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Expression of apoptosis related genes in buffalo embryos produced through *in vitro* fertilization and parthenogenetic activation

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ABSTRACT

In present study immature oocytes were obtained from buffalo slaughterhouse ovaries and were subjected to *in vitro* maturation (IVM) in TCM-199 + 10% FBS + 5 µg/ml pFSH + 0.81 mM sodium pyruvate + 5% buffalo follicular fluid for 24 h in a CO_2 incubator (5% CO_2, 90–95% relative humidity) at 38.5°C. The maturer oocytes were used for IVF and the cleaved embryos were cultured for 8 days, while the parthenotes were produced with exposure of 7% ethanol, 6-dimethyl aminopurine and cultured for 8 days in mCR2aa medium. The relative abundance of *BCL-XL* transcripts in immature, matured oocytes; and embryos produced through IVF i.e. 2-cell, 4-cell, 8-16-cell, morula and blastocyst stage were 25.33±0.90, 12.67±1.20, 37.67±0.90, 30.67±0.30, 23.67±0.90, 18.33±0.90 and 27.00±1.20 respectively, while in parthenogenesis these values were 23.67±0.88, 13.67±1.20, 23.67±1.20, 22.34±0.88, 24.34±0.88, 33.67±0.88, 45.34±1.20 respectively. Relative abundance of *BAX* transcripts by IVF were 23.0±0.60, 0.33±0.10, 4.00±0.60, 5.00±0.60, 0.37±0.06, 13.0±0.66 and 56.7±0.90; and by parthenonenesis were 22.3±0.90, 0.13±0.03, 13.67±0.90, 14.0±0.60, 15.33±0.90, 64.67±2.20 and 55.0±2.10 respectively. In conclusion, expression pattern of apoptosis related genes revealed that incidence of apoptosis was significantly higher in IVF and parthenogenetically produced buffalo embryos at stages like immature oocytes, morula and blastocyst stage than the early cleavage stage embryos.

Key words: Apoptosis, Buffalo, Embryo, Parthenogenesis

Except cloning in all the cases 2 types of cells i.e. oocyte and spermatozoa contribute their inheritance to offspring, but if only elite mother produces offspring without help of male partner, the produced animal is completely clone to the mother is known as parthenogenesis (Beatty 1967). The fertilization is not essential and absolutely necessary for the propagation in certain cases. Parthenogenesis is common mode of reproduction in lower organisms in nature and is reported in about 70 vertebrate species (about 0.1%). There is no report on birth of parthenogenetic mammal but mammalian oocytes can be activated by using chemicals like ethanol, ionomycin, strontium chloride etc. (Singh *et al.* 2012). These activated oocytes have never been reported to complete the development to terms. But due to manipulation if possible, it opens a new era and may become a milestone in reproductive biology.

Apoptosis or programmed cell death is an evolutionarily highly conserved mechanism that allows the organism to tightly control cell numbers, tissue size and protect itself from dangerous cells that threaten homeostasis (Wyllie *et al.* 1995, Rich *et al.* 1999). The first observations of apoptotic cells in normally developing embryos were reported immediately after EGA. All mammalian species show the highest level of spontaneous apoptotic processes at the blastocyst stage. Apoptotic morphological changes were observed in 70–80% of all *in vivo* or *in vitro* produced blastocysts from mice (Hardy 1997) and humans (Hardy *et al.* 1989) and in practically all *in vivo* and *in vitro* produced blastocysts from cattle and buffalo (Elamaran *et al.* 2012). Death of cells occurs due to differential expression of Bcl-2 family including pro-apoptotic (Bax, Bcl-X_s, Bak, Bad, Bik and Bid) members and anti-apoptotic (Bcl-2, Bcl-X_L, Bcl-W, Mcl-1 and A1) members (Krajewski *et al.* 1993, Elamaran *et al.* 2012). Bcl-2 family plays a crucial role in determining the cellular sensitivity to apoptosis more than (Gjorret *et al.* 2003 and Byrne *et al.* 1999) the expression level of individual members (Betts and King 2001). Expression level of the pro-apoptotic or apoptotic genes depend on initial oocyte health, status of gene activation during embryogenesis and culture conditions (Lonergan *et al.* 2003).

