REDUCED REPRODUCTIVE OUTCOME AND EXTENDED GESTATIONAL LENGTH IN PREGNANT RATS EXPOSED TO PREDATOR-INDUCED PSYCHOSOCIAL STRESS

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ABSTRACT

Maternal stress is commonly cited as a potential cause for idiopathic pregnancy loss. However, the mechanisms through which stress affects pregnancy are yet to be totally known. This study was designed to determine the effect of predator-induced psychosocial stress on implantation and pregnancy outcome in rat, as well as the hormonal changes associated with it. Cycling rats (n=60) in proestrus phase were paired overnight with sexually matured male in ratio 2:1. Following confirmation of mating in the morning, rats were randomly assigned to either control (n=30) or stress (n=30) group. Stress was induced by exposing rats to cat for 60 minutes/day for 14 consecutive days. Subsequently, six animals from each group were sacrificed on days 4 (embryo counting), 6, 8 (implantation studies), and 19 (for foetal parameters). Blood was collected through cardiac puncture for hormonal analysis and implantation sites (IS) determined by injection of Evans blue dye. Remaining six animals in each group were allowed to deliver at term. Significant ($p<0.05$) reduction in number of implants was observed on days 6 and 8 compared with control while weight of IS was reduced on day 8 compared with control. There was significant ($p<0.05$) reduction in the number and weight of foetuses and litters on day 19 and at term compared with their corresponding days in control. Corticosterone was elevated in the stress groups compared with control. Progesterone-oestradiol ratio was significantly ($p<0.05$) lower in stress group compared with control on days 4 and 8. Also, length of gestation was significantly ($p<0.05$) extended in the stress group compared with control. This study suggests that exposure to predator-induced psychosocial stress reduced reproductive outcome and prolonged gestation length by interfering with the pituitary-adrenal and pituitary-ovarian axes.

***Keywords**: stress, predator, implantation, progesterone:oestradiol ratio, corticosterone, gestation.*

INTRODUCTION

Infertility is defined as the inability to achieve pregnancy after 12 months or more of regular unprotected sexual intercourse (Zegers-Hochschild *et al.,* 2009). In another words, it is also the inability of a woman to naturally conceive and deliver a healthy child (Deka and Sarma, 2010). Globally, about 48.5 million couples experienced infertility (Mascarenhas *et al*., 2012) of which 30% is attributed to female factor (Liebmann-Smith, 2005).

Psychosocial stress has become one of the hallmarks of life in the modern society and is responsible for many of the health challenges happening nowadays (Kudielka and Wust, 2010). Although stress response plays an important role in ensuring the survival of an organism, when stress is prolonged, the impact can become pathological (Kalantaridou *et al.,* 2004). One of the most adverse consequence of prolong stress experience is disruption of reproductive physiology and behaviour (Wingfield and Sapolsky, 2003; William *et al.,* 2007). Common finding in stress-response is the activation of hypothalamic-pituitary-adrenal (HPA) axis leading to elevation of glucocorticoid that can directly or indirectly suppress reproduction (Tilbrook *et al.,* 2000; Chatterjee and Chatterjee, 2009). Women of reproductive age are especially known to be more affected by stress. This is because the female brain operates in a constantly changing chemical milieu caused by cyclical changes in gonadal hormones which can pass readily from the plasma to the brain where they can influence neuronal function (Legato, 2010; Lovick, 2012).

The idea that maternal psychosocial stress can have detrimental effects on reproductive outcome is not new (Brunton, 2013). Stressful experiences, such as experiences of people living in war-torn regions of the world, abusive or violent relationship, lack of a stable partnership/relationship, lack of support from family members, fear of losing career opportunities, uncertainty about the economic situation after delivery, *etc*. are some of life challenges constituting and/or predisposing to psychosocial stress in pregnant women (Hocher, 2007).

Although some studies could not find an association between psychosocial stress and a woman's ability to attain and maintain pregnancy (Klonoff-Cohen, 2005; de Klerk *et al.,* 2008), many studies have established a negative association between stressful life events or emotional stress and poor reproductive outcome (Facchinetti *et al.,* 1997; Demyttenaere *et al.,* 1998; Gallinelli *et al.,* 2001; Klonoff-Cohen and Natarajan, 2004; Boivin and Schmidt, 2005; Ebbesen *et al.,* 2009; Li *et al.,* 2011; Liu *et al.,* 2012). This is evident from poor clinical outcome in infertile patients seeking for fertility treatment from fertility centres (Chi *et al.,*

2016). Also, pregnant women who experienced terrorist attack on the World Trade Centre in the United States gave birth to babies with low birthweight (Berkotwiz *et al.,* 2003). In animal studies, there are varying reports on the impact of maternal stress on pregnancy which is dependent on the type of stressor, frequency and severity of stress, and the period during pregnancy when stress occur (Brunton, 2013; Zhao *et al.,* 2013).

