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—Original Article—

Stress exposure during the preimplantation period affects blastocyst lineages and offspring development

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Abstract. We found retardation of preimplantation embryo growth after exposure to maternal restraint stress during the preimplantation period in our previous study. In the present study, we evaluated the impact of preimplantation maternal restraint stress on the distribution of inner cell mass (ICM) and trophectoderm (TE) cells in mouse blastocysts, and its possible effect on physiological development of offspring. We exposed spontaneously ovulating female mice to restraint stress for 30 min three times a day during the preimplantation period, and this treatment caused a significant increase in blood serum corticosterone concentration. Microscopic evaluation of embryos showed that restraint stress significantly decreased cell counts per blastocyst. Comparing the effect of restraint stress on the two blastocyst cell lineages, we found that the reduction in TE cells was more substantial than the reduction in ICM cells, which resulted in an increased ICM/TE ratio in blastocysts isolated from stressed dams compared with controls. Restraint stress reduced the number of implantation sites *in uteri*, significantly delayed eye opening in delivered mice, and altered their behavior in terms of two parameters (scratching on the base of an open field test apparatus, time spent in central zone) as well. Moreover, prenatally stressed offspring had significantly lower body weights and in 5-week old females delivered from stressed dams, fat deposits were significantly lower. Our results indicate that exposure to stress during very early pregnancy can have a negative impact on embryonic development with consequences reaching into postnatal life.

Key words: Behavior, Eye opening, Preimplantation period, Restraint stress

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Stress mobilizes adaptive behavior and peripheral functions and restricts energy-costly and vegetative functions such as growth, digestion and reproduction. Stressors impacting the maternal organism during gestation may transform optimal conditions of the maternal environment into suboptimal conditions, and these can be a cause of poor or inappropriate embryonic development. Stress can activate two major response systems, the hypothalamo-pituitary-adrenal (HPA) axis and the sympathoadrenal system, resulting in elevated glucocorticoid and catecholamine secretion. Activation of the HPA axis has an inhibitory effect on the hypothalamic–pituitary–ovarian (HPO) axis, resulting in decreased circulating levels of gonadotropins [1–3]. Stress-induced pregnancy failure is therefore likely to be due to compromised hormone secretion and its downstream consequences [4].

There are data indicating that glucocorticoids and catecholamines may directly influence mammalian preimplantation embryos by binding to their respective receptors [5–7]. In addition, several animal studies have shown that glucocorticoid and catecholamine administration *in vitro or in vivo* can negatively affect developmental capacity and quality of preimplantation embryos [6, 8–13]. In humans, it has been demonstrated that pregnancies characterized by increased maternal cortisol during the first 3 weeks after conception are more likely to result in spontaneous abortion [14]. Some studies have shown that maternal stress in mice around the time of implantation can change blastocyst implantation rates [15, 16]; however, there is no information about how maternal stress affects two cell lines, trophectoderm (TE) and inner cell mass (ICM) cells involved in implantation of the blastocyst. It has been demonstrated that the number of ICM cells during implantation is linked to implantation success [17–21], while the TE is responsible for communication with the uterine environment during implantation [22]. The mutual proportion of these two cell lines is maintained within relatively narrow limits, indicating their importance to future development [18].

Only limited data are available about the effect of maternal stress during the preimplantation period on later development of individuals, although early pregnancy appears to be very susceptible to adverse impacts affecting offspring [4]. The major focus has so far been directed towards a mid or late pregnancy window of vulnerability, but there are data indicating that changes in metabolic pathways and development of the neural and cardiovascular systems may be set up even earlier [18, 23–28]. The aim of this study was to find out whether maternal restraint stress, acting during the narrow period of preimplantation development, can alter the cellular organization of the

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blastocyst and have long-term effects on the phenotype of offspring.

Materials and Methods

Animals

Female mice (ICR strain, Velaz, Prague, Czech Republic; 33–38 days old) were maintained under standard conditions (temperature 22 ± 2 C, humidity 65 ± 5%, 12/12-h light-dark cycle with lights on at 0500 h, free access to food and water), and housed at 10 animals per cage. Spontaneously ovulating female mice were mated with males during one or more nights; mating was confirmed by checking for a vaginal plug every morning at 0800 h, and this time was designated 0 h post plug (day 1 of pregnancy). After vaginal plug appearance, spontaneously ovulating mice were divided into two groups: stressed and control.

Restraint stress

Dams in the stressed group were subjected to restraint stress by being placed individually into adjusted and perforated 50 ml plastic tubes (without squeezing or compression). Each animal was isolated in a cell made of cardboard located outside of the animal colony during the stress exposure. Restraint stress was applied three times a day (at 0800 h, 1200 h, 1600 h) for 30 min during the light phase of the day from day 1 to day 4 of pregnancy (D1 to D4). After stress exposure, mice were returned to their home cages and provided free access to food and water. A portion of the dams selected for offspring delivery remained undisturbed until birth in their home cages. The rest of the dams were subjected to blood collection, embryo isolation or implantation rate evaluation at the appropriate time.

Experiment 1 – preimplantation period

Corticosterone measurement: Females exposed to the last 30 min period of restraint stress (on D4) and control females were decapitated for blood collection. Blood samples were centrifuged to obtain serum. Serum was transferred to clean vials and stored at –80 C until the measurement of corticosterone concentrations. Corticosterone was measured using a commercially available EIA kit, according to the manufacturer's instructions (Corticosterone EIA kit, Enzo Life Sciences).

