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Mobile Electromagnetic Radiation Affects Vitelline Vessels development in Chick Embryo: A Morphometric Study

Fatma Al-Qudsi[1] and Asmaa Al-Qahtani[2]

[1] Biology Department, Science Faculty, King Abdulaziz University. Tel; 009666400000 P.O. Box 42650 Jeddah 21551 **Saudi Arabia.**

[2] Biology Department, Science Faculty, King Abdulaziz University· P.O. Box 8742 Jeddah 21351 **Saudi Arabia**

***Correspondence:** falqudsi@kau.edu.sa, aalgahtani0053@stu.kau.edu.sa **Received 05-02-2020, Revised: 05-03-2020, Accepted: 06-03-2020 e-Published: 08-03-2020**

Smart mobile phone use has increased dramatically in the last five years. Electromagnetic radiation (EMR) emitted from mobile phones might affect embryonic development. However the mechanism of this effect is not completely understood. Vitelline vessels are the first blood vessels formed, playing a vital role in embryonic nutrition during development. The aim of this research was to study the effect of mobile phone EMR (450–2100 MHz) on the formation of vitelline vessels in chick embryo (*Gallus gallus domesticus*) and detect the resulting congenital malformations. Fertilized chicken eggs were divided into three groups: control (C), exposed without call (EO) and exposed with call (EW). In EW group the mobile phone was called every 6 hours for 15 minutes, (60min/24hr.). While the EO group was exposed to a mobile phone connected through the Wi-Fi to the internet only. Embryos were extracted on day 2, 3, 4 and 5 of incubation. A major decrease in the formation of vitelline vessels was seen in the treated groups compared to the controls causing bleeding seen in several sites of the treated embryos. Congenital malformations increased in treated groups compared to the controls of all experimental ages. The congenital malformations seen were growth retardation, bleeding, clotting and neural tube defects. It was concluded that mobile phone EMR prevented proper formation of vitelline vessels resulting in deformed embryos.

Keywords: Vitelline vessels; chick embryo; mobile phones; electromagnetic radiation; morphometry; growth retardation.

INTRODUCTION

The effect of mobile radiation on human health is a subject of interest and study worldwide (Velmurugan, 2017). Any electromagnetic field can cause harmful effect on human, depending on the frequency. Electromagnetic fields (940 MHz) can make changes in structure of DNA (Hekmat et al. 2013). Exposure to radiation from cell phones may cause serious problems such as attention and hearing deficits, autism, behavioral changes, insomnia, tinnitus, Parkinson's disease (chronic progressive neurological disease), Alzheimer's disease and a broad array of nervous system disturbances (Davis et al. 2013). Many studies suggested that prenatal radio frequency radiation exposure affected the behavior of animal offspring (Aldad et al. 2012, Haghani et al. 2013, Zhang et al. 2015). Exposure to pulsed square wave at duration of 0.5 msec (millisecond) at the first 48 hours of incubation of chick embryos at 100 Hz showed significant abnormalities in the heart and blood vessels (Ubeda et al.1983). Large numbers of experimental studies showed that microwave radiation from mobile phone causes damage to the embryonic development (Salama et al. 2010, Roda et al. 2011). Permanent exposure to mobile phone

during incubation of the chick embryo, caused higher mortality rates compared to the control (Bastide et al. 2001, Batellier et al. 2008, Ye et al. 2016)

Electromagnetic fields potentially affects embryonic development, but the mechanism of effect is still not completely understood. Studies on the effects of 50-100 Hz electromagnetic fields in embryos of different species of animals (fish, chicken, rat and mouse) shows that the early stages of embryonic development are sensitive to the electromagnetic field radiation. TV and mobile phone use during the first trimester of pregnancy increase the risk of fetal growth restriction, especially in women with a history of high-risk pregnancy. Exposure to extremely low frequency electromagnetic fields during pregnancy can cause adverse effects on pregnancy and fetal development in female rats. Also, reduction in the fetal weight in late pregnancy where abnormalities in the fetus were observed (Khaki et al. 2016). Extremely Low Frequency Electromagnetic fields can cause brain tumors, childhood leukemia, genotoxicity, neurodegenerative diseases, congenital malformations, miscarriage and infertility (Infante-Rivard and Deadman, 2003, Sambucci et al. 2010, Khaki et al. 2016). Studies result suggests that the use of mobile phones can be related to the early spontaneous abortions (Mahmoudabadi et al. 2015).

The chick embryo has many advantages as a model to study developmental biology because the chick embryos are similar to mammals in the morphological complexity, however the chick embryos are easily accessible also easier to observe and obtain, also the chick embryos can be used for visualization complex processes.

The vitelline vessels are found over the surface of the yolk sac, the vitelline vein takes nutrients from the yolk sac to the embryo and the blood is returned to the yolk sac via the vitelline artery, vitelline vessels are connected to the vitellus and this is where they get their name (Wolpert et al. 2002). Malformation and malfunction of vitelline vessels might lead to abnormal nutrients delivery to embryonic cells leading to abnormal embryonic development. Therefore, the development of vitelline vessels could be considered an important key factor in studying the teratogenicity of environmental factors.

Many studies were done to investigate the effects of mobile phone EMR on chick embryos, however none studied the effect of mobile phone EMR on the vitelline vessels. Therefore, the aim of this research was to detect the changes that occur in the early formation of the vitelline vessels of the chick embryo (*Gallus gallus domesticus*) and the general congenital malformations that might be seen due to the exposure to mobile phone EMR.

MATERIALS AND METHODS

All experimental procedures were approved by the Biology Department at King Abdul-Aziz University, and were done at post graduate labs in the female section at King Abdul-Aziz University. Fertilized chicken eggs were obtained from Aljamom farm chicken in Jeddah. Plastic egg Incubators were purchased from AlHakeem Foundation, model number WQ –(56 egg incubators) incubation Specification, automatic temperature, automatic egg turning (every 2 hours), 220 V, power 80 watt. The mobile phone used in this study was a smart phone, it emitted low levels of Radiofrequency in the microwave range (450–2100 MHz). The EMR detector used in the present study was electrosmog meter a dual mode device for quick measurement of both high and low frequency purchased from amazon.

Experimental design

The amount of EMR emitted from the chosen mobile phone within the incubator was measured using the EMR detector. The mobile phone was placed in the center above the eggs. The eggs layout in the incubator is shown in (Figure1 A&B). The fertilized chicken eggs were divided into three main groups control (C), exposed without call (EO) and exposed with call (EW).. Fertilized chicken eggs in EW group were exposed to mobile phone EMR that was called every 6 hours for 15 minutes, (60min/24hr). Fertilized chicken eggs in EO group were exposed to a mobile phone connected to Wi-Fi internet without calls. See (table 1) for the amount of electromagnetic radiation each group was exposed to.

Table .1. Showing the egg number according to figure 1 and the distance between the eggs and the mobile phone for both treated groups. The amount of EMR absorbed by the egg for EO group it was 0.06 mw/m²and for EW group it was 18 mw/m².

