FETAL OOCYTES: A POTENTIAL RESOURCE IN EMBRYO TRANSFER? PROGRESS AND PROSPECTS.

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ABSTRACT

The technique of in vitro oocyte maturation, fertilization and embryo transfer (IVMF-ET), which was started about 25 years ago is now an integral part of dairy cattle breeding in some countries. However, its extensive application is amongst other reasons, being hindered by the small number of follicles from which the oocytes are obtained. In fetal ovaries, there is a large population of primordial follicles and which could form an alternative source of oocytes for IVMF-ET. Utilization of fetal oocytes would eliminate the necessity for adult animals in passing from one generation to the next, and thus, shorten to a big extent, the generation interval. In vivo, an interaction between follicular and body reproductive hormonal levels, and oocyte morphology, is critically important for the control of oocyte maturation and development competence.To understand and evaluate the potential use of bovine fetal oocytes in IVMF-ET, studies are being conducted to evaluate the onset and progress of Follicle Stimulating Hormone (FSH), estradiol 17b (E2) and progesterone (P4) hormones secretion, meiosis onset and progression, and morphology of fetal follicles and oocytes. Preliminary gross morphological findings from 73 fetuses indicate that 32% of those that were >150 days old (or about 11% of the total) had vesicular follicles. There is immunohestochemical evidence that FSH secretion starts during the fetal period and in addition, with advancing age, there was follicular and oocyte growth, and progression in meiotic maturation.

***Keywords**: fetus, oocytes, use, embryo production*

INTRODUCTION

The techniques of *in vitro* maturation, fertilization and embryo transfer have enabled the production of live animals from oocytes derived from antral follicles. These techniques have developed rapidly in the past 10-15 years, and are now an integrating part of dairy cattle breeding programmes in some countries (Iritani, 1988). In cattle, the technique relies on follicles obtained from superovulated cows and mature or immature heifers (Armstrong et al., 1992) as well as from slaughtered animals. In tropical Africa, genetic improvement of the indigenous cattle through cross breeding (either by natural mating or artificial insemination) has been in use for some decades. In addition to these, several embryo transfers (ET) indicate an achievement of 50-63% pregnancy rates and has thus been suggested to be a useful additional breeding tool (Iritani, 1988; Jordt and Lorenzini, 1988; Cumming et al., 1994).

However, the yield of viable embryos per cow and thus the extensive use of ET technology is restricted to the small number of suitable follicles from which oocytes for in vitro maturation and fertilization (IVMF) are obtained. This has resulted into a growing interest in the use of a

much larger population of immature follicles, specifically the population of follicles that is present in fetal gonads (Betteridge et al., 1989), since high numbers of these structures are present during fetal life. The use of fetal-derived oocytes for in vitro fertilization could eliminate the necessity for adult animals in passing from one generation to the next. Besides shortening the interval between generations, this would mean that the oocytes are never exposed to environmental influences like toxins that they may encounter in the adult ovary. However, there is very little information to date on the usefulness of oocytes derived from fetuses for *in vitro* production of embryos (Barns, 1994; Renard, 1994). There is therefore, a need for more data on the prenatal development of the ovarian activity in cattle including the age at which a majority of the oocytes enter meiosis, the developmental potential of the fetal oocytes as judged by their morphology and the maturation of the pituitary-ovarian axis activities.

The objective of the present work is therefore to acquire the above data. The prenatal secretion of FSH will be monitored by immunohistochemistry while the ovarian and oocyte structures will be studied by light and transmission electron microscopy. Reproductive hormonal state will be evaluated by assaying fetal follicular fluid and peripheral blood plasma for estradiol and progesterone. In addition, meiosis onset and progress will be determined by evaluating the morphology of the chromosomes.

MATERIALS AND METHODS

Gravid uteri were collected from Small East African Zebu (*Bos indicus*) cattle slaughtered at the Morogoro abattoir. Fetuses were obtained within 30 minutes after death of the dam.

The approximate age of the fetus (>42 days) was estimated by using both the crown-rump length (CRL) measurement and the external morphological features (Evans and Sack, 1973; El Sayed et al., 1987; Roberts, 1986).

