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Effect of Ultra High Frequency Radiation from 2G & 3G Cell Phone on Histology of Chick Embryo Retina – A Comparative Study

Mary Hydrina D'Silva[1], Rijied Thompson Swer[2], J. Anbalagan[3], Rajesh B[4]

[1]Assistant Professor of Anatomy, Mahatma Gandhi Medical College & Research Institute, Pillaiyarkuppam (Cuddalore-Pondy Main Road), Puducherry – 607402, India

[2]Associate Professor of Anatomy, Mahatma Gandhi Medical College & Research Institute, Pillaiyarkuppam (Cuddalore-Pondy Main Road), Puducherry – 607402, India

[3]Professor of Anatomy, Mahatma Gandhi Medical College & Research Institute, Pillaiyarkuppam (Cuddalore-Pondy Main Road), Puducherry – 607402, India

[4]Associate Professor of Anatomy, Sri Lakshminarayana Institute of Medical Sciences, Bharath University, Puducherry, India

Abstract: ***The mobile phones have become popular due to faster communication, convenience and lower costs. The electromagnetic fields emitted by them are absorbed into the user's body. The scientific reports on the possible health effects of these radiations on both human and animal models are contradictory. The present study is undertaken to evaluate the possible tissue damage in developing retina of chick embryo following chronic exposure of radiation emitted from 2G and 3G cell phone. Fertilized chick embryos were incubated in four groups - Group A-experimental group exposed to 2G radiation, Group B- experimental group exposed to 3G radiation, Group C- sham exposed control group and Group D – control group. After the scheduled duration, the embryos were processed for routine histological studies. The thickness of each layer of retina was measured using oculometer and statistically compared using one way ANOVA.The eyes of one batch of eggs of all groups were processed for assessment of DNA damage using the alkaline comet assay technique. Our study conclude that the 2G and 3G cell phone radiation caused significant changes in the thicknesses of different layers of retina and structural changes in the form of increased intercellular spaces and disintegration of optic nerve fibre.The DNA damage was highly significant in the experimental groups. The changes were more pronounced in 3G group.***

Keywords: Radiofrequency radiation, retinal pigment epithelium, melanogenesis, Comet assay, DNA damage, double strand breaks (DSB).

1. Introduction

The cell phones are the most important source of radiofrequency radiation. Gadgets like tablets, smartphones are multiplying at a rate of five times faster than global human population. The US Census Bureau puts global human population between 7.19 and 7.22 billion. According to data from digital analysts at GSMA Intelligence (Groupe Speciale Mobile Association), the number of active mobile devices has crossed 7.22 billion mark. At present, it is the fastest growing manmade phenomenon ever. (The Times of India, Oct 10,2014).

The Global system mobile communication (GSM /2G) cellular phones functions in frequency range of 900- 1800 MHz and 3G cell phone works in the frequency range of 1900- 2100 MHz. 4G cell phones that works in the frequency of 2300 MHz has been introduced recently in few selected cities in India. Whenin operation, the cell phones emit a pulsed radiofrequency electromagnetic wave that is absorbed into the user's body.The scientific reports on the health effects of UHF/RFR (ultrahigh frequency/ radiofrequency radiation) on biological tissues in both animals & humans are contradictory. Exposure to electromagnetic fields from base stations and cell phones are associated with depressive symptoms, head ache, dizziness, memory changes, tremors and sleep disturbances.[1-3]. Leung S et al.[4]reportedacute exposure to 2G and 3G affected human cognitive functions. The mortality rate was significantly increased in chick embryos on exposure to RFR emitted from cell phone.[5-8]. Exposure also caused congenital malformations,[9, 10] and structural changes in developing kidneys.[11]

Exposure of chick embryos to electromagnetic radiation of 900- 1800MHz caused enhanced body growth & eye development till 10th day of incubation and further radiation resulted in brain malformations with reduced body and eye growth.[9]The chronic exposure of chick embryos to RF radiation from 2G cell phone resulted in increased retinal thickness, early retinal differentiation and structural changes.[12]Khaki et al, on exposing rats to electromagnetic waves of 50 - 60 Hz for 4 weeks reported increased retinal thickness.[13]However, Zareen et al reported that RFR emitted from GSM mobile phone caused retarded retinal growth of chick embryos of 10 days and enhanced retinal growth and pigmentation of embryos of 15 days.[14]

Though RFR/UHF emitted from cell phone is a non-ionizing radiation, over exposure could cause health hazards due to oxidative stress (FCC, 1999). Kesari K K.[15]et al observed in wistar rats exposed to 3G cell phone radiation, a transient increase in phosphorylation of HSP 27,HSP 70 and P38 mitogen–activated protein kinase (P38MAPK) whichleads to mitochondrial

dysfunction that induced apoptotic cell death. RFR/UHF caused an increase inHSP-70 and HSP-27 protein expression in lens epithelial cells of human and animal models.[16, 17]There are numerous reports on structural damage of lens epithelial cells due to RF exposure that affected its transparency leading to cataract formation.[17-22]RF exposure from 2G cell phone caused microstructural changes in lens epithelium, with appearance of cystic cells and spaces and distorted arrangement of lens fibers in the chick embryo.[23]

Contradictory reports are available on the effect of RFR on antioxidant activities. Dasdag et al. [24] observed changes in antioxidant capacity and catalase enzyme activity in rat brain due to 900MHz radiation and alterations on apoptosis of glial cells. However, Dogan M et al.[25] reported no significant change in antioxidant activities due to 3G mobile phone exposure. Denirel S et al.[26] reported no change in the catalase and glutathione peroxidase enzymes level after exposure of Wistar albino rats to electromagnetic radiation from 3G mobile phone.

RF exposure can cause physiological changes in a celleven at molecular level. It is reported to produce single and double stranded DNA breaks and inhibition of DNA synthesis and mitosis of lens epithelial cells.[27, 28]Various exogenous factors such as UV, ionizing and nonionizing radiation and chemicals can cause DNA strand breaks. [29].