However, despite numerous investigations, the mechanism through which stress, especially psychosocial stress, affects female reproductive outcome are far from total elucidation and therefore requires further investigations. Reports on the impact of psychosocial stress as a result of exposure to predator on reproductive outcome are limited. Therefore, this study was designed to investigate the effect of predator-induced psychosocial stress on reproductive outcome in an animal model of Sprague-Dawley rat.

MATERIALS AND METHOD

Sixty (60) female Sprague-Dawley rats were used for this study. They were purchased from the Animal House of the College of Medicine, University of Lagos, housed in the animal room of the Department of Physiology, College of Medicine, University of Lagos and fed with standard rat chow purchased from Livestock Feeds, Ikeja, Lagos, Nigeria. Throughout the period of experiment, the animals had access to feed and water *ad libitum* and were kept under standard condition of 12-hour light and 12-hour dark cycle. The animals were allowed two weeks of acclimatization before the commencement of the experiment. Experiment was conducted in accordance to the National Institute of Health Guide for the Care and Use of Laboratory Animals (1996).

EXPERIMENTAL DESIGN

After acclimatization, animals were randomly divided into control and stress groups. Female rats in proestrus phase of the oestrous cycle were paired overnight with mature males in the ratio of 2:1 to allow for free mating. On the following day, the presence of sperm cells in the vaginal smear confirms mating. This day was recorded and taken as day 1 of pregnancy and the beginning of implantation and pregnancy study. Six animals in each group were sacrificed on gestation day 4 (embryo counting), 6 and 8 for implantation study and 19 to assess foetal parameters. The remaining animals in each of the groups were allowed to deliver at term to study pregnancy outcome. Embryo counting was done according to the method described by Vercheval *et al.* (1990) while implantation sites (IS) were determined according to the method of Dey (2004).

STRESS INDUCTION

Pregnant rats were stressed by exposure to domestic cat (*Felis catus*) (Figueiredo *et al.*, 2003). This was achieved by using a 3-compartment cage. Cat was placed in the middle compartment of the cage, while pregnant rats were placed on either side. This allowed for visual, olfactory, and auditory stimuli input but prevented physical contact between the cat and rats. Rats were exposed to cat for 60 minutes every day between the hours 8-9am or 9-10am local time for 14 consecutive days. Time of exposure to cat was varied to reduce the possibility of habituation to repeated exposure. Animals in the stress group were exposed to cat starting from Day 1 of pregnancy.

ANIMAL EUTHANASIA AND BLOOD SAMPLING

Animals were euthanized by cervical dislocation on gestation day 4, 6, 8, and 19. Following euthanasia, blood samples were collected through cardiac puncture into plain sample bottles and centrifuged at 3000 rpm for 15 minutes to obtain serum for hormonal analysis. Serum was kept frozen at -20 °C.

HORMONAL ASSAYS

Assays were done using enzyme-linked-immunosorbent assay (ELISA) techniques in compliance with the manufacturers' instruction. Biochemical assay kit for corticosterone was obtained from Enzo-life Science while assay kits for progesterone and oestradiol was obtained from Monobind Inc, USA.

DETERMINATION OF FOOD INTAKE, ANIMAL WEIGHT, AND WEIGHT OF IMPLANTS

Food consumption by each animal was measured daily (in grams) in the morning. Food intake was determined by subtracting the food leftover on the following day from the food administered the day before. The beddings were checked for food spill over which was added to the leftover feed. This allowed 24 hour monitoring of food consumption. Weight of animals were also measured (in grams) daily. Food and animal weights were determined using a weighing balance. Weights of implants were determined using a sensitive electronic weighing balance.

STATISTICAL ANALYSIS

Analysis of data was done using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California). Results are presented as Mean± Standard Error of Mean (SEM). Differences between groups were analysed by one-way ANOVA followed by Student's Newman-Keuls post-hoc test and were considered significant when $p<0.05$.

