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Assessment of immunolocalization of Fas antigen in the ovarian follicle and the role of IGF-I in Fas-L induced granulosa cell apoptosis in water buffalo

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ABSTRACT

Primordial follicle pool size in the ovary is small and rate of follicular atresia is high in water buffaloes. The objective of this experiment was to study the expression of Fas antigen in granulosa cells of water buffalo ovarian follicle, and to know the role of insulin like growth factor-1(IGF-I) on Fas-ligand (Fas-L) mediated apoptosis. The Fas expression was observed mainly in the granulosa cells of the follicles. The expression was higher in the granulosa cells, which were denuded from basement membrane, indicating apoptosis. Healthy follicles which had intact multilaminar granulosa cell layer showed negative reaction for Fas antigen. The granulosa cells from 3–6 mm diameter follicles of the buffalo ovaries collected from the slaughterhouse ovaries were cultured *in vitro* to assess the effect of Fas-L at 10, 20 and 50ng/ml on granulosa cell viability. The Fas-L even at the lowest dose tested (10ng/ml) significantly decreased granulosa cells viability as compared to control; whereas addition of IGF-I to the culture significantly improved granulosa cell viability. Addition of IGF-I could significantly improve the granulosa cells viability in the Fas-L treated culture. Overall this study demonstrated the existence of Fas and Fas mediated apoptosis in the granulosa cells of the buffalo follicle, and also suggested that IGF-I could rescue granulosa cells from Fas-L mediated apoptosis.

Key words: Buffalo, Fas, IGF-I, Granulosa cells, Immunohistochemistry

Apoptosis is the underlying mechanism of follicular atresia in the mammalian ovary. A small pool size of primordial follicles (Aboul-Ela 2000) and a higher rate of follicular atresia (Sreejalekshmi *et al*. 2011) as compared to cattle (Grimes *et al*. 1987) could be some of the basic reasons for poor reproductive performance in buffalo. Yang and Rajamahendran (2000) showed that atresia occurs at all stages of follicle development. During follicular development, gonadotropins, together with local intra ovarian growth factors, cytokine as well as estrogens influence growth of ovarian follicles (Quirk *et al*. 2004). The death of granulosa cells associated with follicular atresia occurs through apoptosis. The signals that induce granulosa cells to undergo apoptosis have not been defined but may include lack of growth factors and/or induction of cytotoxic stimuli such as tumor necrosis factor (TNF) or Fas-L (Quirk *et al*. 1998). Fas, a cell surface receptor, when engaged by Fas-L or specific agonist antibodies triggers apoptosis. Expression of Fas or Fas-L or both was demonstrated in ovarian follicles of rats, mice, cows and pigs (Quirk *et al*. 1998, Kim *et al*. 1999, Vickers *et al*. 2000). Cultured follicle cells respond to treatment with Fas-L or agonistic anti-Fas antibodies by undergoing atresia (Quirk *et al.*1998). Factors like the gonadotropins, together with IGF-I and estrogens are antiapoptotic and rescue follicles from atresia (Quirk *et al.* 1998). In contrast, TNFa, Fas-L and androgens are proapoptotic. These diverse signals probably converge on selective intracellular pathways including genes like Bcl-2, caspase 3, etc. to regulate apoptosis.

Apparently there are few or no reports on Fas/Fas-L mediated apoptosis in buffalo ovarian follicles. The present study was undertaken to identify the expression of Fas antigen and Fas mediated apoptosis in granulosa cells of buffalo ovarian follicle and the possible role of IGF-I in its regulation.

MATERIALS AND METHODS

Immunohistochemistry: The ovaries from apparently non-pregnant (as assessed by uterine examination) buffaloes were procured from the corporation slaughterhouse, Bengaluru.

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The ovaries, collected immediately after slaughter, were transported in sterile normal saline at ice cold condition to the laboratory. The ovaries were washed in sterile normal saline. Longitudinal slices of ovary approximately 4–5 mm thickness were made and fixed in 10% neutral buffered formalin for further histological processing processing. Sections of 5µm thickness were cut and every 10th section from each slice was used for immunohistochemical staining. The sections were deparaffinized and rehydrated. The slides were incubated in 0.3% H_2O_2 in methanol for 30 min. The slides were washed thrice in 0.25 M Tris HCl at pH 7.5, for 20 min and were placed in buffer A (0.25 M Tris HCl at pH 7.5) for 5 min. The sections were blocked in blocking buffer (Tris HCl + 2% fetal calf serum and 0.5% BSA) for 5 min. The slides were incubated overnight with primary antibody (1:100 dilution, Fas monoclonal mouse antibody) in a humidified chamber. The slides were rinsed gently and incubated with secondary antibody (1:200, goat anti mouse secondary antibody) in a humidified chamber for 45 min. The sections were washed, incubated in freshly prepared diaminobenzidine (DAB) solution and the reaction was stopped by washing in running water when a uniform brown color was visible on the slide. Finally the sections were counterstained with haematoxylin for 6 sec (to aid in the morphological evaluation and characterization of normal and apoptotic cells), dehydrated through increasing concentrations of alcohol and cleared in xylene. The slides were mounted using DPX and viewed under phase contrast microscope. Sections from the spleen of the rat served as positive controls. Negative control sections were processed identically with the omission of primary antibody. Brownish colour of the granulosa cells was considered positive for the presence of Fas antigen. No brown colour was observed in the granulosa cells of negative control sections.

