HISTOLOGICAL EXPLORATION OF GRAAFIAN AND ATRETIC FOLLICLES OF BUFFALO OVARY: A SEASONAL STUDY

Jasvinder Singh Sasan[1], Varinder Uppal[2], Neelam Bansal[2] and Anuradha[2]

ABSTRACT

The present research work was conducted to study distribution of Graafian and atretic follicles in buffalo ovary during different seasons of year. For this purpose, 100 ovaries of adult Murrah buffaloes during different seasons (20 in each season) of year viz; winter (November - January), spring (February - March), summer (April - June), rainy (July - August) and autumn (September - October) were collected from slaughter house. The paraffin sections of 10 µm were cut and every 20th section was stained with hematoxylin and eosin. In present study, two types of follicles were observed viz. young and mature Graafian follicles. Highest number of normal follicles was during autumn season whereas lowest number of normal follicles was during summer season. The most common type of atresia was antral. The ratio of normal to atretic follicle was maximum in summer (1: 3.10) and minimum in autumn season (1: 2.19).

Keywords: buffalo, follicular atresia, histomorphometry, ovarian follicles, seasons

INTRODUCTION

Buffaloes are known for the poor reproductive performance such as silent heat, low conception rate and long calving interval which causes heavy economic losses to farmers (Madan, 1988). Further, the embryo transfer technique in buffalo has not proved to be much successful, mainly due to very poor superovulatory response and poor recovery of viable embryo (Taneja *et al.,* 1991). The success of *in vitro* production of buffalo embryos has been hampered by factors such as low quality of follicles on ovaries and poor oocyte recovery rate. In past it has been shown that environmental temperature plays an important role and buffaloes exhibit a distinct reproductive performance in different seasons (Shah, 1988). The number of graafian follicles produced on the ovary may be one of the important factor to determine the reproductive efficiency of the animal as it is affected by the environmental temperature. Although, the number of mature follicles have been counted during follicular and luteal phases of estrous cycle (Danell, 1987 and Bansal, 2002) but seasonal study on such a count is lacking. So the present research was planned to count and to correlate the number of mature follicles in each season of year.

[1]Sher-e-Kashmir University of Agricultural Sciences and Technology (SKUAST), Jammu, India, E-mail: jssasan216@gmail.com

[2]Department of Veterinary Anatomy, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University (GADVASU), Ludhiana, Punjab, India

MATERIALS AND METHODS

Collection of ovaries

Left and right ovaries of 100 adult Murrah buffaloes were collected during different seasons (20 in each season) of year viz; winter (November - January), spring (February – March), summer (April - June), rainy (July - August) and autumn (September - October). Immediately after collection, the ovaries were fixed in 10% neutral buffered formalin (NBF).

Processing of ovaries

Ovaries were processed by acetone benzene schedule (Luna, 1968). The whole ovary was serially sectioned with a rotary microtome at a thickness of 10 µm. The serial sections were placed in a sequence on clean glass slides keeping track of section numbers and slide numbers. Every 20th section of ovaries was stained with hematoxylin and eosin (Luna, 1968). Subsequent sections were stained with Masson's trichrome for collagen fibres, Gridley's stain for reticular fibres and Verhoeff stain for elastic fibres.

Number of follicles

The follicles which were ≥ 1mm in diameter were observed and counted by a method reported by Danell (1987). Every 20th section of ovary was placed in an enlarging apparatus and projected 5 times larger onto normal photographic paper. After development, the photographs were arranged in a series and fixed on a white sheet. For describing the size of follicles, each particular follicle was given a similar number on all the photographs where it appeared. The mean follicle diameter was determined as the average of three measurements: the largest diameter (D_1), the diameter perpendicular to D_1 (D_2) and diameter perpendicular to the plane of section (D_3). Third diameter D_3 was calculated as

$$D_3 = N \times 10 \times 20$$

where

"N" represents number of sections in which the particular follicle appeared

"10" represent the thickness of section in µm

"20" represents every 20th serial section of ovary at which diameter was measured.

Actual estimated diameter was calculated by dividing the mean follicle diameter by five.