The fetuses were opened through a midline incision through the *linea alba* followed by separation of the pelvic symphysis. Blood samples were collected from the femoral vessels into additive-free vials, and later (within 2 hrs) centrifuged at 6,000 rpm for about 15 minutes and the plasma stored at -20^{o}C.

For each fetus, one ovary was fixed in 10% neutral buffered formaldehyde, processed and embedded in either historesin or paraffin for light microscopic studies. One half of the other ovary was stored in 1% acetic acid for use in the evaluation of the structure of the chromosomes. The acetic acid-fixed ovary sample was later in the laboratory, mechanically macerated in fresh 1% acetic acid, centrifuged and the sediment spread over glass slides followed by staining with giemsa. The other one half of the ovary was cut into tiny pieces and fixed for 1-3 hours at room temperature in 3 % glutaraldehyde (in 0.1 M phosphate buffer at pH 7.2). Thereafter, the samples were preserved in 0.1M phosphate buffered saline pH 7.2 (PBS) at 4^{o}C until further processed and embedded in epon using a standard procedure (Hayat, 1989). These samples will later be studied with transmission electron microscopy (TEM).

In each fetus with vesicular vesicles, the follicular fluid was aspirated and stored at -20^{o}C until assayed for estradiol 17ß (E2) and progesterone (P4). Oocytes that might be isolated from the follicular contents will be processed and studied accordingly (Assey et al., 1994).

The pituitary gland was collected within 2 hrs. It was fixed in 10% neutral buffered formaldehyde for about 48 hrs. There after, it was divided into two portions, one which was embedded in historesin for light microscopic morphological studies while the other half was stored in 0.1M phosphate buffer into which sodium azide had been added to reduce fungal growth. The latter sample was used for the immunohistochemical localization of FSH.

A three step indirect immunoenzymatic staining method using the avidin-biotin horse-radish peroxidase method was used to study FSH immunoreactivity in the pituitary glands. The primary antibody was a monoclonal mouse IgG antibodies against bFSH (Eurodiagnostica, Sweden). The secondary (link) antibody was biotinylated affinity-isolated rabbit anti-mouse IgG (DAKO, Denmark). Streptavidin-biotin complex coupled to horse-radish-peroxidase (HRP), 3-diaminobenzidine (DAB) and hydrogen peroxide was used for visualization of the reaction products. The immunohistochemical reaction will assist in the determination of the onset and progress in FSH secretion by assessing the presence, distribution, mean size and number of FSH secreting cells in the gland.

RESULTS

General.

At about 45 days age, the external and internal reproductive organs could be identified as of the female type. In many occasions, the age of fetus as estimated by CRL measurement was lower by 5-20 days than that estimated by the external features, especially before day 180. A total of 73 fetuses belonging to the following age categories have so far been collected: 42-60 days (n=13); 61-90 days (n=12); 91-120 days (n=10); 121-150 days (n=8); 151-180 days (n=9); 181-210 days (n=7) and 210-230 days (n=14) days age.

A total number of 8 follicular fluid samples have also been collected. These were obtained from fetuses of 151-180 days (n=1), 181-210 days (n=2) and >210 days (n = 5).

Preliminary findings on the chromosomal morphology indicate onset of meiosis at a very early age and which progress with advancing age. A detailed evaluation awaits the arrival of a x15 objective and a green filter.

For TEM studies, some representative samples have been embedded in epon, and the general evaluation of some of these samples shall be done at the University of Dar es salaam.

For immunohistochemistry, preliminaries to evaluate the methodology and reactivity have so far been done. The initial findings indicate the methodology to be working and there is an indication of FSH secretion in the fetal period. The secretion seems to begin in one part of the gland and there are also signs of the reaction intensity (thus FSH secretion) to be directly related to the presence of ovarian follicles.

Light microscopic structure of the ovaries.

At about 45 days, the ovary measured about 3.5 mm in length and was bean-shaped with a hilus where the mesovarium was attached.

