

Morphological classification of bovine ovarian follicles

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Abstract

Follicle classification is an important aid to the understanding of follicular development and atresia. Some bovine primordial follicles have the classical primordial shape, but ellipsoidal shaped follicles with some cuboidal granulosa cells at the poles are far more common. Preantral follicles have one of two basal lamina phenotypes, either a single aligned layer or one with additional layers. In antral follicles <5 mm diameter, half of the healthy follicles have columnar shaped basal granulosa cells and additional layers of basal lamina, which appear as loops in cross section ('loopy'). The remainder have aligned single-layered follicular basal laminae with rounded basal cells, and contain better quality oocytes than the loopy/columnar follicles. In sizes >5 mm, only aligned/rounded phenotypes are present. Dominant and subordinate follicles can be identified by ultrasound and/or histological examination of pairs of ovaries. Atretic follicles <5 mm are either basal atretic or antral atretic, named on the basis of the location in the membrana granulosa where cells die first. Basal atretic follicles have considerable biological differences to antral atretic follicles. In follicles >5 mm, only antral atresia is observed. The concentrations of follicular fluid steroid hormones can be used to classify atresia and distinguish some of the different types of atresia; however, this method is unlikely to identify follicles early in atresia, and hence misclassify them as healthy. Other biochemical and histological methods can be used, but since cell death is a part of normal homeostasis, deciding when a follicle has entered atresia remains somewhat subjective.

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Introduction

The adult ovary contains a reserve of inactive primordial follicles. Each contains a small non-growing oocyte and a layer of non-dividing pregranulosa cells encapsulated by the follicular basal lamina. Every day a number of primordial follicles become active, and the oocyte commences growing while the granulosa cells begin to divide. As the granulosa cells divide, the number of layers of cells (called the membrana granulosa or follicular epithelium) around the oocyte increases, and the follicular basal lamina expands. Later in development, a fluid-filled cavity or antrum forms and specialized stromal layers, the theca interna and externa, develop. Only follicles that reach the stage of having a large antrum, and in the follicular wave following regression of corpora lutea, can ovulate an oocyte in response to the surge release of LH. Following ovulation, the granulosa cells and thecal cells differentiate into the large and small luteal cells of the corpus luteum, and the vascular supply of the corpus luteum is derived from the capillaries of the theca interna. All non-ovulating follicles undergo atresia and regression.

Since the development and regression of follicles are associated with major structural and functional changes, it is important to classify follicles accurately as healthy or atretic at all stages of development and to subclassify

them further into the different isoforms that exist at most stages. Classification enables us to better compare studies and also assists us to focus on subtleties in follicles that might otherwise have gone unnoticed. Unfortunately, numerous studies have been conducted on follicles or their cells with no effort to ensure these are from similar follicular types and this has led to misinformation about follicular development. Even though the follicles have been classified into healthy and atretic, a number of different methods have been used to classify atresia, including histology, biochemical markers and ultrasonography; and while there may be no consensus on the best method for classifying atresia, the use of different methods leads to different groupings of follicles from one method to another. Here, we review the approaches to classifying follicles from cattle. Cattle are not only important agricultural species but their ovarian follicle dynamics makes them ideal models for humans (Adams & Pierson 1995), including the changes that occur on ageing at menopause (Malhi *et al.* 2005).

Primordial to preantral follicles

Primordial follicles in most species are identified histologically on the basis of a small non-growing oocyte, without a zona pellucida and surrounded by flattened granulosa cells (Fig. 1; Fair *et al.* 1997).