The exposure of human LEC to microwaves resulted in repairable DNA damage. [16]. Exposure of Wistar rats to 3G radiation resulted in DNA double strand breaks, increased micronuclei, capase 3 and apoptosis.[15]Philips et al.[30]on exposing Molt-4 human lymphoblastoid cells to low intensity EMF showed both increased and decreased DNA damage. Their study showed that the outcome of EMF exposure depends on the type of signal, intensity and duration of exposure. Theintermittent exposure schedule is reported to produce significantly more DNA damage than continuous exposure.[31] Hydrina et al.[23] reported increased DNA damage in the eyes of chick embryo on chronic exposure to 2G cell phone radiation. However, absence of DNA damage was reported in human peripheral blood culture,[32, 33, 34]and also in rat brain on exposure to RFR.[35, 36]

The mobile phonehas become an essential gadget in human life. In order to provide better network coverage, the cell phone towers are placed haphazardly on commercial buildings, hospitals, college campuses and terraces of densely populated urban residential areas.[37]The public are unaware about the possible health hazards from the long term electromagnetic radiation exposure from these sources. At present, there is no literature availableon long term effect of RF exposure on Indian population.We have undertaken the present study to evaluate the possible effects of chronic exposure of RFR emitted from 2G and 3G mobile phones in developing chick embryo retina.

2. Materials and Methods

This study was done after getting the clearance from Institutional Animal Ethical Committee (IAEC).Fertile hen eggs (Gallus domesticus) were procured from Rajiv Gandhi college of Veterinary and Animal sciences, Puducherry. The eggs were incubated in 16 batches of 12 eggs each (total-192 eggs) in a standard egg incubator at 37±0.5°c and 50-55% of humidity and ventilation. The eggs were rotated manually 2 times a day and checked with a Candler for the viability of embryos.

The first batch (12 eggs) was treated as control (Group –D) and they were incubated without any external factors interfering with their developmental process. Next 4 batches (48eggs) 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 hung from above with 5 cm distance separating the egg and kept in null status (switched off). Morphological features and structure of retina of both these groups were similar. So we have considered the sham exposed group as the control group for the present study.

The experimental groups (Group –A and B) were also incubated (48+ 48 eggs) in a similar manner with the cell phone kept in silent mode with head phones plugged in (switched on) (Fig:1). This arrangement ensured that the cell phone gets switched on automatically each time it receives a call.

A popular service provider is used for network connection for both 2G and 3G exposure. For exposure, the cell phone is rung from another cell phone for duration of 3 minutes each, every half an hour, with the first exposure given at 12th hour of incubation (4.30am-4.30pm). The total exposure for a 12 hour period is 72 minutes followed by 12 hour of exposure-free period. This is repeated daily.

Six embryos per day were sacrificed from 5thday to 12thday. The embryos were fixed in 10% formalin and then processed for routine histological studies. 5 micron thick sections were cut in sagittal plane, coronal plane and in transverse plane and stained with H&E. The thicknesses of each layer of retina in all the groups were measured using calibrated oculometer and the values obtained were statistically analyzed using one way ANOVA using Graph Pad Instat 3.

The eyes of 5th batch of embryos of all the 3 groups (12+12+12) were subjected to alkaline comet assay technique developed by N.P.Singh,[38] with modifications in staining procedure,[39] for assessing the DNA damage. The eyes of 9th – 12th day embryos were removed and minced in Hanks Balanced Salt Solution (HBSS). The cell suspension was used for the assay. The slides were stained with silver nitrate and then analyzed using automated comet scoring software (Comet Score IV) to assess and quantify the levels of DNA damage in 3 groups. The mean comet length, the mean tail length, mean % of DNA in the tail and mean tail moment of all 3 groups were statistically compared using one way ANOVA with Graph Pad Instat 3.

3. Observations

Histological examination of retina of 5 days old control group showed 3 layers; the layers being pigment layer, germinative or proliferative layer & inner marginal layer (putative optic nerve fibre). The pigment layer showed mild pigmentation and neural retina showed closely packed cells without spaces between them. (Fig.2). Experimental group A (2G) and B (3G) showed thin pigment retinal layer with mild pigmentation and neural retina showed 2 layers – germinative or proliferative layer showing spaces between the cells & inner marginal layer (Fig.3,4,). The thicknesses of all the 3 layers of 2G and 3G group weremore when compared with control group. However, this increase was significantin pigment layer and germinative layerfor 2G group (P<0.05, 0.001 respectively) and only in pigment layer for 3G group (P<0.001). On comparing between 2G and 3G group, it was found that 3G group showed increased thickness of pigment layer (P = 0.001) and 2G group showed increased thickness of germinative and inner marginal layer .(P=0.01, 0.001respectively) (Table1)

6 days control embryo showed similar features for retina as 5 day old control. The 2G group and 3G group also showed 3 distinct layers. The pigment layer showed mild pigmentation, cleft like spaces were seen between cells ofgerminative or proliferative layer and inner marginal layer was disintegrated in some of the embryos. The mean thickness of pigment layer of all 3 groups was same. The thickness of germinative or proliferative layer was significantlymore in 2G group(p value < 0.001) and 3G showed non-significant change when compared with control & inner marginal layer showed no significant difference in all 3 groups. But the total retinal thickness of experimental groups A and Bwas more when compared with control group. However, the increase was significant only for 2G group (p value < 0.001).On comparing between the 2G and 3G groups, 2G groupshowed significant increase in germinative layer and total retinal thickness (p value < 0.001) and non-significant increase in inner marginal layer. (Table1)

Retina of 7 day old control embryo showed mild to moderate pigmentation with same3 layers. The experimental groups also showed similar 3 layers with pigment layer showing moderate - intense pigmentation and thickness of neural retina was more compared to control group. The germinative or proliferative layer also showed spaces between cells & inner marginal layer showed disintegrated optic nerve fibres. The thickness of all the 3 layers were significantly more in 2G and 3G groupwhen compared with control group except the pigment layer of 3G group that showeddecreased thickness that was statistically significant (p value < 0.001). Total retinal thickness of 2G group and 3G group showed increased thickness than control group which was highly significant(p value< 0.001) (Table1). On comparing between the 2G and 3G groups, it was found that 2G group showed increased thickness of pigment layer and inner marginal layer (p value < 0.001, 0.01 respectively) .3G showed increased thickness of germinative layer and total retinal thickness which was significant (p value < 0.001, 0.05 respectively).