RESULTS AND DISCUSSION

Depending upon the size of antrum, two types of follicles were observed viz. young and mature Graafian follicles. Mostly the follicles contained single oocyte but occasionally 2 or 3 oocytes were also observed (Figure 1). The oocyte of the graafian follicle was round to oval with mean diameter of 71.47±8.18 µm. The nuclei were centrally or eccentrically placed. The antrum was filled with eosinophillic colloidal fluid, the liquor folliculi. The membrana granulosa was covered by basement membrane. The theca layer was completely differentiated into theca interna and theca externa (Figure 2) as reported earlier in cattle (Rajakoski, 1960) and buffalo (Bansal, 2002). Theca interna composed of mainly epithelial cells with vesicular nuclei and small amount of fibroblasts and connective tissue fibres whereas theca externa consisted of fibroblasts, connective tissue and muscle fibres. Theca interna was highly vascular as reported earlier (Danell, 1987; Bhardwaj and Roy, 1998).

The mean size of Graafian follicle was 443.18±72.1 µm. The average thickness of zona pellucida was 5.34±0.48 µm. The mean size of antrum was 395.83±39.26 µm. The membrana granulosa, which enclosed the antrum was having mean thickness of 38.22±2.52 µm. The mean thickness of theca interna and externa was 63.03±3.4 µm and 59.25±5.45 µm, respectively.

Graafian follicles (> 1 mm) were classified into five categories on basis of size viz. 1 – < 2 mm, 2 – < 3 mm, 3 – < 4 mm, 4 – < 5 mm and ≥ 5 mm and has been depicted in Figure 13. The present study revealed that the follicles falling in 1 – < 2 mm group were present in maximum number and these constituted 65.27%, 64.79%, 61.16%, 53.33% and 64.89% of the total follicles during winter, spring, summer, rainy and autumn season, respectively. These follicles represented more than half of the total number of follicles (≥ 1 mm in diameter). The findings corroborates well with the earlier findings of Danell (1987) and Bansal (2002) during different seasons.

Percentage distribution of total number of Graafian follicles (normal and atretic) in the ovaries during different seasons has been summarized in Table 1 which revealed highest number of normal follicles was during autumn season whereas lowest number of normal follicles was during summer season. Similar types of findings were observed by Sadeghinezhad and Hasanzadeh (2010) in river buffalo. Also, it was observed that although the number of normal and atretic follicles were more on the right sided ovary but the difference between distribution of follicles in left and right ovary was non-significant. The maximum per cent of atresia (75.62) was observed during summer followed by spring (72.89), rainy (71.25), winter (69.79) and autumn (68.73). These findings are almost similar to Danell (1987) who reported 70.60 per cent atresia in Surti buffalo and Bansal (2002) who reported 71.77 per cent of atresia in buffalo ovary.

The ratio of normal and atretic follicles was calculated during different seasons. It was 1:2.31 in winter, 1:2.68 in spring, 1:3.10 in summer, 1:2.48 in rainy season and 1:2.19 in autumn, respectively. The data revealed that the ratio was highest in summer season as compared to other seasons.

Follicular atresia

As the primordial follicles progress through different stages of development, most of them never reach maturity and thus got degenerated at certain point along the way. This process of follicular degeneration is called follicular atresia and depends upon the variable degree of susceptibility of different cells to death (Rodgers and Irving-Rodgers, 2010). In the present study, atresia was seen in follicles at all stages of development. The highest atresia was also observed in the follicles of range 1 - < 2 mm (Figure 14) as reported earlier by Danell (1987) and Bansal (2002) with maximum value of 76.35% during summer season.

The follicular atresia of large follicles was categorized as obliterative and cystic. The obliterative atresia was further subdivided into first degree and second degree as described by Danell (1987) and Bansal (2002). In first degree of atresia, a number of pyknotic nuclei were observed either in the layers of membrana granulosa close to antrum or in the antrum itself but in close proximity to the membrana granulosa whereas the cells closest to the basal lamina were tightly packed and appeared healthy (Figure 3). In some of the follicles, the upper layer of granulosa cells was detached from the underlying granulosa cells (Figures 4, 5). The pyknotic nuclei were rarely seen in the granulosa cells close to the basement membrane. The fibroblast present in the theca layer

were spindle shaped and were orientated parallel to the membrana granulosa. This type of atresia has been described as antral atresia by Irving-Rodgers *et al.* (2001) and is comparable to first degree of obliterative atresia described by Danell (1987) and Bansal (2002). With further advancement, there was degeneration of granulosa layers with few pyknotic nuclei in the antrum, granulosa cells and cumulus. Later on, cumulus disappeared leaving a naked oocyte followed by in growth of connective tissue in the antrum (Figures 6, 7) as described earlier by Danell (1987) and Bansal (2002) in buffalo ovary. When the antrum was completely filled with connective tissue, the degenerated follicle was termed as corpus atreticum (Figure 8).