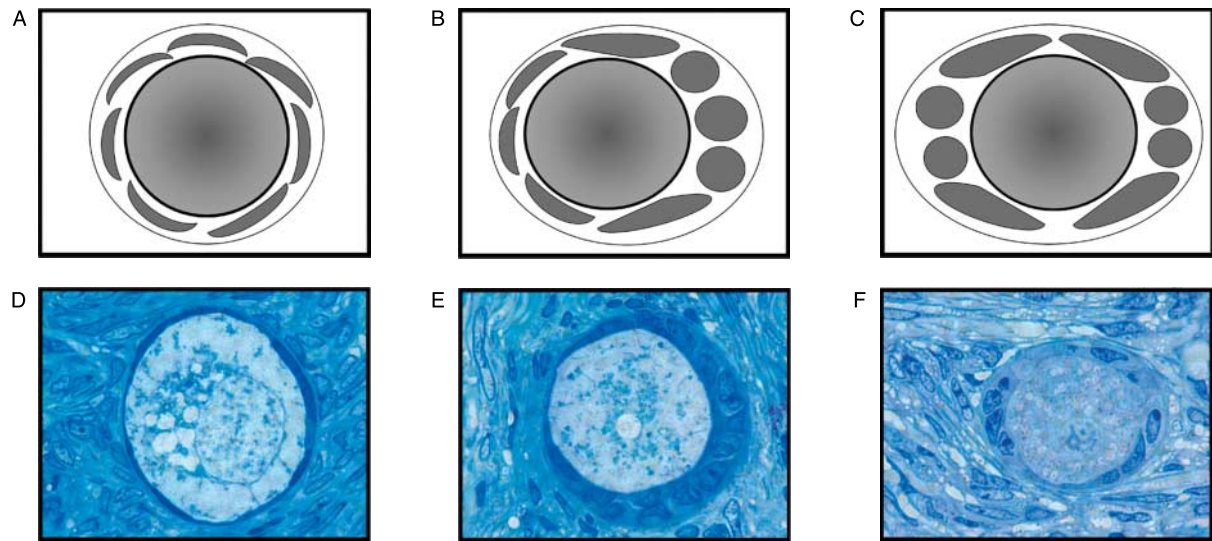


Figure 1 Cartoons and light micrographs (methylene blue-stained section of plastic embedded tissues) of human (D and E) and bovine (F) follicles illustrating the classical primordial follicle shape (A and D), the human transitional shape (B and E) and the common ellipsoid shape of bovine follicles (C and F).

The follicles appear this way because the oocyte is not enlarging and the granulosa cells are not replicating. Once activated, the primary follicles have an enlarging oocyte around which the zona pellucida will form, and a layer of granulosa cells that have become cuboidal in shape (Fair *et al.* 1997). The cells are presumably cuboidal because they have commenced replicating and cells 'round-up' at the prophase and metaphase allowing spindle formation for division to subsequently occur (Boucrot & Kirchhausen 2008, Rosenblatt 2008). In support of this, it has been observed directly in the mouse ovary that the cuboidal granulosa cells divide more than the flat cells (Da Silva-Buttkus *et al.* 2008).

In both bovine (van Wezel & Rodgers 1996) and human ovaries (Gougeon & Chainy 1987), other morphological variations of primordial and primary follicles are also observed. In bovine ovaries, 83% of follicles that are less mature than the primary stage are ellipsoid in shape with cuboidal cells located at the poles of the follicle (Fig. 1). These have been interpreted to be primordial follicles with an ellipsoid shape imposed upon them by the surrounding bundles of collagen fibrils (van Wezel & Rodgers 1996). In the humans, an intermediate form has been observed in which some granulosa cells (usually on one side of the follicle) are cuboidal in shape (Fig. 1; Gougeon & Chainy 1987). These follicles are as numerous as the true primordial follicles and of the same size, with oocytes of a similar size, but with more granulosa cells seen in cross section (Gougeon & Chainy 1987). The terms 'transitional' or 'intermediate' are commonly used to describe these follicles (Stubbs *et al.* 2007, Westergaard *et al.* 2007, Rice *et al.* 2008). This is potentially misleading if it is interpreted to mean that these follicles are in a transition stage from primordial to primary. Furthermore,

primordial follicles that have been activated but not yet developed to the primary stage would be expected to be few in number, unless the rate of cell division was very slow. This is very unlikely to be the case as evidence from rat studies would indicate (Meredith *et al.* 2000). Thus, we believe the use of terms intermediate or transitional should not be used to necessarily indicate transition from primordial to primary follicle.

Preantral follicles in bovine (Irving-Rodgers & Rodgers 2000) and also human (Irving-Rodgers *et al.* 2008) can be classified into two groups depending on the morphological phenotype of the follicular basal lamina. In both species, some follicles have a conventional basal lamina of a single layer aligned to the surface of the basal granulosa cells, which at the preantral stage in both species are substantially thicker or even partially laminated than either the primordial or the antral follicles. Preantral follicles with additional layers of basal lamina have also been observed. Loops of basal lamina are seen in cross sections and are connected to additional layers closer to the granulosa cell surface (Fig. 2). Cellular projections emanating from the basal surface of the basal granulosa cells and membrane-bound vesicles often at the end on these processes and adjacent to the basal lamina are also present (Irving-Rodgers & Rodgers 2000). No studies comparing these two types of follicles at the preantral stage have been conducted in either bovine or human, but there are clear differences at the antral stages as discussed below.

Healthy antral follicles

During antral follicle growth, there is both replication of granulosa cells and formation and expansion of the follicular antrum containing follicular fluid. The concept