Embryo isolation: Dams from both stress and control groups were killed by cervical dislocation on D4. This day, preceding the start of implantation, was chosen to avoid embryo loss (at D5, expanded hatched blastocysts start to implant in the endometrium, and at this stage, they cannot be flushed from the uterus effectively). Embryos were recovered by flushing the oviduct and uterus 80 h post plug using a flushing–holding medium (FHM) [29] containing 0.1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA). Classification of blastocysts was performed by stereomicroscopy.

Detection of CDX2-positive cells (TE cells) by immunostaining: Isolated blastocysts were fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) in PBS for 30 min at room temperature and stored in 1% paraformaldehyde in PBS at 4 C for up to one week. The fixed blastocysts were washed twice in PBS containing 0.1% BSA and transferred into PBS with 0.5% Triton X-100 (Sigma–Aldrich, Germany) for 1 h. Nonspecific immunoreactions were blocked with 10% normal goat serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. After blocking, the blastocysts were further incubated with the primary antibody (rabbit anti-mouse CDX2 polyclonal antibody, 1:100 dilution; Cell Signaling Technology, Danvers, MA, USA) diluted in blocking solution at 4 C overnight. Blastocysts were washed twice in the blocking solution and then incubated for 1 h with Texas Red-X goat anti-rabbit IgG (1:200 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Afterwards, blastocysts were washed twice in 0.1% BSA–PBS, and cell nuclei were counterstained with Hoechst 33342 in PBS/BSA (20 mg/ml; Sigma-Aldrich, Germany). Blastocysts were then mounted in ProLong Gold antifade reagent (Invitrogen, Life Technologies) on glass slides, sealed with coverslips and observed using an epifluorescent microscope (BX51, Olympus, Tokyo, Japan). The total number of nuclei and the number of cdx-2-positive nuclei were determined using the ImageJ software in each blastocyst (two or more sections) (Supplementary Fig. 1: online only). The specificity of immunostaining was proven by the absence of signals in samples processed without the primary antibody.

Experiment 2 – implantation period

Implantation rate: Uteri were dissected from stressed and control dams on D6 after conception. Implantation sites were morphologically distinguishable at this time, and their numbers were evaluated by visual observation.

Experiment 3 - offspring

Birth weight and sex ratio: On the day of birth, the number of pups, natal body weight and sex of the pups were assessed**,** and the sex ratio was calculated (number of male / number of female pups in each group).

Eye opening in offspring: Delivered pups were checked for eye opening every morning (0800 h) and afternoon (1700 h) before lights were turned off from day 12 to 16 of age.

Body weight and fat deposits on day 30 after birth: On day 30 of age, weaned offspring of stressed and control dams were individually weighed and scanned with EchoMRI (Whole Body Composition Analyzer, Echo Medical System, Houston, TX, USA) for evaluation of the exact amount of body fat deposits (in grams). Percentage of body fat was calculated as body fat (g)/body weight (g) × 100.

Behavior in offspring (open field test): To investigate changes in behavior, 5-week-old offspring of stressed and control dams were assessed using the open field test. In each group, 40 animals (20 males and 20 females) were randomly chosen for evaluation (to eliminate parental impact on dataset, only one male and one female offspring were taken per mother for the test). Open field tests were conducted in the afternoon in a normally lit room, and the mice were habituated to the behavior room for 2 h prior to the test. The open field test apparatus consisted of a wooden base covered with washable waterproof black foil and transparent acrylic walls (60 × 45 × 36 cm). A color CCD camera (Panasonic WV-CP484) was installed in the center above the apparatus. A red dot was painted on the backs of the mice, and the color CCD camera tracked down the dot. Mice were placed in the center of the open field, and the behavior of the mice was recorded for 5 min. After each recording, the mice were returned to their home cages and the open field arena was wiped down with disinfectant solution and paper towels. Data were analyzed with

EthoVision XT 7.0 (Noldus Information Technology BV, Wageningen, The Netherlands), software for automatic behavioral scoring, to assess the total distance traveled by the animal and the time spent in the central or peripheral zone. An observer scored the number of rears, the number of scratching acts on the base of the apparatus, the rest duration, the cleaning frequency and the cleaning duration.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). The Student's *t*-test was used to detect differences in serum corticosterone concentration, cell counts of blastocysts, number of implantation sites, litter size, behavioral parameters of offspring**,** and body weight and fat deposits of offspring between the control and experimental groups. The Mann-Whitney test was used to evaluate the difference in ICM/TE ratio. The χ^2 test with 3 degrees of freedom was used for eye opening analysis. Differences of $P < 0.05$ were considered significant.

All animal experiments were reviewed and approved by the Ethics Committee for Animal Experimentation of the Institute of Animal Physiology, approved by the State Veterinary and Food Administration of the Slovak Republic, and performed in accordance with Slovakian legislation based on EU Directive 2010/63/EU on the protection of animals used for experimental and other scientific purposes.

Results

Experiment 1 – preimplantation period

Corticosterone concentration: On D4 of pregnancy, the blood serum corticosterone concentration was significantly higher in the females exposed to restraint stress when compared with control females (Fig. 1).

Cell numbers in blastocysts: Exposure of mothers to stress significantly decreased cell numbers in blastocysts ($P < 0.001$; Fig. 2), but the extent of the reduction was not the same for TE and ICM cells. The reduction in TE cells (16%) was more substantial than the reduction in ICM cells (9.12%), resulting in increased ICM/TE ratios in blastocysts isolated from stressed dams compared with controls (0.473 ± 0.014, N=170 *vs*. 0.434 ± 0.014, N=144, $P < 0.05$).

Experiment 2 – implantation period

Implantation sites: On gestation day 6, we found a significant reduction in implantation sites *in uteri* obtained from stressed mothers compared with controls (Table 1).

Experiment 3 - offspring

Litter size, birth weight and sex ratio: We found no difference in litter size, birth weight or sex ratio in the delivered pups (Table 1).