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MATERIALS AND METHODS

In vitro *embryo production*

Collection and in vitro *maturation of oocytes:* Buffalo ovaries were obtained immediately after slaughter and oocytes were collected by aspiration of surface follicles (2–8 mm diameter) in aspiration medium (TCM-199 + 0.3% BSA + 0.81mM sodium pyruvate + 50 µg/ml gentamicin sulphate). Cumulus-oocyte complexes (COCs) having >2 layers of unexpanded cumulus cell layers with homogenous ooplasmic granulation were searched and were processed for *in vitro* maturation. For *in vitro* maturation, the oocytes were washed 4–6 times with the washing medium (TCM-199 supplemented with 2mM HEPES buffer, 10% FBS, 0.81 mM sodium pyruvate and 50 mg/ml gentamicin sulphate), and then twice with the IVM medium (TCM 199 + 10% FBS + 5 µg/ml porcine FSH + 0.81 mM sodium pyruvate + 5% buffalo follicular fluid + 50 µg/ml gentamicin sulphate). Groups of 15–20 COCs were placed in 100 µl droplets of the IVM medium, covered with mineral oil in a 35 mm Petri dish and cultured for 24 h in a humidified CO_2 incubator (5% CO_2 in air) at 38.5°C.

In vitro *production of parthenogenic embryos:* The matured oocytes were subjected to parthenogenetic activation (PA) as described by Kumar *et al.* (2012). For production of different stages of embryos the activated oocytes were cultured for 9 days in mCR2aa medium containing 0.6% BSA fraction-V in a Co_2 incubator (5% Co_2 in air) at 38.5°C.

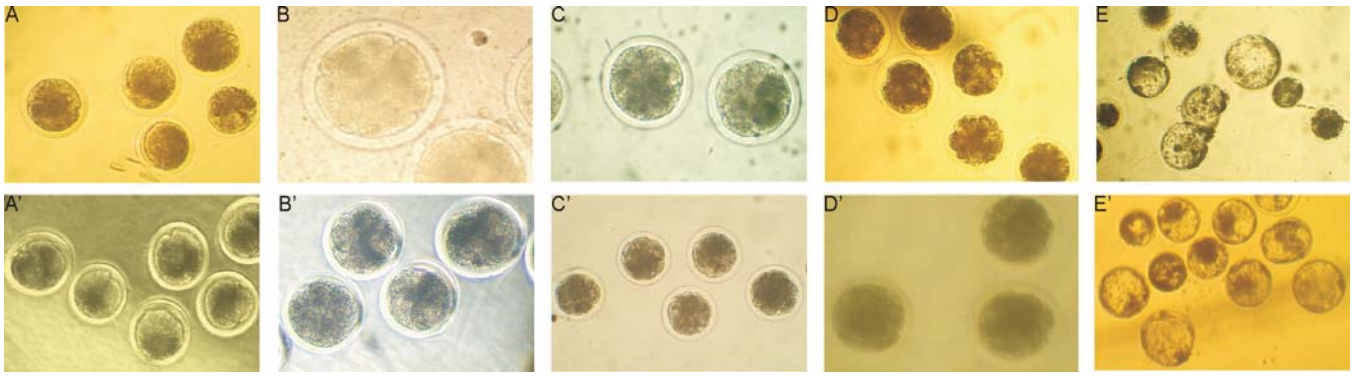
In vitro *fertilization and culture of presumed zygote:* The spermatozoa were prepared for fertilization (Chauhan *et al.* 1997). The presumed zygote were cultured in 100 µl droplets of mCR2aa medium. The cleavage rate was recorded on day 2 (42–44 h post insemination) in terms of oocytes that cleave to the 2 cell stage or beyond. At 48 h post insemination, the cleaved embryos were washed with the mCR2aa medium containing 10% FBS + 0.6% BSA fraction-V and cultured for up to 9 days in a CO_2 incubator (5% Co_2 in air) at 38.5°C.