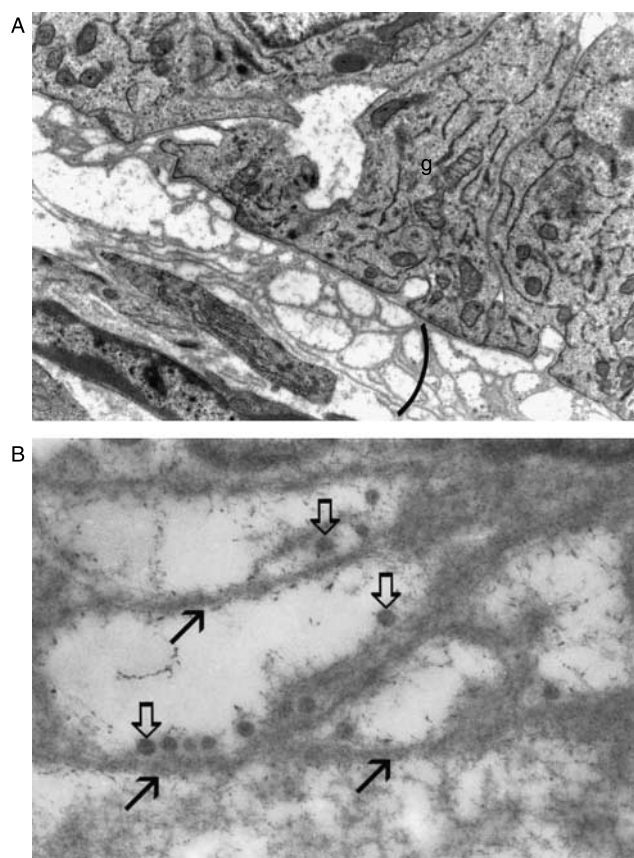


Figure 2 Electron microscopic images of (A) a follicle with a loopy basal lamina (bracket) located at the base of granulosa cells (g) and of (B) a loopy follicular basal lamina with matrix vesicles (arrowheads) located on the granulosa cell side of basal lamina layers (arrows).

that granulosa cell replication and follicular antrum expansion can be regulated differentially (Rodgers *et al.* 2001) should not be contentious. On the one hand, granulosa cell replication involves regulation of cell cycle and other genes, and can be stimulated by a number of growth factors, and, on the other hand, the formation of follicular fluid is biochemically very different and it has been suggested in bovine to involve the production of osmotically active proteoglycans hyaluronan and versican (Clarke *et al.* 2006). Evidence of differential regulation also comes from theoretical considerations. It has been argued (Rodgers *et al.* 1999) that during follicular development there is on average a net 19 doublings in the surface area of the follicle (from a primordial to an 18 mm bovine follicle, calculated from van Wezel & Rodgers (1996)), and a net 21 doublings of granulosa cell numbers (if 40 million granulosa cells were present as reported by McNatty *et al.* (1984) – see van Wezel & Rodgers (1996)). Thus, it is predicted in bovine that cell layers in the membrana granulosa would increase from one layer, as in primordial follicles, to four (calculated as $(21-19)^2=4$) layers (Rodgers *et al.* 1999), and this is close to the number of layers observed in bovine preovulatory

follicles (Irving-Rodgers *et al.* 2001). However, there is considerable variation in the numbers of layers per follicle during follicle growth (van Wezel *et al.* 1999b), consistent with a reported variation in the number of granulosa cells obtained from follicles of the same size (McNatty *et al.* 1979). On the basis of these observations, it was suggested that the rate of granulosa cell proliferation and maturation is not tightly or co-ordinately regulated with the rate of antrum expansion (Rodgers *et al.* 2001).

The concept of differential regulation of granulosa cell replication and follicular fluid accumulation has been used (Rodgers *et al.* 2001) to explain two follicle phenotypes (Irving-Rodgers & Rodgers 2000). These phenotypes in bovine can be identified on morphological and functional criteria. One phenotype has the 'loopy' basal lamina and columnar basal granulosa cells (Irving-Rodgers & Rodgers 2000) and expression of the RNA subunit of telomerase (TERC) in the antrally situated cells (original data in Lavranos *et al.* (1999) and discussed in Rodgers *et al.* (2001)). The other phenotype has an aligned single layer of follicular basal lamina and rounded basal cells that express TERC. In bovine antral follicles up to 5 mm in diameter, both phenotypes occur in equal proportions, while over 5 mm in diameter only the latter phenotype is observed (Irving-Rodgers & Rodgers 2000).

It has been suggested that the shape of the basal granulosa cells reflects the dynamics of the follicle as a whole, rather than individual differences between the cells within the membrana granulosa (Rodgers *et al.* 1999, van Wezel *et al.* 1999b). It is known that bovine follicles can expand at different rates whether this is calculated from replication rates of cells (Lussier *et al.* 1987) or monitored directly by ultrasonography (Fortune *et al.* 1991, Ginther *et al.* 1996). It was predicted that if the follicular antrum expansion is slow relative to granulosa cell replication, the layers become compacted leading to one or more of the basal layers of cells being columnar (Fig. 3; Rodgers *et al.* 2001). This concept linking basal granulosa cell shape and follicular basal lamina phenotype with the rate of follicular antrum expansion is also supported by the observation that columnar basal granulosa cells and loopy basal laminae are not observed in follicles approaching or past the size of dominance where the rate of antrum expansion is greater (>5 mm (van Wezel *et al.* 1999a, Irving-Rodgers & Rodgers 2000)), and in follicles identified as dominant by ultrasonography (Irving-Rodgers *et al.* 2002).