Fas-L and IGF-I on granulosa cell viability: Freshly excised buffalo ovaries were obtained from the abattoir, transported in ice cold saline within 1.5 h of slaughter. Granulosa cells were obtained by aspirating the follicles of 4–6 mm diameter with a 21-gauge needle, followed by flushing of the follicles with Dulbecco modified Eagle medium (DMEM). Cells were collected by centrifugation, washed, viability was assessed using 0.4% trypan blue and cultured in a cell culture incubator at 5% CO_2, and 37ºC as described earlier (Quirk *et al*. 2000). The cells were cultured in a basal media, DMEM medium (supplemented with 1 mM pyruvate, 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone) containing 10% fetal bovine serum (FBS) for 2 days (day 0, start of the experiment). Cells were plated at a concentration of approximately 5×10^4 viable cells/well in 96-well plates. On day 2, the basal media with insulin (100 ng/ml), apotransferrin (5 µg/ml), selenium (20 nM), 0.1% bovine serum albumin and 0 or 100ng/ml IGF-I was used for culturing granulosa cells. On day 3, the cultures were treated in the same media with 0, 10, 20 or 50 ng/ml Fas-L. On day 4, the media were removed and the cells were harvested by adding 0.25% trypsin-1 mM EDTA solution and incubated for 6–8 min with. The cells were washed in DPBS and the viability (per cent) of cells was assessed using 0.4% trypan blue. Trypan blue (200µl) was added to the granulosa cells with media (200µl) and incubated for 1 min. The live (cells excluding trypan blue) and dead (cells with blue colour) cells were counted in a hemocytometer within 4 min. A minimum of 200 cells were counted in each sample and percentage of viability was calculated.

Statistical analysis: The cell culture experiment was repeated four times. Statistical analysis was performed after arcsine transformation of percentage data by using the SPSS 15.0 software. One-way ANOVA and Tukey's post-hoc test were used for comparison between the extent of apoptosis in terms of live granulosa cells between the control and experimental groups at the end of the culture. Data were expressed as mean±SEM. A probability of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Detection of Fas antigen in granulosa cells by immunohistochemistry: Using the immunohistochemistry technique, localisation of Fas antigen (a transmembrane protein) was detected in the granulosa cells (cell membrane along with cytoplasm) of the ovarian follicles. Positive (rat spleen) and negative (without primary antibody) controls were used along with the test samples for comparison (Fig. 1). The follicles which had intact, and well-organized multilaminar granulosa cell layer were negative. Fas localization was observed in the cytoplasm along the membrane of the granulosa cells that were partially detached

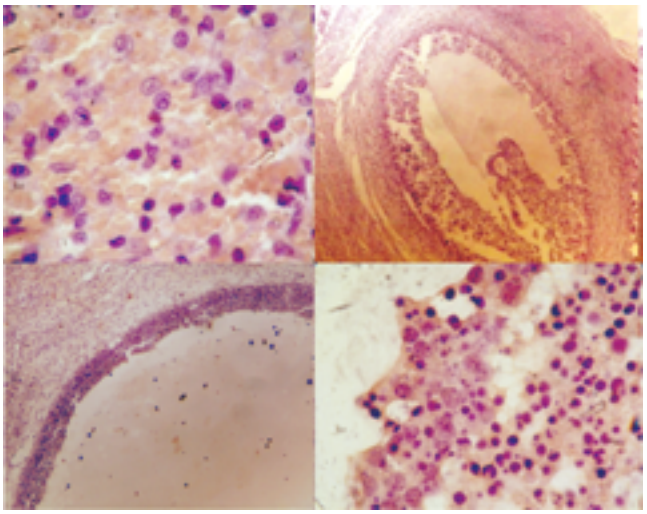

Fig. 1(a-d). Expression of Fas in the buffalo ovarian follicle. The expression was studied using (**a.** rat spleen, 1000×) positive control and (**b.** omitting primary antibody, 400×) negative control. The expression was mainly observed in the atretic follicles of the granulosa cell cytoplasm and membrane (**c**, 100×; and **d**. 1000×).

from the basement membrane. Fas reaction was more intense in the granulosa cells that were denuded into the follicular fluid. Fas protein was seen on the antral side of denuded granulosa cells, which is characteristic of atretic follicle.