Eye opening: Table 2 shows that nearly one-quarter of the control pups opened their eyes on day 13, more than half of them opened their eyes on day 14 and one-fifth of them opened their eyes on day 15. Unlike controls, only one-tenth of the prenatally stressed pups opened their eyes on day 13, nearly half opened their eyes on day 14, and nearly two-fifths of them opened their eyes on day 15. The statistically significant difference in the distribution between the control and stressed groups ($P < 0.001$) indicates a delay in eye opening in offspring delivered by stressed dams.

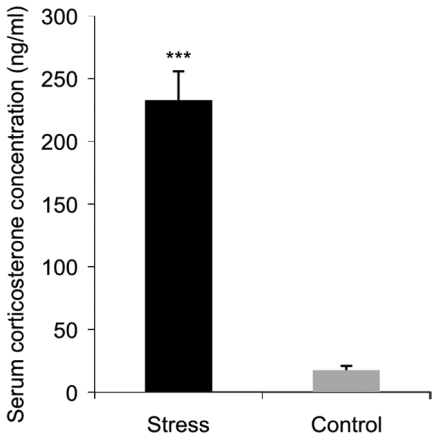

Fig. 1. Blood serum corticosterone concentration after exposure to the last restraint stress. The black column represents stressed dams (N = 10), and the grey column represents control dams (N = 11). Values are arithmetical means ± SEM. Statistical differences between stressed and control females were assessed using the Student's *t*-test. *** $P < 0.001$.

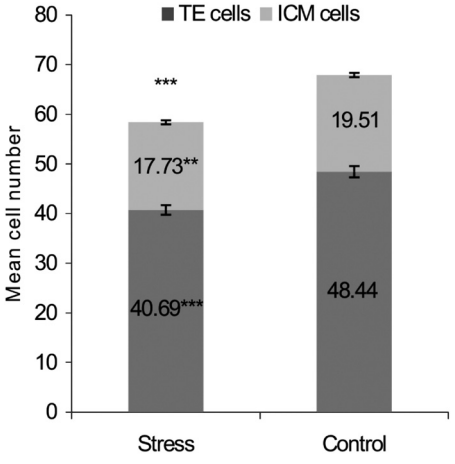

Fig. 2. Cell numbers of ICM and TE lineages in blastocysts isolated from stressed and control dams. Values are arithmetic means ± SEM. Total number of evaluated blastocysts in the experimental groups: stress, 170; Control, 144. Statistical differences between stressed and control females were assessed using the Student's *t*-test. ** $P < 0.01$; *** $P < 0.001$.

Body weight and fat deposits: At day 30 of age, the average body weight of prenatally stressed females and males was significantly lower compared with the control group. We did not find any changes in average body fat deposits in prenatally stressed males. However, the average body fat deposits in prenatally stressed females were significantly lower compared with the control group (Table 3).

Behavioral test: Our results showed significant changes in two behavioral parameters caused by preimplantation restraint stress in delivered offspring (Fig. 3). We observed a higher frequency of scratching on the base of the open field test apparatus (1.82 ± 0.34

Table 1. Number of implantation sites, litter size, birth weight and sex ratio of pups after exposure to maternal restraint stress

	No. of implantation sites	Litter size	Birth weight (g)	Sex ratio (%)
Stress	11.61 ± 0.62* (N = 31 dams)	12.48 ± 0.5 (N = 22 dams)	1.59 ± 0.01 (N = 205 pups)	50.00% male: 50.00% female (N = 205 pups)
Control	13.59 ± 0.40 (N = 34 dams)	12.21 ± 0.44 (N = 27 dams)	1.58 ± 0.01 (N = 238 pups)	49.82% male: 50.18% female (N = 238 pups)

Values in the first three columns are arithmetic means ± SEM. Statistical differences between stress and control groups were assessed using the Student's *t*-test. * $P < 0.05$. Values in the last column are expressed as percentages, and the χ^2 test with 1 degree of freedom was used for analysis of distribution.

Table 2. Eye opening in the progeny after exposure to maternal restraint stress

	No. of offspring	D13 (%)	D14 (%)	D15 (%)	D16 (%)	Mean day of eye opening
Stress	122	10.66	47.54	39.34	2.46	14.34 ± 0.05***
Control	166	23.49	55.42	19.88	1.20	13.99 ± 0.06

Numbers in columns D13 to D16 are expressed as a percentages, and the χ^2 test with 3 degrees of freedom was used for analysis of distribution ($P < 0.001$). Numbers in the last column are arithmetic means ± SEM, and the Mann-Whitney test was used to evaluate differences between them. *** $P < 0.001$.

Table 3. Body weight and fat deposits of progeny (on day 30 after birth) after exposure to maternal restraint stress

	Weight of males (g)	Weight of females (g)	Fat deposits of males (%)	Fat deposits of females (%)
Stress	21.71 ± 0.35* (N = 61)	18.15 ± 0.32** (N = 54)	7.89 ± 0.10 (N = 61)	8.64 ± 0.12* (N = 54)
Control	22.74 ± 0.27 (N = 92)	19.86 ± 0.29 (N = 71)	7.74 ± 0.10 (N = 92)	9.10 ± 0.12 (N = 71)

Values are arithmetic means ± SEM. Statistical differences between stress and control group were assessed using the Student's *t*-test. * $P < 0.05$; ** $P < 0.01$.

times *vs*. 0.88 ± 0.26 times, $P < 0.01$) and a shorter time spent in the central zone (12.69 ± 1.34 sec *vs*. 17.99 ± 1.89 sec, $P < 0.05$) in the experimental group. Furthermore, prenatally stressed progeny had a tendency to spend a shorter amount of time resting (25.17 ± 4.88 sec *vs*. 38.71 ± 7.57 sec, $P = 0.1667$) and had fewer entries to the central zone (13.75 ± 1.32 times *vs*. 17.50 ± 1.57 times, $P = 0.0714$); however, the differences between these values were not statistically significant. We found no differences in the remaining five evaluated behavioral parameters (distance traveled, rears, jumps, cleaning frequency, cleaning duration).

