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Anesthesia and Acoustic Stress-Induced Intra-Uterine Growth Retardation in Mice

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Abstract. Stress interferes with reproduction, adversely influencing implantation and fetal growth, and sometimes even leading to abortion. Here, we attempted to evaluate the early gestational effects of uncomfortable sound on pregnant mice and their offspring. Ten-week-old pregnant Jcl:ICR mice were exposed to sound (100 dB, random frequency between 9–34 kHz) for 8 hours on the 3rd, 5th and 7th gestational days (GD). The effects of general anesthesia were also investigated, with or without acoustic stress. All groups were examined on the 18th GD for fetal growth. Fetal weight, number of ossified sacrococcygeal vertebrae and placental weight were all significantly reduced ($P < 0.0001$) when stress was induced on the 7th GD, but not on the 3rd or 5th GD. This intra-uterine growth retardation (IUGR) was significantly inhibited by general anesthesia ($P < 0.0001$), although general anesthesia alone induced significant IUGR ($P < 0.0001$) when compared with control mice. This suggests that acoustic exposure indirectly exerts an effect on fetal growth, possibly via a psycho-maternal pathway. We also found that analysis of the number of ossified sacrococcygeal vertebrae is the most sensitive tool for the study of IUGR.

Key words: Acoustic stress, Intra-uterine growth retardation, Mice, Ultrasound, Anesthesia, Sacrococcygeal vertebrae

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Stress has been reported to adversely influence reproductive function and may result in increased intrauterine mortality, impaired fetal development and behavioral changes [1, 2]. Among these problems, fetal intrauterine growth retardation (IUGR) is a frequent and serious complication in human pregnancy. IUGR-related risk factors include smoking, alcohol, low socioeconomic status, and heavy workload [3–7]. However, psychological factors such as anxiety, fear, and emotional stress can also lead to IUGR [8, 9]. In experimental research, pregnant rodents

have been exposed to various psychological stressors in order to determine the causes of IUGR [10, 11]. In the present study, we used acoustic stress (100 dB, random frequency between 9–34 kHz) as a candidate psychological stressor on pregnant mice at an early gestational age because 20 ± 10 kHz is within the specific range that rodents emit during fighting and because the equipment was originally designed for use as a pesticide (the “ultrasonic rodent exterminator”). We initially investigated whether the stress induces abortion, IUGR or neither. In addition, when an adverse effect was noted, we attempted to elucidate the most effective gestational day (GD) to apply such

stress. We used the number of sacrococcygeal vertebrae on the 18th GD as a sensitive candidate for IUGR qualification. We then investigated the effects of general anesthesia on the observed outcomes, and attempted to elucidate the nature of ultrasound as a stressor on pregnancy.

Materials and Methods

Ten-week-old pregnant Jcl:ICR mice (Clea Japan Inc.) were used in this study and were maintained under environmentally controlled conditions, (ambient temperature: 24 ± 2 C; light/dark cycle: 0800 h–2000 h). Food and water were provided ad libitum. After verifying three consecutive regular estrous cycles, mice were mated on the first proestrous day. On the following morning, the vaginal plug was confirmed and that day was designated as the 1st GD.

Experiment 1

Pregnant mice were exposed to sound (100 dB, random frequency between 9–34 kHz) for 8 hours from 0900 h to 1700 h on the 3rd (N=7), 5th (N=8) and 7th (N=6) GD. Mice were sacrificed on the 18th GD by cervical dislocation and fetal growth maturity was estimated by live fetal weight, placental weight and the number of sacrococcygeal bones [12]. Staining procedures for the fetal skeleton are described below. Nine Jcl:ICR mice without acoustic stress were used as a control group and also analyzed on the 18th GD.

Experiment 2

Four Jcl:ICR mice were exposed to acoustic stress for 8 hours (0900 h to 1700 h) on the 7th GD. During acoustic exposure, the group was anesthetized with Isoflurane (Rhodia Organique Fine Ltd., England) in nitrous oxide (N₂O 60~70%) and oxygen. Inhalation of Isoflurane (2–1.5% inspired) was maintained using an individual mask. Body temperature was monitored by rectal thermometer and was maintained at about 25 ± 0.5 C using a heat lamp. A second group of 5 pregnant mice was also exposed to isoflurane for 8 h on the 7th GD. Pregnant mice of both groups were sacrificed and analyzed in the same manner as described for experiment 1.