In mammalian ovaries, atresia of ovarian follicles occurs through a process of physiological and programmed cell death called apoptosis. Although several follicles develop in each oestrous cycle, only a few follicles reach ovulation in multiovulatory species and only one in monovulatory species. The role of Fas-L and Fas expression was implicated in apoptotic process, causing atresia of ovarian follicles in bovine (Vickers *et al.* 2000). The present study was conducted to confirm the role of Fas mediated apoptosis in granulosa cells of buffalo ovarian follicles, in which the information on the molecular mechanisms of follicular atresia is not thoroughly studied. In this study, Fas protein (antigen) was detected in the granulosa cell layer and in cells present in the follicular fluid, similar to the characteristics of atretic follicle in the bovine (Rouillier *et al*. 1996, Roughton *et al.* 1999). Expression of the Fas-antigen was not observed on the granulosa cells which were intact, and well-organized multi-laminar granulosa cell layer, a characteristic feature of healthy follicle. These findings were in agreement with observations of Roughton *et al*. (1999).

In mice, rats, cows and sows, both Fas-L and Fas are expressed in granulosa cells, and apoptosis is inducible by their interaction and is the major molecular mechanism of follicular atresia (Vickers *et al*. 2000). Fas antigen was localized in oocyte and Fas-L on granulosa cells, interaction of these molecules culminated in apoptosis of the follicles in mice (Dharma *et al*. 2003). Apoptotic cells appeared to be disseminated throughout the granulosa cell layer. However, a higher prevalence of apoptotic cells and apoptotic bodies was observed in the antral granulosa cell layer. These findings were similar to the observations made in bovine antral granulosa cells (Rouillier *et al.* 1996). The role of differences in steroidogenic activity among buffalo and bovine antral and mural granulosa cells and the significance of basement membrane in transmitting the survival signals are vital in arresting the apoptotoic process (Amsterdam *et al*. 1998). The interaction of Fas and Fas-L is of utmost importance in granulosa cell apoptosis resulting in atresia of ovarian follicles and also for the selection of follicles for ovulation (Matsui *et al*. 2003, Manabe *et al*. 2004). The follicle is regarded as immune privileged site. In rats, the theca cells expressed relatively more Fas-L that may function as a barrier by inducing apoptosis in the invading immune cells possessing Fas protein and preventing them from reaching the antral cavity (Jong *et al*. 1998). The immune privilege in buffalo ovarian follicles remains to be investigated.

Fas-L and IGF-I on granulosa cell viability: Addition of IGF-I (100ng/ml) could significantly (P<0.01) improve granulosa cells viability as compared to control (Fig. 2). The Fas-L, even at the lowest dose tested (10 ng/ml) significantly

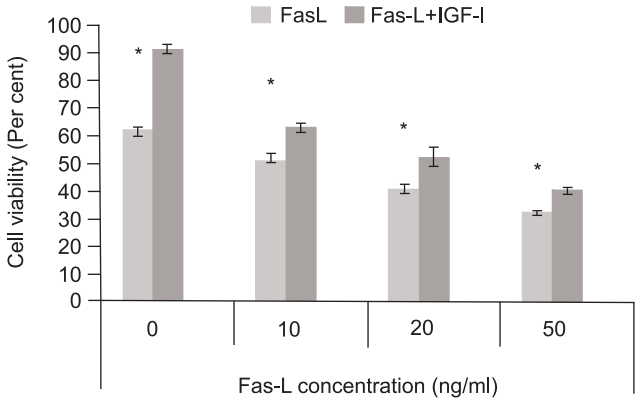

Fig. 2. Role of IGF-I in Fas-L induced apoptosis as assessed by granulosa cell viability (day 4) in granulosa cells culture. The percentage of granulosa cell viable at the start of the treatment (day 0) was considered as 100% (*P<0.01).

(P<0.01) decreased granulosa cells viability as compared to control. Addition of IGF-I (100 ng/ml) in the lowest dose of Fas-L (10 ng/ml) could be able to rescue Fas-L mediated apoptotic effect. IGF-I significantly improved (P<0.01) the granulosa cells viability in the Fas-L (20 and 50 ng/ml) treated culture. However the granulosa cells viability was significantly lower than the control.