Egg number	The distance between the eggs and the mobile phone
1, 2, 8, 9	15.2 cm
3, 7, 10, 14	14 cm
4, 13	16.5 cm
5, 6, 11, 12	16 cm

This Empty space should be deleted All groups were weighed before incubation to insure weight homogeneity of eggs. Eggs of all groups were incubated under the standard condition temperature 37.5℃, humidity 80%. The chick eggs at the days 2, 3, 4 and 5 of incubation were collected. One batch was left till hatching.

Sample collection

Eggs were weighted on extraction day. All chick embryos were extracted from all groups on the following incubation days 2, 3, 4, 5. Eggs were opened by knocking the egg shell by scissors, then the top of the egg shell was removed and embryos were extracted at day 2, 3, 4 by cutting around the edge of area pellucida and were put in a petri dish and washed with normal saline. On day 5 only the embryo was extracted and washed with normal saline without cutting the edge of area pellucida. Chick embryos were then photographed under the mobile phone and microscope.

Morphological studies

To perform morphological studies, we compared our control embryos to the normal stages of chick embryo in Hamburger and Hamilton (1951). Control day2 with stage 13, control day3 with stage 20, control day4 with stage 24 and control day5 with stage 27. The percentage of congenital malformations and hatchability was calculated.

Photographing

Each embryo was photographed using an iPhone 7 Plus camera 12 Megapixel the iPhone was attached to a tripod, zoom and distance from specimen was unified. A ruler was put near the embryo to be used as a scale when preforming morphometrics using the photos. (Figure 1D). Also, videos by iPhone 7 plus were taken to all embryos on day 3 and 4 of incubation to record heartbeat per minute. Then each embryo was photographed by a Dissecting Microscope BX51M connected with iPhone holder to highlight any malformation in embryo and vitelline vessels (Figure 1C).

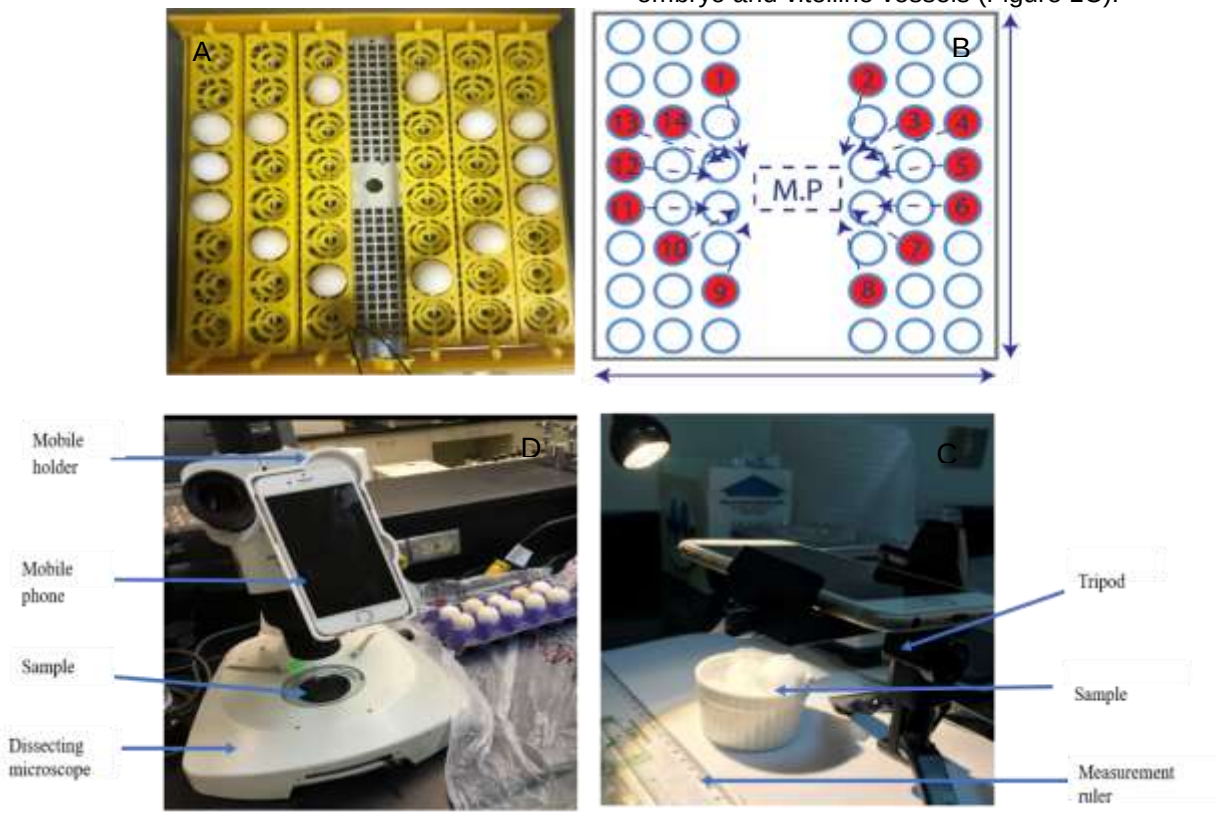

Figure 1. (A,B) Places of eggs in the incubator. (C) Whole embryo photography and videos. (D) Dissecting microscope photography.

Morphometric method

All chick eggs were weighted before and after the incubation. At day 2 the measurements taken were whole body length (WBL), area of area pellucida (AAP), length and width of area pellucida (Figure 2A). In day 3 and 4 embryos the measurements taken were length and angles of the right and left basic arteries, length of the right and left first generation branches of artery, length of the anterior and posterior veins, heart beats per minute (HBPM) for each embryo and WBL. The numbers of the artery branches have been set starting from top to bottom (Figure 2B&C). At day 5 the measurement taken was WBL only (Figure 2D). All measurements were taken from the photos taken by the iPhone camera using Image tool (a free computer software downloaded from (http://cme.msu.edu/cmeias/). HBPM was calculated from the video taken from the embryos.

Hatchability method

For each experimental group 10 eggs were placed in the incubator (Figure 1A.B). EW group and EO group were exposed for 21 days. On day 19 the incubator was opened; the egg holder was removed and the eggs were placed randomly on the incubator floor to facilitate the hatching process (Figure 3.A). Eggs were left until hatching from day 21 to day 28, then chicks were examined and photographed to detect any congenital malformations (wings, beak, legs, feather… etc.) (Figure 3.B)

Statistical analysis

Data was analyzed using SPSS 22. Anova, Student-Neuman Keul test, was used with normal distribution. In case of irregular distribution Man-Whiteney U test from the non-parametric test was used. Significance was at $p < 0.05$.

