryo.
   - **Appearance of yolk**: Whether the yolk were looking smooth or looking turbid. Turbid yolk indicated dead embryo.

5. **Physical condition of embryo**: Whether the heart beats and movements were visible or not. Embryos without heart beat and movements indicated moribund state and were discarded.
   
   Total number of dead embryos in control and experimental groups from 5th - 12th day were recorded to compare the mortality rate.

6. The embryo along with the part of vasculosa was cut carefully using a pair of fine scissors and was scooped by a small spoon and removed from the shell (Plate 2-C).

7. Embryo was then transferred carefully to a small petridish containing normal saline (Plate 2-D). The following features in the embryos were observed and recorded.
   - **Curvature of embryo**: Healthy embryos showed normal body flexure whereas moribund and dead embryos showed decreased or absence of flexure.
   - **Congenital anomalies**: The embryos were examined for any apparent congenital anomalies in the form of absence of limb buds and any eventration of thoracic or abdominal viscera
   - **Condition of eye balls**: Normal embryos showed prominent, bulging and large eyeballs compared to the size of the embryo. Deviations from normal appearance, if any, were noted.
   - **Condition of brain vesicles**: The early stages of normal embryo showed very large, bulging and translucent brain vesicles. Any abnormalities in the brain vesicles were noted.
PLATE 2: Extraction of chick embryo from the egg.

A. Removing the shell in piecemeal and exposing the vasculosa.
B. Egg showing healthy looking blood vessels in the vasculosa.
C. Scooping the embryo with a spoon.
D. Transferring the embryo in normal saline.
GROWTH PARAMETERS:

The volume, the weight and the crown rump (CR) length of embryos were recorded to compare the growth parameters in the control group and both experimental groups.

- **Volume:** The volume of small embryo was measured by fluid displacement method. In a syringe of 5ml capacity, normal saline was taken up to certain milliliters mark (2ml; V₁). The embryo was gently dropped in the normal saline in the syringe, which raised the level of saline through a fraction of milliliter which was not possible to be measured accurately. Hence, a tuberculin syringe with 1.0 ml of saline in it was used to add saline drop by drop to the 5ml syringe till the level touched next ml mark (say for eg. 3 ml; V₂). The volume of saline added from tuberculin syringe was recorded (say it was 0.58 ml; V₃). The volume of the embryo was calculated as follows: Volume of the embryo = (V₂ - V₁) - V₃ = (3-2) - 0.58 = 0.42ml.

For larger embryos a measuring cylinder of 50 ml capacity was used. The saline was filled to a certain milliliter mark (say 5 ml; V₁). The embryo was gently submerged in the cylinder and the raised level of the saline was noted (say 7 ml; V₂). The volume was then calculated as V₂ - V₁ = 7-5 = 2ml.

- **Weight:** Before weighing, the embryos were gently kept on a blotting paper and blotted dry. The weight of the embryo was then measured using a digital weighing balance (Denver instruments).

- **CR length:** CR length was measured by placing the embryo on a paper and marking the two poles. The distance between the points was considered as CR length.

The readings were recorded and analyzed statistically for all the groups.
2. HISTOLOGICAL STUDY:

Four batches of eggs from each group, Group A - exposed to 2G radiation, Group B - exposed to 3G radiation and Group C - sham control and Group D control group were used subjected for histological study (Table 2).

Table 2: Total number of eggs incubated in each batch

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of eggs incubated per batch</th>
<th>Number of batches</th>
<th>Total number of eggs incubated</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (2G)</td>
<td>12</td>
<td>4</td>
<td>48</td>
</tr>
<tr>
<td>B (3G)</td>
<td>12</td>
<td>4</td>
<td>48</td>
</tr>
<tr>
<td>C (sham control)</td>
<td>12</td>
<td>4</td>
<td>48</td>
</tr>
<tr>
<td>D (control)</td>
<td>12</td>
<td>4</td>
<td>48</td>
</tr>
</tbody>
</table>

All the chemicals used in the histological studies are of analytical grade procured from Qualigens, Merck and Hi media.

FIXATION:

Soon after harvesting the embryos, the volume was measured. The embryos were then fixed in 4% formal saline in a petridish to prevent the autolytic changes. The weight and CR length were recorded after the initial fixation. The embryos were then put into a large quantity of fixative, 10% formal saline, for further fixation (20 times more than the volume of the embryo). 5 - 7 days old embryos were allowed to remain in the fixative for 48 hours. 8 – 12 days old embryos were subjected to fixation for 72 hours. For bigger embryos (10 - 12 days) the head and torso were dissected and then fixed for better perfusion. After fixation the embryos were washed in running tap water for 24 hours and subjected to graded dehydration.

DEHYDRATION:

Embryos were put in 70% alcohol overnight followed by a second change in 70% alcohol for one hour. The embryos were then further dehydrated with two changes of 90% alcohol followed by three changes of absolute alcohol for one hour each (Plate 3-A).
CLEARING:

After dehydration, the embryos were cleared with two changes of xylene for half hour duration. Precautions were taken while clearing small size embryos were as they required less time for clearing.

PARAFFIN EMBEDDING:

After clearing, the embryos were subjected to paraffin embedding with three changes of 100% paraffin maintained at 62° C. i.e. 2° C above the melting point of paraffin used. For smaller embryos that were 5 – 7 days old, each change lasting for half an hour duration was performed. But for larger embryos (8- 12 days old) each change lasted for one hour duration.

After embedding, paraffin blocks were prepared using Lukhardt’s ‘L’ molds. Out of six embryos sacrificed each day, two embryos in each group were oriented in such a way as to cut in coronal plane, transverse plane and sagittal plane. For large sized embryos 10 – 12 days old, two separate blocks of head and torso were prepared.

SECTIONING:

Five micron thick sections were cut in sagittal, coronal and transverse plane using a rotary microtome (Senior Rotary microtome, model: RMT- 30). The sections were then serially mounted on the slides using floatation bath according to standard technique and allowed to dry completely.

STAINING:

The sections were stained using haematoxylin and eosin and periodic acid Schiff’s (PAS) staining and examined under Trinocular research microscope fitted with micro photographic attachment (Olympus, Japan). (Plate 3–B and C).
PLATE 3.

A. Tissue processing.
B. Staining of the slides.
C. Trinocular research microscope with micro photographic attachment (Olympus, Japan).
1. **Haematoxylin and Eosin staining** (Bancroft, J.D and Layton, C., 2012)

a) **Preparation of Delafield’s haematoxylin:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematoxylin</td>
<td>4 g</td>
</tr>
<tr>
<td>95% alcohol</td>
<td>125 ml</td>
</tr>
<tr>
<td>Saturated aqueous ammonium alum</td>
<td>400 ml</td>
</tr>
<tr>
<td><em>(15 g/100 ml of distilled water)</em></td>
<td></td>
</tr>
<tr>
<td>Glycerin</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

The Haematoxylin powder was dissolved in 25 ml of alcohol and then added to alum solution. The mixture was ripened for 5 days and then filtered. Glycerin and 100 ml of 95% of alcohol were added to the solution which was further ripened for 3–4 months. The solution was then filtered and stored in a tightly stoppered bottle.

b) **Preparation of Eosin Y (1%):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eosin Y</td>
<td>1 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>Thymol crystal</td>
<td>1</td>
</tr>
</tbody>
</table>

The eosin Y was dissolved in distilled water and Thymol crystal was added to prevent fungal growth. The solution was then filtered and stored in a tightly stoppered bottle.

**Staining procedure:**

- Sections were deparaffinized with xylene (2 changes of 5 minutes each).
- Sections were then passed through descending grades of alcohol from absolute alcohol through 90%, 70% to 50% alcohol for one minute each.
- Washed in distilled water.
- Stained with Delafield’s Haematoxylin for 10 minutes.
- Washed in running tap water for 10 minutes (bluing).
- Differentiated in 1% acid alcohol (1% HCl in 70% alcohol) for 5-10 seconds.
- Sections were washed again in running tap water for 10 minutes.
- Stained with eosin Y for 3-5 minutes.
- Washed in distilled water.
• Dehydrated in ascending grade of alcohol from 70% alcohol through 90% alcohol to absolute alcohol for one minute each.
• Cleared in xylene (2 changes of five minutes each)
• Mounted in D.P.X.

2. **Periodic acid Schiff’s (PAS) staining** (Drury & Wallington., 1980)
   a) **Preparation of periodic acid solution:**
      
      Periodic acid 1 g
      Distilled water 100 ml
      
      The periodic acid was dissolved in distilled water, filtered and stored in refrigerator.

   b) **Preparation of Schiff’s reagent:**
      
      Basic fuchsin 1 g
      Distilled water 200 ml
      
      When dissolved, it was cooled and filtered and stored in refrigerator.

   c) **Preparation of Harris’s Haematoxylin** (Bancroft, J.D and Layton, C., 2012):
      
      Haematoxylin 2.5 g
      Absolute alcohol 25 ml
      Potassium alum 50 g
      Distilled water 500 ml
      Mercuric oxide 1.25 g
      Glacial acetic acid 20 ml
      
      Alum was dissolved in warm distilled water in a 2 litre flask. Haematoxylin was dissolved in absolute alcohol and then added to alum solution. The mixture was then rapidly brought to boil and the mercuric oxide was slowly and carefully added. The stain was rapidly cooled by plunging the flask into cold water and acetic acid was added. The stain was then filtered and stored.
**Staining procedure:**

- Sections were deparaffinized.
- Sections were then passed through descending grades of alcohol from absolute alcohol through 90%, 70% to 50% alcohol for one minute each.
- Sections were washed in distilled water.
- Oxidized for 5 minutes in 1% aqueous solution of periodic acid.
- Washed in running tap water for 5 minutes and then rinsed in distilled water.
- The sections were placed in Schiff’s reagent for 20 minutes, which was brought to room temperature, followed by washing in running tap water for 10 minutes.
- Counterstained with Harris’s Haematoxylin for 2 minutes.
- Differentiated in 1% HCl in 70% alcohol by a short dip in for 5-10 seconds.
- Washed again in running tap water for 10 minutes.
- Dehydrated in ascending grades of alcohol, cleared in xylene and mounted in D.P.X.

**SELECTION OF THE ORGANS FOR THE STUDY:**

The stained sections of embryos were observed under research microscope. Scanning of the tissue was done to identify the location and differentiation of different organs at different periods of incubation and harvested age in all the four groups. Since the solid organs absorb more radiation than the hollow organs, the following solid organs were selected for our histological study.

**A. Kidney:**

The mesonephric kidney of chick embryo starts its development at around 55 hours of incubation and its functional activity is at its height from the 5th – 11th day. After 11th day the mesonephros degenerates and metanephros begins to become active. Hence, any developmental insult due to RFR exposure on mesonephros at an early stage of embryonic period might manifest at the cellular level as structural changes and could be observed.

A general examination of the sections under light microscope, showed kidney tubules of sham control embryos (Group C) with sporadic appearance of vacuolations in the cytoplasm and pyknotic nuclei which could be a part of apoptosis in the developing organs. These changes were
very prominent in both experimental Group A and Group B embryos. The following indices were taken into consideration for assessing cellular degeneration in PCT.

- Cytoplasmic vacuolations.
- Nuclear changes in the form nuclear fragmentation (karyorrhexis) and pyknotic changes.
- Disruption of brush border
- Disruption of basement membrane.

B. Liver:

The hepatic primordium becomes recognizable in chick embryos as early as 22 – somite stage (72 hours). Its rapid cell division and differentiation into metabolically and functionally active cells makes it an ideal organ to study the teratogenicity of RFR, if any, at structural level. Degenerative changes in hepatocytes in the form of cytoplasmic vacuolations, pyknotic changes and sinusoidal congestion were observed and compared in all the groups.

C. Eye:

The primary optic vesicles in chicks develop at about 30 hours of incubation and lens primordium at 40 hours of incubation. The close proximity of the eye to the cell phone while in use has prompted us to choose it for histological study to assess the damaging effects of RFR on developing the eye. The gradation of retinal pigment epithelium, intercellular spaces in each layer of retina, the arrangement of lens fibers and any other structural changes were observed and compared in all the groups.

D. Brain:

The neural plate develops as early as 16 – 18 hours of incubation. Hence the effect of radiation, if any, at the stage of cell division and differentiation would be evident at cellular level.

3. HISTOMORPHOMETRY (ELIAS AND HYDE 1980)

Histomorphometry is a known technique for the quantification of alterations in tissue elements under experimental conditions. It involves the usage of quantitative data to describe structural elements. Accepted morphometric procedures were followed in our study to obtain
quantitative information about the thickness of retina and lens and the extent of cellular damage in the kidney and liver.

In the present study, every third section from each slide was analyzed and the data was collected in such a way that they are representative of the whole organ. The study was done using the ocular micrometer (1cm scale), stage micrometer (1mm scale) and reticule (1-cm$^2$ grid of square lattice containing 441 intersections) as described by Elias and Hyde(1980) (Figure:3).

**Figure: 3**

Calibration of Ocular Micrometer:

The ocular micrometer consists of a 1 cm scale engraved on a circular lens and stage micrometer consists of a 1 mm scale engraved on a glass slide. Both scales were divided into hundred divisions. The distance between two divisions of stage micrometer was 0.01 mm. The image of ocular micrometer which was placed in the focal plane of the eye piece was superimposed over that of the stage micrometer until the zero lines of each scale were coinciding with each other. The number of divisions was counted between the zero line and the line coinciding to the far right on both scales. Finally the number of stage divisions was divided by
the ocular divisions to determine the number of mm in each ocular unit. This value, when multiplied by 0.01mm gives the calibration constant in mm.

For example, under 40x, 100 lines of oculometer coincided with 25 lines of stage micrometer. Thus the calibration constant was calculated as follows.

\[
\text{Calibration constant} = \frac{25}{100} \times 0.01\text{mm} = 0.0025\text{mm}
\]

This constant was calculated for 10x, 40x and 100x objective and the calibration constant value was used for morphometric analysis.

The following parameters were observed and analyzed statistically in the control group and both the exposed groups.

**A. Kidney:**

a) **Measurement of lining cell height of PCT and DCT**

The standard epithelial height (SEH) in both PCT and DCT was measured from the basement membrane to the tubular lumen using calibrated ocular micrometer under 40x magnifications. The measurement was taken from every third section of each slide using ocular micrometer and 20 such sections were examined from each embryo. Thus a total of 120 readings (20 x 6) were taken for each day. The data was compared and analyzed statistically.

b) **Measurement of nuclear diameter of cell lining PCT and DCT**

The nuclear diameter of both PCT and DCT was measured under oil immersion using calibrated ocular micrometer in a similar manner used for the measurement of epithelial height of PCT and DCT. The data obtained were analyzed statistically for all the groups.

c) **Calculation of diameter of glomerulus and urinary space**

The outer diameter (A) and inner diameter (B) of glomerulus were also measured in a similar manner under 40x magnifications. The urinary space was then calculated (A – B) and the data were analyzed statistically.
d) Calculation of karyorrhexia in kidney

For comparing nuclear changes in the form of fragmentation (karyorrhexia), the number of nuclei showing karyorrhexia was counted in a randomly selected field using a square reticule from every third section of each slide. This study was done under oil immersion and a total of 50 such fields were analyzed statistically for control and both experimental groups. The data were recorded and analyzed statistically.

B. Liver:

For comparing the effect on the liver, two parameters were taken into consideration.

a) Measurement of nuclear diameter of hepatocytes

The nuclear diameter of hepatocytes was measured with the help of ocular micrometer under oil immersion. The healthy nuclei showing prominent nucleoli were used for the measurement. The diameter was measured from a randomly selected field of every fifth section from each slide. A total of 100 such readings were taken from control and both experimental group of each day and compared statistically.

b) Calculation of karyorrhexia:

The study was done in a similar manner done for the calculation of karyorrhexia in kidney. The data were recorded and analyzed statistically.

C. Eye:

a) Measurement of retinal thickness:

The thickness of each layer of the retina was measured in every fifth section of each slide using an ocular micrometer and 20 such sections were examined from each embryo. The measured values were then multiplied with calibration constant. Thus a total of 120 retinal readings (20 x 6) were taken for each day. The observations were scanned under 40x magnifications. The data were analyzed statistically.

b) Measurement of Lens diameter

The transverse diameter and thickness of the lens were also measured from every third section using ocular micrometer under 10 x magnifications. More than 100 such readings were taken for each day and analyzed statistically for all the groups.
4. ESTIMATION OF ENZYMATIC ANTIOXIDANTS

One batch of fertilized eggs (12+12+12) Group A, Group B and Group C were used for the estimation of activity of antioxidants SOD and GPx. Six embryos from each 3 groups were sacrificed on 9th day and 12th day. The eyes, the brain and the liver of embryos were dissected out and washed carefully in ice cold normal saline and stored in low temperature freezer at -80°C (U 410, New Brunswick Scientific, Germany) for enzyme study (Figure 4).

![Low temperature freezer](image)

Figure 4: Low temperature freezer

Out of six embryos sacrificed, three embryos were used for the estimation of SOD activity and remaining three were used for the estimation of GPx activity. EnzyChrome ™ Super oxide Dismutase and Glutathione Peroxidase assay kits were used (BioAssay Systems, USA) for the estimation (Figure 5 and 6).
1. QUANTITATIVE COLORIMETRIC ESTIMATION OF SOD ACTIVITY

**PRINCIPLE:**

In the assay, super oxide \( (O_2^\cdot) \) was provided by Xanthine oxidase (XO) catalyzed reaction. \( O_2^\cdot \) reacts with WST -1 dye to form a colored product. SOD scavenges the \( O_2^\cdot \) so that \( O_2^\cdot \) available for chromogenic reaction will be less. The color intensity (OD\(_{440nm}\)) was used to determine the SOD activity in a sample.

**KIT CONTENTS:**

- Assay Buffer - 20 ml
- SOD enzyme - 120 µl
- Xanthine - 600 µl
**Dilutent** - 20 µl

**XO Enzyme** - 120 µl

**WST – 1** - 600 µl

**PREPARATION OF TISSUE SAMPLES AND REAGENTS:**

a. **Preparation of tissue samples**

The tissue was perfused thoroughly with cold PBS to remove any red blood cells. It was homogenized in cold lysis buffer at 5ml/g in tissue homogenizer (ULTRA TURRAX - IKA®, Germany)(Figure 7) (Plate 4-A) and centrifuged at 12,000 rpm in high-speed refrigerated centrifuge to pellet any debris (REMI CPR -24 plus, India) (Figure 8) (Plate 4- B and C) for 5 minutes at 4°C. The supernatant was used for the assay.

![Figure 7: Tissue homogenizer (ULTRA TURRAX - IKA®, Germany)](image)

![Figure 8: High-speed refrigerated centrifuge (REMI CPR -24 plus, India).](image)

b. **Preparation of cold lysis buffer**

Potassium phosphate - 50 mM

EDTA - 0.1mM

Triton X-100 - 0.5%

The reagents were mixed well and stored in refrigerator.
c. Preparation of SOD standard solution

SOD enzyme - 8 µl
Dilutent - 392 µl

The reagents were mixed together and the solution was then diluted according to the table given below (Table 3).

Table 3: Preparation of SOD standard solution

<table>
<thead>
<tr>
<th>No</th>
<th>3U/mL SOD + Dilutant</th>
<th>Standard (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 µL + 0 µL</td>
<td>3.0</td>
</tr>
<tr>
<td>2</td>
<td>80 µL + 20 µL</td>
<td>2.4</td>
</tr>
<tr>
<td>3</td>
<td>60 µL + 40 µL</td>
<td>1.8</td>
</tr>
<tr>
<td>4</td>
<td>40 µL + 60 µL</td>
<td>1.2</td>
</tr>
<tr>
<td>5</td>
<td>18 µL + 82 µL</td>
<td>0.54</td>
</tr>
<tr>
<td>6</td>
<td>8 µL + 92 µL</td>
<td>0.24</td>
</tr>
<tr>
<td>7</td>
<td>4 µL + 96 µL</td>
<td>0.12</td>
</tr>
<tr>
<td>8</td>
<td>0 µL +100 µL</td>
<td>0.0</td>
</tr>
</tbody>
</table>

d. Preparation of working reagent

Assay buffer - 160 µl
Xanthine - 5 µl
WST-1 - 5 µl

The reagents were mixed thoroughly and were prepared for each well.

PROCEDURE: (Plate 4).

- All the reagents were brought to room temperature (25°C) before assay.
- The enzyme tubes were centrifuged briefly before the preparation of reagents.
- 20 µl of SOD standard solution were transferred to separate wells of 96-well plate.
- 20 µl of tissue sample were also transferred to separate wells.
160 µl of working reagent were transferred to each well and the plate was tapped well to mix the contents.

The XO enzyme was diluted with the Diluent in the ratio 1:20 for each well.

20 µl of this diluted XO enzyme were added quickly to each assay well and the plate was tapped well to mix the contents.

Optical density (OD $440$ nm) were then read immediately (OD $420-460$ nm) (OD$_0$) using UV-Vis spectrometer (SPECTROstar $Nano$ BMG Labtech, Germany).

Contents were then incubated in dark for 60 minutes at room temperature and then OD$_{420}$nm was read again (OD$_{60}$) (Plate 4-D, E and F).

The SOD activity was expressed in units/g of protein. The readings were recorded and analyzed statistically for all the groups.
PLATE 4: Procedure for enzyme study

A. Homogenizing the tissue.
B. Vials are kept inside the High-speed refrigerated centrifuge.
C. Centrifuged at 12,000 rpm.
D. Assay wells are prepared by filling with tissue sample and reagents.
E. Loaded in UV- Vis spectrometer.
F. Optical density was read at (OD $440\text{ nm}$) and (OD$_{600}$).
2. QUANTITATIVE COLORIMETRIC ESTIMATION OF GPX ACTIVITY

PRINCIPLE:

GPx prevent lipid peroxidation of cellular membranes by removing free peroxide in the cell. GPx catalyzes the following reaction with glutathione reductase (GR):

\[
GPx \rightarrow 2\text{GSH} + \text{ROOH} \rightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O}
\]

\[
GR \rightarrow \text{GSSG} + \text{NADPH} \rightarrow 2\text{GSH} + \text{NADP}^+
\]

The assay directly measures NADPH consumption in the enzyme coupled reactions. The measured decrease in optical density at 340 nm is directly proportional to enzyme activity in the sample.

KIT CONTENTS:

- Assay buffer - 25 ml
- GR enzyme - 1 ml
- Glutathione - 240 µl
- NADPH - 40 µl
- Calibrator - 100 µl
- Cumene Hydroperoxide - 50 µl
- Positive control - 9 µl GPx.

