Middle East Fertility Society Journal 22 (2017) 132–135

Contents lists available at ScienceDirect

Middle East Fertility Society Journal

journal homepage: www.sciencedirect.com

Original Article

No cytotoxic effects from application of pentoxifylline to spermatozoa on subsequent pre-implantation embryo development in mice

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ARTICLE INFO

Article history:
Received 21 July 2016
Accepted 4 December 2016
Available online 28 December 2016

Keywords:
Pentoxifylline
Fertilization
Blastocyst
DNA fragmentation index
Mouse

ABSTRACT

The aim was to assess the effect of spermatozoa exposed to PTX on the rates of fertilization and embryo development and apoptotic cells within blastocysts in an animal model. Mice Oocytes were inseminated with spermatozoa exposed to 3.6 mmol PTX for 30 min, or with neat spermatozoa. Then fertilization and embryo development rate, blastocyst formation and quality, as well as total cell number of blastocyst, and DNA fragmentation index (DFI) in blastocysts were surveyed in both groups. Fertilization and embryo development rate were similar between the groups. The rates of blastocyst formation did not differ significantly between control and PTX groups (52.4% vs. 51.8%). The average of total cell count in blastocysts and DFI in control and PTX groups were also insignificant (31.08 ± 1.5 vs. 34.14 ± 1.5 and 9.76 ± 5.0 vs. 11.77 ± 5.4). Application of PTX for enhancing sperm motility does not cause a cytotoxic effect on subsequent embryo development and embryo genome integrity.

1. Introduction

Successful fertilization depends on the quality of spermatozoa, including the motion characteristics. The diminished sperm motility and poor binding capacity to zona pellucida (ZP) are some causes of male infertility. It has been reported that agents such as (PTX) and other Methylaxenthins can noticeably improve sperm motility in in vitro condition [1,2]. These chemicals block cyclic nucleotide phosphodiesterase and increase level of intracellular cAMP, then phosphorylation of tyrosine in sperm tail occurs for its activation [3]. Many studies have investigated the effect of PTX on different aspect of spermatozoa, including motion characteristics and viability [4,5]. However, there are only a few studies about the effect of this agent on in vitro embryo development from spermatozoa that were already exposed to PTX. In this regards, Tournaye and co-workers investigated the impact of washed sperms after exposing to 3.6 mmol PTX on mice embryo formation. Their results expressed that PTX exposed spermatozoa had no adverse effects on fertilization and embryo development rates [6]. Also, Rashidi and associates showed that PTX increased fertilization and 2-cell cleavage rates after IVF in mice, when compared with controls [7]. Since then, no other reports have been presented regarding the efficacy as well as the safety of PTX on generation of mammalian embryos in vitro.

One important physiological phenomenon in reproductive biology is cell apoptosis. During apoptosis, unwanted or useless cells are generally eliminated during development and other normal biological processes. Regulation of apoptosis is very important, especially in pre-implantation embryo development that contains only few vital cells. Inner cell mass (ICM) within blastocysts has been considered as an indicator of embryo quality and loss of cells within ICM below a critical threshold can compromise subsequent fetal development [8,9]. Also, total blastocyst cell numbers is correlated with embryo implantation potential in process of reproduction [10]. Therefore, the aim of this experimental study was to assess the effect of in vitro exposure of spermatozoa to PTX on the rates of fertilization and embryo development as well as apoptotic cells within blastocysts in an animal model.

2. Materials and methods

This study was approved by our institute research committee. Reported experiments were performed according to the appropriate ethical and legal standards.

2.1. Collection and preparation of oocytes

Female Balb/C mice aged 8–10 weeks were induced for superovulation by i.p. injection of 10 IU pregnant mare serum

Peer review under responsibility of Middle East Fertility Society.
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http://dx.doi.org/10.1016/j.mefs.2016.12.001
1110-5690/

gonadotropin (PMSG, Sigma, USA). Approximately 48 h later, they were injected with 10 IU of human chorionic gonadotrophin (hCG, Serono, Swiss; i.p.) to trigger ovulation. After 15 h, the oviducts were excised under aseptic conditions and the cumulus-oocyte complexes (COCs) were collected in IVF medium (vitrolife, Sweden). They were cultured in IVF medium (vitrolife, Sweden) in an atmosphere of 5% CO_2 in air at 37 °C. The oocytes were randomly allocated into two groups of control and PTX. In control group, oocytes were inseminated with sperm suspension only. However, in PTX group, insemination was done with sperm suspension exposed to 3.6 mmol PTX.