Isolation of mRNA and reverse transcription polymerase chain reaction (RT-PCR): The total RNA was extracted from immature, matured oocytes and embryos at different stages of development (2 cell, 4 cell, 8–16 cell, morula and blastocysts). All stages of embryos were washed with ice cold 1× PBS, 4 to 5 times; 50 µl of lysis buffer was added, and then vortexed for 3 min. The homogenized mixture was incubated for 10 min at 75°C followed by digestion with DNase-I for 30 min at 37°C. After incubation, DNase-I was inactivated by immediate heating the mixture to 75°C for 5 min. For synthesis of cDNA, reaction mixture (10 µl cell lysate, 4 µl dNTP mix and 2 µl random hexamer) was heated for 1 min at 78°C. Then mixture was immediately cooled on ice, and 2 µl 10 × RT buffer, 1 µl MMLV and 1 µl RNase inhibitor was added. Reverse transcription was carried out for 1 h at 42°C, followed by incubation at 95°C for 10 min. Negative RT control with all the reaction components except for the reverse transcriptase was also set in each experimental batch. The PCR cycle included heating to 94°C for 2 min, followed by repeated cycles of 94°C for 30 sec, annealing temperature 60–62°C for 30 sec, and extension at 72°C for 45 sec cycle was repeated 35 times with final extension for 72°C for 10 min. The following primers were used for PCR: primers specific for *BAX* F-5'- TGCTTCAGGGTTTCATC CAG-3', R-5'- GTGTCCCAAAGTAGGAGAGG-3'(427bp, 60°C); *BCL-XL* F-5'- GGATAGCCCTGCTGTGAATGG-3', R-5'- TGAGCCCAGCAGAACCACA-3' (505bp, 62°C) and housekeeping gene 18srRNA F-5'-GGACACTCAGCTAA GAGCATCG-3', R- 5'-GAGAAACGGCTACCACATCC-3' (337bp, 60°C). For detection and semi-quantification of RT-PCR products, electrophoretic fragments were visualized on a UV-trans illuminator and densitometry data for band intensities was generated using AlphaDigiDoc™ AD-1201 software under Windows™ environment. The relative abundance of different transcripts was estimated by dividing the intensity of the band of the transcript of interest by the intensity of 18S rRNA band from the same sample and data analyzed with help of SYSTAT software.

TUNEL protocol: TUNEL was carried out using an *in situ* cell death detection kit as per the manufacturer's protocol. Briefly, day 8 parthenogenetic and IVF blastocysts were collected separately, washed 3 times with PBS+0.3% polyvinyl alcohol (PVA) and fixed in 4% paraformaldehyde for 1 h at room temperature. Fixed blastocysts were washed with PBS+0.3% PVA and then permeabilized with 0.5% Triton-X 100 for 1 h at room temperature. Blastocysts were then divided into 3 groups: treatment group, positive control and negative control. The blastocysts of the positive control group were treated with DNase solution (2 µl DNase + 38 µl PBS+0.3% PVA) for 40 min and were then washed 2–3 times with PBS+0.3% PVA. The blastocysts of treatment group and positive control group were incubated with 1 µl of enzyme solution + 9 µl label solution while negative control blastocysts were incubated with 10 µl label solution only and kept in dark chamber at 38°C for 1 h. After incubation, all the blastocysts were washed 3 times with PBS+0.3% PVA and were treated with RNase solution: (1µl RNase A + 100 µl RNase buffer) for 1 h at 38°C, then washed with PBS+0.3% PVA. Finally, the blastocysts were incubated with propidium iodide (5 µg/ml) for 15 min at 38°C, washed with PBS+0.3% PVA and transferred to slides, which were observed under a fluorescence microscope.

RESULTS AND DISCUSSION

Embryos produced through parthenogenesis: In embryo production through parthenogenesis, a sum of 373 immature oocytes were subjected to *in vitro* maturation and activation. The cleavage rate observed was 75.81±3.64% (n=281, Fig. 1A) after 24 h of culture. When these cleaved or two cell embryos further cultured, they developed in to 4- cell (Fig.



Figs 1 (A-E;A'-E'). A group of *in vitro* produced embryos through IVF: 2- cell (A), 4- cell (B), 8–16 cell (C), morula (D) and blastocyst (E). A', B', C', D' and E' are respective stages produced parthenogenetically.

1B), 8–16 cell (Fig. 1C), morula (Fig. 1D) and blastocyst (Fig. 1E).

Embryos produced through in vitro *fertilization:* In embryo production through *in vitro* fertilization, a sum of 366 immature oocytes were subjected to *in vitro* maturation and *in vitro* fertilization. The cleavage rate was 50.51±3.31% (n=184, Fig. 1A'). When these cleaved or 2 cell embryos cultured for further development, they developed into 4- cell (Fig. 1B'), 8–16 cell (Fig. 1C'), morula (Fig. 1D') and blastocyst (Fig. 1E').

The embryo production rate i.e. 2- cell, 4-cell and blastocyst stage was significantly higher ($P<0.05$) when produced through parthenogenesis compared to IVF. There is no significant difference at 8–16 and morula stage of embryos (Table 3).