PREPARATION OF TISSUE SAMPLES AND REAGENTS:

a) Preparation of tissue samples

10 mg of the tissue was homogenized in 200 µl of cold 1X PBS in tissue homogenizer (ULTRA TURRAX - IKA®, Germany) and then centrifuged at 14,000 rpm for 10 minutes in
high-speed refrigerated centrifuge to pellet any debris (REMI CPR -24 plus, India) (Figure 7 and 8). The clear supernatant was used for the assay.

b) Preparation of NADPH reagent

NADPH - 40 µl

Distilled water - 360 µl

The contents were mixed well by vortexing in the NADPH tube and the tube was stored on ice.

c) Preparation of reconstituted reagent

Assay buffer - 500 µl

Positive control - 9 µl GPx

The contents were mixed in the positive control tube by vortexing and then stored on ice.

d) Preparation of standard solution (calibrated stock solution)

Calibrator - 12 µl

Distilled water - 188 µl

They were mixed well and this calibrated stock solution was then diluted based on the following table. (Table 4)

<table>
<thead>
<tr>
<th>No:</th>
<th>6 mM calibrator + H2O</th>
<th>Volume (µL)</th>
<th>[Equivalent NADPH] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100 µL + 0 µL</td>
<td>100</td>
<td>6.0</td>
</tr>
<tr>
<td>2</td>
<td>60 µL + 40 µL</td>
<td>100</td>
<td>3.6</td>
</tr>
<tr>
<td>3</td>
<td>30 µL + 70 µL</td>
<td>100</td>
<td>1.8</td>
</tr>
<tr>
<td>4</td>
<td>0 µL + 100 µL</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>
e) Preparation of working reagent

Assay buffer - 84 µl

Glutathione - 2 µl

35 mM NADPH - 3 µl

GR enzyme - 8 µl

Working reagent was prepared by mixing these reagents for each well.

f) Preparation of Cumene hydroperoxide substrate solution

Cumene hydroperoxide - 5 µl

Distilled water - 1495 µl

The cumene hydroperoxide was transferred into an empty 1.5 ml Ependorff tube and distilled water was added into it. The contents were mixed well by vortexing for 30 sec. This solution was then diluted in the ratio of 1:10 in distilled water to generate 1X substrate solution.

PROCEDURE: (Plate 4)

- All the reagents were brought to room temperature (25°C) before assay.

- The enzyme tubes were centrifuged briefly before the preparation of reagents.

- 10 µl of standard solution (calibrated stock solution) were transferred into wells of a clear flat bottom 96- well plate.

- 190 µl of assay buffer were then added to all standard wells.

- 10 µl of prepared tissue sample and 10 µl of reconstituted GPx positive control were transferred into separate wells of 96- well plate.

- In addition to these, a back ground control containing 10 µl of assay buffer were also included for each assay run.
- 90 µl of working reagent were added quickly to the sample and control well and the plate was tapped to mix the contents.

- 100 µl of 1X cumene hydroperoxide substrate solution were added to all sample and control wells and the plate was tapped to mix the contents thoroughly (Plate 4- D).

- Optical density (OD \(340 \text{ nm}\)) were then read immediately (time zero, OD\(0\)) and again at 4 minutes (OD\(4\)) using UV- Vis spectrometer (SPECTROstar\(^{Nano}\) BMG Labtech, Germany). (Plate 4- E and F)

The GPx activity in the tissues was expressed in U/mg protein. One unit is the amount of GPx that produces 1 µ mole of GS-SG per minute at pH 7.6 and room temperature. The readings were recorded and analyzed statistically for all the groups.

**5. ESTIMATION OF DNA DAMAGE USING COMET ASSAY.**

One batch of fertilized eggs (12+12+12) from Group A, Group B and Group C were subjected for the estimation of DNA damage using alkaline comet assay technique (Singh NP et al., 1988) with modifications in staining procedure (Rajesh B., 2014). Three embryos form each group were collected from 9\(^{th}\) to 12\(^{th}\) day. The eyes, brain and liver were dissected and minced in Hanks Balanced Salt Solution (HBSS w/ Phenol Red w/o ca and mg, Cat.No.55021C. SIGMA\(^{®}\)). The cell suspension was used for the comet assay (Plate 5- A, B, C and D)

**PRINCIPLE:**

The single cell gel electrophoresis (SCGE) / comet assay, developed by N.P. Singh is a simple, reliable and sensitive technique used for quantitation of low level DNA damage and repair in individual cells. The damaged DNA fragments with negative charge move outside the cell towards the anode leaving a trail resembling a comet’s tail and measurement of this tail gives the extent of DNA damage (Figure 9). The low current used in this electrophoresis does not cause the movement of normal cell DNA. Thus the degree of DNA damage can be quantified by this migration.
PREPARATION OF REAGENTS

a) Preparation of Ca\(^{++}\), Mg\(^{++}\) free Phosphate Buffered saline (PBS)

Sodium Chloride (NaCl) - 8.0 g  
Potassium Chloride (KCl) - 0.2 g  
Disodium Orthophosphate (Na\(_2\)HPO\(_4\)) - 1.15 g  
Potassium Dihydrogen Phosphate (KH\(_2\)PO\(_4\)) - 0.2 g

The reagents were weighed and dissolved in 500ml of distilled water and then made up to 1000ml. The pH was adjusted to 7.4, filtered and stored at 4°C.

b) Preparation of 0.5% Low Melting point Agarose (LMA)

Agarose - 125 mg  
PBS - 25 ml

These were mixed and heated until near boiling to dissolve the agarose and the mixture was stored in 5ml aliquots at 4°C.
c) **Preparation of 0.67% Normal Melting point Agarose (NMA)**

Agarose - 167 mg

PBS - 25 ml

These were mixed and heated until near boiling to dissolve the agarose and the mixture was stored in 5ml aliquots at 4°C.

d) **Preparation of Lysing Solution – Stock Solution**

The stock (890ml) was prepared by dissolving

- NaCl - 146.1 g
- EDTA - 37.2 g
- Tris - 01.2 g

These were dissolved in 700ml double distilled water and stirred. 10 gms Sodium Lauryl Sarcosinate was added and the contents stirred again. To dissolve the contents, 12gms of NaOH was added. The pH was adjusted to 10. The lysing stock solution thus prepared was filtered and stored at room temperature.

e) **Preparation of working Lysing solution**

- Triton X-100 - 1%  

  *(1ml dissolved in 100ml of distilled water)*

- Dimethyl Sulphoxide (DMSO) - 10%

  *(10 ml dissolved in 100ml of distilled water)*

This was added to the fresh stock solution and refrigerated 1 hour prior to use.

f) **Electrophoresis Buffer (Alkaline)**

1. **Preparation of 10N NaOH stock solution**

- NaOH - 200 g

This was dissolved in 500ml of double distilled water and was stored at room temperature in dark bottle.
2. Preparation of 200mM (EDTA) solution

EDTA - 14.89 g

It was dissolved in 200ml double distilled water; pH was adjusted to 10, filtered and stored at 4°C.

3. Preparation of working buffer

NaOH stock solution - 7.5 ml
EDTA stock solution - 1.25 ml

The solutions were mixed and the volume was adjusted to 250ml with double distilled water, pH>13.

(The working solution is to be made fresh before each run)

g) Preparation of Neutralizing buffer

TRIS - 14.55 g

It was dissolved in 300ml of distilled water; pH was adjusted to 7.5, filtered and stored at 4°C.

h) Preparation of Fixing Solution – 500ml

Trichloroacetic acid - 75 g
Zinc Sulphate - 25 g
Glycerol - 25 g

The reagents were mixed in 500ml of double distilled water.

i) Preparation of Staining Solution

Staining solution (A) – 500ml

Sodium Carbonate - 25 g

It was dissolved in 500ml of distilled water and stirred vigorously.

Staining solution (B) – 500ml

Silver Nitrate - 100 mg
Tungstosaliclyc acid - 500 mg
Formaldehyde - 250 µl
These were dissolved in 500ml double distilled water.

**Staining solution (C) – 100ml**

Solution A  -  32 ml  
Solution B  -  68 ml  
They were mixed together fresh before each staining procedure.

**j) Preparation of stopping solution**

Glacial Acetic acid  -  1%  
(1ml glacial acetic acid dissolved in 100ml of double distilled water)

**PROCEDURE:**

- The organs selected (eyes, brain and liver) were minced in ice cold Hanks balanced salt solution (HBBS) in a petridish (Plate 5- A, B, C and D)
- The cell suspension was then used to prepare the slides as mentioned below (Plate 5- E)

1. **Preparation of slides**
   
   The slides were prepared in triplicates for each embryo.
   
   i. **First layer**
      
      Dust free, plain slides were covered with a layer of 140µl of 0.67% NMA and allowed to dry for 10 minutes in hot air oven.  
      
      *(This layer serves as an anchor for additional layers to prevent the slippage)*
   
   ii. **Second layer**
      
      20µl of cell suspension was mixed with 110µl of warm 0.5% LMA and this mixture was layered as second layer and gelled at 4°C for 10minutes.  
      
      *(At 4°C, the removal of the cover slip is facilitated with ease)*
   
   iii. **Third layer**
      
      An additional layer of 110µl of LMA was added on top and gelled again in a similar way to the one mentioned above.  
      
      *(To sandwich the middle sample layer and to prevent the loss of sample)*
2. **Lysing of cells**
- After the third layer of gel was set, the slides were treated overnight in freshly prepared chilled lysis buffer solution at 4 °C. (*With this treatment the cell membrane and nuclear membrane were lysed and the majority of proteins were removed to expose the nucleoids*)
- The slides were then removed from the lysing solution, drained and placed in a horizontal gel electrophoresis tank (Tarsons Electrophoresis power supply unit (code: 7090) and Midi Submarine Electrophoresis unit (7020), India) side by side avoiding in between spaces with the agarose end of the slide facing the anode. The tank was filled carefully with fresh electrophoresis buffer solution to a level approximately 0.25cm above the slides (Plate 5- F and G)
- The slides were left in the high pH (>13) buffer for 30mins. (*to allow unwinding of DNA and expression of alkali labile sites before electrophoresis*)
- Electrophoresis was carried out at room temperature for 30mins at 300mA, 20V. (*DNA fragments if any, due to DNA damage, migrate into gel*) (Figure:9)

3. **Neutralizing**
- After electrophoresis the slides were flooded gently with chilled neutralizing buffer (Tris pH 7.5) for 5mins thrice. (*to remove any traces of detergents and alkali which would otherwise interfere with staining*)

4. **Silver nitrate staining**
- The slides were washed thrice with distilled water and air dried completely.
- The air-dried slides were briefly immersed in the fixing solution for 10mins.
- Washed gently with double distilled water several times, the slides were allowed to air dry for about 1 hour before staining.
- Just before staining 68ml of staining solution (B) was mixed with 32ml of staining solution (A) and poured over the dried slides so as to cover the slides uniformly. This step was repeated with a fresh mixture of staining solutions until a greyish color developed on the slides. (*no need of treatment with stopping solution*)
- The whole procedure of Comet Assay was carried out in dim light to minimize artificial DNA damage.
Randomly selected 100 cells from the tissues were then analyzed using automated comet scoring software (Comet Score IV) to assess and quantify the levels of DNA damage in control group and the experimental groups. (Plate 5- H)
PLATE 5: Procedure for comet assay.

A. Eyes of chick embryo dissected out for comet assay.
B. Brain of chick embryo dissected out for comet assay.
C. Liver of chick embryo dissected out for comet assay.
D. The tissues are minced in HBSS.
E. Supernatant of the tissues are collected for assay.
F. Preparation of the Agarose gel.
G. Electrophoresis is carried out in electrophoresis tank.
H. Prepared slides are read using using automated comet scoring software (Comet Score IV).
The mean comet length, the mean tail length, mean % of DNA in the tail and mean tail moment were analyzed to assess the DNA damage in the form of double strand breaks (DSB). The data has been compiled and statistically analyzed.

**Statistical analysis**

The observed data were subjected to one-way ANOVA and the significance was determined using a —Tukey's post-hoc with P<0.05 for statistical significance. The statistical tests were performed using software Graph Pad Instat 3. All the data were expressed as Mean ± SEM.
RESULTS

1. GROSS MORPHOLOGICAL OBSERVATIONS:

A. MORTALITY RATE:

In our study, the mortality rate was found to be high for both 2G and 3G exposed groups than the control group. The number of dead embryos found each day in all the three groups is tabulated in Table 5; Graph 1a and b. The mortality rate was found to be 5.55% for the control group (Group - C), 13.8% for 2G exposed group (Group – A) and 9.7% for 3G exposed group (Group – B). In the control group, the embryos were found dead during 5th - 8th day of incubation. In the 2G exposed groups, the mortality of was higher between 6th – 10th days and in 3G exposed groups the mortality rate was higher between 7th – 12th days of incubation.

B. CONGENITAL ANOMALIES:

In the control group, most of the embryos were normal without any observable congenital anomalies (Plate - 6). However, in 2G exposed groups, 16.6% of embryos showed congenital malformations while in 3G exposed groups 15.2% showed congenital malformations (Table 6; Graph 2a and 2b). The anomalies observed in our study were monophthalmia, microphthalmia, limb bud anomalies, ectopia cordis and development of conjoint embryos (Table 7, Graph 3a, b and c) (Plate - 7). In addition to that, most of the embryos of both 2G and 3G exposed group showed subcutaneous hemorrhage.

C. GROWTH PARAMETERS:

i. Volume:

The volume of all the three groups of embryos increased gradually in proportion to the days of incubation. The 2G group embryos showed volume increase in comparison with the control group, however the increase was significant only on 7th and 10th day of incubation (p<0.01). But the 3G group embryos showed non-significant change with the control group embryos except on 6th day of incubation where they showed a significant decrease in the volume (p<0.001). On comparing 2G and 3G embryos volume, the 3G embryos showed decreased
Table 5: Mortality rate of chick embryos in all the 3 groups.

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Number of chicks sacrificed N = 72</th>
<th>Number of dead chick embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control group (Group-C) 5.55%</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>(6 + 6 + 3 = 15)</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>(6 + 3 = 9)</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>(6 + 3 = 9)</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>(6 + 6 + 3 = 15)</td>
<td>-</td>
</tr>
</tbody>
</table>

Graph 1a: Number of dead embryos in each day in all the 3 groups of chick embryo

Graph 1b: Mortality rate in all the three groups of chick embryo
Table 6: Number of chick embryo with congenital anomalies in all 3 groups

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Number of chicks sacrificed N = 72</th>
<th>Number of embryos showing anomalies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group (Group-C) 16.6%</td>
<td>2G group (Group-A) 15.2%</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>7</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>9</td>
<td>(6 + 6 + 3 = 15)</td>
<td>4</td>
</tr>
<tr>
<td>10</td>
<td>(6 + 3 = 9)</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>(6 + 3 = 9)</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>(6 + 6 + 3 = 15)</td>
<td>-</td>
</tr>
</tbody>
</table>

Graph 2a: Number of chick embryos showing congenital anomalies in each day.
Graph 2b: Percentage of chick embryos showing congenital anomalies.
Table 7: Congenital anomalies observed in all 3 groups of chick embryos

<table>
<thead>
<tr>
<th>Congenital anomalies</th>
<th>Number of embryos showing anomalies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control group (Group-C)</td>
</tr>
<tr>
<td>Eye anomalies</td>
<td>-</td>
</tr>
<tr>
<td>Limb bud anomalies</td>
<td>-</td>
</tr>
<tr>
<td>Ectopia cordis</td>
<td>-</td>
</tr>
<tr>
<td>Conjoint embryo</td>
<td>-</td>
</tr>
</tbody>
</table>

Graph 3a: Number of chick embryos showing different types of anomalies
Graph 3b: % of different congenital anomalies observed in 2G group chick embryos

Graph 3c: % of different congenital anomalies observed in 3G group chick embryos
PLATE 6: Shows control group (Group –C) Chick embryos of various gestational ages with normal features.

A. 5 day old control embryo.
B. 6 day old control embryo.
C. 7 day old control embryo.
D. 8 day old control embryo.
E. 9 day old control embryo.
F. 10 day old control embryo.
G. 11 day old control embryo.
H. 12 day old control embryo.
PLATE 7: Experimental group (Group – A and B) Chick embryos showing various congenital anomalies.

A. 2G group embryo showing fused head (arrow) and separate trunk.
B. 2G group embryo showing fused trunk (arrow) and separate head.
C. 2G group embryo showing microphthalmia (yellow arrow) and limb bud anomalies (red arrow)
D. 2G group embryo showing microphthalmia (yellow arrow) and beak anomaly (red arrow)
E. 2G group embryo showing subcutaneous haemorrhage (red arrow)
F. 2G group embryo showing abnormal fluid filled enlarged body (red arrow)
G. 2G group embryo showing ectopia cordis (red arrow)
H. 3G group embryo showing cloudy appearance of eye (yellow arrow) and limb bud anomalies (red arrow)
I. 3G group embryo showing microphthalmia (red arrow)
J. 3G group embryo showing anophthalmia (red arrow) on right side.
K. 3G group embryo showing ectopia cordis (red arrow)
L. 3G group embryo showing extensive subcutaneous haemorrhage (red arrow).
volume that was significant on 6th, 7th, 9th and 10th day of incubation (p<0.001, p<0.01, p<0.05 and p<0.01 respectively). (Table 8, Graph 4).

ii. **Weight:**

The weight of all the three groups of embryos showed a gradual increase as the age advanced. The 2G group embryos showed an increase in weight than the control group embryos and weight was significant on 7th, 8th and 10th day of incubation (p<0.05, p<0.001 and p<0.001 respectively). But the 3G group embryos showed significant decrease in weight on 5th and 6th day (p<0.01) and on the 10th day they showed a significant increase in body weight than the control group embryos (p<0.05). Other days showed non-significant changes in correlation with the control embryos. On comparing 2G and 3G group embryos, 3G group embryos showed significant decrease in body weight on 5th, 6th, 7th, 8th and 10th day of incubation (p<0.05, p<0.01, p<0.05 and p<0.01 respectively). (Table 9, Graph 5).

iii. **CR length:**

The CR length of all the three groups of embryos also showed gradual increase as the age advanced. The 2G group embryos showed increased CR length than the control group embryos but the increase was significant only on the 10th day of incubation (p<0.05). The 3G group embryos showed decreased CR length than control group embryos that was significant only on 6th day of incubation (p<0.01). On comparing 2G and 3G group embryos, the 3G group showed decreased CR length than 2G group embryos that was significant on 6th, 7th and 8th day of incubation (p<0.05, p<0.05, and p<0.01 respectively). (Table 10, Graph 6).
### Table 8: Mean volume of chick embryos in all the 3 groups

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.12 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.09 ± 0.008</td>
</tr>
<tr>
<td>6</td>
<td>0.3 ± 0</td>
<td>0.3 ± 0</td>
<td>0.18 ± 0.01***</td>
</tr>
<tr>
<td>7</td>
<td>0.53 ± 0.03</td>
<td>0.8 ± 0.08**</td>
<td>0.5 ± 0**</td>
</tr>
<tr>
<td>8</td>
<td>1 ± 0</td>
<td>1.02 ± 0.025</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>9</td>
<td>1.21 ± 0.09</td>
<td>1.76 ± 0.13</td>
<td>1 ± 0.20*</td>
</tr>
<tr>
<td>10</td>
<td>1.5 ± 0</td>
<td>2.35 ± 0.16**</td>
<td>1.6 ± 0.16**</td>
</tr>
<tr>
<td>11</td>
<td>2.16 ± 0.10</td>
<td>2.5 ± 0.12</td>
<td>2.2 ± 0.12</td>
</tr>
<tr>
<td>12</td>
<td>3.41 ± 0.15</td>
<td>3.25 ± 0.17</td>
<td>3.3 ± 0.12</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 72 chick embryos) (p value˂ 0.05* significant, ˂ 0.01** highly significant and ˂ 0.001 *** extremely significant)

### Graph 4: Effect of RFR on mean volume of chick embryos in all the 3 groups

* indicates P value ≤ 0.05 with control group considered significant.

# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
Table 9: Mean weight of chick embryos in all the 3 groups

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.143 ± 0.007</td>
<td>0.129 ± 0.01</td>
<td>0.087 ± 0.002**</td>
</tr>
<tr>
<td>6</td>
<td>0.298 ± 0.01</td>
<td>0.316 ± 0.007</td>
<td>0.216 ± 0.01**</td>
</tr>
<tr>
<td>7</td>
<td>0.613 ± 0.009</td>
<td>0.738 ± 0.05*</td>
<td>0.582 ± 0.03</td>
</tr>
<tr>
<td>8</td>
<td>0.864 ± 0.01</td>
<td>1.098 ± 0.02***</td>
<td>0.945 ± 0.03</td>
</tr>
<tr>
<td>9</td>
<td>1.290 ± 0.04</td>
<td>1.643 ± 0.15</td>
<td>1.066 ± 0.20</td>
</tr>
<tr>
<td>10</td>
<td>1.468 ± 0.01</td>
<td>2.239 ± 0.11***</td>
<td>1.837 ± 0.09*</td>
</tr>
<tr>
<td>11</td>
<td>2.189 ± 0.04</td>
<td>2.477 ± 0.08</td>
<td>2.146 ± 0.16</td>
</tr>
<tr>
<td>12</td>
<td>3.433 ± 0.11</td>
<td>3.200 ± 0.14</td>
<td>3.345 ± 0.11</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 72 chick embryos) (p value ˂ 0.05* significant, ˂ 0.01** highly significant and ˂ 0.001 *** extremely significant)

Graph 5: Effect of RFR on mean weight of chick embryos in all the 3 groups

* indicates P value  ≤ 0.05 with control group considered significant.
# indicates P value  ≤ 0.05 on comparing among 2G and 3G group considered significant
Table 10: Mean CR length of chick embryos in all the 3 groups

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>11 ± 0.31</td>
<td>8.98 ± 2.04</td>
<td>11 ± 0.25</td>
</tr>
<tr>
<td>6</td>
<td>13.6 ± 0.4</td>
<td>14 ± 0</td>
<td>12.3 ± 0.49</td>
</tr>
<tr>
<td>7</td>
<td>19.16 ± 0.40</td>
<td>18.75 ± 0.62</td>
<td>16.75 ± 0.25**</td>
</tr>
<tr>
<td>8</td>
<td>21.12 ± 0.31</td>
<td>22.5 ± 0.28</td>
<td>20.16 ± 0.40</td>
</tr>
<tr>
<td>9</td>
<td>23.41 ± 0.41</td>
<td>24.33 ± 1.2</td>
<td>22.37 ± 1.4</td>
</tr>
<tr>
<td>10</td>
<td>25.16 ± 0.87</td>
<td>28 ± 0.44*</td>
<td>27.33 ± 0.49</td>
</tr>
<tr>
<td>11</td>
<td>29 ± 0.73</td>
<td>31.5 ± 0.5</td>
<td>30 ± 1.51</td>
</tr>
<tr>
<td>12</td>
<td>35.16 ± 0.54</td>
<td>34.16 ± 1.4</td>
<td>34.8 ± 0.37</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 72 chick embryos) (p value< 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)

Graph 6: Effect of RFR on CR length of chick embryos

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant
2. HISTOLOGICAL AND HISTOMORPHOMETRICAL OBSERVATIONS: A. KIDNEY

- **Histological observations:**

  On analyzing the sections of kidney tissue, the control group embryos showed well developed mesonephric tubules showing normal structural features. The lining epithelial cells of both proximal convoluted tubule (PCT) and distal convoluted tubule (DCT) showed intact basement membrane and intact brush border in PCT. The lining cells showed less cytoplasmic changes in the form of vacuolations and relatively less nuclear changes in the form of nuclear fragmentation (karyorrhexis) and pyknosis (Plate - 8, 9, 10, 11 and 14). The glomerulus also showed normal features with intact cells lining the parietal and visceral layer with well-developed glomerular capillary network (Plate - 12, 13 & 14). In the 5 and 6 day old embryos, the glomeruli were seen occupying the medial side of mesonephric kidneys. As age advanced, they were seen even in the deeper parts of kidney.

