Journal of Animal and Veterinary Advances 3 (11): 776-780, 2004

Possible Involvement of DNA Topoisomerase 2 Enzyme in the Process of Meiotic Arrest in Mouse Metaphase 2 Oocytes

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Abstract: The effects of etoposide supplementation to mouse gametes co-culture medium on fertilization rate, nuclear events and development to the blastocyst stage were investigated. In vivo matured mouse oocytes were co-cultured with sperms in TYH medium supplemented with 50 ìg/ml etoposide an specific DNA topoisomerase 2 (top 2) blocker. Experiment 1 evaluated the effects of top 2 blocker on fertilization rate and nuclear events in fertilized oocytes whole-mounted 5 hr after gametes co-culture. Top 2 blocking decreased the fertilization rate significantly ($p<0.01$) as compared with the control. Furthermore, the fertilized oocytes were blocked at the metaphase 2 stage and the extrusion of the second polar body an the formation of the female and male pronuclei were completely inhibited. Experiment 2 examined the ability of the oocytes fertilized in the presence of top 2 blocker to cleave and develop to the blastocyst stage in vitro. The cleavage rate of oocytes fertilized in the presence of etoposide was significantly lower ($p<0.001$) than the control (11% vs 86.3%; respectively). Non of the eggs fertilized in the presence of etoposide ($n=154$) developed beyond the 2-cell stage, whereas (76.3%) of the control ($n=145$) developed to the blastocyst stage. It is concluded that the use of top 2 blocker (etoposide) in mouse gametes co-culture medium inhibits exit from meiotic arrest after sperm penetration. Thus, suggesting a possible involvement of top 2 in meiotic arrest.

Key words: DNA topoisomerase 2, Meiotic arrest, Mouse, Etoposide, Fertilization

Introduction

Although it was well known that the cytoplasm of an ovulated oocyte contains a cytostatic factor (CSF) that blocks cell division at the metaphase, little is known about mechanisms underlying release from meiotic arrest. The CSF is assumed to be a short-lived pp39 mos protein, which is degraded upon fertilization (Sagata *et al.*, 1989; Watanabe *et al.*, 1989). On fertilization calcium ions are released from the endoplasmic reticulum, these calcium ions activate the calcium dependant protease calpain 2, which attacks the pp39mos protein, which decline immediately, inactivate the maturation promotion factor (MPF) and reinitiate meiosis (Sagata *et al.*, 1989; Watanabe *et al.*, 1989).

DNA topoisomerase 2 enzyme (top 2) is a structural component of the metaphase chromosomes (Earnshow *et al.*, 1985 and Gasser *et al.*, 1986). Blocking of top 2 in murine metaphase I (M I) oocytes in the presence of protein synthesis inhibitor resulted in complete expulsion of the total chromatin into the first polar body(Elsheikh *et al.*, 1997 and Fulka and Moor 1993), whereas the same treatment of metaphase 2 (M 2) oocytes gave inconsistent results (Fulka and Moor, 1993). Exit from M 2 and mitosis involves separation of chromatids, whereas M I involves separation of homologous chromosomes (Wright and Schatten, 1990). These findings suggest that the role of top 2 during M 2 is different from M 1. Top 2 is required for chromosomes separation, condensation and decondensation during mitosis (Dinardo *et al.*, 1984; Downes *et al.*, 1991; Uemura, *et al.*, 1987; Wright and Schatten, 1990). We Hypothesized that, during meiotic arrest top 2 is not functional to allow separation of the chromatids and release from meiotic arrest. If top 2 is not functional at M 2, upon fertilization top 2 will be activated allowing exit from meiotic arrest, extrusion of the second polar body and the formation of the male and female pronuclei. Since MPF and CSF will drop down upon fertilization, blocking of top 2 during fertilization will prevent exit from meiotic arrest, second polar body and pronuclei formation. Furthermore, top 2 activity was not detected in sperms of Xenopus laevis (Gaudio and Risley, 1994) and rat (Chen and Longo, 1996). Accordingly we hypothesized that using etoposide (an specific top 2 inhibitor) in the fertilization medium of mouse oocytes will not affect the fertilizing ability of sperms.

