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Radio Diagnosis

EFFECT OF COLOR DOPPLER ON OFFSPRING OF COLOR DOPPLER EXPOSED PREGNANT MICE

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ABSTRACT A study on pregnant mice as an experimental animal, to study the effect of color Doppler exposure on the offspring's was performed.

KEYWORDS : Diagnostic ultrasound, autistic-like behavior, risk factor.

INTRODUCTION

Doppler evaluation of the maternal and fetal circulation by color imaging and pulsed wave spectral Doppler imaging has allowed new applications for routine obstetric ultrasound use. Investigation of flow in the uterine artery, umbilical vessels, and fetal arteries and veins are integral to modern assessment of the mother and fetus at risk. However, extensive use of Doppler testing should raise concerns regarding fetal well-being because higher levels of acoustic energy than conventional B-mode imaging can be generated.

As a form of energy, ultrasound has the potential to have bioeffects, the two most likely mechanisms for these being heating and cavitation.

Hyperthermia is a proven teratogen in experimental animals and is considered teratogenic in human fetuses as well, especially during organo-genesis in the first trimester. A general threshold of temperature elevation of 1.5°C to 2°C before any evidence of developmental effects occurs is suggested, and accordingly, the World Federation for Ultrasound in Medicine and Biology has summarized that exposure that produces a maximum temperature elevation of no more than 1.5°C above normal physiologic levels may be used without reservation on thermal grounds. Tissue exposed to ultrasound can absorb energy with the production of heat, thus resulting in a rise of temperature.

Keeping this in mind we thought to evaluate the effect of color Doppler on the off springs of pregnant mice during different exposure duration. The next generation of mice will be studied for different parameters to know the effect of color Doppler on the offspring of pregnant mice for CNS examination by locomotor activities (motor coordination, gait) and behavior changes (conditioned avoidance reflex, MoRaG performance scale) and growth parameters (weight, total body length and head circumference).

In our study we have used mice as an experimental animal because

1. Much easier to study the effect of color Doppler on mice.
2. Closely related in their genome/anatomy/biology to human.
3. Inbred so that the genetic difference between individual mice are minor.
4. Small and we can easily accommodate multiple mice in a cage for efficiency.
5. We can acquire them relatively easily.
6. Gestational period is only 19-21 days, so they can multiply faster and conveniently used for reproductive studies.

There are some reports that diagnostic color Doppler ultrasound induces changes in experimental animals like reduction in prenatal weight, an increase in postnatal mortality in mice, transient changes in body weight, increase in incidence of IUGR.

However some researchers concluded that available data do not provide any conclusive evidence against the perceived safety of color doppler ultrasound exposure during the intrauterine development. Because of inconsistencies in the available reports the present study was carried out to find out the effect of different duration of color Doppler exposure on offspring of pregnant mice.

AIMS AND OBJECTIVES:

- To evaluate any adverse effect of color Doppler of different duration on offspring of color doppler exposed mice.
- To study the birth weight changes on offspring of color doppler exposed mice.
- To study the learning defects in the offspring of color doppler exposed mice.

MATERIAL AND METHODS:

The study was carried out at Jawaharlal Nehru medical college, datta meghe institute of medical science by the department of radio diagnosis, AVBR hospital, Sawangi (Meghe), Wardha. Permission was obtained from institutional animal ethical committee.

Experimental animal

Twenty female Swiss albino mice were taken weighing approximately 20 to 35 gm. The animal were maintained in standard laboratory condition. They were kept in polypropylene cages with husk paddy as the bedding. Diet was provided in the form of pellets and water. Normal cycle of light and dark of 12 hrs were maintained. Mice having any gross abnormality or pathologic conditions were excluded from the study.

Pair of male and female mice were kept for mating for a period of approximately 7-10 days which is the normal duration for the occurrence of pregnancy. Weight of female mice was monitored daily during this period. Daily observation was carried out for confirmation of pregnancy by checking vaginal plug. The day on which vaginal plug is seen was considered as day 0 of gestation. After confirmation of pregnancy, pregnant female were divided into 2 groups, each contain 10 mice.