  However, both 2G and 3G exposed embryos showed moderate to intense histopathological changes in the cytoplasm and nucleus of lining cells of PCT and DCT. The changes observed were increased vacuolations in the cytoplasm of lining columnar cells, disruption of luminal border and basement membrane of PCT (Plate - 8, 9, 10, 11 and 14). The nuclei showed increased pyknosis and karyorrhexis in comparison with the control. The intensity of these changes increased as the age advanced. The cuboidal cells lining the DCT and collecting ducts appeared normal during the initial days of incubation. But as the age advanced, they also showed mild histopathological changes in the form of vacuolations, disruption of basement membrane and karyorrhexis. Moreover, inflammatory changes in the interstitial spaces were observed. Interstitium showed diffused interstitial edema with variable infiltrates of lymphocytes, plasma cells, monocytes and macrophages and also were engorged with RBCs indicating hemorrhagic changes (Plate - 8, 9, 10, 11 and 14). The glomerulus of both exposed groups also showed degenerative changes with the distorted glomerular capillary network and increased urinary space (Plate - 12, 13 and 14).
PLATE 8: Photomicrograph showing kidney of control, 2G and 3G group embryos at 400X magnification (5th - 8th day). A, B, C, and D – photomicrograph of control group embryos showing normal features. E, F, G and H – photomicrograph of 2G group embryos and I, J, K and L – photomicrograph of 3G group embryos. Control group embryos showed normal cells lining PCT (red arrow). Both 2G and 3G group embryos showed vacuolations (red arrow) in PCT (P), disruption of brush border (black arrow) and interstitial edema with infiltrations (blue asterix). DCT (D) and glomerulus (G) is also seen. (H&E staining)
PLATE 9: Photomicrograph showing kidney of control, 2G and 3G group embryos (9th – 12th day) at 400X magnification. Control group embryos showed normal normal cells lining PCT(red arrow) with intact brush border (black arrow) and basement membrane (green arrow). Both 2G and 3G group embryos showed vacuolations (red arrow) in PCT (P), disruption of brush border (black arrow), disruption of basement membrane (green arrow) and interstitial edema with infiltrations (blue asterix). DCT (D) and glomerulus (G) is also seen. (H&E staining)
PLATE 10: Photomicrograph showing kidney of control, 2G and 3G group embryos (5th – 8th day) at 1000X magnification. Control group embryos showed normal cells lining PCT (red arrow) with intact brush border (black arrow) and basement membrane (green arrow). Both 2G and 3G group embryos showed vacuolations (red arrow) in PCT (P), disruption of brush border (black arrow), disruption of basement membrane (green arrow) and interstitial edema with infiltrations (blue asterix). DCT (D) and glomerulus (G) is also seen. (H&E staining)
PLATE 11: Photomicrograph showing kidney of control, 2G and 3G group embryos (9th – 12th day) at 1000X magnification. Control group embryos showed normal cells lining PCT (red arrow) with intact brush border (black arrow) and basement membrane (green arrow). Both 2G and 3G group embryos showed vacuolations (red arrow) in PCT (P), disruption of brush border (black arrow), disruption of basement membrane (green arrow) and interstitial edema with infiltrations (blue asterix). DCT (D) is also seen. (H&E staining)
PLATE 12: Photomicrograph showing glomerulus of control, 2G and 3G group embryos (5th – 8th day) at 1000X magnification. Control group embryos showed glomerulus with intact capillary plexus (red arrow) and normal urinary space (blue asterix). Both 2G and 3G group embryos showed distorted glomerular capillary network (red arrow) and increased urinary space (blue asterix). (H&E staining)
PLATE 13: Photomicrograph showing glomerulus of control, 2G and 3G group embryos (9th – 12th day) at 1000X magnification. Control group embryos showed glomerulus with intact capillary plexus (red arrow) and normal urinary space (blue asterix). Both 2G and 3G group embryos showed distorted glomerular capillary network with endothelium showing vacuolations (red arrow) and increased urinary space (blue asterix). (H&E staining)
PLATE 14: Photomicrograph showing PCT and glomerulus of control, 2G and 3G group embryos (9th and 12th day) at 1000X magnification. Control group embryos showed normal looking PCT with intact brush border (black arrow) and basement membrane (green arrow) (A and B). Glomerulus showed intact capillary plexus with normal urinary space (blue asterix) (C and D). Both 2G and 3G group embryos showed vaculoations (red arrow) in PCT, disruption of brush border (black arrow), disruption of basement membrane (green arrow) (E and F., I and J respectively) and distorted glomerular capillary network with endothelium showing vacuolations (red arrow) and increased urinary space (blue asterix) (G and H., K and L respectively). (PAS staining)
• **Histomorphometric observations:**
  
i. **Cellular height of PCT**

The standard epithelial height (SEH) of lining columnar cells of PCT showed a gradual increase as the age advanced in all the three groups. The epithelial height was found to be significantly increased on 7\(^{th}\), 8\(^{th}\), 9\(^{th}\), 10\(^{th}\) and 12\(^{th}\) day for 2G group embryos (p<0.001, p<0.001, p<0.001 and p<0.01 respectively) and on all days for 3G group embryos when compared with control group embryos (p<0.001). On comparing 2G and 3G embryos, the 3G embryos showed increased height that was significant on all days except on 7\(^{th}\) and 10\(^{th}\) day that showed a non-significant increase (p<0.01, p<0.001, p<0.001, p<0.001, p<0.01 respectively). (Table 11, Graph 7)

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.007 ± 0.0000</td>
<td>0.007 ± 0.0002</td>
<td>0.008 ± 0.0000**</td>
</tr>
<tr>
<td>6</td>
<td>0.007 ± 0.0000</td>
<td>0.007 ± 0.0001</td>
<td>0.009 ± 0.0000***</td>
</tr>
<tr>
<td>7</td>
<td>0.008 ± 0.0000</td>
<td>0.010 ± 0.0001***</td>
<td>0.010 ± 0.0001***</td>
</tr>
<tr>
<td>8</td>
<td>0.009 ± 0.0000</td>
<td>0.010 ± 0.0001***</td>
<td>0.011 ± 0.0001***</td>
</tr>
<tr>
<td>9</td>
<td>0.009 ± 0.0001</td>
<td>0.011 ± 0.0001***</td>
<td>0.012 ± 0.0001***</td>
</tr>
<tr>
<td>10</td>
<td>0.011 ± 0.0001</td>
<td>0.012 ± 0.0001***</td>
<td>0.013 ± 0.0001***</td>
</tr>
<tr>
<td>11</td>
<td>0.012 ± 0.0001</td>
<td>0.012 ± 0.0001</td>
<td>0.013 ± 0.0001***</td>
</tr>
<tr>
<td>12</td>
<td>0.0121 ± 0.0000</td>
<td>0.013 ± 0.0001**</td>
<td>0.013 ± 0.0001***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value< 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)
### ii. Cellular height of DCT

The standard epithelial height (SEH) of lining cuboidal cells of DCT showed a gradual increase in height as the age advanced in all the three groups. The epithelial height was found to be significantly increased on 5th, 6th, 7th, 8th, 9th, 10th and 11th day for 2G group embryos (p<0.001) and on all day for 3G group embryos when compared with control group embryos (p<0.001). On comparing 2G and 3G embryos, the 2G embryos showed increased height that was significant on 5th, 6th and 7th day (p<0.05, p<0.05 and p<0.001 respectively) and 3G group embryos showed significantly increased height on the 12th day of incubation (p<0.001). (Table 12, Graph 8)
Table 12: Mean height of lining cells of DCT in all the 3 groups of chick embryo

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.003 ± 0.00004</td>
<td>0.0049 ± 0.00006***</td>
<td>0.0045 ± 0.00004***</td>
</tr>
<tr>
<td>6</td>
<td>0.004 ± 0.00005</td>
<td>0.0049 ± 0.00006***</td>
<td>0.0047 ± 0.00005***</td>
</tr>
<tr>
<td>7</td>
<td>0.0045 ± 0.00007</td>
<td>0.0052 ± 0.00007***</td>
<td>0.0048 ± 0.00008***</td>
</tr>
<tr>
<td>8</td>
<td>0.0046 ± 0.00007</td>
<td>0.0053 ± 0.00005***</td>
<td>0.0052 ± 0.00004***</td>
</tr>
<tr>
<td>9</td>
<td>0.0048 ± 0.00003</td>
<td>0.0053 ± 0.00009***</td>
<td>0.0052 ± 0.00004***</td>
</tr>
<tr>
<td>10</td>
<td>0.0048 ± 0.00005</td>
<td>0.0060 ± 0.00007***</td>
<td>0.0061 ± 0.00006***</td>
</tr>
<tr>
<td>11</td>
<td>0.0049± 0.00006</td>
<td>0.0062 ± 0.00006***</td>
<td>0.0061 ± 0.00005***</td>
</tr>
<tr>
<td>12</td>
<td>0.0057± 0.00004</td>
<td>0.0058 ± 0.00006</td>
<td>0.0063 ± 0.00007***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value < 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)

Graph 8: Effect of RFR on height of lining cells in DCT in all the 3 groups of chick embryo

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
iii. **Nuclear diameter of the cells lining PCT**

The nuclear diameter of all the three groups showed a gradual increase in diameter as the age advanced. Both the 2G and 3G exposed groups showed an increased nuclear diameter than the control group and was significant on all day (p<0.05, p<0.01, p<0.001 and p<0.001 respectively). On comparing 2G and 3G group, the 3G group, embryos showed an increased diameter that was significant on 5th, 6th, 8th, 9th and the 11th day (p<0.01, p<0.05, p<0.05, p<0.01 and p<0.05 respectively). (Table 13, Graph 9)

**Table 13: Mean nuclear diameter of lining cells of PCT in all 3 the groups of chick embryo**

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.0037 ± 0.00004</td>
<td>0.0039 ± 0.00005*</td>
<td>0.0041 ± 0.00004***</td>
</tr>
<tr>
<td>6</td>
<td>0.0038 ± 0.00003</td>
<td>0.004 ± 0.00004**</td>
<td>0.0042 ± 0.00004***</td>
</tr>
<tr>
<td>7</td>
<td>0.0038 ± 0.00003</td>
<td>0.0041 ± 0.00004***</td>
<td>0.0042 ± 0.00004***</td>
</tr>
<tr>
<td>8</td>
<td>0.0039 ± 0.00002</td>
<td>0.0041 ± 0.00002**</td>
<td>0.0042 ± 0.00003***</td>
</tr>
<tr>
<td>9</td>
<td>0.0040 ± 0.00002</td>
<td>0.0041 ± 0.00003*</td>
<td>0.0043 ± 0.00004***</td>
</tr>
<tr>
<td>10</td>
<td>0.004 ± 0.00001</td>
<td>0.0042 ± 0.00006***</td>
<td>0.0043 ± 0.00004***</td>
</tr>
<tr>
<td>11</td>
<td>0.0041 ± 0.00002</td>
<td>0.0042 ± 0.00004*</td>
<td>0.0044 ± 0.00005***</td>
</tr>
<tr>
<td>12</td>
<td>0.0042 ± 0.00004</td>
<td>0.0045 ± 0.00004***</td>
<td>0.0046 ± 0.00004***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value< 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)
iv. **Nuclear diameter of the cells lining DCT**

The nuclear diameter of DCT of all the three groups showed a gradual increase in diameter as the age advanced. The 2G embryos showed an increased diameter than the control group, embryos that were statistically significant on 5th, 6th, 7th, 9th and 10th day of incubation (p<0.001, p<0.01, p<0.05, p<0.05 and p<0.05 respectively). The 3G group also showed an increased nuclear diameter on comparing with the control group and the increase was significant only on 5th and 6th day (p<0.05). On comparing 2G and 3G group, embryos, the 2G group showed a significant increase in diameter on 5th, 9th, 10th and 11th day of incubation (p<0.05, p<0.001, p<0.05 and p<0.05 respectively). (Table 14, Graph 10)
Table 14: Mean nuclear diameter of lining cells of DCT in all the 3 groups of chick embryo

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.0035 ± 0.00004</td>
<td>0.0038 ± 0.00003**</td>
<td>0.0036 ± 0.00004*</td>
</tr>
<tr>
<td>6</td>
<td>0.0036 ± 0.00004</td>
<td>0.0038 ± 0.00003**</td>
<td>0.0037 ± 0.00004*</td>
</tr>
<tr>
<td>7</td>
<td>0.0037 ± 0.00004</td>
<td>0.0039 ± 0.00004*</td>
<td>0.0038 ± 0.00005</td>
</tr>
<tr>
<td>8</td>
<td>0.0038 ± 0.00003</td>
<td>0.0039 ± 0.00002</td>
<td>0.0038 ± 0.00003</td>
</tr>
<tr>
<td>9</td>
<td>0.0039 ± 0.00002</td>
<td>0.0040 ± 0.00002*</td>
<td>0.0038 ± 0.00004</td>
</tr>
<tr>
<td>10</td>
<td>0.0039 ± 0.00001</td>
<td>0.0040 ± 0.00004</td>
<td>0.0039 ± 0.00002</td>
</tr>
<tr>
<td>11</td>
<td>0.004 ± 0.00003</td>
<td>0.0041 ± 0.00003*</td>
<td>0.0040 ± 0.00001</td>
</tr>
<tr>
<td>12</td>
<td>0.0041 ± 0.00003</td>
<td>0.0041 ± 0.00003</td>
<td>0.0041 ± 0.00003</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value < 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)

Graph 10: Effect of RFR on nuclear diameter of lining cells in DCT of all 3 groups

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
v. **Diameter of urinary space**

The urinary space also showed a gradual increase in diameter as the age advanced in all the three groups. Both the 2G and 3G groups showed statistically significant increase in the urinary space on all days as compared with control group, embryos (p<0.001 and p<0.001 respectively). On comparing 2G and 3G group, the 3G groups, embryos showed an increased diameter of urinary space that was significant on all day (p<0.001, p<0.01and p<0.05 respectively). (Table 15, Graph 11)

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.007 ± 0.0002</td>
<td>0.011 ± 0.0003***</td>
<td>0.013± 0.0004***</td>
</tr>
<tr>
<td>6</td>
<td>0.007 ± 0.0002</td>
<td>0.011 ± 0.0005***</td>
<td>0.015 ± 0.0005***</td>
</tr>
<tr>
<td>7</td>
<td>0.008 ± 0.0003</td>
<td>0.013 ± 0.0004**</td>
<td>0.016 ± 0.0005***</td>
</tr>
<tr>
<td>8</td>
<td>0.008 ± 0.0002</td>
<td>0.015 ± 0.0003***</td>
<td>0.017 ± 0.0003***</td>
</tr>
<tr>
<td>9</td>
<td>0.009 ± 0.0003</td>
<td>0.015 ± 0.0006***</td>
<td>0.018 ± 0.0005***</td>
</tr>
<tr>
<td>10</td>
<td>0.009 ± 0.0003</td>
<td>0.015 ± 0.0005***</td>
<td>0.019 ± 0.0005***</td>
</tr>
<tr>
<td>11</td>
<td>0.010 ± 0.0004</td>
<td>0.018 ± 0.0005***</td>
<td>0.020 ± 0.0004***</td>
</tr>
<tr>
<td>12</td>
<td>0.010 ± 0.0004</td>
<td>0.018 ± 0.0003***</td>
<td>0.020 ± 0.0008***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value< 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)
vi. **Karyorrhexis in kidney tubules**

Both 2G and 3G group, embryos showed increased karyorrhexis than the control group embryos. The increase was significant on 8th, 9th and the 11th day for 2G group, embryos (p<0.001, p<0.05 and p<0.05 respectively) and for all day for 3G group embryos (p<0.001 and p<0.01 respectively). On comparing between 2G and 3G group, 3G group showed increased karyorrhexis than 2G group, however the increase was significant only on 5th, 6th and 12th day (p<0.001). (Table 16, Graph 12).
Table 16: Karyorrhexis in nuclei of kidney in all 3 groups of chick embryo

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.74 ± 0.10</td>
<td>0.94 ± 0.08</td>
<td>1.72 ± 0.16***</td>
</tr>
<tr>
<td>6</td>
<td>0.529 ± 0.08</td>
<td>0.711 ± 0.10</td>
<td>1.5 ± 0.11***</td>
</tr>
<tr>
<td>7</td>
<td>0.549 ± 0.10</td>
<td>0.862 ± 0.09</td>
<td>0.96 ± 0.09**</td>
</tr>
<tr>
<td>8</td>
<td>1.163 ± 0.06</td>
<td>1.66 ± 0.09***</td>
<td>1.89 ± 0.08***</td>
</tr>
<tr>
<td>9</td>
<td>0.74 ± 0.09</td>
<td>1.14 ± 0.10*</td>
<td>1.28 ± 0.11**</td>
</tr>
<tr>
<td>10</td>
<td>0.86 ± 0.12</td>
<td>1.1 ± 0.10</td>
<td>1.38 ± 0.10**</td>
</tr>
<tr>
<td>11</td>
<td>0.58 ± 0.11</td>
<td>1.04 ± 0.11*</td>
<td>1.08 ± 0.10**</td>
</tr>
<tr>
<td>12</td>
<td>0.943 ± 0.12</td>
<td>1.22 ± 0.08</td>
<td>1.88 ± 0.09***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value< 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)

Graph 12: Effect of RFR on Karyorrhexis of kidney nuclei in all the 3 groups

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
B. LIVER

- **Histological observations:**

Histological evaluation of control embryo liver showed normal architecture with hepatocytes arranged in the form of hepatic cords and sinusoids separating them. The sinusoids showed mild to moderate numbers of RBC’s. The Hepatocytes showed eosinophilic granular cytoplasm and centrally placed large vesicular nuclei with prominent nucleoli. The cytoplasmic changes in the form of vacuolations and nuclear changes (karyorrhexis, pyknosis) were indistinct in the control group (Plate 15, 16, 17, 18 and 19).

