THE DEVELOPMENTAL COMPETENCE OF BOVINE OOCYTES MATURED *IN VITRO* USING THYMOSIN BETA 4*

Joanna Romanek*, Joanna Jurkiewicz, Agnieszka Wieruchoś-Hilczer, Jolanta Opiela

Department of Reproductive Biotechnology and Cryoconservation,
National Research Institute of Animal Production, 32-083 Balice n. Kraków, Poland

*Corresponding author: joanna.romanek@izoo.krakow.pl

Abstract

The aim of this study was to examine the effect of thymosin beta 4 (Tβ4) during *in vitro* maturation (IVM) of oocytes and subsequent embryonic development after *in vitro* fertilization as well as to assess the quality of obtained blastocysts. Cumulus-oocyte complexes (COCs) were matured *in vitro* in 4 different media: 1. control medium; 2. control media supplemented with 50 ng/mL Tβ4; 3. control media supplemented with 0.5 mg/mL Tβ4; and 4. control media supplemented with 1 mg/mL Tβ4. The quality of the developed blastocysts was analysed by the TUNEL method. The number of cleaved eggs was significantly higher ($P<0.05$) when gametes were matured in the presence of 50 ng/mL Tβ4 than it was using the other types of media. Additionally, the largest number of blastocysts was observed when 0.5 mg Tβ4 was added to the medium ($P<0.05$). No significant difference was noted in the mean number of apoptotic nuclei per blastocyst or in the mean number of nuclei per blastocyst in any of the analysed groups. In conclusion, Tβ4 supplementation (50 ng/mL) in maturation medium increased the number of cleaved oocytes, and the number of blastocysts obtained increased when 0.5 mg/mL Tβ4 was used. This positive effect was not observed when a higher concentration of Tβ4 (1 mg/mL) was used.

Key words: thymosin, maturation, bovine oocyte, IVF

The final stage of female gamete development is maturation. Appropriate regulation of the maturation process is crucial for obtaining a good quality oocyte. Techniques such as *in vitro* maturation, *in vitro* fertilization and *in vitro* embryo culture are regularly used in bovine reproduction. Relative to the initial number of eggs, the proportion of bovine blastocysts obtained *in vitro* rarely exceeded 40% (Lucas et al., 2015). The use of supplements is an alternative method used to increase *in vitro* maturation. These additives improve the efficiency of maturation in the culture medium and permit immature oocytes to obtain competence for fertilization and embryogenesis (Guimarães et al., 2016).

*Scientific work was financed by the statutory activity of National Research Institute of Animal Production #02-013.1 and 01-19-09-11.

Tβ4 is a 43-amino acid small peptide that is ubiquitously expressed in all types of cells and tissues except for red blood cells (Lee et al., 2019). Moreover, Tβ4 is involved in many cellular processes, such as apoptosis (Zhao et al., 2011), angiogenesis (Dubé and Smart, 2018), preservation of the balance of actins (Namgoong and Kim, 2016), wound healing (Kim and Kwon, 2017), and tumorigenesis (Lee et al., 2019). Nemolato et al. (2010) further described the role of Tβ4 in developing foetal salivary glands (Nemolato et al., 2010). Moreover, during kidney development in foetal mice, the level of Tβ4 increases, with levels reaching a peak during active nephrogenesis (Guinobert et al., 2006). Tβ4 gene transcript levels are also high in the human embryonic kidney (Hall, 1991).

Tβ4 also plays an important role in the development of the reproductive system. Tβ4 expression was observed in the rat ovary. The expression of Tβ4 can be stimulated by human chorionic gonadotropin (Hall et al., 1991 a). Moreover, Tβ4 is associated with luteinization (Hall et al., 1991 b) and has been found to stimulate the release of luteinizing hormone releasing hormone and LH (Hall et al., 1992). Until 2010, the roles of Tβ4 in the bovine ovary had not been elucidated. The results of Salhab et al. (2010) showed that in bovine cumulus cells, Tβ4 intracellular localization is restricted mostly to the cytosol. Tβ4 expression is upregulated in bovine cumulus cells during meiotic maturation. Tβ4 mRNA was more abundant in immature cumulus cells of adult cows than it was in calf cumulus cells. Moreover, supplementation of IVM medium with EGF resulted in increased thymosin expression (Salhab et al., 2010). Labas et al. (2018) showed that Tβ4 could be a marker of maturation that could indicate bovine cumulus-oocyte complex quality.