- Group A:- mice exposed to color Doppler for 5 mins
- Group B:- mice exposed to color Doppler for 10 mins
- Pregnant mice in the same group were numbered 1to 10 like A 1, A2 , A3 A 10, same applied for B group.

Equipment's

1. Siemens color Doppler ultrasound machine Model —Siemens acuson X300
2. Card board
3. Sticking tape
4. Digital weighing machine
5. Measuring tape
6. Vernier calipers
7. Cook's apparatus
8. Traction wire apparatus.
9. MoRaG apparatus

Color doppler exposure

The ventral surface of the abdomen of the mice was shaved using hair removal cream and was fixed to the cardboard with sticking tape. Simens color Doppler ultrasound machine was used to give Doppler exposure between 1st to 8th day of gestation on alternate day for 5mins to group A and 10 min to group B animals each time using 3-5 MHz frequency. An attempt was made to minimize attenuation of ultrasound signal by applying coupling agent in form of ultra gel. Care was taken to apply uniform pressure as far as possible. Animals of both the groups were left to complete gestation and parturition. The pregnant mice were observed till the delivery of offspring. The offspring of both groups were evaluated for growth parameters and the CNS activity.

Growth parameters

1. Weight of offspring: Weight of offspring was taken at birth, at 1st , 2nd, 3rd, and 4th post partum weeks by using digital weighing machine.
2. Total body length: It was taken from tip of the nose to base of the tail by using measuring tape weekly from the date of birth till 4th week post partum.
3. Head circumference: Head circumference was measured using vernier's caliper calibrating the distance between two ears weekly from the date of birth till 4th week post partum.

CNS Activity

1. Motor Co-Ordination Traction wire test was used for this purpose. Mice were left to hang on wire manually with only forepaw. Time required forming a loop around wire and number of falls from wire in 5 min were measured.
2. Conditioned Avoidance Response(CAR) CAR is observed using cook's apparatus consisting shocking grid floor and wooden pole in the centre of the apparatus. For response, mice was kept on the grid floor and allowed to acclimatize for 30 min . in the first phase, buzzer was applied and after 30 seconds electric shock was applied. This procedure was done continuously till the mice learned to climb the pole. After that mice climbs the pole even on ringing of the buzzer only without electric shock. This was taken as positive response.
3. MoRaG Response(mouse reaching and grasping performance scale) The ability to use limb and digit movements to reach, grasp and retrieve food is widespread among mammal, including mice. The control of multi-joint arm movements, such as placing the hand on a visually detected target, grasping and retrieving requires the transformation of visually derived information (position of the target) into a motor command to position the hand and perform the grasping response. Reaching, grasping and releasing functions are goal-directed movements. Injury or disease affecting the central nervous system (CNS) may diminish the ability to execute effectively goal-directed movements. The Mouse Reaching and Grasping Performance Scale (MoRaG) consists of a qualitative and quantitative assessment of reaching, grasping and retrieval behavior in the mouse. Individual animals are placed in a Plexiglas chamber 10.5 cm high by 6 cm deep by 6 cm wide. Outside the front wall of each cubicle a Plexiglas feeding platform, accessible through a 9 mm hole is attached 5.4 cm from the floor. Small food pellets are placed on the feeding platform so that the mouse can withdraw food by using a single paw. Reaching, grasping and retrieval in animals are monitored. The reaction time between when the mouse sees the food pellet and reaches it (reaching time) is considered for all the trials. A successful grasping is scored if the mouse grasps and holds the pellet. A successful retrieval is scored if the mouse brings the food toward the mouth to consume it.

Statistical analysis

All the data of our study were grouped to study the statistical significant. For quantitative data P value was calculating using "Z test". For qualitative data P value was calculated using "Chi-Square test". For the P value having value of <0.05 considered as significant for the given parameters while P>0.05 implies that given parameters are not significant.

RESULTS

This study done in the department of Radio Diagnosis for Effect of Color Doppler showed following observation. The total number of pregnant mice in the study was 20. They were exposed to color Doppler examination and the weight and CNS activity of the offspring were studied.