RESULTS

Exposure to predator (stress) caused no significant difference ($p>0.05$) in number of embryo(s) counted on day 4 of pregnancy in both groups. However on days 6 and 8 of gestation, IS were significantly reduced ($p<0.05$) in pregnant rats exposed to predator compared with control. Weight of implants was significantly reduced ($p<0.05$) on day 8 of gestation compared with control and rats sacrificed on day 6 (Table 1).

Table 1: Effect of Predator-induced Stress on number and weight of Implantation Sites

	Control	**Stress**
Number of embryo (Day 4)	11.17±0.70	9.67±0.49
Number of Implants (Day 6)	10.83±0.48	5.30±0.56*#
Number of Implants (Day 8)	10.50±0.56	6.40±0.72*#
Weight (g) of Implants (day 6)	0.047±0.005	0.036±0.003
Weight (g) of Implants (day 8)	0.064±0.010	0.035±0.002*

Values are expressed as Mean ± SEM. *shows significant difference to control at $p<0.05$; # shows significant difference compared with stress day 4 at $p<0.05$.

Effect of Predator-induced Stress on Pregnancy Outcome in Rats

Predator-induced stress significantly reduced ($p<0.05$) the mean number and mean weight of foetuses in animals sacrificed on gestation day 19 compared with control. Similarly, the mean litter size and mean weight of offspring was significantly reduced ($p<0.05$) compared with control. There is no significant difference in the number of foetuses and mean litter size between the stressed groups on day 19 and term (Table 2).

Table 2: Effect of exposure to predator on Pregnancy Outcome in Rats

	Control	**Stress**
Number of foetus (Day 19)	9.00±0.37	6.00±0.37*
Weight (g) of foetus (Day 19)	1.61±0.17	1.15±0.09*
Number of Resorption sites (Day 19)	0.00±0.00	0.00±0.00
Weight (g) of Placenta (Day 19)	0.47±0.02	0.33±0.06*
Litter size (Term)	9.83±0.54	6.17±1.01*
Weight (g) of litters (Term)	5.64±0.15	4.09±0.14*
Birthweight:Placental weight Ratio	12.00±0.01	12.39±0.02
Gestation length (days)	21.33±0.21	22.83±0.17*

Values are expressed as Mean ± SEM. *shows significant difference compared to control at $p<0.05$.

EFFECT OF PREDATOR-INDUCED PSYCHOSOCIAL STRESS ON CORTICOSTERONE, PROGESTERONE AND OESTRADIOL DURING PREGNANCY

Corticosterone level increased significantly (p<0.05) in animals exposed to predator (stress) compared with control. Progesterone was significantly reduced (p<0.05) on day 4, 6, and 8 in stressed animals compared with corresponding days in control, but no significant difference in serum progesterone was observed on day 19 in both groups. Serum oestradiol was significantly increased (p<0.05) in stressed exposed animals sacrificed on day 4 and 8 compared with control and corresponding days in stressed groups.

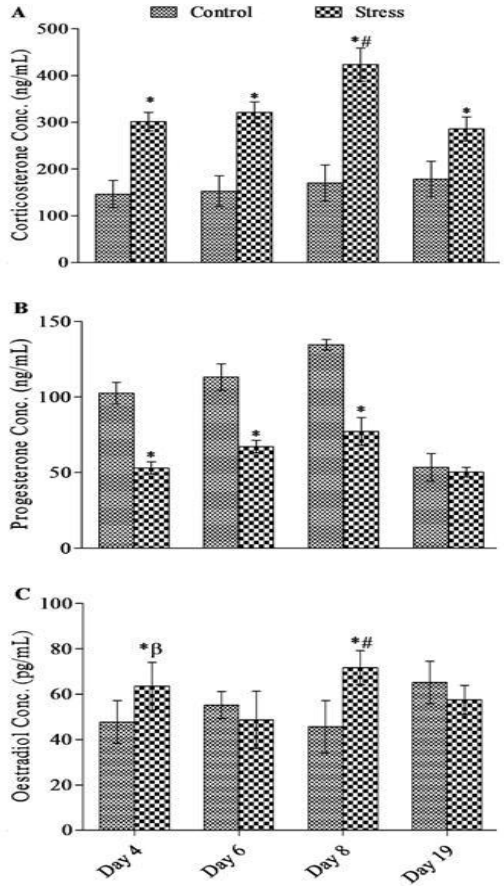

Figure 1: Effect of exposure to predator stress on (a) corticosterone (b) progesterone and (c) oestradiol levels during pregnancy. Values are expressed as Mean ± SEM. * shows significance compared to control. # shows significance compared with stress groups.