Another function of Tβ4 is its antiapoptotic effect. On the one hand, Tβ4 itself reduces apoptosis, but Tβ4 also induces the expression of survival genes (Hinkel et al., 2008; Zhao et al., 2011; Wierzchoś-Hilczner, 2017). In endothelial progenitor cells, Tβ4 treatment upregulated the expression of anti-apoptotic Bcl-2 protein and simultaneously decreased the expression of pro-apoptotic Bax protein, inhibiting the release of mitochondrial cytochrome c (Zhao et al., 2011). Furthermore, Tβ4 treatment decreased the activity and expression of caspase-9 and caspase-3 (Zhao et al., 2011). Tβ4 was also reported to promote cell survival by upregulating antioxidative enzymes, including SOD, copper/zinc SOD, and catalase (Zhao et al., 2011).

Due to the properties of Tβ4 presented above, we decided that the addition of this supplement to the medium for oocyte maturation could have a beneficial effect on oocyte developmental competence. In 2016, Skowronek et al. analysed the effect of Tβ4 supplementation on oocyte meiotic entry. However, these are the first studies conducted to assess the effect of thymosin on the developmental capacity of bovine oocytes.

In our study, we tested thymosin beta 4 (Tβ4) as an oocyte maturation medium supplement.

The aim of the present study was to investigate the effect of Tβ4 supplementation during oocyte maturation on embryonic development and to assess the quality of the obtained blastocysts.

Material and methods

Chemicals and supplies

The reagents used in the presented experiments were purchased from Sigma-Aldrich, Poland, unless otherwise indicated.

Collection of cumulus-oocyte complexes (COCs)

Bovine ovaries were obtained from a local slaughterhouse and were transported to the laboratory within two hours. After 3 washes in physiological saline (PBS) containing 0.5 mL of kanamycin solution (0.075 g/L PBS), bovine COCs were collected by aspiration from follicles (diameter, 2–5 mm). COCs with intact cumulus granulosa cells and evenly granulated cytoplasm were selected.

In vitro maturation

COCs were washed in IVM medium, which consisted of M-199 medium that was buffered with sodium bicarbonate and supplemented with L-glutamine, 20% FBS, 0.15 IU/mL of FSH and 10 μ g/mL of β -oestradiol. *In vitro* maturation was performed with 3 different concentrations of T β 4 (Lipopharm, Poland): 50 ng, 0.5 mg and 1 mg per mL of maturation medium. As a control group, COCs were matured in IVM medium without T β 4. Each group of 40–50 COCs was introduced into a 500 μ L droplet of IVM medium in 4-well plates; they were then covered with mineral oil and incubated for 22–24 hours in 5% CO $_2$ with humidified air at 38.5°C.

In vitro fertilization

IVF was performed as described by Opiela et al. (2014). Briefly, frozen-thawed semen was washed with Talp Ca $^{2+}$ -free medium and isolated by centrifugation (300 g, 30 minutes) over a two-step gradient with 1 mL of 90% Percoll (Pharmacia, Sweden) and 1 mL of 45% Percoll. After washing, the mature oocytes were transferred in groups of 10–12 into 50 μ L of fertilization medium containing 10 μ g/mL of heparin and a mixture of penicillamine (20 μ M), hypotaurine (10 μ M), and epinephrine (1 μ M) and then inseminated with the washed spermatozoa (1×10^6 cells/mL). Coincubation of oocytes with sperm was performed at 38.5°C under 5% CO $_2$ in air at high humidity for approximately 20 hours.

