J Endocrinol Reprod 13 (2009, 1 : 1-6)

Variation in gonadal steroid and melatonin during gestation of a diurnal seasonal breeder Indian palm squirrel *Funambulus pennanti*

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SUMMARY

Very little information is available to delineate the role of melatonin during the complex process of gestation in seasonally breeding mammals. The present study was aimed at understanding the physiology of gestation and fetal growth in relation to maternal hormonal levels, including melatonin, of a tropical seasonal breeder, *Funambulus pennanti*. Through assessment of the vaginal smear during breeding season (March) we selected the early pregnant females (~5 days) from nature and provided them adequate facility in laboratory as in nature. The total length of gestation period i.e., ~45 days, comparatively a long gestation period for a diurnal rodent, was recorded. The litter size was small (3-4), with the maximum number of pregnancies during March to May (in August to September, very less) depending upon the availability of food. The intrauterine growth of the fetus is divisible into three phases, i) implantation and cell division, ii) organ formation, and iii) growth and completion of intrauterine development. A method for the quantitative analysis of fetal growth was devised. A correlation with the levels of maternal hormones suggests that a direct relationship of progesterone and melatonin exists during pregnancy, which declined immediately after parturition. The high melatonin level during gestation, having an almost inverse relationship with estradiol level, suggests that melatonin might play a role in maintenance of pregnancy. Variations, if any, in the melatonin receptor expression in the uterus throughout the gestation period may throw some light on the role of melatonin in maintenance of pregnancy.

Introduction

In general, the number of litters per year in seasonal breeders, such as squirrel, varies from 3-4. Prolonged maternal care during embryonic and neonatal periods substitutes for huge numbers of unattended offspring as the means for increasing the likelihood of survival. After lodging firmly within the uterine lining and gaining access to the maternal circulation, the animal secretes protein and steroidal hormones that ensure continued maternal acceptance, and facilitate maternal function to provide for embryonic development. The establishment of pregnancy involves maternal recognition of pregnancy and implantation (Geisert et al., 2006; Spencer et al., 2007). In most mammals progesterone production by the corpus luteum is required for successful pregnancy (Tajima et al., 2006; Stocco et al., 2007). Progesterone acts on the uterus to stimulate and maintain uterine functions that are permissive to early embryonic development, implantation, placentation and successful fetal and placental development to term (Ghaemi et al., 2008). Prolonged lifespan of the corpus luteum is a characteristic feature of mammalian pregnancy in species with a gestation period that exceeds the length of a normal estrous or menstrual cycle, for example, domestic animals, laboratory rodents and humans.

A great many number of wild species follow a seasonal pattern of reproduction in order to give birth to young ones at the most favorable period of the year so as to allow the newborn to grow and develop under favorable temperature and conditions of food availability. In this very complex process of gestation that reflects on activity of numerous endocrine pathways, melatonin plays a central role. Melatonin is necessary for entrainment of seasonal photoperiodic responses to the annual cycle of day length and probably regulates reproduction by acting at three levels *viz*., i) the hypothalamic GnRH neurons, ii) the pituitary, and iii) the gonads and reproductive tissues (Kennaway and Rowe, 1995).

Until a little more than a decade ago no information was available on whether the pineal gland has any influence

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on gestation, or *vice versa*, whether pregnancy has any effect on pineal metabolism and function (Bishnupuri and Haldar, 2001). The first observation on such an interrelationship was published by Huang and Everitt (1965). Later, the ultrastructural observation of "light" and "dark" pinealocytes during the second half of the pregnancy provided supportive evidence for heightened pineal activity during that period (Gil et al., 2005).

To date, reports on the gestational physiology of wild animals that are seasonal breeders are very scanty, and little information is available pertaining to the seasonal breeders that inhabit the tropical zone. Here in we made an attempt to study in detail the physiology of gestation and fetal growth in relation to maternal peripheral melatonin and other hormonal levels of a tropical seasonal breeder, the Indian palm squirrel *Funambulus pennanti*.