Table 1: Mean Thickness of Each Layer of Retina in all 3 Groups

Age (days)	*Pig. Layer (mm)*	*Germinative layer (mm)*	*Optic nerve fibre (mm)*	*Total thickness (mm)*
5 (CON)	0.003	0.048	0.005	0.056
5(2G)	0.004*	0.055***	0.007	0.066***
5(3G)	0.007***	0.05	0.004	0.061
6(CON)	0.005	0.056	0.005	0.066
6(2G)	0.005	0.068***	0.006	0.079***
6(3G)	0.005	0.058	0.005	0.068
7(CON	0.005	0.072	0.006	0.082
7(2G)	0.005	0.083***	0.008***	0.095***
7(3G)	0.004***	0.091***	0.006	0.101***

(* P value < 0.05, *** P value < 0.001)

Retina of 8 day old control embryo showed moderate pigmentation with less intercellular spaces.Most of the control retina showed only 3 layers – pigment layer, germinative layer & inner marginal layer (Fig.5). Two controlgroup embryosshowed 5 layers of retina (33.2%). The layers were 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. The entire 2G experiment group embryo showed 5 layers of retina. The pigment layer showed mainly moderate pigmentation of retina. They also showed increased intercellular spaces in inner neuroblasticlayer and disintegrated optic nerve fibre (Fig 6). The 3G group retina showed 5 layers with intense pigmentation of pigment retina. The structural changes were similar to that of 2G group (Fig 7). On comparing the thickness of all the layers between 3 groups, it was found that the pigment cell layerhad same thickness in control & both experimental groups. The thickness of outer neuroblastic layer, Chievitzlayer, optic nerve fibre layer and total thickness of retina of 2G and 3G group was more when compared with control group. However, this change was significant for 2G group(p value < 0.001) and for 3G group the change was significant only for outer neuroblastic and Chievitz layer (p value < 0.001 and 0.01 respectively).The thickness of inner neuroblastic layer was significantly less in both 2G and 3G group than control group (p value < 0.001).On comparing between 2G and 3G groups, 2G group showed significant increasein thickness than 3G group in all the layers.(Table 2).

Table 2: Mean Thickness of Each Layer of Retina in all 3 Groups

Age (Days)	*Pigment layer (mm)*	*Outer neuroblastic layer (mm)*	*Transient layer of chievitz(mm)*	*Inner neuroblastic layer (mm)*	*Optic nerve fibre layer(mm)*	*Total thickness (mm)*
8 (CON	0.005	0.075	0.004	0.016	0.009	0.106
8 (2G)	0.005	0.0868***	0.005***	0.012***	0.011***	0.121***
8 (3G)	0.005	0.0802***	0.005**	0.01***	0.009	0.109

(** P value < 0.01,*** P value < 0.001)

9 days old control and both experimental embryos showed well-formed 8 layers. Ext.plexiform layer were clearly seen from 9th day onwards separating external nuclear layer &internal nuclear layer. Pigment layer of control group showed moderate pigmentation with well-formed layersshowing little space between cells. 9 days 2G group embryos showed intense pigmentation of retina with well differentiated 8 layers and spaces were visible between the cells in external nuclear layer, internal nuclear layer & ganglion cell layer. The 3G group showed similar changes and the pigment retina showed moderate to intense pigmentation. The thickness of pigment layer, rods and cones, external nuclear layer, external plexiform layer, inner plexiform layer and ganglion layer were found to be almost same for control & both experimentalgroups except theinternal nuclear layer which was found to be significantly more in thickness in boththe experimental groups (p value < 0.01 and 0.001 respectively) .However, optic nerve fibre layer and total retinal thicknesses was found to be significantly more in 3G group (p value < 0.001). On comparing between 2G and 3G group, it was found that the thicknesses of outer nuclear layer and outer plexiform layer was significantly more in 2G group (p value <0.05 and 0.001 respectively). However, the thicknesses of inner nuclear layer, ganglionic layer, optic nerve layer and total retinal thickness was significantly more in 3G group (p value <0.001, 0.05, 0.001 and 0.001 respectively). (Table3).

10 days old control showed moderate pigmentation of retina and other normal features. All the embryos of 2G group showed intense pigmentation and increased intercellular spaces in internal nuclear layer and ganglion cell layer and optic nerve fibre layer was disintegrated. Internal plexiform layer were well developed when compared with control group. Moreover, internal limiting membrane was also visible. The 3G group showed moderate pigmentation with increased space between the cells, outer plexiform layer was not distinct and optic nerve fibre layer showed disintegration.The thickness of pigment layer, layer of rods and cones, external nuclear layer & external pexiform layer of control and 2G group showed same thickness. However, 3G group showed same thickness as control for pigment layer and rods and cones. But, external nuclear layer showed increased thickness and external plexiform layer showed decreased thickness than control group which was statistically significant (p value <0.01 and 0.001 respectively). The thickness of internal nuclear layer, internal plexiform layer, ganglion cell layer & optic nerve fibre layer of both 2G and 3G group were more when compared with control group which was significant (p value <0.001, and 0.01). The total thickness of both experimental group also showed significant increase in thickness (p value<0.001) (Table 3). On comparing between the 2G and 3G groups, the 2G group showed increased thickness of external plexiform layer, internal nuclear layer, internal plexiform layer, ganglion cell layer, optic nerve fibre layer and total retinal thickness. But, this increase was significant only for ganglion cell layer, optic nerve fibre layer and total retinal thickness (p value <0.05, 0.001 and 0.05 respectively). 3G group showed increased thickness of external nuclear layer (p value <0.01).

11 days old control embryos showed moderate to intense pigmentation of retina and normal histological features. 2G group showed intense pigmentation of retina with spaces in inner nuclear and ganglionic cell layer. Optic nerve fibres showed disintegration in some areas. Internal plexiform layer was well formed. The 3G group showed moderate to intense pigmentation. The structural changes were similar to 2G group except that the internal plexiform layer was not formed properly. The thickness of pigment layer, layer of rods and cones and external nuclear layer were similar in all the three groups. The 2G group showed non-significant change inexternal plexiform layer, internal nuclear layer and total retinal thickness when compared with control group. The 2G group also showed a significant increase in internal plexiform layer and optic nerve fibre layer and decrease in ganglion cell layer than control group (p value < 0.001, 0.05 and 0.001 respectively). However, 3G group showed statistically significant decrease in all these layers including the total retinal thickness than control group (p value <0.01, 0.001, 0.05, 0.001 and 0.01 respectively).On comparing between the 2G and 3G groups, it was found that 2G group showed increased thickness of these layers. However, the increase was significant for external plexiform layer, internal nuclear layer, internal plexiform layer and total retinal thickness (p value <0.001, 0.01, 0.001 and 0.001 respectively) (Table 3).

