Original Article

The Effect of the Duration of *In Vitro* Maturation (IVM) on Parthenogenetic Development of Ovine Oocytes

Abolfazl Shirazi [1,2*], Amin Bahiraee [1], Ebrahim Ahmadi [1], Hassan Nazari [1], Banafsheh Heidari [2], and Sara Borjian [1]

1. Research Institute of Animal Embryo Technology, Shahrekord University, Shahrekord, Iran
2. Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

Abstract

The aim of this study was to compare the effect of time of parthenogenetic activation (22 *hr* versus 27 *hr* after *In Vitro* Maturation-IVM) on *in vitro* development of ovine oocytes using either single (Ionomycin 5 μM for 5 *min* or Ethanol 7% for 7 *min*) or combined (ionomycin and ethanol with 6-DMAP 2 *mM* for 3 *hr*) activation treatments. The abattoir-derived *in vitro* matured activated oocytes were cultured in modified synthetic oviductal fluid and assessed for the cleavage, blastocyst, and hatching rates. The single-activated oocytes had a reduction in cleavage, blastocyst and hatching rates compared to the combined-activated oocytes (except for the cleavage at 27 *hr*). In single-treated groups the rates of cleavage and blastocyst were increased as the maturation time was extended from 22 *hr* to 27 *hr*. The numbers of total cells and Inner Cell Mass (ICM), though insignificant, were greater in combined-treated groups compared to the single treatment. The number of ICM in Eth+6-DMAP group activated at 27 *hr* was lower than 22 *hr*. Nonetheless, irrespective of the activation protocol, development to the blastocyst stage, the numbers of total cell, ICM, and cell allocation (ICM/total cells) were significantly lower in parthenogenetic than fertilized embryos. In conclusion, though the cleavage and blastocyst rates in single-treated groups were positively influenced by the extension of duration of IVM (27 *hr*), there was a trend of decreased numbers of total cells and ICM in slightly aged oocytes. Moreover, developmental potential of ovine parthenotes, especially in young oocytes, was improved by the addition of 6-DMAP to the activation regimen.

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*** Corresponding Author:**
Abolfazl Shirazi, D.V.M., Ph.D., Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran, P.O. Box: 19615-1177
Tel: +98 21 22432020
Fax: +98 21 22432021
E-mail:
shiraziabbas@yahoo.com
a.shirazi@avicenna.ac.ir
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Introduction

Oocyte activation is one of the essential elements that determine the success of nuclear transfer and the subsequent development of cloned embryos. Failure to activate oocytes efficiently, in a simple manner constitutes one of the limiting steps for the success of cloning by nuclear transfer. Oocyte activation allows synchronization of cell cycle phase between the cytoplasm of the oocyte and the transferred nucleus, promoting nuclear reprogramming and maintenance of normal ploidy [(1)]. The study of parthenogenetic activation also permits a greater understanding of the mechanisms of spontaneous activation, preventing it in *in vitro* fertilization systems, and allows investigating the comparative roles of paternal and maternal genomes in controlling early embryo development [(2)].

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Artificial stimuli through elevating the cytoplasmic levels of calcium ions of oocytes aim to mimic the action of sperm cells during fertilization [3]. Some artificial activation treatments promote an increase in intracellular free calcium concentrations by the release of calcium from cytoplasmic stores, such as Strontium [4] and Ionomycin [5] while there are elements that promote influx of calcium from the extra-cellular medium, such as electrical stimulus. There are, however, elements that promote both effects, such as ethanol [5].

These treatments are commonly followed by the application of an inhibitor of protein phosphorylation (6-dimethylaminopurine; 6-DMAP) which prevents Maturation Promoting Factor (MPF) activation or an inhibitor of protein synthesis (cycloheximide, CHX) that prevents cyclin synthesis [6-9].