In present study it is clearly shown that high efficiency of oocyte activation in the buffalo can be achieved by sequential treatment with ethanol followed by incubation in 6-DMAP. Our results were in agreement with the previous findings that a combination treatment of chemical agents, which increase intracellular calcium (calcium ionophore or ethanol), and a protein phosphorylation inhibitor, 6-DMAP, are more effective in inducing activation and the subsequent development of bovine oocytes (Liu *et al.* 1998, Singh *et al.* 2012). In goats, a combination treatment of MII oocytes by ethanol and 6-DMAP results in 58% cleavage (Ongeri *et al.* 2001).

The percentages of cleavage and blastocyst development in ethanol-activated oocytes were significantly higher ($P<0.05$) than those of IVF as controls (Table 1). In present study the production of *in vitro* fertilized embryo is presented on the basis of their cleavage rate and the overall blastocyst production was 15.31±1.52, which is comparable to the findings of other workers (Palta and Chauhan 1998, Gasparrini 2002, Gasparrini *et al.* 2004). In cattle, the percentage of blastocyst development obtained from oocytes that were activated by the ethanol or calcium ionophore followed by 6-DMAP was also similar to that of the IVF controls (Liu *et al.* 1998).

Expression of BAX gene in different stages of embryos: Relative abundance of *BAX* transcripts by semi quantitative RT-PCR analysis in immature, mature oocytes; and embryos produced by parthenogenesis i.e. 2-cell, 4-cell, 8–16-cell, morulae and blastocyst stage were 22.3±0.90, 0.13±0.03, 13.67±0.90, 14.0±0.60, 15.33±0.90, 64.67±2.20 and 55.0±2.10 respectively. The relative abundance of *BAX* transcripts in immature, matured oocytes; and embryos produced by IVF i.e. 2-cell, 4-cell, 8–16-cell, morulae and blastocyst stage were 23.0±0.60, 0.33±0.10, 4.00±0.60, 5.00±0.60, 0.37±0.06, 13.0±0.66 and 56.7±0.90 respectively (Figs 2a-2b).

Relative abundance of *BAX* transcripts was significantly higher in the blastocyst stage produced through IVF than the other stages, but the relative abundance of *BAX* transcripts was significantly higher in morula and blastocyst stage of embryos produced parthenogenetically. When the level of *BAX* expression was compared between IVF and parthenogenetic embryos, the expression was significantly higher in parthenogenetic embryo than IVF embryo at 2-cell to morula stage. This leads to less cell number in parthenogenetic blastocyst then IVF blastocyst.

Expression of BCL-XL gene in different stages of embryos: Relative abundance of *BCL-XL* transcripts by semi quantitative RT-PCR analysis in immature, matured oocytes;

Table 1. Buffalo embryos produced through parthenogenesis and IVF

Method of embryo production	Oocytes for IVM	Cleavage n (% ± SEM)	4 cell n (% ± SEM)	8–16 cell n (% ± SEM)	Morula n (% ± SEM)	Blastocyst n (% ± SEM)
Parthenogenesis	373	281[a] (75.81±3.64)	229[a] (81.49±2.93)	158 (56.77±5.10)	103 (36.65±0.97)	79[a] (27.89±1.90[a])
IVF	366	184[b] (50.51±3.31)	117[b] (63.89±4.50)	92 (50.20±3.06)	60 (32.70±2.91)	28[b] (15.31±1.52[b])