It has been speculated as illustrated in Fig. 4 that follicles with a loopy follicular basal lamina are slower growing, producing excess basal lamina that is subsequently shed (Rodgers *et al.* 2001) from the basal surface of the granulosa cells by budding off areas of the surface membrane containing focal adhesions and thus producing the observed membrane-bound vesicles as occurs on release of fibroblasts from collagen matrix

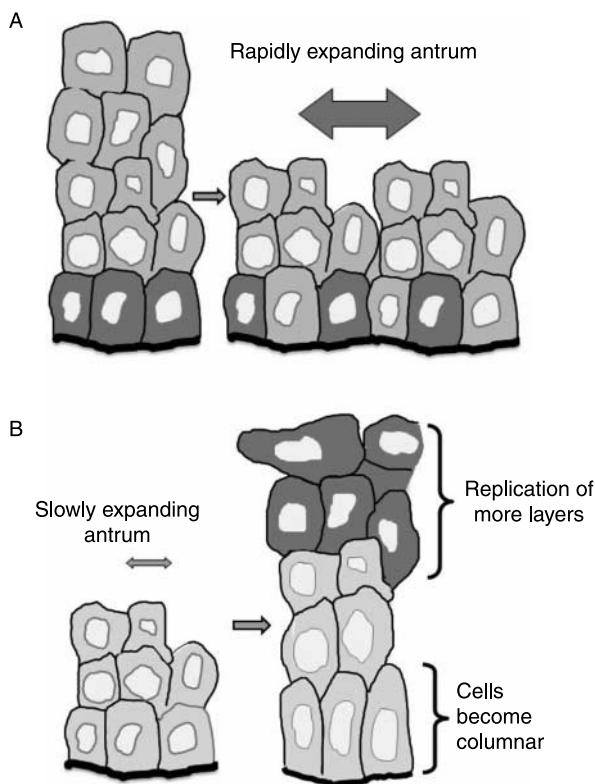


Figure 3 A cartoon illustrating the effects of differential rates of antrum expansion. (A) A fast rate reduces the numbers of layers and, with ample room to accommodate the cells, they take up their natural relaxed rounded shape. (B) A slow rate of antrum expansion leads to a build-up of layers and, with little room to accommodate cells, the basal cells pack in and become columnar in shape.

(Lee *et al.* 1993). Upon resynthesis of basal lamina at the basal cell surface, a loop in cross section is produced boarded by the new and the shed basal lamina. The corollary of this is that for follicles of the same size, the one with a loopy basal lamina has been growing for longer. It was on this basis that we sought to compare the quality of the oocytes in the two types of follicles. Follicles with loopy follicular basal lamina were found to have significantly and substantially poorer quality oocytes with only 28% developing to blastocyst following *in vitro* maturation, fertilization and culture to blastocyst, compared with 65% for the follicles with an aligned basal lamina (Irving-Rodgers *et al.* 2009b). No differences in the concentrations of insulin-like growth factor-binding proteins (IGFBPs; Irving-Rodgers *et al.* 2003a) or steroid hormones (Irving-Rodgers *et al.* 2003b) in follicular fluids have been observed between these follicles types. That the oocyte quality is poorer may stem from the possibility that these follicles may be slower growing and hence 'older' in terms of time since activation.

Alternatively, oocytes in follicles with a loopy basal lamina may already be of poor quality. Oocyte-derived growth factors, such as BMP15 and GDF9, are known to

affect follicular cells (Hussein *et al.* 2005, McNatty *et al.* 2006). It could be that oocytes from follicles with a loopy follicular basal lamina might not adequately stimulate replication of granulosa cells or antrum expansion leading to the observed loopy follicular basal lamina phenotype. If this was the case, then these follicles with a loopy follicular basal lamina could represent an early form of atresia prior to any recognizable evidence of cell death. Since the oocytes of older women are of poorer quality (Broekmans *et al.* 2009) and since in cows follicle numbers in a wave decline with age (Malhi *et al.* 2005), it would be interesting to know whether such ovaries contain an increased proportion of follicles with loopy basal lamina.

Dominant and subordinate follicles

In cows, there are two or three waves of follicular development during an oestrous cycle (Fortune *et al.* 1991, Ginther *et al.* 1996). Each wave consists of the contemporaneous appearance, observable by ultrasonography, of three to six follicles > 5 mm in diameter

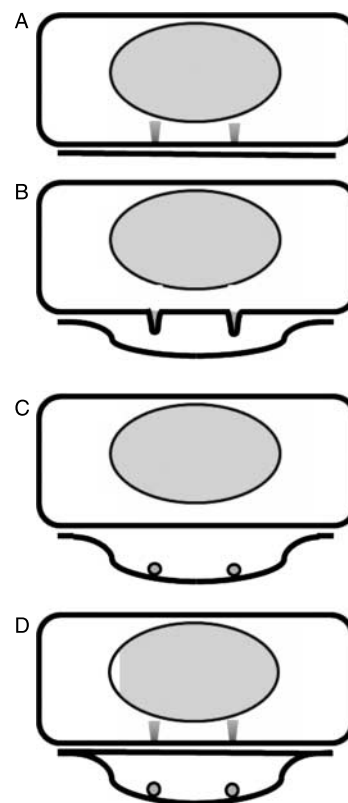


Figure 4 Cartoon illustrating sequentially (A–D) how the loopy follicular basal lamina phenotype could develop. (A) Aligned single layer of basal lamina with focal adhesions on cell surface. (B) Blebbing of cell surface at location of focal adhesions. (C) Release and formation of matrix vesicles from the cell surface of the area occupied by focal adhesions. (D) Synthesis of a new basal lamina at the granulosa cell surface with the original basal lamina with attached matrix vesicles located further away from the cell surface.