Among the deficiencies in the process of artificial activation, the age of oocyte (maturation time) could be an important contributing factor [6,10]. Activation is more easily achieved in aged oocytes due to the spontaneous reduction on MPF activity with aging of oocytes [11,12] and changes in sensitivity of metaphase II (MII) oocytes to the internal calcium perturbation provoked by an artificial stimulus [13]. However, it is known that such aging, apart from its negative cytoplasmic changes, causes alterations in components of the cytoskeleton of the oocyte [14-16], impairs enucleation through changes in the location and organization of the second meiotic spindle [17,18], affects embryonic development after fusion [10-19] and increases the frequency of fragmentation and caspases activation, responsible for cell apoptosis [20]. In this context, dependence on oocyte age for parthenogenetic activation can be minimized by promotion of multiple intracellular calcium pulses [21] or combination of different artificial activation treatments [22,23].

In some species the response of young oocytes to parthenogenetic activation is low [24,25] and aged oocytes are often used as recipient oocytes for nuclear transfer of embryonic nuclei [18]. The potential of aged mouse [26] and rabbit [16] NT oocytes receiving embryonic nuclei to develop into blastocyst is higher than that of young oocytes. In contrast, there are reports indicating the lower developmental potential of aged bovine [27] and mouse [28] oocytes after parthenogenetic activation compared to the young oocytes.

Although an age-dependent activation response has been described previously in several species, including the mouse [29], cow [24,30], and pig [31], there are no relevant studies on the effect of oocyte age on parthenogenetic activation in sheep. In the current study, as a primary objective, the effect of maturation time (22 *hr* vs 27 *hr*) of ovine oocytes on efficiency of activation treatments, and as a secondary objective the comparison between the effect of single or combined activation treatments on ovine parthenogenesis were evaluated. In this evaluation, the cleavage and blastocyst rates, the capacity of the blastocyst to hatch from the zona pellucida, blastocyst cell numbers and the cell allocation were analyzed.

Materials and Methods

Except where otherwise indicated, all chemicals were obtained from the Sigma (St. Louis, MO, USA).

Oocyte collection and in vitro maturation

Prepubertal and adult ovine ovaries were collected at a local slaughterhouse and transported to the laboratory within 2 to 3 *hr* in normal saline at temperature between 25 and 35°*C*. The ovaries were washed 3 times with prewarmed (37°*C*) fresh saline, and all visible follicles with a diameter of 2 to 6 *mm* were aspirated using gentle vacuum (30 *mmHg*) via a 20 gauge short beveled needle connected to a vacuum pump. The follicle content was released in preincubated hepes-buffered TCM 199, supplemented with 50 *IU/ml* heparin.

The method for *in vitro* maturation and production of sheep embryo was the same as described by Thompson et al [32] with minor modification.

Briefly, the oocytes, with at least 3 layers of cumulus cells (COCs: Cumulus-Oocyte

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Complexes), with a uniform granulated cytoplasm and homogenous distribution of lipid droplets in the cytoplasm, were selected for the experiments. Before culturing, the oocytes were washed in Hepes-buffered TCM199 (H-TCM199) supplemented with 5% FBS (Fetal bovine serum, Gibco 10270), and 2 *mM* glutamine. The Oocyte Culture Medium (OCM) consisted of bicarbonate-buffered TCM199 with 2 *mM* L-glutamine supplemented with 0.02 *mg/ml* cysteamine, 1 *IU/ml* hCG, 0.05% *IU/ml* FSH, 1 *µg/ml* E_2, 100 *IU/ml* penicillin, 100 *µg/ml* streptomycin, 10% FBS (Fetal bovine serum, Gibco 10270), and 0.2 *mM* Na-Pyruvate. The selected COCs were pooled and randomly distributed in maturation droplets (15 oocytes in 50 *µl*) and covered by sterile paraffin oil in a 60 *mm* Petri dish (Falcon 3004; Becton & Dickinson, Franklin Lakes, NJ) and were then incubated for 22 and 27 *hrs* at 39 *ºC* under an atmosphere of 5%CO_2 and 100% humidity.

