Effect of Predator Stress on the Reproductive Performance of Female Mice after Nonsurgical Embryo Transfer

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Predator stress can exert detrimental effects on female mammals, leading to disrupted reproduction. Although many studies have addressed the effects of predator stress on reproductive output in rodents, few studies have focused on the effect of visual or auditory stress on pregnant females. In this study, we investigated the possible effect of predator stress, either visual only or combined visual and auditory (visual+auditory), on the reproductive performance of female mice after nonsurgical embryo transfer. Reproductive performance was assessed as pregnancy rate, implantation rate, gestation length, live pup rate, and neonatal birth weight. Moreover, serum cortisol and progesterone levels in dams were measured by using electrochemiluminescence immunoassay. Exposure to predator (cat) stress did not lead to a significant change in pregnancy rates in the tested mice. However, the stressed mice showed significantly decreased implantation rates compared with the control group. Similarly, the live pup rate and neonatal birth weight were significantly lower in the group exposed to predator stress than in the control group. Furthermore, mice exposed to visual+auditory stress showed a significant reduction in gestation length compared with the control mice. Our data showed that predator visual+auditory stress as combined stimuli significantly increased serum cortisol level. In contrast, progesterone levels did not significantly vary among the experimental groups. Taken together, our findings imply that predator stress adversely affects the reproductive efficiency of pregnant mice by decreasing the implantation rate, live birth rate, and neonatal birth weight and by prolonging gestation length.

Abbreviations: dpc, days postcoitum; NSET, nonsurgical embryo transfer

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Along with a variety of other stressors, noise induces psychologic and physiologic changes in mothers that may affect pregnancy.³⁵ Reproductive traits in rodents are affected by several environmental factors,⁴² and diverse stressors, including loud noise, predator exposure, heat, and physical restraint, can cause early pregnancy to fail.³⁹ Biologic evidence has shown reproductive failure induced by immune–endocrine disequilibrium in response to stress.²⁸ In addition, predator stress during the oocyte prematuration stage can affect oocyte maturation in vivo and impair oocyte developmental potential.²³ Furthermore, many reproductive events in early pregnancy, including embryonic development and implantation, can be disrupted by restraint stress.⁴³ Mice exposed to stress showed incidences of failure to maintain pseudopregnancy, degenerated embryos in oviducts, implantation failure, and postimplantation losses.⁴³ In addition, pregnant female rodents exposed to predator odors during the periimplantation period may give birth to smaller

litters.^{3,34} Studies have indicated that exposure of Norwegian rats to domestic cat odor inhibited their reproductive performance and resulted in reduced litter size.⁴¹ Similarly, there were increased rates of abortion and cannibalism in mice exposed to construction noise.³³ Moreover, maternal stress can exert inhibitory effects on reproduction and induce fetal anomalies,²⁶ low birth weight, and spontaneous preterm birth.²³ Exposure of day 7 pregnant mice to continuous noise for 6 h significantly increased the number of malformed fetuses.²⁷ In addition, nursing mothers frequently discontinued nursing during exposure to noise.¹⁰ Several investigators have indicated that various types of maternal stress may contribute to the production of abnormal growth patterns in offspring.¹⁶

An imperative question existing across all cultures and since ancient times is whether maternal stress is linked to human reproductive failure.²⁸ In women, psychologic stress can exert unfavorable effects on reproduction.²³ The mother's perceived stress is frequently alleged to be a cause of infertility, miscarriages, late pregnancy complications or impaired fetal development.²⁸ Moreover, the mean birth weight in pregnant women exposed to high noise was lower than that of women exposed to low noise.²² Laboratory mice are the most widely used animal model for studying human diseases in medical research, in part because of their short reproductive cycle, short lifespan, small size, and low cost of maintenance. In addition, predator-induced stress in mice is a potentially useful model of human stress and its physical consequences.³ Furthermore, over the past decade, surgical embryo transfer has been considered

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the standard method for producing transgenic mice and chimeric blastocysts and transferring cryopreserved embryos.³⁶ However, surgery puts animals at risk of infection and increases recipients' stress due to the painful surgical procedure.³⁸ Recently, a novel device that can be used for transcervical transfer of embryos into recipient mice (that is, nonsurgical embryo transfer [NSET]) is an alternative that avoids disadvantages of surgical embryo transfer (including postoperative pain and the need for postoperative analgesia).¹⁷