(ovulatory size is 12–20 mm) and, several days after the emergence of a wave, one follicle becomes larger than the rest at a time called deviation which occurs when the follicle is about 8 mm in diameter (Fortune *et al.* 1991, Ginther *et al.* 1996). The larger, dominant follicle continues to grow at a faster rate (Ginther *et al.* 2001), while the smaller follicles in the cohort, referred to as subordinate follicles, will eventually regress (Fortune *et al.* 1991, Ginther *et al.* 1996). The corollary of these dynamic changes in follicle growth is that healthy antral follicles are clearly of two types, dominant and subordinate, characterized by whether they are actively growing or growing slowly respectively.

It has been shown in cows that as follicles grow from sizes smaller to larger than deviation, the expression in granulosa cells of FSH receptor declines, while LH receptor, CYP11A1 (cholesterol side-chain cleavage), 3 β -hydroxysteroid dehydrogenase (HSD3B) and CYP19A1 (aromatase) increase as do the concentrations of oestradiol (E₂) and progesterone in the follicular fluids (reviewed in Bao & Garverick (1998), Webb *et al.* (1999), Fortune *et al.* (2001), Knight & Glister (2003) and Beg & Ginther (2006)). In comparison with subordinate follicles, dominant follicles have elevated follicular fluid E₂ concentrations (Fortune *et al.* 2001, Ouellette *et al.* 2005, Ryan *et al.* 2007, Sisco & Pfeffer 2007) and synthetic capacity (Fortune *et al.* 2001, Rhodes *et al.* 2001), and increased free insulin-like growth factor 1 and decreased IGFBP4 and -5 (Beg & Ginther 2006). The dominant follicle in a first wave of the cycle is also slightly larger than one in a second wave (Manikkam *et al.* 2001), and these have higher follicular fluid E₂ concentrations (Wolfenson *et al.* 1999). No difference in plasma E₂ concentration was found between cows with two or three follicular waves (Parker *et al.* 2003), while the ovulatory follicle is larger in cows with two versus three follicular waves (Townson *et al.* 2002, Parker *et al.* 2003); however this is not due to differences in growth rates (Townson *et al.* 2002). A significant negative relationship between transforming growth factor β 1 (TGFB1) in follicular fluid and follicle diameter occurs at 6.5 mm (before selection), but not at later stages (Ouellette *et al.* 2005). In addition, TGFB1 inhibits FSH-stimulated E₂ secretion from granulosa cells of small antral follicles (Ouellette *et al.* 2005, Zheng *et al.* 2008) as well as CYP19A1 expression, but not CYP11A1 (Zheng *et al.* 2008).

The use of ultrasonography of cows, carried out at least daily, is highly recommended for identifying dominant and subordinate follicles for *in vivo* measurements. However, there are some caveats with this approach. The subordinate follicles undergo atresia some time post deviation when expansion ceases. However, there is clearly a window before this change in growth is observed by ultrasonography when the follicle is atretic by histological or biochemical observation and from our

own observations when a follicle has been observed to have ceased growth on daily ultrasounds; histologically, the follicle can be well advanced into atresia with substantial death of granulosa cells (Irving-Rodgers *et al.* 2001). Furthermore, many studies are conducted on follicles in an artificially induced first wave of a cycle. While this makes the conduct of the experiments easier, these follicles may not be identical to normal cyclic follicles. A more physiological approach is to synchronize the cycle, monitor oestrous expression and examine the follicles in the first wave of the subsequent cycle (Ireland *et al.* 2008). In this case, the animals are still relatively well synchronized, but the follicles are not artificially induced.

Ovaries from non-stimulated cows collected at an abattoir can also be informative (Irving-Rodgers *et al.* 2009a) especially if additional information is available on the ages, breed and husbandry. If pairs of ovaries from each animal are collected separately and the three largest follicles identified and a sample of the follicle wall examined histologically, it is possible to identify dominant follicles (largest) that can be subdivided further into partially dominant if the subordinate follicles are healthy, or fully dominant if the subordinate follicles are atretic. This subdivision of dominant follicles clearly identifies follicles earlier and later post deviation respectively. Furthermore, the subordinate follicles can be classified as healthy or atretic by this approach. When using non-pregnant animals, the age of the corpora lutea in these pairs of ovaries can be assessed by gross (Ireland *et al.* 1980) or histological (Rodgers *et al.* 1986, Fields & Fields 1996) examination. This can be used to assign follicles into either the first or subsequent (second or third) waves of the cycle. Importantly, the sizes of follicles collected this way will differ from those recorded from ultrasound and estimates suggest they can be up to 3 mm greater (Sirois & Fortune 1988). The latter method measures diameters from the granulosa–follicular fluid interface and the former measures the dissected size of the follicle, which includes the theca interna and part of the externa at least (unpublished histological observations), and these differences should be considered when comparing data obtained by these different methods.