In vitro culture

After gentle pipetting, presumptive zygotes were denuded from cumulus cells and transferred into 50 μ L droplets of synthetic oviductal fluid medium (SOF) supplemented with 0.8 mg/mL BSA. Droplets were covered with mineral oil to protect against evaporation, and the presumptive zygotes were cultured in a humidified atmosphere at 39°C with 5% CO $_2$, 5% O $_2$, and 90% N $_2$ for 24 h. On Day 2 following fertilization, the number of cleaved embryos was recorded. Cleaved embryos were placed into 50 μ L droplets of SOF medium supplemented with 10% FBS and 0.8 mg/mL BSA covered with mineral oil in a humidified atmosphere at 39°C with 5% CO $_2$, 5% O $_2$, and 90% N $_2$. Embryos were cultured *in vitro* for 7–9 days (Figure 1). The medium was changed every 48 hours. Data are from six independent experiments.

Figure 1. *In vitro*-produced bovine embryos from *in vitro* matured oocytes at the blastocyst stages on Day 8 of *in vitro* culture (Day 0 = day of *in vitro* fertilization). Images were taken at 400 $\times$ magnification

Terminal deoxynucleotidyl transferase dUTP nick-end labelling

Terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) was performed according to the protocol described by Warzych et al. (2013) using a Deadend Fluorometric TUNEL System (Promega, Poland). Briefly, the blastocysts were fixed with 4% paraformaldehyde (PFA) for 1 h at room temperature, and then they were transferred to 1% PFA for storage at 4°C for no longer than 3 weeks. On the day of analysis, embryos were washed three times with PVP/PBS and permeabilized with a 0.2% Triton X-100 solution for 5 minutes at room temperature. Samples were washed with PVP/PBS and then were incubated in a reaction mixture consisting of equilibration buffer, nucleotide mix containing fluorescein-dUTP, and terminal deoxynucleotidyl transferase. Embryos were incubated for 1 hour at 37°C in a humidified chamber. After this incubation, blastocysts were placed in a solution of 2 $\times$ SSC for 15 minutes and then were washed 3 times with PVP/PBS. Finally, embryos were mounted with Vectashield mounting solution (Vector Labs, Burlingame, CA). Detection of fragmented DNA was then observed with a Nikon Eclipse E600 fluorescence microscope (Figure 2). The death cell index (DCI) was determined by calculating the percentage of TUNEL-positive cells to DAPI-labelled cells.

Figure 2. Evaluation of cytological quality of *in vitro* cultured bovine blastocysts based on the detection of cells in late-apoptosis by TUNEL analysis of simultaneously 4',6-diamidino-2-phenylindole (DAPI) and fluorescein isothiocyanate (FITC)-stained cell subpopulations. In each analysed cell subpopulation, the nuclei per blastocyst had been labelled with DAPI counterstain and subsequently fluoresced blue. The nuclei per blastocyst of late-apoptotic cells displaying internucleosomal DNA fragmentation were dyed with FITC, causing them to fluoresce bright green. In each photograph, the DAPI-derived blue and FITC-derived green fluorescent signals were merged. Images were taken at 400 $\times$ magnification: A – control medium; B – control media supplemented with 50 ng/mL T β 4; C – control media supplemented with 0.5 mg/mL T β 4 and D – control media supplemented with 1 mg/mL T β 4

Statistical analysis

Differences in cleavage and blastocyst rates were assessed using a chi-square test (χ^2). Significant differences in TUNEL results regarding all blastocyst nuclei, apoptotic nuclei, and DCI were calculated by using ANOVA followed by Tukey's post hoc test.