In addition to gonadotropins, the development of ovarian follicles is also controlled by locally produced intraovarian regulators such as growth factors and cytokines, which act in a paracrine/autocrine manner. IGFs are most prominent apoptosis inhibitory cytokines and their regulated expression in various tissues play a pivotal role in regulating cellular homeostasis (Yin *et al*. 1998). In this study, addition of Fas-L induced apoptosis in buffalo granulosa cells in a dose dependent manner, which is in accordance with observation made in rodents (Kim *et al*. 1999, Roughton *et al.* 1999) and in, mice, rat, cows and sows (Vickers *et al*. 2000). When cells were cultured in media containing Fas-L and IGF-I, there were significantly more number of live cells in the media containing IGF-I than in Fas-L alone. This strongly suggested that IGF-I is at least one of the growth factors that blocks the Fas mediated apoptosis in culture. The IGFs promote follicular growth and differentiation of follicular cells, which is essential for follicle selection, dominance and ovulation (Poretsky *et al*. 1999, Quirk *et al.* 2000). IGF-I was able to rescue the granulosa cells from atresia/apoptosis or maintain granulosa cells viability to greater degree in the absence of Fas-L, that resembles *in vivo* biological system (i.e., IGF is in greater concentration in dominant follicle compared to that of sub-ordinate follicle) which is in agreement with the earlier observations (Fortune *et al*. 2001).

Several studies reported that the bio-availability of IGF-I to granulosa cells may be reduced by elevated levels of low molecular weight IGF-binding proteins (IGFBP) in follicular fluid of atretic follicles relative to that observed in

healthy follicles (Poretsky *et al*. 1999). The presence of the binding proteins is controlled at the level of synthesis (Armstrong *et al*. 1998) and by the presence of specific proteases in healthy antral follicles, which breakdown low molecular weight IGFBP (Rivera *et al*. 2001). Though we did not study the effect of IGF-I in presence or absence of IGFBP's, the added IGF-I analogue in the culture is did not bind with the IGFBP in culture (Grimes and Hammond 1992). Hence the added IGF-I would have acted to its maximum potency.

The effect of IGF-I in suppressing the apoptosis of granulosa cells in the present study is similar to the observation made in bovine (Yang and Rajamahendran 2000) and porcine (Mao *et al*. 2004) species. The protective activity of IGF-I was mediated through the PI3K (phosphatidylionositol 3-kinase)/ Akt pathway to protect granulosa cells against Fas-L induced apoptosis (Quirk *et al*. 2004). IGF-I also increased the cells mitosis by progression from G0/G1 to -S phase in cells from Fas-L induced apoptosis, and is regulated by the PI3K/Akt pathway (Hu *et al*. 2004). The granulosa cells are most susceptible to apoptosis at G1/S transition phase. These findings are consistent with the increased availability of IGF-I in dominant follicles compared with subordinate follicles at the time of follicle selection and may explain their critical role to support follicle development *in vivo* (Quirk *et al*. 2004). The rescuing effect of IGF-I on granulosa cells against Fas-L mediated apoptosis, may be due to its mitotic effect by progressing through G1/S transition phase (phase at which cells are most susceptible to apoptosis) and protect granulosa cells from Fas-L mediated apoptosis.

The present study demonstrated the presence of Fas mediated apoptosis in the granulosa cells of the buffalo follicles. The study also suggested that IGF-I could rescue Fas-L mediated apoptosis in granulosa cells of the buffalo ovary. Modulating IGF-I levels in the follicle could rescue the follicles undergoing apoptosis and could be used to improve superovulatory response in buffalo. This approach might potentially be utilized to increase reproductive efficiency in other domestic animals.

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REFERENCES

Aboul-Ela M B E. 2000. Super-ovulation in the buffalo: constraints and manipulation. *Buffalo Journal* **1**: 1–20.

Amsterdam A, Dantes A, Hosokawa K, Schere-Levy C P, Kotsuji F and Aharoni D. 1998. Steroid regulation during apoptosis of ovarian follicular cells. *Steroids* **63**: 314–8.

Dharma S J, Kelkar R L and Nandedkar T D. 2003. Fas and Fas ligand protein and mRNA in normal and atretic mouse ovarian follicles. *Reproduction* **126** : 783–9.

Fortune J E, Rivera G M, Evans A C O and Turzillo A M. 2001. Differentiation of dominant versus subordinate follicles in cattle. *Biology of Reproduction* **65**: 648–54.