Cell death in follicles

Atresia of follicles leads to loss of the whole follicle and not just death of single cells, even if death of one cell, such as the oocyte, is observed early in the process. Additionally, even though the focus is often on cell death, atresia is also an active cellular process with resorption of the follicle involving macrophage infiltration, phagocytosis, migration of fibroblasts from the theca and production of collagen, which are some of the processes observed in wound healing (Martin 1997, Schultz & Wysocki 2009). Which cell(s) die first and

perhaps initiate atresia varies during follicular development. During follicle growth, different cell(s) may vary in their susceptibility to death and if these cells are irreplaceable then this would result in follicular atresia. At the bovine preantral stage, oocytes are reported to be the first to die (Rajakoski 1960), in contrast to the antral stage where granulosa cells die first, and in one form of atresia (discussed below) thecal cells, including steroidogenic and endothelial cells (Clark *et al.* 2004), also die very early in atresia. When atresia is initiated by a large amount of cell death, this could be due to loss of growth factor support such as TGF α (Wang *et al.* 2002), or that expression of Fas or Fas ligand (Porter *et al.* 2000, Quirk *et al.* 2004) could be involved in initiating the process. But where one cell, such as the oocyte, is involved in the initiation of atresia, this is likely to include a failure in development of the oocyte, whereas failure of a few granulosa cells would not have the same consequences. In fact, cell death is a normal part of tissue homeostasis. Therefore, a limited amount of cell death of granulosa or thecal cells would not be unexpected in a healthy follicle (Jolly *et al.* 1994), contributing to the difficulty of defining precisely when a follicle has commenced atresia, and estimation of the required level of cell death to indicate atresia is imprecise.

Modes of cell death include apoptosis, autophagy, cornification and necrosis; and all forms have been observed in granulosa cells (Jolly *et al.* 1994, van Wezel *et al.* 1999a, D'Haeseleer *et al.* 2006); however, their occurrence does not necessarily indicate atresia. Hence, the term apoptosis should not be used when the term atresia is meant. A consensus statement on the nomenclature of cell death (Kroemer *et al.* 2009) also recommends that the methods by which cell death has been observed should always be reported when reporting the degree of cell death, e.g. apoptosis as observed by DNA fragmentation or karyorrhexis. Examples of why this is important in follicles include the observation in bovine follicles of morphological dead pyknotic nuclei that can be TUNEL negative (Braw-Tal & Roth 2005) or not have DNA ladders by COMET (van Wezel *et al.* 1999a). Using the latter two methods would not have identified all the dead cells.

Historically, atresia was first classified by gross and morphological criteria. These criteria assessed follicles that were then used to identify biochemical changes, such as steroid hormones in follicular fluid. Concentrations of steroid hormones in follicular fluids have since been used as surrogate markers of atresia; however, this approach should be applied with caution as clearly the levels of hormones change with both growth and development, as well as with atresia. Later, direct physiological observation of follicle size using ultrasound was able to identify follicles decreasing in size, and hence in advanced stages of atresia. Recently, with the interest in the mechanisms of cell death, a number of

additional biochemical methods often in combination with histology or FACS analysis have been used. Since characteristics of atresia differ with different sizes of follicles, atresia at different sizes of antral follicles is considered separately below.

Atresia of antral follicles <5 mm in diameter

Many studies of atresia in the bovine ovary have been confounded because there is more than one type of atresia, and a seminal report on follicular atresia (Rajakoski 1960) misdescribed one form by misidentifying granulosa cells as theca cells in what is now called 'basal atresia'. Marion *et al.* (1968) accurately described this major type of atresia misidentified by Rajakoski, but described incorrectly how this form of atresia originated. On re-examination of these forms, they were renamed as 'antral (apical may have been a better

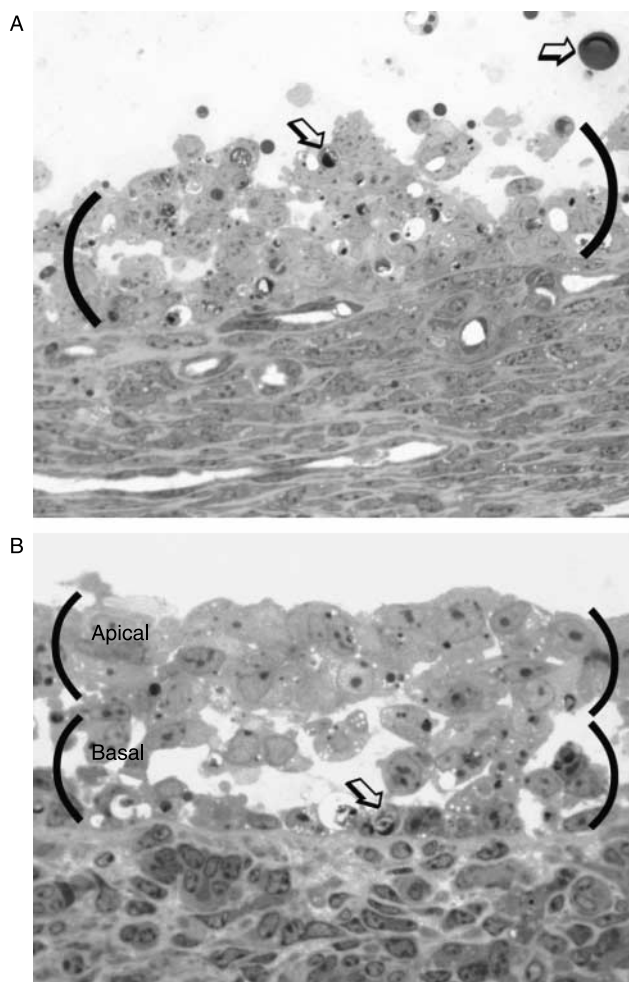


Figure 5 Light micrographs of bovine antral (A) and basal (B) atretic follicles showing the location of granulosa cells (brackets) and examples of dying cells (arrowheads), which in the basal atretic follicles are in the basal areas of the membrana granulosa, while the apical area shows granulosa cells of increased size.