Table 1: Number of mice and their offspring

Group A	No of offspring	No. of death	Group B	No of offspring	No. of deaths
A1	10	2	B1	3	3
A2	6	1	B2	5	2
A3	3	2	B3	8	3
A4	6	2	B4	6	2
A5	5	1	B5	9	1
A6	2	1	B6	2	2
A7	6	2	B7	4	1
A8	8	1	B8	5	3
A9	6	1	B9	8	2
A10	7	2	B10	6	1
Total	59	15		56	20

Table 2: Wight of offspring comparison of weight of mice offspring in group A and B at birth, week 1,2,3,4 (z-test)

	Group	N	Mean	Std. deviation	Std. error mean
At birth	Group A	59	1.20	0.25	0.03
	Group B	56	1.03	0.14	0.01
At 1st week	Group A	59	3.97	0.68	0.08
	Group B	56	3.00	0.24	0.03
At 2nd week	Group A	59	6.37	0.76	0.09
	Group B	56	6.23	0.77	0.10
At 3rd week	Group A	59	9.47	0.57	0.07
	Group B	56	9.44	1.02	0.13
At 4th week	Group A	59	13.93	1.08	0.14
	Group B	56	14.35	1.27	0.17

Z-test

	Z	p-value	Mean difference	Std. error difference	95% confidence interval of the difference	
					Lower	Upper
At birth	4.37	0.000 S, p<0.05	0.16	0.03	0.09	0.24
At 1st week	10.08	0.000 S, p<0.05	0.97	0.09	0.78	1.17
At 2nd week	0.95	0.341 NS, p>0.05	0.13	0.14	-0.14	0.42
At 3rd week	0.19	0.847 NS, p>0.05	0.02	0.15	-0.27	0.33
At 4th week	1.86	0.064 NS, p>0.05	-0.41	0.22	-0.84	0.02

Table 3: Head Circumference Comparison of head circumference of mice offspring in group A and B at birth, week 1,2,3,4 (Z-test) Descriptive statistics

	Group	N	Mean	Std. deviation	Std. error mean
At birth	Group A	59	6.93	0.57	0.07
	Group B	56	6.79	0.48	0.06
At 1st week	Group A	59	10.61	0.90	0.11
	Group B	56	10.71	1.07	0.14
At 2nd week	Group A	59	11.52	1.40	0.18
	Group B	56	11.37	0.92	0.12
At 3rd week	Group A	59	13.19	0.86	0.11
	Group B	56	13.00	1.00	0.13
At 4th week	Group A	59	15.14	1.47	0.19
	Group B	56	14.97	1.04	0.13

Z-test

	Z	p-value	Mean difference	Std. error difference	95% confidence interval of the difference	
					Lower	Upper
At birth	1.360	0.177 NS, p>0.05	0.13	0.09	-0.06	0.33
At 1st week	0.555	0.580 NS, p>0.05	-0.10	0.18	-0.46	0.26
At 2nd week	0.682	0.497 NS, p>0.05	0.15	0.22	-0.28	0.59
At 3rd week	1.136	0.258 NS, p>0.05	0.19	0.17	-0.14	0.54
At 4th week	0.704	0.483 NS, p>0.05	0.16	0.24	-0.30	0.64

Table 4: Total body length comparison of total body length of mice offspring in group A and B at birth, week 1,2,3,4 (Z-test) descriptive statistics

	Group	N	Mean	Std. deviation	Std. error mean
At birth	Group A	59	39.16	2.84	0.36
	Group B	56	38.55	2.13	0.28
At 1st week	Group A	59	63.89	4.05	0.52
	Group B	56	64.39	2.52	0.33
At 2nd week	Group A	59	83.38	7.33	0.95
	Group B	56	81.59	5.16	0.69
At 3rd week	Group A	59	106.79	9.14	1.19
	Group B	56	103.80	7.22	0.96