Discussion

In our experiment, we applied short restraint (30 min) three times a day to prevent animal adaptation to the stress stimulus. We measured corticosterone, the main glucocorticoid involved in regulation of stress responses in rodents [30], and found significantly higher blood levels of this hormone after the last exposure of females to restraint stress (on day 4 of pregnancy). The corticosterone concentration was about 13-times higher in stressed females than in controls. We found a similar increase in the corticosterone level after the first exposure of females to the 30-min period of restraint stress (on day 1 of pregnancy) in our previous experiment [31]. These results prove the validity of our stress model and exclude habituation of mice to restraint stress exposure over time.

In our previous article [31], we discovered that restraint stress applied to mouse females during the preimplantation period had an adverse effect on embryo development, and we recorded the impaired embryo growth. The present study shows that the maternal environment altered by restraint stress significantly reduces the average cell numbers in both the ICM and the TE lineages of the blastocyst. It is known that proliferation of embryonic cells can be negatively influenced by suboptimal conditions in the maternal environment; for example, Kwong *et al.* [25] showed the same effect on ICM and TE lineages in dams fed a low-protein diet. In our study, we found that maternal stress decreased the number of ICM and TE cells unevenly, exerting a stronger effect on TE cells, thus resulting in a higher ICM/TE ratio in blastocysts isolated from prenatally stressed mothers. To our knowledge, this is the first report showing that maternal stress can affect the ICM/TE ratio. Since a reduction in TE cells may result in the development of a smaller interface for communication of the embryo with the uterine environment [22], we can assume that the reduction in TE cells in blastocysts could be one of the factors responsible for the decreased implantation rate found in our experiment. Several authors have demonstrated that the number of ICM cells is linked to implantation success and pregnancy outcome as well [17–21]. Since we found a decrease in the number of ICM cells as well (though to a lower extent than in TE cells), we assume that both these events could contribute to the lower implantation rate of blastocysts developing in stressed mothers.

According to Fleming *et al.* [18], the early mouse embryo is equipped with self-regulating mechanisms to maintain ICM and TE cells and their ratio within relatively narrow limits, indicating their importance to future development. Thus we can hypothesize that

