Full Length Research Paper

Effects of elevated ambient temperature on embryo implantation in rats

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Implantation is a crucial step in mammalian reproduction as it is a gateway to further embryonic development and successful pregnancy. Changes in the environmental factors, such as temperature have adverse effects on reproduction. However, the impact of elevated temperature on the implantation process is not well defined. The objective of this study was to investigate the possible effect of elevated ambient temperature on implantation time and rate. The results revealed that exposure to elevated ambient temperature leads to a delayed implantation and reduced number of implantation sites in Sprague Dawley rats. Moreover, the exposure to elevated temperature resulted in change in the progesterone and estradiol patterns during the implantation time. These findings indicate that elevated temperature disturbs the implantation process.

Key words: Elevated temperature, implantation time, number of implantation sites, progesterone and estradiol.

INTRODUCTION

Implantation is the most important step in mammalian reproduction and development. It is the first challenge in the life; either to be or not to be. Successful implantation needs an intricate succession of molecular and genetic interactions. These reciprocal interactions between the embryo and the uterus must be executed within a limited period known as the window of implantation (Psychoyos, 1973). Any breach in the communication between the endometrium and embryo during this time leads to implantation failure. Implantation failure in human is an unresolved problem in reproductive medicine and is considered as an important cause of infertility.

Assisted reproductive technology (ART), such as *in vitro* fertilization and embryo transfer (IVF-ET), is widely used to overcome many causes of infertility, such as tubal scaring and male factors. Nevertheless, the rate of successful pregnancies is only 30% (Wang and Dey, 2006) and subsequent delivery rates are only 10-30% (Adamson et al., 2006). The ART disappointing result is due to implantation failure when the embryos are transferred to non-receptive uteri.

Implantation failure is a major obstacle affecting the reproductive efficiency in both human and animals. In farm animals, implantation failure is considered as the main source of reproductive wastage and hence has enormous economic implications. In cattle, for instance, around 40% of total embryonic losses occur during early pregnancy, that is, from day 8 to 17 of pregnancy (Thatcher et al., 2001). Currently, *in vitro* embryo production is widely used in cattle as an excellent tool for genetic improvement (Camargo et al., 2006). However, the implantation rate and pregnancy outcome remain low due to implantation failure. Two-thirds of implantation

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failure is related to inadequate endometrial receptivity and only one-third is related to the embryo itself (Simon et al., 1998; Ledee-Bataille et al., 2002).

Stress is one of the factors that disturb natural fertility (Campagne, 2006). An interview-based study has revealed that psychological stress has adverse results on IVF-ET outcomes (Eugster et al., 2004). Recent study of Kondoh et al. (2009) has shown that the implantation rates decrease after exposure to stress. One of the most important stresses that affects reproductive performance is heat stress. Heat stress is often unavoidable and, because of recent climate changes, it may unfortunately be experienced over a wide range of geographical regions. The most economically important reproductive performance traits of farm animals may be endangered by heat stress (Bloemhof et al., 2008). For instance, lactating cows are more sensitive to elevated temperatures; that is, hyperthermia in lactating cows can occur at ambient temperatures as low as 27°C (Berman et al., 1985) and causes a decreased conception rate (Udompraset and Williamson, 1987; De Rensis and Scaramuzzi, 2003). Heat stress has adverse effect on embryo production (Hansen et al., 2001). Moreover, several studies in mice and cows have shown that exposure to heat on the day 1 of pregnancy compromises pre-implantation embryo development (Ealy et al., 1993; Matsuzuka et al., 2005 Ozawa et al., 2002, 2003; Sakamoto et al., 2008).

Since implantation is considered as the most critical step in reproduction, it is crucial to investigate the possible effect of heat stress on the implantation. Therefore, the current study was intended to determine the effects of exposure to elevated ambient temperature on implantation time, number of implantation sites and concentrations of plasma progesterone and estradiol.

