Histogenesis, development and atresia of ovarian follicles in different developmental stages of Makuii ewe fetuses

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Summary

This study was conducted to determine the histogenesis, growth and atresia of ovarian follicles in Makuii sheep fetuses at different developmental stages. Forty fetuses in four ages group of ≤35, 36–70, 71–100 and 101–150 intrauterine days old were taken for study. The fetuses were collected and preserved in 10% neutral buffered formalin solution. After recognition and harvesting of ovaries from fetuses, they were processed through routine paraffin embedding. Serial 5–7 µm thick sections were taken and stained with Hematoxylin-Eosin, Verhoeff, Van Gieson, Toluidne blue and PAS. Histologic and histomorphometric (point count) studies were adopted. In fetuses ≤35 days old, the necrotic primordial germ cells (PGCs) were identified by pyknotic, condensed or karyolytic nuclei. In these fetuses, the undifferentiated gonads were recognized at the caudal aspects of the developing metanephrous kidneys. The healthy PGCs, were large with euchromatic nuclei, without any somatic cell surrounding them. In 36–70 days old fetuses , the germ cells were observed in clusters, which surrounded by simple squamous cells. The hilus cells were seen in mesovarium. In the 71–100 days old fetuses, primordial follicles were observed. In the 84 days old fetuses, primary follicles were seen for the first time. In the ovaries of 101–150 days old fetuses, the secondary follicles with zona pellucida and theca folliculi were seen. Tertiary follicles were not seen in any age groups. The mean number of germ cell population at 0.25 mm^2 surface area of ovary, was the highest in 36–70 days old and lowest in 101–150 days old age group fetuses. It was revealed that in all age groups, the population of healthy follicles and/or germ cells is higher than population of atretic follicles and that there was significant difference ($P<0.001$) between them. There were significant ($P<0.001$) differences in the mean distribution of healthy follicles and or germ cells among all age groups. There were significant ($P< 0.001$) differences in atretic follicles among all age groups, except between 36–70 days old and 71–100 days old fetuses. We concluded that by increasing age, a decrease in population of healthy as well as atretic follicles in fetuses takes place. However, in all age groups, the population of the healthy follicle is greater than the population of atretic follicles.

Key words: Makuii ewe, Fetus, Follicular development, Follicular atresia

Introduction

The knowledge on gonadal growth and development in fetus, appearance of germ cells and subsequently follicles in them, and follicular atresia in fetal life has outstanding values in different fields of veterinary sciences such reproductive science/diseases, in vitro fertilization (IVF), animal production and management, animal selection and genetic sciences. The ovaries are not recognizable microscopically in the first few weeks of development. The gonads of sheep fetus are observed at 24th day of intrauterine life. The total oocyte reservoir of the adult animal ovaries originates from the limited primordial germ cells (PGCs) which are originating from cells of the inner cell mass of developing embryo.

Oocyte and its surrounding follicular cells are known as primordial follicle, and their population are present in the sheep fetus and humans fetus at 75th and 158th days of gestation, respectively (Gosden and Spear, 1997). Antenatal developmental period of sheep fetus is 143–150 days. After birth, new primordial follicles are no longer exist in ovaries, and as a result of follicular atresia the population of oocytes and

follicles decrease so that approximately 30,000–50,000 of them remain. In fetal period, some of these follicles may undergo growth and then degenerate by the process of follicular atresia.

The developing follicles of fetus are situated in the cortico-medullary region of ovaries, which is highly vascular (Van Wezel and Radyers, 1996). It has been suggested that, follicular cells are producing a substance, which is preventing follicular development and it directly acts on the primordial follicles (Gougeon and Chainey, 1987). By changing the squamous and flat granulosa cells in primordial follicles into cuboidal cells, such follicles change to primary follicles and in the sheep, it is when at least 15 cubical granulosa cells are surrounding the oocyte (Challi and Mauleon, 1981). Before birth, follicular growth is brought about by growth factors such as fibroblastic growth factor (FGF) or epidermal growth factor (EGF) along with two proteins, *i. e.*, C-kit (present in the oocyte plasma membrane) and its ligand, KL protein, which is produced in granulosa cells (Driancourt and Thuel, 1998).