The liver structure in 2G and 3G experimental groups showed marked dilation of sinusoidal spaces (peliosis) with moderate to more numbers of RBC’s indicating hemorrhage in the parenchyma. The cytoplasm of hepatocytes showed vacuolations rendering them a foamy appearance. The nuclei showed karyorrhexis and pyknotic changes. (Plate 15, 16, 17, 18 and 19)

- **Histomorphometric observations:**
  
i. **Nuclear diameter of hepatocytes**

The nuclear diameter of hepatocytes in all the three groups showed a gradual increase in diameter as the age advanced. Both the 2G and 3G exposed groups showed increased nuclear diameter than the control group which was significant on all days (p<0.001 and p<0.001 respectively). On comparing 2G and 3G groups, the 3G group embryos showed increased diameter that was significant on all days (p<0.01, p<0.05 and p<0.001 respectively). (Table 17, Graph 13)
PLATE 15: Photomicrograph showing liver of control, 2G and 3G group embryos at 400X magnification (5th - 8th day). A, B, C, and D – photomicrograph of control group embryos showing normal features. E, F, G and H – photomicrograph of 2G group embryos and I, J, K and L – photomicrograph of 3G group embryos. Control group embryos showed normal looking hepatocytes (black arrow) and sinusoids (blue asterix). Both 2G and 3G group embryos showed vacuolations (black arrow) of hepatocytes and increased sinusoidal space with infiltrations (blue asterix). Central veins (CV) are also seen. (H&E staining)
PLATE 16: Photomicrograph showing liver of control, 2G and 3G group embryos at 400X magnification (9th -12th day). Control group embryos showed normal looking hepatocytes (black arrow) and sinusoids (blue asterix). Both 2G and 3G group embryos showed vacuolations (black arrow) of hepatocytes and increased sinusoidal space with infiltrations (blue asterix). Central veins (CV) are also seen. (H&E staining)
PLATE 17: Photomicrograph showing liver of control, 2G and 3G group embryos at 1000X magnification (5th - 8th day). Control group embryos showed normal looking hepatocytes (black arrow) and sinusoids (blue asterix). Both 2G and 3G group embryos showed vacuolations (black arrow) of hepatocytes and increased sinusoidal space with infiltrations (blue asterix). Central veins (CV) are also seen. (H&E staining)
PLATE 18: Photomicrograph showing liver of control, 2G and 3G group embryos at 1000X magnification (9th -12th day). Control group embryos showed normal looking hepatocytes (black arrow) and sinusoids (blue asterix). Both 2G and 3G group embryos showed vacuolations (black arrow) of hepatocytes and increased sinusoidal space with infiltrations (blue asterix). Central veins (CV) are also seen. (H&E staining)
PLATE 19: Photomicrograph showing liver of control, 2G and 3G group embryos at 400X and 1000X magnification (5th and 12th day). A and B – control group liver at 400X, C and D at 1000X, E and F- 2G group liver at 400X, G and H- at 1000X, I and J- 3G group liver at 400X, K and L at 1000X. Control group embryos showed normal looking hepatocytes (black arrow) and sinusoids (blue asterix). Both 2G and 3G group embryos showed vacuolations (black arrow) of hepatocytes and increased sinusoidal space with infiltrations (blue asterix). Central veins (CV) are also seen. (PAS staining)
Table 17: Mean nuclear diameter of hepatocytes in all the 3 groups of chick embryo

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.0039 ± 0.00002</td>
<td>0.0041 ± 0.00003**</td>
<td>0.0042 ± 0.00004***</td>
</tr>
<tr>
<td>6</td>
<td>0.0039 ± 0.00002</td>
<td>0.0042 ± 0.00004***</td>
<td>0.0043 ± 0.00004***</td>
</tr>
<tr>
<td>7</td>
<td>0.0040 ± 0.00002</td>
<td>0.0042 ± 0.00003***</td>
<td>0.0043 ± 0.00004***</td>
</tr>
<tr>
<td>8</td>
<td>0.0040 ± 0.00001</td>
<td>0.0042 ± 0.00004***</td>
<td>0.0044 ± 0.00004***</td>
</tr>
<tr>
<td>9</td>
<td>0.0041 ± 0.00002</td>
<td>0.0043 ± 0.00004***</td>
<td>0.0045 ± 0.00004***</td>
</tr>
<tr>
<td>10</td>
<td>0.0041 ± 0.00003</td>
<td>0.0044 ± 0.00004**</td>
<td>0.0045 ± 0.00005***</td>
</tr>
<tr>
<td>11</td>
<td>0.0042 ± 0.00004</td>
<td>0.0044 ± 0.00004*</td>
<td>0.0047 ± 0.00004***</td>
</tr>
<tr>
<td>12</td>
<td>0.0043 ± 0.00004</td>
<td>0.0046 ± 0.00004***</td>
<td>0.0049 ± 0.00004***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value˂ 0.05* significant, ˂ 0.01** highly significant and ˂ 0.001 *** extremely significant)

Graph 13: Effect of RFR on nuclear diameter of hepatocytes in all the three groups.
ii. **Karyorrhexis in hepatocytes**

Both 2G and 3G group embryos showed increased karyorrhexis than the control group embryos. The increase was significant on 5\textsuperscript{th} and 12\textsuperscript{th} day for 2G group embryos (p<0.01) and for 3G group embryos the increase was significant on 5\textsuperscript{th}, 7\textsuperscript{th}, 9\textsuperscript{th}, 11\textsuperscript{th} and 12\textsuperscript{th} day of incubation (p<0.001, p<0.01, p<0.01, p<0.001 and p<0.001 respectively). On comparing between 2G and 3G group, 3G group showed increased karyorrhexis than 2G group but the increase was significant only on 7\textsuperscript{th}, 9\textsuperscript{th} and 11\textsuperscript{th} day (p<0.05, p<0.05, p<0.05 and p<0.001 respectively). (Table 18, Graph 14)

**Table 18: Karyorrhexis in nuclei of hepatocytes in all the 3 groups of chick embryo**

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.84 ± 0.08</td>
<td>1.42 ± 0.13**</td>
<td>1.5 ± 0.12***</td>
</tr>
<tr>
<td>6</td>
<td>1.14 ± 0.09</td>
<td>1.32 ± 0.13</td>
<td>1.5 ± 0.13</td>
</tr>
<tr>
<td>7</td>
<td>1.06 ± 0.16</td>
<td>1.22 ± 0.14</td>
<td>1.72 ± 0.12**</td>
</tr>
<tr>
<td>8</td>
<td>1.5 ± 0.14</td>
<td>1.5 ± 0.10</td>
<td>1.92 ± 0.16</td>
</tr>
<tr>
<td>9</td>
<td>1.1 ± 0.11</td>
<td>1.3 ± 0.11</td>
<td>1.74 ± 0.13**</td>
</tr>
<tr>
<td>10</td>
<td>1.21 ± 0.11</td>
<td>1.22 ± 0.11</td>
<td>1.38 ± 0.13</td>
</tr>
<tr>
<td>11</td>
<td>1.27 ± 0.10</td>
<td>1.32 ± 0.09</td>
<td>2.02 ± 0.16***</td>
</tr>
<tr>
<td>12</td>
<td>0.72 ± 0.13</td>
<td>1.32 ± 0.11**</td>
<td>1.62 ± 0.10***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value< 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)
C. **EYE**

a) **RETINA**

- **Histological observations:**

  Histological examination of retina of 5 - 7 days old embryos of all the three groups showed 3 layers: the pigment layer, germinative or proliferative layer and inner marginal layer (putative optic nerve fiber), whereas, 8 day old embryos showed five layers: the pigment layer, outer neuroblastic layer, inner neuroblastic layer and a layer of tangled cell processes demarcating them (transient layer of chievitz) and inner marginal layer (Plate- 20). The 9th – 12th day old embryos showed well-formed eight layers. The layers were pigment layer, layer of rods and cones, external nuclear layer, external plexiform layer, inner nuclear layer, inner plexiform layer, ganglion layer and layer of optic nerve fiber (Plate- 21).
PLATE 20: Photomicrograph showing retina of control, 2G and 3G group embryos at 1000X magnification (5th -8th day). A, B, C, and D – photomicrograph of control group embryos showing normal features. E, F, G and H – photomicrograph of 2G group embryos and I, J, K and L – photomicrograph of 3G group embryos. 5th – 7th day embryos showed three layered retina. 8th day embryos showed five layered retina. Control group embryos showed intact inner marginal layer (black arrow) and compactly arranged cells (blue asterix). Both 2G and 3G group embryos showed disintegrated inner marginal layer (black arrow) and increased intercellular spaces (blue asterix).
PLATE 21: Photomicrograph showing retina of control, 2G and 3G group embryos at 1000X magnification (9th - 12th day). All the embryos showed eight layered retina with external and internal limiting membranes. Control group embryos showed intact optic nerve fiber layer (black arrow), compactly arranged cells in inner nuclear layer (blue asterix) and ganglion cell layer (red arrow). Both 2G and 3G group embryos showed disintegrated optic nerve fiber layer (black arrow) and increased intercellular spaces in inner nuclear layer (blue asterix) and ganglion cell layer (red arrow). Ganglion cell layer was found to be decreased in thickness on 11th day and 12th day for both experimental group (G and H of 2G group and K and I of 3G group).
i. **Retinal changes in 5th – 7th day embryos:**

The retina of 5th – 7th day old control group, embryos showed mild pigmentation of pigment layer and neural retina showed 2 layers – germinative or proliferative layer showing closely packed cells without spaces between them and inner marginal layer (Plate- 20).

However, the pigment layer of 2G and 3G group, embryos showed mild pigmentation up to 6th day and on the 7th day they showed moderate pigmentation. The neural retina showed spaces between the cells in the germinative or proliferative layer. 7 day old embryos of both 2G and 3G groups showed disintegrated inner marginal layer. (Plate- 20).

ii. **Retinal changes in 8th day embryos:**

Retina of 8 day old control embryo showed mild pigmentation and few spaces between the cells. Most of the control group retina showed only 3 layers and in two embryos of control group (33.2%), five layered retina was observed (Plate- 20).

The entire experiment group embryos (2G and 3G) showed 5 layered retina. The pigment layer of 2G group embryos showed mainly moderate pigmentation of the retina. They also showed increased spaces between the cells in inner neuroblastic layer and disintegrated optic nerve fiber layer. The 3G group embryos showed intense pigmentation of pigment layer and also similar changes were observed in 2G group embryos (Plate - 20).

iii. **Retinal changes in 9th – 12th day embryos:**

9th – 12th day control group, embryos showed well-formed 8 layered retina. The external plexiform layer was clearly seen from 9th day onwards separating external and internal nuclear layer. Pigment layer showed moderate pigmentation. All layers were well-formed with little space between cells of various layers (Plate- 21).

Both 2G and 3G group embryos also showed well differentiated 8 layers but increased intercellular spaces were visible between the cells in external nuclear layer, internal nuclear layer and ganglion cell layer. The optic nerve layer was found to be disintegrated in many places. The 2G group embryos showed moderate pigmentation on 9th day followed by intense pigmentation on 10th – 12th day. However, 3G group embryos showed intense pigmentation on 9th day and 10th day followed by decreased pigmentation on 11th and 12th day (moderate pigmentation) (Plate-21) (Table 19)
Table 19: Pigmentation grade in all 3 groups of chick embryo

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>9</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>10</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>11</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>12</td>
<td>++</td>
<td>+++</td>
<td>++</td>
</tr>
</tbody>
</table>

(+ mild, ++ moderate, +++ intense pigmentation)

- **Histomorphometric observations:**
  
i. **Thickness of retinal layers in 5th – 7th day old embryos:**

  On comparing the thicknesses of each layer of retina in all the three groups, it was found that the layers increased in thickness as the age advanced except for 3G group embryos. The 3G group embryos showed a decrease in the thickness of pigment layer that was highly significant on 7th day (p<0.001).

  The 2G and 3G group showed significant increase in thickness of all the three layers in comparison with the control group embryos (p<0.001 and p<0.001 respectively) except for the pigment layer of 3G group embryos. The pigment layer was found to be significantly decreased in thickness than the control group embryos on 7th day (p<0.001).

  On comparing 2G and 3G group embryos, it was found that the 3G group showed significantly increased thickness of germinal layer on all the days (p<0.05, p<0.001, and p<0.001 respectively) than the 2G groups but significant decrease in pigment and marginal layer (p<0.001 and p<0.001 respectively). (Table 20, Graph 15)
Table 20: Mean thickness of each layer of retina in all the 3 groups (5–7 days)

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Pigment Layer (mm)</th>
<th>Germinative layer (mm)</th>
<th>Optic nerve fibre (mm)</th>
<th>Total thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (CON)</td>
<td>0.004 ± 0.0001</td>
<td>0.052 ± 0.0002</td>
<td>0.003 ± 0.0001</td>
<td>0.06 ± 0.0003</td>
</tr>
<tr>
<td>5 (2G)</td>
<td>0.005 ± 0.0002**</td>
<td>0.055 ± 0.0005***</td>
<td>0.006 ± 0.0002***</td>
<td>0.066 ± 0.0007***</td>
</tr>
<tr>
<td>5 (3G)</td>
<td>0.007 ± 0.0001***</td>
<td>0.068 ± 0.0016***</td>
<td>0.005 ± 0.0001***</td>
<td>0.08 ± 0.0017***</td>
</tr>
<tr>
<td>6 (CON)</td>
<td>0.0049 ± 0.0001</td>
<td>0.063 ± 0.0003</td>
<td>0.005 ± 0.0001</td>
<td>0.072 ± 0.0003</td>
</tr>
<tr>
<td>6 (2G)</td>
<td>0.0053 ± 0.000**</td>
<td>0.069 ± 0.0006**</td>
<td>0.008 ± 0.0002***</td>
<td>0.082 ± 0.0008</td>
</tr>
<tr>
<td>6 (3G)</td>
<td>0.0048 ± 0.0000</td>
<td>0.074 ± 0.0014***</td>
<td>0.006 ± 0.0002*</td>
<td>0.084 ± 0.0014***</td>
</tr>
<tr>
<td>7 (CON)</td>
<td>0.005 ± 0.0000</td>
<td>0.082 ± 0.001</td>
<td>0.006 ± 0.0001</td>
<td>0.093 ± 0.0011</td>
</tr>
<tr>
<td>7 (2G)</td>
<td>0.005 ± 0.0000</td>
<td>0.083 ± 0.001</td>
<td>0.009 ± 0.0002***</td>
<td>0.097 ± 0.0012***</td>
</tr>
<tr>
<td>7 (3G)</td>
<td>0.004 ± 0.00013***</td>
<td>0.093 ± 0.0006***</td>
<td>0.007 ± 0.0002***</td>
<td>0.103 ± 0.0007***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value˂ 0.05* significant, ˂ 0.01** highly significant and ˂ 0.001 *** extremely significant)

Graph 15: Effect of RFR on thickness of retinal layers in all the 3 groups (5-7 days)

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
ii. **Thickness of retinal layers in 8 day old embryos:**

The 2G group embryos showed insignificant change in thickness of pigment layer and transient layer of Chievitz in comparison with the control group embryos. However, the outer neuroblastic layer and the optic nerve layer were significantly increased in thickness (p<0.001 and p<0.001 respectively) and the inner neuroblastic layer was significantly decreased in thickness than the control group embryos (p<0.001).

The 3G group embryos showed no significant change in thickness of optic nerve fiber layer when compared with control group embryos. The thicknesses of the pigment layer, outer neuroblastic layer and transient layer of Chievitz were significantly increased than control group embryos (p<0.001, p<0.001 and p<0.05 respectively). But, the inner neuroblastic layer showed significant decrease in thickness on comparing with the control group embryos (p<0.001).

On comparing the 2G and 3G group embryos, it was observed that the 3G group showed no significant change in the transient layer of Chievitz but a significant increase in the thickness of pigment layer, outer neuroblastic layer and inner neuroblastic layer (p<0.001, p<0.01, and p<0.001 respectively). The optic nerve fiber layer showed a significant decrease in thickness than 2G group embryos (p<0.001). (Table 21, Graph 16)

**Table 21: Mean thickness of each layer of retina in all 3 the groups of 8 day old embryo**

<table>
<thead>
<tr>
<th>Age (Days)</th>
<th>Pigment layer (mm)</th>
<th>Outer neuroblastic layer (mm)</th>
<th>Transient layer of chievitz (mm)</th>
<th>Inner neuroblastic layer (mm)</th>
<th>Optic nerve fibre layer(mm)</th>
<th>Total thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 (CON)</td>
<td>0.005 ± 0</td>
<td>0.073 ± 0.0007</td>
<td>0.0035 ± 0.0001</td>
<td>0.016 ± 0.0006</td>
<td>0.009 ± 0.0004</td>
<td>0.107 ± 0.001</td>
</tr>
<tr>
<td>8 (2G)</td>
<td>0.005 ± 0</td>
<td>0.083 ± 0.0008***</td>
<td>0.004 ± 0.0001</td>
<td>0.010 ± 0.0001***</td>
<td>0.012 ± 0.0001***</td>
<td>0.113 ± 0.001</td>
</tr>
<tr>
<td>8 (3G)</td>
<td>0.0056 ± 0.00***</td>
<td>0.085 ± 0.0005***</td>
<td>0.004 ± 0.0001*</td>
<td>0.012 ± 0.0003***</td>
<td>0.0097 ± 0.0002</td>
<td>0.117 ± 0.0007***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value< 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)
iii. Thickness of retinal layers in 9 day old embryos:

No significant change in the thickness of pigment layer, layer of rods and cones and internal nuclear layer was observed between 2G and control group embryos. However, 2G group embryos showed significant increase in thickness of external nuclear layer, external plexiform layer and ganglion cell layer (p<0.001, p<0.001, and p<0.001 respectively) but significant decrease in internal plexiform layer and optic nerve fiber layer on comparing with the control group embryos (p<0.001 and p<0.01 respectively).

The 3G group embryos also showed no significant change in the thickness of the pigment layer and layer of rods and cones on comparing with control group embryos. However, they showed a significant increase in thickness of internal nuclear layer, ganglion cell layer and optic nerve fiber layer (p<0.001, p<0.001 and p<0.001 respectively) and a significant decrease in the thickness of external nuclear layer, external plexiform layer and internal plexiform layer on comparing with the control group embryos (p<0.01, p<0.05, and p<0.001 respectively).
On comparing between 2G and 3G group embryos, it was observed that there was no significant change in thickness of pigment layer, layer of rods and cones and internal plexiform layer. However, 3G group embryos showed a significant increase in thickness of internal nuclear layer and optic nerve layer (p<0.001 and p<0.001 respectively) and a significant decrease in the thickness of external nuclear layer, external plexiform layer and ganglion cell layer (p<0.001, p<0.001 and p<0.01 respectively). (Table 22, Graph 17)

**Graph 17: Effect of RFR on thickness of retinal layers of 9 day old chick embryos**

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
iv. **Thickness of retinal layers in 10 day old embryos:**

On comparing 2G group embryos with control group embryos, no significant change was observed in the thickness of the pigment cell layer, layer of rods and cones, external nuclear layer and external plexiform layer. However, there was a significant increase in the thickness of internal nuclear layer, internal plexiform layer, ganglion cell layer and optic nerve fiber layer (p<0.001, p<0.001, p<0.001 and p<0.001 respectively).

The 3G group embryos showed no significant change in the thickness of pigment cell layer, layer of rods and cones and external plexiform layer on comparing with control group embryos. However, the thickness of the external nuclear layer, internal nuclear layer, internal plexiform layer, ganglion cell layer and optic nerve layer was significantly increased on comparing with control group embryos (p<0.001, p<0.001, p<0.001, p<0.001 and p<0.001 respectively).

However, on comparing 2G and 3G group embryos, no significant change was observed in the thickness of the pigment layer, layer of rods and cones, external plexiform layer, internal plexiform layer and ganglion cell layer of both groups. There was a significant increase in the thickness of external nuclear layer and internal nuclear layer (p<0.001 and p<0.001 respectively) and a significant decrease in the thickness of optic nerve fibre layers of 3G group embryos (p<0.001). (Table 22, Graph 18)
v. **Thickness of retinal layers in 11 day old embryos:**

On comparing 2G group embryos with control group embryos, it was observed that the thickness of the pigment layer, the layer of rods and cones, external nuclear layer and external plexiform layer showed no significant change. However, the other layers showed significant increase in thickness on comparing with the control group embryos (p<0.001).

3G group embryos also showed no significant change in the pigment layer, layer of rods and cones and ganglion cell layer on comparison with the control group embryos. The remaining layers showed significant decrease in thickness than the control group embryos (p<0.001, p<0.05, p<0.01, p<0.001 and p<0.001 respectively).

On comparing 2G and 3G group embryos, the pigment layer, the layer of rods and cones and ganglion cell layer showed no significant change. However, other layers showed a significant decrease in thickness in 3G groups (p<0.001, p<0.05, p<0.001, p<0.001, p<0.01 and p<0.001 respectively). (Table 22, Graph 19)
vi. **Thickness of retinal layers in 12 day old embryos:**

On comparing 2G group embryos and control group embryos, no significant change was observed in the thickness of the pigment layer and layer of rods and cones. However there was a significant increase in the thickness of external plexiform layer, internal nuclear layer and optic nerve fiber layer (p<0.001, p<0.01 and p<0.001 respectively) and a significant decrease in external nuclear layer, internal plexiform layer and ganglion cell layer of 2G group embryos (p<0.001, p<0.001 and p<0.001 respectively).

The 3G group embryos also showed no significant changes in the thickness of the pigment layer, layer of rods and cones and external plexiform layer on comparing with control group embryos. However, the other layers showed a significant decrease in thickness on comparing with control group embryos (p<0.001, p<0.001, p<0.001 p<0.001, p<0.01 and p<0.001 respectively).
On comparing 2G and 3G group embryos, no significant change was observed in the thickness of the pigment layer and layer of rods and cones. The other layers showed a significant decrease in thickness in 3G group than 2G group embryos (p<0.001). (Table 22, Graph 20)

Graph 20: Effect of RFR on thickness of retinal layers of 12 day old chick embryos

vii. **Total retinal thickness on 5th – 12th day embryos**

The total retinal thickness showed a gradual increase in thickness in all the three groups as the age advanced except for the 3G group. The 3G group showed a non-significant decrease in total retinal thickness on the 11th day and a significant decrease on the 12th day.

The total retinal thickness was found to have increased in both 2G and 3G group embryos when compared with control group embryos from 5th to 10th day of incubation (p<0.001 and p<0.001 respectively). However on comparing 2G and 3G group embryos, it was observed that the 3G group embryos showed a significant increase in thickness than 2G group embryos (p<0.05, p<0.001, p<0.001, p<0.01, p<0.001 and p<0.001 respectively).
On the 11\textsuperscript{th} day, however, the 2G group showed a significant increase in total retinal thickness than the control group embryos (p<0.001) and 3G group embryos showed a non-significant decrease in total retinal thickness. On comparing 2G and 3G group embryos, the 3G group showed a non-significant decrease in total retinal thickness (p<0.001).