MATERIALS AND METHODS

Animals

Mature virgin females Sprague Dawley rats were used in this study. These in-house bred rats were 2-3 months old and weighed 200-220 g. The animals were maintained under controlled lighting regime of 12 h light and 12 h dark. The light-on at 0700 h and off at 1900 h. The rats were maintained under a temperature of 23±1°C and relative humidity of 55-65%. The rats were allowed free access to chow and water. The animal care and handling throughout the study were approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, University Putra Malaysia, Malaysia. Only females that showed at least two consecutive, regular four-day estrous cycles immediately before the study were used. Proestrus females were caged overnight with a male from the same strain aged 4-5 months, a weight of 400-500 g and proven to be fertile. The morning of the following day, successful copulation was confirmed by the presence of spermatozoa in the vaginal smear, and that day was designated as day 1 of pregnancy.

Influence of the parents was controlled by mating each male with two littermate females. On day 2 of pregnancy, each rat was randomly assigned either to 1) control group, the rats were maintained under optimal temperatures or 2) experimental group, the rats were exposed to heat stress. Animals of both control and experimental groups were euthanized on days 5, 6, 7, and 8 of pregnancy, and the reproductive organs were excised through a midline incision and examined macroscopically.

Heat application

This study was conducted in May, when the ambient temperature was high. The pregnant rats were kept in a room without an air conditioner or fan. The ambient temperature and relative humidity were 33±2°C and 82%, respectively. This temperature is in the range that causes heat stress in rats (Godsil et al., 2003).

Blue dye reaction

Blue dye was used to determine the time of implantation. An amount of 0.3 ml of 1% Chicago Sky Blue 6B (Sigma Aldrich, Inc., St. Louis, USA) in 0.9% NaCl was injected intravenously, under anesthesia using a combination of Rompun and Ketamin (7mg/ kg Rompun and 65 mg/kg Ketamin). The dye was allowed to circulate for 15 min. The animals were then euthanized under anaesthesia by decapitation. Blue bands in the uterus indicated the implantation sites.

Implantation time

The animals were euthanized after intravenous injection of blue dye in order to determine the implantation initiation time in control and experimental groups. The blue dye injection started on day 5 of pregnancy at 1400 h. The rats were euthanized at 2 h intervals (n = 5) until appearance of distinct blue bands.

On days 6, 7 and 8 of pregnancy, the rat uterine horns (both control and experimental groups, n = 8 at each stage) were examined for the presence of implantation sites (IS). The intensity of the blue dye and the thickness of the bands were also observed.

Implantation sites (IS)

The IS and the corpora lutea on the corresponding ovaries were counted and compared between the control and experimental groups. Numbers of IS in rats exposed to elevated temperature were counted on days 6, 7 and 8 of pregnancy and compared with the number of IS of corresponding control group. The number of IS that was observed on day 6 of pregnancy (peri-implantation) in exposed rats was also compared with those observed on day 8 of pregnancy (post-implantation) in order to estimate the rate of embryonic loss during implantation.

Blood collection and radioimmunoassay

Radioimmunoassay (RIA) was used to estimate the estradiol and progesterone levels at day 5 in the afternoon (at 1400 h), day 5 in the evening (at 2000 h), and day 6 in the afternoon (at 1400 h) of pregnancy. One milliter (1 ml) of blood was collected from the heart of anesthetized rats into EDTA tubes and immediately centrifuged at 3000 rpm for 20 min at 4°C. The plasma was then transferred to another clean tube and stored at -30°C until analysis.

