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# Histopathological alterations in the antral ovarian follicles in dairy cows with a tendency to emaciation

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**Summary.** The aim of the study was to define interrelationships between histopathological alterations in ovarian antral follicles and body condition in dairy cows with a tendency to emaciation (BCS 1 and 2) compared with dairy cows with normal body condition (BCS 3). The ovaries were recovered from slaughtered cyclic dairy cows (at the luteal phase of the cycle) of Czech Fleckvieh and Holstein breeds at different times of the *post-partum* period. The animals were estimated as belonging to certain grade of body condition score (BCS) according to a 5-point scale. Only dairy cows with BCS1 (emaciation; n=6), BCS2 (tendency to emaciation; n=5) and BCS3 (optimal body condition status; n=6) were available for the experiment.

The ovarian samples were embedded into Technovit 7100 resin; the tissue sections were stained with buffered basic fuchsine with toluidine blue. For acidic mucopolysaccharides (aMPS) a combination of PAS-technique with Alcian blue was used. Histological analysis showed that emaciation was associated with an increased occurrence of late (cystic) and luteinization-related atresia in granulosa and theca cells and increased levels of aMPS in small atretic follicles. Our observations indicate that dairy cows with a tendency to emaciation (BCS 2) or emaciated (BCS 1) have elevated occurrence of late atresia and atresia with luteinization, while initial atresia is less. This expands our basic knowledge of ovarian histopathology providing new insight into the association of antral follicle atresia and body condition status in dairy cows.

**Key words:** Cattle, Emaciation, Body condition score, Ovarian follicle, Atresia

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## Introduction

Decreased feed intake in the *post-partum* period, associated with energy deficit, causes rapid loss in body weight, increased occurrence of metabolic disorders and changes in the blood levels of glucose, insulin and IGF-I, which are important factors of follicle development (Butler and Smith, 1989; Spicer and Echternkamp, 1995). These events may lead to a negative energy balance (NEB), which restricts the production of gonadotropins (FSH and LH) and, therefore, adversely affects normal ovarian follicle development, causing follicle atresia (Butler et al., 1981; Butler, 2000). Several studies indicate that the NEB at early lactation can also cause excessive mobilization of fatty acids in cow liver tissue. Such cows are less fertile, have significantly longer interval to calving and a greater number of inseminations per conception compared to cows with moderate fattening (Reid et al., 1979; Reid and Collins, 1980).

Microscopic and histochemical signs of both normal and atretic bovine ovarian follicles have been described previously (Rajakoski, 1960; Marion et al., 1968; McKenzie and Kenney, 1973; Irving-Rogers et al., 2001, 2009). Irving-Rodgers et al. (2001) studied patterns of follicular atresia in bovine antral follicles and defined two types of follicular atresia. According to their study, the first type, antral atresia, is characterized by the presence of dead and pyknotic cells within the antral granulosa layer, whilst the basal layer remains intact. Atresia in the antral follicles is characterized by the destruction of the *membrana granulosa* layer close to the *antrum* and by the occurrence of pyknotic nuclei and the nuclei of dying cells in the *antrum* close to the *membrana granulosa* (Irving-Rodgers et al., 2001). The second type, basal atresia, is characterized by the occurrence of dead cells (apoptotic bodies) in the basal

granulosa layer, whilst the antral layers are intact. The basal lamina of atretic follicles is often breached by macrophages, which phagocytise dying basal granulosa cells. The theca is characterized by an increased deposition of collagen and the cells are orientated randomly.

The body condition of dairy cows, evaluated by the BCS grades, influences their reproductive characteristics, time of oestrus onset, conception rate and embryonal mortality (Silke et al., 2002). However, morphological changes in ovarian follicle structure within individual grades of BSC have not been reported. The aim of the study was to define interrelationships between histopathological alterations in ovarian antral follicles and body condition in dairy cows with a tendency to emaciation or emaciated (BCS 1 and 2) in comparison with dairy cows of average (BCS 3) condition.