Microscopic examination revealed that the ovary was surrounded by a pseudostratified layer of cuboidal or low columnar mesothelial cells. These mesothelial cells formed cell cords that penetrated the ovarian mesenchyme and which were being separated by dividing primitive germ cells. Within the ovarian stroma, numerous vascular channels occurred. However, most of the vascular channels in the outer part of the ovary were empty while deep in the ovary these were filled with blood. Within the ovarian mesenchyme, descendants of the mesothelial cells were characterised by their oval-shaped nuclei, and were mixed with round-shaped oogonia and differentiating or fully differentiated fibrocytes. The oogonia measured 15-24 µm in diameter and were undergoing extensive mitosis especially in the middle part of the ovary. By the end of the second month, a greater part of the ovary was filled with a high population of dividing oogonia and descendants of the mesothelial cells (epithelial somatic cells) to the extent that in a large number of the sections, cell cords were less apparent.

During the third month, the outer part of the ovary had a dense population of oogonia mixed with epithelial somatic cells. Within the deeper part of the ovary, oogonia and epithelial somatic cells were organized into clusters that were in many cases surrounded by few fibrocytes. Scattered narrow empty vascular channels were still evident in the outer part of some of the ovaries, while in the inner part, these were wider and filled with blood cells.

At the second half of the third month, some oogonia were surrounded by epithelial cells of rete origin. This was the first sign of follicular organization. Such arrangements were located deep in the ovary in the region of the disintergrating rete tubules and close to blood vessels. In such early follicles, the germ cells either had condensed chromosomes, inactive nucleus or were in the early stage of meiotic prophase. By the end of the third month, distinct follicles located deep in the ovary were observed in the majority of the fetuses.

During the first half of the fourth month, a section of the ovary revealed four distinct regions. Below the mesothelium was a loosely packed area that was occupied by epithelial somatic cells mixed with germ cells, and which showed little mitotic activity. Below this was an area that showed high mitotic activity. Clusters of oogonia and epithelial somatic cells formed the third region, and in which the germ cell clusters were separated by epithelial somatic cells. Deep in this region, separation of germ cells by epithelial somatic cells and forming follicles were evident. The innermost region consisted of well-organised follicles and fully differentiated fibrocytes. Ova enclosed in follicles measured about 15-30 µm in diameter. In most of the ova that were surrounded by a complete layer of granulosa cells, the chromosomes were thread-like and along their lengths were points of condensation.

During the second half of the fourth month, the region of the ovary with follicles was broader while that with abundant mitotic figures was narrower. At the low power, cortex, medulla and cortico-medullary regions of the ovary were easily distinguishable. The mesothelial covering, especially around the concave side of the ovary was of low cuboidal. Short cell cords, few vascular channels and clusters of dividing oogonia were occasionally seen in the cortex. The cortico-medullary region was occupied by a high number of follicles whose lining epithelium was of either cuboidal, squamous or mixed type and enclosed oocyte measured up to 35 µm in diameter. The oocytes had thread-like chromosomes and prominent nucleoli. Degenerating oogonia that were characterized by pyknotic or degenerating nuclei were also observed. The medulla consisted of vascular channels, differentiating mesothelial and mesenchymal cells, few follicles and disintegrating rete system.

During the fifth month, the surrounding mesothelial covering was of low cuboidal. Short cell cords occupying a thin outer part of the ovary were still evident in some of the ovaries and occurred mainly on the convex side of the ovary. The middle cortex was filled with follicles that were surrounded by either cuboidal, flat (squamous) or mixed epithelium. In addition, there was a corresponding increase in the size of the medulla that was devoid of follicles, but with large blood vessels. Thus, a majority of the follicles were increasingly located in mid and outer cortex. At this age, secondary and tertiary follicles could be seen deep in the cortex. By day 180, oocytes had grown to about 49 µm in diameter and the ovary presented a thin outer cortex and a larger medulla. Nevertheless, in some ovaries, the rete was still evident.

From the sixth month onwards, the ovary was covered by either a low cuboidal or squamous epithelium and had a thin outer cortex and a wider inner medulla. Follicles of all categories occurred only in the cortex, and an increasing number of follicles that were surrounded by a squamous epithelium could be seen. In addition, scattered short cell cords could still be observed in the outer convex side of some of the ovaries. Tertiary follicles occurred in about 32 % of the ovaries of the fetuses older than 150 days. In some cases, large oocytes with a loose zona pellucida were seen. However, some of the tertiary follicles lacked the theca layer while in some with the theca layer, the granulosa layer was only 3-5 cells thick .