Plasma progesterone and estradiol concentrations were measured by a commercial solid-phase radioimmunoassay (RIA) kit (Coat-A-Count; Siemens Medical Solutions Diagnostics, Los Angeles, USA) according to the manufacturers' procedure. The intra- and inter-assay coefficients of variations were 5.52 and 8.21% for progesterone assay, and 6.3 and 8.45% for estradiol assay, respectively.
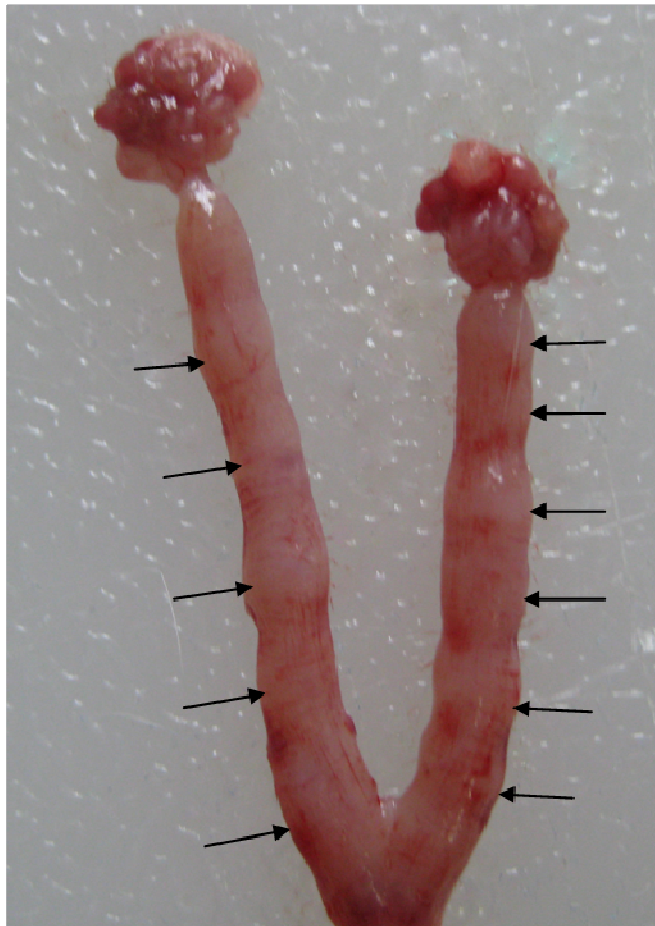
Figure 1. The uterus of non-exposed rat in the afternoon of day 5 of pregnancy. Note the embryonic sites (arrows).

Statistical analysis

The number of IS and corpora lutea and the concentrations of plasma progesterone and estradiol were analyzed and compared between the control and exposed groups using independent *t*-test. The statistical analysis was carried out using the PASW statistics 18 package (SPSS Inc., Chicago, IL, USA) with the significance level set at $P < 0.05$. All the data are presented as mean ± SEM.

RESULTS

Effect of elevated ambient temperature on implantation time

The implantation time was determined in the control group (females not exposed to elevated temperature) and in experimental group (females exposed to elevated ambient temperature).

On day 5 of pregnancy, before appearance of the blue bands, there were distinct differences observed between uteri of exposed and non-exposed rats. In the afternoon of day 5, the uterus of non-exposed rat became elongated and very rich in blood vessels. In this stage, the blastocysts distribution and spacing occurred. There were enlargements at the embryonic sites. These enlargements were seen along the uterus, that is, from the utero-tubal end to the cervix. The spaces between the enlargements were approximately equal (Figure 1).

At 1800 h on day 5, dilation was noticed in the upper part of the exposed-rat uterus (Figure 2). Most of the blastocysts were present in this part of the uterus and no organized distribution was observed. Blastocysts distribution and spacing were delayed compared with the non-exposed rats; this phenomenon was observed as early as at 2200 h and thereafter.

In the control group, the blue dye reaction was observed as a diffused dye (not as distinct bands) in the lower part of the uterus in the afternoon of day 5 of pregnancy. This reaction was observed in only two out of five rats euthanized at that time, whereas all the rats euthanized in the evening of day 5 (1800 - 1900 h) showed distinct blue bands. By contrast, exposed rats experienced delayed implantation. No blue bands were observed in the afternoon or evening of day 5 of

Figure 2. The uterus of rat exposed to elevated temperature in the evening of day 5 of pregnancy. Note the dilation at the upper part of the uterus (open arrow).

pregnancy. The first appearance of the blue dye reaction was reported at 0000-0100 h on day 6 of pregnancy. However, only two out of five rats exhibited blue bands. These bands were few and very pale.

On day 6 of pregnancy, the non-exposed rats showed very thick bands (Figure 3A), which darkly stained with the blue dye, while the exposed rats exhibited thin and pale bands (Figure 3B). Moreover, three out of eight exposed rats did not show any blue bands.