12 day old control embryo showed normal retina with moderate pigmentation (Fig 8). The 2G group showed intense pigmentation of retina with spaces in inner nuclear layer and disintegrated optic nerve fibre(Fig 9). The 3G group showed similar changes. The cells were less in external nuclear layer and external plexiform layer was not developed properly (Fig 10). The thickness of pigment layer and layer of rods and cones were same for all the three groups. The external nuclear layer & ganglion cell layer ofcontrol and 2G group showed non-significant changes. The External plexiform layer, internal nuclear layer , optic nerve fibre layer and total retinal thickness of 2G group showed significantly increased thickness than control group (p value <0.01, 0.001,0.001and 0.001 respectively). However, the thickness of internal plexiform layer was significantly less in 2G group (p value <0.001). The 3G group showed decreased thickness of all the layers and decreased total thickness than control which was statistically significant (p value <0.001). On comparing between 2G and 3G group , the 2G group showed increased thickness in all layers which was statistically significant (p value <0.001).

Table 3: Mean Thickness of Each Layer of Retina in all 3 Groups

Age (days)	Pig. Layer (mm)	Rods& Cones (mm)	Ext.nu. Layer (mm)	Ext.pl. Layer (mm)	In.nu. Layer (mm)	In.pl. Layer (mm)	Gan. Layer (mm)	Op. Nerve (mm)	Total thickness (mm)
9 (con)	0.005	0.0025	0.0103	0.0033	0.0588	0.0068	0.0164	0.0095	0.112
9 (2G)	0.005	0.0025	0.0109	0.0038	0.0694**	0.0067	0.0156	0.009	0.123
9 (3G)	0.005	0.0025	0.009	0.0025	0.086***	0.005	0.0187	0.015***	0.144***
10(con)	0.005	0.0025	0.0098	0.0044	0.0666	0.0046	0.0160	0.0116	0.120
10(2G)	0.005	0.0025	0.0098	0.0043	0.076***	0.007***	0.023***	0.017***	0.144***
10(3G)	0.005	0.0025	0.011**	0.003***	0.076***	0.006***	0.0193**	0.0128	0.135***
11(con)	0.005	0.0025	0.010	0.0038	0.0801	0.0073	0.0263	0.0108	0.146
11(2G)	0.005	0.0025	0.01	0.0039	0.0789	0.009***	0.0225**	0.0154*	0.147
11(3G)	0.005	0.0025	0.01	0.0025**	0.072***	0.0061*	0.021***	0.0131	0.132**
12(con)	0.005	0.0025	0.0120	0.0029	0.0809	0.0128**	0.0240	0.0155	0.155
12(2G)	0.005	0.0025	0.0118	0.0037**	0.093***	0.009***	0.0234	0.022***	0.171***
12(3G)	0.005	0.0025	0.009***	0.0025	0.071***	0.008***	0.019***	0.013*	0.130***

(* P value < 0.05, ** P value < 0.01, *** P value < 0.001)

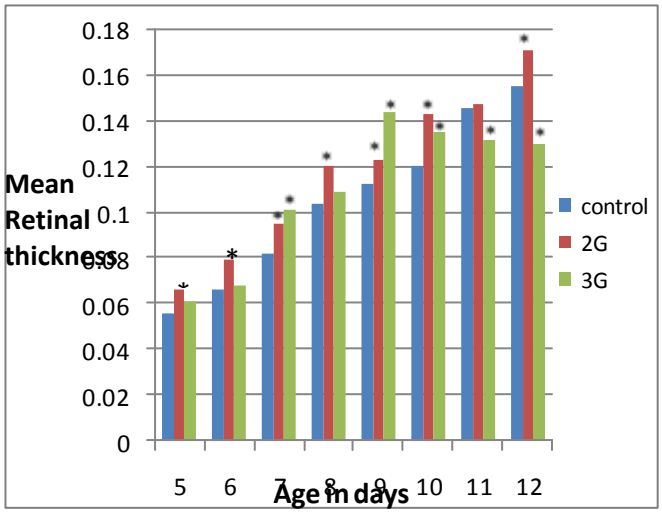
On comparing the total thickness of retina of all the 3 groups, the 2G group and 3G group showed increased thickness up to 10th day than control group. 11th day embryos of 2G group showed non-significant increase and on 12th day, the retina showed significant increase in thickness. However, the 3G group embryos showed significant decrease in total retinal thicknesson 11th and 12th day.On comparing between 2G and 3G groups, it was found that 2G group showed increased thickness than 3G group except on 7th and 9th day where 3G group showed increased total retinal thickness than 2G group(Table-4)

Table 4: Mean Total Retinal thickness in all 3 Groups.

TABLE : 4

Age in days	Mean retinal thickness (mm)		
	Control group	2G group (Group-A)	3G group (Group- B)
5	0.0558	0.066***	0.061
6	0.0660	0.0793***	0.068
7	0.0824	0.0951***	0.101***
8	0.1044	0.1206***	0.109
9	0.1126	0.1231*	0.144***
10	0.1205	0.1436**	0.135***
11	0.1460	0.1477	0.132**
12	0.1558	0.1712**	0.130***

(p value< 0.05* significant,< 0.01** highly significant and < 0.001 *** extremely significant)



Graph showing the effect of electromagnetic fields from 2G and 3G cell phone on the total retinal thickness. Values are means ± SE taken from 6 samples per day for control and both experiment groups (total sample size of 48 embryos each for control group & both experiment group). * represents p value statistically significant.