## Materials and methods

### *Biological material*

The ovaries were recovered at slaughter of cyclic dairy cows of Czech Fleckvieh and Holstein breeds at different times during the *post-partum* period. Cyclic cows were identified on the basis of visual inspection of the ovaries (presence of developing follicles with a cavity and corpora lutea). The ovaries selected for histological analysis were approximately at the luteal phase of the ovulatory cycle, which was determined according to the presence of corpus luteum. The cows with no pathological changes on sexual organs were identified and were kept under normal feed regime. The animals were estimated as belonging to a certain grade of body condition score (BCS) according to a 5-point scale of BCS (Edmonson et al., 1989). In this experiment only dairy cows of BCS1 (emaciation; n=6), BCS2 (tendency to emaciation; n=5) and BCS3 (average body condition status; n=6) were available. Data on groups of these cows were taken from the farm records of individual cows as following: average age - 6.2 years, 4.1 years and 5.7 and post-partum period in the range of 6-9, 6-11 and 7-13 months for BCS1, BCS2 and BCS3 respectively.

### *Histological analyses*

For histological analysis ovarian samples were fixed in 10% neutral buffered formalin (Sigma-Aldrich), dehydrated in rising set of ethanol solutions (70% and 96% for 2 hours and 100% for 1 hour) and embedded in Technovit 7100 resin (Heraeus GmbH, CoKG, Werheim/Ts., Germany) according to the producer's manual. For light microscopy 1-5 $\mu$m sections were cut using AC-820 rotation microtome (American Corporation, USA). Each ovary was cut into 5 fragments and 15 sections were done sequentially from each fragment. The sections were stained with buffered basic fuchsine with toluidine blue ratio 3:2 prior to use (Bourne and St. John, 1978). For acidic mucopolysaccharides (aMPS) a combination of PAS-technique with Alcian blue staining using Alcian blue-PAS kit (Merck, Darmstadt, Germany) was used. Stained sections were mounted on Entelan and analyzed under a Jenaval light microscope (Carl Zeiss, DDR).

The number of follicles examined in each section varied from 0 to 7 follicles, on average about 3.5 follicles per section. Small follicles were distinguished from large follicles according to the number of layers of granulosa cells: large follicles had up to 8-10 cell layers, whilst small follicles had a smaller number of cell layers (4-6).

Atresia of antral follicles in histological specimens was evaluated on the basis of histopathological image of altered non-ovulated follicles, classified into four categories according to McKenzie and Kenney (1973) with partial modifications as follows:

Three forms of atresia without luteinization: (1) atresia of a first instance (initial atresia); (2) obliterative atresia; (3) late (cystic) atresia;

One form of atresia associated with luteinization: (4) initial atresia with luteinization of granulosa and theca cells.

### *Statistics*

The data for histopathological changes in non-ovulated antral follicles were statistically evaluated by Chi-square test using procFreq software (SAS/STAT statistical package). At the comparison of differences between different BCS grades a zero hypothesis was cancelled.

## Results

Using morphological assessment we observed the following histopathological alterations, which characterize atresia of antral follicles in comparison to follicles without changes (Fig. 1A). The initial form of atresia is characterized by degenerative changes in the nuclei of granulosa cells, more often in the form of pyknosis (Fig. 1B). Degenerated cells with pyknotic nuclei are observed on the surface and within each layer of *membrana granulosa* (Fig. 1C), and also on the basal membrane (Fig. 1D,E). Altered cells are very often released into the follicular cavity (Fig. 1C,D). The basal membrane of atretic follicles becomes separated from the basal layer of granulosa cells; it is swollen and often forms visible folds and undulations (Fig. 1D,E). Waving of *membrana granulosa* (Fig. 1D) and swelling of the basal membrane (Fig. 1E) indicate changes in intrafollicular pressure and also altered physic-chemical properties of the basal membrane. Apoptotic bodies occurred in both basal and surface layers of *membrana granulosa* in the initial form of atresia in antral follicles