In other type of atresia, there was destruction of the most basal layer of the granulosa cells whereas the most antral granulosa cells remained healthy and closely apposed to each other. There were very few pyknotic nuclei in the antrum or in membrana granulosa closest to antrum. The cells nearer to antrum were often flattened so that overall antral surface of membrana granulosa appeared smooth and regular. The cells in the basal layer of membrana granulosa were separated from each other and from basal lamina by intercellular spaces. With further advancement of atresia, there was degeneration of granulosa cells (Figure 9) and large apoptotic bodies (Figure 10) were observed in the antrum. The spindle shaped fibroblasts of the theca interna were arranged randomly. This type of atresia observed during present study fits well as basal atresia described earlier by Irving- Rodgers *et al* (2001) and is comparable to the second degree of obliterative atresia as observed by Danell (1987) and Bansal (2002).

In cystic type of atresia, both granulosa and theca layers atrophied or only granulosa layer atrophied and theca layer may be luteinized, fibrosed or hyalinized. There was decrease in follicular size and antrum. In some of the follicles only 1-2 layers of granulosa cells was observed so giving the follicle a classic string of pearl orientation as described by Marion *et al.* (1968) who assumed that these follicles had begun to expand on atresia. Rodgers *et al.* (2001) reported that number of layers of granulosa cells decreases as follicles enlarge to reach a plateau. The number of granulosa cell layer is both a function of net rate of granulosa cell replication and the rate of antrum expansion. Thus, the string of pearls description used by Marion *et al.* (1968) arised by reduction in the number of layers of granulosa cells as the antrum expands during follicular growth and not during atresia. Luteinized cystic type of atresia occurred infrequently in which there was degenerated granulosa cells followed by luteinization of whole of the theca interna cells (Figure 11). Since atretic follicles produces high amount of progesterone as compared to non-atretic follicles (Westhof *et al.,* 1991) and also dibutyryl cyclic AMP significantly stimulates progesterone production by cells of atretic follicles. The increased concentration of progesterone leads to luteinization of follicles which is accompanied by cellular hypertrophy, formation of diffusely distributed lipo-proteins and increased number of lipid droplets (Guraya, 1997). Another type of cystic atresia observed in present study was fibrosed type in which there was degeneration of granulosa layer along with increased number of fibroblasts in theca interna as reported earlier by Bansal (2002) in buffalo ovary. This type of atresia was maximum among all the types of cystic atresia. In third type of cystic atresia, theca cells were hyalinized along with degeneration of granulosa cells (Figure 12). The atresia occurs in a particular sequence as reported earlier by Guraya (1979) and Danell (1987) i e. first