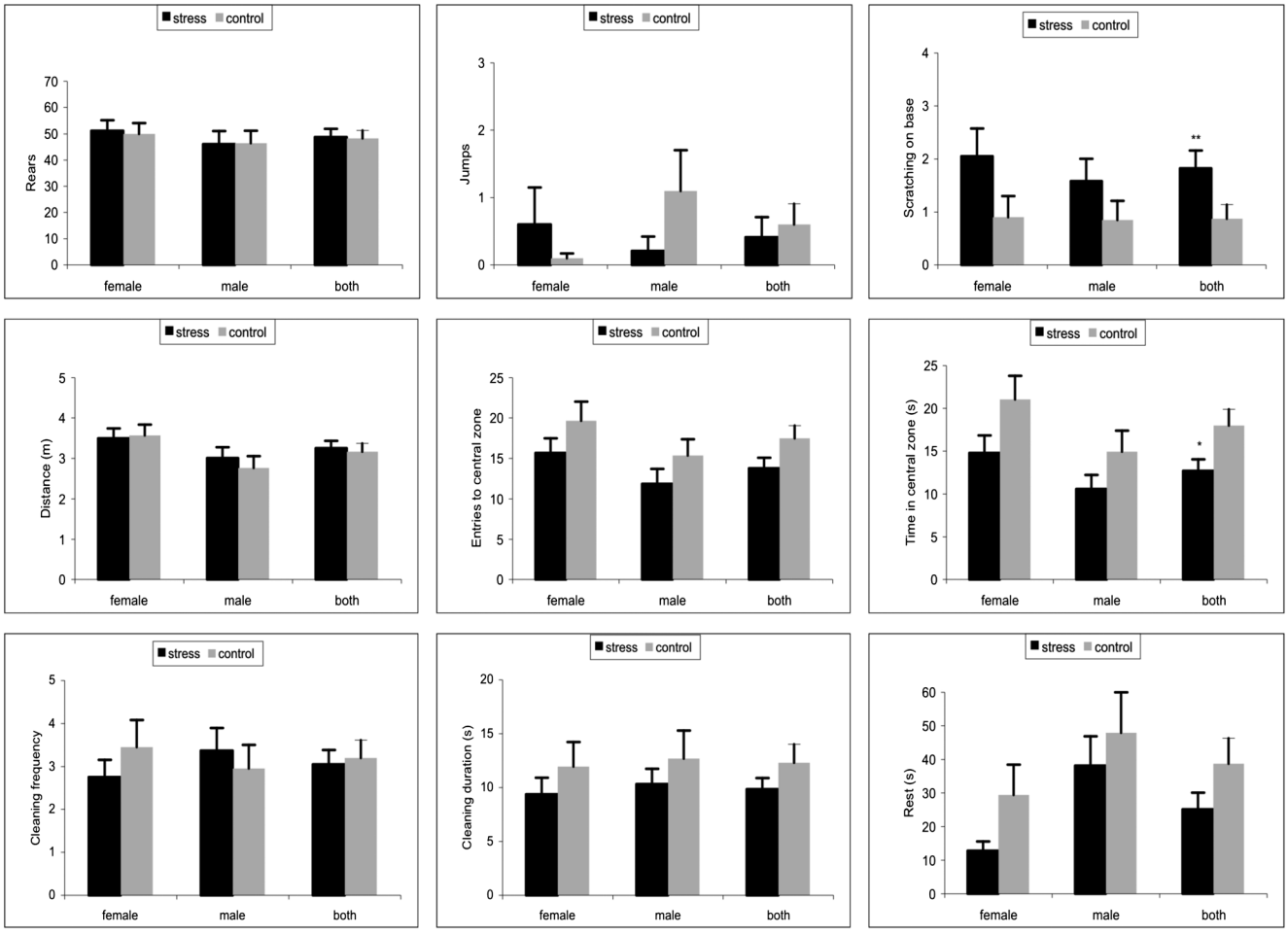

Fig. 3. The impact of maternal restraint stress on behavior of offspring tested in the open field test for 5 min. Values are arithmetic means ± SEM. Statistical differences between stressed and control females were assessed using the Student's *t*-test. * $P < 0.05$; ** $P < 0.01$.

embryos exposed to suboptimal conditions in the maternal environment caused by restraint stress prefer to invest limited resources in developing relatively normal ICM cells at the expense of a reduced number of TE cells. Besides altered cell differentiation, there is another possible way in which preimplantation stress might affect the implantation process. Previous studies have indicated that the window of implantation is very narrow and under strict regulation by ovarian hormones [32]. It is believed that the window of receptivity in rodents lasts for about 24 h, after which the uterus becomes non-receptive [33]. The attachment reaction occurs in the mouse around midnight on day 4 of pregnancy [34, 35], and according to a review by Wang and Dey [36], the mouse uterus becomes receptive between 2000 h on day 3 and 0730 h on day 5, whereas the attachment reaction is initiated at 2000 h on day 4 of pregnancy. Since the last stress session was performed before initiation of the attachment reaction in our experiment, we might assume that restraint stress postponed the attachment reaction by inhibiting blastocyst activation and impaired implantation by preventing blastocyst hatching in a similar way as in the experiment of Zhao *et al.* [16].

Surprisingly, although we found a lower implantation rate of blastocysts in stressed dams, we did not find a difference in the number of delivered pups compared with controls. The main reason could be the relatively high disproportion between implantation and birth rates in control animals (13.59 ± 0.45 implantation sites *vs*. 12 ± 0.58 pups/l) caused probably by smaller numbers of animals in the control groups. Reduced implantation sites and litter sizes following maternal preimplantation stress were found in the experiments by deCatanzaro *et al.* [15] and Zhao *et al.* [16]. Other authors did not find any significant influence of early maternal stress on the number of implantation sites in mice [37], hamster [38] and rats [39], but they found significantly smaller litters in stressed females (except Lee *et al.* [39], who did not examine this parameter in their study). In contrast, Pawluski *et al.* [40] and Sanches *et al.* [41] did not find any difference in litter size in rats after preimplantation stress exposure compared with controls. Finally, Kondoh *et al*. [42] observed a reduction in implantation sites after embryo transfer of blastocysts developed in females exposed to sonic stress (rodent repellent device), but litter size was not examined in their study. Since there are too many contradictory results among several authors in these two parameters, a bigger population study should be performed to draw a final conclusion.

In our experiment, maternal stress during the preimplantation period resulted in lower body weights in male and female offspring compared with controls. Similar results were reported by Bustamante *et al.* [43], while Abdul Aziz *et al.* [44], Franco *et al.* [45] and Pankevich *et al.* [24] observed decreased body weights only in prenatally stressed male offspring. In contrast, Schultz *et al.* [46] reported increased body weights in prenatally stressed male offspring, and Tamashiro *et al.* [47] did not find any changes in body weight in prenatally stressed offspring. In addition to reduced weight, we also observed reduced adiposity in prenatally stressed dams. Pankevich *et al.* [24] found decreased adiposity only in stressed males, and Franco *et al.* [45] did not find any differences in fat depots between stressed and control offspring at all. Our results and the results of Pankevich *et al.* [24] indicate that stress acting even during very early pregnancy might have a long-term impact on feeding behavior and energy metabolism in offspring. This probably results from changes in placental function. It seems that placental function may be altered even during the blastocyst stage, with stress retarding TE outgrowth and changing the proportion of TE and ICM cells in the blastocyst, as shown in our results. It is well known that the TE provides nutrients to the embryo, is responsible for embryo-uterine communication during implantation and develops into a large part of the placenta. Thus interference to TE outgrowth may have negative consequences for offspring development.

Several studies have shown impairment of neurodevelopment and changes in offspring behavior induced by prenatal stress in later pregnancy stages. But can maternal restraint stress cause changes in neurodevelopment and subsequently alter behavior even when it occurrs during the preimplantation period? Our results show that stress applied during the preimplantation developmental period delays eye opening. According to Koehler *et al.* [48] and Gandhi *et al.* [49], this delay implies a mild impairment of neurodevelopment in animals. Moreover, in mature offspring, we found altered behavior in two open field parameters (higher frequency of scratching on the base of the open field apparatus and shorter time spent in the central zone) and also a tendency toward altered behavior in two other parameters (shorter time resting and fewer entries into the central zone). In summary, we can say that prenatal stress, acting during the preimplantation developmental period, caused a reduction in comfort behavior and increased anxiety-like behavior compared with controls. Several authors [50–53] have reported that stress applied to rodents during neurodevelopment caused some variances in behavior of offspring (anxiety-like behavior, decreased rearing, decreased crossing and decreased locomotor activity). Our results indicate that maternal stress can influence offspring neurodevelopment and behavior even when applied during preimplantation period, before neurons are formed.