On day 7 of pregnancy, the implantation in the non-exposed rats (control) was established. On the other hand, three exposed rats showed distinct dark blue bands, while the rest of the animals did not exhibit any IS.

On day 8 of pregnancy, one rat of the exposed group showed blue bands, while another rat had no IS; however, the implantation in the rest of the animals was completed. But the implantation sites were smaller in size than those of control.

Effect of elevated ambient temperature on the number of implantation sites

The number of IS decreased after exposure to heat. On

days 6 and 7 of pregnancy, the exposed groups showed significantly less number of IS than the control groups ($P < 0.01$). On day 8 of pregnancy, the exposed rats exhibited a dramatic decrease ($P < 0.0001$) in the numbers of IS when compared with the non-exposed rats (Figure 4). Furthermore, exposed rats showed a significant reduction (28.6%) in the number of IS at post-implantation (day 8) when compared with those at peri-implantation (day 6) ($P < 0.05$).

No difference was observed between the control and experimental groups in the number of corpora lutea.

Concentration of plasma progesterone

Plasma progesterone levels in the control group were significantly increased from afternoon to evening of day 5 ($P < 0.05$), followed by slightly non significant decrease from in the evening of day 5 to afternoon of day 6. In the exposed group, however, there were no significant differences in the progesterone levels between day 5 afternoon and evening or between day 5 evening and day 6 afternoon ($P > 0.05$).