At 4th week	Group A	59	129.69	7.32	0.95
	Group B	56	127.75	5.63	0.75

Z-test

	Z	p-value	Mean difference	Std. error difference	95% confidence interval of the difference	
					Lower	Upper
At birth	1.308	0.194 NS, p>0.05	0.61	0.47	-0.31	1.5
At 1st week	0.780	0.437 NS, p>0.05	-0.49	0.63	-1.75	0.76
At 2nd week	1.560	0.122 NS, p>0.05	1.85	1.18	-0.50	4.20
At 3rd week	1.941	0.055 NS, p>0.05	2.99	1.54	-0.06	6.04
At 4th week	1.590	0.4115 NS, p>0.05	1.94	1.22	-0.47	4.36

Table 5: Motor Co-ordination comparison of motor co-ordination of mice offspring in group A and B at 45 days of age (Z-test) Descriptive Statistics

	Group	N	Mean	Std. deviation	Std. error mean
Time required to form a loop on wire	Group A	59	1.23	0.46	0.06
	Group B	56	1.73	0.98	0.13
No. of fall from wire	Group A	59	1.83	1.40	0.18
	Group B	56	3.57	1.77	0.23

Z-test

	Z	p-value	Mean difference	Std. error difference	95% confidence interval of the difference	
					Lower	Upper
Time required to form a loop on wire	3.48	0.001 S, p<0.05	-0.49	0.14	-0.77	-0.21
No. of fall from wire	5.84	0.000 S, p<0.05	-1.74	0.29	-2.33	-1.15

Table 6: Comparison of conditioned avoidance response of mice offspring in group A and B at 45 days of mice

CAR	Group A	Group B	X^2-test	p-value
Present	54	42	5.68	0.01 S,p<0.05
Absent	5	14		
Total	59	56		

Table 7: Mouse reaching and grasping reflex (MoRaG) Comparison of MoRaG of mice offspring in group A and B at 45 days of mice Chi-square Test

MoRaG	Group A	Group B	X^2-test	p-value
Present	55	38	11.95	0.0005 S,p<0.05
Absent	4	18		
Total	59	56		

DISCUSSION

This study was carried out in the Department of Radio-Diagnosis and imaging at Acharya Vinobha Bhave Hospital attached to Jawaharlal Nehru Medical College, Sawangi (Meghe), Wardha, as the continuation project to the study done by Dr. Tehzeeb Motala. She helped us to evaluate the effect of ultrasound on the offspring of pregnant mice exposed to ultrasound antenatally. In our study we went a step further to evaluate the effect of color Doppler on the offspring of mice exposed to color Doppler antenatally.

Experimental animal

Human study are associated with ethical issue, long study period, long follow up period is required to study behavioral effect, which is not feasible in our set up as it rural based. That's why we carried our research on mice expecting the same mechanism applied to human fetus.

Because of the potential advantage, mice were taken as the experimental animal in our study. Many studies also used rats, lamb and rabbit to assess the effect of color Doppler.

In a study by Allison S. Duckett et al[1]. They performed study on mice ex- vivo and in-vivo to see biological effect of ultrasound biomicroscopy and Doppler in the embryo and at soft tissue interface. Stone PR et al[2]. investigated the heating effects of pulsed Doppler ultrasound on fetal lamb brain tissue. Nakagawa K et al[3] used rabbit to study the long term effect of the transcranial color Doppler. Animal studies may not co-relate that well with the human finding hence few workers also carried human studies. Newnnam JP[8]. carried out human study to see the effect of ultrasound imaging and continuous wave Doppler flow studies on fetus. Another human study by Sharon Evans et al[4].

Study Population

In our study we took 20 female mice out of which 10 mice were subjected to color Doppler for 5 minutes and other 10 mice were subjected to color Doppler for 10 minutes. A total of 115 offspring of both groups were evaluated for growth para meters and CNS activity. As seen in a study by Jianping Zhu et al[5] who used 72 healthy hybridized Sprague-Dawley rats to see the effect of color Doppler on cell cycle of newborn rats.