At the present time, many authors are looking for a link between changes in the maternal environment that occur during the preimplantation and peri-implantation periods and changes in somatic functions that occur later in postnatal life. Epigenetic factors are one of the possible options. The periconceptional period of mammalian development has been identified as an early "developmental window" during which environmental conditions may influence the pattern of future growth and physiology [54–58]. The role of epigenetic modifications in DNA and chromatin organization has been identified as a likely mechanism through which environmental perturbations can affect gene expression patterns, resulting in phenotypic changes [26, 59]. The latest findings of Yao *et al.* [60] concur with descriptions of intergenerational stress impacts caused by human migration, natural disasters and poverty, which may program maternal health preconceptionally via the maternal lineage.

Our data reveal that maternal restraint stress during the preimplantation period of development may not only influence early embryo growth, blastocyst cellular organization and implantation rate but may also have long-term effects reaching into postnatal life. Thus the impact of exposure to mental stress during very early pregnancy should not be underestimated and should be regarded as a potential risk factor in animal as well as human reproduction.

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References

1. **Calogero AE, Burrello N, Negri-Cesi P, Papale L, Palumbo MA, Cianci A, Sanfilippo S, D'Agata R.** Effects of corticotropin-releasing hormone on ovarian estrogen production in vitro. *Endocrinology* 1996; **137**: 4161–4166. [Medline]
2. **Chrousos GP, Torpy DJ, Gold PW.** Interactions between the hypothalamic-pituitary-adrenal axis and the female reproductive system: clinical implications. *Ann Intern Med* 1998; **129**: 229–240. [Medline] [CrossRef]
3. **Tetsuka M, Milne M, Simpson GE, Hillier SG.** Expression of 11beta-hydroxysteroid dehydrogenase, glucocorticoid receptor, and mineralocorticoid receptor genes in rat ovary. *Biol Reprod* 1999; **60**: 330–335. [Medline] [CrossRef]
4. **Douglas AJ.** Mother-offspring dialogue in early pregnancy: impact of adverse environment on pregnancy maintenance and neurobiology. *Prog Neuropsychopharmacol Biol Psychiatry* 2011; **35**: 1167–1177. [Medline] [CrossRef]
5. **Siemieniuch MJ, Majewska M, Takahashi M, Sakatani M, Lukasik K, Okuda K, Skarżyński DJ.** Are glucocorticoids auto- and/or paracrine factors in early bovine embryo development and implantation? *Reprod Biol* 2010; **10**: 249–256. [Medline] [CrossRef]
6. **Čikoš S, Rehák P, Czikková S, Veselá J, Koppel J.** Expression of adrenergic receptors in mouse preimplantation embryos and ovulated oocytes. *Reproduction* 2007; **133**: 1139–1147. [Medline] [CrossRef]
7. **Čikoš Š, Czikková S, Chrenek P, Makarevich AV, Burkuš J, Janštová Ž, Fabian D, Koppel J.** Expression of adrenergic receptors in bovine and rabbit oocytes and preimplantation embryos. *Reprod Domest Anim* 2014; **49**: 92–100. [Medline] [CrossRef]
8. **Čikoš S, Veselá J, Il'ková G, Rehák P, Czikková S, Koppel J.** Expression of beta adrenergic receptors in mouse oocytes and preimplantation embryos. *Mol Reprod Dev* 2005; **71**: 145–153. [Medline] [CrossRef]
9. **Van Merris V, Van Wemmel K, Cortvrindt R.** In vitro effects of dexamethasone on mouse ovarian function and pre-implantation embryo development. *Reprod Toxicol* 2007; **23**: 32–41. [Medline] [CrossRef]
10. **Zhang SY, Wang JZ, Li JJ, Wei DL, Sui HS, Zhang ZH, Zhou P, Tan JH.** Maternal restraint stress diminishes the developmental potential of oocytes. *Biol Reprod* 2011; **84**: 672–681. [Medline] [CrossRef]
11. **Brandt Y, Madej A, Rodríguez-Martínez H, Einarsson S.** Effects of exogenous ACTH during oestrus on early embryo development and oviductal transport in the sow. *Reprod Domest Anim* 2007; **42**: 118–125. [Medline] [CrossRef]
12. **Mihalik J, Kravcuková P, Spakovská T, Mareková M, Schmidtová K.** Study of high deprenyl dose on the preimplantation embryo development and lymphocyte DNA in rat.

Gen Physiol Biophys 2008; **27**: 121–126. [Medline]