Materials and Methods

Animals and maintenance

Sixty pregnant female Indian palm squirrels *Funambulus pennanti* during their 1st week of pregnancy (as judged by the presence of dead spermatozoa in the vaginal smear) were collected from the vicinity of Varanasi (Lat.25^0 18'N; Long. 83^0 1'E) in the month of March. A large number of female squirrels (body weight 115 $\pm$ 5g) during this part of the year showed the least variation in the gestation period (i.e., +/- 2-4 days), and, hence, it was easy to select the animals for the experiments. The non-pregnant and all pregnant females were kept in separate wire net cages (25" x 25" x 30") in the animal room fully exposed to ambient conditions of light, temperature and humidity and were provided with soaked gram (*Cicer arietnum*) and water *ad libitum*.

All the experiments were conducted in accordance with the institutional practice and within the framework of Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) and the rules of Government of India 2001 on animal welfare.

Experimental design

The experiment was conducted during March – May (the environmental variations were: temperature maximum 30.4 ^{0}C to 34.6 ^{0}C, minimum, 14 ^{0}C to 18.7 ^{0}C; humidity maximum 52.5 to 50.2%, minimum 34.5 to 32.2%; photoperiod 11.45 to 13.15 hours) for a complete gestation period till delivery.

Sampling

During the experiment six pregnant female squirrels were sacrificed at the interval of 5, 10, 15, 20, 25, 30, 35, 40 days of gestation and after delivery (~45 days). The body weight of squirrels was noted. The ovary and uterus were dissected out, weighed in a digital balance and fixed in Bouin's fluid for histological observation.

Fetal growth rate analysis

The fetuses of different stages were isolated from the uterus to judge the fetal development. The isolated fetuses were cleaned and weighed. The body length (from snout to vent), head length (from snout to the base of head) and head diameter (from one ear base to the other) were measured using a divider and a scale as shown in figure 1.

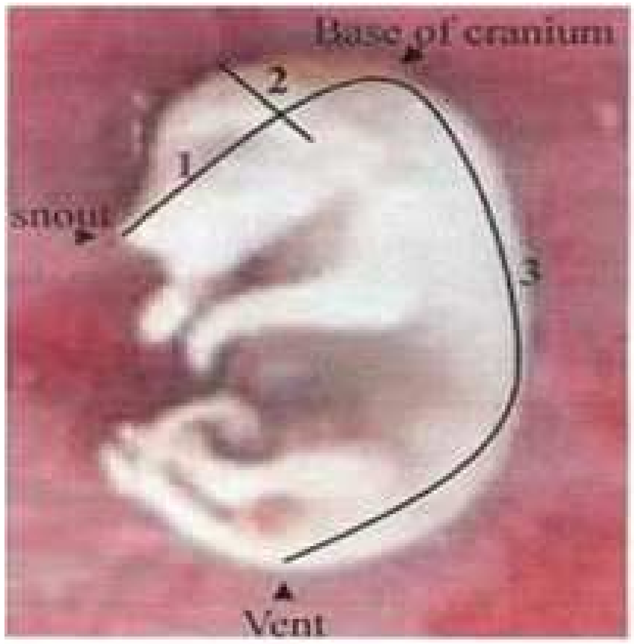

Fig. 1. Photograph of a fetus showing details of fetal growth analysis. 1. Head length in cm (Snout to base of cranium); 2. Head diameter in cm (From one ear base to another); 3. Body length in cm (Snout to vent)

Hormone analysis

For the RIA of hormones (melatonin, estradiol and progesterone), blood (0.5 ml) was collected in heparinized tubes from the subclavian of each of the pregnant females during the night hours, under dim red light, prior to the day of sacrifice, centrifuged at 3000 x g to separate the plasma, and stored at –20 ^{0}C until analysis.