The 5th& 6th day control & experimental groups showed mild pigmentation of pigment retina. 7th& 8th day control embryo showed mild pigmentation, whereas, 2G group of same age showed moderate pigmentation and 3G group showed moderate pigmentation on 7th day and intense pigmentation on 8th day. 9th – 12th day control embryo showed moderate pigmentation while 2G group showed intense pigmentation of pigment retina and 3G group showed moderate to intense pigmentation.(Table-5)

Table 5: Pigmentation Grade in all 3 Groups

Age in days	*Pigmentation*		
	Control group	*2G group (Group-A)*	*3G group (Group- B)*
5	+	+	+
6	+	+	+
7	+	++	++
8	+	++	+++
9	++	+++	++
10	++	+++	++
11	++	+++	++
12	++	+++	++

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

On comparing the thicknesses of each layer of retina for both control & experimental groups the following changes were noticed.The thickness of pigment layer & rods and cones of control &both experimental groups for all age group didn't show much difference and they remained constant at 0.005& .0025mm respectively except on 5th day where both experimental embryos showed a significant increase in thickness of pigmentlayer (Table 1). The thicknesses of ext. nuclear layer & external plexiform layer were showing almost same value for control &2G group. The thicknesses of int. nuclear layer, int. plexiform layer, ganglion cell layer & optic nerve fibre layer showed an increased thickness in 2G group except on 11th& 12th day. On 11th day the thickness of ganglion cell layer of control group was significantly more than 2Ggroup. On 12th day inner plexiform layer of control group was significantly more than 2Ggroup.The 3G group showed decreased thickness of external plexiform layer and increased thickness of other layers except on 11th and 12th day.The 3G group showed significant decrease in all the layers on 11th and 12th day when compared with control group and 2G group. (Table -3)

On assessing the DNA damage using alkaline comet assay technique, we found an extremely significant increase in the mean comet length, the mean tail length, mean % of DNA in the tail and mean tail moment in the eyes of both the experiment groups. (Table 6, Fig: 11). They further showed moderate to severe DNA damage when compared with the control group that showed minimal damage (Fig.12, 13). On comparing between the 2G and 3G group, 3G group showed increased damage in all the days.

Table 6: Level Of DNA Damage in Control, 2G and 3G Group

Age in days	Mean comet length (µm)	Mean tail length (µm)	% of DNA in tail (µm)	Mean tail moment (µm)
9 (con)	6.95	5.85	34.69	174.7
9 (2G)	8.8***	7.29***	42.91*	286.18***
9 (3G)	7.18	5.942	50.74***	273.68**
10(con)	4.36	2.84	27.82	71.11
10(2G)	7.27***	6.009***	34.40*	174.97***
10(3G)	6.04***	4.20**	45.01***	167.55***
11(con)	6.90	5.19	27.71	131.77
11(2G)	8.56***	6.66***	43.64***	256.61***
11(3G)	9.18***	7.27***	56.54***	360.03***
12(con)	6.5	4.82	24.71	108.73
12(2G)	7.15	5.3	35.23***	170.83**
12(3G)	7.63	6.07*	55.52***	296.21***

(p value< 0.05* significant,< 0.01** highly significant and < 0.001 *** extremely significant)

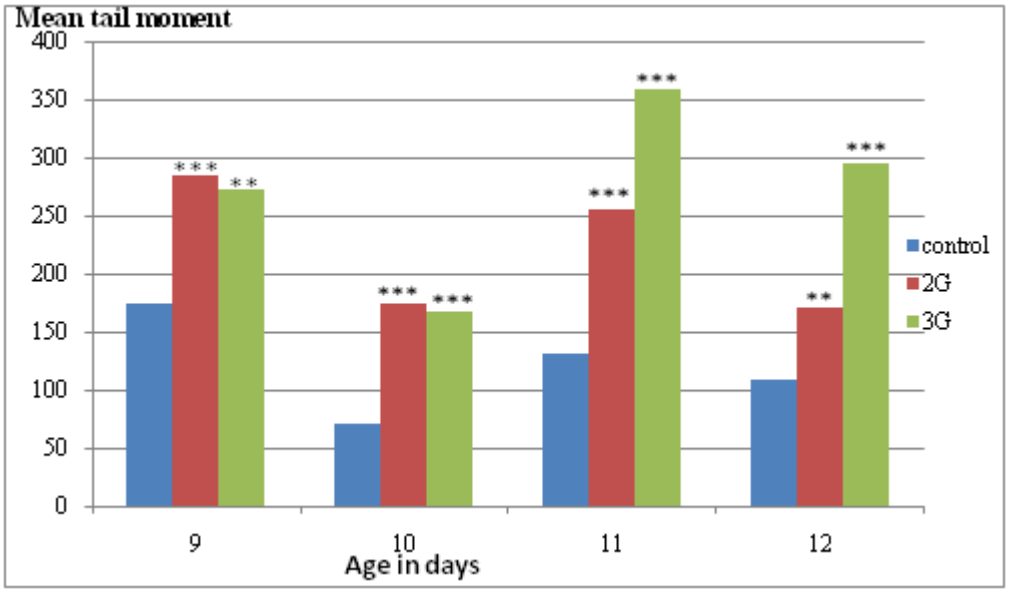


Figure 11:

Fig.11. A Graph showing the effect of electromagnetic fields from 2G and 3G cell phone on DNA damage. Values are means ± SE taken from 3 samples per day for control and both experiment groups (total sample size of 12 embryos each for control group & both experiment group). p value < 0.05* significant,< 0.01** highly significant and < 0.001 *** extremely significant)

4. Discussion

In our study, 5th – 8th day embryos of 2G group showed increased thickness in all the layers than the control group. The 9th – 10th day embryos showed significant increase in thickness ofinternal nuclear layer, internal plexiform layer, ganglion cell layer and optic nerve fibre layer than the control group. The neural retina showed increased spaces between the cells of inner nuclear layer & ganglion cell layer and disintegrated optic nerve fibre.Similar findingswas reported by Fatima Al Qudsi et al.[9] However,11th embryo showed decrease in ganglion layer and a decrease in inner plexiform layer was observed on the 12th day of 2G groups.

The 3G group also showed increased thickness of all the layers of retina from 5th -8th day and non-significant changes on 9th day.There was a significant increase in all the layers on 10th day and highly significant decrease on 11th and 12th day in comparison with control group.Moreover, structural changes in the form of increased spaces between the cells of internal nuclear layer , ganglion cell layer and disintegrated optic nerve fibre were much conspicuous than 2G group.

The internal plexiform layer was reduced in thickness in 3G group. Thedifferences in the growth parameters of different layers of retina might be due to different cellular responses to EMF during different embryological periods as cells might be trying to rebalance their growth & differentiation rate.[9]

On continuing the exposure, we found that the total retinal thickness in 2G group increased on 11th and 12th day. However, 3G group showed highly significant decrease in total retinal thickness on these days. It was in accordance with the findings of Fatima Al Qudsi et al.[9]who reported similar decrease in thickness of retina on 11-14th day on exposing chick embryos to 2G radiations. The increased intercellular spaces in the retinal layers might be due to shrinkage of cells or it might be due to cell death caused by chronic exposure of embryos to RF radiation that resulted in oxidative stress rendering the cells vulnerable to damaging effects of RF radiation.[12]

The present study also showed early differentiation of different layers of retina in both experimental groups. The retina showed five layers on 8 days old 2G and 3G embryos while control embryo showed mainly 3 layers. The layers were 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.[40]Moreover, the thickness of ganglion cell layer in 11th& 12th day 2G and 3G embryosshowed decreased thickness and was more pronounced in 3G group. This change is probably due to natural cell death or apoptosis that normally happens in ganglion cell layer towards the end of gestation. [40]This probably would have resulted in decreased thickness of inner plexiform layer due to loss of synaptic contact between ganglion cells and cells of inner nuclear layer.These changes show an early onset of maturation of retina in exposed groups than the control group.