The disruptive effects of exposing pregnant rodents to acute stress for a short duration on reproductive function are well-documented, but surprisingly few studies exist that demonstrate the effect of predator stress throughout the entire length of gestation on reproduction performance. We hypothesized that exposure of pregnant mice to visual and auditory predator stress throughout gestation after NSET would reduce reproductive performance and disturb maternal hormone balance. To address this hypothesis, we modified a previously described^{18,23} model of cat and mouse predatory stress to induce either auditory or combined visual and auditory (that is, visual+auditory) stress. Therefore, the aim of the present study was to identify the effect of maternal stress on embryo development *in vivo* by examining levels of pregnancy-related hormones, specifically progesterone and cortisol, and outcomes from embryo transfer. The number of surviving offspring was an integrated index of reproductive success.

Materials and Methods

Ethics approval and consent to participate. All of the methods and experimental procedures were conducted according to the approved protocol (approval ID: GAR-110502-X0017) and the guidelines and regulations of the IACUC of the Division of Applied Life Sciences, Department of Animal Science at Gyeongsang National University, South Korea. According to the guidelines of the committee, the staff must be trained before handling or using animals.

Experimental design. Experimental mice were randomly assigned to 3 groups ($n = 16$ per group): auditory, visual+auditory, and control. In each group, 8 mice were euthanized at 6.5 d postcoitum (dpc) to evaluate implantation sites, by visualizing the morphologic features of the uterus, and serum cortisol and progesterone levels; the remaining mice were used to assess reproductive parameters. To avoid effects on the pregnancy rate due to cases of pseudopregnancy, which may result from failure of fertilization, and from adverse events associated with surgical embryo transfer, we decided to use the NSET method. After NSET, mice were placed in a cage with 2 filter tops to avoid olfactory stimuli¹⁸ and gently transferred to the cat rearing room. For the auditory stress group, all mouse cages were kept approximately 1 m from the cat cage to avoid variation in exposure to auditory stimuli¹⁹ and were separated from the cat cage by using opaque fabric squares to block visual stimuli; all cages for the visual+auditory stress group was placed inside the cat cage (Figures 1 and 2). Recipient female mice were weighed once daily after embryo transfer. Mouse pups were weighed on the day of birth.

Chemicals. Unless otherwise noted, all chemicals and reagents were obtained from Sigma-Aldrich (St Louis, MO).

Animals and housing conditions. All mice were SPF (Koatech, Gyeonggi-do, Republic of Korea; list of excluded agents at <http://www.koatech.co.kr/sub03/05.php>), housed at a constant temperature (22 to 25 °C) and humidity (45% to 55%), and maintained on a 14:10-h light:dark cycle (lights on, 0500). Mice were housed in transparent, double-filter-covered cages (floor,

area, 503 cm²; Threeshine, Yuseong-gu Daejeon, Republic of Korea) at 3 to 5 mice per cage, with free access to food (Koatech, Gyeonggi-do, Republic of Korea) and water. Donor mice were female C57BL/6NHsd (age, 4 to 6 wk) mice, and Hsd:ICR(CD1) (age, 8 to 10 wk) female mice were the recipient mice.

Vasectomy and preparation of pseudopregnant female recipients. Vasectomy was performed approximately at 8 to 10 wk of age through an aseptic operation as previously described.⁶ Male mice ($n = 6$) were anesthetized by using intraperitoneal tribromoethanol (250 mg/kg; Sigma) and were used 1 wk after recovering. To start the pseudopregnancy as previously described, 2 female ICR mice were placed with one vasectomized ICR mouse in a single cage for 3 consecutive days. Every morning, mice were checked for a copulatory plug. Only females with easily visible plugs (0.5 dpc) were chosen as embryo recipients. To calculate the implantation rate, female mice were euthanized by CO₂ inhalation on pregnancy day 6.5 and implantation sites were visualized through the uterus.

Cat housing. The cat used as the predator was housed in a sound-attenuating room during the predator exposure experiment and maintained under the same light cycle, temperature, and humidity as the mouse room. The cat was caged in a stainless wire cage measuring 50 × 150 × 50 cm with water available without restriction and feed daily at 1000 and 1600.