Radioimmunoassay

RIA of estradiol was performed using a commercial kit (Leuco Diagnostic Inc., Miss., USA). The recovery and sensitivity of estradiol were 102.2% and

1-45 pg/ml, respectively. Intra- and inter-assay variations of estradiol were 9.2% and 4.3%, respectively. Progesterone was assayed using a commercial RIA kit (Binax, Portland, Maine, USA). The sensitivity of the assay was 50 pg/tube and the intra- and inter-assay coefficients of variation were 6.5% and 8.7%, respectively. RIA for melatonin was performed according to Rollag and Niswender (1976), using anti-melatonin antibody (Stock Grand, Surrey, UK). The recovery, accuracy and sensitivity for the melatonin RIA were 92%, 0.98 and 10 pg/ml, respectively. Intra- and inter-assay variations of melatonin were 9.0% and 15%, respectively.

Statistical analysis

The data are presented as means ± S.E.M. (M ± S.E.M.). The data were analyzed by one-way analysis of variance (ANOVA; Brunning and Knitz, 1977) followed by Student's *t*- test. Differences of mean were considered significant when $P<0.05$.

Results

Fetal growth rate

There was a slow but steady increase in fetal weight, length, head diameter and length upto the 30th day of gestation, which then slowed down up to delivery (Fig. 2).

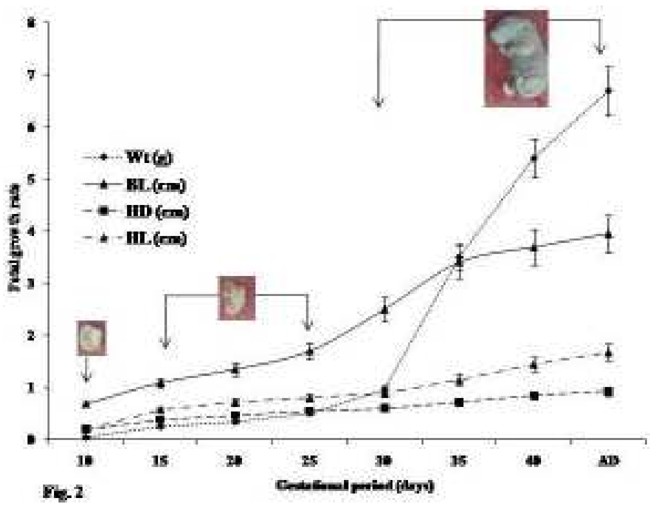

Fig. 2. Graph showing fetal growth rate as measured by body length (BL, snout to vent, cm), head diameter (HD, cm), head length (HL, snout to occipital junction, cm) and body weight (g) during different gestational periods of squirrel. Upper panel shows the actual photograph of the growing fetus till delivery. (Vertical bar on each point presents mean ± S.E.M.).

Body, uterus and ovary weight of pregnant squirrel

Body weight of the pregnant females started to increase from the 5th day of pregnancy. Thereafter, a steady increase in body weight was noted up to the 40th day. Generally, after the 40th days, the pregnant squirrels delivered and, therefore, a sudden decrease in body weight was noticed following delivery. The uterine weight increased gradually from the 5th day onwards until 25th day of gestation, where onwards up to the 40th day of gestation there was a dramatic increase in the uterine weight. Subsequent to delivery, the uterine weight decreased. On the other hand, the ovarian weight showed a slow and steady decrease up to the 40th day of gestation and the trend was continued even after delivery (Fig. 3).

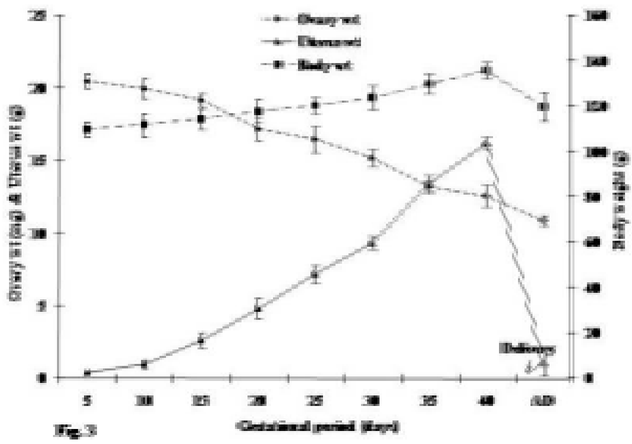

Fig.3. Graph showing changes in body weight and relative uterus and ovary weights of pregnant females during the gestation period and after delivery (~45 days) of squirrel. (Vertical bar on each point presents mean ± S.E.M.)