In our study, control group showed mild pigmentation of pigment retina upto 8th day followed by moderate pigmentation till 12th day. The melanin pigmentation of 2G and 3G groups were mild up to 6th day followed by moderate to intense pigmentation for 2G group and moderate pigmentation for 3G group up to 12thday(Table 5). Thus an early onset of increase in melanogenesis in both the experimental groups as compared with control group was observed.

RF exposure results in DNA damage,[16, 27, 29] in the form of single strand breaks (SSB) and double strand breaks (DSB). DNA strand breaks results in melanogenesis as a part of the repair mechanisms.[41]In the present study, the RF exposure would have induced DNA damagein both the experimental groups resulting in early onset of increased melanogenesis as indicated by the pigment gradation (Table:5).Melaninpresent in retinal pigment epithelium (RPE) plays a very important role in differentiation of neural retina,[14, 42]DOPA, which is a melanin precursor present in RPE is important for regulating retinal cell mitosis.[43]This would have caused increased retinal thickness and early differentiation of neural retina in the present study. This is in accordance with the findings of Zareen et al,[14] who observed mild pigmentation with retarded growth and differentiation of neural retina due to 2G cell phone exposures. Whereas, on prolonged exposure it resulted in intense pigmentation of RPE due to increased melanin production that resulted in increased growth of retina.

However, 3G group showed decreased thickness in all the layers and total retinal thickness on 11th and 12th day. This might be due to moderate pigmentationas compared with 2G group that showed intense pigmentation in those days.

RF radiation causes Fenton reaction in the cells resulting in free radical formationthat kills the cells by damaging macromolecules such as DNA and proteins.[27] This impairs DNA repair mechanism resulting in DNA damage in the form of DNA strand breaks and DNA cross links.[29] DNA strand breaks are associated with cell death, aging and cancer.

In the present study, we assessed the DSB in the developing eye of the chick embryo following chronic exposure to RF radiation from 2G and 3G cell phoneusing the alkaline comet assay. Our study showed significantly increased DNA damage in the both experiment groups than the control group (Table 6, Fig: 11). Similar findings were reported in human lens epithelial cells on exposing to 1.8 GHz fields at 3 and 4 W/Kg.[28, 44]An increase inDNA doublestrand breaks in the rat brain exposed to 3G cell phone radiations was cited by Kesari et al.[15]

In the present study, the damage was seen in 9th – 12th day 2G and 3Ggroups in the form of increased mean comet length, the mean tail length, mean % of DNA in the tail and mean tail moment except for 12th day (Fig 12,13). The mean comet length and the mean tail length of both groups on 12th day didn't show any significant change but mean % of DNA in tail and mean tail moment showed an increase in both groups and was highly significant (Table 6, Fig:11).On comparing DNA damage between the exposed groups, it was found that on 9th day 2G group showed more DNA damage than 3G group ($p< 0.05$). This is correlated with our histological findings where the total retinal thickness of 2G group was significantly less than 3G group. However, the DNA damage was less in 2G group than 3G group on 10th – 12thday ($p< 0.001$,0.01 and 0.001 respectively) that resulted in increased thickness of all layers of retina and total retinal thickness of 2G group than 3G group. Thismight be due to the protective mechanism of eye by activating enzyme pathways to protect its components from oxidative stress caused by RF radiation and maintain homeostasis.[16,17, 45]In the case of 3G group, this protective mechanism would have come into play earlier itself as indicated by its increased total retinal thickness on 7th and 9th day. But, on prolonged exposure it would have induced cellular apoptosis due to increased DSB as reported by Kesari et al,[15]resulting in decreased thickness of retinal layers as age advanced.

5. Conclusion

In the present study, the chronic exposure of chick embryos to RF radiation from 2G and 3G cell phone resulted in increasedDNA damage, with increased melanogenesis in RPE as repair mechanism. This could have resulted in

increased retinal thickness and earlyretinal differentiationin both the experimental groups except for 3G group where they showed significant decrease in retinal layers on 11th and 12th day.Exposed group also showed structural changes in the form of increased spaces between the cells in the different layers of retina and also disintegrated optic nerve fibre layer.The DNA damage and structural changes in retina were more pronounced in 3G group that resulted in their decreased thickness in all the retinal layers and total retinal thickness on 11th and 12th day.Thus, the chronic exposure of chick embryo retinae to RF radiation emitted from the 3G cell phone are more damagingthan the 2G cell phone.

6. Future Scope

Whether the reported structural changes in eye are reversible or not upon withdrawal of radiation source from 2G and 3G cell phone requires further study. The upcoming new generation phones (4G and 5G) widens the scope for future investigations to find out their possible effects on developing tissues and to compare it with other existing network systems.

7. Conflicts of Interest

The authors had no conflicts of interest todeclare in relation to this article.

8. Acknowledgment

We express our sincere thanks to Dr. S. Arunchandra Singh, Head of the department of Anatomy, Dr. Sudha Rao, Professor of Anatomy, MrChandrasekar M, Technician department of Anatomy, Mahatma Gandhi Medical College and Research Institute, Puducherry, for their valuable suggestions and support.

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Figure:1

Fig:1 A Photograph showing the experimental set up. The Mobile phone (red arrow) is hung with a distance of 5cms separating it from the fertilized chicken eggs. A RF meter is kept inside the incubator to check the intensity of radiation (yellow arrow).