2.2. Sperm preparation

Male Balb/C mice (10–14 weeks) were killed, and the cauda epididymis were removed immediately. For control group, epididymis was dissected in pre-warm Ham' F10 medium containing 5% serum albumin. For PTX group, it was dissected in aforementioned medium supplemented with 3.6 mmol PTX. Both suspensions were incubated for 30 min to ensure sperm capacitation. Then, for washing, 5 ml Ham' F10 containing 5% serum albumin was added to both suspensions and centrifuged in 300*g* for 5 min. The supernatants were discarded and the pellets were washed again. The final pellets were used for MII oocytes insemination.

2.3. IVF and embryo cultures

A total of 221 MII oocytes were collected from fallopian tubes of mice, then divided randomly to either the control or PTX groups. For IVF, the oocytes were exposed to spermatozoa for 5 h. Then, oocytes in both groups were washed and cultured in G1 medium (vitrolife, Sweden) for 48 h. The cleaved embryos were cultured in G2 medium (vitrolife, Sweden) until day 5. The rates of fertilization, cleavage, and blastocyst formations were evaluated and compared in both groups. Blastocyst grading was done according to Gardner protocol [11]: (1) early blastocyst: the blastocoels is less than half the embryo volume; (2) blastocyst: the blastocoels is greater than half of the embryo volume; (3) full blastocyst: the blastocoel occupy more than half the volume of the embryo; (4) expanded blastocyst: the blastocoel volume is larger than early embryo and the zona is thinning; (5) hatching blastocyst: the blastocyst has started to exit though the zona; (6) hatched blastocyst; the blastocyst has completely exited the ZP.

2.4. Assessment of apoptosis

For fixation, embryos were incubated in 3.7% paraformaldehyde/PBS for 1 h at room temperature (RT). The embryos were washed in PBS/PVP twice and incubated in 0.5% Triton X-100/PBS for 1 h at RT. After washing in PBS/PVP, the embryos were incubated in 10–15 ml dUTP-FITC labeling mix (Roche, Germany) for 10 min at RT. Then, these were incubated in 10–15 ml of TUNEL mix (Roche, Germany) for 1 h/37 °C in dark. After washing, for nuclear counter stain, embryos were exposed to PBS with Hoechst stain (Sigma). Finally, washing were repeated again and embryos were analyzed immediately in drops of glycerol on glass slides with conventional fluorescence microscopy [11].

2.5. Statistical analysis

SPSS 19 was used for statistical analysis. Differences between data for comparison of the two groups were analyzed by chi-square test and independent *t*-test. P value < 0.05 was statistically significant.

3. Results

3.1. Fertilization and embryo development

The findings generated from this study showed that the rates of fertilization and embryo development were similar between the control and PTX groups. Also, the rates of blastocyst formations did not differ significantly between two groups (Table 1). However, the data demonstrated that both early and expanded blastocyst formations were significantly higher in PTX group when compared with control (Table 2). Fig. 1 shows blastocysts grading in this study.

3.2. Blastocyst cell count and apoptosis

The average of total cell counts in blastocyst stage between two groups were insignificant. However, the DFI was higher in PTX group when compared with control, but this difference was insignificant (Table 3). Fig. 2 shows Hoechst and TUNEL staining of

Table 1
Comparison of fertilization, embryo development and blastocyst rates in control and PTX group.

IVF data	Control	PTX	P value
MII Oocyte (No.)	110	111	
Fertilization (%)	103 (93.6)	108 (97.2)	0.2
4 Cell embryo (%)	91 (88.3)	92 (85.1)	0.5
Morola (%)	79 (76.6)	82 (75.9)	1.0
Blastocyst (%)	54 (52.42)	56 (51.8)	1.0

Table 2
Comparisons of blastocysts grading in control and PTX groups.