term) atresia' and basal atresia, based upon which granulosa cells die first (Irving-Rodgers *et al.* 2001). Antral atresia is characterized by early destruction of the layers of the membrana granulosa closest to the antrum, while the most basal cells remained intact until later (Fig. 5). Numerous pyknotic nuclei are first observed in the most antral or apical layers and in the antrum close to the membrana granulosa. This is the classic description of atretic follicles and is observed in many species and occurs at all sizes of follicle development in the bovine and almost universally in large follicles (>5 mm in diameter), including non-ovulating dominant follicles. Basal atretic follicles are as prevalent as the antral atretic follicles in sizes up to 5 mm in diameter (Irving-Rodgers *et al.* 2001) and are characterized by initial destruction of the most basal layer of granulosa cells, whereas the cells in the most antral layers remain associated with each other and are predominantly healthy until later in atresia (Fig. 5).

The phenotype of the basal atretic follicles observed in bovine is substantially different to the antral atretic follicles (illustrated in Fig. 6). The follicular basal lamina of basal atretic follicles is often breached by macrophages, which then phagocytose dying basal granulosa cells (Irving-Rodgers *et al.* 2001). Importantly, in these basal atretic follicles, the surviving antral granulosa cells, now detached from the basal lamina, express CYP11A1 and HSD3B (healthy and antral atretic follicles

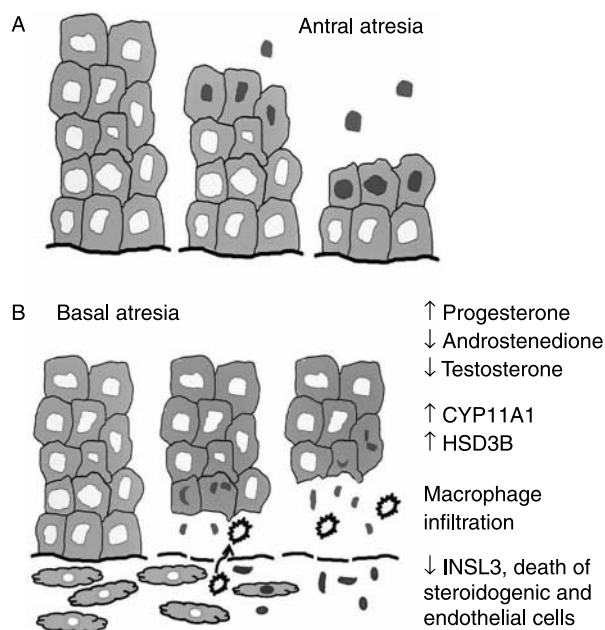


Figure 6 A cartoon of antral (A) and basal (B) atresia illustrating the changes over three time points as cell death progresses either from the apical regions or from the basal region. In basal atresia, the known changes in follicular fluid hormone, expression of steroidogenic enzymes in the apical cells, breaching of the follicular basal lamina and migration of cells from the theca including macrophages, and death of steroidogenic cells and endothelial cells in the theca and changes in insulin-like factor 3 in the thecal interna are indicated.

of the same size do not), and these follicles have substantially elevated progesterone levels in follicular fluid (Irving-Rodgers *et al.* 2003b). In basal atretic follicles, there is also an increased deposition of collagen within the theca, and early death of endothelial and steroidogenic thecal cells as observed by TUNEL labelling and morphology (Clark *et al.* 2004) and reduced expression of insulin-like factor 3 in the theca (Irving-Rodgers *et al.* 2003a). The early decline of the theca in these follicles is also consistent with reduced levels of thecal-derived androstenedione and testosterone in the follicular fluid compared with healthy and antral atretic follicles of the same size (Irving-Rodgers *et al.* 2003b). It has been suggested that the basal atretic follicles develop from antral atretic follicles later in the process of atresia (Braw-Tal & Roth 2005); however, in the absence of an intermediate phenotype between antral and basal atresia as would be expected if one morphed into the other, together with the restriction of basal atresia to sizes <5 mm, and with two distinct phenotypes of healthy follicles that correspond to the same size ranges as for the two atretic phenotypes, it is more likely that these atretic follicle phenotypes are separate identities.

Considering bovine follicles <5 mm only, the elevated levels of progesterone in follicular fluid indicate basal atresia (Irving-Rodgers *et al.* 2003b) and this was also observed in an earlier study of bovine follicles >5 mm diameter, which were classified histologically as either healthy or atretic (Grimes *et al.* 1987). In that study, ~6% of the follicles were classified as 'atretic-luteinized' and described as having elongated and hypertrophied granulosa cells and a disorganized theca. These follicles had progesterone levels four to eightfold greater in the follicular fluid in comparison with healthy follicles or those classified as atretic by standard criteria (antral atresia description). Additionally, these atretic-luteinized follicles had a low ratio of progesterone to E_2 . The atretic-luteinized follicles were probably basal atretic follicles as their description is very similar to the description of basal atretic follicles (Irving-Rodgers *et al.* 2003b).