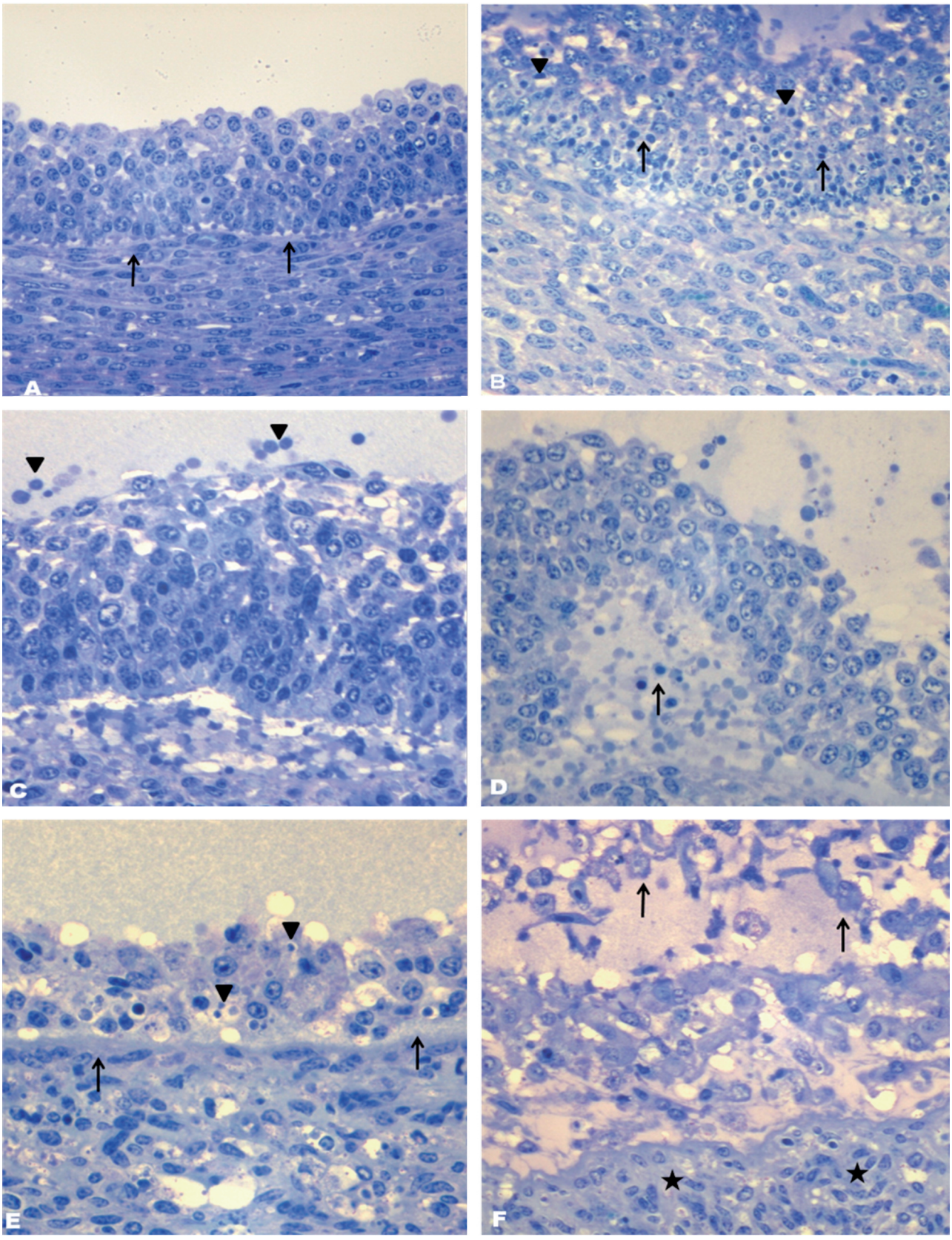


**Fig. 1.** Histopathological picture of cow ovarian antral follicles. **A.** Tertiary (Graafian) follicle of control cow. Follicular multilayered epithelium of *membrana granulosa*, which is formed by follicular cells and by external and internal theca cells without histopathological changes. The lower cell layer is settled on a thin basal membrane - *membrana propria* (arrows). **B.** Numerous pyknotic nuclei of the antral follicular cells (arrows) manifested as cell shrinkage, hyperchromatosis of the nucleus and occurrence of apoptotic bodies (arrowheads). **C.** Atrophy of follicular cells in surface layers of the antral follicle *membrana granulosa* at initial stage of atresia (arrowheads). **D.** Waved *membrana granulosa* with edematosis in the basal membrane and occurrence of apoptotic bodies (arrows). **E.** Swollen basal membrane (arrows), necrotic-like changes of follicular cells in the basal and surface layers of *membrana granulosa* and the occurrence of apoptotic bodies (arrowheads). **F.** Obliterative atresia of the antral follicle at initial phase. Numerous follicular cells released into the follicle cavity (arrows) and surrounded by fibrotic tissue (asterisks). Basic fuchsine and toluidine blue staining. x 500