Group	Early	Full	Expand	Hatched
Control (%)	31.4	35.18	11.11	22.2
PTX (%)	7.1	33.9	33.9	25
P value	0.001	1	0.005	0.8

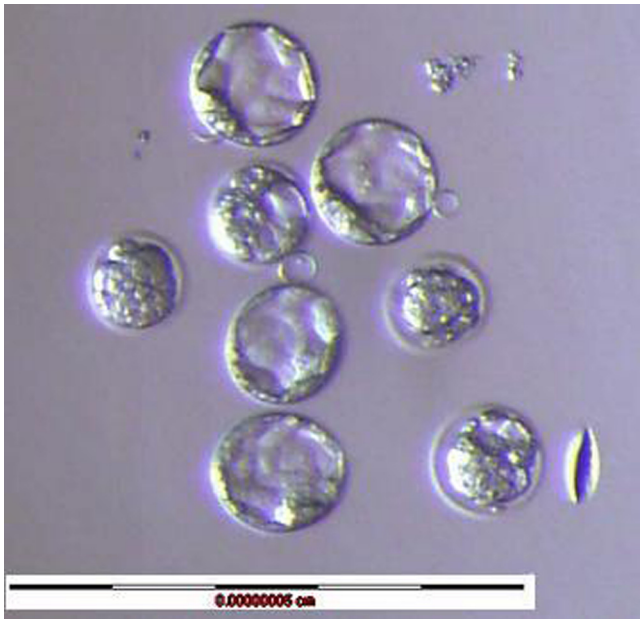

Fig. 1. Stereo photomicrograph of mouse blastocysts in different stages of development.

Table 3
Comparison of blastocyst total cell count and DFI in control and PTX groups.

Group	Control	PTX	P value
Cell number	31.08 ± 1.5	34.14 ± 1.5	0.16
DFI	9.76 ± 5.0	11.77 ± 5.4	0.07

Values are mean ± SE. DFI = DNA fragmentation index.

4. Discussion

PTX was first used for IVF program prior to introducing ICSI technology. Over the years, it was used aiding fertilization in selected IVF cases, also for certain IUI protocol [12]. Although, this drug was not applied in IVF cycles anymore, but it was used for diagnosis of viable sperms in cases with totally immotile spermatozoa in ICSI cycles [13]. Multiple reports have been published regarding the live birth of healthy children after using PTX in ICSI cycles, even in case of Kartagener's syndrome [14]. There are many reports about the effect of PTX on sperm parameters and functions. It has been demonstrated that PTX was successful in enhancing sperm motility in all groups of infertile patients, especially in men with PESA/TESE samples [15,16]. But, only a few studies have been published about the effect of this agent on embryo development and physiology. Therefore, it seems essential doing more surveys about the safety of this drug in assisted reproduction program. In this regard, Tournaye et al. [6] concluded that if spermatozoa were washed free from PTX, this agent had no adverse effect on embryo development in IVF procedure in mice. However, they warned that if the zygotes or embryos are exposed to PTX, this can cause perturbation in further embryo viability and development [6]. Also, in another study, no adverse effect of PTX on fertilization and development rate was reported in animal model [7].

We used 3.6 mmol PTX and washed the epididymal spermatozoa twice after 30 min of exposure time. It is well known that mouse spermatozoa are very sensitive compared with sperm from other mammalian species, especially against mechanical stresses during pipetting or centrifugation [17]. Therefore, we did sperm washing in control group as well, in order to have similar conditions in both groups. We noticed similar rates of fertilization, cleavage and blastocyst in PTX and control groups. Also, we did blastocyst grading in groups to ensure the safety of this chemical in ART facilities. Surprisingly, we realized that the speed of blastocysts development in PTX group were accelerated, so that expanded blastocysts were more in PTX group. Because, sperm cells had been hyperactivated in PTX than control groups, sperm penetration in oocytes may had occurred at an earlier period of time. As a result, the speed of blastocyst development was subsequently faster in PTX group. In clinical ICSI settings, it was shown that PTX has significantly increased fertilization rates from 55.9% in untreated to 66.0% in the treatment group [18]. Mangoli et al. also compared the efficacy of hypoosmotic swelling test (HOS) and PTX test for selection of viable spermatozoa in immotile sperm population obtained from testicular biopsies [16]. They concluded that use of viable immotile spermatozoa after PTX exposure showed a marked increase in the fertilization rates (62% vs. 41%) and clinical pregnancies of 32% vs. 16% as well. They did not show any adverse effects on embryo cleavage or morphology in PTX group. Therefore, they concluded that PTX has no cytotoxic and genotoxic effects on human embryos generated in ICSI program [16]. In a similar study, Cook and colleagues reported no detrimental effect of PTX on asthenozoospermia samples in ICSI cycles. Their results showed that selection of viable spermatozoa with PTX resulted in a significant increase in both clinical pregnancy and implantation rates in >38 years old patients [19].