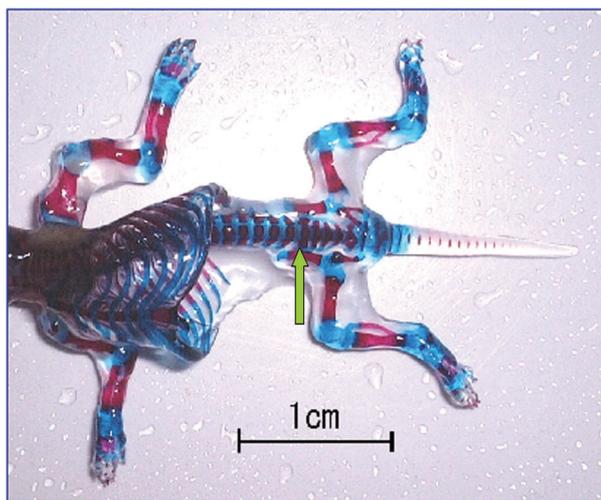


Fig. 1. Skeletal system of the mouse fetus on the 18th gestational day. Specimens were placed in acetone to remove fat and then stained in a solution of alcian blue, alizarin red, acetic acid and ethanol. Specimens were kept in 1% aqueous solution of KOH and then stored in glycerin. Red color indicates ossified bone and blue color indicates cartilage. For precise methodology, refer to Materials and Methods.

Staining of fetal skeleton

After murine fetuses were fixed in 95% ethanol for at least 4 d, the skin, viscera and adipose tissue were removed. Specimens were then placed in acetone for at least 2 d to remove fat and were then stained for 2–3 d in a solution of 0.3% filtered alcian blue 8GS (C.I 74240, Chroma) in 70% ethanol, 0.1% filtered alizarin red S (C.I 58005, Merck) in 95% ethanol, acetic acid and 70% ethanol (1:1:1:17 v/v). Specimens were rinsed with water and kept in 1% aqueous solution of KOH for 12–48 h. Skeletons were clearly visible through the surrounding tissue and specimens were placed in an aqueous solution of 20% glycerin containing 1% KOH for 1–5 d. The resulting stained specimens were transferred from 50% to 80% glycerin and stored in 100% glycerin. In stained specimens (Fig. 1), red is indicative of ossified bone and blue is indicative of cartilage. The green arrow shows from where counting of sacrococcygeal vertebrae was initiated.

Statistical analysis

One-way analysis of variance was used with Scheffe test as a post-hoc comparison, when necessary. Unpaired t-test or Mann-Whitney U-test was used for comparison between groups. A p

value of less than 0.05 was considered statistically significant.

Results

Experiment 1

Fetal body weight in the group stressed on the 7th GD was significantly lower ($p < 0.0001$) when compared with the other groups (Fig. 2). There were also significant differences between the control group and those stressed on the 3rd or 5th GD ($p < 0.01$), but there were no significant differences between the groups stressed on the 3rd or 5th GD. Placental weight was significantly reduced in the groups stressed on the 5th ($p < 0.001$) or 7th ($p < 0.0001$) GD when compared with the control group (Table 1), but there were no significant differences between the group stressed on the 3rd GD and the control group. The number of ossified sacrococcygeal bones were also lowest (Fig. 3) in the group stressed on the 7th GD ($p < 0.0001$, *vs.* 3rd GD, 5th GD and control), but there were no significant differences between the control and the groups stressed on the 3rd or 5th GD.

Experiment 2

There were significant differences in the live fetal body weight ($p < 0.0001$), placental weight ($p < 0.01$; Table 2) and the number of ossified sacrococcygeal bones ($p < 0.0001$) between the groups exposed to anesthesia on 7th GD and those that were only acoustically stressed (Figs. 4 and 5). While there were no significant differences in fetal growth between the anesthetized groups, either with or without acoustic stress, these groups exhibited

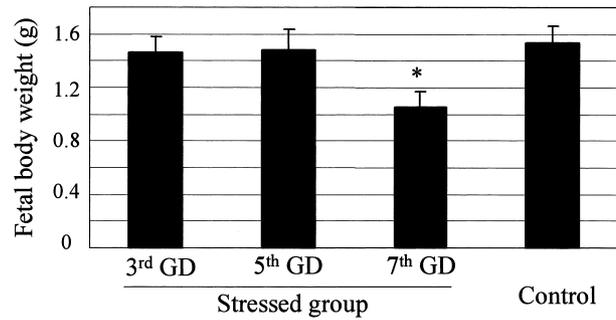


Fig. 2. Fetal body weight on the 18th gestational day (GD) when stressed on an earlier GD. Fetal body weight was significantly reduced when stressed on the 7th GD in comparison with other groups (3rd, 5th GD and control). * indicates $P < 0.0001$ *vs.* other groups. Values are expressed as mean \pm SD.

significant differences in the fetal body weight ($p < 0.0001$) and the number of ossified sacrococcygeal bones ($p < 0.0001$) when compared with the control group. In addition, anesthesia itself resulted in IUGR of fetal body weight ($p < 0.05$ *vs.* control) and the number of ossified sacrococcygeal bones ($p < 0.0001$ *vs.* control).