13. **Mihalik J, Mašlanková J, Špakovská T, Mareková M, Hodorová I, Kušnír J, Rybárová S, Ferenc P, Schmidtová K.** Impact of 2 doses of clorgyline on the rat preimplantation embryo development and the monoamine levels in urine. *Reprod Sci* 2010; **17**: 734–741. [Medline] [CrossRef]
14. **Nepomnaschy PA, Welch KB, McConnell DS, Low BS, Strassmann BI, England BG.** Cortisol levels and very early pregnancy loss in humans. *Proc Natl Acad Sci USA* 2006; **103**: 3938–3942. [Medline] [CrossRef]
15. **deCatanzaro D, MacNiven E, Goodison T, Richardson D.** Estrogen antibodies reduce vulnerability to stress-induced failure of intrauterine implantation in inseminated mice. *Physiol Behav* 1994; **55**: 35–38. [Medline] [CrossRef]
16. **Zhao LH, Cui XZ, Yuan HJ, Liang B, Zheng LL, Liu YX, Luo MJ, Tan JH.** Restraint stress inhibits mouse implantation: temporal window and the involvement of HB-EGF, estrogen and progesterone. *PLoS ONE* 2013; **8**: e80472. [Medline] [CrossRef]
17. **Januário DA, Perin PM, Maluf M, Lichtenfels AJ, Nascimento Saldiva PH.** Biological effects and dose-response assessment of diesel exhaust particles on in vitro early embryo development in mice. *Toxicol Sci* 2010; **117**: 200–208. [Medline] [CrossRef]
18. **Fleming TP, Kwong WY, Porter R, Ursell E, Fesenko I, Wilkins A, Miller DJ, Watkins AJ, Eckert JJ.** The embryo and its future. *Biol Reprod* 2004; **71**: 1046–1054. [Medline] [CrossRef]
19. **Tam PP.** Postimplantation development of mitomycin C-treated mouse blastocysts. *Teratology* 1988; **37**: 205–212. [Medline] [CrossRef]
20. **Binder NK, Hannan NJ, Gardner DK.** Paternal diet-induced obesity retards early mouse embryo development, mitochondrial activity and pregnancy health. *PLoS ONE* 2012; **7**: e52304. [Medline] [CrossRef]
21. **Richter KS, Harris DC, Daneshmand ST, Shapiro BS.** Quantitative grading of a human blastocyst: optimal inner cell mass size and shape. *Fertil Steril* 2001; **76**: 1157–1167. [Medline] [CrossRef]
22. **Russell DF, Baqir S, Bordignon J, Betts DH.** The impact of oocyte maturation media on early bovine embryonic development. *Mol Reprod Dev* 2006; **73**: 1255–1270. [Medline] [CrossRef]
23. **Williams CL, Teeling JL, Perry VH, Fleming TP.** Mouse maternal systemic inflammation at the zygote stage causes blunted cytokine responsiveness in lipopolysaccharide-challenged adult offspring. *BMC Biol* 2011; **9**: 49. [Medline] [CrossRef]
24. **Pankevich DE, Mueller BR, Brockel B, Bale TL.** Prenatal stress programming of offspring feeding behavior and energy balance begins early in pregnancy. *Physiol Behav* 2009; **98**: 94–102. [Medline] [CrossRef]
25. **Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP.** Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development* 2000; **127**: 4195–4202. [Medline]
26. **Watkins AJ, Ursell E, Panton R, Papenbrock T, Hollis L, Cunningham C, Wilkins A, Perry VH, Sheth B, Kwong WY, Eckert JJ, Wild AE, Hanson MA, Osmond C, Fleming TP.** Adaptive responses by mouse early embryos to maternal diet protect fetal growth but predispose to adult onset disease. *Biol Reprod* 2008; **78**: 299–306. [Medline] [CrossRef]
27. **Ceelen M, van Weissenbruch MM, Vermeiden JP, van Leeuwen FE, Delemarre-van de Waal HA.** Cardiometabolic differences in children born after in vitro fertilization: follow-up study. *J Clin Endocrinol Metab* 2008; **93**: 1682–1688. [Medline] [CrossRef]
28. **Sakka SD, Loutradis D, Kanaka-Gantenbein C, Margeli A, Papastamataki M, Papassotiriou I, Chrousos GP.** Absence of insulin resistance and low-grade inflammation despite early metabolic syndrome manifestations in children born after in vitro fertilization. *Fertil Steril* 2010; **94**: 1693–1699. [Medline] [CrossRef]
29. **Lawitts JA, Biggers JD.** Culture of preimplantation embryos. *Methods Enzymol* 1993; **225**: 153–164. [Medline] [CrossRef]
30. **Thaete LG, Nesbitt MN, Malkinson AM.** Genetic analysis of the distribution of corticosterone-containing cells in mouse adrenal cortex. *Proc Soc Exp Biol Med* 1990; **194**: 97–102. [Medline] [CrossRef]
31. **Burkuš J, Cikoš S, Fabian D, Kubandová J, Czikková S, Koppel J.** Maternal restraint stress negatively influences growth capacity of preimplantation mouse embryos. *Gen Physiol Biophys* 2013; **32**: 129–137. [Medline] [CrossRef]
32. **Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, Yoshinaga K.** Embryo implantation. *Dev Biol* 2000; **223**: 217–237. [Medline] [CrossRef]
33. **Paria BC, Huet-Hudson YM, Dey SK.** Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. *Proc Natl Acad Sci USA* 1993; **90**: 10159–10162. [Medline] [CrossRef]
34. **Enders AC.** Anatomical aspects of implantation. *J Reprod Fertil Suppl* 1976; **1**: 1–15. [Medline]
35. **Das SK, Chakraborty I, Paria BC, Wang XN, Plowman G, Dey SK.** Amphiregulin is an implantation-specific and progesterone-regulated gene in the mouse uterus. *Mol Endocrinol* 1995; **9**: 691–705. [Medline]
36. **Wang H, Dey SK.** Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet* 2006; **7**: 185–199. [Medline] [CrossRef]
37. **Wiebold JL, Stanfield PH, Becker WC, Hillers JK.** The effect of restraint stress in early pregnancy in mice. *J Reprod Fertil* 1986; **78**: 185–192. [Medline] [CrossRef]
