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SHORT COMMUNICATION

Effect of thymosin β on maturation of pig oocytes and quality of *in vitro* produced embryos

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ABSTRACT. Thymosin (TMS) is a peptide hormone that has regenerative qualities. To date, the regenerative qualities of thymosin have been tested only on somatic cells and bovine oocytes. The objective of this study was to determine the effect of thymosin on the *in vitro* maturation of pig oocytes and on *in vitro* fertilization (IVF), which would be used to assess the quality of obtained embryos. Pig oocytes (n = 463) were matured *in vitro* to the Met II stage in medium supplemented with TMS. After IVF the zygotes were cultured *in vitro* until they reached the blastocyst stage. The number of cells and the level of nuclear DNA fragmentation were evaluated. The addition of TMS to the maturation medium resulted in a significant increase in the percentage of competent oocytes that, after fertilization, developed to the blastocyst stage. Moreover, supplementing the maturation medium with TMS had a positive effect on the quality of obtained blastocysts by limiting the occurrence of apoptosis.

Introduction

In recent years, significant improvements have been made in *in vitro* oocyte maturation methods in many mammalian species, including pigs. Despite using morphological selection as well as creating an environment that is very similar to *in vivo* conditions, the obtained *in vitro* matured oocytes still display a lower quality than oocytes obtained *in vivo*. Methods for *in vitro* maturation of pig oocytes have been significantly improved in recent years, resulting in effectiveness of about 50–90% of oocytes reaching the Met II stage (Nguyen et al., 2017; Chen et al., 2020). This is why attempts are being made to modify maturation conditions, with the goal of producing mature *in vitro* porcine oocytes similar to those obtained *in vivo*.

Thymosin (TMS) is a peptide hormone first described following its discovery by Goldstein (Goldstein et al., 1966). Apart from its immunomodulatory functions, TMS promotes angiogenesis and prevents inflammation, apoptosis and tumorigenesis (Li et al., 2010; Kumar and Gupta, 2011). It demonstrates regenerative effects such as stimulation of hair regrowth, acceleration of collagen production, healing of wound burns and dermal damage and heart muscle regeneration (Tseng et al., 2002; Hinkel et al., 2015). To date, the regenerative qualities of TMS have been tested only on somatic cells and bovine oocytes (Skowronek et al., 2016; Wierzchoś-Hilczer, 2017). Taking into consideration TMS regenerative qualities, it was assumed that utilizing TMS for porcine oocyte maturation – especially oocytes with a lower morphological quality – would result in improved quality of the oocytes and developing embryos.

So, the objective of this study was to determine the effect of TMS on the *in vitro* maturation of pig oocytes and on *in vitro* fertilization (IVF), which would be used to assess the quality of obtained embryos.

Material and methods

Chemicals

Thymosin was purchased from Lipopharm (Gdańsk, Poland). All other chemicals were obtained from Sigma Aldrich (Sigma Chemical, St. Louis, MI, USA) unless stated otherwise.

All experimental procedures used in this study were carried out in accordance with the European Directive 2010/63/EU and were approved by the II Local Ethical Commission in Krakow 1181/2015, May 21, 2015.

In vitro maturation

The experiment was carried out on immature porcine oocytes obtained from sow and gilt ovaries after slaughter. Oocytes were cultured for 42–44 h up to the Met II stage in modified tissue culture medium (TCM)-199.

Maturation took place in two phases:

- pre-maturation: oocytes were cultured for 20–22 h in medium 1 containing: TCM-199 supplemented with $NaHCO_3$, cysteine, fetal calf serum (FCS), porcine follicular fluid, dibutryl cAMP (dbcAMP), equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG),
- maturation: culture for 20–22 h in medium 2 containing: TCM-199 supplemented with $NaHCO_3$, L-cysteine, porcine follicular fluid, FCS and kanamycin.

Oocytes were cultured in plastic dishes, in 100 µl medium droplets under mineral oil. Each droplet contained 10–12 oocytes. Culture was carried out at 39 °C, 5% CO_2 and maximum humidity. The maturation medium was described in detail in previous study (Poniedziałek-Kempny et al., 2020). The oocytes were randomly divided into 3 groups:

A. Experimental I – maturation in medium supplemented with 0.5 mg/ml TMS,
B. Experimental II – maturation in medium supplemented with 1 mg/ml TMS,
C. Control – maturation in the medium without TMS.