In the afternoons of day 5 and 6 of pregnancy, the
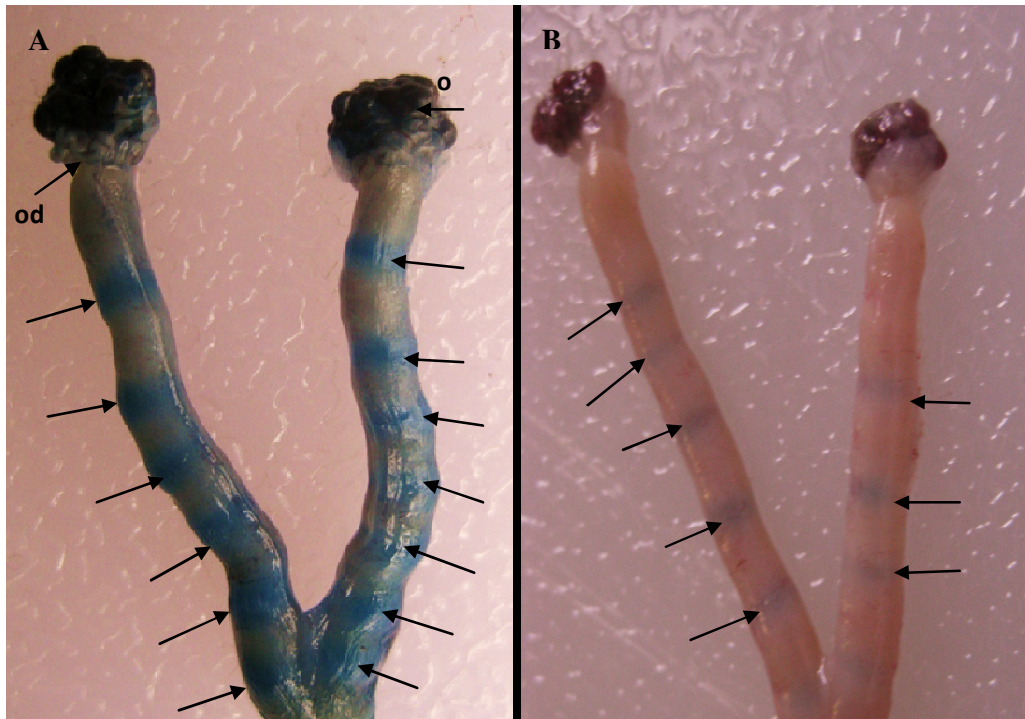
Figure 3. The implantation sites on day 6 of pregnancy. (A) Non exposed - rat uterus, and (B) exposed - rat uterus. The blue bands indicate the implantation sites (arrows). (o) – Ovary; (od) – oviduct. Note the variation in the blue dye intensity and bands thickness.
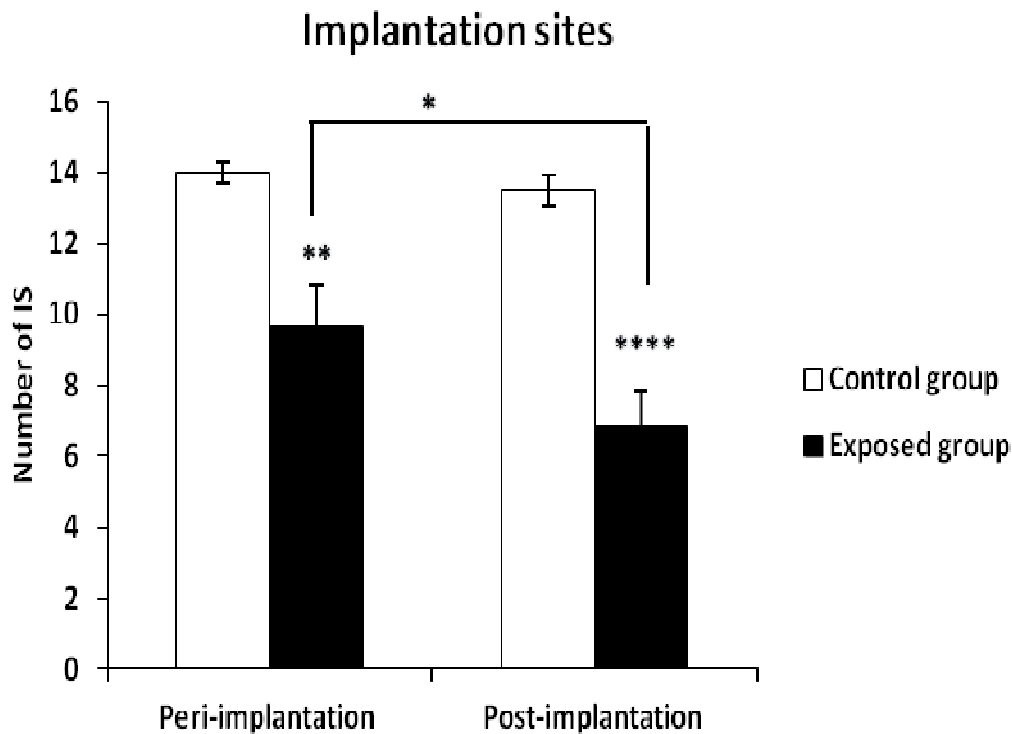
Figure 4. The number of the implantation sites in the control group and in the group exposed to elevated temperature at peri-implantation and post-implantation. The