**In situ** evaluation of granulosa cells during apoptosis in caprine ovary

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**Abstract**

To evaluate the frequency of apoptotic cells in early phases, the transmission electron microscopic analyses of intact cells from the membrana granulosa of antral follicles from goat ovary were carried out vis-à-vis 3' end labeling in situ. Morphologically, apoptotic granulosa cells revealed the pyknotic appearance with marginated chromatin, multiple, smaller densely staining nuclear fragments, indentations of nuclear membrane, swollen and vacuolated mitochondria. The specific apoptotic in situ 3' end labeling procedure endorsed the fragmentation of DNA in granulosa cells of atretic follicles. The use of these two methods compliment, confirm and provide freedom to assess the numerical decline in ovarian follicular granulosa cell population.

**Keywords:** Apoptosis, Granulosa cells, ovary, Follicular atresia.

**INTRODUCTION**

The molecular mechanism of atresia in granulosa cells is largely explained by the phenomenon of apoptosis in mammals (Tilly et al., 1996). The morphological manifestations of apoptosis include cell shrinkage, membrane blebbing, cytoplasmic fragmentation etc. (Wu Ji et al., 2000; Chaube et al., 2005) and biochemical changes involve DNA ladder formation on gel electrophoresis. The DNA fragmentation in ovarian tissues has been explained in situ using DNA fluorescence flow cytometry (Guthrie et al., 1995) and autoradiography (Tilly et al., 1991, 1992; Manabe et al., 1996). The only limitations that this method encompasses is the requirement of large amount of DNA and that it is difficult to investigate the apoptotic changes in the specific tissue compartments or at the single cell level. Hence, the use of terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) is a powerful tool for evaluating DNA fragmentation in an individual cell (Ansari et al., 1993). Although, morphology provides the basic criteria for apoptosis, detection of early apoptotic changes are not traced. Alternatively, it is well known that apoptosis may occur without evidence of DNA fragmentation (Zamai et al., 1996) and therefore its detection becomes beyond the scope of terminal deoxynucleotidyl transferase nick-end labeling (TUNEL) assay. Therefore, in present investigation morphological criterion in combination with TUNEL assay is employed to examine apoptosis in caprine ovary.

**MATERIALS AND METHODS**

**Collection of ovaries and classification of follicles**

Ovaries from Jammanapari breed of goat showing secondary and tertiary ferning pattern (follicular phase) of vaginal smear (Noonan et al., 1975) were collected from the slaughter houses of Delhi (28° 38’ N, 77° 12’E) and around Kurukshetra (29° 6’ N, 76° 50’E) and brought to lab in normal saline at 4°C. The ovarian follicles were manually separated with the help of fine pair of forceps. The follicles were classified into healthy and atretic on morphometric basis (colour, turbidity of follicular fluid and vascularity) (Yang and Rajamahendran, 2000). The healthy follicles were characterized by highly vascularised (pink or red) theca interna and clear amber colored follicular fluid with no debris whereas the atretic follicles possessed pale theca interna with diminished blood vessels and flocculent highly turbid follicular fluid with a number of dark gobbets (Yang and Rajamahendran, 2000).
Granulosa cell isolation and Giemsa staining

The antral follicles of the size of 2-8mm in diameter were aspirated with a 20-gauge needle on a 2ml syringe containing a small volume of phosphate buffer saline (PBS). Cumulus oocyte complexes (COCs) and granulosa cell aggregates were allowed to settle from the follicular aspirate and COC was removed under microscopic examination. The granulosa cell aggregates were re-suspended and washed twice in PBS. Thereafter, GC smear was prepared on Mayer’s albumin coated slides. After air drying, the slides were stained with Giemsa, followed by dehydration through a series of alcoholic grades; the slides were cleared in xylene and mounted in DPX (Distyrene, Plasticizer and Xylene). The photographs were taken under Olympus microscope at the magnification of 1000x.

In Situ end labelling (TUNEL) assay

TUNEL was carried out as per manufacturer’s (R&D system Inc., Minneapolis, USA) instructions. Briefly, pre-washed cells were smeared on Poly-L-Lysine coated slides and fixed in 4% formaldehyde. Cells were treated with cyanin for 10min followed by quenching with H2O2. Biotinylated nucleotides were incorporated into the 3’ OH ends of the DNA fragments by TdT, and detected by using Streptavidin-HRP. The colour was developed by DAB (Diaminobenzidine) solution and later counter stained with methyl green. TUNEL positive cells were examined using a Olympus microscope.