Although in each reproductive cycle of mammals several follicles start their growth and development, in each species only a limited number of follicles are able to ovulate and the rest of them undergo degeneration by the process of follicular atresia (Farookhi, 1981). This process begins when the primordial follicles are recruited for the growth (Hsueh and Erickson, 1979). As a result of atresia, 99% of ovarian follicles undergo degeneration and death (Peluso *et al*., 1980). Factors affecting follicular atresia are follicular recruitment (Mary *et al*., 1976), hormones and apoptosis or programmed cell death (Neubourg *et al*., 2000).

This study was conducted to determine the histogenesis, growth and atresia of ovarian follicles in Makuii sheep fetuses at different developmental stages.

Materials and Methods

Forty sheep fetuses with different intrauterine developmental age were collected from abattoir (Fig. 1). Their ages were assessed using the Crown-Anus formula (Arthur *et al*., 1996), *i.e.,* $Developmental\ age_{(days)} = 2.1 \times (crown\text{-}anus\ length_{(cm)} + 17)$.

The fetuses were divided into four age groups of ≤35, 36–70, 71–100, and 101–150 intrauterine days old. Immediately after slaughter of animals, the fetuses were taken out and fixed in 10% formaldehyde solution.

In the laboratory using a stereomicroscope, ovaries of fetuses were identified. The right and left ovaries were collected separately. The fetuses aged ≤ 35 days were processed totally. The specimens were processed routinely. After paraffin embedding, several staining techniques, *i.e.,* Hematoxylin-Eosin (H&E), Toluidine blue, Van Gieson, Verhoeff and periodic acid schiff (PAS) were used to study the fibrocellular structure, mast cells, follicular structures, collagen and elastin fibers and carbohydrate compounds, respectively (Gretchen, 1979).

Fetuses in each age group studied histomorphologicaly and histomorphometricaly. In the histomorphometric study, a special lens device was used and populations of the germ cells were assessed in a 0.25 mm^2 wide area of gonadal tissue. In this study, we adopted a point count pattern assessment.

Results

Histomorphology

In the study of complete serial section of fetuses aged ≤35 days, the undifferentiated gonads were recognized at caudal aspect of mesonephric kidneys in two sides of median line as ridges protruded into the celomic cavities of fetuses (Fig. 2A). The surface epithelium of these ridges was simple squamous to simple cuboidal. These undifferentiated gonads were highly cellular and the large PGCs were microscopically differentiated from smaller somatic cells (Fig. 2B). At this age, the germ cell distribution was approximately uniform throughout the gonads. In fetuses aged 36–70 days, the ovaries were differentiated and the distribution of germ cell was uniform throughout the regions. Clusters of germ cells were covered with simple squamous cells. At this stage, ovarian follicles were not seen, but the medullar and cortical regions of ovaries were identified. In the medullar

region, the population of germ cells was significantly decreased, but the blood vessels were highly distributed. The hilus cells were seen at mesovarial tissue. In fetuses aged 70 ± 2 days, germ cell clusters were dispersed, thus this age could be considered as the beginning of primordial follicles appearance. In the fetuses aged 71–100 days, the medullar region of ovaries had well-developed blood vessels. At the hili of ovaries, remnants of mesonephric ducts with simple cubiodal epithelium were seen. In the medullar region of ovaries some germ cells were still seen. The tunica albuginea of ovaries was comparatively well-developed and seen for the first time at this age. The demarcations of cortex and medulla were more obvious. For the first time, in 75-day-old fetuses, the primordial follicles were seen at medullae of ovaries. In 77-day-old fetuses, most of germ cells were converted into primordial follicles. However, some primordial germ cells were observed. In the 84-day-old fetuses, for the first time, primary follicles were seen at medullar regions of ovaries. In 86-day-old fetuses, clusters of germ cells were seen in the cortex in a syncytial form; all were atretic.