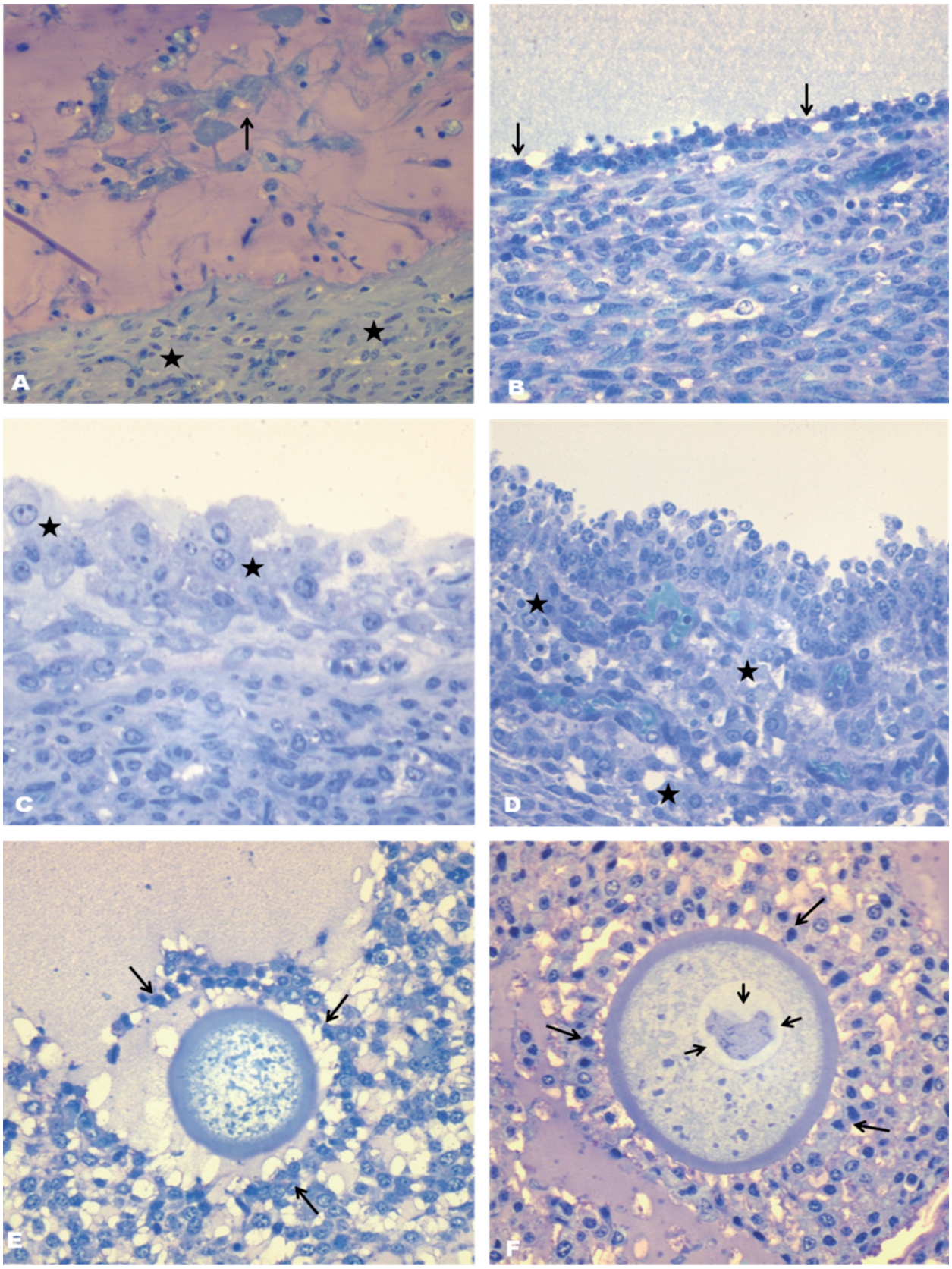


**Fig. 2.** Histopathological picture of cow ovarian antral follicles and oocytes. **A.** Obliterative atresia at advanced phase: numerous fibrotic tissue cells (asterisks) surrounding follicular cell remnants; the area of degenerated (necrotic-like) cells (arrow). Neutral mucopolysaccharides in the extracellular matter filling the cavity of obliterating follicle (violet colour) after staining with basic fuchsine and toluidine blue. **B.** Late atresia of the antral follicle with the epithelioid cell layer (arrows). **C.** Atresia of the antral follicle associated with luteinization of granulosa cells (asterisks). **D.** Atresia of the antral follicle associated with luteinization of both *theca interna* and *theca externa* cells (asterisks). **E.** The oocyte in the atretic antral follicle surrounded by cumulus cells (arrows) without tight connection with the degenerating ooplasma. **F.** Prominent pyknosis of the nuclei of cumulus cell (arrows) surrounding the oocyte with collapsed germinal vesicle (short arrows) and the occurrence of dark bodies in the ooplasma. Basic fuchsine and toluidine blue staining. x 500

**Table 1.** Histopathological alterations in the antral ovarian follicles from cow with different BCS.

| Parameters | Body condition score of cows | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Totally follicles studied (n) | 227 | 205 | 243 |