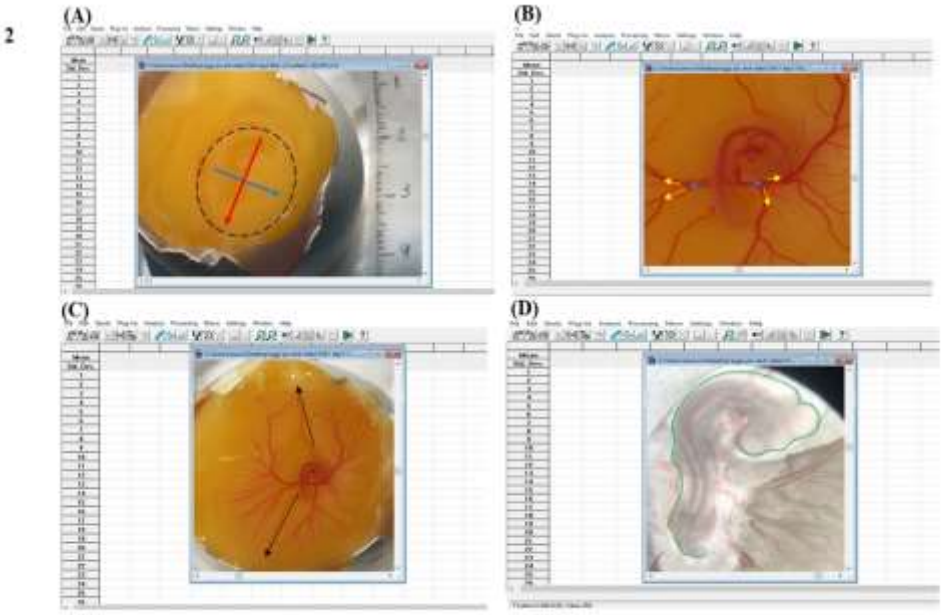

Figure 2: Showing the morphometry method for chick embryos. (A) Day2 area of area pellucida (black), Length of area pellucida (red) and width of area pellucida(blue). (B) Day3 and 4 Length of the right and left basic artery (blue), Length of the right and left branches of artery (yellow) and angles of the right and left artery (red). (C) length of the anterior and posterior vein (black) at day 3,4. (D) Whole body length (yellow) using Image tool.

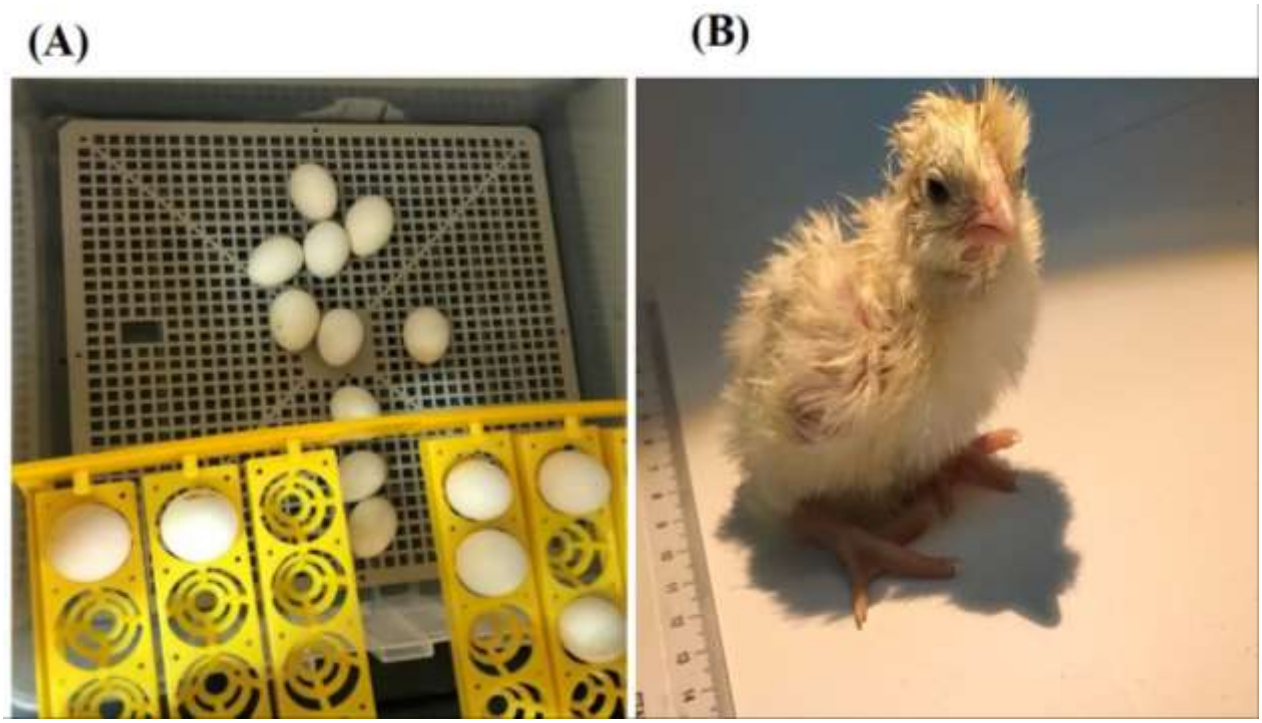

Figure 3: Hatching stage experiment. (A) The egg holder was removed from the incubator and eggs were placed randomly on the floor of the incubator. (B) All chicks were photographed and examined.

RESULTS

Effect of EMR on chick embryo Morphology:

The control chick embryo at day2 had a question mark shape (?) and was surrounded by area pellucida. The head was starting the fold to the right. The brain was divided into (diencephalon, mesencephalon, and metencephalon). Auditory vesicles and optic cup and lens were clear. The lateral and caudal amniotic folds were beginning to form. The heart tube shifting from C shape to S shape with outflow tract and ventricle was detected and start to beat. The heart tube was not yet developed to all chambers. At 48 hours of incubation about 27 pairs of somites were seen. The blood vessels were present in the vitelline membrane. Also, the vitelline artery appeared in the area vasculosa. The neural tube, tail bud, tail fold and branchial arches were seen clearly in this stage.

The exposed chick embryos with and without calls at day 2 were similar to the control chick embryos. However, some malformations were seen such as growth retardation in most embryos. Increased growth was seen only in one embryo. Bleeding was seen in some parts of the area vasculosa, surrounding the embryo and on the edges of area pellucida. Also, clotting was seen at the edge of the area pellucida and area vasculosa. One embryo seemed to have increased blood vitelline vessels branching compared to the controls. Neural tube defects such as (curvature and unclosed neural tube) were seen in some embryos (Figure 4).

The control chick embryo at day3 had the shape of letter (C) and was surrounded by the area pellucida. The heart started establishing the onset of blood circulation. Parts of the brain appeared clearly (telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon). The auditory vesicles were clear. Optic cup formed of neural and pigmented retina and lens were formed. The heart increased in size compared to the previous age. The main blood vessels found in area vasculosa were the right and left vitelline artery, anterior vein and posterior vein. The neural tube, tail bud, wing bud, leg bud, allantois, amnion and branchial arches were also seen clearly in this stage.