Preparation of sperm and In Vitro Fertilization (IVF)

After IVM, the oocytes were washed four times in H-SOF (HEPES- synthetic oviductal fluid) and once in fertilization medium and were then transferred into the fertilization droplets. Fresh semen was collected from a Lori-Bakhtiari ram of proven fertility. For swim up, 80-100 *µl* of semen was kept under 1 *ml* of BSA-HSOF in 15 *ml* conical tube at 39 *ºC* for up to 45 *min*. After swim up, the 700-800 *µl* of medium was gently taken off the top of the suspension and were then added into 15 *m1* conical tube containing 3 *ml* of BSA-HSOF, centrifuged twice at 200×*g* for 3 *min* and the final pellet was re-suspended with BSA- HSOF. The oocytes were inseminated with 1.0×10^6 normal, motile spermatozoa/*ml*. The fertilization medium was SOF (as originally described by Tervit et al) [33] and enriched with 20% heat inactivated estrous sheep serum. A 5 *µl* aliquot of sperm suspension, (1.0×10^6 *sperm/ml*), was added into the fertilization droplets (45 *µl*) containing 10 oocytes. Fertilization was carried out by co-incubation of sperm and oocytes for 22 *hr* at 39 *ºC* in an atmosphere of 5% CO_2 in humidified air. After IVF, presumptive zygotes were denuded of surrounding cumulus cells by vortexing for 2 *min* in H-SOF containing 0.1% hyaluronidase and were then transferred to culture drops.

Activation of oocyte

Methods for activation of oocytes were modified from Susko-Parrish et al [34]. After IVM (22 and 27 *hrs*), cumulus cells were removed by incubation in H-SOF containing 0.1% hyaluronidase at 39 *°C* for 2 *min* followed by vortexing for 3 *min*. Denuded oocytes were pooled and randomly allocated into single or combined treatment groups. In single treatment groups the oocytes were treated with either Ionomycin (5 *µM* for 5 *min*) or ethanol (7% for 7 *min*). After 5 *min* exposure to Ionomycin, the oocytes were then rinsed in H-SOF containing 30 *mg/ml* BSA to stop activation. All of the chemicals for oocyte activation were dissolved in H-SOF medium supplemented with 1 *mg/ml* Bovine Serum Albumin (BSA), except ethanol which was supplemented with 0.1 *mg/ml* PVP. In combined treatment, after oocyte activation with the same concentrations of Ionomycin or ethanol as in the single treatment, the oocytes were then incubated in 2.0 *mM* 6-DMAP for 3 *hr*. Following activation, the oocytes were washed twice in H-SOF medium and transferred to the culture medium.

A summary of the treatment groups is presented in the below. Each treatment consisted of at least 5 replicates:

Group IVF: The *in vitro* matured oocytes for 22 and 27 *hrs* were fertilized with fresh semen (control)

Group Io: Denuded oocytes activated with 5 *µm* Ionomycin for 5 *min*

Group Io+6-DMAP: Denuded oocytes activated with 5 *µm* Ionomycin for 5 *min*+ 2 *mM* 6-DMAP for 3 *hr*

Group Eth: Denuded oocytes activated with 7% ethanol for 7 *min*

Group Eth+6-DMAP: Denuded oocytes activated with 7% ethanol for 7 *min*+2 *mM* 6-DMAP for 3 *hr*

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In vitro culture

Presumptive zygotes in IVF group and activated oocytes in parthenogenetic groups were allocated to 20 *µl* culture drops (five to six embryos/drop) containing SOF supplemented with 2% (*v/v*) BME-essential amino acids, 1% (*v/v*) MEM-nonessential amino acids, 1 *mM* glutamine and 8 *mg/ml* fatty acid free BSA. Embryos were cultured for 8 *days* at 39 *ºC* under mineral oil in a humidified atmosphere of 5% CO2, 7% O2. On the third and fifth *days* of culture (Day 0 defined as the day of fertilization), 10% charcoal stripped Fetal Bovine Serum (FBS) was added to the medium. The rates of cleaved embryos (day 3) and blastocysts (day 7) were expressed on the basis of the number of oocytes at the onset of culture. The percentage of hatched blastocysts on day 8 was expressed on the basis of the total number of blastocysts present on day seven.