bars represent mean±SEM. Levels of significant are shown by asterisks (* $P < 0.05$; **$P < 0.01$; ****$P < 0.0001$) compared with the control.
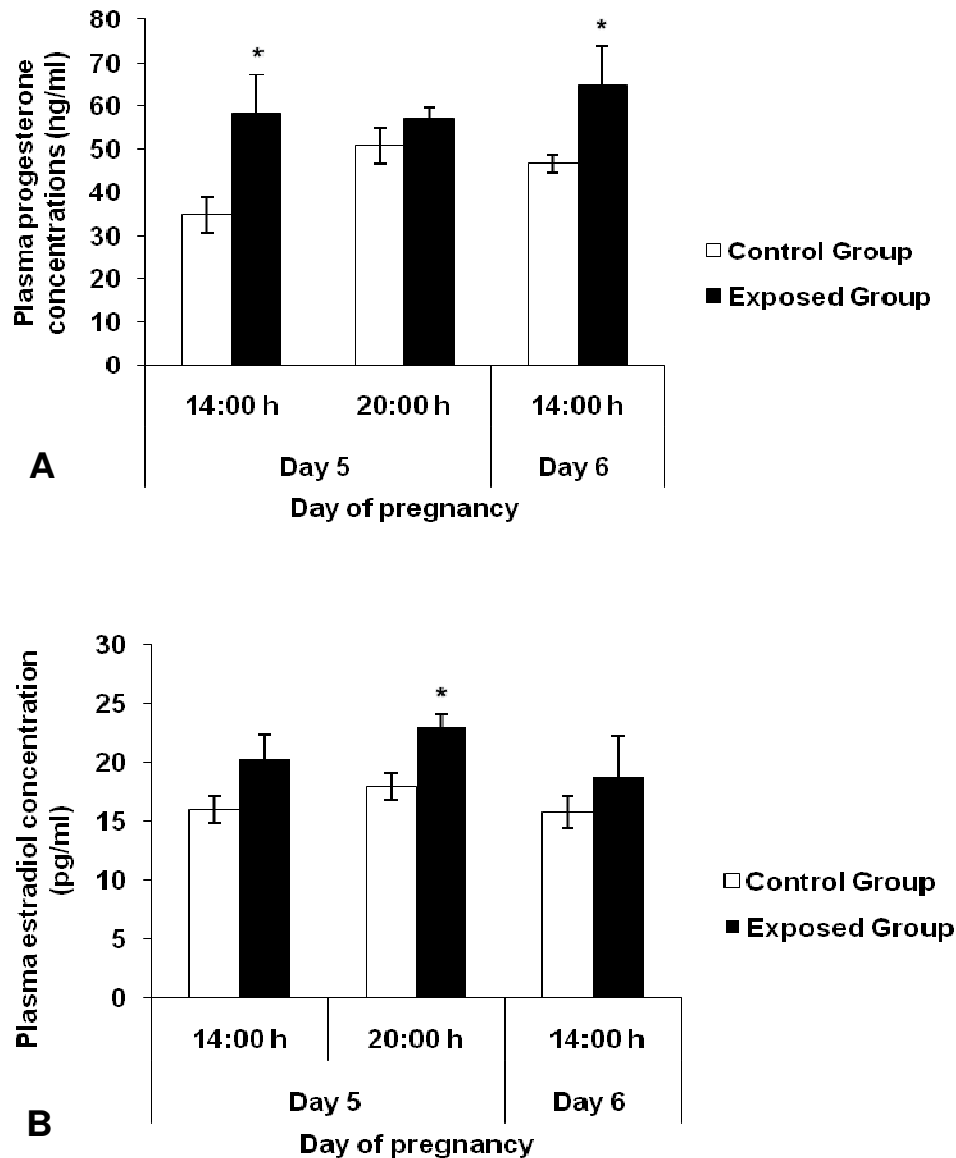
Figure 5. Graphs represent progesterone and estradiol concentrations and P4:E2 ratio in the control group and in the group exposed to elevated temperature at three stages: day 5 afternoon, day 5 evening and day 6 of pregnancy. (A) Progesterone concentrations (ng/ml). (B) Estradiol concentrations (pg/ml). (C) P_4:E_2 ratio. The bars represent mean±SEM. *P < 0.05.