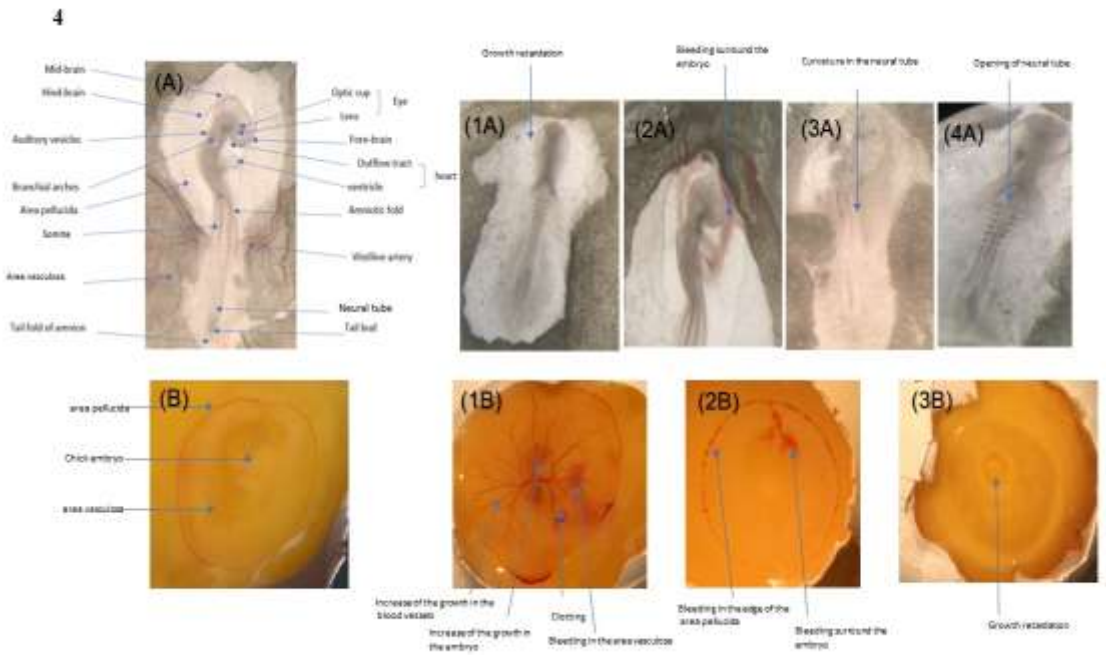

Figure.4. Morphology of chick embryo at day 2. (A) Under the dissecting microscope (2.0X). (B) Under smart phone camera. (A)Control embryo. (1A)EW embryo had growth retardation. (2,3,4A) EO embryo. Note: (2A) Bleeding surrounding the embryo, (3A) Curvature in the neural tube. And (4A) opening of neural tube. (B)Control embryo. (1,2B) EO embryo. (3B)EW embryo. Note: (1B) Bleeding in the area vasculosa with increase of the growth in the blood vessels & embryo, (2B) Bleeding in the edge of the area pellucida and surround the embryo. And (3B) Growth retardation.

The exposed chick embryos with and without calls at day 3 were similar to the control chick embryos. However, some malformations were seen such as growth retardation in most embryos. Bleeding was seen in some parts of the area vasculosa, surrounding the embryo and on the edges of area pellucida. Also, clotting was seen at the edge of area pellucida and area vasculosa. Curvature in the neural tube was seen in some embryos (Figure 5).

The control chick embryo at day4 of incubation had a body shape similar to the letter (C). All parts of the brain were clearly seen (telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon). The auditory vesicles were also clear. Eyes became pigmented the heart at day 4 seemed to have lost the tubular shape. Blood vessels; right and left vitelline artery, anterior and posterior vein became very much thickened compared to the previous stage. Wing buds and leg buds increased in size and became elongated compared to 4-day embryos. The neural tube, tail bud, allantois, amnion and branchial arches were seen clearly in this stage. (Figure6).

The exposed 4-day old chick embryos with and without calls groups were similar to the control 4 day old chick embryos. However, some malformations were seen such as growth retardation in all embryos. Bleeding was seen in some parts of the area vasculosa, surrounding the embryo. Also, clotting was seen in the area vasculosa. Curvature in the neural tube was also seen in some embryos. In the exposed without call group, special cases were seen as two conjoined twins joined in the head and upper abdomen region. Another egg had two chick embryos one of them was small and the other one large (Figure 6).

The control chick embryo at day 5 had a body shape similar to the letter (C). All parts of the brain were seen clearly (telencephalon, diencephalon, mesencephalon, metencephalon and myelencephalon) and auditory vesicles. Eyes became larger compared to the previous age 4 days. The heart was clearly seen. Blood vessels were more extended than 4-day old embryos.

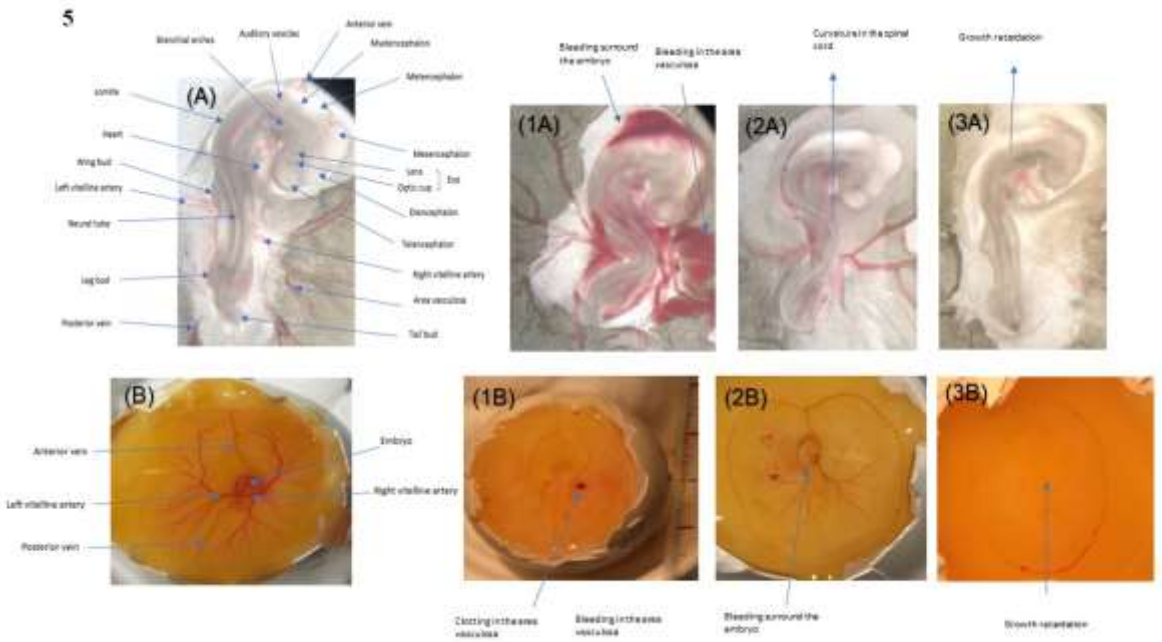

Figure.5: Morphology of chick embryo at day 3. (A) Under the dissecting microscope (2.0X). (B) Under smart phone camera. (A)Control embryo. (1A&2A)EW embryo. (3A) EO embryo. Note: (1A) had Bleeding in the area vasculosa and surround the embryo, (2A) Curvature in the neural tube. And (3A) Growth retardation. (B) Control embryo. (1B)EO embryo. (2B & 3B)EW embryo. Note: (1B) had Bleeding with clotting in the area vasculosa, (2B) Bleeding around the embryo. And(3B)Growth retardation.