On the 12\textsuperscript{th} day, the 2G group showed a non-significant increase of total retinal thickness on comparing with control group embryos and 3G group embryos showed a significant decrease in total retinal thickness than the control group embryos (p<0.001). On comparing 2G and 3G group embryos, the 3G group showed a significant decrease in total retinal thickness (p<0.001). (Table 23, Graph 21)

Table 23: Mean total retinal thickness in all 3 the groups (5 – 12 days)

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.06 ±0.0003</td>
<td>0.066 ± 0.0007***</td>
<td>0.08 ± 0.0017***</td>
</tr>
<tr>
<td>6</td>
<td>0.072 ± 0.0003</td>
<td>0.082 ± 0.0008</td>
<td>0.084 ± 0.0014***</td>
</tr>
<tr>
<td>7</td>
<td>0.093 ± 0.0011</td>
<td>0.097 ± 0.0011***</td>
<td>0.103 ± 0.0007***</td>
</tr>
<tr>
<td>8</td>
<td>0.107 ± 0.0013</td>
<td>0.113 ± 0.0010***</td>
<td>0.117 ± 0.0007***</td>
</tr>
<tr>
<td>9</td>
<td>0.117 ± 0.0007</td>
<td>0.123 ± 0.0015***</td>
<td>0.141± 0.0007***</td>
</tr>
<tr>
<td>10</td>
<td>0.115 ± 0.0007</td>
<td>0.140 ± 0.0011***</td>
<td>0.147± 0.001***</td>
</tr>
<tr>
<td>11</td>
<td>0.129 ± 0.0012</td>
<td>0.143 ± 0.0008***</td>
<td>0.128 ± 0.0008</td>
</tr>
<tr>
<td>12</td>
<td>0.155 ± 0.0007</td>
<td>0.158 ± 0.0015</td>
<td>0.132 ± 0.0009***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value< 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)
b) LENS

- **Histological observations:**

  The lens of control group embryos showed normal histological features. The lens capsule was intact covering the lens. Deep in the capsule the anterior surface was lined by lens epithelial cells (LEC) which were low cuboidal cells. These cells as they migrated towards the lens equator became columnar with elongated nuclei to form lens fibers that formed the core of the lens. The nuclei of lens fibers were densely packed at the equator and nuclei of the inner fibers were displaced anteriorly giving it a lens bow appearance as —c/l/l1 shaped curve. Very few spaces were able to make out in between the regularly arranged lens fibers. (Plate - 22, 23 & 24)
PLATE 22: Photomicrograph showing ocular lens of control, 2G and 3G group embryos at 400X magnification (5th - 8th day) Control group embryos showed lens fibers (LF) arranged regularly (black arrow), normal appearance of lens bow (LB) and mostly single layered anterior lens epithelium (red arrow). Both 2G and 3G group embryos showed disintegrated lens fibers with fragmentation and irregular arrangement (black arrow), increased number of cystic cells and spaces (blue asterix) and multilayered anterior lens epithelium (red arrow) (H & E staining)
PLATE 23: Photomicrograph showing ocular lens of control, 2G and 3G group embryos at 400X magnification (9th - 12th day) Control group embryos showed lens fibers (LF) arranged regularly (black arrow), normal appearance of lens bow (LB) and mostly single layered anterior lens epithelium (red arrow). Both 2G and 3G group embryos showed disintegrated lens fibers with fragmentation and irregular arrangement (black arrow), increased number of cystic cells and spaces (blue asterix) and multilayered anterior lens epithelium (red arrow). Few experimental embryos showed cortical clefts filled with degenerating proteins (Morgagnian globules) (green arrow) (H & E staining)
PLATE 24: Photomicrograph showing ocular lens of control, 2G and 3G group embryos at 1000X magnification. Control group embryos showed lens fibers (LF) arranged regularly (black arrow), normal appearance of lens bow (LB) and mostly single layered anterior lens epithelium (red arrow). Both 2G and 3G group embryos showed disintegrated lens fibers with fragmentation and irregular arrangement (black arrow), increased number of cystic cells and spaces (blue asterix) and multilayered anterior lens epithelium (red arrow) (H & E staining)
However, in both the 2G and 3G groups, most of the embryos showed a multilayered epithelium on the anterior surface. The lens fibers showed fragmentation with cystic spaces and at some places the lens fibers had swollen to form cystic cells/balloon cells. In the central part of the lens, the nuclei appeared small, spherical and condensed. There were vacuoles or clefts in the lens cortex. Whereas, in the anterior part of lens, the nuclei were elongated and arranged in the form lens bow and posteriorly the lens fibers showed irregular orientation with folded lens fibers with spaces. Cystic spaces and cells were found to be largely towards anterior part. Few experiment embryos showed cortical clefts filled degenerated lens protein (Morgagnian globules) and wrinkled capsule on the posterior part. (Plate- 22, 23 and 24)

**Histomorphometric observations:**

i. **Equatorial diameter of ocular lens of chick embryos:**

The transverse diameter/ equatorial diameter showed a gradual increase as the age advanced in all the three groups of embryo. Both 2G and 3G group embryos showed a significant increase in the equatorial diameter on all days as compared with control group embryos (p<0.001 and p<0.001 respectively). On comparing 2G and 3G group embryos, it was found that the 3G group showed increased equatorial diameter on all days but the increase was significant only on 7th, 8th, 9th and 10th day (p<0.001). (Table 24, Graph 22)

ii. **Axial sagittal width / thickness of ocular lens of chick embryos:**

The thickness of lens also showed a gradual increase as the age advanced in all the three groups of embryo. The thickness of the ocular lens of 2G group embryos was found to be more up to 9th day on comparing with control group embryos. This increased thickness was significant only on the 6th and 7th day (p<0.001 and p<0.001 respectively) and from the 10th day onwards there was a decrease in thickness which was significant on the 11th and 12th day (p<0.05 and p<0.05 respectively).

However, 3G group embryos showed a significant decrease in thickness of lens in all days on comparing with control group embryos (p<0.001). On comparing the 2G and 3G group embryos, the 3G group embryos showed a significant decrease in thickness of ocular lens on all days (p<0.001). (Table 25, Graph 23)
Table 24: Mean lens equatorial diameter in all the 3 groups of chick embryo

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.328 ± 0.007</td>
<td>0.369 ± 0.006***</td>
<td>0.378 ± 0.003***</td>
</tr>
<tr>
<td>6</td>
<td>0.374 ± 0.003</td>
<td>0.534 ± 0.008***</td>
<td>0.517 ± 0.005***</td>
</tr>
<tr>
<td>7</td>
<td>0.545 ± 0.008</td>
<td>0.586 ± 0.003***</td>
<td>0.818 ± 0.007***</td>
</tr>
<tr>
<td>8</td>
<td>0.628 ± 0.01</td>
<td>0.681 ± 0.007***</td>
<td>0.813 ± 0.006***</td>
</tr>
<tr>
<td>9</td>
<td>0.740 ± 0.01</td>
<td>0.731 ± 0.01</td>
<td>0.875 ± 0.01***</td>
</tr>
<tr>
<td>10</td>
<td>0.846 ± 0.007</td>
<td>0.927 ± 0.008***</td>
<td>0.978 ± 0.008***</td>
</tr>
<tr>
<td>11</td>
<td>0.905 ± 0.013</td>
<td>0.984 ± 0.011***</td>
<td>1.0198 ± 0.006***</td>
</tr>
<tr>
<td>12</td>
<td>1.052 ± 0.01</td>
<td>1.109 ± 0.006***</td>
<td>1.106 ± 0.006***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value˂ 0.05* significant, ˂ 0.01** highly significant and ˂ 0.001 *** extremely significant)

Graph 22: Effect of RFR on mean equatorial diameter of ocular lens in all the 3 groups

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
Table 25: Mean lens thickness in all the 3 groups of chick embryo

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Control group (Group-C)</th>
<th>2G group (Group-A)</th>
<th>3G group (Group-B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.178 ± 0.004</td>
<td>0.187 ± 0.003***</td>
<td>0.138 ± 0.002</td>
</tr>
<tr>
<td>6</td>
<td>0.239 ± 0.004***</td>
<td>0.274 ± 0.003***</td>
<td>0.209 ± 0.006</td>
</tr>
<tr>
<td>7</td>
<td>0.307 ± 0.006***</td>
<td>0.354 ± 0.004***</td>
<td>0.252 ± 0.004</td>
</tr>
<tr>
<td>8</td>
<td>0.375 ± 0.007</td>
<td>0.362 ± 0.012***</td>
<td>0.262 ± 0.004</td>
</tr>
<tr>
<td>9</td>
<td>0.439 ± 0.005</td>
<td>0.457 ± 0.006***</td>
<td>0.338 ± 0.008</td>
</tr>
<tr>
<td>10</td>
<td>0.534 ± 0.005</td>
<td>0.526 ± 0.004***</td>
<td>0.471 ± 0.004</td>
</tr>
<tr>
<td>11</td>
<td>0.575 ± 0.007*</td>
<td>0.547 ± 0.006***</td>
<td>0.521 ± 0.005</td>
</tr>
<tr>
<td>12</td>
<td>0.666 ± 0.007*</td>
<td>0.634 ± 0.004***</td>
<td>0.603 ± 0.008</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 6 samples per day for control, 2G and 3G group (n = 48 chick embryos) (p value< 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)

Graph 23: Effect of RFR on mean thickness of ocular lens in all the 3 groups

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
D. BRAIN

Since the neurons in the brain were not differentiated fully during the 5th – 12th days, a general scanning of different areas of brain showed no significant structural changes in control and exposure groups.

3. ENZYMATIC ANTIOXIDANT ACTIVITY

A. SUPEROXIDE DISMUTASE (SOD) ACTIVITY

i. SOD activity in liver

The SOD activity was found to be significantly reduced for both the 2G and 3G group embryos on 9th day when compared with control group embryos (p<0.05 and p<0.01 respectively). However, no significant change was observed in SOD activity for both 2G and 3G group embryos on comparing with control group embryos on 12th day. On comparing between 2G and 3G group embryos, no significant change was observed in SOD activity on both days (Table 26, Graph 24)

ii. SOD activity in eye

No significant change was observed in SOD activity for both the 2G and 3G group embryos on comparing with control group embryos on 9th day. However, on the 12th day, the SOD activity was significantly reduced for 2G group and 3G group embryos on comparing with the control group embryos (p<0.05 and p<0.05 respectively). On comparing between 2G and 3G group embryos, no significant change was observed in SOD activity on both days (Table 26, Graph 25)

iii. SOD activity in brain

On the 9th day, no significant change was observed in SOD activity for 2G group embryos on comparing with control group embryos, whereas, 3G group embryos showed a significant reduction in SOD activity on comparing with control group embryos (p<0.05). However, on the 12th day 2G group showed significant reduction in SOD activity on comparing with control group embryos (p<0.001) and 3G group embryos showed no significant change in SOD activity on comparing with control group embryos. On comparing between 2G and 3G group embryos, no significant change was observed in SOD activity on both days (Table 26, Graph 26)
Table 26: Estimation of superoxide dismutase (SOD) in all the 3 groups

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Tissue</th>
<th>Control group (Group-C) U/g of protein</th>
<th>2G group (Group-A) U/g of protein</th>
<th>3G group (Group-B) U/g of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Liver</td>
<td>714.1 ± 1</td>
<td>361.7 ± 119*</td>
<td>262.4 ± 8**</td>
</tr>
<tr>
<td>12</td>
<td>Liver</td>
<td>211.4 ± 9.9</td>
<td>176 ± 42.4</td>
<td>194.4 ± 14.7</td>
</tr>
<tr>
<td>9</td>
<td>Eye</td>
<td>308.99 ± 111</td>
<td>217.28 ± 44</td>
<td>248.3 ± 59.7</td>
</tr>
<tr>
<td>12</td>
<td>Eye</td>
<td>256.3 ± 10</td>
<td>160.4 ± 27.2*</td>
<td>158.04 ± 0.49*</td>
</tr>
<tr>
<td>9</td>
<td>Brain</td>
<td>219.7 ± 65.9</td>
<td>81.2 ± 11.3</td>
<td>35.3 ± 3.2*</td>
</tr>
<tr>
<td>12</td>
<td>Brain</td>
<td>52.6 ± 0.17</td>
<td>24.1 ± 2.6**</td>
<td>36.6 ± 6.5</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 3 samples per day for control, 2G and 3G group (n = 18 chick embryos) (p value < 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)

Graph 24: Effect of RFR on SOD activity of liver in all the 3 groups

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
Graph 25: Effect of RFR on SOD activity of eye in all the 3 groups

Graph 26: Effect of RFR on SOD activity of brain in all the 3 groups

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
B. GLUTATHIONE PEROXIDASE (GPx) ACTIVITY

i. **GPx activity in liver**

The GPx activity was found to be significantly reduced for both 2G and 3G group embryos on the 9th day when compared with the control group embryos (p<0.01 and p<0.01 respectively). However, on the 12th day, the GPx activity was significantly reduced for 2G group embryos (p<0.05) but 3G group embryos showed non-significant reduction in GPx activity on comparing with control group embryos. On comparison between the 2G and 3G group embryos, no significant change was observed in GPx activity (Table 27, Graph 27).

ii. **GPx activity in eye**

The GPx activity was significantly reduced for both 2G and 3G group embryos on the 9th day when compared with control group embryos (p<0.05 and p<0.05 respectively). However, on the 12th day, the GPx activity showed non-significant changes in GPx activity for both 2G and 3G group embryos on comparing with control group embryos. On comparing between 2G and 3G group embryos, no significant change was observed in GPx activity (Table 27, Graph 28).

iii. **GPx activity in brain**

No significant change was observed in GPx activity for both 2G and 3G group embryos on comparing with control group embryos on 9th and 12th day. On comparing between 2G and 3G group embryos, no significant change was observed in GPx activity (Table 27, Graph 29).
Table 27: Estimation of glutathione peroxidase (GPx) in all the 3 groups

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Tissue</th>
<th>Control group (Group-C) U/mg of protein</th>
<th>2G group (Group-A) U/mg of protein</th>
<th>3G group (Group-B) U/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Liver</td>
<td>2330 ± 223</td>
<td>760 ± 188***</td>
<td>1136 ± 28**</td>
</tr>
<tr>
<td>12</td>
<td>Liver</td>
<td>1413 ± 188</td>
<td>569 ± 119*</td>
<td>875 ± 170</td>
</tr>
<tr>
<td>9</td>
<td>Eye</td>
<td>2099 ± 251</td>
<td>706 ± 219*</td>
<td>850 ± 293*</td>
</tr>
<tr>
<td>12</td>
<td>Eye</td>
<td>1226 ± 251</td>
<td>1268 ± 104</td>
<td>1136 ± 82</td>
</tr>
<tr>
<td>9</td>
<td>Brain</td>
<td>646 ± 184</td>
<td>752 ± 263</td>
<td>215 ± 18</td>
</tr>
<tr>
<td>12</td>
<td>Brain</td>
<td>439 ± 88</td>
<td>381 ± 66</td>
<td>153 ± 63</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 3 samples per day for control, 2G and 3G group (n = 18 chick embryos) (p value ≤ 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)

Graph 27: Effect of RFR on GPx activity of liver in all the 3 groups

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
Graph 28: Effect of RFR on GPx activity of eye in all the 3 groups

Graph 29: Effect of RFR on GPx activity of brain in all the 3 groups

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
4. DNA DAMAGE

A. LIVER

i. **Comet length**

The comet length was found to be significantly increased for both 2G and 3G group embryos in all the days (9\textsuperscript{th} – 12\textsuperscript{th} days) when compared with the control group embryos (p<0.001 and p<0.001 respectively). On comparing between 2G and 3G group embryos, it was found that 3G group embryos showed increased comet length than the 2G group embryos which was statistically significant on 11\textsuperscript{th} and 12\textsuperscript{th} day (p<0.001 and p<0.001 respectively). (Table 28, Graph 30) (Plate - 25).

ii. **Tail length**

The tail length of comets was also found to be significantly increased for both the 2G and 3G group embryos in all the days (9\textsuperscript{th} – 12\textsuperscript{th} days) when compared with the control group embryos (p<0.001 and p<0.001 respectively). On comparing between 2G and 3G group embryos, it was found that 3G group embryos showed increased tail length than the 2G group embryos which was statistically significant on 11\textsuperscript{th} and 12\textsuperscript{th} day (p<0.001 and p<0.001 respectively). (Table 28, Graph 31).

iii. **% of DNA in tail**

The % of DNA in the tail of comets was also found to be significantly increased for both 2G and 3G group embryos in all the days (9\textsuperscript{th} – 12\textsuperscript{th} days) when compared with the control group embryos (p<0.001 and p<0.001 respectively). On comparing between 2G and 3G group embryos, it was found that 3G group embryos showed increased % of DNA in the tail than the 2G group embryos which was statistically significant on 9\textsuperscript{th}, 10\textsuperscript{th} and 11\textsuperscript{th} day (p<0.001, p<0.001 and p<0.001 respectively). (Table 28, Graph 32).

iv. **Tail moment**

The tail moment of comets was also found to be significantly increased for both 2G and 3G group embryos in all the days (9\textsuperscript{th} – 12\textsuperscript{th} days) when compared with the control group embryos (p<0.001 and p<0.001 respectively). On comparing between 2G and 3G group embryos, it was found that 3G group embryos showed increased tail moment than the 2G group embryos which
was statistically significant on all days (p<0.001, p<0.001, p<0.01 and p<0.05 respectively) (Table 28, Graph 33).

Table 28: Estimation of DNA damage in liver in all the 3 groups (9 – 12 days)

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Mean comet length µm</th>
<th>Mean tail length µm</th>
<th>Mean % of DNA in tail µm</th>
<th>Mean tail moment µm µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 (CON)</td>
<td>4.03 ± 0.15</td>
<td>4.18 ± 0.1</td>
<td>21.63 ± 0.6</td>
<td>80.55 ± 3.0</td>
</tr>
<tr>
<td>9 (2G)</td>
<td>7.9 ± 0.18***</td>
<td>6.06 ± 0.1***</td>
<td>31.76 ± 1.6***</td>
<td>184.89 ± 6.2***</td>
</tr>
<tr>
<td>9 (3G)</td>
<td>7.8 ± 0.22***</td>
<td>6.12 ± 0.2***</td>
<td>47.16 ± 1.7***</td>
<td>258.69 ± 11.5***</td>
</tr>
<tr>
<td>10(CON)</td>
<td>4.6 ± 0.19</td>
<td>3.90 ± 0.1</td>
<td>21.23 ± 0.5</td>
<td>84.49 ± 3.1</td>
</tr>
<tr>
<td>10(2G)</td>
<td>7.3 ± 0.14***</td>
<td>6.40 ± 0.2***</td>
<td>34.05 ± 1.3***</td>
<td>157.13 ± 5.8***</td>
</tr>
<tr>
<td>10(3G)</td>
<td>7.4 ± 0.15***</td>
<td>6.39 ± 0.2***</td>
<td>48.07 ± 1.4***</td>
<td>260.53 ± 12.3***</td>
</tr>
<tr>
<td>11(CON)</td>
<td>5.2 ± 0.18</td>
<td>3.70 ± 0.1</td>
<td>22.46 ± 0.6</td>
<td>92.48 ± 3.0</td>
</tr>
<tr>
<td>11(2G)</td>
<td>8.3 ± 0.24***</td>
<td>6.35 ± 0.2***</td>
<td>42.43 ± 1.2***</td>
<td>240.54 ± 15.1***</td>
</tr>
<tr>
<td>11(3G)</td>
<td>9.5 ± 0.14***</td>
<td>8.30 ± 0.2***</td>
<td>48.94 ± 1.5***</td>
<td>292.16 ± 11.6***</td>
</tr>
<tr>
<td>12(CON)</td>
<td>6.0 ± 0.13</td>
<td>4.38 ± 0.1</td>
<td>23.10 ± 0.6</td>
<td>93.99 ± 3.2</td>
</tr>
<tr>
<td>12(2G)</td>
<td>8.9 ± 0.17***</td>
<td>7.15 ± 0.1***</td>
<td>47.14 ± 1.8***</td>
<td>248.08 ± 14.1***</td>
</tr>
<tr>
<td>12(3G)</td>
<td>9.8 ± 0.15***</td>
<td>8.24 ± 0.2***</td>
<td>49.67 ± 1.5***</td>
<td>298.69 ± 15.3***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 3 samples per day for control, 2G and 3G group (n = 36 chick embryos) (p value˂ 0.05* significant, ˂ 0.01** highly significant and ˂ 0.001 *** extremely significant)
Graph 30: Effect of RFR on mean comet length in liver (9-12 days)

Graph 31: Effect of RFR on mean comet tail length in liver (9-12 days)

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
Graph 32: Effect of RFR on mean % of DNA in comet tail in liver (9-12 days)

Graph 33: Effect of RFR on mean comet tail moment in liver (9-12 days)

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
PLATE 25: Photomicrograph showing comets in liver of control, 2G and 3G group embryos at 200X magnification (9th - 12th day). Control embryo comets showed minimal DNA damage with large head diameter (yellow arrow) and tail length is shorter (red arrow). Both 2G and 3G group embryos showed comets with severe DNA damage. Head diameter is decreased (yellow arrow) and tail length is increased indicating migration of damaged DNA (red arrow) (Silver nitrate staining)
B. **EYE**

i. **Comet length**

Both the 2G and 3G group embryos showed an increase in the mean comet length when compared with the control group embryos. The increase was statistically significant for 2G group on all days (p<0.001, p<0.01, p<0.001 and p<0.001 respectively) and for 3G group on 9th, 10th and 12th day (p<0.01, p<0.001 and p<0.001 respectively). In comparing between 2G and 3G group embryos, no significant change was observed except for 3G group embryos which showed a significant decrease in comet length on the 9th day (p<0.001) (Table 29, Graph 34) Plate-26.

ii. **Tail length**

Both the 2G and 3G group embryos showed an increase in the mean tail length of comets when compared with the control group embryos. The increase was statistically significant for 2G group on all days (p<0.001, p<0.05, p<0.001 and p<0.01 respectively) and for 3G group on 10th, 11th and 12th day (p<0.01, p<0.05 and p<0.01 respectively). On comparing between 2G and 3G group embryos, no significant change was observed except for 3G group embryos which showed a significant decrease in tail length on 9th day (p<0.001) (Table 29, Graph 35).

iii. **% of DNA in tail**

The % of DNA in the tail of comets in eye was also found to be significantly increased for both 2G and 3G group embryos in all the days (9th – 12th days) when compared with the control group embryos (p<0.001). On comparing between 2G and 3G group embryos, it was found that 3G group embryos showed increased % of DNA in the tail than the 2G group embryos which was statistically significant on 10th, 11th and 12th days (p<0.001, p<0.01 and p<0.01 respectively) except on 9th day where 2G group embryos showed a significant increase in % of DNA in the tail of comets (p<0.05) (Table 29, Graph 36)

iv. **Tail moment**

The tail moment of comets in eye was also found to be significantly increased for both 2G and 3G group embryos in all the days (9th – 12th days) when compared with the control group embryos (p<0.001 and p<0.001 respectively). On comparing between 2G and 3G group embryos, no significant change was observed except for 3G group embryos which showed a significant increase in tail moment on 10th day (p<0.001) (Table 29, Graph 37).
<table>
<thead>
<tr>
<th>Age in days</th>
<th>Mean comet length ( \mu m )</th>
<th>Mean tail length ( \mu m )</th>
<th>Mean % of DNA in tail ( \mu m )</th>
<th>Mean tail moment ( \mu m )</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 (CON)</td>
<td>5.09 ± 0.22</td>
<td>3.70 ± 0.23</td>
<td>29.04 ± 1.4</td>
<td>96.42 ± 8.3</td>
</tr>
<tr>
<td>9 (2G)</td>
<td>7.15 ± 0.17***</td>
<td>5.37 ± 0.19***</td>
<td>44.55 ± 1.4***</td>
<td>170.83 ± 12.1***</td>
</tr>
<tr>
<td>9 (3G)</td>
<td>6.01 ± 0.14**</td>
<td>4.25 ± 0.13</td>
<td>39.32 ± 2.1***</td>
<td>167.54 ± 12***</td>
</tr>
<tr>
<td>10 (CON)</td>
<td>5.32 ± 0.29</td>
<td>4.56 ± 0.34</td>
<td>24.4 ± 2</td>
<td>102.2 ± 11.08</td>
</tr>
<tr>
<td>10 (2G)</td>
<td>6.88 ± 0.37</td>
<td>5.69 ± 0.35*</td>
<td>33.5 ± 1.56**</td>
<td>162.7 ± 12.03*</td>
</tr>
<tr>
<td>10 (3G)</td>
<td>7.18 ± 0.22</td>
<td>5.94 ± 0.22**</td>
<td>50.73 ± 1.9***</td>
<td>273.68 ± 16.7***</td>
</tr>
<tr>
<td>11 (CON)</td>
<td>6.92 ± 0.21</td>
<td>5.21 ± 0.19</td>
<td>27.1 ± 2.1</td>
<td>129 ± 13.2</td>
</tr>
<tr>
<td>11 (2G)</td>
<td>8.66 ± 0.20***</td>
<td>6.79 ± 0.19***</td>
<td>43.7 ± 2.2***</td>
<td>264 ± 18.3***</td>
</tr>
<tr>
<td>11 (3G)</td>
<td>7.59 ± 0.27</td>
<td>6.01 ± 0.25*</td>
<td>56.07 ± 1.3***</td>
<td>295.7 ± 14.7***</td>
</tr>
<tr>
<td>12 (CON)</td>
<td>6.95 ± 0.2</td>
<td>5.85 ± 0.19</td>
<td>34.69 ± 2</td>
<td>174.7 ± 10.7</td>
</tr>
<tr>
<td>12 (2G)</td>
<td>8.81 ± 0.37***</td>
<td>7.29 ± 0.37**</td>
<td>42.91 ± 2.3**</td>
<td>286.18 ± 27.1***</td>
</tr>
<tr>
<td>12 (3G)</td>
<td>9.17 ± 0.24***</td>
<td>7.26 ± 0.20**</td>
<td>56.54 ± 2.3***</td>
<td>360.02 ± 19.1***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 3 samples per day for control, 2G and 3G group (n = 36 chick embryos) (p value < 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)
Graph 34: Effect of RFR on mean comet length in eye (9-12 days)

