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Morphometric changes in the *corpus luteum* of pregnant hypokinetic rats

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The aim of this work was to study the influence of hypokinetic conditions on the ovary and corpus luteum *of pregnant rats. The rats were kept in hypokinetic conditions for 5 days in the period between the 13th and 18th days of pregnancy. A three-dimensional reconstruction of the ovary and* corpora lutea *and also a stereological evaluation of the luteal cells and their nuclei were performed using serially cut material. Hypokinesia caused a decrease in the mean volume of the ovary and individual* corpus luteum *and in the total volume of* corpora lutea *per ovary in immobilised animals as compared to the control. Moreover, a decrease was observed in the mean number of luteal cells and an increase in the size of these cells, as well as in the mean volume fraction of their nuclei. These results indicate that immobilisation of pregnant rats for 5 days considerably influences the morphology of the* corpus luteum *and luteal cells.*

Key words: immobilisation, stereology, ovary, luteal cells

INTRODUCTION

Hypokinesia induces a large number of morphological and functional changes in many organs, including the female reproductive system. Hypoplastic changes have been found during the oestrus cycle in immobilised animals, resulting in a decrease in the number of *corpora lutea* and strong expression of atresia of the follicles [11].

Immobilisation stress during different periods of pregnancy in rats [6, 10] and mice [26] significantly reduced the number of implantation sites *in utero* [10, 26] and increased intrauterine mortality and foetal loss. It also impaired foetal development [6], which caused a delay in postnatal growth [1], reduced fertility and fecundity in female offspring [8] as well as feminisation and demasculinisation of male offspring [20]. Of the rats studied 50% showed that implantation failed to deliver [10].

To our knowledge there are only a few fragmentary reports in the literature on changes to the *corpus luteum* of pregnant animals induced by immobilisation. The aim of our study therefore was a complex morphometric analysis of the *corpus luteum* of rats kept in hypokinetic conditions. Animals were immobilised from Day 13 to Day 18 of pregnancy. A three-dimensional (3-D) reconstruction disclosed a decrease in the mean volume of the ovary and individual *corpus luteum* as well as in the total volume of all *corpora lutea* in the ovary of animals subjected to hypokinetic conditions as compared to the control. Moreover, stereological evaluation showed that in the experimental group there was a decrease in the mean number of luteal cells and an increase in the mean size of these cells and in the mean volume fraction of their nuclei.

MATERIAL AND METHODS

Virgin female Wistar rats at 4 months of age and weighing 240–280 g were kept individually overnight with males of the same strain. The day on which

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spermatozoa were found in a vaginal smear was designated as Day 1. The pregnant rats were divided into control and experimental groups, each consisting of 9 animals. The control animals were housed individually in standard animal colony conditions during the entire period of the study. For the first 12 days of pregnancy the experimental rats were kept under the same conditions as the control animals. From the 13th to 18th day of pregnancy they were placed in cylindrical cages (20 cm × 7 cm) which restricted their movements.

All animals were fed a standard diet. Water and food was present inside the cages and was available *ad libitum.* They were housed under a constant temperature and humidity and were under a 12-hour light-dark cycle.

The principles of laboratory animal care were followed and the study was approved by the Animal Ethical Committee of the Medical University of Warsaw.

On the 18th day of pregnancy all animals were killed by i.p. injection of Nembutal (60 mg/kg body weight). The left ovaries were taken for histological procedures and the right ovaries were processed for separate electron microscopic studies.

In each dam the number of foetuses was counted and in each ovary, the number of *corpora lutea* was preliminarily evaluated. This value was then verified on serial sections.

Histology

The left ovaries were fixed in 10% buffered formalin solution and were processed according to standard histological technique. After being embedded in paraffin, the ovary from each rat was cut serially at 5 μm and the sections stained with haematoxylin and eosin (H&E).

Morphometry

Morphometric evaluation involved three-dimensional reconstruction and volume measurement of the ovary and *corpora lutea* and luteal cell stereological analysis and was performed using different sampling procedures and different microscopic magnifications.

For three-dimensional reconstruction and volume measurement of the ovary and *corpora lutea*, every 3rd serial section was inspected using a Documator (Zeiss) projector at a magnification of 17.5 ×. The ovary and the profiles of particular *corpora lutea* were drawn by hand. Next, the drawings were traced on a Summagraphic ComGraph digitise connected to a microcomputer.

The volume of the ovary, individual *corpora lutea* and the total volume of all *corpora lutea* in the ovary was then calculated using values taken from the areas of the traced profiles and taking into consideration the distance between the sections analysed (15 μm in this study). The volume fraction occupied by the *corpora lutea* in the ovary was also calculated.