Atresia was observed in all types of follicles as well as germ cells. In 88-day-old fetuses, follicular atresia was highly pronounced at medulla. The spaces between

Fig. 1: A- Group of sheep fetuses with ≤35 days intrauterine life, B- Sheep fetus with 88 days intrauterine life. The bar is 2 cm long

Fig. 2: A- Cross section of a fetus with less than 35 days old. Right and left genital ridges (←), metanephrous kidneys (🡻), Celomic cavity (🡾), abdominal wall (➔). (H&E, ×40), B- Cross section through primary gonad of a 35-day-old sheep fetus. Primordial germ cells (PGCs) (↓), stromal cells (➔), mesothelium of gonad (⇨). (H&E, ×1000)

primordial follicles were considerably increased and they were arranged at juxta-medullary region of the cortex. The larger primordial follicles were oriented deeply near the medulla, but the smaller ones were situated superficially (Fig. 3A). In those aged 101–150 days, some primary follicles were seen at cortical region of ovaries. Most of the primary follicles were at medulla or juxta medullary regions of the cortex.

Fig. 3: A- Cross section of the cortical region of 115-day-old sheep fetus. Primordial follicles, which are individually surrounded by a simple squamos follicular cells (↓). (H&E, ×400), B- Cross section through the medullar region of a 128-day-old sheep fetus. A secondary follicle in advanced atresia in which granulose cells having pyknotic nuclei (↓). (H&E, ×250)

Numerous primary follicles at medulla were entered the atretic process High reduction in follicular population was seen in medulla. For the first time, the zona pellucida (ZP) was observed in the structure of the multi-laminar primary follicles. In 109-day-old fetuses, for the first time, the theca layer became observable at multi-laminar primary follicles. At this age, the population of primary follicles was enhanced in deeper regions of the cortex. In ovaries of 128-day-old fetuses, secondary follicles were seen (Fig. 3B). The toluidine blue staining could not reveal any mast cells in the fetal ovarian tissue. The tertiary follicles were not observed. The collagen and elastin fibers of the fetal ovarian tissues were thin and delicate.

Histomorphometry

The mean ± SE of the healthy follicles in the 36–70, 71–100 and 101–150 days old group fetuses were 76.09 ± 3.36, 34.37 ± 2.57 and 7.22 ± 0.39 in a 0.25 mm^2 wide area of ovarian tissue, respectively. In the same region, in these age groups, the mean ± SE of atretic follicles were 5.66 ± 0.56, 6.09 ± 0.29 and 2.89 ± 0.21, respectively. The statistical analysis revealed significant ($P<0.001$) differences in mean distribution of healthy follicles among all age groups. The difference in mean distribution of the atretic follicles between the first and second groups was not statistically significant ($P>0.001$). The mean distribution of the atretic follicles between the first and third, as well as between the second and third groups were statistically significant ($P<0.001$), (Fig. 6). The differences in mean distribution of healthy and atretic follicles among studied groups were significant ($P<0.001$) (Fig. 5). There was no significant difference between the mean distribution of healthy follicles of the third group with that of atretic follicles of the second group. No significant difference was also seen between the mean distribution of healthy follicles of the third group with the mean distribution of atretic follicles of the first group (Fig. 5).

Discussion

Due to the lack of information on gonadal growth and development in fetuses of Iranian sheep breeds such as Makuii ewes, we conducted this study. In the way of migration, entrance and settlement in the ovaries, some of the PGCs committed to atresia and death (Picton *et al*., 1999). In this study, the PGCs observed in ≤35 days old fetuses were larger than the somatic cells.

The distribution of both cell types was more or less uniform throughout the gonads. The primordial germ cells are organized in clusters, which are surrounding by a squamous type of cells. Some times, the clusters of PGCs create a syncytium and this is due to the failure of their separation in a rapid mitotic division (Motta *et al*., 1997). Such structures were observed in this study too and most of them were necrotic.

If any of the germ cells fail to be surrounded and supported by the pregranulosa cells, itwill commit atresia and death. In this study, for the first time, we observed primordial follicles on the 76th day of fetal development that was in agreement with reports of Gosden and Bownes (1995) and McNatty *et al*., (1995). They stated that in the sheep fetus on 75th day of the prenatal life, the number of germ cells is 900,000; on the 90th day, it decreases to a value between 170,000 and 200,000; and on the 135th day it declines more to 82,000. The removal of follicular cells of cumulus oophorus from around the oocyte, will affect its nucleo-cytoplasmic maturation (Sun *et al*, 2001).