Estradiol, progesterone and melatonin levels

The peripheral levels of estradiol and progesterone increased from the 5th day onwards until 40th day of gestation. This was followed by a sudden decrease of progesterone level. The estrogen level increased gradually from the 40th day. Melatonin level was low on the 5th day of pregnancy. There was a slow but steady increase in melatonin level up to the 40th day of gestation, followed by a sudden decrease in melatonin level after delivery (Fig. 4).

Discussion

Annual cycle of external light, temperature and food availability plays an important role in the seasonal reproduction (Nelson et al., 1997) and causes changes in

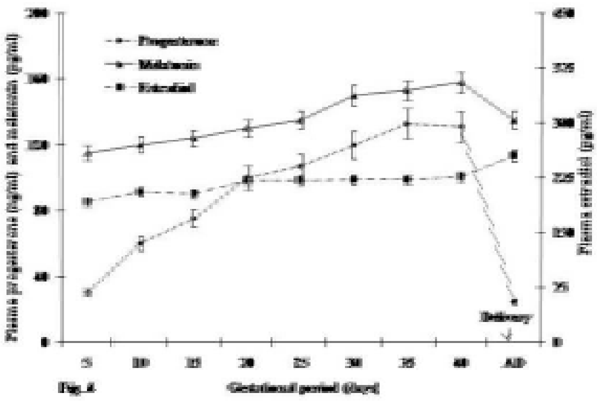

Fig.4. Graph showing variation in peripheral level of melatonin (pg/ml), progesterone (ng/ml) and estradiol (pg/ml) of pregnant females during the gestation period and after delivery (~45 days) of squirrel. (Vertical bar on each point presents mean ± S.E.M.).

reproductive behavior, weight gain, appetite, body fat storage and energy metabolism, growth of fibers and horns and/or hibernation (Bartness et al., 1993). The squirrel model of our study is a seasonal breeder. It breeds twice a year. Pregnancy occurrs from March to May in a large number of squirrels, with the largest litter size (3 to 4) while a few pregnancies were also recorded in August-September with a smaller litter size (1 or 2). This would be due to the abundant availability of food, such as papaya, banana, ground nuts, grams, etc., in nature during spring (March to May) while monsoon rainfall (August to September) would limit availability food, shelter and movement. We recorded the total length of gestation period i.e., ~45 days, which is a fairly long period for a diurnal rodent.

It has been suggested that the females gain body weight during the breeding season due to most favorable environmental conditions such as availability of food and sufficient place for their movement and mating (Inns and Miller, 1981). Fertilization of the ova by the spermatozoa occurs in different parts of the uterine tubes and, after certain developmental stages, the embryos are implanted in the uterus. After implantation the pregnant squirrels accumulate fat in the lower abdomen and gain weight. The fat thus accumulated serves as a cushion for the developing embryos when the squirrels jump from tree to tree and run in search of food. Later on, this fat becomes the source of energy for lactation during postpartum. After parturition, the females show a decreasing trend in their body weight due to delivery of pups and utilization of abdominal fat for lactation. It usually takes ~60 days to restore the normal body weight.

The initiation and maintenance of pregnancy depends primarily on the interactions of neuronal and hormonal factors. Proper timing of these neuroendocrine events within and between the placental, fetal and maternal compartments is critical in directing fetal growth and development as well as in coordinating the timing of parturition. The secretions from the endometrial epithelia influence conceptus (embryo/fetus and associated extra-embryonic membranes), survival, growth and development in all mammals (Gray et al., 2001; Burton et al., 2002; Bagchi et al., 2003). The intra-uterine growth of fetuses of the Indian palm squirrel can be divided into three phases:

Implantation and cell division: Beginning with implantation of embryos in the uterine wall there would be cellular division, continued approximately up till ~7 days. Up to this period of gestation the embryos look like a cell mass (pennicule).