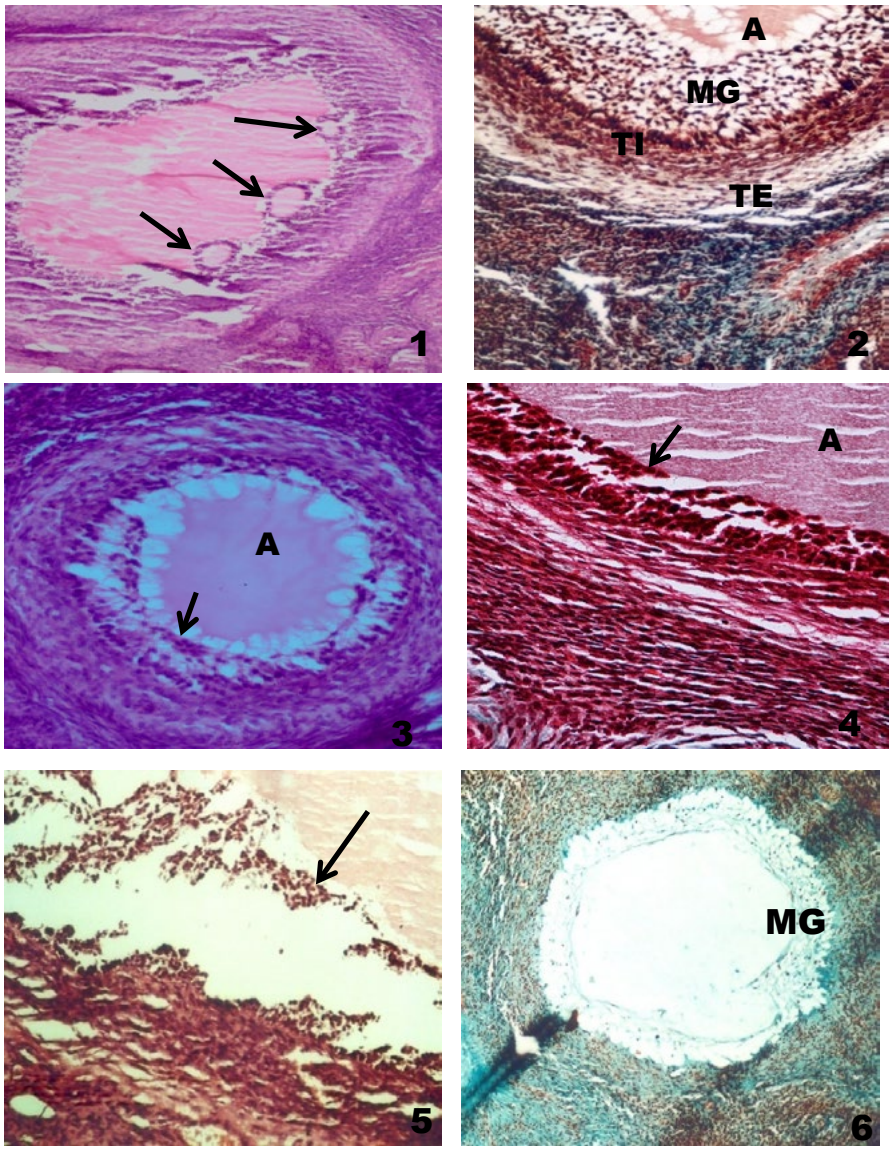

Figure 1. Growing follicle with three oocytes (arrow). H & E stain X10.

Figure 2. Section of buffalo ovary showing layers of normal Graafian follicle; antrum (A), membrana granulosa (MG), theca interna (TI) and externa (TE). Masson's trichrome stain X20.

Figure 3. Photomicrograph of paraffin section of ovary showing sloughing of granulosa cells (arrow) close to antrum (A). H & E stain X40.

Figure 4. Section of ovary showing loosening and sloughing of granulosa cells (arrow) close to antrum (A). Masson's trichrome stain X40.

Figure 5. Section of ovary showing complete sloughing of granulosa cells (arrow). Masson's trichrome stain X20.

Figure 6. Section of ovary showing degeneration of membrana granulosa (MG) cells. Masson's trichrome stain X10.