Results

The effect of T β 4 on the developmental competence of bovine oocytes

The number of cleaved eggs was significantly higher ($P < 0.05$) when oocytes were matured in medium supplemented with 50 ng/mL T β 4 than in the control group (Table 1). Moreover, supplementation with 50 ng/mL T β 4 highly significantly

($P<0.01$) increased the cleavage rate of embryos compared to embryos matured in the presence of 1 mg/mL T β 4 in the IVM medium (Table 1). However, the number of blastocysts obtained was the highest when 0.5 mg/mL T β 4 was used. The addition of 0.5 mg/mL T β 4 increased the rate of blastocyst formation on day 7 after fertilization ($P<0.05$) compared with that of control oocytes and oocytes matured with 1 mg/mL T β 4 (Table 1). No significant difference was noted in the number of cleaved eggs and obtained blastocysts between the 50 ng/mL T β 4 group and the 0.5 mg/mL T β 4 group. It should be mentioned that a positive trend was observed in the number of blastocysts in the group treated with 50 ng/mL T β 4 compared to that of the control group. A statistically significantly higher number of blastocysts was obtained compared to the control after adding a 1000-times higher concentration of T β 4 (0.5 mg/mL T β 4) to the IVM medium.

Table 1. The effect of T β 4 supplementation on the developmental competence of bovine oocytes (6 replicates per group)

Group	No. fertilized oocytes (%)	No. cleaved oocytes (%) (mean $\pm$ S.D.)	No. blastocysts (%) (mean $\pm$ S.D.)
50 ng T β 4	224	199 (88.8) Aa (63 $\pm$ 22.80)	38 (17.0) c (11.4 $\pm$ 3.64)
0.5 mg T β 4	273	228 (83.5) c (58.3 $\pm$ 22.01)	56 (20.5) a (14.6 $\pm$ 10.76)
1 mg T β 4	298	238 (79.9) Bc (69.0 $\pm$ 11.68)	37 (12.4) b (17.01 $\pm$ 17.49)
Control	332	272 (81.9) b (66.0 $\pm$ 20.62)	44 (13.2) b (10.3 $\pm$ 5.45)

Values within column with different letters differ significantly: a:b – $P<0.05$, A:B – $P=0.006$ (test χ^2). c: a, b, c – $P>0.05$.

Table 2. The effect of T β 4 supplementation on blastocyst quality, as measured by DNA fragmentation of blastomeres

Group	No. blastocysts	Mean number of nuclei per blastocyst $\pm$ S.D.	Mean number of apoptotic nuclei per blastocyst $\pm$ S.D.	Mean DCI per blastocyst $\pm$ S.D.
50 ng T β 4	38	76 $\pm$ 26 a	1.4 $\pm$ 1.2 a	2.18 $\pm$ 2.39 a
0.5 mg T β 4	41	79 $\pm$ 20 a	1.6 $\pm$ 1.6 a	2.04 $\pm$ 2.17 a
1 mg T β 4	33	73 $\pm$ 29 a	1.2 $\pm$ 1.2a	1.71 $\pm$ 1.70 a
Control	42	71 $\pm$ 33 a	1.9 $\pm$ 2.0 a	2.86 $\pm$ 2.97 a

a:a – $P>0.05$.

The effect of T β 4 on blastocyst quality, as measured by TUNEL.

There were no significant differences among the blastocysts in any of the groups according to the analysed parameters: the mean number of apoptotic nuclei per blastocyst, the mean number of nuclei per blastocyst, and the dead cell index (DCI) (Table 2).

Discussion

In the present study, we observed an increased cleavage rate and an increased number of obtained blastocysts after the addition of T β 4 in maturation medium (50 ng and 0.5 mg, respectively), so the addition of T β 4 into IVM medium could increase the developmental potential of matured oocytes *in vitro*. Salhab et al. (2010) suggested that the expression of T β 4 was directly correlated with oocyte competence for fertilization and early development *in vivo* but not *in vitro*. This appears to be consistent with the findings of the present study but also *in vitro*.

Because there are few reports in the literature on the influence of T β 4 supplementation in IVM medium on embryonic development and the quality of obtained blastocysts, comparing the results obtained here with other data is rather difficult. Relative to this study, a similar study was carried out on pig oocytes (Gajda et al., 2015; Poniedziałek-Kempny, unpublished data). In contrast to our results, the authors observed no difference in the cleavage rate or number of blastocysts obtained from pig oocytes cultured in maturation medium containing 0.5 mg/mL or 1 mg/mL T β 4. The differences between the results obtained in pigs and cattle may be explained by the species-specific response to T β 4.