Discussion

A number of studies have investigated the risk factors of IUGR [3–7], and various types of stress lead to different pregnancy outcomes. Psychological stress is one risk factor that results in adverse pregnancy outcome. In an effort to elucidate the gestational stage that is most sensitive to acoustic stress, we selected the 3rd, 5th and 7th GD.

Table 1. Effect of acoustic stress on fetal growth at different gestational days

	Control (N ^a =9)	Acoustic stress		
		3 rd GD (N=7)	5 th GD (N=8)	7 th GD (N=6)
Number of live fetuses	16.1 \pm 1.6	16.7 \pm 1.7	17.0 \pm 2.4	16.5 \pm 1.4
Placental weight (g)	0.115 \pm 0.067 (n ^b =143)	0.105 \pm 0.019 ^e (n=118)	0.096 \pm 0.019 ^d (n=134)	0.088 \pm 0.014 ^{ce} (n=96)
Uterine weight (g)	1.669 \pm 0.389	1.466 \pm 0.146	1.543 \pm 0.249	1.224 \pm 0.218
Aborted fetuses	0.026 \pm 0.031	0.071 \pm 0.063	0.026 \pm 0.039	0.036 \pm 0.056
Sex ratio (M/M+F) ^f	0.54 \pm 0.09	0.49 \pm 0.08	0.50 \pm 0.07	0.50 \pm 0.11

Parameters indicating fetal well-being were analyzed in four groups on the 18th GD. Three groups were exposed to acoustic stress for eight hours on the 3rd, 5th or 7th GD. All data are expressed as mean \pm SD. Precise procedures are described in the text. a: N= number of mothers, b: n= number of fetuses. c: $P < 0.0001$ *vs.* control. d: $p < 0.01$ *vs.* control, e: $p < 0.05$. f: M=number of male fetuses/M+F= number of live fetuses.

Table 2. Effect of anesthesia during acoustic stress on fetal growth

	Control (N ^a =4)	Acoustic stress		7 th GD Anes. (N=5)
		7th GD (N=4)	7th GD Anes. (N=4)	
Number of live fetuses	168 ± 2.1	17.0 ± 3.2	15.0 ± 2.6	14.4 ± 4.3
Placental weight (g)	0.124 ± 0.095 (n ^b =66)	0.088 ± 0.022 ^c (n=65)	0.098 ± 0.028 (n=57)	0.101 ± 0.038 (n=72)
Uterine weight (g)	1.739 ± 0.259	1.124 ± 0.145 ^{cd}	1.586 ± 0.156 ^d	1.363 ± 0.218

Parameters indicating fetal well being were analyzed in four groups on the 18th GD. Anesthesia and/or acoustic stress were applied to the indicated groups for eight hours. Precise procedures are described in the text. All data are expressed as mean ± SD. a: N= number of mothers, b: n= number of fetuses. c: p<0.01 *vs.* control. d: p<0.05.

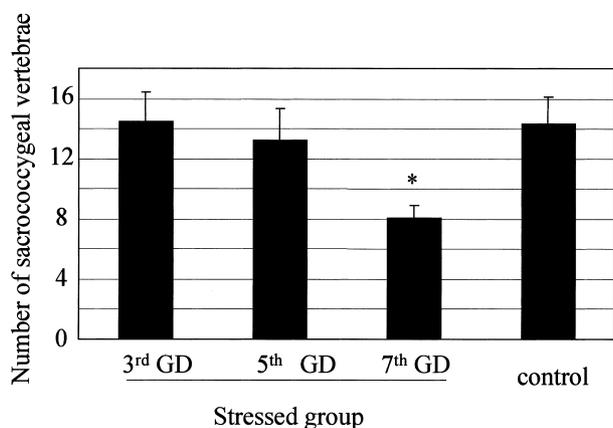


Fig. 3. Number of sacrococcygeal vertebrae on the 18th gestational day (GD) in the groups stressed on an earlier GD. Number of sacrococcygeal vertebrae was significantly lower when stressed on 7th GD in comparison with other groups (3rd, 5th GD and control). * indicates p<0.0001 *vs.* other groups. Values are expressed as mean ± SD.

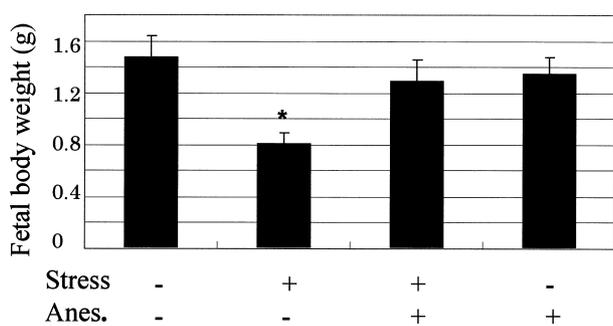


Fig. 4. Fetal body weight on the 18th gestational day (GD) when stressed under anesthesia on the 7th GD. Fetal body weight was significantly reduced in the group stressed on the 7th GD when compared with the two groups under anesthesia and the control group. * indicates p<0.0001 *vs.* other groups. Values are expressed as mean ± SD. "Anes" and "Stress" indicate anesthesia with Isoflurane and acoustic stress, respectively.