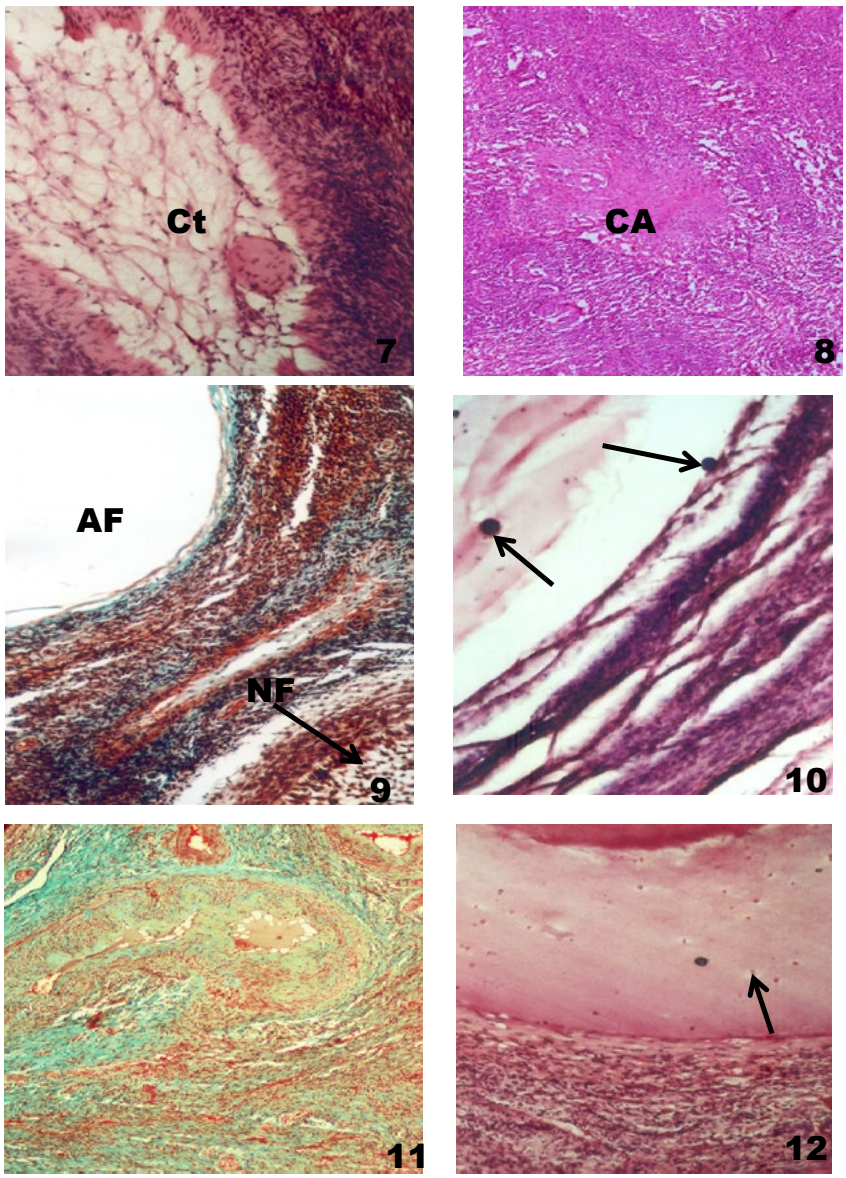

Figure 7. Section of ovary showing connective tissue (Ct) in antrum. H & E stain X20.

Figure 8. Section of ovary showing connective tissue scar, corpus atreticum (CA). H & E stain X10.

Figure 9. Section of ovary showing wall of normal (NF) and atretic follicle (AF). Masson's trichrome stain X20.

Figure 10. Figure38 Photomicrograph of paraffin section of ovary showing apoptotic bodies (arrow) in basal type of atresia. H & E stain X 20.

Figure 11. Photomicrograph of paraffin section of ovary showing luteinization of follicle. Masson's trichrome stain X10.

Figure 12. Photomicrograph of paraffin section of ovary showing hyalinization of follicular wall. Numerous pyknotic nuclei (arrow) can be seen. H & E stain X20.