DISCUSSSION

The initial sign in ovary differentiation is the organization of the inner epithelial tissue (epithelial somatic cells) surrounding the primordial germ cells into cord-like structures, and which is accompanied by degeneration of the mesonephric tubules (Jost et al., 1973). According to Marion and Gier (1970) and Roberts (1986), sexual differentiation of the bovine (taurine cattle) gonad occurs at around 41-45 days post conception. In the present study, samples collected from fetuses that were estimated to be 45 days old showed both of the above morphological features indicating that the gonad was already differentiated. It is very likely therefore, that differentiation of the reproductive organs in the zebu cattle occurs at a slightly earlier age than in the taurine cattle.

The first sign of follicular organization was in the medullary region of the ovary close to blood vessels and involved epithelial cells from the disintegrating rete system. Later on, granulosa cells of the developing follicles originated from epithelial somatic cells. It seem therefore that both rete and ovarian mesothelial cells participate in the formation of follicular granulosa cells, and which is according to other authors (Peters, 1978; Sadler, 1990).

By the end of the third month, distinct follicles were observed in the inner part of the ovary. This is slightly earlier than the reported 95-100 days age for the taurine cattle (Vigier et al., 1976; Peters, 1978; Roberts, 1986). Therefore, compared to the taurine cattle, ovarian follicles seem to form at a slightly earlier age in the zebu cattle, and which support the above observation of an early differentiation of the ovary in this breed of cattle. However, the observation of folliculogenesis beginning in the inner part of the ovary and gradually spreading towards its outer part is in agreement to what is considered to be the general concept in mammalian folliculogenesis (Peters, 1978).

The first follicle to appear in the ovary is the primordial follicle, and which in the literature is referred to have a flat or squamous epithelium. When the surrounding epithelial (granulosa) cells become cuboidal, the follicle is referred to as a primary follicle (Dellmann and Brown, 1981; Fair et al., 1997). Apparently, the present study shows that the earliest follicles are surrounded by a cuboidal epithelium and that follicles surrounded by flat or squamous epithelium (to fit the description for resting primordial follicles, Fair et al., 1997) appear later. Therefore, at least in the zebu cattle, the first follicles to appear in the ovary are surrounded by cuboidal granulosa cells, and resting primordial follicles appear later.

The mechanism behind the changing of the shape of the granulosa cells from cuboidal to squamous, and then reverting to cuboidal again in the early growing (primary) follicles is not known. During the 4th month, nuclei of some gametes had a mass of fine threads of chromatin and which in some cases had a beaded appearance. According to Bakken and McClanahan (1978), transitory stages of meiosis may be recognized by chromosomal configurations and the above appearance of chromosomes indicate leptotene and zygotene stages of meiosis. This observation is supported by the few chromosome morphologies that indicate presence of preleptotene, leptotene and zygotene stages of meiosis. It appears that by day 120, meiosis is already in progress, and which is more or less in agreement to other reports (Roberts, 1986).

Amongst other factors, pituitary gonadotrophins and various paracrine factors including follicular regulatory protein, inhibin, polypeptide growth factors such as epidermal growth factor (EGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF) and growth differentiation factor 9 (GDF-9) are believed to play role in folliculogenesis (Driancourt, 1991;

Badinga et al., 1992, Vitt et al., 2000). However, the exact mechanism of this process is not yet very clear. Similarly, a number of factors including cAMP and others generally known as maturation inhibition or promoting factors are believed to play role in the control of meiosis in vertebrate oocytes. Nevertheless, the exact causative relationships and mechanisms between entrance into meiotic prophase and folliculogenesis are not well known. In the taurine cattle, it has been indicated that meiosis begins earlier than folliculogenesis (Ohno and Smith, 1964; Erickson, 1966a; Mauleon, 1967; Roberts, 1986).