Embryo collection. Donor mice ($n = 72$) were superovulated by intraperitoneal injection of 10 IU equine chorionic gonadotropin (Daesung Microbiologic Labs, Gyeonggi-do, Republic of Korea), followed 44 to 48 h later by intraperitoneal injection of 10 IU human chorionic gonadotropin (Daesung Microbiologic Labs). After injection of human chorionic gonadotropin, female mice were caged individually with a male overnight and checked for a vaginal plug on the following morning. When the vaginal plug was present, females were assumed to be at day 0.5 of pregnancy. Donor mice were euthanized by CO₂ inhalation. At 3.5 dpc, blastocysts were flushed from the uterine horns by using M2 medium (Sigma-Aldrich, St Louis, MO) and washed 3 times in M16 medium (Sigma-Aldrich).

NSET. Embryo transfer was carried out as previously described with slight modification.⁷ In brief, 2.5 dpc recipient female mice were anesthetized by using intraperitoneal tribromoethanol (250 mg/kg). The cervical opening was visualized by inserting a speculum into the vagina, and then approximately 12 blastocysts were loaded into the NSET device (Partech, Lexington, KY) and deposited in one uterine horn.

Cat noise level. Cat noise was measured every 0.5 h during the light phase by using a sound-level meter (Extech Instruments, Nashua, NH). The meter was placed within the mice cage to help to gauge sound heard by stressed mice. Depending on their subgroup, mice were exposed to cat noise daily directly after NSET throughout the gestation length. The cat noise level ranged from 69 to 86 dB, with the peak noise before feeding from 0800 to 1000 and afternoon from 1500 to 1600. For visual+auditory exposure, the mouse cage was kept within the cat cage throughout gestation. Mice were housed individually and observed in the morning and afternoon for signs of parturition during the last 24 h of gestation.

Hormone assay. Mice were euthanized by CO₂ inhalation, which was unlikely to confound cortisol measurements, as recommended by the American College of Laboratory Animal Medicine.⁵ Blood samples of approximately 1 mL were collected from every mouse through cardiocentesis; the collection tubes were stored overnight at -4 °C and then centrifuged at 1000 × g, 4 °C for 15 min. The collected serum samples were stored at -80 °C until the hormone assay. Serum cortisol and progesterone levels

Figure 1. Experimental schedule. To evaluate effects on the live pup rate, mice were exposed to cat stress after NSET either until 6.5 dpc or throughout gestation, after which the mice were euthanized and the implantation sites recorded.

Figure 2. Schematic diagram showing housing design for different experimental groups. Mice were placed either (A) inside a cat cage (visual+auditory group) or (B) beside the cat cage after the side was covered with an opaque fabric squares (auditory group). All of the mouse cages were transparent but had filter tops to block olfactory stimuli. Cat cages were made of steel-wire mesh. Food and water are not shown in the photo.

were measured by electrochemiluminescence immunoassays (Cobas 8000 with Cobas e801 module and Elecsys Progesterone III and Cortisol II kits, Roche Diagnostics International, Penzberg, Germany). Assays were performed according to the manufacturer's recommendations.

Statistical analysis. Statistical analyses were performed by using SPSS software version 18.0 (IBM, Armonk, NY). Data were tested for normal distribution and homogeneity of variance and underwent arcsine transformation when these criteria were not met. One-way ANOVA was used to compare reproductive parameters (pregnancy rate, implantation rate, live pup rate, and gestation length), neonatal birth weight, and hormonal concentrations. The Duncan multiple range test was used to compare treatment groups. Data for recipient body weight gain were compared by using 2-way ANOVA. Data are presented as mean $\pm$ SE. The level of statistical significance was set at a P value of less than 0.05.

Results

Assessment of reproductive performance after NSET. To study the effect of predator stress during pregnancy on reproductive performance, mice were either exposed to cat stress after NSET until 6.5 dpc, euthanized, and the implantation sites recorded, or they were exposed throughout gestation to evaluate effects on the live pup rate (Figure 1). For each experimental group, the total number of offspring per female was counted, and the

gestational period was measured. Exposure to stress, either auditory or visual+auditory, did not lead to a significant change in the pregnancy rates of the tested mice (Table 1). However, the results showed a significant ($P < 0.05$) decrease in implantation rate in both the auditory and visual+auditory groups ($41.7\% \pm 5.9\%$ and $22.9 \pm 7.1\%$, respectively) compared with the control group ($87.5 \pm 12.5\%$; Figure 3 A). Moreover, exposure of mice to visual+auditory stimuli resulted in fetal resorption after implantation (Figure 3 D). Similarly, a significant ($P < 0.05$) effect on embryo death was found in terms of reduction in the live pup rate in the group exposed to cat noise only ($25.5\% \pm 4.1\%$) and the visual+auditory group ($22.5\% \pm 6.6\%$) compared with the control group ($50.1 \pm 8.7\%$, Table 1). Furthermore, exposure of mice to visual+auditory stimuli significantly ($P < 0.01$) extended the duration of gestation compared with the control group (17.7 ± 0.2 d compared with 16.6 ± 0.2 d, respectively), which was not different from the auditory stress group (16.8 ± 0.4 d, Table 1).