38. **Pratt NC, Lisk RD.** Effects of social stress during early pregnancy on litter size and sex ratio in the golden hamster (Mesocricetus auratus). *J Reprod Fertil* 1989; **87**: 763–769. [Medline] [CrossRef]
39. **Lee YE, Byun SK, Shin S, Jang JY, Choi BI, Park D, Jeon JH, Nahm SS, Kang JK, Hwang SY, Kim JC, Kim YB.** Effect of maternal restraint stress on fetal development of ICR mice. *Exp Anim* 2008; **57**: 19–25. [Medline] [CrossRef]
40. **Pawluski JL, Valença A, Santos AI, Costa-Nunes JP, Steinbusch HW, Strekalova T.** Pregnancy or stress decrease complexity of CA3 pyramidal neurons in the hippocampus of adult female rats. *Neuroscience* 2012; **227**: 201–210. [Medline] [CrossRef]
41. **Sánchez DJ, Bellés M, Albina ML, Gómez M, Linares V, Domingo JL.** Exposure of pregnant rats to uranium and restraint stress: effects on postnatal development and behavior of the offspring. *Toxicology* 2006; **228**: 323–332. [Medline] [CrossRef]
42. **Kondoh E, Okamoto T, Higuchi T, Tatsumi K, Baba T, Murphy SK, Takakura K, Konishi I, Fujii S.** Stress affects uterine receptivity through an ovarian-independent pathway. *Hum Reprod* 2009; **24**: 945–953. [Medline] [CrossRef]
43. **Bustamante C, Henríquez R, Medina F, Reinoso C, Vargas R, Pascual R.** Maternal exercise during pregnancy ameliorates the postnatal neuronal impairments induced by prenatal restraint stress in mice. *Int J Dev Neurosci* 2013; **31**: 267–273. [Medline] [CrossRef]
44. **Abdul Aziz NH, Kendall DA, Pardon MC.** Prenatal exposure to chronic mild stress increases corticosterone levels in the amniotic fluid and induces cognitive deficits in female offspring, improved by treatment with the antidepressant drug amitriptyline. *Behav Brain Res* 2012; **231**: 29–39. [Medline] [CrossRef]
45. **Franko KL, Forhead AJ, Fowden AL.** Differential effects of prenatal stress and glucocorticoid administration on postnatal growth and glucose metabolism in rats. *J Endocrinol* 2010; **204**: 319–329. [Medline] [CrossRef]
46. **Schulz KM, Pearson JN, Neeley EW, Berger R, Leonard S, Adams CE, Stevens KE.** Maternal stress during pregnancy causes sex-specific alterations in offspring memory performance, social interactions, indices of anxiety, and body mass. *Physiol Behav* 2011; **104**: 340–347. [Medline] [CrossRef]
47. **Tamashiro KL, Terrillion CE, Hyun J, Koenig JI, Moran TH.** Prenatal stress or high-fat diet increases susceptibility to diet-induced obesity in rat offspring. *Diabetes* 2009; **58**: 1116–1125. [Medline] [CrossRef]
48. **Koehler CL, Akimov NP, Rentería RC.** Receptive field center size decreases and firing properties mature in ON and OFF retinal ganglion cells after eye opening in the mouse. *J Neurophysiol* 2011; **106**: 895–904. [Medline] [CrossRef]
49. **Gandhi SP, Cang J, Stryker MP.** An eye-opening experience. *Nat Neurosci* 2005; **8**: 9–10. [Medline] [CrossRef]
50. **Laloux C, Mairesse J, Van Camp G, Giovine A, Branchi I, Bouret S, Morley-Fletcher S, Bergonzelli G, Malagodi M, Gradini R, Nicoletti F, Darnaudéry M, Maccari S.** Anxiety-like behaviour and associated neurochemical and endocrinological alterations in male pups exposed to prenatal stress. *Psychoneuroendocrinology* 2012; **37**: 1646–1658. [Medline] [CrossRef]
51. **Van den Hove DL, Blanco CE, Aendekerk B, Desbonnet L, Bruschettini M, Steinbusch HP, Prickaerts J, Steinbusch HW.** Prenatal restraint stress and long-term affective consequences. *Dev Neurosci* 2005; **27**: 313–320. [Medline] [CrossRef]
52. **Liu Y, Zhuang X, Gou L, Ling X, Tian X, Liu L, Zheng Y, Zhang L, Yin X.** Protective effects of nizofenone administration on the cognitive impairments induced by chronic restraint stress in mice. *Pharmacol Biochem Behav* 2013; **103**: 474–480. [Medline] [CrossRef]
53. **Miyagawa K, Tsuji M, Fujimori K, Saito Y, Takeda H.** Prenatal stress induces anxiety-like behavior together with the disruption of central serotonin neurons in mice. *Neurosci Res* 2011; **70**: 111–117. [Medline] [CrossRef]
54. **Reik W, Constância M, Fowden A, Anderson N, Dean W, Ferguson-Smith A, Tycko B, Sibley C.** Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. *J Physiol* 2003; **547**: 35–44. [Medline] [CrossRef]
55. **Faulk C, Dolinoy DC.** Timing is everything: the when and how of environmentally induced changes in the epigenome of animals. *Epigenetics* 2011; **6**: 791–797. [Medline] [CrossRef]
56. **Feil R, Fraga MF.** Epigenetics and the environment: emerging patterns and implications. *Nat Rev Genet* 2011; **13**: 97–109. [Medline]
57. **Denomme MM, Mann MR.** Genomic imprints as a model for the analysis of epigenetic stability during assisted reproductive technologies. *Reproduction* 2012; **144**: 393–409. [Medline] [CrossRef]
58. **El Hajj N, Haaf T.** Epigenetic disturbances in in vitro cultured gametes and embryos: implications for human assisted reproduction. *Fertil Steril* 2013; **99**: 632–641. [Medline] [CrossRef]
59. **Watkins AJ, Lucas ES, Fleming TP.** Impact of the periconceptional environment on the programming of adult disease. *J Dev Orig Health Dis* 2010; **1**: 87–95. [Medline] [CrossRef]
60. **Yao Y, Robinson AM, Zucchi FC, Robbins JC, Babenko O, Kovalchuk O, Kovalchuk I, Olson DM, Metz GA.** Ancestral exposure to stress epigenetically programs preterm birth risk and adverse maternal and newborn outcomes. *BMC Med* 2014; **12**: 121. [Medline] [CrossRef]