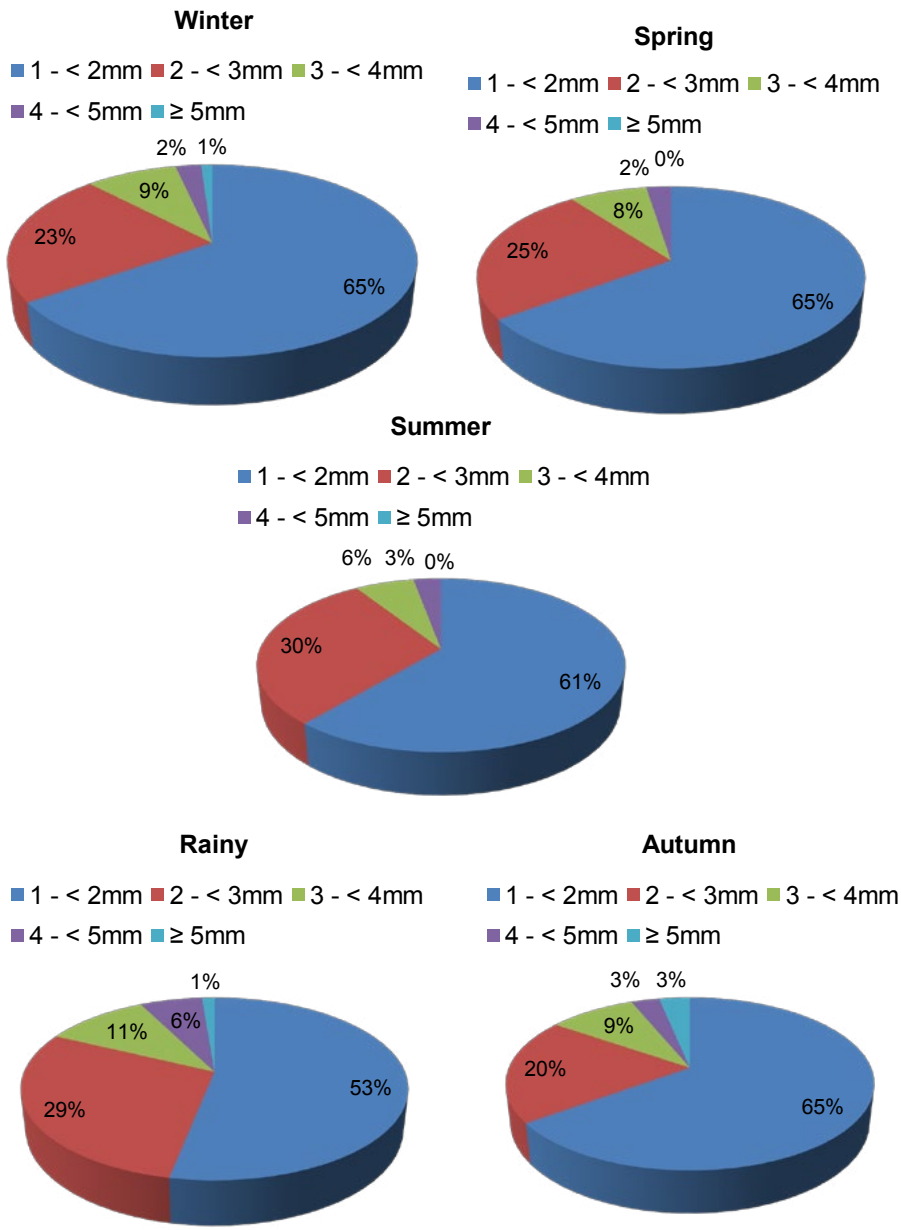

Figure 13. Percentage of different categories of follicles (≥ 1 mm) during different seasons.

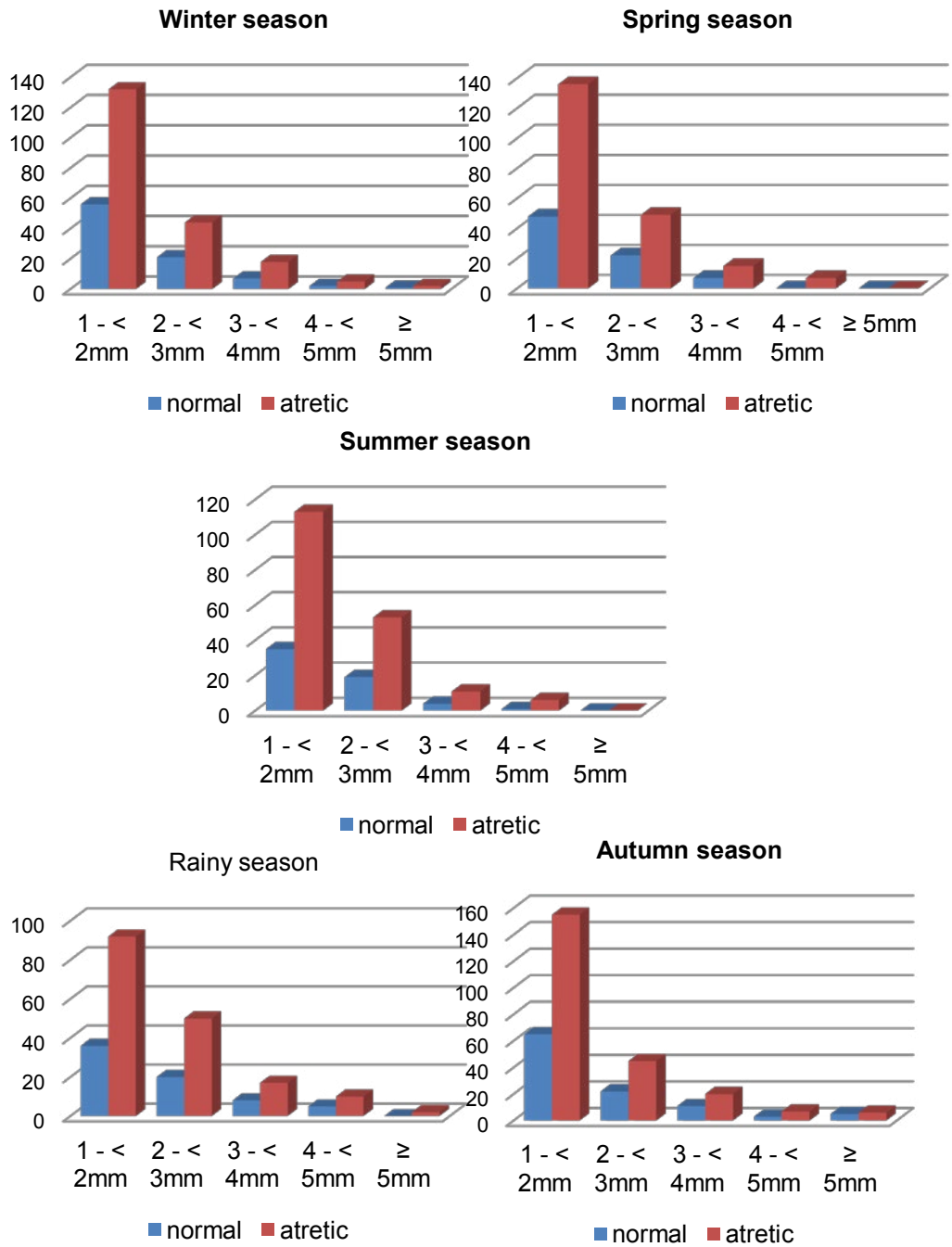

Figure 14. Number of normal and atretic follicles (≥ 1 mm) in different categories during different seasons.