| Non-ovulated atretic follicles, n (%) | 184 (81.0)[a] | 157 (76.6)[a,b] | 167 (68.7)[b***] |
| Of them: | | | |
| Atresia without luteinization | | | |
| -initial | 36 (19.6)[a] | 43 (27.4)[a,b] | 57 (34.1)[b***] |
| -obliterative | 60 (32.6) | 58 (36.9) | 63 (37.7) |
| -late (cystic) | 26 (14.1)[a] | 15 ( 9.5)[a,b] | 12 ( 7.2)[b***] |
| Atresia with luteinization | 62 (33.7)[a] | 41 (26.1)[b**] | 35 (20.9)[b***] |

Values with different superscripts within rows are significantly different (Chi-square test); **: p<0.01; ***: p<0.001

**Table 2.** Intensity of occurrence of acidic mucopolysaccharides (aMPS) in small and large antral ovarian follicles of cows with different BCS.

| Antral follicles | Body condition score of cows | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Small (0.3-1 mm) | +++ | +++ | ++ |
| Large (5-8 mm) | +++ | ++ | + |

Small follicle with initial antrum formation; Large follicle with expanded antrum and differentiated *cumulus oophorus*; intensity of aMPS occurrence: +: small; ++: moderate; +++: abundant

(Fig. 1D,E). Of initial changes in the *membrana granulosa* cells, obliterative or late form of atresia may occur. These types of atresia lead to the destruction of epithelial granulosa and theca cells.