Frequency

The frequency used for medical imaging are generally in the range of 1 to 18MHz. Higher frequency have a smaller wavelength and can be used to make sonogram with smaller details. However, the attenuation of the sound wave is increased at higher frequency, so in order to have better penetration of deeper tissue, a lower frequency (3-5MHz) is used.

In our study we have used frequency of 3-5MHz delivered through ultrasound machine. As Jianping Zhu et al[5]. have also used similar wavelength frequencies (0, 2.5, 3.5, 7.0, MHz) in their study. Nakagawa K[3] also used the frequency of 2 MHz in their study. So the frequency in our study is nearly similar to the frequency routinely used in antenatal ultrasound.

Exposure period

After mating the mice pair overnight, we observed female mice daily for vaginal plug and weight. On the day when vaginal plug was found it was taken as day 0 of gestation. In our study color Doppler was applied to pregnant mice on alternate day from day 1 to 8th day of gestation.

As in a study by Jianping Zhu et al[5] day on which sperm were found was defined as gestation 0 and the pregnant rats were insonated with color Doppler ultrasound of different frequencies and insonation time on 7th day of pregnancy.

In another study by Allison S. Duckett et al[1]. to performed study on mice ex-vivo and in-vivo to see biological effect of ultrasound biomicroscopy and Doppler in the embryo and at soft tissue interface. High frequency ultrasound (40MHz) was used on pregnant mice on embryonic day 8 and 10 for 1 hour ultrasound biomicroscopy imaging and 3minutes per embryo for Doppler imaging.

In our study the exposure period correlated with the organogenesis and early fetal period.

Technique of color Doppler application

To apply color Doppler on mice, they were stick to card board with adhesive tape. Abdomen was exposed through hole on the card board. The hairs over the abdomen were removed using a hair removing cream. Then color Doppler ultrasound probe was applied with ultragel applied on the abdomen as a coupling agent. In a study by P.Uma Devi et al [6]. animal were restrained by adhesive tape on a flat Perspex sheet with their abdomen facing probe just like our study. They also used coupling agent in their study. In a study by Sidney J. Stolzen berg et al[7]. They used holding frame to apply ultrasound.

Weight of offspring

In our study the weight of offspring were measured at birth and then weekly for 4 weeks in both group A and group B. and it was found that the offspring of group B who were exposed to color Doppler for 10 minutes shows low weight at birth and first week of life as compared to group A who were exposed to color Doppler for 5 minutes. But after 2nd week of age and subsequently the offspring were gaining weight and by 4th week of age the weight of the offspring in both group A and group were not significantly different.

As was seen in one study by P.Uma Devi et al[6] in which there is reduction in weight observed which continued up to 5th week of age when ultrasound was applied on organogenesis period while reduction in weight was observed for 1 week only if ultrasound was applied in pre

implant period.

In another study by Newnnam JP et al[8] they found that there were significantly higher intrauterine growth restrictions in the intensive group. Their finding suggest that five or more ultrasound imaging and Doppler flow studies between 18 and 38 weeks gestation when compared with single imaging study at 18 week gestation , increase the proportion of growth restricted fetuses by about one third.

Other growth parameters

In our study we found that head circumference and total body length were not affected which were measured at birth and thereafter weekly for 4 week of age. In a study by M.P.Hande and P.Uma Devi et al[16] they noticed there was no change in the head circumference, body length when ultrasound was applied on 6 and 11 day of gestation.

Johan Newnham et al[4]. also performed study to evaluate relative reduction in bone and soft tissue growth , principal component analysis were performed on the measurement of soft tissue size. In the intensive group, normally formed babies of term gestation age, tended to be shorter when measured at birth. There were statically insignificant reduction in the circumferences of the chest, abdomen, mid-arm and in the skin fold thickness of the triceps, para-scapular and sub-scapular region. Component analysis showed a trend for reduction for the skeletal component but not for the soft tissue component.

Newnnam JP et al[8] in a human follow up study confirmed and indicated that the primary effect was on bone growth compared with soft tissue. There was strong trend for the intensive group to have smaller long bone measures.