5 DAY OLD CONTROL EMBRYO RETINA

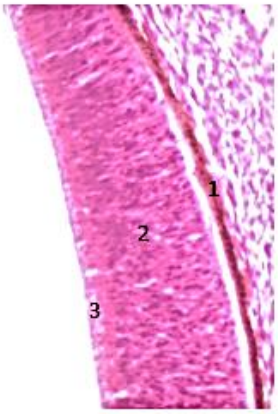


Fig:2 Photomicrograph of 5 day old control embryo retina showing 3 layers -
1. Pigment layer - mild pigmentation.
2. Germinative layer.
3. Inner marginal layer (H&E x 400)

5 DAY OLD 2G EMBRYO RETINA

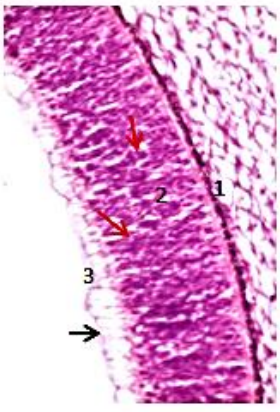


Fig:3 Photomicrograph of 5 day old 2G embryo retina showing 3 layers -

1. Pigment layer- mild pigmentation.
2. Germinative layer showing spaces between the cells (red arrow)
3. Disintegrated Inner marginal layer (black arrow) (H&E x 400)

5 DAY OLD 3G EMBRYO RETINA

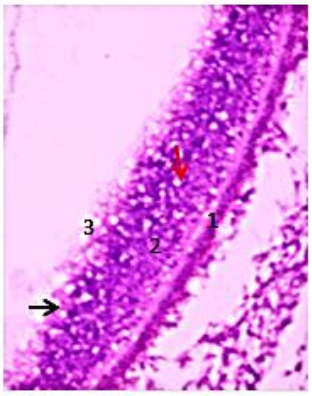
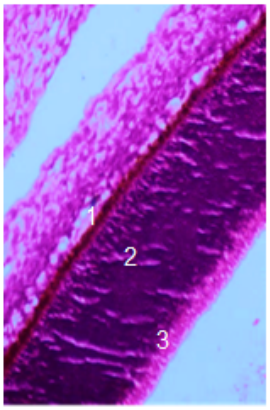
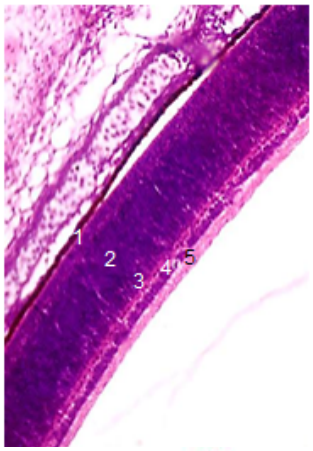


Fig:4 Photomicrograph of 5 day old 3G embryo retina showing 3 layers –
1. Pigment layer- mild pigmentation.
2. Germinative layer showing spaces between the cells (red arrow)
3. Disintegrated Inner marginal layer (black arrow) (H&E x 400)



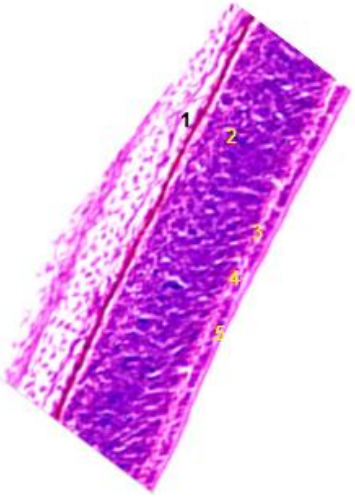
8 DAY OLD CONTROL EMBRYO RETINA

Fig:5 Photomicrograph of 8 day old control embryo retina showing 3 layers –
1. Pigment layer- moderate pigmentation.
2. Germinative layer.
3. Inner marginal layer (H&E x 400)



8 DAY OLD 2G EMBRYO RETINA

Fig:6 8 day old 2G experimental embryo retina showing 5 layers –
1. Pigment layer- intense pigmentation.
2. Outer neroblastic layer.
3. Transient layer of Chievitz
4. Inner neuroblastic layer.
5. Inner marginal layer. (H&E x 400)



8 DAY OLD 3G EMBRYO RETINA

Fig:7 8 day old 3G experimental embryo retina showing 5 layers –
1. Pigment layer- moderate pigmentation.
2. Outer neuroblastic layer with spaces.
3. Transient layer of Chievitz
4. Thin Inner neuroblastic layer.
5. Inner marginal layer. (H&E x 400)

12 DAY OLD CONTROL EMBRYO RETINA

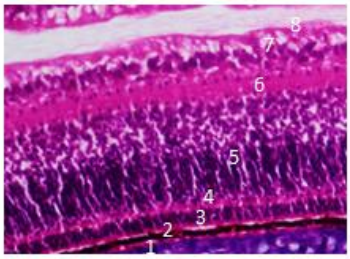


Fig:8 Photomicrograph of 12 day old control embryo retina showing 8 layers –
1. Pigment layer 2. Rods & cones 3. External nuclear layer 4. Ext. plexiform layer 5. Internal nuclear layer 6.well developed internal plexiform layer 7. Ganglion cell layer 8. Optic nerve fibre layer (H&E x 400)

12 DAY OLD 2G EMBRYO RETINA

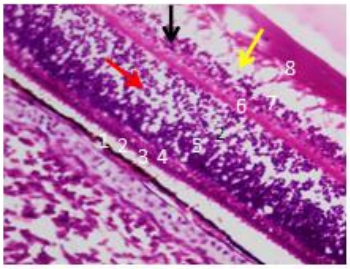


Fig:9 Photomicrograph of 12 day old 2G experimental embryo retina showing 8 layers –
1. Pigment layer 2. Rods & cones 3. External nuclear layer 4. Ext. plexiform layer 5. Internal nuclear layer showing spaces (red arrow)) 6. Internal plexiform layer 7. Ganglion cell layer showing spaces between cells (black arrow) 8. Disintegrated Optic nerve fibre layer (yellow arrow) (H&E x 400)

12 DAY OLD 3G EMBRYO RETINA

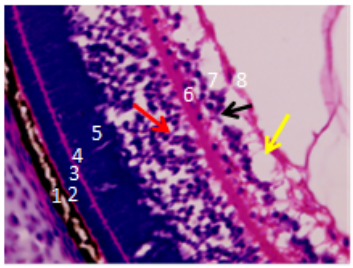


Fig:10 Photomicrograph of 12 day old 3G embryo retina showing 8 layers –
1. Pigment layer 2. Rods & cones 3. External nuclear layer 4. Ext. plexiform layer 5. Internal nuclear layer showing spaces (red arrow)) 6. Internal plexiform layer 7. Ganglion cell layer showing spaces between cells (black arrow) 8. Disintegrated Optic nerve fibre layer (yellow arrow) (H&E x 400)