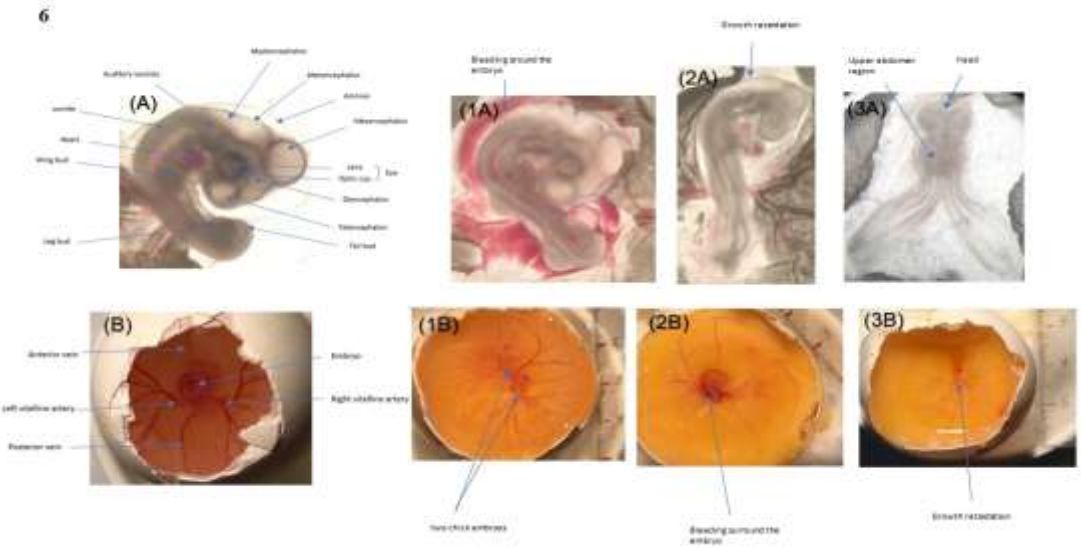

Figure.6. Morphology of chick embryo at day 4. (A) Under the dissecting microscope (2.0X). (B) Under smart phone camera. (A)Control embryo. (1,3A)EO embryo. (2A)EW embryo. Note: (1A) Growth retardation, (2A) Curvature in the neural tube. And (3A) Bleeding surround the embryo and in the area vasculosa. (B)Control embryo. (1B)EW embryo. (2,3B)EO embryo. Note: (1B) Growth retardation, (2B) Bleeding surround the embryo. And (3A and 3 B) two chick embryos one of them was small and the other one larger.

Wing buds and leg buds were larger than the previous age. Tail bud, allantois, amnion and branchial arches were seen clearly in this stage. Beak at day 5 was hardly recognizable (Figure 7).

The exposed 5-day old chick embryos with and without calls were similar to the controls. However, some malformations were seen such as growth retardation in all embryos, Bleeding around the embryo, and clotting was seen in the area vasculosa. Table 2 shows the percentage and type of the malformations seen in the chick embryos in each age treatment group in this study.

Effect of EMR on chick embryo WBL:

The mean WBL of control chick day 2 embryos in this study was = 28.51 mm, 56.25 mm for day 3 ,79.79 mm for day 4 and 136.39 mm for day 5. There was a highly significant decrease in the WBL of the EW and EO groups of day 2,4 and 5 (p= 0.000) compared to the C group. For day 3 embryos there was a significant decrease in the EO group (p= 0.001) compared to the C group. While there was a non-significant decrease in the WBL in EW group compared to the C group see (Figure 8A).

Effect of EMR on chick embryo area pellucida of day 2:

Mean length of the area pellucida (LAP) of control chick embryos in this study was = 31.08 mm. There was a non-significant decrease in both treated groups compared to the C group.

Mean width of the area pellucida (WAP) of control chick embryos in this study was = 29.11 mm. There was a significant decrease in the WAP in the EW group (p=0.003) compared to the C group. While a non-significant decrease was seen in EO group compared to the C group see (Figure 8B)

Mean AAP of control chick embryos in this study was = 746.95 mm². There was a significant decrease in the AAP in exposed with call group (p=0.005) compared to the C. While there was a non-significant decrease in EO compared to the C group see (Figure 8C).

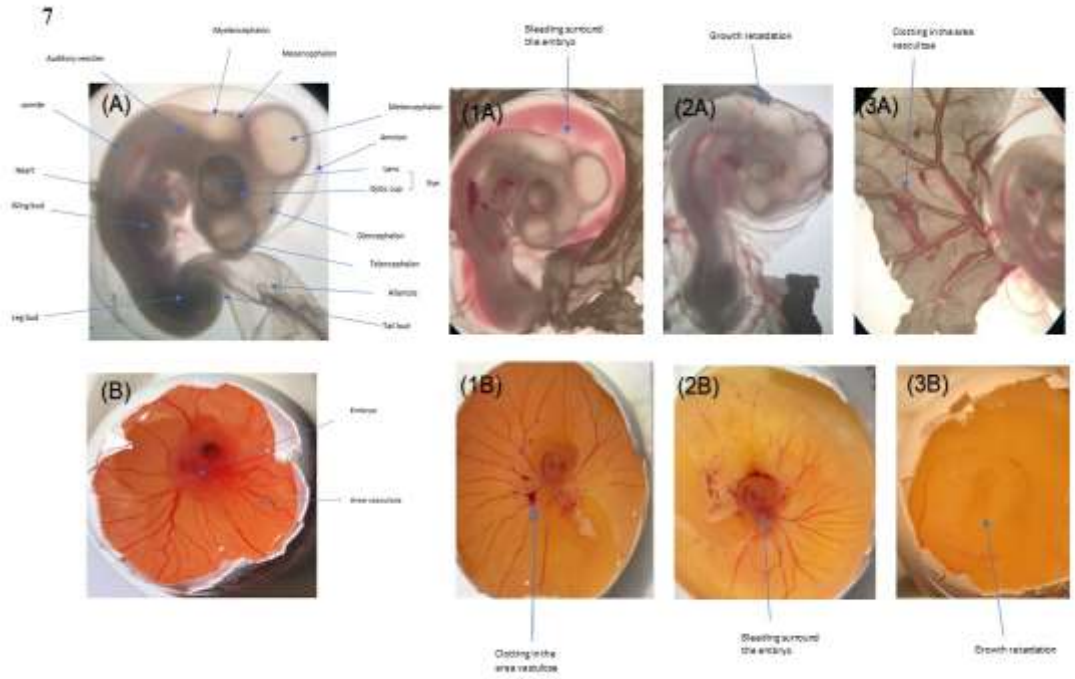

Figure.7. Morphology of chick embryo at day 5. (A) Under the dissecting microscope (2.0X). (B) Under smart phone camera. (A)Control embryo. (1,3A)EO embryo. (2A)EW embryo. Note: (1A) Bleeding surround the embryo. (2A) Growth retardation. (3A) Clotting in the area vasculosa. (1B)EO embryo. (3B)EW embryo. Note: (1B) Clotting in the area vasculosa. (2B) Bleeding surround the embryo. (3B) Growth retardation.