Differential staining

Differential staining of Inner Cell Mass (ICM) and Trophectoderm (TE) compartments was carried out on day 7 blastocysts. Briefly, blastocysts were incubated in Triton X-100 prepared in the base medium (H-SOF containing 5 *mg/ml* BSA) for 20 *sec*. The blastocysts were then stained in the base medium containing 30 *µg/ml* Propidium Iodide (PI) for 1 *min*. After two washes in the base medium, the blastocysts were transferred in ice-cold ethanol containing 10 *µg/ml* Hoechst 33342 for 15 *min*. The blastocysts were directly mounted into the small droplet of glycerol on glass slide and examined under an epifluorescent microscope (IX71 Olympus, Tokyo, Japan). The ICM nuclei appeared blue, caused by DNA labeling with the membrane permeable Hoechst 33342, and trophoblastic cells appeared red due to staining of nuclear DNA with the membrane impermeable PI.

Statistical analysis

Data were collected over at least five replicates. All proportional data were subjected to an arc-sine transformation, and the transformed values were analyzed using one-way ANOVA. When the ANOVA revealed a significant effect, comparison of the means between the groups was performed using Tukey test. When normality test was failed the Kruskal-Wallis One Way Analysis of Variance on Ranks was applied. A $p<0.05$ level was considered significant (SPSS version 11.5).Data was expressed as mean±SEM.

Results

Effect of oocyte age on development of parthenotes

As shown in Table 1, two maturation times were considered to compare the effect of four activation regimens on parthenogenesis of ovine oocytes matured *in vitro*. The cleavage rates of artificially activated oocytes after 22 *hr* of culture, in groups receiving either Io or Eth was lower than groups receiving Io+6-DMAP and Eth+6-DMAP ($p<0.001$). How ever, the difference between Eth and Eth+6-DMAPgroups was not significant ($p>0.05$). The cleavage rate in the combined treatment groups (Io+6-DMAP and Eth+6-DMAP) was comparable with the IVF group. There was not significant difference between the IVF and the artificially activated oocytes after 27 *hr* of culture in terms of cleavage rate

Table 1. Comparison of *in vitro* development of ovine oocytes activated by Ionomycin or Ethanol alone or in combination with 6-DMAPat 22 or 27 *hr* after in vitro maturation

Experimental Groups	Oocyte No.		Cleavage (mean±SEM)		Blastocyst (mean±SEM)		Hatched blastocyst (mean±SEM)	
	22 *hr*	27 *hr*	22 *hr*	27 *hr*	22 *hr*	27 *hr*	22 *hr*	27 *hr*
IVF[1]	156	167	82.2 ± 3.2^{a}	85.6 ± 3.6	41.0 ± 3.2^{a}	42.6 ± 3.4^{a}	71.1 ± 1.7^{a}	77.0 ± 2.6^{a}
Io[2]	147	149	$46.6\pm6.9^{b,A}$	75.4 ± 5.9^{B}	$3.8\pm2.1^{b,A}$	$15.2\pm1.9^{b,d,B}$	0.0 ± 0.0^{b}	5.0 ± 5.0^{b}
Io+6-DMAP	149	151	80.8 ± 5.4^{a}	82.8 ± 3.3	26.0 ± 2.9^{c}	29.6 ± 2.2^{c}	$37.8\pm4.6^{c,A}$	$59.2\pm2.9^{a,B}$
Eth[3]	151	153	$50.4\pm6.9^{b,c,A}$	73.5 ± 3.3^{B}	$3.6\pm2.0^{b,A}$	$14.6\pm1.3^{b,B}$	8.3 ± 8.3^{b}	4.0 ± 4.0^{b}
Eth+6-DMAP	139	159	$70.7\pm3.8^{c,a}$	80.2 ± 4.5	18.8 ± 2.1^{c}	$24.4\pm1.4^{d,c}$	41.7 ± 8.6^{c}	54.7 ± 12.1^{a}

[a,b,c,d] Means±SEM; different lowercase letters indicate statistical differences into columns ($p<0.001$)

[A,B] Means±SEM; different uppercase letters indicate statistical differences in the same embryonic development stage ($p<0.05$)