Top 2 blocking induces DNA breaks and DNA denaturation in Xenopus spermatocytes (Gaudio and Risley, 1994). The DNA breaks induced by top 2-targeted drug in Surf Clam (Spisula solidissima) oocytes, treated with top 2 inhibitor (teniposide) one hour before fertilization, inhibited the development beyond the 2-cell stage (Wright and Schatten, 1990).

Accordingly, queries are raised. Is top 2 functioning during meiotic arrest and in sperms head? Does top 2 activity

appear in the metaphase spindles and sperm head after fertilization? Does top 2 blocking during gametes co-culture inhibit exit from meiotic arrest? And finally do oocytes fertilized in the presence of top 2 blocker (etoposide) develop to blastocysts?
The objectives of the current study are to answer these queries by co-culturing mature mouse oocytes with sperms in a medium supplemented with a top 2 blocker (etoposide).

Materials and Methods

Preparation of Fertilization and Culture Media: Media were prepared for each trial weekly with Milli-Q water, distilled water was passed through a Milli-Q 4-bowel system (Organex type, Nihon Millipore Ltd., Tokyo, Japan). The fertilization medium used was TYH medium (Toyoda *et al.*, 1971) supplemented with 4 mg/ml bovine serum albumin (BSA, Sigma Chemical Co., U.S.A). At time of use TYH supplemented with or without etoposide (Sigma) (50 μg/ml), was filtered through a filter sterilizer (Gelman Sciences, Michigan, U.S.A) and 0.4 ml (for fertilization) or 0.5 ml (for sperm suspension) was placed in the center of sterilized tissue culture dishes (35 x 10 mm, Nunc, Roskilde, Denmark) and covered with paraffin oil. For culturing the fertilized oocytes in vitro KSOM supplemented with 1mg/ml BSA (KSOM + BSA) was prepared (Erbach *et al.*, 1994), filtered through a filter sterilizer and 30 μl drops were made on tissue culture dishes (35 x 10 mm, Nunc) and covered with oil. The media prepared were kept over night in CO_2 incubator at 37°C under 5% CO_2 in air until used.

Oocytes Collection: ICR female mice were superovulated by i.p. injection of 5 IU pregnant mare serum gonadotropin (PMSG; serotropin; Teikoku Zoki, Tokyo, Japan) followed by an i.p. injection of 5 IU human chorionic gonadotropin (hCG; gonatropin; Teikoku Zoki) 48 h after. Superovulated mice were sacrificed by cervical dislocation 14 - 15 hr after hCG injection the oviducts were excised and placed into the paraffin oil beside the fertilization drops. The ampullae of the oviducts were torn with the aid of 25 gauge needle and the oocytes cumulus complexes were introduced into the fertilization drops. The fertilization drops containing the oocytes cumulus complexes were returned to the incubator and kept there for 2 hr until the sperms were capacitized.

Sperm Preparation and Gametes Co-Culture: Sperms were obtained from cauda epididymidis of ICR males of proven fertility as described previously (Toyoda *et al.*, 1974). For each trial a male was killed by cervical dislocation and its cauda epididymidis were removed aseptically and placed into the paraffin oil beside the sperm suspension drop and punctured with 25 gauge needle. One to three drops of the dense sperm mass from each cauda epididymidis were introduced into the sperm suspension drop. Capacitation was allowed to proceed for 2 hr at 37° C under 5% CO_2 in air.
Sperm suspension showing vigorous motility and sperm concentration of 1 - 3 x 10^7/ml was used. Twenty micro-litres of the sperm suspension was introduced into the fertilization drops that contain the oocytes cumulus complexes, to have a final sperm concentration about 0.4 - 1.4 x 10^6 spermatozoa /ml. The gamete co-culture was allowed to continue for 5 hr in CO_2 incubator at 37°C under 5% CO_2 in air.

Culture of the Fertilized Oocytes: The oocytes fertilized in the presence or absence of etoposide were thoroughly washed in several drops of KSOM + BSA and cultured for 96 hr in 30 μl drops of the same medium at 37°C under 5% CO_2 in air. The cleavage rate was assessed 24 hr after culture and the developmental progress towards the blastocyst stage was recorded every 24 hr.