After 42–44 h of culture, oocytes were evaluated morphologically.

In vitro fertilization

Ejaculated, fresh semen from one boar was used. After diluting in the commercial extender (Biosolwens Plus, Biochefa, Poland) the motility and concentration of spermatozoa were estimated. For IVF spermatozoa with a mean motility of 70% and a mean concentration of 26×10^9/ml were used. Spermatozoa were capacitated *in vitro* in modified mM-199 medium which consisted of TCM-199 and was supplemented with calcium chloride, sodium pyruvate, glucose, HEPES and bovine serum albumin (BSA). A semen was centrifuged (25 s, 6000 rpm) in a phosphate buffered saline (PBS) with 0.001 g/ml BSA. After washing,a capacitation medium was added to the sperm pellet and centrifuged at 6000 rpm for 25 s. Sperm were resuspended in the capacitation medium to 1×10^5/ml and pre-incubated for 1 h at 39 °C. The basic medium for IVF was mM-199 supplemented with calcium chloride, sodium pyruvate, glucose, HEPES, caffeine and BSA. Matured oocytes were transferred in groups of 10–12 to IVF medium drops under mineral oil. A portion of 50 µl sperm (concentration 5×10^4/ml) was added to each droplet with oocytes. Oocytes and spermatozoa were co-incubated for 4 h in humidified atmosphere containing 5% CO_2 in the air, at 39 °C. The IVF procedure was described in details by Poniedziałek-Kempny et al. (2020). The IVF experiments were performed in 6 repetitions.

In vitro culture

Presumptive zygotes were cultured in North Carolina State University-23 (NCSU-23) medium (Petters and Wells, 1993) that was supplemented with 4 mg/ml BSA. The culture procedure was demonstrated in a previous study by Poniedziałek-Kempny et al. (2020). The number of presumptive zygotes, morulae and expanded blastocysts was evaluated.

TUNEL assay

A TUNEL assay was used to detect DNA fragmentation through nuclear staining (Trzcińska et al., 2008; Gajda et al., 2011). In the blastocyst, the number of cells and the level of nuclear DNA fragmentation were evaluated (Figure 1).

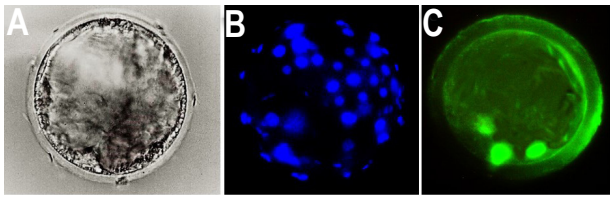

Figure 1. Porcine blastocyst obtained as a result of *in vitro* fertilization of mature oocytes with thymosin. A: Light microscope image (20x). B: Fluorescent microscope image after DAPI staining (20x). Blue fluorescence indicates cell nuclei. C: Fluorescence microscope image after fluorescein staining (20x). TUNEL analysis reveals the green fluorescence of cell nuclei with DNA fragmentation.

Statistical analysis

Statistical analysis was performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). Data were analysed with the chi^2 test and the one-way ANOVA with a post-hoc LSMEANS test. *P*-values of <0.05 and <0.01 were considered significant.

Results

The effect of thymosin on *in vitro* oocyte maturation and fertilization

It was demonstrated a statistically higher percentage of oocytes maturing in the group supplemented with 0.5 mg ($P = 0.0003$) and 1 mg ($P = 0.0314$) of TMS when compared to oocytes from the control group (Table 1). *In vitro* fertilization was carried out on matured oocytes in a medium supplemented with 0.5 mg/ml and 1 mg/ml of TMS. A tendency was observed for a higher proportion of zygotes, morulae and blastocysts in the group where oocytes matured with 0.5 mg of TMS in comparison to group with 1 mg of TMS and control one (differences statistically non-significant) (Table 1).

The quality of blastocysts obtained after *in vitro* fertilization of oocyte matured with thymosin

In blastocysts obtained from oocytes matured with 0.5 and 1 mg of TMS supplementation a statistically significant ($P = 0.0029$ and $P = 0.0071$, respectively) lower number of apoptotic nuclei was counted when compared to blastocysts from the control group. At the same time the total number of cells in the blastocysts obtained from oocytes cultured with TMS supplementation of 0.5 and 1 mg as well as without supplementation was similar. Statistically significant differences were noted in the apoptotic index of blastocysts obtained from oocytes matured with TMS supplementation of 0.5 mg ($P = 0.0016$) and 1 mg ($P = 0.0026$) in comparison to embryos form the control group (Table 2).