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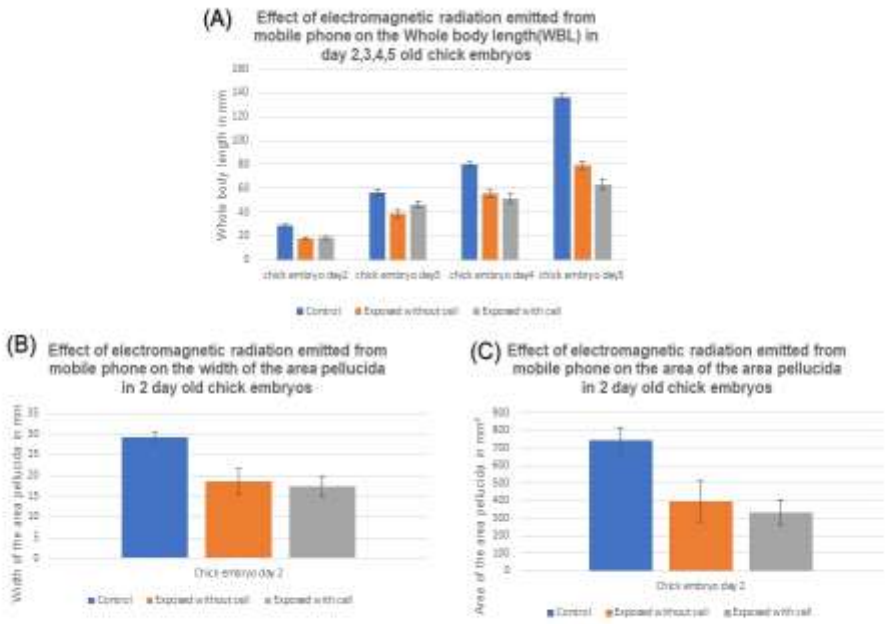

Figure .8. Graph showing the effect of EMR emitted from mobile phone in chick embryos.(A) on the (WBL). (B) on the width of the area pellucida. (C) on the area of area pellucida. Values are mean ± SE taken from 14 samples for each group age treatment. (*) p< 0.05 with control.

Table 2. Showing the percentage and type of the malformations seen in the chick embryos in each age treatment groups in this study.

Embryonic age		Congenital malformations seen				
	Treatment	Growth retardation	Bleeding	Clotting	Neural tube defect	Increase The growth in the embryo
		%	%	%	%	%
2 Days	Control	-	-	-	-	-
	EO	64%	28%	35%	14%%	14%
	EW	57%	42%	-	7%	-
3 Days	Control	-	-	-	-	-
	EO	100%	14%	21%	7%	-
	EW	92%	21%	21%	14%	-
4 Days	Control	-	-	-	-	-
	EO	100%	50%	50%	28%	-
	EW	92%	64%	42%	7%	7%
5 Days	Control	-	-	-	-	-
	EO	100%	7%	14%	-	-
	EW	100%	-	7%	-	-

Effect of EMR on vitelline vessels morphometry:

Mobile phone EMR affected the length of the vitelline arteries and veins in 3 and 4 day chick embryos. As it caused a decrease in all measured vitelline vessels compared to the controls. The decrease was significant in several arteries and veins. The angles measured were also decreased. Table 3 summarizes the mean and statistical

differences of the length and angles of the measured vitelline vessels for 3 and 4 day embryos for all experimental groups in this study, and figure 9 shows the graphs of the measurements that had significant differences.

Hatchability

Hatching rate in C was 100%, EO 10% and EW 0%. Chick embryos started hatching on day 20 until day 26. Control group hatching started at day 20 where 3 hatched. Then at day 21, 4 chicks hatched, and at day22, 3 chicks hatched (Figure 10A). In the EO group, only one embryo hatched on day 26, and 2 died when hatching (Figure 10C). While the rest 7 embryos did not hatch and were dead within the egg. In the EW group no hatching occurred until day 28. As 5 embryos did not hatch and were dead with malformations and 5 embryos had stopped growth in early stage (Figure 10B).

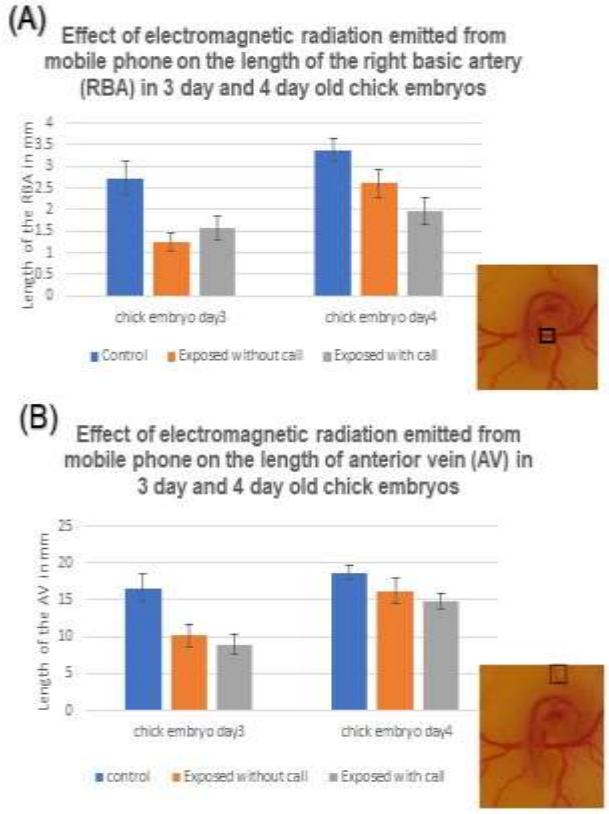

Figure .9. Graph showing the effect of EMR emitted from mobile phone in day 3 and 4 old chick embryos. (A) on the length of (RBA). (B) on the length of (AV). Values are mean ± SE taken from 14 samples for each group age treatment. (*) p< 0.05 with control.

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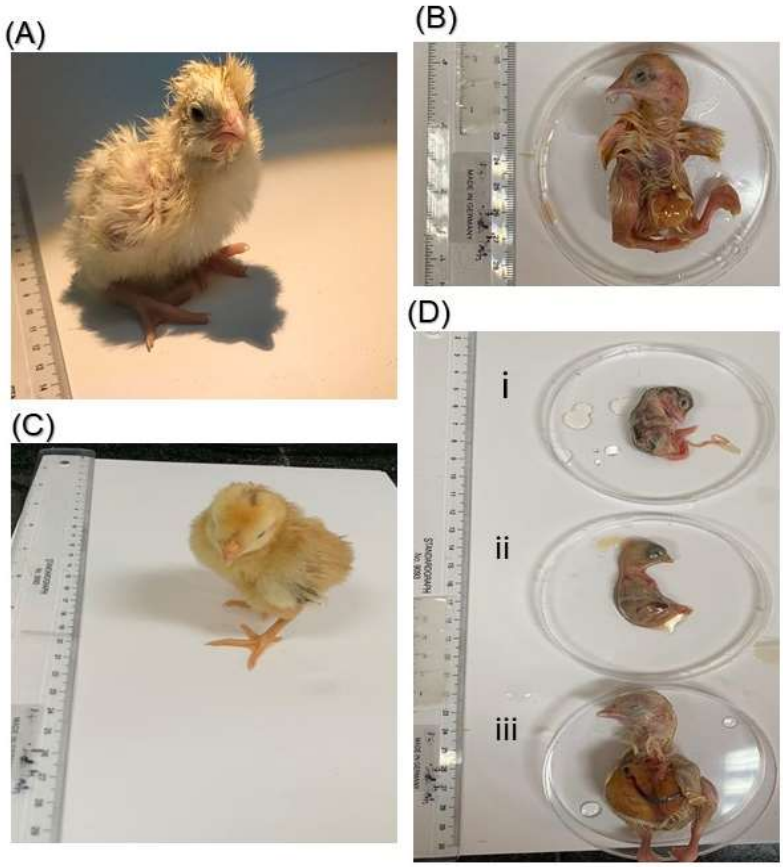

Figure .10. (A) Control chick embryo. (B) Exposed without call chick embryo show hernia with abnormal front and hind limbs, incomplete wings and eye malformation. (C) One exposed without call chick embryo hatch without malformation only delayed hatching time and was similar to the control group. (D) Exposed with call chick embryos (i) embryo had growth retardation and eye malformation. (ii) the embryo was like a lump and had growth retardation and bleeding. (iii) the embryo had growth retardation, abdominal hernia, wing malformation and abnormal front and hind limbs. Note: all embryos seen in B and D were within the egg.