exposed rats showed significantly increased plasma progesterone concentrations when compared with the non-exposed rats at the same stages of pregnancy (P < 0.05). Although, the progesterone levels in the exposed group on the evening of day 5 were higher than those in the control group at the same stage of pregnancy with no statistically significant difference (Figure 5A).

Concentration of plasma estradiol

The mean estradiol concentration for the non-exposed

rats showed statistically no significant differences between day 5 afternoon and evening or between day 5 evening and day 6 afternoon (P > 0.05), although slight increase was observed on day 5 evening compared with day 5 afternoon and day 6 afternoon. In the exposed group, also, estradiol levels were higher in the evening of day 5 than in the afternoons of day 5 and day 6 of pregnancy with no statistically significant differences (Figure 5B).

The exposed rats showed higher concentrations of plasma estradiol than the non-exposed rats in the afternoons of days 5 and 6 of pregnancy, but these
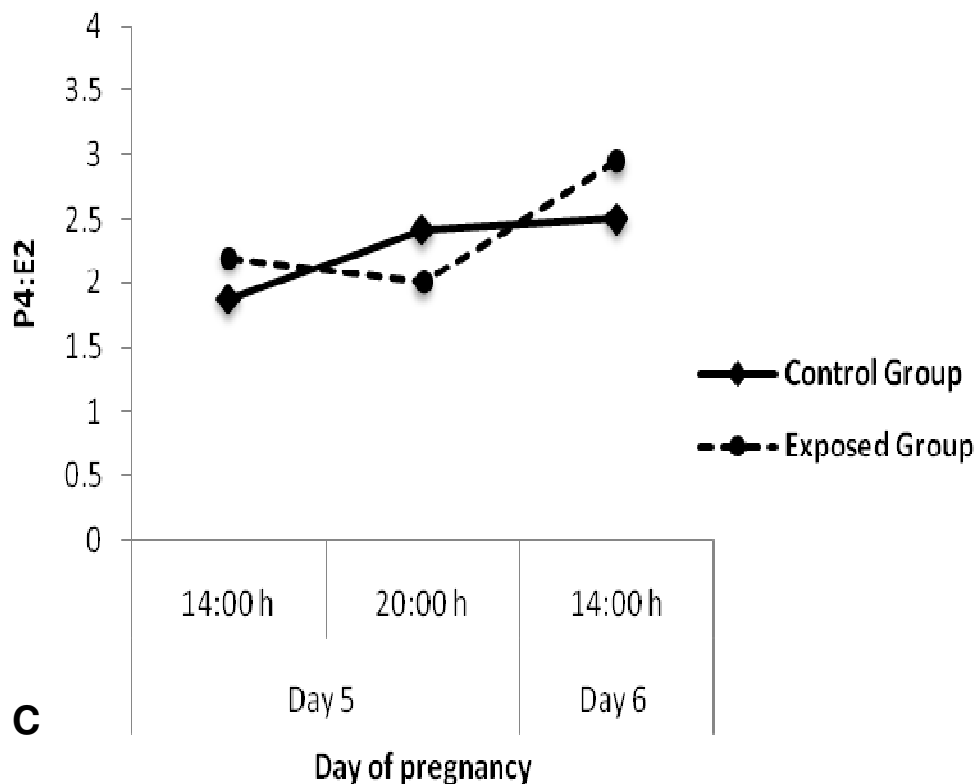
Figure 5. Contd.

differences were not statistically significant ($P > 0.05$). On day 5 evening, the concentrations of plasma estradiol in the exposed group were significantly ($P < 0.05$) higher than in the control group (Figure 5B).

Progesterone: Estradiol ratio ($P_4:E_2$)

The progesterone: estradiol ratio was calculated by dividing the P_4 value in nm/ml by the E_2 value in nm/ml. The exposed group showed higher $P_4:E_2$ ratio at day 5 and day 6 afternoons than in the control group, whilst in the evening of day 5, the $P_4:E_2$ ratio was lower in the exposed group than in the control group (Figure 5C).

DISCUSSION

Maternal and paternal traits are strongly related to reproductive efficiency. To control the parental factors, therefore, all the rats used in this study were bred in our laboratory, and each of the two littermate females were mated with the same male before being randomly assigned to one of the two groups.

In this study, the effect of exposure to elevated ambient temperature on implantation time was evaluated. Chicago Sky Blue 6B was used to determine the initiation time of implantation. Appearance of distinct blue bands in the uterus, after intravenous injection of macromolecular blue dye solution indicates the initiation of implantation (Psychoyos, 1986; Dey, 2003). In the control group (non-exposed rats), implantation was initiated in the evening of day 5 and completed on day 7 of pregnancy. This finding concurs with the results of previous studies (Enders and Schlafke, 1967; Garside et al., 1996). In the exposed group, the implantation delayed. Delayed implantation is a phenomenon found in almost 100 mammals in seven different mammalian orders, including rodents (Renfree and Shaw, 2000; Lee and DeMay, 2004). Delayed implantation is one of the mechanisms that can prolong the gestation period (Renfree and Shaw, 2000) in cases of stressful conditions. The significance of implantation time is not confined only to the gestation length, but implantation is of specific interest in the embryo-fetal developmental toxicity studies because it is the starting point of the treatment period (Sivaraman et al., 2008).