Graph 35: Effect of RFR on mean comet tail length in eye (9-12 days)

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
Graph 36: Effect of RFR on mean % of DNA in comet tail in eye (9-12 days)

Graph 37: Effect of RFR on mean comet tail moment in eye (9-12 days)

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
PLATE 26: Photomicrograph showing comets in eye of control, 2G and 3G group embryos at 200X magnification (9th - 12th day). Control embryo comets showed minimal DNA damage with large head diameter (yellow arrow) and tail length is shorter (red arrow). Both 2G and 3G group embryos showed comets with severe DNA damage. Head diameter is decreased (yellow arrow) and tail length is increased indicating migration of damaged DNA (red arrow) (Silver nitrate staining)
C. **BRAIN**

i. **Comet length**

Both the 2G and 3G group embryos showed an increase in comet length on comparing with control group embryos. The increase was significant for both 2G group and 3G group on all days (p<0.001 and p<0.001 respectively). On comparing between 2G and 3G group embryos, the 3G group embryos showed statistically significant increase of comet length on 11th and 12th day (p<0.01 and p<0.001 respectively) but a significant decrease was observed on 9th day (p<0.05) (Table 30, Graph 38, Plate -27).

ii. **Tail length**

Both the 2G (p<0.001, p<0.001, p<0.001 and p<0.01 respectively) and 3G group embryos (p<0.05, p<0.001, p<0.001 and p<0.001 respectively) showed significant increase in tail length of comets on comparing with control group embryos on all days. On comparing between 2G and 3G group embryos, the 3G group embryos showed statistically significant increase of tail length on 12th day (p<0.001) (Table 30, Graph 39).

iii. **% of DNA in tail**

The % of DNA in the tail of comets in brain was also found to be significantly increased for both 2G and 3G group embryos in all the days (9th – 12th days) when compared with the control group embryos (p<0.001 and p<0.001 respectively). On comparing between 2G and 3G group embryos, it was found that 3G group embryos showed increased % of DNA in the tail than the 2G group embryos which was statistically significant on 10th and 12th days (p<0.01) (Table 30, Graph 40).

iv. **Tail moment**

Both the 2G and 3G group embryos showed an increase in the mean tail moment of comets when compared with the control group embryos. The increase was statistically significant for 2G group on 9th, 10th and 12th day (p<0.001, p<0.001, p<0.01 respectively) and for 3G group on all days (p<0.001). On comparing between 2G and 3G group embryos, 3G group embryos which showed a significant increase in tail moment on 11th day and 12th day (p<0.001 and p<0.01 respectively). (Table 30, Graph 41).
Table 30: Estimation of DNA damage in brain in all the 3 groups (9 – 12 days)

<table>
<thead>
<tr>
<th>Age in days</th>
<th>Mean comet length µm</th>
<th>Mean tail length µm</th>
<th>Mean % of DNA in tail µm</th>
<th>Mean tail moment µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 (CON)</td>
<td>5.5 ± 0.13</td>
<td>4.15 ± 0.08</td>
<td>24.88 ± 1.1</td>
<td>119.1 ± 6.9</td>
</tr>
<tr>
<td>9 (2G)</td>
<td>7.4 ± 0.22</td>
<td>5.48 ± 0.2***</td>
<td>35.66 ± 1.5***</td>
<td>169.9 ± 9.1***</td>
</tr>
<tr>
<td>9 (3G)</td>
<td>6.8 ± 0.11</td>
<td>5.87 ± 0.17***</td>
<td>35.31 ± 1.5***</td>
<td>173.2 ± 8.6***</td>
</tr>
<tr>
<td>10 (CON)</td>
<td>5.5 ± 0.13</td>
<td>4.17 ± 0.1</td>
<td>25.72 ± 1.1</td>
<td>123.8 ± 6.8</td>
</tr>
<tr>
<td>10 (2G)</td>
<td>6.5 ± 0.20***</td>
<td>5.45 ± 0.2***</td>
<td>33.73 ± 1.4***</td>
<td>179.9 ± 8.5***</td>
</tr>
<tr>
<td>10 (3G)</td>
<td>6.7 ± 0.14***</td>
<td>5.13 ± 0.1***</td>
<td>41.03 ± 1.6***</td>
<td>177.1 ± 7.2***</td>
</tr>
<tr>
<td>11 (CON)</td>
<td>4.9 ± 0.09</td>
<td>3.12 ± 0.08</td>
<td>34.9 ± 2.19</td>
<td>149.7 ± 7.3</td>
</tr>
<tr>
<td>11 (2G)</td>
<td>6.5 ± 0.20***</td>
<td>5.86 ± 0.25***</td>
<td>48.56 ± 2.8***</td>
<td>182.1 ± 11.7</td>
</tr>
<tr>
<td>11 (3G)</td>
<td>7.6 ± 0.21***</td>
<td>6.2 ± 0.2***</td>
<td>48.57 ± 1.7***</td>
<td>250 ± 16.07***</td>
</tr>
<tr>
<td>12 (CON)</td>
<td>5.63 ± 0.11</td>
<td>4.25 ± 0.01</td>
<td>38.54 ± 1.3</td>
<td>158.1 ± 6.5</td>
</tr>
<tr>
<td>12 (2G)</td>
<td>6.65 ± 0.16***</td>
<td>4.89 ± 0.14**</td>
<td>55.39 ± 1.3***</td>
<td>214.47 ± 13.5**</td>
</tr>
<tr>
<td>12 (3G)</td>
<td>8.6 ± 0.14***</td>
<td>6.5 ± 0.13***</td>
<td>61.83 ± 1.2***</td>
<td>267.6 ± 9.5***</td>
</tr>
</tbody>
</table>

Values are means ± SEM taken from 3 samples per day for control, 2G and 3G group (n = 36 chick embryos) (p value < 0.05* significant, < 0.01** highly significant and < 0.001 *** extremely significant)
Graph 38: Effect of RFR on mean comet length in brain (9-12 days)

Graph 39: Effect of RFR on mean comet tail length in brain (9-12 days)

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
Graph 40: Effect of RFR on mean % of DNA in comet tail of brain (9-12 days)

Graph 41: Effect of RFR on mean comet tail moment in brain (9-12 days)

* indicates P value ≤ 0.05 with control group considered significant.
# indicates P value ≤ 0.05 on comparing among 2G and 3G group considered significant.
PLATE 27: Photomicrograph showing comets in brain of control, 2G and 3G group embryos at 200X magnification (9th - 12th day). Control embryo comets showed minimal DNA damage with large head diameter (yellow arrow) and tail length is shorter (red arrow). Both 2G and 3G group embryos showed comets with severe DNA damage. Head diameter is decreased (yellow arrow) and tail length is increased indicating migration of damaged DNA (red arrow) (Silver nitrate staining)
DISCUSSION

Radiofrequency fields (RFR) are the part of electromagnetic spectrum and their frequency ranges from 9 KHz – 300 GHz (FCC., 1999, ARPANSA., 2011). The interaction of RF radiation emitted from cell phone on biological system has been studied for decades. The possible mechanisms by which these radiations interact with biological systems could be of various ways.

Biophysical mechanisms of interaction of RF fields

- Radiofrequency electromagnetic field when strikes on a biological body, the waves are reflected, transmitted, refracted or scattered by the body (Allen., S et al., 2009). The transmitted and refracted fields then induce electric and magnetic fields in the biological systems that interact with cells and tissues in a variety of ways, depending on the frequency, waveform and strength of the induced fields and the energy deposited or absorbed in the biological systems.

- RF radiation has low energy level photons carrying energy as low as $1.24 \times 10^{-5}$ ev which are incapable of producing ionization. Ionization is produced by the ejection or promotion of orbital electrons from the atoms or materials through which an electromagnetic wave propagates. The minimum energy required for producing ionization in water, atoms of carbon, hydrogen, nitrogen and oxygen are between 10 and 25 ev. Hence, RF radiation are considered as non-ionizing radiations of electromagnetic spectrum along with electric fields, magnetic fields, microwaves, infrared rays, ultra violet rays and visible light (FCC., 1999, ARPANSA., 2011). However, when RF fields are strong, more and more low energy RF photons are absorbed into the body that could potentially produce ionization in biological materials (Lin., 1978). The ionizing radiation of electromagnetic spectrum consists of x-rays and gamma rays and they are hazardous to health. The Non-ionizing radiations are less dangerous than ionizing radiations, but on prolonged exposure can cause health hazards (FCC., 1999).

- Under the influence of RF fields, the molecules and cells would rearrange and form chains along the direction of the field. This is known as Pearl chain effect and the chains formed might be single stranded or multi stranded (Schwan, H.P., 1982, Takashima, S et al., 1985).
- Stronger fields of RF radiation can alter the cellular shape, electroporation or permeabilization of cells (Gehl, J., 2003, Weaver, J.C., 1993).

- RF radiation can produce microwave auditory effect. Absorption of pulsed microwave energy produces a rapid rise in temperature that creates a thermo-elastic expansion of the soft tissue which then launches an acoustic wave of pressure that travels to cochlea. These are detected by the hair cells and relayed to the central auditory system for perception. A single microwave pulse can be perceived as an acoustic click, and a continuous microwave pulse can be sensed as a buzz (Lin, J.C., 1978, 1980, 2007a).

- The absorbed RF radiation can produce tissue heating from oscillating electric fields. Due to bioelectrical nature of cells, the electrical fields exert a force on the charged ions present in the body causing them to move, resulting in electric currents. The electrical resistance of the material in which the currents are flowing results in heat (thermal effect) (Lin, J.C et al., 2007b). This increased temperature in the body is then reduced by various heat dissipating mechanisms in the body (Hamada, A.J et al., 2011). Exposure to RF radiations may result in an imbalance between the heat generation and heat dissipation that result in thermal effects which can adversely affect the functioning of biological body (Deepinder, F et al., 2007, Habash, R.W.Y., 2008).

**Biochemical mechanisms of interaction of RF fields (nonthermal effects)**

Cells require a homeostatic environment for its survival and function. It maintains its homeostatic internal environment at the expenditure of energy (Na⁺ K⁺ ATPase pumps) by means of various control mechanisms. This depends on the integrity of various membranes, organelles, gene products and ions, intracellular and extracellular signals. Cells would die when they can no longer maintain the homeostasis due to loss of membrane integrity, disruption of ionic transport across the membrane and disruption of potential energy needed for ionic transport (Anjana, V.Y et al., 2009). The RF radiations would interact with the cells and alter their homeostatic environment by
- Electroporation of plasma membrane (Neumann, E et al, 1982).
- Alterations in Gap junction intercellular communication (GJIC) (Alberts, B et al., 1994, Li, C.M et al., 1999, Ye, J et al., 2002).
- Activation of secondary intracellular lyzosomes in the cytoplasm to release its hydrolytic enzymes leading to apoptosis (Neil, C., 2000).

Thus RFR / RF fields could adversely affect the functioning and survivability of cells by causing these adverse mechanisms.
1. GROSS MORPHOLOGICAL PARAMETERS:

A. MORTALITY RATE

Our study showed a considerable increase in mortality rate in chick embryos of both the 2G and 3G exposed groups than the control group embryos. The mortality rate was found to be 5.55% for the control group (Group - C), 13.8% for 2G exposed group (Group – A) and 9.7% for 3G exposed group (Group – B). The increased mortality rate of chick embryos on RFR exposure was also reported by other researchers (Bastide, M et al., 2001, Grigov‘ev Iug., 2003, Batellier, F., 2008, Ingole, I.V et al., 2006a, Zareen N et al., 2009b, Fatima Al Qudsi et al., 2012, Lotfi, A et al., 2012). Their studies showed the mortality rate ranging from 4.5 % - 77.78 % in exposed group embryos as compared with control group embryos that showed a mortality rate of 1 % – 16 %.

The variations in the mortality rate in each study might be due to the differences in methodologies followed by various researchers. In the present study, the 2G group embryos were exposed to RFR of 900 -1800 MHz and 3G group embryos to 1900 - 2100 MHz at a power density of 2 W/m² with a SAR of 0.37 W/kg for 12 days. The duration of exposure was 72 minutes per day. However, in earlier studies by various researchers, the embryos were exposed to RFR ranging from 428 MHz – 900 MHZ with SAR ranging from 47.1 mW/kg to 0.37 W/kg. The duration of exposure also varied from 4 to 21 days and power density ranged from 5.5 mW/cm² to 2 W/ m².

Moreover, in our study, 2G and 3G group embryos showed increased fatality towards the latter half of our experiment when compared with control group embryos. This might be due to the cumulative effect of repeated RFR exposure to the embryos (Lai, H et al., 2009, Carpenter, R.L et al., 1960 and Carpenter, R.L et al., 1968). We also observed that the maximum fatality was on those embryos placed near the antennae of the cell phone used and a similar observation was also reported by Ingole, I.V et al., 2006a. This probably might be due to increased power intensity near the antenna causing increased mortality rate (Ingole, I.V et al., 2006a).

The present study along with these previous studies proves the correlation of lethality with multifactor like the frequency and intensity of transmission, the duration of exposure, the
number of exposure and also the distance from the radiation source (Philips, J.L., 2009, Sivani, S et al., 2012).

**B. CONGENITAL ANOMALIES:**

The RFR emitted from a cell phone is a known environmental stress factor and are injurious to rapidly dividing tissues. It can affect the dynamic developmental process in an embryo making it susceptible to radiation induced disorders of growth and development (Anderson, R.E et al., 2009). They can cause cellular damage by means of various non-thermal mechanisms mainly due to increased free radical production.

In our study, we observed a number of congenital malformations in both 2G and 3G group embryos. The anomalies observed were mainly subcutaneous hemorrhage, monophthalmia, microphthalmia, limb bud anomalies, ectopia cordis and development of conjoint embryos. Various researchers have reported teratogenicity of RFR on developing chick embryos. The exposure of chick embryos to 900-1800 MHz RFR from cell phone for 14 days resulted in subcutaneous hemorrhage, anophthalmia, head abnormalities and abdominal hernia (Fatima Al Qudsi et al, 2012). Lahijani, M.L et al., 2007 and 2011 reported hemorrhage in various tissues, sinusoidal denaturation, increased lymphoid tissue, spina bifida, anophthalmia, monophthalmia, microphthalmia, growth retardation, brain malformation and increased apoptotic cells. The exposure of chick embryos to EMF resulted in neural tube defects (Farrell, J.M et al., 1997), in increased blood brain barrier permeability, increased cellular apoptosis and torn blood vessels (Kalantari, S et al., 2014).

However, in the present study we also observed ectopia cordis and conjoint embryos which were not reported in earlier studies. Moreover, brain malformations were not observed in the present study in both 2G and 3G group embryos but were reported by many researchers in their earlier studies. We also observed that RFR emitted from 3G cell phone (1900 MHz – 2100 MHz) caused similar congenital anomalies as caused by RFR emitted from a 2G cell phone. The previous authors have reported the teratogenicity of electromagnetic radiations in the frequency range of 50 MHz – 1800 MHz.

Majority of the live embryos exposed to 2G and 3G radiations in our study showed subcutaneous hemorrhage on comparing with control. This probably might be due to injury of
endothelial cells lining the blood vessels as they are highly sensitive to electromagnetic radiations (Anderson, R.E et al., 2009). This probably would have resulted in vasodilation and increased vascular permeability resulting in subcutaneous hemorrhage.

Batellier F., 2008, in his study observed that the lethal effects of chick embryo development in the experimental group were mainly observed between 9th and 12th day of incubation. In our study also 2G group embryos showed maximum congenital anomalies on 9th day (66.6%) and 3G group embryos showed maximum anomalies on 10th and 11th day (50%). Maximum mortality rate (50%) was also observed for both 2G and 3G group embryos on 9th day of incubation similar to his findings.

Organogenesis period of a developing embryo is considered as maximally radiosensitive period. Any insult (endogenous or exogenous) including RFR during this period of remarkable growth and differentiation is associated with the formation of various congenital malformations (Anderson, R.E et al., 2009). Thus our study reports that RF radiations from cell phone do have a negative impact, probably due to genotoxicity, on the developing chick embryo that would have caused various congenital anomalies.

C. GROWTH PARAMETERS:

In the present study, the growth parameters – volume, weight and CR length of the embryo was found to be increased for 2G group embryos exposed to RFR of 900 – 1800 MHz up to 10th day of incubation and on 11th and 12th day the growth rate became more or less similar to control group embryos. However, the changes were significant only from 7th – 10th day. This increase in growth parameters of 2G group embryos could be due to increased cellular proliferation on exposure to RFR (Parivar, K.M et al., 2006 and Zareen, N et al., 2009b). This cell proliferation coupled with $\text{ca}^{2+}$ influx into the cells that would have increased their survivability might have resulted in increased growth rate in our study. The increased survivability of damaged cells might later turn into neoplasms on prolonged exposure (Bawin, S.M., 1976, Blackman, C.F et al., 1982, 1990, Neil, C., 2000, Rao, V.S et al., 2008). Similar observation was reported earlier by Fatima Al Qudsi et al., 2012. In their study, the exposure of chick embryos to 900-1800 MHz increased the growth parameters (the CR length, body length and beak length) and also increased the eye development (eye weight, eye diameter, eye area,
eye perimeter) on the tenth day of incubation and a decrease in these parameters was observed on further exposure (Fatima Al Qudsi et al., 2012).

However, in the present study there was a decrease in growth parameters for 3G group embryos exposed to RFR of 1900 – 2100 MHz when compared with control group embryos. The decrease was significant only up to 7th day of incubation and thereafter it showed non-significant changes and by 10th day the embryos seemed to show an increase in rate of growth to balance its growth pace similar to that of control group embryos. This probably could be due to RFR interactions at cellular level (free radical production) and molecular level (DNA damage) resulting in genotoxicity. This in turn might affect cell proliferation either by increasing or reducing the proliferation rate and thus plays an important role during early embryonic development (Panagopoulos, D.J et al., 2011, Zareen, N et al., 2009b). A number of studies are available reporting retarded growth on exposure to RFR of similar frequency. Decreased fetal weight and crown-rump length was observed in intrauterine exposure of rat and mouse animal models to RFR ranging from 27.12 MHz to 2450 MHz (Lary, J. M et al., 1982, Marcickiewicz, J et al, 1986., O’Conner, M.E et al, 1999., Amer, F.I et al, 2013).

On comparing 2G and 3G group embryos, we observed that the 3G group embryos showed a decrease in growth parameters that was significant only up to 8th day of incubation. Also, the observed changes in growth parameters for both 2G and 3G group embryos were only up to 10th day. On 11th and 12th day all the three groups of embryos showed more or less similar values for volume, weight and CR length. This probably could be due to difference in cellular responses to RFR at different embryological periods and the cells might be trying to rebalance their growth and differentiation rate to normal by activating various cellular stress response mechanisms (Fatima Al Qudsi et al., 2012).

Thus, in the present study, we observed a correlation between the frequencies of radiation on growth parameters. The higher frequency range (1900 – 2100 MHz) of 3G spectrum might be causing more deleterious effects on developing tissues of chick embryos resulting in decreased growth parameters as observed in our study.
2. HISTOLOGICAL AND HISTOMORPHOMETRICAL PARAMETERS:

All living organisms respond to various noxious agents (external and internal) at cellular level that could result in cell injury and cell death. Electromagnetic radiations are also included in this list of potentially harmful substances that can compromise cellular safety and initiate cellular stress responses. The cellular reactions to these noxious agents include cytoplasmic changes, nuclear changes and vascular changes. They are the indication of compromised cellular safety conveyed by the cells in their language (Blank, M et al., 2009). The injured cell usually recovers once there is cessation of the noxious stimuli (reversible injury). But the prolonged exposure to these harmful stimuli causes irreversible injury to the cell resulting in apoptosis and necrosis (Anjana, V.Y et al., 2009).

A. KIDNEY

Kidneys are moderately sensitive to radiations. The effects of radiations are mainly seen in convoluted tubules and glomerulus (Anderson, R.E et al., 2009).

i. Cytoplasmic changes

In our study, both 2G and 3G group embryos showed cytoplasmic changes in the form increased vacuolations and alterations in cellular morphology in the form of focal disruption of brush border and basement membrane of lining cells of PCT. Cellular debris were also visible in the lumen due to sloughing of lining cells. The interstitium showed edema with variable infiltrates of lymphocytes, plasma cells, monocytes and macrophages and also were engorged with RBCs. These necrotic changes were visible from 5th day onwards with increasing severity as the age of embryos advanced. These findings were similar to previous reports published by a number of researchers in various animal models.

Al-Glaib, B et al., 2008 reported the presence of atrophied glomerulus, vacuolations and dilatation of renal tubules and interstitial edema showing mononuclear leukocyte infiltration in mice on exposure to RFR (900 MHz) from mobile phone for one hour per day for 10 days. Similar changes were also reported by Latifa Ishaq Khayyat., 2011 on exposing mice to EMF for 8 hours for 3 days and 12 days. The author also reported congested and dilated renal veins and inter-tubular inflammation with the histopathological changes more pronounced in 12th day. The exposure of rat to 1 hour per day for 4 weeks resulted in atrophied glomeruli and extravasation of blood in the interstitial space between the renal tubules (Laila, K et al., 2010). On exposing the
chick embryos to RFR of 900 MHz for 4 hours, 5 hours and 6 hours for 6, 8 and 10 days resulted in narrowed bowman’s space of renal corpuscles, increased vacuolations, pyknotic nuclei, and disruption of luminal border and basement membrane in lining cells of PCT (Ingole, I.V et al, 2006b).