Table 3 - Showing the mean and statistical differences of the length and angles of the measured vitelline vessels for 3 and 4 day embryos for all experimental groups in this study. Significance is shown as (*), p <0.05

Day3

Day 3		N	Mean	Standard Error
Length of RBA	C	14	2.7214	.39859
	EO	14	1.2429	.20814
	EW	14	1.5650	.27497
Length of 1 RBA	C	14	2.1429	.32835
	EO	14	1.5329	.36786
	EW	14	1.4950	.35766
Length of 2 RBA	C	14	7.6664	5.26851
	EO	14	1.9336	.38154
	EW	14	2.0971	.47714
Angle of RBA	C	14	71.9757	7.21568
	EO	14	75.6271	4.82806
	EW	14	71.8014	4.18701
length of LBA	C	14	1.8521	.29337
	EO	14	.9386	.15151
	EW	14	1.3264	.22598
Length of 1 LBA	C	14	2.5400	.32417
	EO	14	1.2664	.31961
	EW	14	2.6664	.50397
Length of 2 LBA	C	14	1.2814	.20793
	EO	14	1.6400	.30419
	EW	14	1.4336	.23726
Angle of LBA	C	14	67.8757	4.27681
	EO	14	69.5143	5.57484
	EW	14	71.1550	5.90617
length of AV	C	14	16.5543	1.90517
	EO	14	10.1386	1.48434
	EW	14	8.9700	1.37893
Length of PV	C	13	17.0238	1.47160
	EO	12	13.3350	2.09354
	EW	10	13.5620	2.23350

Groups		Significance
C	EO	.028
	EW	.004 *
EO	C	.028
	EW	.736
C	EO	.402
	EW	.445
EO	C	.403
	EO	.997
C	EO	.411
	EW	.391
EO	C	.411
	EO	.999
C	EO	1.000
	EW	.888
EO	C	1.000
	EO	.878
C	EO	.254
	EW	.021
EO	C	.254
	EO	.468
C	EO	.972
	EW	.068
EO	C	.972
	EO	.041
C	EO	.905
	EW	.580
EO	C	.905
	EO	.833
C	EO	.900
	EW	.974
EO	C	.900
	EO	.974
C	EO	.005*
	EW	.020
EO	C	.005*
	EO	.865
C	EO	.428
	EW	.347
EO	C	.428
	EO	.996

Day4

Day 4		N	Mean	Standard Error
Length of RBA	C	14	3.3736	.25242
	EO	14	2.6071	.32459
	EW	14	1.9557	.30824
Length of 1 RBA	C	14	3.2307	.38207
	EO	14	2.8064	.49975
	EW	14	2.0693	.36320
Length of 2 RBA	C	14	9.2807	5.84165
	EO	14	2.7464	.38482
	EW	14	3.2686	.73519
Angle of RBA	C	14	63.8350	6.30725
	EO	14	65.1700	4.17105
	EW	14	72.4043	5.42109
Length of LBA	C	14	2.1271	.28275
	EO	14	2.1721	.33719
	EW	14	1.9529	.63446
Length of 1 LBA	C	14	3.1843	.46319
	EO	14	2.3571	.32052
	EW	14	3.1536	.61977
Length of 2 LBA	C	14	8.9071	4.86118
	EO	14	3.3464	.70882
	EW	14	3.1879	.47209
Angle of LBA	C	14	56.8107	5.73578
	EO	14	70.3929	5.50559
	EW	14	73.1907	6.34496
Length of AV	C	14	18.6379	.96940
	EO	14	16.2229	1.66931
	EW	14	14.8600	1.05302
Length of PV	C	14	21.1650	1.11170
	EO	14	19.3507	1.22039
	EW	14	19.9136	2.02680

Groups		Significance
C	EO	.174
	EW	.005 *
EO	C	.174
	EW	.278
C	EO	.756
	EW	.136
EO	C	.756
	EW	.436
C	EO	.373
	EW	.433
EO	C	.373
	EW	.994
C	EO	.983
	EW	.503
EO	C	.983
	EW	.611
C	EO	.997
	EW	.959
EO	C	.997
	EW	.936
C	EO	.455
	EW	.999
EO	C	.455
	EW	.481
C	EO	.361
	EW	.341
EO	C	.361
	EW	.999
C	EO	.243
	EW	.133
EO	C	.243
	EW	.939
C	EO	.379
	EW	.102
EO	C	.379
	EW	.730
C	EO	.675
	EW	.828
EO	C	.675
	EW	.962

DISCUSSIONS

In this study chick embryo was used as a model to investigate the effect of mobile phone EMR on the early development of vitelline vessels. In this study the control group morphology and body length were similar to other studies. (Rahman et al. 2014). In the present study EMR mobile phone caused several malformations to chick embryos.

In this study mobile phone EMR caused growth retardation in all experimental ages it was seen as a decrease of WBL. This was seen in several other studies. Diode laser applied three times for one minute each time showed growth retardation in chick embryos at day 7, 10 and 14 of incubation. It was concluded that diode laser affected cell proliferation (Al-Qudsi and Al-Beladi, 2019). Also, studies found that exposure to EMR emitted from mobile phone (1800 MHz) for 20 minutes (calls) per day affected the growth of the chick embryo by decreasing body weight at day 10 of incubation. It was concluded that exposure to EMR mobile phone caused DNA damage, reactive oxygen species production and apoptosis which caused growth retardation (Siddiqi et al. 2016). Also, chick eggs exposure to mobile phone EMR during incubation decreased growth at day 14 of incubation. The study concluded that different cellular responses to EMF occur during different embryological periods as cells might be trying to rebalance their growth and differentiation rate (Al-Qudsi and Azzouz, 2012).

The embryo is most sensitive to exposure in the first 24 h of incubation. The process of embryo development includes cell multiplication (cellular division), proliferation, differentiation, relocation, and programed cell death. Any alterations in incubation environment influences the growth of embryos. Effect of EMR on living cells depends on dose and duration. It seems that EMR might have reduced the cellular division during embryonic development and caused growth retardation.