This study reveals that exposure to elevated ambient temperature reduces the number of IS in the rats. The number of IS in the exposed group on days 6, 7 and 8 were less than that in the controls. Reduced IS may be due to the effects of elevated temperature on the embryonic development or/and uterine receptivity. Several studies in mice have shown that maternal hyperthermia for 12 h soon after mating strongly disturbs normal embryonic development (Matsuzuka et al., 2005; Ozawa et al., 2002, 2003; Sakamoto et al., 2008). In this

experiment, to avoid the severe effect of maternal heat exposure on day 1 on early embryonic development, the rats were exposed to elevated temperature on day 2 onwards. Thus, the possibility that the reduced number of IS of stressed rats is due to early embryonic mortality, become low, but not excluded. Furthermore, the current findings indicate that heat stress can also affect the uterus. Heat stress, in this study, affected the morphological aspects of the uterus during pre- and peri-implantation periods. In addition, there was a decrease in the expression levels of several genes in the uterus of exposed rat (Yahia-Hamid et al., unpublished work), suggesting that exposure to elevated temperature may has adverse effects on the uterine receptivity and thus reduced the number of IS. The dramatic decrease in the number of IS from day 6 to day 8 (28.6% reduction) indicates that the implantation process may have been ceased at least after attachment (blue bands). This finding suggests that exposure to elevated temperature during implantation may attenuate or block some molecules that are important in the subsequent steps of implantation process.

Uterine receptivity is regulated by ovarian hormones estrogen and progesterone. Progesterone is important for the implantation and maintenance of pregnancy in all mammals, whereas requirement of estrogen is species-specific (Wang and Dey, 2006). In rats, implantation requires both estrogen and progesterone (Wang and Dey, 2006). In the present study, plasma progesterone concentrations in the control group were low in the afternoon of day 5 of pregnancy followed by a dramatic increase at the initiation point of implantation (day 5 evening) and maintained at higher levels on day 6 of pregnancy. However, the exposed group showed higher levels of progesterone before implantation (that is, on day 5 afternoon), which would lead to dyssynchrony between the embryo age and endometrium receptivity. In addition, the exposed rats showed high levels of estradiol on day 5 of pregnancy, particularly in the evening. High levels of estrogen inhibit the implantation (Gidley-Baird et al., 1986; Kramer et al., 1990; Ma et al., 2003) and reduce the pregnancy rates in IVF-treated women (Basir et al., 2001). Thus, the absence of implantation sites at this stage may be due to increased levels of estradiol.

The exposed group showed, beside the high levels of estradiol, low $P_4:E_2$ ratio in the evening of day 5; suggesting that low $P_4:E_2$ ratio may also have contributed to implantation delay/failure in this group. By contrast, decrease in estradiol levels and raise of $P_4:E_2$ ratio on day 6 of pregnancy may have given the viable embryos a late opportunity for implantation. Delayed implantation and reduced number of implanted embryos in the exposed rats, besides the low $P_4:E_2$ ratio at the implantation initiation time, support the previous findings of Gidley-Baird et al. (1986) and Kramer et al. (1990), who reported that change in $P_4:E_2$ ratio affects the number of implanting embryos in the mice and rats. Thus,

the $P_4:E_2$ ratio can be used as a good predictor for implantation success or failure rather than the absolute levels of either estrogen or progesterone.

In conclusion, elevated ambient temperatures can delay or prevent the initiation of implantation, and can also cease the implantation process even after it starts. Elevated ambient temperature changes the pattern of estradiol and progesterone during implantation. Changes in the progesterone and estradiol levels may affect the implantation directly or indirectly through their effects on the morphology of the endometrium and/or on the expression of some molecules important in the implant-tation process. The $P_4:E_2$ ratio is a good reflector for the endometrium receptivity status. Impact of temperature on implantation should be considered when IVF-ET treatment is used in the tropical areas or during hot season.

ACKNOWLEDGEMENT

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