Although systematic chromosomal studies are not yet done, light microscopic morphological results indicate that follicular formation started during the third month while meiosis started during the 4th month. This finding suggest that exclusion of oogonia from other cells by the follicular cells may be a prerequisite for meiotic division. Accordingly, separation of the oocyte from the surrounding tissue secures its survival and creates and sustains a defined micro milieu that sustains further growth and differentiation. It was also interesting to find that within the prenatal period, the oocytes grow from about 10-24 µm at the second month to about 50 µm at the end of the sixth month, and that formation of zona pellucida is also possible in growing fetal oocytes. Although a majority of such oocytes are likely to degenerate before the sexual maturity of the animal, it is worth noting that these changes, and which are also observed during the final growth and maturation of the oocytes (Assey et al., 1994; Fair, 1995) can occur, even before birth. Tertiary or vesicular follicles were observed as early as day 150 post-conception. This is much lower than the 250-270 days reported for taurine cattle (Erickson, 1966b, Russe, 1983) and slightly earlier than the 180 days reported by Rakha and Igboeli (1971), but is within the 150-180 days reported by Mbassa (1989).

Experimental fetal hypophysectomy and neutralization of circulating gonadotrophins with antibodies show that pituitary hormones play a key role in follicles entering the vesicular stage (Gulyas et al., 1977). The presence of FSH in the fetal pituitary glands support these observations and suggest that fetal follicular development may also be FSH-dependent.

CONCLUSION

In conclusion, preliminary findings on the study of the developmental potential of fetal oocytes indicate onset and advancement in oocyte meiosis during prenatal period. They also indicate fetal FSH secretion and morphological changes of the oocytes that are associated with attainment of developmental competence. While waiting for all the data that will emanate from the study, these preliminary findings are giving signs of optimism in the establishment of some degree of developmental competence in tetal oocytes.

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REFERENCES.

Armstrong, D.T., Holm, P., Irvine, B., Petersen, B.A., Stubbings, R.B., Mclean, D., Stevens, G.F. and Seamark, R.F., (1992). Pregnancies and live births from in vitro fertilization of calf oocytes collected by laparoscopic follicular aspiration. *Theriogenology,* 38: 667 - 678.

Assey, R.J., Hyttel, P., Greve, T., Purwantara, B. (1994). Oocyte morphology in dominant and subordinate follicles. *Molecular Reproduction and Development,* 37: 335 - 344.

Bakken, A.H. and Mc Clanahan, M., (1978). Patterns of RNA synthesis in early meiotic prophase oocytes from fetal mouse ovaries. *Chromosome,* 67: 21 - 40.

Badinga, L., Driancourt, M.A., Savio, J.D., Wolfenson, D., Drost, M., de La Sota, R.L. and Thatcher, W.W., (1992). Endocrine and ovarian responses associated with the first follicular wave dominant follicles in cattle. *Biology of Reproduction* 47: 871 - 883.

Barnes, F.L., (1994). Aspects of mammalian embryo production in vitro. Embryotechnology Conference, *22- 26th August 1994, RVAU. Abstracts. p.3.*

Betteridge, K.J., Smith, C., Stubbings, R.B., Xu, K.P. and King, W.A. (1989). Potential genetic improvement of cattle by fertilization of fetal oocytes in vitro. *Journal of Reproduction and Fertility Supplement,* 38: 87 - 98.

Cumming, I., Friend, A. and Aguma, C.O., (1994). Use of indigenous breeds of cattle and their crosses in Uganda as recipients for imported *Bos taurus* embryos. *Tropical Animal Health and Production,* 26: 119 -126.

Dellmann, H.D. and Brown, E.M., (1981). *Textbook of Veterinary Histology,* Ist ed. Lea and Febiger, Philadelphia p. 314.

Driancourt, M.A., (1991). Follicular dynamics in sheep. *Theriogenology,* 35: 55 - 79.

El Sayed, M.A.I., Kessy, B.M. and Maurya, S.N., (1987). Developmental changes in the gravid uterus of the Tanzanian short horn zebu cattle. *Alexandria Journal of Veterinary Science,* 3: 255 - 268.

Erickson, B.H., (1966a). Development and radio-response of the prenatal bovine ovary. *Journal of Reproduction and Fertility,* 10: 97 - 105.

Erickson, B.H., (1966b). Development and senescence of the postnatal bovine ovary. *Journal of Animal Science,* 25: 800 - 805.