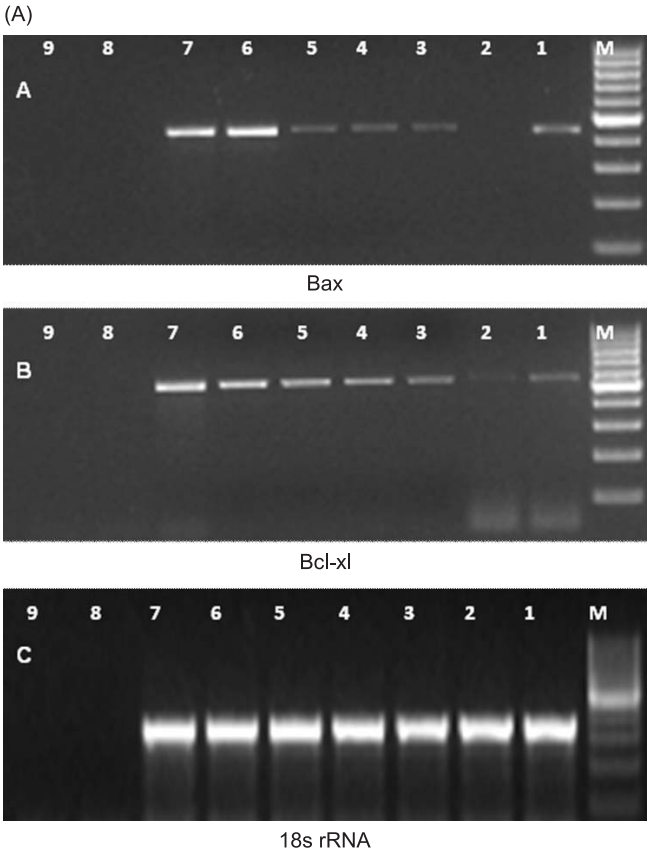


Fig. 2a. Expression of (A) *Bax* (427bp), (B) *Bcl-xl* (505bp) and C) *18s* rRNA (337bp) in oocytes and parthenogenetically produced embryos. Lane 1: Immature oocytes; Lane 2: *In vitro* matured oocytes; Lane 3: 2-cell stage embryos; Lane 4: 4-cell stage embryos; Lane 5: 8- to 16-cell stage embryos; Lane 6: morulae; Lane 7: blastocysts; Lane 8: -ve RT; Lane 9: -ve PCR and M: markers of 100 base pairs.

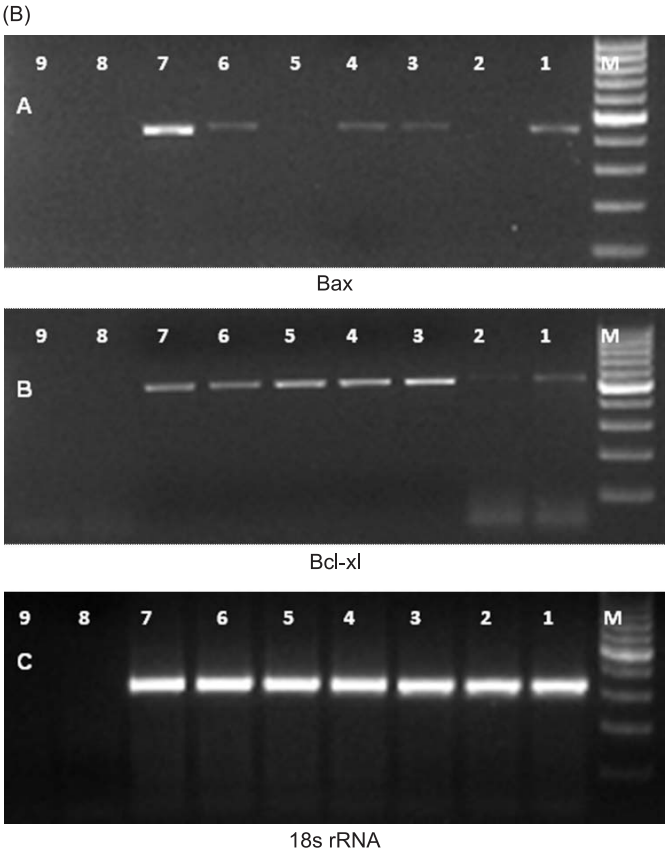


Fig. 2b. Expression of (A) *Bax* (427bp), (B) *Bcl-xl* (505bp) and (C) *18s* rRNA (337bp) in oocytes and IVF embryos. Lane 1: Immature oocytes; Lane 2: *In vitro* matured oocytes; Lane 3: 2-cell stage embryos; Lane 4: 4-cell stage embryos; Lane 5: 8- to 16-cell stage embryos; Lane 6: morulae; Lane 7: blastocysts; Lane 8: -ve RT; Lane 9: -ve PCR and M: markers of 100 base pairs.

and embryos produced by parthenogenesis i.e. 2-cell, 4-cell, 8–16-cell, morulae and blastocyst stage were 23.67±0.88, 13.67±1.20, 23.67±1.20, 22.34±0.88, 24.34±0.88, 33.67±0.88, 45.34±1.20 respectively. The relative abundance of *BCL-XL* transcripts in immature, mature oocytes; and embryos produced by IVF i.e. 2-cell, 4-cell, 8–16-cell, morulae and blastocyst stage were 25.33±0.90, 12.67±1.20, 37.67±0.90, 30.67±0.30, 23.67±0.90, 18.33±0.90 and 27.00±1.20 respectively (Figure 2a, 2b).