We also observed mild cytoplasmic changes in DCT of older embryos that showed isolated vacuolations and focal disruption of brush border. Previous authors reported changes in PCT alone. However, in PCT the vacuolations were present in almost entire cells lining the tubules indicating necrotic changes. The vacuolations in the cytoplasm might be due to fatty metamorphosis or steatosis as they were PAS negative (Anjana, V.Y et al., 2009).

ii. **Vascular changes**

The edema and presence of RBCs in the interstitium of kidney tissue observed in our study might be due to vascular damage caused by RFR resulting in vascular dilatation, vacuolations of the endothelium of peritubular capillaries with focal necrosis resulting in hemorrhage and exudation of blood plasma containing variable infiltrates of lymphocytes, plasma cells, monocytes and macrophages indicating changes similar to acute interstitial nephritis (Anderson, R.E et al., 2009, Arthur, H.C et al., 2009). Interstitial edema in the kidney tissue after RFR exposure were also reported by Al-Glaib, B et al., 2008, Laila, K et al., 2010 and Latifa, I.K., 2011.

iii. **Changes in glomerulus**

In our study, we also observed that the glomerulus showed a non-significant increase in diameter and significantly increased urinary space in both 2G and 3G exposed embryos when compared with the control group. The increased glomerular diameter might be due to cytoplasmic changes caused by RFR. RFR emitted from cell phone causes degeneration or weakening of plasma membrane due to non-thermal effects (Blackman, C.F et al., 1982, 1990, Lew, V.L et al., 1988, Ha, B.Y., 2001). This would have resulted in increased influx of water resulting in severe swelling (hydropic changes / oncosis) of cells lining the parietal and visceral layer of glomerulus and also endothelial cells lining the glomerular capillaries which would have caused an increase in glomerular diameter (Anderson, R.E et al., 2009, Anjana, V.Y et al., 2009). Moreover, the endothelial cells lining the blood vessels are highly sensitive to radiation effects and it manifests as vasodilatation and increased vascular permeability. In our study, vasodilatation and increased vascular permeability of glomerular capillaries would have caused
the plasma exudate to escape into the urinary space resulting in increased urinary space (Anderson, R.E et al., 2009, Arthur, H.C et al., 2009). This observation was similar to the findings of Mugunthan, N et al., 2014, who reported dilated glomeruli and urinary space on exposing mice to RFR of 900 – 1900 MHz for 48 minutes per day for a period of 30 to 180 days. However, other previous studies reported glomerular atrophy on RFR exposure on rat and mice models (Al-Glaib, B et al., 2008, Laila, K et al., 2010, Latifa, I.K., 2011, Hanafi, N et al 2012). In their studies, the duration of exposure was short ranging from 3 – 12 days. In our study, though the experiment duration was for 12 days, the animal model used was chick embryo which was much smaller in size than rat and mice. This would have resulted in better penetration and interaction of RFR with chick embryo tissues. Mugunthan, N et al exposed the mice for a longer duration (30-180 days) that would have resulted in cumulative effect of RFR on the mice kidney. Thus the effect of RFR exposure might manifest differently depending on the duration of exposure, frequency and intensity of transmission, the number of exposure, the shape and size of exposed organism, the water and mineral content of the organism and also the distance from the radiation source (Philips, J.L., 2009, Sivani, S et al., 2012).

On comparing between 2G and 3G group embryos, it was found that 3G group embryos showed significantly increased urinary space which might be an indicator of increased lethality of 3G radiations.

iv. **Changes in standard epithelial height of PCT and DCT**

Another observation that warrants our attention was the increase in standard epithelial height (SEH) of lining cells (cytomegaly) in PCT and DCT of both 2G and 3G group embryos as compared with control group embryos. This increased height might be again due to hydropic changes / oncosis resulting in cellular swelling (cytomegaly) on RFR exposure (Anderson, R.E et al., 2009, Anjana, V.Y et al.,2009). On comparing both 2G and 3G group embryos it was observed that 3G group embryos showed significantly increased SEH in PCT and on 12th day in DCT. This probably might be due to increased lethality of RFR emitted from 3G cell phone.

v. **Nuclear changes**

In addition to cytoplasmic changes and vascular changes, nuclear changes were also observed in our study. The radiations are known to cause nuclear swelling (nucleomegaly), mild to moderate pyknosis, karyorrhexis (nuclear fragmentation) and karyolysis (dissolution of chromatin) (Anderson, R.E et al., 2009). The nuclear changes observed in our study were
increased nuclear diameter in both 2G and 3G group embryos which might be due to hydropic changes and clumping of chromatin (Anderson, R.E et al., 2009, Anjana, V.Y et al., 2009). Some of the nuclei also showed pyknosis and karyorrhexis (nuclear fragmentation) that was significantly more on comparing with control. On comparing between 2G and 3G group embryos, 3G group embryos showed significantly increased nuclear diameter in PCT but 2G group embryos showed increased diameter in DCT but on 12th day showed non-significant change with 3G group embryos. Karyorrhexis also was significantly more in 3G group embryos. These observations again points to increased lethality of 3G radiation.

B. LIVER

Liver also is moderately sensitive to radiations. The effects of radiations are mainly seen in endothelial cells of small hepatic veins resulting in collagen deposits and delicate fibrin strands within lumen resulting in venoocclusive disease. This might lead to variable amounts of centrilobular congestion and necrosis in parenchyma (Anderson, R.E et al., 2009).

i. Cytoplasmic changes

The cytoplasmic changes that we observed in hepatocytes of 2G and 3G group embryos were the presence of vacuolations rendering them with a mild foamy appearance. These vacuolations were PAS negative that indicated fatty changes (steatosis) (Anjana, V.Y et al., 2009, Kamal, G.I et al., 2009). A number of similar observations were reported earlier by a number of researchers. The exposure of white leghorn chicken embryos to 50 Hz electromagnetic fields showed fibrotic bands in hepatocytes, severe steatohepatitis, degenerated hepatocytes, abnormal lipid accumulation and lipid droplets pushing hepatocytes nuclei to the corner of the cell (Lahijani, M.L et al., 2009). The exposure of pregnant albino rats to 900 MHz for 1 hour per day from 13th to 21st day of pregnancy resulted in histopathological changes in the liver of 21 day old neonatal rats. The changes observed were necrotic hepatocytes with hydropic changes in the liver parenchyma especially near peri-central area and irregular nuclei (Zehra, T et al., 2015). Tarantino, P et al., 2005 reported vacuolations in cytoplasm and presence of granules in hepatocytes of rabbit liver on exposure to 650 MHz electromagnetic fields for 18 months continuously. However, in the present study necrotic changes were not observed. In one of the studies, the exposure of mice to electromagnetic fields of 50 MHz and intensity of 0.5 mT for 4
hours daily for 2 months showed no significant increase in necrotic cells and kuffer cells and no structural changes in liver (Rajaei, F et al., 2013).

ii. **Vascular changes**

The sinusoidal spaces appeared dilated (peliosis hepatis) and congested with RBCs in both the 2G and 3G group embryos than control group embryos. This probably might be due to endothelial injury caused by RFR that would have allowed blood to accumulate in spaces of Disse with resultant formation of the cavities (Kamal, G.I et al., 2009). Sinusoidal dilatations in hepatic parenchyma have been reported earlier by other researchers. The exposures of young male rats to electromagnetic radiations lead to sinusoidal dilatation in the parenchyma and periportal area of liver tissue (Gokcimen, A et al., 2002). Sinusoidal expansion and irregular sinusoidal lumen diameter was observed in rabbit liver on exposure to 650 MHz radiation for 12 and 18 months respectively. Moreover, infiltrations within the sinusoids of chick embryo liver were also reported by Lahijani, M.L et al., 2009. In our study also infiltrations were there in sinusoids and the sinusoids were engorged with RBCs.

iii. **Nuclear changes**

The hepatic nuclei in 2G and 3G group embryos appeared swollen (nucleomegaly) which might be due to hydropic changes. Glycogenated nuclei or hydropic nuclei could be encountered in various pathological conditions (Kamal, G.I et al., 2009). In the present study, RFR might have triggered the formation of hydropic nuclei in the hepatocytes of both 2G and 3G group embryos. The nuclear diameter was found to be significantly increased in both 2G and 3G group embryos on comparing with control group embryos. This coincides with our observation of swollen appearance of hepatic nuclei due to hydropic changes. Previous studies have reported the presence of dentated nuclei in chick embryo liver on exposure to 50 Hz electromagnetic radiations (Lahijani, M.L et al., 2009) and necrotic hepatocytes with irregular nuclei (Zehra, T et al., 2015).

On comparing the hepatic nuclear diameter of 2G and 3G group embryos, we observed that the 3G group embryos showed significantly increased nuclear diameter. This might be due to increased lethality of 3G radiations.
C. **EYE**

The eyes are highly sensitive to radiations especially the ocular lens. A correlation between the radiation exposure and formation of cataracts has been long established (Lipman, R..M et al., 1988, Cutz, A. 1989., Gordon, K.K et al., 2009). According to ICNRP (International Commission on Non-ionizing Radiation Protection) guidelines, the microwave exposure limit for eyes is set at 5 mW/cm² (Yu, Y et al., 2010, Mary, H.D et al., 2014b). Exposure to RFR below this level will not cause any thermal stress, as the body could tolerate the thermal changes through various heat dissipating mechanisms without any possible damage. But numerous scientific studies have shown that even though the exposure level to RFR is well within this set limit, the exposed retina and lens showed histopathological changes which might be probably due to non-thermal effects of RFR. (Mary, H.D et al., 2014b).

**a) RETINA**

i. **Histological changes in chick embryo retina:**

In our study we observed an increase in intercellular spaces in all the cellular layers of retina in both 2G and 3G group embryos. The spaces were seen in the germinative layer of 5th - 7th day retina, in outer and inner neuroblastic layer of 8th day retina and external nuclear layer, internal nuclear layer and ganglion cell layer of 9th – 10th day old chick embryo retina. Moreover, disintegration of optic nerve fibers was observed in both 2G and 3G group embryos. Similar observations were also reported in previous studies also. The exposure of the pregnant CD-1 mice to RFR from 900- 1800 MHz for 2 hours per day from 7th – 14th day of gestation showed increased vacuolations in the cytoplasm of outer and inner nuclear layers, pyknotic nuclei and DNA fragmentation in the retina of exposed fetuses on the 18th day as compared with control group (Amer, F.I et al., 2013). In another study, exposure of chick embryos to RFR of 900- 1800 MHz caused disintegrated optic nerve fiber and disorganized cells in inner nuclear layer (Fatima Al Qudsi et al., 2012).

The increase in intercellular spaces observed in our study might be due to alterations in plasma membrane integrity caused by non-thermal effects on RFR exposure. This would have caused either cytomegaly due to hydropic changes rendering an appearance of increased intercellular space between the cells or subsequent cell death caused by the failure of Na –K ATPase pumps to prevent the cells from reaching —Gibbs–Donnan equilibrium (Anjana, V.Y et
al., 2009). Once the Gibbs–Donnan equilibrium is reached, massive cellular swelling due to influx of water and eventual death occurs.

### ii. Changes in thickness of Retinal layers in 5th – 7th day embryos:

On comparing the thickness of each layer we observed that the pigment layer showed significantly increased thickness in 3G group embryos than control group and 2G group embryos on 5th day but this increase later changed to decreased thickness by 7th day. This might be due to the cells trying to rebalance their growth and differentiation rate to normal by activating various cellular stress response mechanisms (Fatima Al Qudsi et al., 2012).

The germinative layer in both 2G and 3G group showed significantly increased thickness in all the three days. Two possible explanations for this increase could be an increased cellular proliferation due to RFR exposure (Parivar, K.M et al., 2006 and Zareen, N et al., 2009b) and hydropic changes in the cytoplasm rendering the cells to swell (Anderson, R.E et al., 2009, Anjana, V.Y et al., 2009). The latter could also give an appearance of increased intercellular spaces among the cells.

The inner marginal layer was also significantly increased in thickness in both experimental groups which probably might be due to their disintegration on RFR exposure.

On comparing between 2G and 3G group, we observed a significant increase in thickness of germinative layer of 3G group embryos. This again might be due to increased lethality of 3G radiations. Since literatures are not available to compare the changes in retina in this age group, ours might be a maiden study on the effect of RFR in retina in this age group.

### iii. Changes in thickness of Retinal layers in 8 day old embryos:

The thickness of outer neuroblastic layer was significantly increased in both experimental groups than control group which might be due to cytoplasmic changes (hydropic changes) (Anderson, R.E et al., 2009, Anjana, V.Y et al., 2009) and increased cellular proliferation (Parivar, K.M et al., 2006 and Zareen, N et al., 2009b). However, the inner neuroblastic layer showed a decreased thickness than control group probably might be due to decreased cellular proliferation. These differences in the growth parameters among the layers of retina might be due to different cellular responses of each layer to RFR exposure (Fatima Al Qudsi et al., 2012).
On comparing the 2G and 3G group embryos, the thicknesses of pigment layer, outer neuroblastic layer and inner neuroblastic layer was found to be significantly increased in 3G group and also histological changes were more pronounced. These observations might be yet another proof for the increased lethality of 3G radiations.

iv. **Changes in thickness of Retinal layers in 9th and 10th day embryos:**

On examining the thicknesses of each layer we found that in both experimental groups, the cellular layers - external nuclear layer, internal nuclear layer and ganglion cell layer showed increased thickness in both days which might be due to increased cell proliferation and hydropic changes in the cell (Parivar, K.M et al., 2006 and Zareen, N et al., 2009b, Anderson, R.E et al., 2009, Anjana, V.Y et al., 2009). However, the layer of nerve plexuses – external plexiform layer and internal plexiform layer showed a decreased thickness in both 2G and 3G group on comparing with control on 9th day which probably might be due decreased synaptic contacts between the cells of different layers. On 10th day no significant change was observed for external plexiform layer for all three groups but the internal plexiform layer showed slight increase in thickness for both 2G and 3G group embryos. Previous study by Fatima Al Qudsi et al., 2012 has shown increased thickness of external plexiform layer, internal nuclear layer, internal plexiform layer and ganglion cell layer on 10th day and 14th day of chick embryo retina. Amer, F.I et al., 2013 reported the appearance of vacuolations in the cytoplasm of cells in outer nuclear layer, inner nuclear layer and ganglion cell layer with pyknotic nuclei. However, in the present study the external and internal plexiform layer showed decreased thickness but internal nuclear layer increased in thickness similar to the observations of Fatima Al Qudsi et al., 2012.

On comparing between 2G and 3G group embryos, it was observed that no significant change in the thickness of pigment layer and layer of rods and cones on both days. However, 3G group embryos showed significant increase in thickness of internal nuclear layer in both days which might be due to increased cellular proliferation or hydropic changes of cells under RFR exposure. But significant decrease was observed in the thickness of external nuclear layer, external plexiform layer and ganglion cell layer on 9th day followed by non-significant changes on 10th day. This could be due to the activation of various cellular stress response mechanisms under the influence of which the cells might be trying to rebalance their growth and differentiation rate to bring back to normal growth rate (Fatima Al Qudsi et al., 2012).
v.  **Changes in thickness of Retinal layers in 11\textsuperscript{th} and 12\textsuperscript{th} day embryos:**

The pigment layer and the layer of rods and cones showed no significant change in thickness in the entire three groups. An interesting observation we noticed in these days were the decreased thicknesses of remaining layers in 3G group embryos when compared with control and 2G group embryos. A possible explanation for this decrease could be a decreased cellular proliferation and decreased synaptic contact between the cells due to the cumulative effect of RFR on prolonged exposure (Lai, H et al., 2004., Carpenter, R. L et al., 1960 and Carpenter, R. L et al., 1968).

However, 2G group embryos continued to show increased thickness of internal nuclear layer in both days similar to the observation of Fatima Al Qudsi et al., 2012 who also reported increased internal nuclear layer thickness on 10\textsuperscript{th} and 14\textsuperscript{th} day of chick embryo retina exposed to 2G radiation. Moreover, our study showed decreased thickness of other layers on 12\textsuperscript{th} day except the pigment layer and layer of rods and cones that showed non-significant changes.

On comparison between 2G and 3G group embryos, there was a decrease in thickness in different layers of retina in 3G group embryos on 11\textsuperscript{th} and 12\textsuperscript{th} day.

Thus the RFR exposure caused cellular proliferation during the initial days of incubation in the cellular layers of retina but on prolonged exposure resulted in different cellular responses. Prolonged exposure caused either in decreased or increased cellular proliferation causing different thicknesses in various layer of retina in both 2G and 3G group embryos. These changes might be due to activation of various cellular stress response pathways that would have caused the cells to rebalance their growth and differentiation by altering the cell proliferation (Fatima Al Qudsi et al., 2012).

vi.  **Changes in pigment layer and total retinal thickness:**

Pigment layer is made up of retinal pigment epithelium (RPE). They contain melanin pigment that plays an important role in maturation of neural retina, provides protection against oxidative stress and cytotoxicity and also detoxifies peroxides (Jeffrey, G et al., 1994, Akeo et al., 2000, Lia, M et al., 2000, Agar, N et al., 2005, Zareen, N et al 2009a). Developing retinal pigment epithelium contains dopa which is a melanin precursor. Dopa regulates retinal cell mitosis and its absence causes retinal deficits (Lia, M et al., 2000).
In the present study, the RPE showed mild pigmentation up to 8\textsuperscript{th} day of incubation followed by an increase in pigmentation rendering the RPE with moderate pigmentation till 12\textsuperscript{th} day of incubation for control group embryos. Whereas, 2G and 3G group showed mild pigmentation till 6\textsuperscript{th} day and moderate pigmentation on 7\textsuperscript{th} day. This increase in melanin pigment would have resulted in increased growth and development of cellular layer of neural retina in both the experimental group embryos and their increased total retinal thickness on 5\textsuperscript{th} - 7\textsuperscript{th} day when compared to control group embryos.

From the 8\textsuperscript{th} day onwards, 2G group embryo continued to show moderate pigmentation up to 9\textsuperscript{th} day followed by intense pigmentation on 10\textsuperscript{th} – 12\textsuperscript{th} day. Probably this would have resulted in decreased thickness of neural retina of 2G group embryos up to 10\textsuperscript{th} day followed by increased thickness on 11\textsuperscript{th} and 12\textsuperscript{th} day due to intense pigmentation on comparing with 3G group embryos. This observation was similar to the findings of Zareen, N et al., 2009a. They reported impaired growth of retina with hypopigmentation in chick embryos till 10\textsuperscript{th} day of incubation and further exposure up to 15 days showed growth enhancement and hyperpigmentation of retinal pigment epithelium (RPE) on exposing the embryos to RFR emitted from 1800 MHz GSM mobile phone for 15 minutes twice a day.

However, 3G group showed intense pigmentation from 8\textsuperscript{th} day onwards up to 10\textsuperscript{th} day followed by decrease in pigmentation rendering them moderate pigmentation on 11\textsuperscript{th} and 12\textsuperscript{th} day. This could have caused increased neural retinal thickness of 3G group embryos up to 10\textsuperscript{th} day than control and 2G group embryos. Thereafter, on 11\textsuperscript{th} and 12\textsuperscript{th} day 3G group embryos showed decrease in neural retinal thickness than control and 2G group embryos due to decrease in pigmentation (moderate pigmentation). This decreased pigmentation might be due to pathophysiological changes caused by the overproduction of various heat shock proteins HSP-70, HSP-27 on prolonged RFR exposure (Lixia, S et al., 2006, Yu, Y et al., 2008). Our observation for 3G group embryos is similar to the findings of Fatima Al Qudsi et al., 2012. In their study, the retina of chick embryos showed enhanced development up to 10\textsuperscript{th} day of incubation and prolonged exposure till 14\textsuperscript{th} day showed impaired retinal growth on exposing them to RFR of 900- 1800MHz. Another study showed an increased retinal thickness on exposing the rats to electromagnetic waves of 50 - 60 Hz for 4 weeks (Khaki, A.A et al., 2011)
Previous studies have correlated hypopigmentation of pigment retina with deranged retinal development. Hypopigmentation resulted in failure of central retina development in mammals (Jeffrey, G et al., 1994). It also caused age-related macular degeneration causing blindness (Schraermeyer, U et al., 1999). Local ocular hypopigmentation caused regional abnormalities in retina (Gimenez, E et al., 2005). In our study, we observed hyperpigmentation and increased retinal thickness in both experimental groups than control group embryos. However, on 11th and 12th day 3G group showed decreased thickness due to moderate pigmentation when compared to control and 2G group embryos.

Thus, in the present study, an early onset of increased melanogenesis was observed in both the 2G and 3G group embryos as compared with control group embryos. RF exposure is known to cause DNA damage (Lai, H et al., 1996, Lixia, S et al., 2006, Philips, J.L et al., 2009) in the form of single strand breaks (SSB) and double strand breaks (DSB). DNA strand breaks could trigger melanogenesis as one of its repair mechanisms (Agar, N et al., 2005). In our study, the RF exposure would have caused DNA damage in the retina of both the 2G group and 3G group embryos resulting in early onset of melanogenesis as indicated by the pigment gradation (Mary, H.D et al., 2014a).