In this study mobile phone EMR caused neural tube defects such as curvature of neural tube, that was seen in all experimental ages. Also, open neural tube was only seen in EO group day 2. This was seen in several other studies. Effect of 30-90 mg/kg of metamizole Sodium (common analgesic) on chick embryo caused opening in neural tube at 48h. The researchers thought that metamizole Sodium cause stress in the embryo and blocked neurulation(Guvenc et al. 2016). A study found that chick embryo exposed to mobile phone EMR caused open neural tube at 30, 40h of incubation the researchers concluded that EMR affected the molecules responsible for the apoptotic mechanism during the closing of the neural tube (Umur et al. 2013). Studies also showed that neural tube defect appeared in 48h chick eggs injected by high dose progesterone. They showed that the mechanism of progesterone was to inhibit folate transporters at the cellular level, which can lead to neural tube defects(Iqbal et al. 2011).

In the present study EMR might have delayed neural tube closure as beyond 48h the neural tube was closed. Also, EMR might have disturbed cells development which might have caused the curvature of the neural tube.

In this study mobile phone EMR caused bleeding and clotting in all experimental ages it was seen in some parts of the area vasculosa, surrounding the embryo and on the edges of area pellucida. Effect of extremely low frequency electromagnetic field (50 Hz, 2 mT) on human umbilical vein endothelial cells reduced the ability of endothelial cells to form new vessels. Studies concluded that most probably extremely low frequency electromagnetic field affected vascular endothelial growth factor (VEGF) signal transduction pathway (Ohkubo and Okano, 2015). Ethanol (30-50%) effect on chick embryo after 48h of incubation was inhibition of normal vascular development. it was concluded that ethanol might inhibit vascular endothelial growth factor protein (VEGF) and basic fibroblast growth factor, VEGF, Flt-1 (expression gene encodes a member of the vascular endothelial growth factor receptor) and Flk-1 (a receptor for vascular endothelial growth factor) mRNA expression (Tufan and Satiroglu-Tufan, 2003). Studies showed that chick eggs exposed to static magnetic field (0.2 Tesla) on the chorioallantois membranes for 3h caused inhibition of angiogenesis (McKay et al. 2007). Studies on chicken eggs showed that exposure to mobile phone radiation may induce myocardium pathological changes and affect vascular development (Ye et al. 2016). In this study it might be that the EMR inhibited angiogenesis but did not affect the blood flow. As bleeding was seen in between vessels and surrounding the embryo. Also, maybe the thermal factor emitted from EMR affected the formation of vitelline vessels. All these reasons might cause bleeding in several parts of area vasculosa and around the embryo, also EMR might have affected the blood islands in some way and caused clotting in several sites in the embryo and prevented endothelial cells from forming new

vessels, therefore inhibiting angiogenesis.

The adult chick vascular system includes arterial, venous and lymphatic compartments. The vascular system has to be developed early in every organ or tissue, so the cardiovascular system is the first system to be established (Eichmann et al. 2005). The major cells responsible for the formation of almost the entire circulatory system including the heart, veins and arteries are the endothelial cells (Cleaver, 2004). Vascular system function is to supply tissues with nutrients and gas exchange and carry away wastes via blood circulation (Gerritsen, 1987, Cleaver, 2004).

VEGF and its receptor vascular endothelial growth factor receptor-2 are expressed in the areas of vascularization (Yancopoulos et al. 2000, Eichmann et al. 2005). After the differentiation of the blood islands in the yolk sac, a capillary meshwork is formed from the endothelial cells surrounding the blood islands and is formed by adhesion, this meshwork is the base for the beginnings of circulation. After the onset of the first beat the blood flow is then established and the yolk sac capillary plexus are remodeled into arteries and veins and a functional circulatory loop is then established (Eichmann et al. 2005).

The morphology of vitelline vessels in this study of the C group had normal formation in size and length. Vitelline arteries and vitelline veins were clearly seen in the C group. In this study mobile phone EMR caused a decrease in the length and angles of vessels in day 3 and 4 exposed chick embryo. To our knowledge, this is the first study using vitelline vessels morphometry of 3 and 4 day old chick embryo (Length of the right and left basic artery, Length of the right and left branches of artery, angles of the right and left artery) therefore we were not able to compare control measurements to other studies. The morphology in this study of area pellucida in control group had normal formation in size and length. Area pellucida was clearly seen in the control group without any malformation that was seen in treated group based on morphological characters of (Hamburger and Hamilton, 1993). In this study mobile phone EMR caused a decrease in the area, length and width of area pellucida in day 2 exposed chick embryo. this was seen in other studies of chick embryo affected by ethanol (Tufan and Satiroglu-Tufan, 2003).A study exposed chick fertilized eggs to EMF at day 10 for 4 h then extracted embryos at day 12 of incubation, showed a significant decrease in the number and length of blood vessels in the test group, it was concluded that EMF inhibited angiogenesis therefore the number of blood vessels was decreased (Balanezhad et al. 2010). In this study it seems that EMR caused blood to leak from the vitelline vessels (arteries and veins) which were shorter than the controls and might have been thinner causing bleeding and clotting in area pellucida.

In the present study there was a significant decrease in the HBPM of the exposed groups compared to the C group. Studies found that exposure to weak electromagnetic field radiation 50Hz frequency from the first to the last day of incubation can increase the heart rate in chick embryo associated with increases in plasma T4 and T3 (thyroid hormone) concentrations, especially from 17 days of incubation. studies concluded that exposure of chick embryos to weak EMF affects protein metabolism and necrotic processes resulting to increase in heart rate (Pawlak et al. 2013). It seems that the difference in EMR level caused the decreased heartbeat seen in this study.

In this study, mobile phone EMR caused a high significant decrease of hatchability in both exposed group. EO group had 90% mortality and EW had 100% mortality. Mobile phone EMR caused malformations seen in chick non-hatching embryos such as growth retardation, hernia, abnormal front and hind limbs, wing and eye malformation. This was seen in several other studies. In a study chick eggs incubated and exposed to mobile phone EMR at day 4, 9, 10, 12, 14 day showed increased mortality of chick embryos in exposed groups, suggesting that EMR might damage the tissues and cause mortality (Ingole and Ghosh, 2006). Studies suggest that changes of temperature and humidity decrease hatchability (Nakage et al. 2003, Al-Qudsi and Al-Beladi, 2019). Chick eggs exposure to low frequency electromagnetic of mobile phone showed increased mortality and malformation such as hernia, suggesting that low frequency EMR affect the rapidly multiplying cells (Siddiqi et al. 2016). In this study it seems that EMR caused premature death of embryos and it might be that EMR affected the cell proliferation and slight changes in temperature and humidity caused several malformations on chick hatchability.

CONCLUSION

To our knowledge this is the first study to explore the effect of mobile phone EMR on the morphometry of chick embryo vitelline vessels during day 3 and 4 of incubation. Our results showed that mobile phone EMR decreased the length and branching angles of the measured vessels, causing bleeding in several sites around

the embryo. We suggest that mobile phone EMR might have prevented proper vasculogenesis and angiogenesis. Resulting in decreasing nutrient flow to the embryo proper therefore producing growth retardation, and low hatchability.

More studies should be done to understand the mechanism of mobile phone EMR on blood vessel formation. These studies could have an importance in preventing congenital malformation or could be used to prevent formation of new vessels during tumor metastasis.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

. F.A. designed and performleed the experiments A.A. performed animal treatments, experiments, sample collection, and data analysis. F.A. and A.A. designed experiments and reviewed the manuscript. All authors read and approved the final version.

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