As mentioned earlier in the performed experiments, the concentration of thymosin 0.5 mg has a beneficial impact on bovine oocyte development competition. It is speculated that one of the possible explanations can be the fact that T β 4 affects actin polymerization and cortical actin cap formation in the oocyte and, on the other hand, influences the formation of a functional mucoelastic extracellular matrix (Nemolato et al., 2009; Salhab et al., 2010). Most cellular actin should exist as filaments because the cellular concentration of total actin is more than 0.1 mM, and actin tends to polymerize. However, 40% of total actin in cells exists as monomers. This can be explained by the fact that most monomeric actin exists as a complex with actin binding proteins such as T β 4 (Namgoong and Kim, 2016). Local regulation of T β 4 binding to monomeric actin regulates actin polymerization along with matrix metalloproteinase (MMP) synthesis to promote bovine endothelial cell motility (Fan et al., 2009). During oocyte maturation, there are three types of actin reorganization processes. The first process involves the change in cytoplasmic actin density. During the germinal vesicle stage, cytoplasmic actin mesh is formed at a density that is higher than what can be disrupted germinal vesicle breakdown, and it is higher than what is present at the early MI stage; further, the mesh is increased during spindle migration and the anaphase-telophase transition. The second process is the formation of the cortical actin cap. The third type of actin reorganization process involves an increase in cortical actin thickness. The process starts at the GV stage and continues to increase through the MII stage (Namgong and Kim, 2016). The rearrangement of F-actin is essential for the synthesis of different components of the mucoelastic extracellular matrix by expanding bovine cumulus cells (Salhab et al., 2010). Moreover, the addition of T β 4 to the medium may have improved the efficiency of actin polymerization in cytoplasm, allowing more effective transport of energy, proteins and other factors which could translate into improvement of oocyte developmental competences that was observed in our studies. Skowronek et al. (2016) observed that supplementation of IVM medium with 50 ng or 0.5 mg T β 4 did not

affect bovine oocyte meiotic maturity and luckily these authors analysed our lowest and middle concentration of T β 4 used. Their result supports our data that 50 ng or 0.5 mg T β 4 does not impact the meiotic maturity of bovine oocytes.

In our study, we observed a significant increase in the development of blastocysts when oocytes were matured with 0.5 mg/mL T β 4, which is the middle concentration of T β 4 that tested. How can this be explained? We think that the positive maturation resulting from T β 4 treatment is connected with its action on actin. However, the dose here is the critical parameter. Previous studies have shown that the actin cytoskeleton plays a role in a surprising number of processes in oocytes, such as long-range vesicle transport, positioning of the germinal vesicle, spindle migration and anchorage, polar body extrusion and chromosome segregation (Uraji et al., 2018). Therefore, actin activity is strictly connected with meiotic maturation, including reaching metaphase II; further, actin is necessary for proper fertilization and cytoplasmic maturation, which generally involves the oocyte state when all necessary proteins or transcripts are synthesised to allow proper development of fertilized eggs. The dose of T β 4 is critical because too high of a dose can accelerate initiation of MII while cytoplasmic maturation is in progress. Disruption of actin by too high or too low of a T β 4 concentration might lead to a decrease in the speed of chromosome segregation and an increase in the number of the lagging chromosome during anaphase. Lagging chromosomes that are mostly moved into spindle pole but are partially in the spindle centre might be divided incorrectly by the cleavage furrow. During metaphase II, actin is also necessary for accurate chromosome alignment. Defective propagation of chromosomes to poles causes such oocytes to form a metaphase plate and enter anaphase with aneuploidy. Mogessie and Schuh (2017) reported that actin promotes the formation of kinetochore fibres. Decreasing or increasing actin in the spindle leads to a reduction or increase in K-fibres, respectively, precluding the accurate alignment and segregation of chromosomes (Mogessie and Schuh, 2017). According to our results, supplementation with 0.5 mg/mL T β 4 may be an adequate concentration for supporting proper cytoplasmic maturation as well as meiotic maturation with accurate matching and segregation of chromosomes. Lower concentration of 50 ng T β 4 has no impact on both cytoplasmic and meiotic maturation, while the highest used concentration of T β 4 (1 mg/mL) could accelerate meiotic maturation and arrest in MII while cytoplasmic maturation is in progress. We have also additional explanation to the question: why did the highest used concentration of T β 4 (1 mg/mL) not have the same positive impact? In our opinion, the results from other authors' study of SW480 cells could elucidate that inconsistency. They found that the overexpression of T β 4 in SW480 cells decreased E-cadherin expression (Wang et al., 2004; Huang et al., 2007). E-cadherin is a protein responsible for cell-cell junction integrity. In the ovary, E-cadherin is responsible for maintaining the primordial follicle structure by facilitating cell-cell adhesive contacts with surrounding pregranulosa cells, and it regulates granulosa cell differentiation in preantral follicles (Yan et al., 2019). As follicle development progresses, the expression of E-cadherin decreases, and granulosa cells lose their polarity in large follicles (Gao et al., 2014). Moreover, E-cadherin participates in cell-cell adhesion events during fertilization (Caballero et al., 2014). According to the results of our study, it is speculated that