Experimental Design: in Experiment 1, the effects of etoposide on fertilization rate and nuclear events during in vitro fertilization of mouse oocytes was examined. The oocytes with cumulus complexes were grouped into two groups; group I is co-cultured with sperms in the presence of etoposide, group 2 (control) is co-cultured with sperms in etoposide-free medium. Gamete co-cultures were allowed to proceed for 5 hr, thereafter, the oocytes were whole-mounted and stained with aceto-orcein to assess the fertilization rate and to examine the changes in the maternal and paternal chromatin. Penetrated oocytes having pronuclei, enlarged or decondensed sperm head were considered as fertilized oocytes. In *Experiment 2* the effects of etoposide supplementation to the co-culture medium on subsequent development of the fertilized oocytes to the blastocyst stage was examined. Oocytes with cumulus complexes were co-cultured with sperms in the presence of etoposide (group I) or co-cultured in etoposide-free medium (group 2, control). The Gamete co-cultures were allowed to continue for 5 hr, thereafter the oocytes were washed several times in KSOM + BSA. The oocytes were cultured in KSOM + BSA for 96 hr. The cleavage rate and development to blastocyst stage were assessed under an inverted microscope (20-40 x) every 24 hr.

Statistical Analysis: The results were statistically evaluated with Chi-square. Differences at a probability of $p<0.05$ were considered to be statistically significant.

Results

Experiment 1: As shown in Table 1 the fertilization rate decreased ($p<0.01$) when the oocytes were co-cultured with sperms in the presence of etoposide as compared with the control (78.9% vs 88.3%; respectively). Polyspermic fertilization was observed only in the oocytes fertilized in the presence of etoposide (Table 1). About 86.6% of the control oocytes developed pronuclei whereas the treated one did not. Chromosome separation and the extrusion of the second polar body were completely inhibited by etoposide. The maternal chromatin remained aligned on the metaphase plate and the penetrating sperm heads enlarged or decondensed , however, the female and male pronuclei were not formed.

Table 1: Sperm penetration and nuclear events after *in vitro* fertilization of mouse oocytes in the presence of etoposidea

Treatment groups[b]	No. of oocytes examined	No. (%) fertilized	No. (%) of oocytes with			
			Pronuclei	Enlarged	Decondensed	Polyspermy
Group 1	327	258 (78.9)*	0(0)**	226 (69.1)**	20 (6.1)*	12 (3.7)*
Group 2	247	218 (88.3)	214(86.6)	0 (0)	4 (1.6)	0 (0)

[a]Data of 5 replicates were pooled. Percentage was based on the number of the examined oocytes
[b]Group 1: oocytes fertilized in the presence of etoposide, Group 2: control oocytes fertilized in etoposide-free medium
*,** Significantly different from group 2 ($p<0.01$, $p<0.001$, respectively)

Table 2: Effects of blocking top 2 with etoposide during in vitro fertilizati9on on cleavage and development of mouse embryos[a].

Treatment group b	No. of eggs cultured	No. (%) of eggs developed to			
		2-cell	4-cell	Morulae	Blastocysts
Group 1	154	17(11.0)*	0 (0)*	0(0)	0 (0)*
Group 2	190	164(86.3)	162 (85.3)	165 (85.3)	145 (76.3)

aData of 4 replicates were pooled. Percentage was based on the number of cultured oocytes
bGroup1: oocytes fertilized in the presence of etoposide, Group 2: oocytes fertilized in etoposide-free medium
*Significantly different from group 2 ($p<0.001$)

Experiment 2: As shown in Table 2 the cleavage rate of the oocytes fertilized in the presence of etoposide was 11% and 86.3% of the control cleaved. Non of the treated eggs ($n=154$) developed beyond the 2-cell stage whereas (76.3%) of the control oocytes ($n=145$) developed to the blastocyst stage.

Discussion

Top 2 activity is detected only in spermatogonia, spermatocytes, round and elongated spermatids of rat (Chen and Longo, 1996) and Xenopus laevis (Gaudio and Risley, 1994), whereas in the mature sperms this activity was not detected. In the present study, although the fertilization rate is reduced by the presence of etoposide in the fertilization medium, the results showed that etoposide has no effect on the sperm fertilizing ability. The reduced rate of fertilization in the current investigation is probably due to the effects of etoposide on the cumulus cells, which is very important for fertilization (Vanderhyden and Armstrong, 1989). The heads of sperms that penetrated the oocytes enlarged and started to decondense. However, further decondensation to form the male pronucleus was inhibited. This result suggest that, top 2 activity appears after enlargement of the sperm head and before decondensation.