Table 1. Percentage distribution of normal and atretic Graafian follicles (≥ mm) in left and right ovaries during different seasons.

Season	Left ovary		Total	Right ovary		Total	Total		Overall total	Normal : Atretic
	Normal	Atretic		Normal	Atretic		Normal	Atretic		
Winter	42 (29.78)	99 (70.22)	141	45 (30.61)	102 (69.39)	147	87 (30.21)	201 (69.79)	288	1 : 2.31
Spring	36 (26.47)	100 (73.53)	136	41 (27.70)	107 (72.30)	148	77 (27.11)	207 (72.89)	284	1 : 2.68
Summer	30 (25.00)	90 (75.00)	120	29 (23.77)	93 (76.23)	122	59 (24.38)	183 (75.62)	242	1 : 3.10
Rainy	29 (28.16)	74 (71.84)	103	40 (29.19)	97 (70.81)	137	69 (28.75)	171 (71.25)	240	1 : 2.48
Autumn	53 (31.36)	116 (68.64)	169	53 (31.18)	117 (68.82)	170	106 (31.27)	233 (68.73)	339	1 : 2.19
Total	**190 (28.40)**	**479 (71.60)**	**669**	**208 (28.73)**	**516 (71.27)**	**724**	**398 (28.57)**	**995 (71.43)**	**1393**	**1 : 2.5**

Values in parentheses indicate percentage calculated from total in a row.

there was dissolution and pyknosis of granulosa cells followed by differentiation of theca interna into fibrous cells. Later on there was hyalinization and dissolution of theca interna layer.

Although atresia may occur due to excessive secretion of gonadotropins (Harman *et al.,* 1975) or insufficient gonadotropins (Hirshfield, 1991), excessive androgens (Harman *et al.,* 1975), excess or insufficient estradiol (McNatty, 1978) and endogenenous GnRH like substance within the ovary (Birnbaumer *et al.,* 1985), yet there are also certain other factors which regulate the cyclic appearance and atresia of dominant follicles and other follicles. These factors may include age, stage of reproductive cycle, pregnancy, lactation, hormones of extra-ovarian and intra-ovarian sources, a genetic programmed nutrition, ischemia and season (Danell, 1987). Sluss *et al* (1983) has suggested the possibility of FSH-binding inhibitors in the induction or propagation of follicular atresia by suppressing the responsiveness of granulosa cells to FSH. Different studies have clearly suggested that the follicles which are destined to undergo atresia in normal estrous cycle of cattle can be rescued by administration of exogeneous gonadotrophins (Drianocourt, 1987). The follicles undergo atresia due to lack of gonadotrophins at a key stage (at which the amount of FSH receptors on granulosa cells and LH receptors on theca layer becomes maximum and also aromatase activity is increased) in folliculogenesis (Guraya, 1997). Actually the balance between estrogens and androgens plays especially an important role in determining whether a follicle becomes atretic or not (Adashi, 1994). It has also been reported that metabolism of granulosa cells can be affected by any interference in the development of their LH receptors which possibly can induce follicular atresia (Guraya, 1997).

So, the most important consequences of follicular atresia is to limit the number of ovulations or select the dominant follicle (Guraya, 1997), thus reducing the number of offsprings. Secondly, follicular atresia contributes thecal type interstitial cells to the ovary which constitute the steroidogenic tissue. Atretic follicles may contribute significantly to intra-ovarian levels of androgens and progesterone which can be utilized by non-atretic follicles to enhance estradiol synthesis (Westhof *et al.,* 1991).

REFERENCES

Adashi, E.V. 1994. Endocrinology of ovary. *Human Reproduction*, **9**: 815-27.

Bansal, N. 2002. *Gross and histomorphological studies on follicular population in ovaries of buffalo (Bubalus bubalis)*. Ph.D. dissertation, Punjab Agricultural University Ludhiana, India.