EFFECT OF PREDATOR-INDUCED PSYCHOSOCIAL STRESS ON PROGESTERONE:OESTRADIOL RATIO (P:E_2)

The P:E_2 was significantly lower (p<0.05) in predator-exposed pregnant rats sacrificed on day 4 and day 8 of gestation compared with respective days in control rats. No significant difference was observed in predator-exposed pregnant rats on day 6 compared with control. This was done by dividing the value of progesterone in ng/mL by the value of oestradiol in ng/mL (Table 3).

Table 3: Effect of predator-induced psychosocial stress on progesterone:oestradiol ratio in rats during gestation.

	Day 4	Day 6	Day 8
Control (/1000)	3.82±0.25	2.73±0.24	3.22±0.25
Stress (/1000)	1.59±0.18*	2.10±0.30	1.79±0.12*

Values are expressed as Mean ± SEM. *shows significance compared to control at p<0.05.

Effect of predator-induced psychosocial stress on body weight and feed intake

Exposure to predator caused significant decrease (p<0.05) in percentage weight gained in stressed animals compared with control (Table 4). Similarly, predator-exposed animals had significant reduction (p<0.05) in feed intake compared with control (Table 5).

Table 4: Effect of predator-induced psychosocial stress on percentage (%) weight gain of rats during gestation

	Day 4	Day 6	Day 8	Day 19
Control (%)	3.12	2.45	4.84	22.51
Stress (%)	1.17*	-0.39*	-0.71*	13.27*

Values are expressed as Mean ± SEM. *shows significant difference to control (p<0.05).

Table 5: Effect of predator-induced psychosocial stress on mean daily feed intake of rats during gestation

	Day 4	Day 6	Day 8	Day 19
Control (g)	19.12±0.77	18.87±0.77	17.26±0.91	18.68±0.66
Stress (g)	15.20±1.13*	13.47±1.13*	13.43±1.22*	14.54±1.65*

Values are expressed as Mean ± SEM. *shows significant difference to control (p<0.05).

DISCUSSION

From this study, the observed elevation in serum concentration of corticosterone confirms stress-induced HPA axis activation in predator-exposed rats (Figueiredo *et al.,* 2003) and has been previously associated with negative reproductive outcome (Brunton, 2013). Exposure of pregnant rats to predator for fourteen consecutive days resulted in reduced implantation sites (IS), decrease in litter size and decrease in litter weight. However, the reduction in weight of IS was only significant in stressed animals on day 8 of pregnancy. From the above observation, it can be inferred that the significant impact of this stress model on weight of IS in rat begins around day 8 of pregnancy. This reduction in weight of IS seems to have been carried over to post-implantation period as observed in foetuses on day 19 and litters at term. In addition, exposure of pregnant rats to predator caused significant increase in length of gestation. This is contrary to most reports in literature implicating stress as one of the aetiologies of miscarriages and preterm birth (Nepomnaschy *et al.,* 2006; Tegethoff *et al.,* 2010, Paris *et al.,* 2011).

The result of this study also revealed significantly perturbed serum concentrations of progesterone and oestradiol. These ovarian hormones are critical in the control of uterine receptivity and are pre-requisite for implantation (Wang and Dey, 2006; Zhao *et al.,* 2013). Certainly, disturbance in the circulating levels of these hormones as a result of stress exposure will have a negative impact on implantation and pregnancy outcome (Arck *et al.,* 2008; Parker *et al.,* 2011). In this study, exposure to predator during implantation significantly decreased progesterone and increased oestradiol levels. Although no pregnancy loss was recorded in this study, significant elevation of oestradiol has been previously reported to reduce implantation and cause pregnancy loss (Basir *et al.,* 2001; Ma *et al.,* 2003; Thorpe *et al.,* 2013). In fact, administration of oestrogens beyond the minimal level has been previously used to mimic the effect of stressors on implantation leading to loss of blastocysts (de Catanzaro *et al.,* 2011).