Grimes R W and Hammond J M. 1992. Insulin and insulin-like growth factors (IGFs) stimulate production of IGF-binding proteins by ovarian granulosa cells. *Endocrinology* **131**: 553–8

Grimes R W, Matton P and Ireland J J. 1987. A comparison of histological and non-histological indices of atresia and follicular function. *Biology of Reproduction* **37**: 82–8.

Hu C L, Cowan R G, Harman R M and Quirk S M. 2004. Cell cycle progression and activation of Akt Kinase are required for IGF-I-mediated suppression of apoptosis in granulosa cells. *Molecular Endocrinology* **18**: 326–38.

Jong M K, David L, Boone Anthony A and Benjamin, K T. 1998. Granulosa cell apoptosis induced at penultimate stage of follicular development is associated with increased levels of Fas and Fas-ligand in the rat ovary. *Biology of Reproduction* **58**: 1170–6.

Kim J M, Yoon Y D and Tsang B K. 1999. Involvement of the Fas/FasL system in p53-mediated granulosa cell apoptosis during follicular development and atresia. *Endocrinology* **140**: 2307–17

Manabe N, Goto Y, Matsuda M F, Inoue N, Maeda A, Sugimoto M, Sakamaki K and Miyano T. 2004. Regulation mechanism of selective atresia in porcine follicles: regulation of granulosa cell apoptosis during atresia. *Journal of Reproduction and Development* **50**: 493–514.

Mao J, Smith M F, Rucker E B, Wu G M, Mccauley T C, Cantley T C, Prather R S, Didion B A and Day B N. 2004. Effect of epidermal growth factor and insulin-like growth factor I on porcine preantral follicular growth, antrum formation, and stimulation of granulosal cell proliferation and suppression of apoptosis in vitro. *Journal of Animal Science* **82**: 1967–75.

Matsui T, Manabe N, Goto Y, Nakayama M, Inoue N, Nishihara S and Miyamoto H. 2003. Changes in the expression and activity of caspase-9 and Apaf1 in granulosa cells during follicular atresia in pig ovaries. *Reproduction* **126**: 113–20.

Poretsky L, Cataldo N A, Rosenwaks Z, Giudice L C. 1999. The insulin-related ovarian regulatory system in health and disease. *Endocrine Reviews* **20**: 535–82.

Quirk S M, Porter D A, Huber S C and Cowan R G. 1998: Potentiation of Fas-mediated killing of murine granulosa cells by interferon-γ, tumor necrosis factor-a and cycloheximide. *Endocrinology* **139**: 4860–9.

Quirk S M, Harman R M and Cowan R G. 2000. Regulation of Fas antigen (Fas, CD95)-mediated apoptosis of bovine granulosa cells by serum and growth factors. *Biology of Reproduction* **63**: 1278–84

Quirk S M, Cowan R G, Harman R M, Hu, C L and Porter D A. 2004. Ovarian follicular growth and atresia: the relationship between cell proliferation and survival. *Journal of Animal Science* **82**: E-Suppl, E40–52.

Rivera G M, Chandrasekher Y A, Evans A C O, Giudice L C and Fortune J E. 2001. A potential role for insulin-like growth factor binding protein-4 proteolysis in the establishment of ovarian follicular dominance in cattle. *Biology of Reproduction*

65: 102–11.

Roughton S, Lareu R and Bittles A. 1999. Fas and Fas ligand messenger ribonucleic acid and protein expression in the rat corpus luteum dur**in**g apoptosis-mediated luteolysis. *Biology of Reproduction* **60**: 797–804.

Rouillier P, Matton P, Sirard M A and Guilbault L A. 1996. Follicle-stimulating hormone-induced estradiol and progesterone production by bovine antral and mural granulosa cells cultured *in vitro* in a completely defined medium. *Journal of Animal Science* **74**: 3012–9.

Sreejalekshmi P, Raghavendra B S, Subramani T S, Murthy V C, Jamuna K V, Prasad R V, Ravindra J P and Selvaraju S. 2011. Detection of follicular apoptosis in water buffalo (*Bubalus bubalis*) ovary by histology and nick end labelling technique. *Reproduction in Domestic Animals* **46**:59–65.

Yang M Y and Rajamahendran R. 2000. Morphological and biochemical identification of apoptosis in small, medium, and large bovine follicles and the effects of follicle-stimulating hormone and insulin-like growth factor-I on spontaneous apoptosis in cultured bovine granulosa cells. *Biology of Reproduction* **62**: 1209–17.

Yin D L, Pu L and Pei G. 1998. Antisense oligonucleotide to insulin-like growth factor II induces apoptosis in human ovarian cancer AO cell line. *Cell Research* **8**:159–65.