[1]In vitro fertilization; [2]Ionomycin; [3]Ethanol

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Table 2. Comparison of cell numbers in parthenogenetically developed blastocyst derived from ovine oocytes activated at 22 or 27 *hr* after *in vitro* maturation

Experimental groups	Blastocyst No.		Cell no. per Blastocyst (mean±SEM)		ICM no. per Blastocyst (mean±SEM)		ICM/total cell (mean±SEM)	
	22 *hr*	27 *hr*	22 *hr*	27 *hr*	22 *hr*	27 *hr*	22 *hr*	27 *hr*
IVF[1]	32	34	145.3±14.9[a]	160.4±14.8[a]	30.0±2.3[a]	32.1±2.4[a]	0.23±0.0[a]	0.22±0.0[a]
Io[2]	6	14	29.5±6.0[b]	28.4±2.3[b]	3.0±0.5[b]	2.6±0.2[b]	0.1±0.0[b]	0.1±0.0[b]
Io+6-DMAP	21	26	63.1±8.8[b]	52.0±7.5[b]	5.3±0.7[b]	4.3±0.6[b]	0.09±0.0[b]	0.09±0.0[b]
Eth[3]	5	14	29.0±5.7[b]	28.7±2.4[b]	3.4±0.5[b]	2.7±0.3[b]	0.12±0.0[b]	0.09±0.0[b]
Eth+6-DMAP	18	21	50.8±5.9[b]	43.3±4.1[b]	4.6±0.6[b,A]	3.1±0.3[b,B]	0.1±0.0[b]	0.08±0.0[b]

[a,b] Means ± SEM; different lowercase letters indicate statistical differences into columns ($p<0.001$)
[A,B] Means ± SEM; different uppercase letters indicate statistical differences into rows of each subject ($p<0.05$).
[1]In vitro fertilization; [2]Ionomycin; [3]Ethanol

($p>0.05$). The cleavage rates were significantly increased in groups Io and Eth when the maturation time was extended from 22 to 27 *hrs* ($p<0.05$).

The blastocyst rates of artificially activated oocytes after 22 *hr* of culture in groups receiving either Io or Eth was lower than groups receiving Io+6-DMAPand Eth+6-DMAP ($p<0.001$). The blastocyst rates in groups receiving Io+6-DMAP and Eth+6-DMAP after 27 *hr* of culture was higher ($p<0.001$) than groups receiving either Io or Eth (except for Eth+6-DMAP and Io). The corresponding value in IVF group, however, was significantly higher than parthenogenetically activated oocytes at both 22 and 27 *hrs* of culture ($p<0.001$). The blastocyst rates were significantly increased in groups Io and Eth when the maturation time was increased from 22 to 27 *hrs* ($p<0.05$).

The hatching rates in combined treatment groups after both 22 and 27 *hrs* of culture were significantly higher than single treatment groups ($p<0.001$). The corresponding rate, however, was significantly higher in IVF group compared to the artificially activated oocytes after 22 *hr* of culture ($p<0.001$). The hatching rate was significantly increased in group Io+6-DMAP as the maturation time was increased to 27 *hr* ($p<0.05$).