We also applied another grading for blastocyst quality using Hoechst staining, to determine cell numbers of blastocysts. Because, it was proven that cell number of blastocysts, specially ICM has an impact on pregnancy outcomes [20,21]. We noticed similar cell numbers of blastocysts in both groups, which indicates that PTX has no adverse effects on blastocyst development. In 2015, Lewis et al. demonstrated that health of spermatozoa influences the health of generated embryos in ART cycles [22]. Also, Virro and colleagues reported that high DFI in semen resulted in both low blastocyst development and initiation of ongoing pregnancy [23]. Another study reported that ART treatment cycles with high sperm DFI, had more chance for cancellation cycles due to the blocked embryo development. Also, miscarriage rates were noticeably higher in these population [24]. One recent study demonstrated that spermatozoa with a low DFI resulted with embryos with faster blastulation and higher pregnancy rates in ICSI cycles [25]. Therefore, this report clearly demonstrates that health of spermatozoa is an important factor for successful ART treatment. In this study, for understanding of the PTX role on health of spermatozoa, the DFI was assayed in blastocysts. Several reports assured the accuracy of their techniques by DFI assessment with application of TUNEL in blastocysts. For example, Grygoruk et al. for determination of best speed for embryo transfer used TUNEL staining for blastocyst viability [26]. Apoptosis is a physiologic

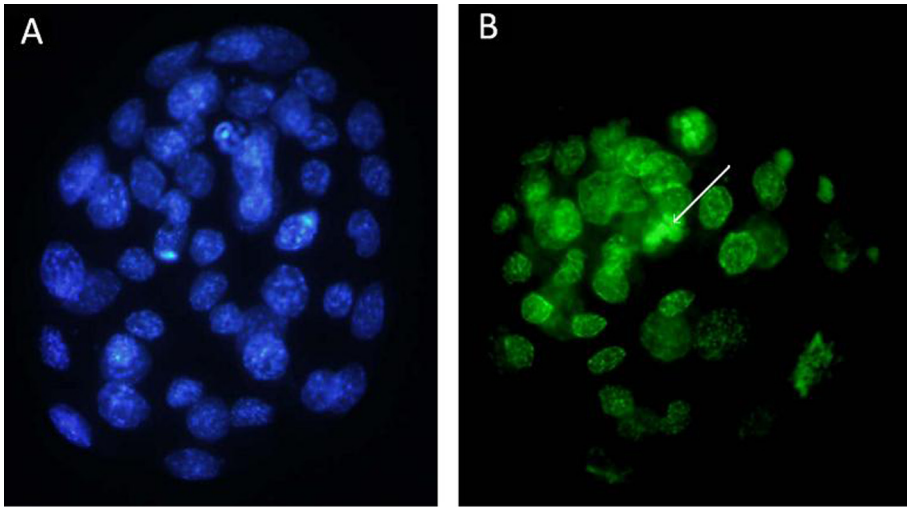

Fig. 2. Hoechst staining (A) and TUNEL staining (B) of blastocyst. Glowing green blastomere shows apoptotic cell (white line).

process that is seen widespread in blastocyst of all mammals. Over 80% of mouse in vivo blastocysts on day 4/5 had one or more dead cells, detected by differential labeling [27]. This process may regulate ICM cell number and protect the genome integrity of embryos with elimination of injured cells with DNA damage [28]. But, its regulation is critical in blastocyst that contains few cells. It has been shown that ICM cell numbers affect subsequent fetal development in mammals [10,29]. In this study, we detected insignificantly more DFI in PTX group (11.7%) than control group (9.7%). This probably indicates that application of PTX does not deteriorate the health of spermatozoa.

In conclusion, application of PTX to enhance sperm motility did not endanger the early process of IVF, if used correctly. So, this chemical may be used safely in ART cycles for motility enhancement, also for selection of viable spermatozoa in ICSI. Further clinical studies may assure the possible application of PTX in ART setting.

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