Transmission electron microscopy

The antral follicles were fixed in 2.5% gluteraldehyde in 0.2M phosphate buffer saline (pH 7.2 to 7.4) at 4°C for 24h. The samples were then post fixed in 1.3% Osmium tetroxide for 2h at 4°C. The fixed material was dehydrated through a graded series of acetone; the samples were cleared with a clearing agent epoxy propane and embedded in epoxy-resin blocks. The blocks were trimmed and the serial 60-90nm thick sections were cut with diamond knives and were mounted on 100 mesh grids. The ultra thin sections were stained with uranyl acetate followed by lead citrate (Hertig and Adams, 1967). The sections were examined and photographed under transmission electron microscope using Morgagni 268 D TEM from FEI, Netherland, installed at AIIMS, New Delhi.
RESULTS

The membrana granulosa of antral follicles revealed the presence of apoptotic cells/Apoptotic bodies amongst the normal cells. Under light microscopic investigation, apoptotic granulosa cells were characterized by multiple small, densely staining nuclear fragments (Fig. 1a), hyalinization of cytoplasm, appearance of vacuoles of varied sizes and membrane blebbing (Fig. 1b). In 3'end labeling, single small densely staining nucleus having pyknotic appearance (Fig. 2a) were frequently observed. A few cells with marginated chromatin material in the nucleus were also occasionally seen (Fig. 2b). The frequency of TUNEL positive apoptotic cells were sparse in granulosa cells of healthy follicles. This number was significantly increased in atretic follicles. Ultrastructurally, the degenerating granulosa cells revealed the presence of different apoptotic characteristic, like increased indentation of the nuclear membrane (Fig. 3a), swollen and vacuolated mitochondria with diffused and degenerate cristae (Fig. 3b), multiple fragmentation of nucleus and vacuolization of cytoplasm (Fig. 3c). Thus, our ultrastructural and in situ 3'end labeling, features of apoptotic granulosa cells show strong correlation with each other and confirm that apoptosis appears to be the predominant form of cell death in membrana granulosa during follicular atresia in caprines.

DISCUSSION

Previous studies have reported apoptotic changes in the porcine ovarian tissues using several molecular techniques (Guthrie et al., 1995; Tilly et al., 1991, 1992; Manabe et al., 1996). These methods require large amounts of DNA and do not allow identification of apoptotic changes at the single cell level. Alternatively, the combined approach proposed by our study has the following advantages: (1) It can be performed on conventional histological sections without disruption of their architecture, (2) It allows direct microscopic visualization and detection of apoptosis at the single cell level and it is a rapid non-radioactive method allowing for the investigation of a large number of cases. Results of this study extend classic histological description of granulosa cell degeneration during follicular atresia in caprines (Nuclear pyknosis, hyperchromatosis, karyorrhexis and the formation of apoptotic bodies) (Hay et al., 1976; Brand et al., 1973; Turnbull et al., 1977) by relating specific morphological features of granulosa cell death to the physiological process of apoptosis. This relationship is supported in this study by in situ histochemical evidence of DNA fragmentation. Quite remarkably, morphological features of degenerating granulosa cells identical to those described and depicted here were first published more than 100 years ago by a German Scientist, Walter Flemming, who studied the involution of ovarian follicles in rabbits (Flemming W, 1885). Flemming's study has been brought to light in a recent review (Majno G et al., 1995), and it has been proposed that this was the first study to introduce the concept of spontaneous cell death as a physiological event. Flemming named the process he described "Chromatolysis" and the drawings he used to illustrate his findings clearly depict all of the morphological features considered to be characteristics of the process now known as ‘apoptosis’ (Majno G et al., 1995). These include cells with a single shrunken and densely basophilic nucleus (pyknotic appearance). While such cells do not display the classically described features of apoptosis (Margination of Chromatin and/or nuclear fragmentation) (Kerr et al., 1972; Wyllie et al., 1980), they probably represent a morphological variant of apoptotic cell death that has been described for a variety of cell types (Bursch et al., 1985; Wyllie et al., 1980; Faa G et al., 1994; Kressel and Groscurth, 1994). Consistent with this view, our illustrations clearly show the presence of fragmented DNA in cells with this morphological appearance, which also agrees with the findings of others using an in vitro model of induced apoptosis in cultured cells (Kressel and Groscurth, 1994).
1994). One notable finding was the presence of apoptotic cells and/or apoptotic bodies in all the follicles that were classified as healthy. This is consistent with similar histological findings in ovine follicles (Hay et al., 1976). However, for a confirmed identification of apoptotic cell death in tissues, the demonstration of DNA fragmentation is not sufficient because chromatin dissolution also affects the necrotic cells (Kressel and Groscurth, 1994; Grazial-kraupp et al., 1995). Thus in situ 3'end labeling technique, in combination with a morphological demonstration at ultrastructural level allows us to distinguish clearly between the two cases. The parallel use of techniques allows us to observe the structural changes taking place in caprine granulosa cells that go on to degeneration, together with methods that use in situ end labeling, have provided the first evidence for a direct link between caprine granulosa cells degeneration and programmed cell death.

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References


Flemming W (1885) Uber die bildung von richtungsfüguren in saugthiereien beim untergang graafscher follikel. Arch Anat Entwickl. 221-244.