The relative abundance of *BCL-XL* gene was significantly higher ($P<0.05$) in 2-cell and 4- cell stages of embryos then the other stages of IVF, but the relative abundance of *BCL-XL* gene was higher in blastocyst and morula than the other stages of parthenotes. When the level of *BCL-XL* expression was compared between IVF and parthenogenetic embryos, the expression was significantly lower in parthenogenetic embryo than IVF embryos at 2-cell to 4-cell, morula and blastocyst stage. This leads to better embryo production rate in parthenogenesis.

The *BAX* gene is the first pro-apoptotic member and when over-expressed, the cellular apoptotic pathway takes place (Yang and Rajamahendran 2002). Byrne *et al.* (1999) reported that blastocysts from late cleaved zygotes had an increased apoptotic pattern compared to those cleaved earlier, and also reported that the *BAX* gene was also significantly higher in the low quality oocytes, denuded oocytes and fragmented embryos. The anti-apoptotic (*BCL-2*) gene expression was higher in the good quality oocytes and embryos, to be responsible for the tendency towards survival and towards a different developmental competence (Elamaran *et al.* 2012). High expression of cell death gene (*BAX*) may confirm that early embryos were inherently biased towards PCD (Kastan *et al.* 1991, Oltvai *et al.* 1993, Yang *et al.* 1995). Transcripts of the anti-apoptotic gene, *BCL-XL*, were detected in all stages of bovine embryo development and displayed a maternal/ embryonic expression profile with a dramatic increase at the blastocyst stage. Expression of this anti-apoptotic gene has previously been reported in bovine blastocysts. Embryos are often lost at, or before the time of the major burst of embryonic transcription (Barnes and First

1991). Jurisicova *et al.* (1998) reported that normal human embryos from 2-cell to morula stage were negative with regard to apoptosis or necrosis. The apoptotic response is thought to depend on the balance of survival and death molecules. The *BCL-2/ BAX* ratio appears to determine the fate of a cell (Wyllie 1995). The detection of apoptosis related genes in buffalo pre-implantation embryos indicated that buffalo embryos are capable of undergoing programmed cell death and constitutively express genes that are required to execute the death programme (Yang *et al.* 1995).

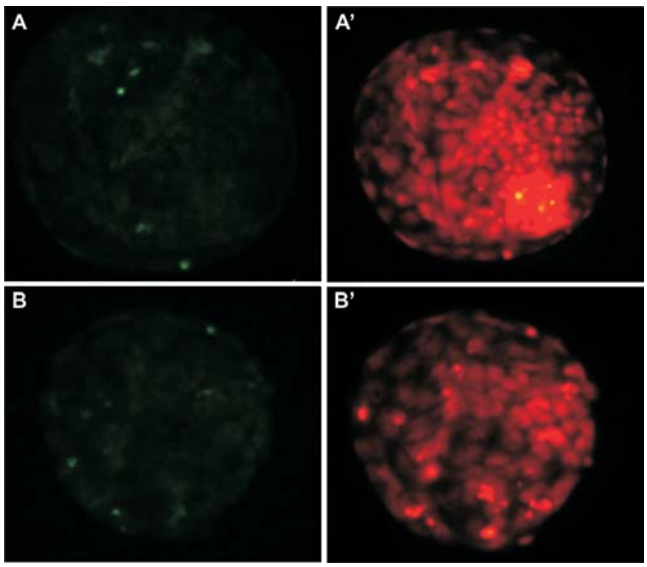


Fig. 3. Detection of apoptotic and all nuclei in IVF blastocyst (A, A') and parthenogenetic blastocyst (B, B') blastocyst by TUNEL (fluroscein isothiocyanate-conjugated dUTP; green channel) propidium iodide (red channel), respectively.

Apoptosis analysis: In the present study, the blastocysts produced through parthenogenesis and via *in vitro* fertilization, were subjected for TUNEL staining, it was observed that parthenogenetic blastocyst had more apoptotic index (5.31±0.72; n=10) than the IVF produced blastocysts (3.53±1.44; n=9) and it was found that the percentage of TUNEL-positive cells was significantly higher ($P<0.05$) in parthenogenetic blastocysts (Fig. 3). This is in agreement with the results of Hao *et al.* (2004) who reported that a significantly higher number of apoptotic cells were found in parthenogenic porcine blastocyst than blastocyst produced through IVF.

Parthengenetically produced embryos are alternate source of *in vitro* embryo production. The efficiency is higher than IVF produced embryos, having less ethical issues and may be useful for early developmental and genomics study. Parthenotes are comparable to embryos and therefore are useful tools for any research aimed at investigating culture conditions, different treatment option exposure to chemicals etc. These are also interesting source of stem cells due to the unique advantage of homozygosity.

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