Similar finding were observed by Newnham JP et al[8] they concluded that exposure to multiple ultrasound exposure from 18 weeks of gestation onwards might be associated with a small effect on fetal growth but when growth and development were followed in childhood, outcome was similar in both regular and in intensive group.

Effect on central nervous system

In our study the central nervous system were evaluated when the mice offspring were 45 days old, as this is the time taken to attain maturity in mice. We evaluated the offspring in locomotor, and learning abilities & goal directed movements. Motor co-ordination which is measured of locomotor activity was impaired in color Doppler exposed mice. Learning behavior & goal directed movements were affected in color Doppler exposed mice as seen by conditioned avoidance reflex and Mouse reaching and grasping reflex.

Study by Harazadira, A et al[9] showed that adult mice who were exposed prenatally twice to diagnostic ultrasound exhibited less locomotor and exploratory activities.

Zhou Ning et al[10] in their study learning and memorizing abilities were estimated via the Morris water maze test when they were 2months old. The mean score of the 30min insonification groups achieved in the Morris water maze test was lower than that of the control group, but there were no difference between the other two exposed group and the control group. Thirty-min exposure to color Doppler ultrasound in utero could adversely affect the learning and memorizing ability of the rat offspring.

Engenius S. B et al[11] explain the effect of ultrasound at ultra structural level in brain. Their study revealed that ultrasound exposure for 30 minutes or longer during period of neuronal migration, a small but statistically significant number of neurons fail to acquire their proper position and remain scattered within cortical layers and in the subjacent white matter. This explains the possible mechanism responsible for CNS effects in mice offspring. Another possible explanation is suggested by N. Margulies et al[12] Their study proved that insonation at 10 days of gestation age produce significant biochemical changes but no morphological differences were found compared to control group.

Barnett SB et a1[13] studied sensitivity of biological tissue to ultrasound and concluded that Actively dividing cells of the embryonic and fetal central nervous system are most readily disturbed. As a diagnostic ultrasound beam envelopes a small volume of tissue, it is possible that the effects of mild disturbance may not be detected unless major neural pathways are involved. Biologically significant temperature increases can occur at or near to bone in the fetus from the second trimester, if the beam is held stationary for more than 30 s in some pulsed Doppler applications.

Helle Keller et al[14] claimed that ultrasound exposure in fetal life increase the risk of left handedness in men suggesting that prenatal ultrasound affects the fetal brain.

Stark CR et al[15] stated that there were no biologically significant difference between exposed and unexposed groups were found at short term or long term in terms of nerve conduction, hearing, visual acuity, color vision, cognitive function, behavior and neurological examination.

So there are varying views and results among various researchers, some show that deleterious effect are seen due to color Doppler ultrasound exposure and few gave no significant results in their study.

As our study was conducted in a limited time with limited resources and sample size hence only the preliminary data regarding the effect of color Doppler on mice offspring can be taken from this study. Final verdict cannot be formulated from this study.

CONCLUSION

- Total of 20 pregnant mice were taken for study and divided into 2 groups of 10 each. Group A was given color Doppler exposure for 5 minutes and Group B for 10 minutes.
- Offspring of both the groups were studied for growth parameters and CNS activities.
- Parameters were measured on 59 offspring of Group A and 56 offspring of Group B.
- Frequency of 3-5 MHz was delivered by convex ultrasound probe on the color Doppler ultrasound machine.
- Color Doppler exposure was given to mice from day 1st to 8th on alternate days.
- It was observed that weight was significantly affected in the during the initial weeks in offspring of group B as compared to group A , but later on they gained weight and values were comparable in both the groups from 3rd week onwards.
- Head circumference and total body length were not affected in the color Doppler exposed mice offspring of different duration.
- Motor co-ordination which is measured of locomotor activity was impaired in mice exposed for longer duration of color Doppler.
- Learning and goal directed movements assessed by conditioned avoidance response and mouse reaching and grasping reflex were seen to be affected in the offspring of mice exposed to long duration of color Doppler.
- Study suggested that long duration of color Doppler exposure during organogenesis period and in early fetal life may affect the post natal behavior.

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