Bhardwaj, R.L. and K.S. Roy. 1998. Age related demographical study on the distribution of follicle type in the ovary of Indian buffalo. *Buffalo. J*., **3**: 395-399.

Birnbaumer, L, N. Shabi, J. Rivier and W. Vale. 1985. Evidence for a physiological role of gonadotropin-releasing hormone (GnRH) or GnRH like material in the ovary. *Endocrinology,* **116**: 1367-70.

Danell, B. 1987. *Oestrus behavior, ovarian morphology and cyclical variation in follicular system and endocrine pattern in water buffalo heifers.* Swedish University of Agricultural Science, Uppsala, Sweden.

Driancourt, M.A. 1987. *Follicular dynamics and intraovarian control of follicular development in the ewe. In* Roche, J. and D.

O'Callanghan (eds.) *Follicular Growth and Ovulation Rate in Farm Animals*. Martinus Nijhoff, The Hague.

Guraya, S.S. 1979. Morphological and histochemical observations on the buffalo ovaries during anoestrus. *Indian. J. Anim. Sci*., **49**: 423-432.

Guraya, S.S. 1997. *Ovarian Biology in Buffaloes and Cattle.* Indian Council of Agricultural Research, New Delhi, India.

Harman, S.M., J.P. Louvet and G.T. Ross. 1975. Interaction of estrogen and gonadotropins on follicular atresia. *Endocrinology*, **96**: 1145-52.

Hirshfield, A.N. 1991. Development of follicles in the mammalian ovary. *International Review of Cytol*ogy, **124**: 43-101.

Irving-Rodgers, H.F., I.L. Van Wezel, M.L. Mussard, J.E. Kinder and R.J. Rodgers. 2001. Atresia revisited: two basic patterns of atresia of bovine antral follicles. *Reproduction,* **122**: 761-775.

Luna, L.G. 1968. *Manual of Histological Staining Methods of Armed Forces Institute of Pathology*, 3rd ed. McGraw Hill Book Company, New York, USA.

Madan, M.L. 1988. Status of reproduction in female buffalo. *In Buffalo Production and Health*: A *Compendium of Latest Research Information Based on Indian Studies*. ICAR publication, New Delhi, India.

Marion, G.B., H.T. Gier and J.B. Choudary. 1968. Micro-morphology of the bovine ovarine follicular system. *J. Anim. Sci*., **27**: 451-465.

McNatty, K.P. 1978. Follicular fluid, p. 215-259. *In* Jones, R. (ed.) *The Vertebrate Ovary.* Plenum Press, New York, USA.

Rajakoski, E. 1960. The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical and left-right variations. Copenhagen. *Acta Endo*., **34**: 1-68.

Rodgers, R.J. and H.F. Irving-Rodgers. 2010. Morphological classification of bovine ovarian follicles. *Reproduction*, **139**: 309-318.

Rodgers, R.J., H.F. Irving-Rodgers, I.L. Van Wezel, M. Krupa and T.C. Lavranos. 2001. Dynamics of the membrana granulosa during expansion of the ovarian follicular antrum. *Mol. Cell. Endocr.,* **171**: 41-48.

Sadeghinezhad, J. and S. Hasanzadeh. 2010. Study of ovarian follicular development during summer and winter seasons in river buffalo. *Global Vet*., **4**: 582-86.

Shah, S.N.H. 1988. Comparative studies of seasonal influence on breeding behavior and conception rate of dairy buffalo and zebu cattle. *In Proceedings of 11th Congress on Animal Reproduction and Artificial Insemination*, **3**: 538.

Sluss, P.M, P.W. Fletcher and L. Richrt Jr. 1983. Inhibition of I^{125} –human follicle-stimulating hormone binding to receptor by a low molecular weight fraction of bovine follicular fluid: inhibitor concentration is related to biochemical parameters of follicular development. *Bio. Rep.* **29**: 1105-1113.

Taneja, M., S.M. Toey, C.H. Pawshe, G. Singh, A. Alli and G. Talwar. 1991. Ultrasonographic monitoring of ovarian follicular dynamics in supraovulatory buffalo, p. 581-585. *In Proceedings of Third World Buffalo Congress,* Vol. 3. Varna, Bulgaria.

Westhof, G., K.F. Westhof, W.L. Braendle and G.S. diZerega. 1991. Differential steroid secretion and gonadotropin response by

individual tertiary procine follicles *in vitro*. Possible physiologic role of atretic follicles. *Bio. Rep*., **44**: 461-468.