Recipient body weight gain and neonatal birth weight. We also investigated the effect of maternal stress on daily body weight gain and neonatal birth weight. The results showed that maternal stress, as auditory only or visual+auditory stimuli, significantly ($P < 0.05$) reduced gestational body weight gain in pregnant dams (Figure 4 A). In addition, there was a significant ($P < 0.05$) decrease in neonatal birth weight in both the auditory and visual+auditory stress groups (1.5 ± 0.03 g and 1.5 ± 0.04 g, respectively) compared with the control, nonstressed group (1.7 ± 0.03 g, Figure 4 B and C).

Table 1. Effect of predator stress on the reproductive performance of mice

	No. of embryos transferred ^a	No. (%) of mice pregnant ^b	No. (%) of live pups ^c	Gestation length ^d
Control group	91	7 (87.5 ± 12.50)	44 (50.1 ± 8.7) ^a	16.6 ± 0.2 ^a
Auditory group	98	6 (75.0 ± 12.50)	22 (25.5 ± 4.1) ^b	16.8 ± 0.4 ^{a,b}
Visual+auditory group	97	6 (75.0 ± 16.37)	14 (22.5 ± 6.6) ^b	17.7 ± 0.2 ^b

Eight replicates were performed, with 8 mice per replicate. Pregnancy rate was calculated as the no. of mice pregnant / no. of total recipients × 100%. The live pup rate was calculated as the no. of live pups/no. of embryos transferred among all female mice × 100%.

Within each column, values with different superscripts differ significantly ($P < 0.05$).

Figure 3. Effect of predator stress on implantation. (A) Implantation rates in different experimental groups. Values with different superscripts are significantly different ($P < 0.05$). Data are presented as mean ± SE (SE). Representative image of implantation sites in (B) control, (C) auditory, and (D) visual+auditory groups.

Figure 4. Effect of predator stress on recipient body weight gain and neonatal birth weight. (A) Recipient body weight gain and (B) neonatal body weight in the various experimental groups. Values indicated with asterisks or different superscripts differ significantly ($P < 0.05$). Data are presented as mean ± SE. (C) Representative image of abnormal birth weight (bottom arrow) in the visual+auditory stress group.

Hormonal analysis. No significant stress-related changes were noted among tested groups in the concentration of serum progesterone (Figure 5 A). To further confirm the possible mechanism of predatory stress on early pregnancy losses, the cortisol concentration in serum was measured in mice after exposure to the cat stress (6.5 dpc). The concentration of serum cortisol was significantly ($P < 0.05$) higher in the visual+auditory stress group (13.1 ± 2.3 ng/mL) compared with the control group (6.2 ± 1.6 ng/mL), which did not differ from the auditory group (12.6 ± 2.9 ng/mL; Figure 5 B).

Discussion

Even though previous studies with both animals and humans suggest adverse effects of stress on reproduction,^{23,47} reports concerning the direct effect of predator stress on reproductive performance are few and conflicting. It has been reported that improvement of the implantation rate is an important goal for successful assisted reproductive technology.²⁵ Here, we studied the effect of predator stress, either auditory or visual+auditory stress stimuli, on implanted embryo development by examin-

ing the pregnancy-related hormonal levels and outcomes from embryo transfer. Our data showed that exposure of pregnant mice to predator stress, as either auditory or visual+auditory stimuli, significantly decreased the implantation and live pup rates, suggesting that embryo losses occurred during implantation or early postimplantation stages. The results are similar to previous studies that reported that exposure of pregnant mice to noise significantly decreased reproductive efficiency by increasing the number of stillborn pups and reducing the number of delivered live pups.³⁴ Furthermore, mice cohoused with rats produced very few litters.¹³ In addition, pregnant mice exposed to extremely high-intensity noise showed a significant decrease in the average number of implantation sites.²⁹ Exposure of sows to heat stress may interfere with early development and implantation of the embryo.³¹ The average number of young per recipient were significantly lower in stressed donor mice exposed to predator stress for 24 h after fertilization.²³ Moreover, predation risk reduced the number of offspring produced per year by 40% in songbirds.⁴⁶ In contrast, a previous study⁴⁴ reported that prolonged exposure of rats to cat odors reduces fear reactions to these odors during subsequent exposure, suggesting