CONTROL EMBRYO - EYE

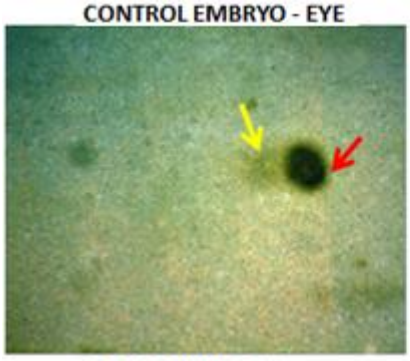


Fig:12 (A) Photomicrograph of 9 day old control embryo comets showing minimal DNA damage. Head diameter is large (red arrow) and tail length is shorter (yellow arrow) 10X

2G EMBRYO - EYE

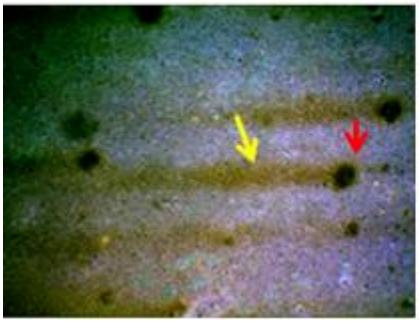


Fig:12 (B) Photomicrograph of 9 day old 2G embryo comets showing severe DNA damage. Head diameter is decreased (red arrow) and tail length is increased (yellow arrow) 10X

3G EMBRYO - EYE

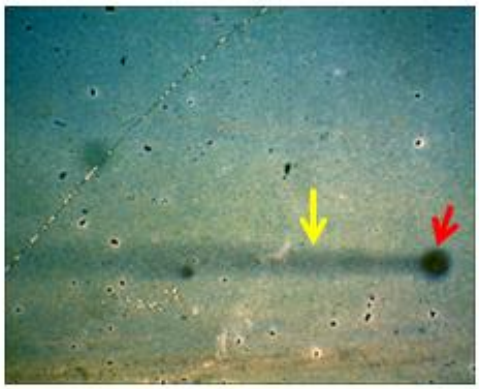


Fig:12 (C) Photomicrograph of 9 day old 3G embryo comets showing severe DNA damage. Head diameter is decreased (red arrow) and tail length is increased (yellow arrow) 10X

CONTROL EMBRYO - EYE

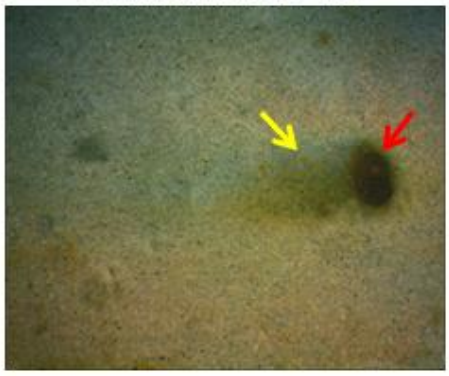


Fig:13 (A) Photomicrograph of 11 day old control embryo comets showing minimal DNA damage. Head diameter is large (red arrow) and tail length is shorter (yellow arrow) 10X

2G EMBRYO - EYE

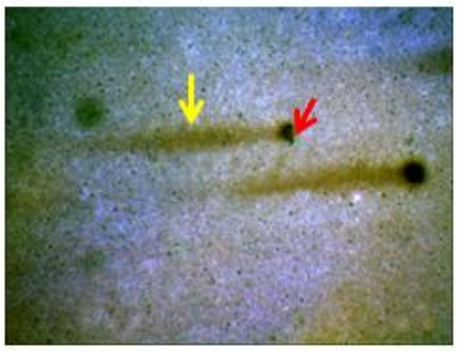


Fig:13 (B) Photomicrograph of 11 day old 2G embryo comets showing severe DNA damage. Head diameter is decreased (red arrow) and tail length is increased (yellow arrow) 10X

3G EMBRYO - EYE

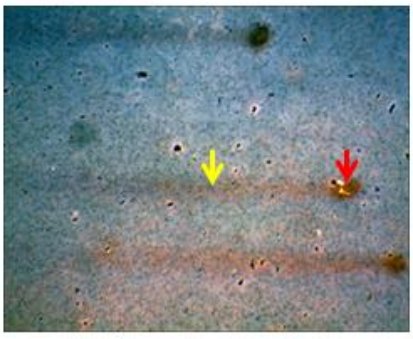


Fig:13 (C) Photomicrograph of 11 day old 3G embryo comets showing severe DNA damage. Head diameter is decreased (red arrow) and tail length is increased (yellow arrow) 10X

Declaration

The Undersigned authors hereby declare that the manuscript "Effect of ultrahigh frequency radiation emitted from 2G cell phone on developing lens of chick embryo- a histological study" has been read and approved and the work has been carried out in the department of Anatomy, MGMC & RI under our supervision. The authors warrant that the article is original and is not under consideration by any other journal and has been previously published and taken responsibility for the context.Furthermore, they warrant that all investigations reported in their publication were conducted in conformity with the recommendation from the declaration of Helsinki and the international guiding principles for biomedical research involving animals.

Author Profile

Mary Hydrina D'Silva has done her MSc Anatomy from Mahatma Gandhi University, Kottayam, Kerala. Currently she is doing her research in cell phone radiation and its effects on developing tissues of chick embryo. At present, she is working as Assistant Professor in Anatomy at MGMC & RI, Pondicherry.

Rijied Thompson Swer has done his MD Anatomy from JIPMER, Pondicherry. At present, he is working as Associate Professor in Anatomy at MGMC & RI, Pondicherry.

J. Anbalagan has done his MSc Anatomy from JIPMER, Pondicherry and PhD in Anatomy from Mahatma Gandhi institute of Medical Sciences, Sevagram. He has co- authored a text book titled "Histology – Text and Atlas" and has published several research papers in various international and national journals. At present, he is working as Professor of Anatomy at MGMC & RI, Pondicherry.

Rajesh B. has done his MSc Anatomy from Mahatma Gandhi University, Kottayam, Kerala and PhD in Anatomy from JIPMER, Pondicherry. At present, he is working as Associate Professor of Anatomy at SLIMS, Pondicherry.