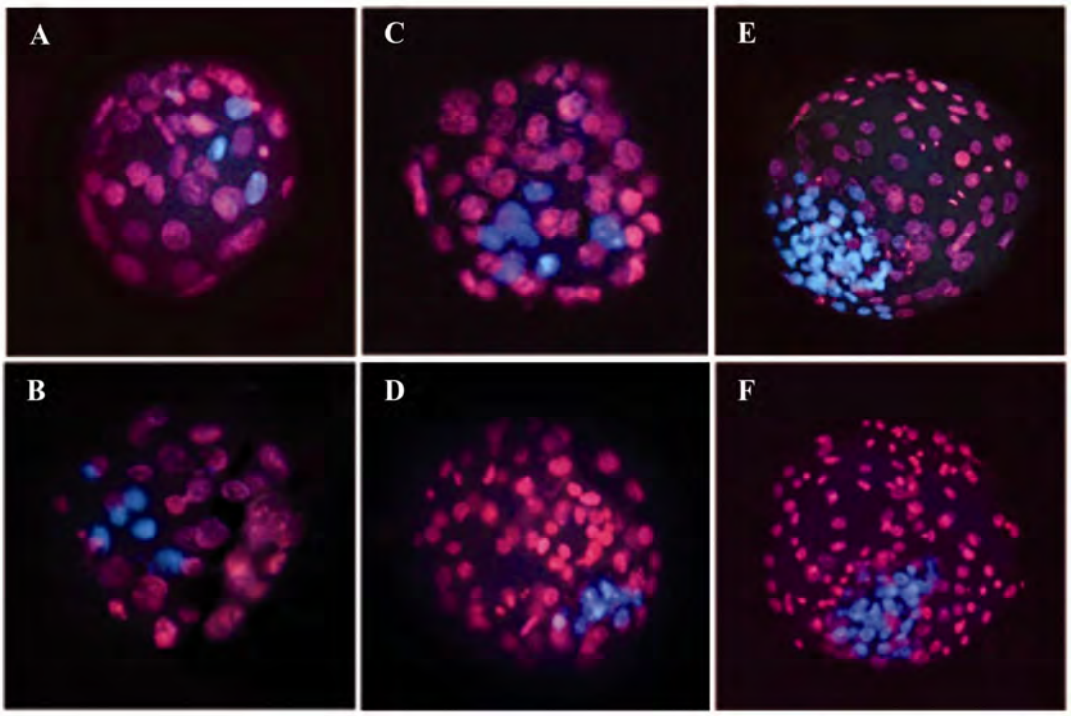

Figure 1. Epifluorescent microscopic images of bovine blastocysts derived from parthenogenetic activation and IVF. Trophectoderm and inner cell mass nuclei were labeled with propidium iodide (red) and Hoechst 33342 (blue), respectively. (A-B) Partenogenetic blastocysts derived from single treated groups (Ionomycin or ethanol). C-D) Partenogenetic blastocysts derived from combined treated groups (Ionomycin or ethanol with 6-DMAP). E-F) IVF-derived blastocysts

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Effect of oocyte age on cell number of parthenogenetically produced blastocysts

The cell numbers in parthenogenetically developed blastocysts were lower than ($p<0.001$) *in vitro* produced embryos for 22 and 27 *hrs* of oocyte maturations (Table 2, Figure 1). A comparison between blastocyst cell numbers in embryos derived from the oocytes matured *in vitro* for 22 and 27 *hrs* did not show any significant difference among experimental groups ($p>0.05$). The number of inner cell mass and its proportion to the total cells were significantly higher in IVF derived blastocysts than parthenogenetically developed blastocysts for 22 and 27 *hrs* of oocyte maturation ($p<0.001$). In group Eth+6-DMAP, the mean number of ICM was significantly higher in oocytes activated after 22 *hr* of culture compared to 27 *hr* ($p<0.05$).

Discussion

The most prominent manifestations of oocyte aging include an increased susceptibility to activating stimuli [13,24], most probably through a decrease in MPF activity [12,35,36], the onset of anaphase II [36,37], and partial exocytosis of cortical granules [38]. It is known, however, that fertilization or artificial activation of aged oocytes resulted in abnormal development [39,40]. In this study, we were interested in testing the effects of the maturation time (oocyte age) on efficiency of chemical activators for activating ovine oocytes matured in vitro. The synergistic effect of Ionomycin and ethanol with 6-DMAP (combined treatments) was also investigated.

The current results indicated that in young oocytes (maturation time; 22 *hr*) the cleavage rate was higher in parthenogenetically activated oocytes treated with combined treatment compared with single treatment (Ionomycin or ethanol alone). In slightly aged oocytes (maturation time; 27 *hr*) there was no such difference between activated oocytes treated by either single or combined treatments. On the other hand, the cleavage rate in the oocytes receiving single treatment (Ionomycin or ethanol) was positively influenced, as the age of oocytes was increased. Similarly, the blastocyst formation and hatching rates were positively influenced by the synergism between Ionomycin or ethanol with 6-DMAP at both 22 and 27 *hrs*.