In our study, we also observed an early differentiation of different layers of retina in both 2G and 3G group embryos. The 8 days old 2G and 3G embryos showed five layers in the retina whereas, the control group embryos showed mainly 3 layers (Mary, H.D et al., 2014a). The ganglion cell layer showed a decreased thickness on 11th and 12th day for both 2G and 3G group embryos that was more prominent in 3G group embryos. The decreased thickness of ganglion cell layer observed in our study could be due to cellular apoptosis that is encountered towards the end of gestation (Antony, J.B et al 1998). This natural cell death probably would have caused loss of synaptic contact between the cells of ganglion cell layer and inner nuclear layer resulting in reduced thickness of inner plexiform layer. These changes indicates an early onset of retinal maturation in both 2G group and 3G group embryos than the control group embryos (Mary, H.D et al., 2014a).
b) **LENS**

Ocular lens is a non-vascularized, non-innervated tissue containing high percentage of water (Mary, H.D et al., 2014b). They have poor heat dissipating mechanism owing to its poor blood supply and they have high percentage of water making the ocular lens highly sensitive to RF radiation (Yu, Y et al., 2010). Moreover, lens fiber cells have limited capacity for repair and fibres towards the center have less metabolic activity. The proteins present in these fibers do not turn over significantly and they are extremely long lived and vulnerable to oxidative stress (Antony, J.B et al 1998).

i. **Histological changes:**

In the present study, most of the 2G and 3G group embryos showed multilayered epithelium on the anterior surface compared to the control group embryos that showed single layered epithelium. The lens epithelial cells located at the center of the lens never undergo mitosis but various external stimuli could initiate mitosis in them making them multilayered as they were the first cells exposed to RFR exposure (Carpenter, R.L., 1979, Antony, J.B et al 1998). The lens fibers showed fragmentation, irregular orientation with folding, cystic spaces and at some places the lens fibers had swollen to form cystic cells/balloon cells. The cystic cells and spaces were seen in both anterior and posterior part of lens and their number and size increased as the age advanced. These cells were formed as a result of RFR exposure and represent an abortive attempt of epithelial cells to undergo normal differentiation into new lens fibers (Carpenter, R.L., 1979). The distortion of lens fibers also resulted in the aberrant arrangement of lens nuclei at the equator leading to irregular —lens bowl arrangement of nuclei and formation of aberrant posterior lens epithelial layer. Few experiment embryos showed cortical clefts filled with degenerated lens protein (Morgagnian globules) and wrinkled capsule on the posterior part. These changes were prominent from 7th day onwards. There were vacuoles or clefts in the lens cortex. All these observations in our study were similar to the findings of Wang, K.J et al., 2003, Dovrat, A et al., 2005, Bormusov, E et al., 2008. They exposed cultured bovine lens, rabbit lens epithelial cells and New Zealand rabbit eyes to RFR ranging from 1.1 GHz – 2.45 GHz.

Carpenter, R.L., 1979 reported structural damages in LEC of New Zealand rabbit eyes in the form of granular degeneration of the lens cell epithelium at the equator, the appearance of
large spherical or ovoid —balloon cells‖ and distortion of lens fibers. Bormusov, E et al., 2008 reported the formation of smaller cells and condensed nuclei (pyknosis) and increased adolase enzyme activities in lens epithelial cells of cultured bovine lens. Dovrat, A et al., 2005 in their study on cultured bovine lens reported that even after the removal of radiation, the microscopic changes still persisted. Wang, K.J et al., 2003 reported distorted arrangement of cultured rabbit lens epithelial cells

Growth and development consists of highly ordered sequence of proliferation of cells, their differentiation and migration followed by programmed cell death (Zareen, N et al, 2009a). The cellular ionic currents and electric fields play an important role in controlling these events. Any disruption in these fields especially due to RFR exposure from cell phone could change the internal environment of cell. Since the ocular lens contains high percentage of water, the RFR interacts with it to form ionizing water molecules, H$_2$O$^+$ and H$_2$O$^–$. This then dissociates to form H$^+$ and OH$^–$ free radicals resulting in oxidative stress and cell injury (Anjana, V.Y et al., 2009).

Oxidative stress is a dominant factor that causes cataract. In our study, the structural changes observed in the ocular lens of chick embryos were similar to cortical cataract (Gordon, K.K et al., 2009) induced by an ionizing radiation (Tokunaga, T., 1963, Kimura, S.J., 1951). Tokunaga, T., 1963, Kimura, S.J., 1951 reported that in radiation cataract, the cystic cells and vacuolations was present in the lens epithelial fibres located beneath the posterior capsule. Nevertheless, in our study the cystic cells and vacuolations were observed even in the anterior sub capsular cortex similar to the findings of Carpenter, R.L., 1979.

ii. **Equatorial diameter of ocular lens of chick embryos**

In the present study, the mean equatorial diameter/ transverse diameter of the lens increased gradually as the incubation period prolonged in both control and experiment group. Both the 2G and 3G group embryos showed significantly increased equatorial diameter than the control group embryos and on comparing 2G and 3G group embryos, 3G group showed increased equatorial diameter that was significant on all days. The increased equatorial diameter in both 2G and 3G group embryos could be due to increased proliferation of LECs at the equator, which is the germinative layer, to form secondary lens fibers. Moreover, the osmotic effect of degenerated cortex causes the lens to imbibe water and swell (Gordon, K.K et al., 2009), increasing the equatorial diameter. The formation of cystic cells and their increase in size and
number with advancing age could also be a reason for increased equatorial diameter observed in our study (Mary, H.D et al., 2014b). In our study the increased equatorial diameter observed in 3G group embryos than 2G group embryos might be due to increased damage of RFR from 3G cell phone.

**iii. Axial sagittal width / thickness of ocular lens of chick embryos:**

In the present study, the thickness of lens also showed a gradual increase as the age advanced in all the three groups of embryo. The thickness of ocular lens of 2G group embryos was found to be significantly increased on the 6th and 7th day and from the 10th day onwards there was a decrease in thickness which was significant on 11th and 12th day.

However, 3G group embryos showed a significant decrease in thickness of lens on all days on comparing with control group embryos. The decrease in thickness in both 2G and 3G group embryos might be due to the fragmentation and folding of lens fibers (Carpenter, R.L., 1979, Wang, K.J et al., 2003) due to RFR exposure. On comparing 2G and 3G group embryos, the 3G group embryos showed a significant decrease in thickness of ocular lens on all the days which might be due to increased lethality of 3G radiation.

**3. ENZYMATIC ANTIOXIDANT ACTIVITY**

Reactive oxygen species (ROS) or free radicals are the byproducts of normal cellular metabolism. They are also produced by polymorphonuclear leucocytes (PML’s), T- lymphocytes and macrophages to kill exogenous biological or physical materials or abnormal cells in the tissue (Anjana, V.Y et al.,2009). Their production is also triggered by a number of endogenous and exogenous factors and RFR is one among them. The ROS include superoxide anion radical (O$_2^{•−}$), hydrogen peroxide (H$_2$O$_2$), peroxyl (ROO$^{•}$) radicals, and very reactive hydroxyl radicals (OH$^{•}$) (Venkatasamy, M et al., 2013). The OH$^{•}$ radicals are more damaging than any other free radicals due to their stability and they can persist long enough to travel through cell structures like membranes, proteins or nucleic acids and attack them (Anjana, V.Y et al.,2009).

The ROS are eliminated by various antioxidant enzymes that provide a defense mechanism for all organisms. The main enzymatic antioxidants are superoxide dismutase (SOD), catalase (CAT), and Glutathione peroxidase (GPx). Failure of antioxidants to detoxify these ROS
and imbalance in the production of ROS will lead to oxidative stress causing enzyme inactivation, protein, DNA and lipid degradation (Di Giulio et al., 1995).

A. SUPEROXIDE DISMUTASE (SOD) ACTIVITY

SOD is an enzymatic antioxidant that plays a very important role in cellular antioxidant defense mechanism. They catalyzes the conversion of superoxide anion ($O_2^\cdot-$) to hydrogen peroxide ($H_2O_2$) and molecular oxygen ($O_2$) (Kanko, M et al., 2005).

$$SOD \quad 2\left(O_2^\cdot-\right) + 2H^+ \rightarrow 2HO_2 \rightarrow H_2O_2 + O_2$$

Decreased SOD activity results in increased cellular accumulation of superoxide anion ($O_2^\cdot-$) and subsequent increase in the level of $H_2O_2$ resulting in oxidative stress.

i. **SOD activity in liver**

SOD activity in the liver was found to be reduced for both the 2G and 3G group embryos on comparing with control group embryos though the decrease was significant only on the 9th day. Comparison between 2G and 3G group did not show any significant change. Our study correlates with a number of previous studies that have reported a decrease in antioxidant activities and increased free radical formation in liver on RFR exposure. Exposure of brain, kidney and liver of adult rats to RFR from 900 MHz fields for 30 minutes per day for 10 days resulted in decreased SOD, CAT and GPx activities (Faruk, O et al., 2005, Martinez, S.J et al., 2010, Merhan, M et al., 2014). On exposure of rats to EMF from GSM base station resulted in increased free radical production and oxidative stress in liver (Yureki, A.L et al., 2006). Increased oxidative stress in liver on exposure to 900 MHz RFR were also reported Amara,S et al., 2007 and Koyu , A et al., 2009. Contradictory reports are also available showing increased SOD activity in liver on exposure to RFR ranging from 50 – 900 MHz on rats and guinea pigs (Emre, M et al 2011., Guler, G et al.,2006., Zehra, T et al., 2015). Thus the literature indicates the persistence of contradiction in the aspect of SOD levels on RFR exposure.

ii. **SOD activity in eyes**

SOD activity in eye was also found to be reduced for both 2G and 3G group embryos when compared with control but the decrease was significant only on the 12th day. On comparing between 2G and 3G group no significant change was observed. Literature shows a number of
studies showing decreased SOD activities in the eyes of rat models. A long term exposure of albino Wistar rats to RFR from 900 MHz mobile phones resulted in decreased concentration of anti-oxidant enzyme glutathione peroxidase, catalase and SOD (Ozuguner, F et al., 2006, Jelodar, G et al 2012., Motawi, T.K et al., 2014). Our study is in congruence with these observations. Conversely, contradicting studies are also available. On exposing albino Wistar rats to RFR emitted 3G cell phone resulted in no significant change in antioxidant activities (Dogan, M et al., 2012, Demirel, S et al., 2012).

### iii. SOD activity in brain

SOD activity was found to be reduced on both the days for 2G and 3G group embryos but the decrease was significant for 3G group embryos on the 9th day and for 2G group embryos on the 12th day. No significant difference was observed on comparing between the 2G and 3G group embryos. There are reports showing decreased SOD activity in rat brain on exposure to 900 MHz microwave radiation (Kesari, K.K et al.,2009 and 2011., Merhan, M et al., 2014). Dasdag, S et al., 2009 reported an increased antioxidant level, increased catalase activity and changes in programmed cell death of glial cells in rat brain on exposure to 900 MHz radiation. Hence literature shows contradictory reports on SOD activity in brain on exposure to RFR. In our study the SOD activity was found to be decreased in chick embryo brain similar to the findings of Kesari, K.K et al.,2009 and 2011., Merhan, M et al., 2014.

### B. GLUTATHIONE PEROXIDASE (GPx) ACTIVITY

GPx also belongs to enzymatic antioxidant along with SOD and CAT (catalase). GPx catalyzes the of \( \text{H}_2\text{O}_2 \) and lipid peroxidation thus preventing lipid peroxidation of cellular membranes by removing free peroxide in the cell.

\[
\text{GPx} \quad 2\text{GSH} + \text{ROOH} \quad \xrightarrow{\text{GPx}} \quad \text{GSSG} + \text{ROH} + \text{H}_2\text{O}
\]

GPx reacts with peroxides and requires reduced GSH (reduced Glutathione) as the reductive substance donating an electron and converting it to GSSG (Saikat Sen et al., 2010). Reduced GPx activity indicates decreased level of GSH or the generation of excess \( \text{O}_2^\bullet \).
i. **GPx activity in liver**

Decreased GPx activity was observed in the liver of both 2G and 3G group embryos on comparing with the control group embryos. The decrease was significant for both 2G and 3G group embryos on 9\textsuperscript{th} day and on 12\textsuperscript{th} day the decrease was significant for only 2G group embryos. No significant change in the GPx level was noted on comparing between 2G and 3G group embryos. Our study correlates with the previous observations made by a number of researchers. In their studies, GPx activity in the rat liver was significantly reduced on exposure to RFR from 2G cell phone (900 MHz) (Martinez, S.J et al., 2010, Hesham, M et al., 2011, Merhan, M et al., 2014, Zehra, T et al., 2015). However, no effect in the levels of GPx, MDA, CAT and SOD in liver and kidney on exposure to 900 MHz electromagnetic radiations was reported by Sihem, C et al., 2006. Thus controversy exists regarding the GPx activity on RFR exposure.

ii. **GPx activity in eye**

GPx activity in the eye was significantly reduced in both 2G and 3G group embryos on comparing with the control group embryos on 9\textsuperscript{th} day. 12\textsuperscript{th} day showed no significant change among all the three groups. Our study shows correlation with previous studies that showed decreased concentration of anti-oxidant enzyme glutathione peroxidase, catalase and SOD in albino rats eyes exposed to 900 MHz RFR (Ozuguner, F et al., 2006, Jelodar, G et al 2012., Motawi, T.K et al., 2014). The repeated exposures of New Zealand rabbit eyes to microwaves for 10, 20 and 30 days (5 minutes per day) at power densities of 5 and 10mW/cm\textsuperscript{2} resulted in decreased glutathione concentrations in the cortex and the core of the lens and increased enzymatic activity of carboxypeptidase A and aminopeptidase (Bernat R., 1985). Contradictory reports showing no effect on the level of antioxidants on exposure to RFR from 3G are also available (Dogan, M et al., 2012, Demirel, S et al., 2012).

iii. **GPx activity in brain**

GPx activity was observed to be reduced in the brain on both the days though the decrease was not statistically significant. There are reports showing decreased GPx activity in rat brain on exposure to 900 MHz microwave radiation (Kesari, K.K et al.,2009 and 2011., Merhan, M et al., 2014). The exposure of rat brain to 900MHz radiation is reported to cause increased antioxidant levels as reported by Dasdag, S et al., 2009. Hence literature shows contradictory reports on GPx activity in brain on exposure to RFR.
Thus a decreased SOD and GPx activity was observed in the liver, eyes and brain of chick embryos on exposure to 2G and 3G radiations in our study. The decreased SOD and GPx activity might probably be due to increased free radical production and their subsequent elimination to keep redox state by the antioxidants resulting in depletion of SOD and GPx level. Excess free radical production could also inactivate various enzymes including enzymatic antioxidants (Di Giulio et al., 1995).

RFR emitted from cell phone enhances the production of free radicals and the magnetic component of RFR could make the free radical stay free for longer time thus giving them the potential to do more damage. The high levels of free radicals could overwhelm the cellular antioxidant capacity and above a certain threshold level could result in cellular damage. The free radicals if added to unsaturated cell membrane lipids can damage membrane lipids thus changing the integrity of membrane. This could lead to influx of water, Na\(^+\) and Ca\(^+\) ions causing swelling and vacuolization of the cytoplasm (cytomegaly), nuclear swelling (nucleomegaly), rupture of nucleus and nucleolus, pyknosis, karyorrhexis, karyolysis and cytolyis. Thus we could establish the role of RFR emitted from 2G and 3G cell phone causing increased free radical production that is correlated with decreased antioxidant activity. This probably would have caused the cellular and nuclear changes observed in our study in liver, kidney and eyes (Anjana, V.Y et al., 2009).

The excess production of free radicals can lead to significant oxidative damage including enzyme inactivation, protein and DNA and lipid degradation (Di Giulio et al., 2008). The SOD converts superoxide anion (O\(_2^–\)) to H\(_2\)O\(_2\) and thus protects GPx and Catalase against inactivation by O\(_2^–\) (Kono, Y and Fridovich, I., 1982). The Catalase and GPx in turn, eliminate H\(_2\)O\(_2\) from the cell and protects SOD from inactivation by excess H\(_2\)O\(_2\) (Sinet, P et al., 1981). This mutual protective function seems to be abolished in our study probably due to excess free radical production on RFR exposure which is shown as reduced SOD and GPx activity in various chick embryo tissues (Venkatasamy, M et al., 2013).

4. DNA DAMAGE

DNA controls growth and function of cells and they are remarkably stable macromolecules. DNA damage is caused by various endogenous factors (free radicals) and exogenous factors (UV, ionizing and nonionizing radiation, chemicals etc.) and they are usually
DNA strand breaks and DNA cross links are the most commonly observed forms of DNA damage (Philips, J.L., 2009). The two types of DNA strand breaks are – Single strand breaks (SSB) and double strand breaks (DSB). Most SSB’s are rapidly repaired with the intact strand serving as a template to direct rejoining process. On the other hand, DSB’s are more lethal and they are believed to be irreparable (Anderson, R.E et al., 2009).

In our study we assessed the DNA damage in the form of double strand breaks (DSB) using alkaline comet assay technique by analyzing the mean comet length, the mean tail length, mean % of DNA in the tail and mean tail moment.

i. DNA damage in liver

In our study, the mean comet length, the mean tail length, mean % of DNA in the tail and mean tail moment of the liver was found to be increased in both 2G and 3G group embryos that is significant statistically on comparing with control group embryos. On comparing between 2G and 3G group embryos, 3G group embryos showed significant increase in these parameters. This observation was in correlation with previous studies that showed increased DNA damage in liver cells of Wistar rats on exposure to 915 MHz radiations with a power density of 2.4 W/m² and SAR of 0.6 W/kg 1 hour per day, seven days per week for 2 weeks (Trosic, I et al., 2011., Yureki, A et al., 2006).

ii. DNA damage in eye

In our study, all the parameters of the comets in eye was found to be increased in both 2G and 3G group embryos on comparing with control group embryos. We observed that these parameters was significantly increased in 2G group embryos on 9th day on comparing with control group and 3G group embryos. It must be recalled that 2G group embryos showed decreased retinal thickness on 9th day and also decreased SOD and GPx levels than control and 3G group embryos.
The 3G group embryos showed increased mean % of DNA in the tail and mean tail moment on 10\textsuperscript{th} – 12\textsuperscript{th} day on comparing with control group and 2G group embryos. The mean comet length and the mean tail length did not show any significant change on comparing with 2G group embryos. This observation must be correlated with our other observations of decreased retinal thickness (statistically significant) and reduced SOD and GPx levels (not significant) on 12\textsuperscript{th} day than control and 2G group embryos.

Increased DNA damage in the eyes was also reported earlier by a number of researchers. Studies on human lens epithelial cells (HLEC) on exposing to 1.8 GHz at SAR of 3 and 4W/Kg field showed increased SSB and DNA damage caused by the field at 4 W/Kg was irreversible (Lixia, S et al., 2006, Sun, L.X et al., 2006., Yao, K et al., 2008). Thus our study correlates with previous findings.

iii. DNA damage in brain

In our study, all the parameters of the comets in brain were found to be increased in both the 2G and 3G group embryos that was significant statistically on comparing with control group embryos. On comparing between 2G and 3G group embryos, 3G group embryos showed significant increase in these parameters. Increased DNA damage in brain on RFR exposure was reported earlier by different authors. The exposure of mice to 2450 MHz microwaves resulted in increased DNA strand breaks and rearrangement of DNA segments in testis and brain (Sarkar, S et al., 1994). The exposure of rat brain cells to a 2450 MHz RFR resulted in an increased SSB and DSB but the effects were blocked by antioxidants (Lai, H et al., 1995, 1996, 1997, 2005). Their study suggested the role of free radicals in producing DNA strand breaks. Exposure of rat brain to 50 GHz microwaves resulted in increased DSBs (Kesari, K.K et al., 2009). Exposure of Wistar rats to RFR ranging from 915 MHz – 2.45 GHz with SAR ranging from 0.6 W/kg – 2.01 W/kg resulted in increased SSBs in brain (Paulraj, R., et al 2006., Trosic, I et al., 2011).

However, these findings were contradicted by other researchers whose studies showed no significant effect of RFR producing DNA damage on brain. No significant change in DNA strand breaks, protein – DNA cross links, DNA – DNA cross links was observed in rat brain cells on exposure to 2450 MHz RFR (Lagaroye, I et al., 2004a, 2004b). The exposure of rat brain to 915 MHz to GSM mobile signal produced no significant DSB (Belyaev, I.Y et al., 2006).
Researchers have postulated different mechanisms by which an EMF interacts with DNA causing damages. The energy associated with RFR cannot directly break the chemical bonds within the molecules. However, it could enhance the free radical activity in the cells by Fenton reaction. In this reaction hydrogen peroxides that are produced either by peroxisomes present in the cells or by dismutation of superoxide anion by SOD (Valko, M et al., 2004) interacts with free iron to form highly reactive hydroxyl radicals (OH•)

\[ \text{Fe}^{++} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{+++} + \text{HO} + \text{HO}^{•} \] (Fenton reaction)

HO• thus produced are unique in that they get added to DNA very rapidly in vivo. They have very high electrophilicity and high reactivity to penetrate the shield of easily abstracted H atom in the sugar moieties and reach DNA bases. They thus attack DNA, yielding altered bases or SSB and DSB (Anjana, V.Y et al., 2009). These free radicals are also known to produce damaging effects on macromolecules such as proteins and membrane lipids (Lai, H et al., 2004, Okten, F et al., 2005, Ozguner, F et al., 2006, Yariktas, M et al., 2005). Another possible mechanism is that, RF radiation is known to produce alterations in the structure of proteins (Bohr, H et al., 2000, Chiabrera, A et al., 2000, George, D. F et al, 2008). Thus, structural alteration in DNA repair enzymes might have caused changes in its function, leading to DNA damage (Philips, J.L., 2009).

From our experimental outcome on DNA damage, we conclude that RFR exposure results in DNA damage in both 2G and 3G group embryos in different tissues of chick embryo, and 3G exposures causes more damage than 2G exposure. The DNA damage observed in the present study might be due to increased free radical production which also correlates with decreased antioxidant levels in various exposed chick embryo tissues.

The differences in experimental outcome if any, observed in our study on comparing with various other studies, are expected as the effect of RFR exposure might manifest differently depending on the duration of exposure, frequency and intensity of transmission, the number of exposure, the shape and size of exposed organism, the water and mineral content of the organism and also the distance from the radiation source (Philips, J.L., 2009, Sivani, S et al., 2012). Sometimes two or more factors interact together giving a totally different result. The type and nature of the cells studied also affects experimental outcome. The type and nature of the mobile
cells studied also affects experimental outcome. Different cells and organisms respond differently to RFR (Di Carlo et al., 1999., Anderson, L.E et al., 2000., Hoyto, A et al., 2007). The cells used in in-vitro studies and laboratory animals in in-vivo studies might show different research outcome mainly because the cells used for in-vitro studies are immortalized in culture media and they might show more resistance to RFR. The cells showing higher proliferation rate, higher content of iron and cells having decreased antioxidant enzymes are more prone for DNA damage due to RFR exposure (Philips, J.L., 2009).

Hence, taking all these factors into consideration, the complex nature of interactions of RFR with biological systems still continues to be an enigma that has to be discovered yet. Further scientific research are needed to understand the underlying principles that determine the nature of interaction of RFR with biological tissues and then to develop strategies to cope with its harmful and cumulative effects.