Bovine antral atretic follicles <5 mm were observed to have no appreciable difference in the levels of progesterone, androstenedione or testosterone to those of healthy follicles of the same size (Irving-Rodgers *et al.* 2003b), and this was in agreement with an earlier study using follicles classified as atretic using features observed on fresh tissues under 10× magnification (McNatty *et al.* 1984). These studies also found either a lower level of E_2 (Irving-Rodgers *et al.* 2003b) or no significant reduction (McNatty *et al.* 1984) respectively at these sizes of follicles. In a latter study, however, significant differences in E_2 production by cultured granulosa cells from follicles classified as healthy and atretic were observed (Henderson *et al.* 1987), indicating that there is a difference in granulosa cells in

their capacity to produce E_2 , at least *in vitro*, at these follicles sizes. However, differences between follicles in E_2 synthetic capacity might not be sufficient to reliably use this method as an indicator of atresia.

What measurements are best to identify atresia in small antral bovine follicles? Histological observations are far less contentious, but a word of warning. When processing a biopsy of a follicle for histology, it is possible to lose a proportion of the granulosa cells during tissue harvesting, which does not occur if the whole follicle is processed without rupture. This is especially true for basal atretic follicles, and it is recommended to only classify follicles when granulosa cells are observed.

Atresia of antral follicles > 5 mm in diameter

In larger bovine follicles, no basal atresia is evident (Irving-Rodgers *et al.* 2001), making classification simpler. That is not to say there are no different types of atresia in these large follicles, and it has been observed that the level of Fas expression in thecal cells was greater in the subordinate follicles undergoing atresia than in dominant follicles undergoing atresia (Porter *et al.* 2001). While this study was not extensive in terms of time points or numbers, it does indicate that some complexity of the atresia processes also occurs in larger follicles.

Numerous studies of bovine follicles have measured steroid hormone concentrations in follicular fluids with different indicators of atresia or cell death used to determine whether the steroid concentrations are indeed good measures of atresia (Ireland & Roche 1983, McNatty *et al.* 1984, Grimes *et al.* 1987, Jolly *et al.* 1994). The consensus is that healthy follicles have higher levels of E_2 than atretic follicles, and atretic follicles have higher levels of progesterone or thecal products such as testosterone or androstenedione for the same size of follicle. The terms 'oestrogen active' or 'inactive' are used to describe healthy or atretic follicles respectively. However, the absolute levels of progesterone, androstenedione and E_2 also increase with increasing follicle size up until ovulation (Ireland & Roche 1983), and hence absolute levels can only be used as a basis of comparison between similar sized follicles. To overcome this problem, ratios of E_2 to progesterone or testosterone were used and shown to decrease substantially on atresia. However, Grimes *et al.* (1987) found no appreciable differences in the concentration of progesterone, testosterone or E_2 in 'intermediate atretic' follicles that, while not as advanced as the 'strongly atretic' follicles, had histologically obviously become atretic. The strongly atretic follicles did have a twofold reduction in E_2 and a twofold elevation in levels of progesterone, but if relying on these measurements alone or ratios thereof, many atretic follicles would be misclassified as healthy. It is thus suspected that as follicles enter atresia, which can be observed

histologically, changes in steroid hormone concentrations occur as a later event. This is not unexpected as death of cells is sequential, starting at the antrum and progressing to the basal lamina over time. This produces a window in which both live and dead cells are present, and presumably the live ones continue their steroid secretory function until dead.

Thus, it is recommended that, if it is needed, a histological approach be undertaken, either by examination of cell morphology, or in combination with markers of cell death, or direct measurement of apoptosis by such methods of DNA end labelling (Jolly *et al.* 1994) or FACS sorting of nuclei (Blondin *et al.* 1996, Lavranos *et al.* 1999, Hendriksen *et al.* 2003).

Conclusions

Since bovine follicles vary greatly in their characteristics during growth and development and atresia, it is necessary to develop criteria for classifying them. A number of different methods have been used to classify follicles and each has its strengths and weaknesses as discussed. Healthy follicles are the easiest to classify and, at nearly all stages of growth, two types of follicles can be identified; primordial follicles of two different shapes but with no known differences between them, preantral follicles of two different follicular basal lamina phenotypes, antral follicles of two different phenotypes based on follicular basal lamina and granulosa cell shape and having oocytes of different qualities but similar levels of follicular fluid steroids and IGFbps, and then larger follicles that are either dominant or subordinate with differential growth rates and sizes. Atretic follicles < 5 mm are of two types and these are also easily identified histologically and biochemically for basal atresia. In larger follicles, atresia can be recognized by a number of methods especially when in advanced stages. However, some methods are better than others at identifying early atresia and these should be used to avoid early atretic follicles being misclassified as healthy.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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