The obliterative form of atresia was characterised by the loss of intrafollicular pressure, which is evidenced by the inoculated follicular wall. Degenerating (necrotic-like) granulosa and theca cells are surrounded by fibrotic connective tissue (Fig. 1F). At the advanced phase of obliterative atresia fibrotic cell mass completely surrounds remnants of follicular cells which subsequently degenerate (Fig. 2A). The final step is a hyalinization of *membrana granulosa* (the wall), which leads to *corpus albicans* formation.

The characteristic sign of late (cystic) atresia is a loss of *membrana granulosa* cells and partial loss of theca epithelial cells. The formation of the follicular cyst is complete when the granulosa basal layer is transformed into epithelial layer (Fig. 2B). The cavity is filled with a fluid under pressure. Late atresia affects the follicles in the size range of 2-20 mm, and this form of atresia must be distinguished from cystic ovarian disease, which affects follicles with more than 20 mm in size, appeared on the ovary surface.

Atresia associated with luteinization is characterized by hypertrophy of granulosa and theca epithelial cells, which leads to local rupture of the basal membrane (*membrana propria*) and to a disturbance of regular layout of granulosa cells. The loss of intrafollicular pressure leads to a cross-mixing of theca and granulosa cells (Fig. 2C,D).

Initial atresia in the antral follicles creates deteriorative conditions for oocyte maturation, leading to oocyte degeneration. Histopathological changes are often manifested as disrupted contacts between *cumulus oophorus* and the ooplasm, or among granulosa cells, as well as expanded intercellular spaces (Fig. 2E). *Vesicula germinativa* of oocytes is often collapsed; the ooplasm has signs of degeneration. The nuclei of *corona radiata* and *cumulus oophorus* cells bear signs of pyknosis (Fig. 2F).

The occurrence of atrophic changes in the non-ovulated ovarian follicles from cows with different body condition scores is shown in Table 1. There was no significant difference in the percentage of the non-ovulated follicles between BCS 1 (81.0%) and BCS 2 (76.6) groups; however this value was significantly lower in BCS 3 (68.7%) animals. Also, no differences in ratios of initial, obliterative and late atresia between BCS1 and BCS2 groups were observed, whilst BCS3 cows showed significantly higher occurence of initial atresia, but a lower percentage of late atresia compared to BCS 1 and 2 groups. No significant differences in the occurrence of obliterative atresia among cows with different BCS grades were observed. The higher occurrence of atresia with luteinization was observed in BCS1 cows, when compared to BCS2 and BCS3 groups. In cows with lower BCS scores (1 and 2) there was a more frequent occurrence of blood and lymphatic capillaries in *theca folliculi interna* when compared to BCS3 cows; these capillaries were dilated and in some cases were filled with blood elements (data not shown).

At subjective evaluation of histochemical reaction using PAS-technique with Alcian blue we observed a higher occurrence of acidic mucopolysaccharides in small (0.3-1 mm) and large (5-8 mm) antral follicles in cows with lower BCS (1 and 2) compared to cows of

BCS 3 (Table 2). In some cases aMPS were observed in the cavity formed during obliterative atresia.

## Discussion

Follicular atresia affects all stages of follicle development in cow ovaries and it is manifested by the arrest or even extinction of further development. Degenerative changes affect the cells of *membrana granulosa*, theca, and their blood capillaries, as well as basal membrane and ovum. Antral follicles can succumb to preterm luteinization or to transformation into luteal cysts. Despite small abnormalities, like anovulation, occurring sometimes, the estrous cycle has a regular pattern. An occurrence of atresia of follicles together with the oocytes can be increased under unfavourable conditions, particularly during weight loss.

The effect of cow's body condition on fertility of dairy cows is widely known (Silke et al., 2002) with the loss of one condition score in the first 5 weeks *post-partum* being associated with delayed onset of oestrus, lowered conception rate and higher rate of embryonal mortality compared to dairy cows with an average condition. Thus, the ratio of oocytes developed to the blastocyst stage in dairy cows with BCS 1.5-2.5 is lower when compared to BCS 3.3-4.0 cows.