Evans, H.E. and Sack, W.O., (1973). Prenatal development of domestic and laboratory mammals : Growth curves, external features and selected references. *Anatomy Histologia Embryologia,* 2: 11 - 45.

Fair, T., (1995). Oocyte growth in cattle. Ultrastructure, transcription and development competence. *PhD Thesis*. Royal Veterinary and Agricultural University, Copenhagen, Denmark p.140.

Fair, T., Hulshof, S.C.J., Hyttel, P., Greve, T. and Boland, M., (1997). Oocyte ultrastructure in preantral and primordial to arly tertiary follicles. *Molecular Reproduction and Development*, 195: 327 - 336.

Gulyas, B.J., Hodgen, G.D., Tullner, W.W. and Ross, G.T., (1977). Effects of fetal and maternal hypophysectomy on endocrine organs and body weight in infant rhesus monkeys (*Macaca mulatta*): With particular emphasis on oogenesis. *Biology of Reproduction*, 16: 216 - 227.

Hayat, M.M., (1989). *Principles and Techniques of Electron Microscopy. Biological Applications*, MacMillan Press. London. pp. 24-124.

Iritani, A., (1988). Current status of biotechnological studies in mammalian reproduction. *Sterility and Infertility* 50: 543 - 551.

Jordt, T. and Lorenzini, E., (1988). Superovulation, collection and transfer of embryos and demi-embryos from Boran (*Bos indicus*) cows and heifers. *Theriogenology*, 30: 355 - 367.

Jost,A., Vigier, B., Prepin, J. and Perchellet, J.P., (1973). Studies on sex differentiation in mammals. In: *Recent progress in Hormone Research,* Vol. 29 (ed. R. O. Greep). Academic Press New York. pp 1-41.

Marion, G.B. and Gier, H.T., (1971). Ovarian and uterine embryogenesis and morphology of the nonpregnant female mammal. *Journal of Animal Science,* 32 Suppl 1: 24 - 47.

Mauléon, P., (1967). Cinétique de I'ovogenése chez les mammiféres. *Archives fur Anatomische und Microscopische Morphologia und Experimentia Supplement,* 3-4: 125 - 150.

Mbassa, G.K., (1989). Studies on the ovarian development in Zebu cattle (*Bos indicus*). *Anatomia Histologia Embryologia*, 18: 143 - 149.

Ohno, S. and Smith, J.B., (1964). Role of fetal follicular cells in meiosis of mammalian oocytes. *Cytogenetics*, 3: 324 - 334.

Peters, H., (1978). Folliculogenesis in mammals. *In: The vertebrate ovary. Comparative Biology and evolution,* ed. R.E. Jones. Plenum Press, New York, London. p 121-140.

Rakha, A.M. and Igboeli, G., (1971). Physiology of pregnancy in tropically adapted cattle. I. Morphological changes in the cow in relation to fetal development. *Journal of Animal Science*, 33: 643 - 646.

Renard, J.P., (1994). Potential of primordial germ cells. *Embryotechnology Conference*, 22-26th August 1994, RVAU. Abstracts. p. 7.

Roberts, S., (1986). *Veterinary obstetrics and genital diseases (Theriogenology)*, 3rd ed. Published by the Author, Woodstock. Vermont p. 5-17, p. 417.

Russe, I., (1983). Oogenesis in cattle and sheep. *Bibliotheca Anatomica*, 24: 77 - 92.

Sadler, T.W., (1990). *Langmans Medical embryology*. 6th ed. Williams and Wilkins, Baltimore, London p. 275.

Vigier,, A.B., Prepin, J. and Jost, A., (1976). Chronologie du development de l'appareil genital du foetus de veau. *Archives fur Anatomische Microscopische und Morphologie Experimentia*, 65: 77 - 101.

Vitt, U.A., Hayashi, M., Klein, C., Hsueh, AJ., (2000). Growth differentiation factor-9 stimulates proliferation but suppresses the follicle-stimulating hormone-induced differentiation of cultured granulosa cells from small antral and preovulatory rat follicles. *Biology of Reproduction*, 62(2): 370 - 377.