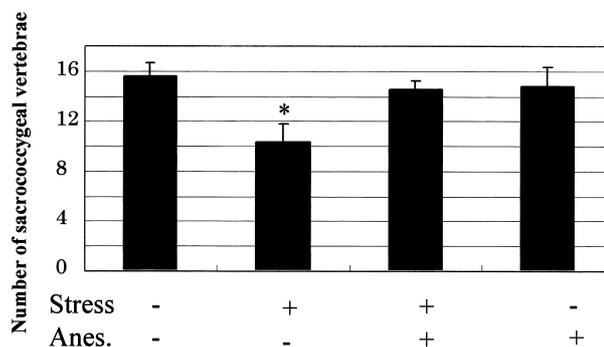


Fig. 5. Number of sacrococcygeal vertebrae on the 18th gestational day (GD) in the groups stressed under anesthesia on 7th GD. Number of sacrococcygeal vertebrae in the group stressed on the 7th GD was significantly lower than the two groups under anesthesia and the control group. * indicates p<0.0001 *vs.* other groups. Values are expressed as mean ± SD. "Anes" and "Stress" indicate anesthesia with Isoflurane and acoustic stress, respectively.

On the 3rd GD in murine embryological development, the fertilized ovum has not yet reached the blastocyst stage and this period is primarily for preparation of the uterus for implantation. On the 5th GD, the embryo is implanted into the uterus, while the 7th GD is after implantation but before full placental formation. The selected days represent early gestational or peri-implantation stages and seem appropriate for evaluating the effects of the stress on the fetus. Our study demonstrated that acoustic stress on the 7th GD causes IUGR in Jcl-ICR mice. On the 7th GD, the embryo is rapidly differentiating at the blastocyst stage and the ectoplacental cone is invaded by blood following the disintegration of uterine epithelium. The original lumen of the uterine crypt has disappeared, and thus the developing placenta achieves solid contact with its surrounding environment and with the mesometrial blood

vessels. In this way, the 7th GD is when important initiation events for establishing the interface between embryo and mother occur. Some studies have reported an association between stress and elevated or impaired uterine artery resistance [8, 13], in which blood flow to the embryo appeared to have been hampered. This may delay cell differentiation in the embryo or restrict placental growth and ultimately result in IUGR. In the present study, we were not able to determine the causes of impaired uterine artery blood flow, but noradrenaline is one of the possible mediators because stress and anxiety are associated with raised plasma noradrenaline levels [14], and infusion of noradrenaline is known to decrease uterine blood flow in pregnant ewes [15]. Acoustic stress may therefore interfere with and diminish trophoblastic invasion.

In the second experiment, we anesthetized pregnant mice during acoustic stress on the 7th GD. As we expected, anesthesia negated the stress by inducing relaxation, and the adverse effects on the fetus were blocked (Fig. 5). Anesthesia may assist in protecting the mother from emotional stress, which in turn improves the conditions for fetal growth. Because there were no significant differences between the anesthetized groups, either with or without acoustic exposure, it appears that anesthesia may completely negate the adverse effects of acoustic stress. These findings confirm that acoustic stress has no direct mechanical effect, but rather has an indirect effect on the fetus via the maternal psycho-endocrine pathway. However, anesthesia itself resulted in IUGR when compared with the control group (Fig. 5), particularly in the sacrococcygeal vertebrae count. In this study, bone development analysis based on sacrococcygeal vertebrae number appeared to be reliable for precise qualification of fetal growth.

We intend to develop a mouse model of human implantation impediment by psychological stress, and thus we selected the 3rd, 5th and 7th GD to apply such stress. We found that auditory stress had the largest effect on the 7th GD in this study, and this GD in the mouse corresponds to the 4th to 5th gestational week in human pregnancy, a putative "critical period" for fetal development, during which time gestation is most sensitive to exogenous agents that may result in prenatal defects. Therefore, it appears logical that mice at the 7th GD were most sensitive to stress in our study, although we did not investigate later gestational days. We also demonstrated a clear reduction in fetal body weight in stressed mothers, however, fetal body weight is a less sensitive indicator than the sacrococcygeal vertebrae number. These results clearly indicate that auditory-stressed pregnant mice accurately mimic an emotionally-stressed human mother, and that adverse fetal effects can be physically examined by analysis of sacrococcygeal vertebrae. Further research is required in order to elucidate the underlying mechanisms of acoustic-induced IUGR through maternal and emotional pathways.

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