Figure 5. Effect of predator stress on hormonal concentrations. Serum (A) progesterone and (B) cortisol concentrations in the various experimental groups. Values with different superscripts differ significantly ($P < 0.05$). Data are presented as mean $\pm$ SE.

that rodents can habituate to cat olfactory stimuli. However, other studies reported that rodents did not show adaptation to cat visual cues.⁸ These observations led to the predator-induced reproductive suppression hypothesis, which postulates that exposure of pregnant mice to cat noise could interfere with the maintenance of pregnancy to term, cause reproductive abnormalities and alterations in fetal growth, and decrease the implantation and live pup rates. Many laboratory rodents emit ultrasonic vocalizations which are likely only to facilitate or inhibit social interactions in adult mice.³² Several studies have reported on procedures for transporting animals between or within facilities. Mice need at least 48 h after transportation by truck or airplane to return to baseline.⁴⁰ However, simply moving mice by carrying the cage from one room to another on the same floor, rather than different floors, can reduce the stress they are exposed to.⁴⁰ Therefore, in our study mice were gently transferred to the cat rearing room located on the same floor.

Furthermore, we showed that exposure of mice to visual+auditory stimuli significantly extended the duration of gestation, providing initial support for the hypothesis that exposure to psychosocial stress during pregnancy may result in prolonged gestation.²⁴ In contrast, several reports suggest that stress reduced the gestational length in rats exposed to restraint and forced swimming during days 12 through 18 of gestation.⁴⁵ It is possible that stress may cause either a decrease or an increase in gestational length, depending on the type, time, and duration of the stressor. We also investigated the effect of predator stress on neonatal birth weight. The results showed a significant decrease in birth weight of offspring delivered from stressed dams exposed to either auditory or visual+auditory stimuli; our findings agree with a previous study that reported that birth weight was decreased in the offspring of stressed mice⁴⁷ and snowshoe hares.³⁷ Our results indicate that exposure to stress throughout gestation can have a negative effect on embryonic development, with consequences reaching into postnatal life. These results support the earlier observation that preimplantation maternal restraint stress-induced pregnant mice to deliver offspring with lower body weights.⁹

Corticosterone, the main glucocorticoid involved in the regulation of immune reactions and stress responses in many species, is often used as an index of stress activation. Several studies reported elevated cortisol levels after stress in mice,²¹ cows,¹ sheep¹¹ and elephants.¹⁵ Moreover, stressed mice showed increased basal serum corticosterone concentrations,¹² and exposure of mice to natural predators induced anxiety-like states and elevated stress hormone levels.²³ Pregnant women with

increased urinary cortisol levels during the first 3 wk after conception were more likely to experience spontaneous abortion.³⁰ We demonstrated that exposure of mice to visual+auditory stress significantly elevated the concentration of serum cortisol.

Progesterone is an endogenous steroid that, through its endocrine and immunologic effects, is involved in the establishment and maintenance of pregnancy.^{4,20} However, the effect of stress on the serum progesterone concentration is unclear and subject to debate. It is possible that stress can cause either a decrease or an increase in progesterone, depending on the time of measurement.¹⁴ Previous studies suggest that the stress-associated increase of premature pregnancy termination in mice results from a reduction in progesterone levels.⁴ Moreover, progesterone concentrations can decrease during chronic stress exposure.² Our results indicated that exposure of mice to visual+auditory stress slightly but nonsignificantly reduced the serum progesterone level, perhaps indicating a role of stress-induced decreases in progesterone in implantation failure. Further studies need to measure relevant stress hormones to better understand the mechanisms by which noise compromises fetal implantation. In our current study, exposure of pregnant mice to a predator cues, either visual or visual+auditory stimuli, throughout gestation after NSET interfered with the maintenance of pregnancy to term, expressed as a reduced pregnancy rate, a significant decrease in the average number of implantation sites, and a lower live pup rate.

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