In rat, the progesterone:oestradiol ($P:E_2$) ratio is a good predictor of implantation which begins on day 4 of gestation and is completed on day 7 or 8 (Gidley-Baird *et al.,* 1984; Hamid *et al.,* 2012; Feuer and Rinaudo, 2012). Therefore, low $P:E_2$ ratio recorded in the morning of day 4, which is preimplantation period, explains the significant decrease in the number of IS. Low $P:E_2$ ratio has been associated with decrease in uterine receptivity and consequently reduced the number of implanting embryo in rats (Hamid *et al.,* 2012). It is of note that no resorption was recorded during implantation period which suggests that the stress model used in

this study did not affect implantation process per se once it has begun. Rather it appears to limit the chances of blastocyst getting implanted. This observation is validated by the fact that there was no significant difference in the number of embryo counted in stressed and control animals that were sacrificed in the morning of day 4 of pregnancy. Stated differently, the number of embryo were significantly higher in stressed animals sacrificed on day 4 compared with other stressed groups on day 6, 8 and 19.

The pre-implantation effect of stress leading to reduced number of blastocyst implanted is an indication of blastocyst wasting (Klonoff-Cohen *et al.,* 2001). This findings support the reprt of Klonoff-Cohen *et al.* (2001) that infertility is not only a result of unfertilised ova but also loss of blastocysts before implantation. In fact, preconception stress has been shown to promote infertility in humans (Lynch *et al.,* 2014). At times, this may be as a result of a non-receptive uterine environment as suggested previously (Hamid *et al.,* 2012; Zhao *et al.,* 2013). The implication of blastocyst wasting is clear if it happens in humans. Subjection of a woman to psychological stress at pre-conception and/or peri-conception period might result in the lost of the zygote or blastocysts. If this were to happen, it is highly unlikely that the woman would be aware that she was ever pregnant. Such fertility case is likely to be, albeit wrongly, diagnosed as idiopathic.

Exposure to elevated level of glucocorticoid in maternal circulation as a result of stress has been previously reported to have adverse effect on foetal development (Field and Diego, 2008; Brunton, 2013). In this study, the significant reduction in foetal weight and birthweight in the offspring of stressed animals is an indicator of intra-uterine growth restriction (IUGR) (Lesage *et al.,* 2004) and might be due to the catabolic effect of elevated glucocorticoid (Valassi *et al.,* 2008; Delaere *et al.,* 2010) or reduced cellular proliferation. Therefore, the combined catabolic effect of elevated glucocorticoid and reduced feed intake during gestation may be part of the mechanisms responsible for the reduction in birthweights of the offspring from stressed animals. This is because pregnancy is a state of increased nutritional demands especially for the growing foetus, failure of which can affect developmental processes in utero (Eskandari, 2007; Amungongo and Hlusko, 2014). Although significant reduction in placental weight was observed in the stressed animals, there was no significant difference in the birthweight:placental weight ratio. This suggests a normal or increased placenta function in supporting pregnancy (Hayward *et al.,* 2016). This is an indicator of placental adaptation to meet foetal nutritional requirement, especially when the environment is suboptimal (Fowden *et al.,* 2009). However, in this study, increase in nutritional

supply could not override the effect of elevated glucocorticoid nor compensate for IUGR in the offspring of stressed dams. Birthweight of offspring is a determining factor of a healthy life style later in life. Low birthweight has been associated with adulthood hypertension, insulin resistance and dyslipidaemia, leading to markedly increased rates of cardiovascular disease and non-insulin-dependent diabetes in adult life (Hocher, 2007, Balci *et al.,* 2010).

Findings in literature showed that stress caused reduced length of gestation in humans (Nepomnaschy *et al.,* 2006; Tegethoff *et al.,* 2010) and rats (Paris *et al.,* 2011) resulting in preterm birth and low birthweight. However, in this study, the extended length of gestation observed in predator-exposed rats may be part of the adaptive compensatory mechanisms for IUGR to allow enough time for foetal development that will guarantee survival in the extra-uterine environment. Parturition in rats involves luteolysis with a decrease in serum and uterine concentration of progesterone starting from day 19 of gestation. This fall has been associated with changes in uterine reactivity towards term (Pepe and Albrecht, 2008). In this study, despite the fall in serum concentration of progesterone stressed animals, gestation was extended. This is suggestive of other factors coming into play which can overrides the drop in progesterone concentration that normally should have led to parturition.

CONCLUSION

The results obtained from this study suggest that exposure to predator-induced psychosocial stress negatively impact reproductive outcome beginning precisely at preconception period through hormonal disturbances consequently resulting in reduced implantation and poor pregnancy outcome. In addition, gestation period was extended possibly as a compensatory adaptive mechanism which could not reverse IUGR in the offspring of stressed dams. Further studies are required to understand the underlying factors that are responsible for the extended gestational length.

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