The unfertilized oocytes contain a store of stable and hyperphosphorylated 39 *mos* protein, which is assumed to be the CSF (Sagata *et al.*, 1989; Watanabe *et al.*, 1989). Upon fertilization, calcium ion influx increases, activates calpain 2 a calcium-activated neutral protease, which degrades 39 *mos* (Sagata *et al.*, 1989). MPF activity and cyclins are also shown to degrade by increasing calcium ions upon fertilization (Masui and Clarke, 1979; Gerhart and Kirschner, 1984). The degradation of cyclins which is assumed to play a crucial role in the activation of the MPF (Westendrof *et al.*, 1989; Murray and Kirschner, 1989) occurs within 10 min of activation (Murry *et al.*, 1989), whereas 39 *mos* degrades 15-30 after egg activation (Watanabe *et al.*, 1989). Although 39 *mos* is proposed to be the CSF responsible for meiotic arrest in vertebrates (Sagata *et al.*, 1989), the difference in the time of degradation of cyclins and 39 *mos* suggests the involvement of other factor(s) in meiotic arrest. In our experiment, the oocytes were examined 5 hr after gametes co-culture. If the eggs are penetrated this time is enough to increase the intracellular level of calcium and to induce complete proteolysis of both CSF and MPF.

However, the M 2 chromosomes remained aligned on the metaphase plates. In our experiment we blocked top 2, which is known to be a structural component of the metaphase chromosomes (Gasser *et al.*, 1986; Earnshow *et al.*, 1985 and Earnshow and Heck, 1985), consequently the exit from meiotic arrest was inhibited. This strongly supported our believe that top 2 is involved in meiotic arrest. During M 2, the chromosomes are aligned in the center of the metaphase plate and are arrested from further segregation until activation of the oocytes. An active top 2 is essential for chromosome separation (Dinardo *et al.*, 1984; Downes *et al.*, 1991; Uemura, *et al.*, 1987 and Wright and Schatten, 1990), and since it is structural component of the metaphase chromosomes this made us to interpret that top 2 is not functioning during meiotic arrest. It appeared from the present findings that the use of etoposide before and during fertilization, maintains meiotic arrest even though the CSF and MPF disappeared. Moreover, the extrusion of the second polar body and the formation the female pronucleus will also be inhibited. Top 2 is known to make cuts in DNA strands (Liu *et al.*, 1980) and its inhibition with top 2 targeted drugs induces DNA break during spermatogenesis in Xenopus laevis (Gaudio and Risley 1994). Furthermore, top2-targeted drugs prevents top 2 from releasing the cuts that the enzyme normally makes in DNA strands, which leads to inhibition of cell division (Wright and Schatten, 1990). On accordance to Gaudio and Risley, (1994) who reported that the DNA breaks induced by top 2-targeted drugs resulted in damaging and denaturation of the DNA, a similar situation may prevailed in experiment two of the present study leading to failure of development. This is in agreement with the findings of Wright and Schatten (1990) who reported that treatment of the oocytes of Surf Calm (*Spisula solidissma*) with top 2 targeted drug (tenoposide) before fertilization inhibited the development beyond the 2-cell stage.

Conclusion

It is concluded that blocking top 2 during gametes co-culture will not interfere with fertilization. However, it will inhibits extrusion of the second polar body; the formation of the female and male pronuclei and the exit from meiotic arrest. The results also suggests that top 2 is not functioning during meiotic arrest and in sperm heads. Top 2 activity appears in sperm head and metaphase spindles after fertilization. Moreover, blocking top 2 during fertilization of mouse oocytes inhibits the development to the blastocyst stage.

Acknowledgement

The authors thank Dr. Badreldin Hago of the department of Surgery and Anaethesia, Faculty of Veterinary Medicine, University of Khartoum for his helpful criticism of this manuscript.

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