In the single treated groups, the blastocyst formation rates were significantly higher in the oocytes parthenogentically activated after 27 *hr* of culture compared with 22 *hr*. The hatching rate was significantly increased in group Ionomycin+6-DMAP in oocytes activated at 27 *hr* of culture compared to the 22 *hr*. These findings was in agreement with earlier studies demonstrating the low response of young oocytes to parthenogenetic activation [24,25,41] and those studies in which aged oocytes were often used as recipient oocytes for nuclear transfer [18,42].

In this context, the potential of the aged mouse [26] and rabbit [16] NT oocytes receiving embryonic nuclei to develop into blastocyst was higher than that of young oocytes. In contrast, in some species following the combined treatment the activation rate and the proportion of parthenogenetically activated oocytes developed to blastocyst stage in young oocytes was higher than aged counterparts [18,28]. The aged oocytes, however, had higher activation rates but lower developmental potential than young oocytes [6]. Similarly, a higher development has been reported in the reconstructed oocytes cloned with young recipient oocytes [18]. In artificially activated bovine oocytes, no differences in the percentages of cleavage and blastocyst development were observed between the oocytes matured for 20 and 24 *hrs* [43].

One possible explanation for this discrepancy between the current result and other reports could be due to the narrow age gap (5 *hr*) between treatment groups (22 and 27 *hrs*). There are, probably, other contributing factors such as the pattern of oocyte maturation (*in vivo* or *in vitro*) and the presence or absence of cumulus cells [44] and inter-species differences which could influence the responsiveness of oocytes to artificial activators. There is even report indicating

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that the effect of oocytes aging on activation rate is strain-dependent [45].

It is known that calcium ionophore (A23187) treatment can induce a single intracellular calcium rise in MII oocytes and its consequence is the activation of several calcium dependent proteolytic pathways, leading to the destruction of cyclin B, reduction of MPF activity, and resumption of meiosis [46]. In young oocytes, A23187 treatment alone caused a slower decrease in H1 kinase activity and no evident of MAPK alteration [19]. In contrast in aged oocytes, activities of both kinases decreased after A23187 treatment, similar to the response in the combined treatments (A23187+6-DMAP). This may explain the higher proportion of cleavage in slightly aged oocytes compared to the young oocytes in single treated groups.

In young, newly matured oocytes, because of the active synthesis of proteins including cyclin B, a recovery of MPF activity would occur after single calcium stimulation because of a quick renewal of cyclin B [13,47]. Alternatively, calcium stimulation followed by treatment with a protein kinase inhibitor such as 6-DMAP could block the activity of newly synthesized MPF and lead to full activation of the newly matured oocytes [23,48].

The current results confirmed the above statement indicating the presence of synergistic effects between Ionomycin or ethanol with 6-DMAP on activation, cleavage and development rates in ovine oocytes [19,23,49]. The fact that the calcium elevation, induced by Ionomycin, destroys cyclin B (and related proteins) and the protein kinase inhibitor (6-DMAP), prevents the renewal of those proteins, could explain the effectiveness of the combined treatment in the current study as supported by previous reports [6,23,48,50].

As documented, for oocyte activation and normal female pronuclear development and subsequent cytokinetics of the oocyte, the presence of high MPF activity is necessary. Moreover, the high frequency of fragmentation in aged oocytes is attributable to their low MPF activity [51]. However, another factor such as pre-MPF, might be involved in the aging phenomena [35]. In the current study, it seems the narrow age gap (5 *hr*) between oocytes in the two age groups has not been big enough to decrease the MPF activity below a critical threshold needed for normal development. Instead the extended maturation time of oocytes (27 *hr*), has probably, resulted in better cytoplasmic maturation which in turn led to the higher cleavage and blastocyst rates in groups receiving either Ionomycin or ethanol. The quality of parthenotes, the numbers of total cell and ICM, however, were not positively influenced in the slightly aged oocytes. There is also report suggesting the occurrence of negative cytoplasmic changes in slightly *in vivo* aged oocytes [16].