Table 1. Effect of thymosin on porcine *in vitro* maturation and development of embryos obtained after *in vitro* fertilization (IVF)

Group	Thymosin concentration (mg/ml)	No. of oocytes for maturation/replicates	No. of mature oocytes (%)	No. of oocytes designated for IVF	No. of presumptive zygotes (%)	No. of embryos developed (%)	
						morulae*	expanded blastocysts*
Experimental I	0.5	269/6	260 (96.6)^a	105	37 (35.2)	20 (54.0)	14 (37.8)
Experimental II	1	216/6	203 (93.9)^b	162	53 (32.7)	22 (41.5)	13 (24.5)
Control	0	246/6	217 (88.2)^c	134	34 (25.4)	15 (44.1)	10 (29.4)

* in comparison to cleaved embryos, ^{abc} – means with different superscripts within a row are significantly different at $P < 0.01$ and $P < 0.05$, respectively

Table 2. Cell number and apoptosis levels in blastocysts obtained after *in vitro* fertilization of oocyte matured with thymosin

Group	Thymosin concentration, mg/ml	No. of evaluated blastocysts	Mean no. of cells/ blastocyst ± SD	Mean no. of apoptotic cells/ blastocyst ± SD	Apoptotic index (TUNEL, %)
Experimental I	0.5	14	38.0 ± 11.27	0.64^a ± 0.72	1.95^a
Experimental II	1	13	38.46 ± 10.99	0.77^b ± 0.8	2.11^b
Control	0	10	32.30 ± 7.54	2.0^c ± 1.41	6.59^c

^{abc} – means with different superscripts within a row are significantly different at $P < 0.01$ and $P < 0.05$, respectively, SD – standard deviation

Discussion

The selected modification of porcine oocyte maturation conditions based on culture medium supplementation resulted in a higher proportion (over 96 and 93%) of mature oocytes cultured with TMS supplementation of 0.5 and 1 mg, respectively than that observed in oocytes cultured in the control group (over 88%). Until now, the functions of TMS have been examined only in bovine oocytes (Skowronek et al., 2016). Therefore, two questions arise: can TMS receptors be present on the surface of porcine cells' zona pellucida, and how can TMS penetrate the oocyte? In this context, a thesis by Rando (2000) should be mentioned. Rando (2000) reported that TMS can penetrate through pores in the cell nucleus by diffusion. The author also suggests that TMS β_4 can be a transcription factor. This, however, is only a hypothesis, and further studies are needed to examine mechanisms of TMS influence on oocyte zona pellucida and thus on the process of porcine oocyte maturation. The addition of TMS β_4 (500 or 50 ng/ml) to bovine maturation medium did not have an impact on bovine oocyte meiotic maturation and DNA fragmentation. However, supplementation of the maturation medium with

50 ng TMS increased the number of cleaved zygotes (Skowronek et al., 2016). Increasing the concentration of TMS in the maturation medium to 0.5 mg/ml enhanced the developmental potential of bovine embryos (Romanek, unpublished data). Consequently, in other experiment the presence of TMS during *in vitro* maturation of porcine oocytes improved the cleavage rate and embryo quality of vitrified IVF embryos (Gajda et al., 2017).

In our study the positive effect of TMS supplementation on *in vitro* porcine oocyte maturation was demonstrated. The addition of TMS to the maturation medium resulted in a significant increase in the percentage of competent oocytes that, after fertilization, developed to the blastocyst stage. Moreover, when compared to the control group, a lower level of apoptosis was observed among blastocysts obtained following TMS supplementation. Therefore, TMS supplementation into maturation medium had a positive effect on the quality of obtained blastocysts by limiting the occurrence of apoptosis. In our other studies, we demonstrated that *in vivo* survivability evaluation of embryos obtained from fertilized oocytes matured with TMS resulted in pregnancy in two recipients and the subsequent birth of 16 live piglets (Poniedziałek-Kempny et al., 2020).

Conclusions

Maturation medium supplementation with thymosin (TMS) results in obtaining a significant proportion of mature oocytes capable to fertilization and development to the blastocyst stage. Moreover, the presence of TMS has a positive effect on embryo quality.

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