Two luteal cell populations, small (also referred to as theca-lutein, Type I or I cells) and large (alternatively called granulosa-lutein, Type II and D cells), have been described in the *corpus luteum* of many species, including rats [13, 14, 27]. In the present study, the term "luteal cells" which we have used actually referred to large luteal cells. In the histological preparation small luteal cells are difficult to recognise. Our analysis therefore only encompassed the population of large luteal cells.

For luteal cell stereological analysis, we used, slightly modified, the sampling procedure applied by Meyer and Bruce [12]. Every 8th section was used and inspected with a Zeiss projection microscope at a magnification of 1060 ×. The diameter of large luteal cells in the pregnant rat *corpus luteum* is approximately 30 μm [13, 27]. Thus, by using every 8th section with a 5 μm thickness, we were able to eliminate the possibility of analysis of more than one profile of the same luteal cell.

In each section, profiles were drawn of luteal cells (and their nuclei) located in two test fields (one near the periphery and the other near the centre of the *corpus luteum*) from 3 *corpora lutea*. Profiles of the luteal cells and their nuclei were then traced using a digitiser connected to a microcomputer. The surface area of the cross-section of the luteal cells and their nuclei, the length of long and short axes of cells, the volume fraction and the number of cells per test area was measured; the area of test field was also recorded.

Using this primary data, the number of luteal cells per unit volume of *corpus luteum* (numerical density) was calculated according to the formula [23]:

$$N_v = \frac{K}{\beta} \times \frac{(N_A)^{3/2}}{(V_v)^{1/2}}$$

where N_V — numerical density; K — factor depending on the size distribution of the measured profiles; β — function of the axial ratio (shape) of evaluated cells; N_A — number of cell profiles per unit area; V_V — volume fraction occupied by cells.

For most biological objects, factor "K" ranges from 1.0 to 1.1 [25]. In the present study the value of 1.05 was applied. Factor "β" was evaluated from the mean axial ratio of the cell profiles [24]. The total number of luteal cells in the ovary was calculated from the numerical density of the luteal cells and also the total volume of the *corpora lutea*. In addition, the volume fraction [23] of the luteal cell nuclei was evaluated.

Statistical analysis

The differences between control and experimental animals were computed using one-way analysis of variance (ANOVA) and Duncan's Range Test. The differences were assumed to be statistically significant at $p < 0.05$.

RESULTS

Though animal weight did not differ statistically at the beginning of our study, we observed significant differences in weight between the control and the experimental rats on Day 18 (Table 1). These differences concerned both weight gained during the entire experiment (Days 1–18) and weight gained/ /lost in the period of immobilisation (Days 13–18).

The mean number of foetuses per rat, while slightly lower in the immobilised group, did not differ statistically from the control group. No clear sign of uterine content resorption was visible.

The mean number of *corpora lutea* per rat was similar in both groups of animals.

Hypokinetic conditions induced a decrease in the volume of the ovary, individual *corpus luteum* and the total volume of *corpora lutea* per ovary (Table 2). The individual *corpus luteum* mean volume decreased by about 28% in the experimental group of rats as compared to the control. The mean volume fraction of the *corpora lutea* in the ovary did not differ between the groups investigated.

These results were accompanied by a decrease of between 27% and 29% in the mean number of luteal cells per ovary and *corpus luteum* (Table 3) of the immobilised rats. The mean surface area of the cross-section of the luteal cells was higher in the *corpora lutea* of animals kept in hypokinetic conditions. The mean volume fraction of luteal cell nuclei was slightly, but statistically significantly, higher in the *corpora lutea* of the experimental animals as compared to the control (Table 3).

Table 1. General characteristics of control and experimental rats kept in hypokinetic conditions from the 13th to the 18th day of pregnancy (results presented as mean ± S.E.M)

General data	Control	Hypokinesia
Animal weight [g] at the beginning of study	265.2 ± 17.8	250.2 ± 6.1
Animal weight gain; Day 1–18 (%)	37.1 ± 2.5	4.9 ± 2.3*
Animal weight gain/loss; Day 13–18 (%)	(+) 12.9 ± 2.4	(–) 7.7 ± 1.8*
No. of foetuses per rat	10.0 ± 1.1	8.0 ± 1.2
No. of *corpora lutea* per rat	13.1 ± 0.7	12.3 ± 0.5

