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Effect of photoperiod on cultured granulosa cells of the bank vole, *Clethrionomys glareolus*

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Abstract

Gonadal function of the bank vole females depends on the photoperiod. This experiment was to show whether photoperiod applied on the whole animal in vivo would affect the function of ovarian cells in vitro. Granulosa cells from large ovarian follicles of bank vole reared in long or short photoperiod were cultured as monolayers in control or luteinizing hormone supplemented media. Formation of cell colonies, activity of Δ^5, 3β-hydroxy steroid dehydrogenase and progesterone secretion were investigated. First colonies of long day cells were formed already on day 1. On day 2 they enlarged and became abundant. Short day cells formed colonies only on day 2. Colonies of similar size to 2 day colonies of long day cells appeared only on day 6. There were also differences in steroid dehydrogenase activity and in progesterone secretion between long and short day control and hormone treated cultures. We conclude that photoperiod applied in vivo affects ovarian cell function in vitro.
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Keywords: Bank vole; Cell culture; Granulosa cells; LH; Ovary; Photoperiod; Progesterone; Steroid dehydrogenase

1. Introduction

Bank voles (*Clethrionomys glareolus* Schreber) breed in the wild usually from March to September. They are generally in an infertile state in late autumn and in winter. Experimental studies have established that in these animals day length or photoperiod are the principal environmental factors regulating gonadal development and function. Bank voles exposed to long day (LD) photoperiod had larger ovaries with more large follicles, larger uteri and showed an increase in the activity of steroidogenic enzymes than those kept in short day (SD) photoperiod (Tähkä, 1980; Clarke, 1985; Kruczek, 1986, 1988; Kruczek and Marchlewska-Koj, 1986; Meek and Lee, 1994; Lee, 1999). It has also been shown that ovarian homogenates of long day bank voles contained more progesterone (P_4) than their short day counterparts (Galas et al., 2003). A similar effect of the photoperiod on steroidogenesis of ovarian follicles isolated from bank vole females has been observed in follicle organ culture (Galas, unpublished) Such changes in the ovaries are no doubt to be related to alterations in the amounts of gonadotropins secreted by the anterior pituitary gland (Clarke and Greig, 1971; Craven and Clarke, 1982; Moffatt et al., 1995).

In large preovulatory follicles granulosa cells (GC) acquire luteinizing hormone receptors (LHRs). Luteinizing hormone (LH) bound to them stimulates P_4 secretion.

Abbreviations: BSA, bovine serum albumin; DHEA, dehydroepiandrosterone; GC, granulosa cell; HSD, hydroxysteroid dehydrogenase; LD, long day; LH, luteinizing hormone; LHR, luteinizing hormone receptor; MEM (Eagle), minimum essential medium; NBT, nitroblue tetrazolium salt; NAD, nicotinamide adenine dinucleotide; P_4, progesterone; ROD, relative optical density; SD, short day; SEM, standard error mean.

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This gonadotropin acts via G protein coupled receptors expressed on follicular cell membranes of the ovary (Tsuchiya et al., 1999; Inoue et al., 2002; Robert et al., 2003). Richards (1980) measured the dynamics of LHR levels during the estrous cycle of the rat using a model of isolated GCs cultured with radiolabeled hCG. The results showed that the hCG binding markedly increased in cells isolated during proestrus. This was then confirmed in vivo in rats injected with labeled hormone (Richards, 1993, Takao et al., 1997). These data proved that LHRs appear and increase in number on GCs in late stages of folliculogenesis thus in large proestrus follicles. In view of those results it is possible to assume that changes in the number of LHR on GCs determine their response to LH.

Szoltys et al. (1982), Stoklosowa et al. (1982) and Stoklosowa (1989) showed that isolated rat ovarian follicles in organ culture, or porcine GC in cell culture were viable and secreted P_4 for a limited time of 2–3 or 4–6 days, respectively, in a manner characteristic for in vivo condition. Beyond this time, cells dedifferentiated which was expressed by the decline in P_4 secretion. Consequently, so long as cells in culture remain differentiated they retain their in vivo functions (Stoklosowa, 1989; Freshney, 2000). These data inclined us to use the model of primary GC cultures which enables the study of homogenous cell population apart from the complex structure of the ovary and from multiple interactions of various cells types within this gland. The aim of this investigation was to determine whether the photoperiod to which animals were exposed in vivo from birth to maturity, would affect the function of GCs in culture.

2. Materials and methods

2.1. Animals

Bank voles (*C. glareolus* Schreber) from wild population living in the Bialowieza National Park have been in captivity for 10 years. Animals used in the present experiment were born and reared under "Osram" white light in LD (18 h light/6 h darkness) or SD (6 h light/18 h darkness) light regimes until the age of 78–80 days. The light intensity at the cages was 250 lux. Animals were housed at 18±2 °C in polyethylene cages (42×27×18 cm) furnished with sawdust for bedding, and given a standard pelletted rabbit chow diet (Lomna, Poland) supplemented with vegetables, apples and cottage cheese, with a constant water supply. The parents of these experimental animals had also been kept in the same lighting regimes.

The young females were separated on day 18–20 of postnatal life, and kept in single sex cages of 3 females for further 60 days. At this time all females both from long and short photoperiods, had perforated vaginas. Animals were sacrificed by cervical dislocation. Average body, uterine and ovarian weights for the LD and SD animals were measured. Ovaries were aseptically excised on ice and collected in Petri dishes containing cold, serum-free culture medium (Eagle MEM). Large LD and SD follicles were aseptically isolated from ovaries under the dissecting microscope. Follicular diameter was measured one by one using an ocular micrometer. LD and SD follicles were separately stored in two Petri dishes with Eagle MEM medium without serum. Follicles were then counted and kept on ice for further GC collection. Altogether 60 LD and 125 SD females were used as follicle donors in the experiment. Slightly more than twice as many SD females as LD females were required since the large follicles from which GCs were collected were about half as frequent in LD ovaries (Table 1b).

2.2. Ethics of experimentation

The experiments were carried out in accordance with the Polish legal requirements, under the license given by the Commission of Bioethics at the Jagiellonian University.

2.3. Cell culture

We used GCs from large follicles of type 7 and 8 (Pedersen and Peters, 1968). Their diameter varied around 750 μm. Healthy, well vascularized, translucent follicles were aseptically isolated. Atretic large follicles, recognized by their opacity and poor vascular supply, were discarded. Under a stereomicroscope, follicles were punctured with an intradermal needle, gauge 26, connected to a 1 mL syringe containing 0.1 mL of serum-free culture medium. After puncturing, the follicles were gently squeezed with small, blunt ended forceps. Released GCs were sucked into the syringe and then collected in a 2.5 mL beaker containing serum-free culture medium. Cells were then rinsed with the medium and centrifuged at 300×*g* for 10 min. Before culture set-up, the cells were counted using a haemocytometer and the cell suspension was diluted to a concentration of 1×10^5 cells/mL. One milliliter of the suspension was added to each LD or SD culture well (Nunc).

For each culture a total of 40 large follicles from 10 LD or 20–25 SD females provided sufficient amount of GCs for initial inoculum for 20 wells. Cells were cultured in a control medium (Eagle MEM) supplemented with 10% calf serum, or in the same medium enriched with luteinizing hormone, LH (LH/NIH/LH-S-8-Ovine, the National Pituitary Agency, NIH, Bethesda, MD, USA), at a concentration of 100 ng/mL. The dose of 100 ng LH has been long known as the most effective one in ovarian cell culture experiments (Channing, 1969; Gregoraszczuk, 1983). Cells were grown as monolayers in 4 well plastic

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