According to Jantosovicova *et al*., (1996), in the sheep fetus, the primordial follicles are appearing on the 9th week of intrauterine life. On the 10th week, their formation gets accelerated. At the meantime, the number of atretic follicles is also increased. From beginning of the 11th week, the primary follicles are formed and the population of atretic follicles gradually decreases. On the 14th or 15th week, the secondary follicles appear in the fetal ovaries and on the 19th–20th week of development, the tertiary follicles are seen. Shortly before the birth, tertiary follicles are seen at deep cortical region but all get committed to atresia. What we found were in part in accord to their reports. In some instances, however, differences were exist. We could not observe any primordial follicles in the 5–7 weeks (36–70 days) old fetuses; this finding is in agreement with to the report of Jantosovicova *et al*., (1996). We observed that during the 11–15 weeks (71–100 days) of intrauterine life, the demarcation established between medullae and cortices of ovaries which is also in accord to the aforementioned report. In this study, the primordial follicles are observed at the end of the 10th week of intrauterine life, while Jantosovicova *et al*., (1996) reported that they appear at the end of the 9th week. We found that the primary follicles were observed at the 84th day (12th week), while according to Jantosovicova *et al*., (1996), they appear at the end of the 11th week of intrauterine life.

The process of follicular growth starts in medullar region of the fetal ovaries and progress towards the cortical zone. On the 109th day of intrauterine life, approximately all of the germ cells are appear in the form of primordial follicles and the medullae of ovaries devoid of follicles. In the cortical zone, however, their population is still high (Fig. 3A). We observed great reduction in follicular population in ovaries of the 110 days old fetuses (Fig. 4). In 128-day-old fetuses, for the first time, secondary follicles were observed. Most of them, however, were atretic. According to the previous reports and what we found we concluded that follicular atresia and degeneration during intrauterine growth and development of sheep fetus is a process, which starts when PGCs and then follicles appear in the ovaries and it can be considered as a physiologic and a natural phenomenon. So far, no theory or hypothesis could fully explain the reasons and complete mechanisms of the follicular atresia. Nevertheless, most of investigators believe that granulosa cells of ovarian follicles, which surround oocyte, are destroyed by an apoptotic mechanism (Bacci *et al*., 1998; Guthrie and Garrett, 2001; Pastor *et al*., 2001). Most likely, the death of oocytes has secondary reasons including lack of nutrients and oxygen and more importantly, the death of surrounding granulosa cells (Ming Yuan and Rajamahendran, 2000).

The morphometric study of this investigation revealed that the highest mean distribution of germ cells in an area 0.25-mm^2 wide of sheep fetus ovarian tissue is during 37–70 days of intrauterine development; during 101–150 days, it is the lowest. Thus, the population of germ cells as well as follicles decrease by advancement of fetus age (Fig. 4). Our study revealed that in all age groups studied, the population of healthy follicles is higher than the population of the atretic follicles (Fig. 5).

Therefore, in the fetal life, there are always a large number of healthy follicles which seems to be reasonable; the fetus must restore its follicular reservoir and protect them from degenerative and atretic processes, so that after birth and throughout its reproductive life, the reproduction of animal carried out normally and the animal does not become sterile. We found that significant differences ($P<0.001$) exist between distributions of healthy follicles in different age groups of fetuses. Therefore, in developmental process of the fetuses, the population of their healthy follicles get decreased (Fig. 5). There was significant ($P<0.001$) difference in atretic follicle distribution among all age groups, except between 37–70- and 71–100-day-old fetuses (Fig. 6). Thereby, by increasing age of intrauterine life of fetuses, the population of atretic follicles also declines (Fig. 5). The probable reason for both of the aforementioned cases, could be a reduction in follicular source of ovaries due to the follicular atresia. We therefore concluded that by reduction in follicular population in fetal ovaries, both the healthy and atretic follicular population also reduce.

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