*Statistically significant difference at $p < 0.05$

Table 2. Effects of 5-day hypokinesia applied from the 13th to the 18th day of pregnancy, on ovary and *corpus luteum* volume (results presented as mean ± S.E.M)

Ovary and corpus luteum volume	Control	Hypokinesia
Ovary volume [mm^3]	59.8 ± 5.7	34.8 ± 2.9*
Volume of *corpus luteum* [mm^3]	6.1 ± 0.7	4.4 ± 0.3*
Volume of *corpora lutea* per ovary [mm^3]	45.4 ± 5.5	25.3 ± 2.5*
Volume fraction of *corpora lutea* in ovary (%)	73.9 ± 3.6	72.0 ± 3.4

*Statistically significant difference at $p < 0.05$

Table 3. Effects of 5-day hypokinesia, applied from the 13th to the 18th day of pregnancy, on the number of luteal cells, the surface area of the cross-section of the luteal cells and the volume fraction of their nuclei (results presented as mean ± S.E.M)

Luteal cells parameters	Control	Hypokinesia
No. of luteal cells per ovary ($\times 10^5$)	18.0 ± 0.1	13.2 ± 0.2*
No. of luteal cells per *corpus luteum* ($\times 10^5$)	2.8 ± 0.1	2.0 ± 0.1*
Surface area of cross-section of luteal cells [μm^2]	245.5 ± 7.1	271.9 ± 6.5*
Volume fraction of luteal cell nuclei (%)	19.5 ± 0.2	22.2 ± 0.2*

*Statistically significant difference at $p < 0.05$

DISCUSSION

The present study shows that numerous changes in the *corpus luteum* and luteal cells are induced in rats kept in hypokinetic conditions for 5 days between the 13th and 18th days of pregnancy. This period comprises the development and full activity of the *corpus luteum* [2, 12, 14, 22]. Its presence until day 20 is essential for the maintenance of pregnancy in the rat [3].

The decrease in volume of individual *corpora lutea* resulted in a reduction in the total volume of all the *corpora lutea* in the ovaries of the experimental rats. It is known that a reduction in *corpus luteum* volume can lead to a proportional decrease in progesterone concentration [5] and that pregnancy maintenance correlates with progesterone level [4]. However, since only about 20% of the luteal mass is sufficient to maintain pregnancy in a rat [3], the observed differences should not affect and did not affect the number of foetuses in hypokinetic rats.

The direct mechanisms in the *corpora lutea* leading to the changes noted by us are unknown, but they may be at least partially related to stress reaction which is induced by immobilisation. In this reaction the endocrine glands and hormones of the hypothalamic-pituitary-adrenal cortex axis (especially ACTH and corticosterone) are involved [19, 21]. Yang et al. [28] demonstrated that administration of ACTH interrupts pregnancy in the rat. It has also been found that ACTH and corticosterone, known as stress-related hormones, may influence the function of the *corpus luteum,* since they inhibit progesterone secretion during mid-pregnancy in rats [9, 18]. Wiebold et al. [26] demonstrated that restraint stress in early pregnancy in mice induces decreased serum progesterone concentration. These functional changes were accompanied by morphological alterations to the *corpus luteum*. On the other hand, Ward and Weisz [21] reported that restraint stress applied from Day 14 to Day 21 of pregnancy in the rat did not affect the concentration of progesterone in the serum.

It was observed by Meyer and Bruce [12] that the number of luteal cells is relatively constant in the period of pregnancy corresponding to the duration of our hypokinetic experiment. It is likely that one of the results of immobilisation stress is interference with the co-operation of luteal cells and non-steroidogenic *corpus luteum* cells, the large majority of which are vascular in origin. The importance of such co-operation, for example for luteal cell steroidogenesis, was demonstrated by Nelson et al. [13]. Interference with this co-operation may induce the release of factors which can cause necrosis or apoptosis, leading to a decrease in cell number.

On the other hand, neither clearly necrotic nor apoptotic changes were observed in the *corpora lutea* on Day 18 of pregnancy, during which material was collected. However, since apoptosis is a very rapid process, its occurrence at the onset of immobilisation could not be excluded [15]. The presence of apoptosis has been reported in luteal cells depending on *corpus luteum* status [16, 29].

The possibility of large to small cell re-differentiation seems to be less likely, but the existence of reciprocal small and large luteal cell differentiation in some species has been suggested by other authors [7, 17].

It is possible that increase in the mean surface area of the cross-section of luteal cells and in the volume fraction of their nuclei in the experimental rats is an expression of elevated activity of these cells in compensation for the reduction in cell numbers.

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