As shown the addition of 6-DMAP to activation protocol could increase the susceptibility of young oocytes to activation. In partially aged oocytes, however, inspite of probably improved cytoplasmic maturation, the addition of 6-DMAP had no extra beneficial effect on the developmental rate. In addition, the blastocyst cell numbers and the numbers of ICM in parthenotes were higher, though not significant, in the combined treated groups compared with single treated groups in both young and partially aged oocytes. Compared to the young oocytes, the number of ICM was significantly decreased only in ethanol+6-DMAP group in aged oocytes. There was, however, a trend of a decreased total cell numbers in combined treatment groups and a decrease in the number of ICM in all the treated groups of aged oocytes.

This might be due to a decreased quality of blastocysts derived from slightly aged oocytes. This finding was in accordance with the report indicating the lower total cell numbers in the parthenogenetic blastocysts developed from aged oocytes compared with young oocytes [28]. It seemed, if the age gap between young and aged oocytes was quite enough, the difference in the quality of parthenogenetically produced blastocysts would become more prominent. However, as aforementioned in some groups of activated

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oocytes the cleavage, blastocyst and hatching rates were significantly higher in slightly aged oocytes and in other groups there was a positive trend towards the slightly aged oocytes.

Another reason for the lower cleavage, blastocyst, and hatched blastocyst rates in single treated groups may be due to the higher incidence of haploidy compared to the combined treated groups. In general, if activated oocytes are allowed to extrude a second polar body, an aneuploid, specifically haploid, parthenote is induced. On the other hand, in single treated groups especially in young oocytes (22 *hr*) the probability of extrusion of the second polar body was increased. Whereas, the polar body extrusion was inhibited in combined-treated oocytes. Indeed, the instant destruction of the spindle in 6-DMAP-treated oocytes could lead to the inhibition of polar body extrusion. Therefore, the oocytes would go directly into interphase and only one diploid pronucleus would be formed [19].

It is also known that the haploid parthenotes were developmentally compromised prior to blastocyst stages in contrast to diploid parthenotes [52-54]. In slightly aged oocytes because of spontaneous reduction of MPF activity, the incidence of haploidy was low. The low rate of haploidy besides, probably, the proper cytoplasmic maturation due to extension of duration of IVM might be the reasons for the higher rates of cleavage and blastocyst in slightly aged oocytes compared to the young oocytes in the single treated groups.

Similarly, the lower total cell number in single treated group may be attributed to the higher incidence of haploid parthenotes in single treated groups compared with combined treated ones. As indicated, the total cell number in haploid parthenotes is significantly less than that in diploid parthenotes [54]. Moreover, the incidence of apoptosis in the haploid parthenotes is significantly higher than that of diploid parthenotes which can explain the decreased total cell number in haploid parthenotes compared to the diploid parthenotes [54].

As expected, in IVF-derived embryos all parameters were obviously higher than corresponding parameters in parthenogenetically developed embryos (except for cleavage at 27 *hr*) indicating the lower potential of parthenogenetically activated oocytes compared with fertilized counterparts. As documented, parthenogenetic embryos exhibit delayed development and reduced total cell and ICM of blastocysts compared with fertilized embryos [55,56]. These developmental defects have been hypothesized to result from genomic imprinting, insufficient parthenogenetic activation, suboptimal *in vitro* culture conditions, or ploidy [54,57].

Another reason for lower cleavage and developmental rate in parthenogenetic embryos might be associated with cell death by apoptosis [58]. Meanwhile, the high levels of cytoplasmic fragmentation due to apoptosis are associated with reduced blastocyst formation and lower blastocyst cell numbers [59,60].

In conclusion, the extension of duration of IVM (from 22 to 27 *hrs*) in ovine oocytes improves the developmental rate of parthenogenetically produced embryos, especially in those activated with either Ionomycin or ethanol. There is, however, a trend of a decreased quality in those embryos developed from slightly aged oocytes. Moreover, the addition of 6-DMAP to activation protocol, improves developmental potential of ovine parthenotes particularly in young oocytes from both quantitative (cleavage, blastocyst, and hatching rates) and qualitative (blastocyst cell number and number of ICM) viewpoints.

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