Organ formation: Fetal organ formation would start from around the 10^{th} day of gestation. Differentiation of the head was the earliest perceptible development. The differentiation of abdominal region, limbs, tail, etc., was apparent on around day 30 of gestation.

Growth and completion of intrauterine development: From ~30^{th} day of gestation the embryo increased in size and weight dramatically. During this phase rapid growth in length, diameter and weight of the fetus occurred. At the time of parturition the average weight of a fetus was 6 ± 1 g .

The maximum weight of the ovary was observed during the mating phase (from first week of March). During pregnancy, the weight of the ovary decreased and again increased following parturition to make the female reproductively active for the next breeding phase. At the same time the plasma level of estradiol and progesterone gradually increased during the gestation period, attaining peak value by the time of parturition. After parturition, the plasma progesterone level sharply declined since progesterone is required essentially for the maintenance of pregnancy, where as the plasma estradiol level increased slowly as it is required to facilitate the lactation in the mothers. Maternal adaptations to hormonal changes that occur during pregnancy directly reflect the development of the fetus and placenta. Gestational adaptations that take place in pregnancy include implantation, maintenance of early pregnancy, providing adequate nutritional support to the developing fetuses, preparation for parturition and subsequent lactation.

Uterus enlarged and the uterine weight increased significantly during the entire gestation period. It varied with the number of implantations in the pregnant squirrels. After parturition the uterus began to shrink and took approximately 20 days to achieve the normal status. The maintenance of uterine condition is a progesterone-dependent phenomenon (while ovarian function is maintained by estradiol); hence, there was an increase in both plasma progesterone level and uterine weight, which is necessary for protection and maintenance of the implanted embryos.

In general, the pineal gland exhibited an inverse relationship with gonads. But, following pregnancy the plasma levels of estradiol and progesterone increased gradually. At the same time pineal gland activity, as judged by plasma melatonin level, indicated an increasing trend throughout the gestation period and exhibited a direct relationship with estradiol level or ovarian steroidogenesis during the gestation period (Haldar and Bishnupuri, 2002). Hence, we present for the first time data to show that the relationship of pineal gland / melatonin level appears to be a gestation phase-dependent phenomenon because, after parturition, the plasma estradiol level increased, while progesterone and melatonin levels decreased.

Multiple neuroendocrine target tissues interpret the daily melatonin signal through the expression of high affinity G-protein-coupled melatonin receptors. Melatonin receptors have a highly restricted distribution in adult mammals, but are more widely expressed in fetal and neonatal tissues, suggesting a specific role for melatonin in maintenance of development (Davis, 1997). Some studies have shown that melatonin sensitivity is shut down by activation of the GnRH system around parturition. This suggests that melatonin's role may depend on the effects exerted during fetal life, before the GnRH system becomes established. However, the degree of negative correlation between the pineal gland and the central nervous system is seasonally dependent in squirrels (Singh and Haldar, 1996; Debrceeni et al., 1997).

The maternal melatonin signal to the fetus and to the developing neonate may be an essential factor in the emerging circadian or seasonal systems of the infant and indeed the adult as well. This hypothesis is supported by the widespread presence of melatonin in rat fetal tissues (Klein, 1972) or melatonin receptors in the human fetus, particularly in the SCN (Yuan et al., 1991). The transient expression of high-density melatonin receptors in gonadotrophs appears to be critical to the fetal programming of the reproductive axis (Vanecek, 1998), i.e., melatonin may affect the onset of puberty (Heideman et al., 2001) through its inhibitory effects on the pubertal activation of the reproductive functions (Ebling et al., 1989). Therefore, a detailed study on the melatonin receptor expression in the uterus throughout the gestation period will be of great interest to establish the correlation of melatonin, estradiol and progesterone with gestation of small rodents.

Acknowledgements

We thank the Council of Scientific and Industrial Research (CSIR), New Delhi, for providing financial support and the Alexander von Humboldt Foundation (Germany) for gift of equipments.

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