In dairy cows of BCS1 and BCS2 a higher occurrence of non-ovulated antral follicles was observed compared with average CS cows. Most of them were subjected to late (cystic) atresia or atresia with luteinization. One possible explanation may be a decreased gonadotropic activity and insufficient LH pulse frequency in cows with a tendency to emaciation at *post-partum* period, which causes atresia rather than ovulation (Savio et al., 1990). Therefore, we may assume that levels of endogenous gonadotropins in cows with a tendency to emaciation (BCS 2) or in emaciated (BCS 1) cows are insufficient for ovulation of follicles, which are then subjected to different forms of atresia.

Many authors studying the problem of ovarian follicle atresia attempted to characterize follicles according to changes in some cell structures. Basic categorization has been done by Marion et al. (1968), who in a micromorphological study described the bovine ovarian follicle system in relation to different grades of atresia. McKenzie and Kenney (1973) studied the histological structure of ovarian follicles in heifers after gonadotropin injections, and they characterized seven grades of atresia. Later, Irving-Rodgers et al. (2001) described two types of atresia: antral and basal atresia. Using histological analysis we detected signs of atresia on surface and basal cells of *membrana granulosa*, which are similar to those described earlier (Irving-Rodgers et al., 2001). We may assume that these changes are preceded by morpho-functional changes in the basal membrane, which in most cases has signs of waving and edematous dilation (Fig. 1). It is supposed that physical-chemical properties of the basal membrane are subjected to changes, which lead to the alteration of their permeability.

Atretic changes affect granulosa cells of both antral and non-antral follicles. Necrotic changes are manifested mostly by pyknotic nuclei and fragmented cytoplasm (Telfer, 1997). Apoptotic changes in granulosa cells occur only in the antral follicles (Yuan and Giudice, 1977). The other sign of atresia is an increase in intercellular spaces (Peluso et al., 1980) and loss of gap junctions between the cells (Merk et al., 1973). In our study all non-ovulated antral follicles were subjected to different forms of atresia, which corresponds to the findings of McKenzie and Kenney (1973). However, atretic changes were detected not only in large antral follicles, but also in small follicles according to the presence of acidic mucopolysaccharides (aMPS). It is known that accumulation of aMPS in ovarian follicular fluid accompanies follicular growth, and the amount may be associated with follicular atresia (Ax and Ryan, 1979). The naturally occurring MPS in biological samples are proteoglycans. In ovarian follicles, classified by morphological and steroidal criteria as atretic, the concentrations of proteoglycans in follicular fluid are higher than in healthy follicles (Salustri et al., 1999). Therefore, histochemical evidence of aMPS is a proper tool for early determination of atretic changes, both in the antral and non-antral follicles (Ax and Ryan, 1979; Salustri et al., 1999). Increased occurrence of aMPS detected in small atretic follicles of emaciated dairy cows in our study confirms already known results. The proportion of ovulated follicles in cows with BCS 1 and 2 in our study was substantially decreased, whilst the proportion of late atresia or luteinization-related atresia was increased.

The increased presence of antral follicle atresia with luteinization in dairy cows with a tendency to emaciation (BCS 1 and 2) may be caused by pre-term action of luteinization-regulated serum factors when permeability of the basal membrane of atretic follicles is changed. The other cause may be an incorporation of capillaries of the basal membrane of atretic follicles into spaces among granulosa cells (Murphy, 2000).

Previous reports described histopathological alterations in ovarian folicles either from gonadotropin-treated (McKenzie and Kenney, 1973) or untreated (basal conditions; Marion et al., 1968; Irving-Rodgers et al., 2001) heifers or cows. A new aspect of our study is that we present histopathological characterization of antral ovarian follicles in relation to body conditions of dairy cows with a tendency to emaciation.

### *Conclusions*

Our observations indicate that in dairy cows with a tendency to emaciation (BCS 1 and 2) the occurrence of late (cystic) atresia and atresia with luteinization is elevated, whilst initial atresia is less frequent. This study expands basic knowledge of ovarian histopathology providing new insights into the association of antral follicle atresia and body condition status of dairy cows.

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