the high concentration (1 mg) of thymosin and the resulting low level of E-cadherin could counteract the positive effect of T β 4 supplementation observed in the other experimental groups. Knockdown of E-cadherin significantly reduced the number of primordial and growing follicles *in vitro* (Yan et al., 2019). Moreover, E-cadherin, by interacting with β -catenin, regulates newborn ovary homeobox (NOBOX) expression. NOBOX is a transcription factor expressed in oocytes, and it is critical for oocyte-specific gene expression, early folliculogenesis and early embryogenesis (Tripurani et al., 2011). Knockdown of E-cadherin in oocytes decreased the level of NOBOX expression (Yan et al., 2019). In our study, the addition of 1 mg T β 4 to IVM medium may have resulted in a decreased level of E-cadherin expression as well as NOBOX genes, which may explain the abolition of the positive effect of thymosin observed in the other analysed groups. Moreover, our result may also be explained by the fact that sperm-oocyte adhesion was impaired when oocytes with strongly reduced expression of E-cadherin were inseminated (Takezawa et al., 2011).

In conclusion, our study adds to the scarce knowledge obtained by other researchers (Nemolato et al., 2009; Salhab et al., 2010) on the effect of T β 4 on the development of oocytes and embryos. In our study, we searched for a concentration of T β 4 that would have a positive effect on development competition and cytoplasmic maturity of oocytes. A 0.5 mg/mL T β 4 concentration improved developmental competences and did not have a negative influence on expanding granulosa cells. However, 1 mg/mL T β 4 has a detrimental effect on cytoplasmic maturity by reducing E-cadherin and causing expansion of the granulosa cells.

To assess the quality of the obtained blastocysts, they were analysed by TUNEL staining. In the present study, we found that T β 4 supplementation did not influence blastocyst quality. The DCI in blastocysts obtained from oocytes matured in medium with the addition of thymosin was slightly lower than that in the control group, but no significant differences were observed.

Additionally, Skowronek et al. (2016) observed that supplementation of IVM medium with 50 ng/mL or 0.5 mg/mL T β 4 did not affect bovine oocyte DNA fragmentation. Our results and those of Skowronek et al. (2016) suggest that T β 4 has no effect on apoptosis in bovine gametes and embryos. Different results were observed in research of pig oocytes. The addition of T β 4 to the porcine oocyte maturation medium had a positive effect on the quality of the obtained blastocysts. Moreover, 0.5 mg and 1 mg T β 4 supplementation significantly decreased ($P < 0.01$, $P < 0.05$, respectively) the number of apoptotic nuclei per blastocyst and the DCI index compared to the values from the control group (Gajda et al., 2015; Poniedziałek-Kempny, unpublished data). This tendency was explained as the result of the anti-apoptotic effect of thymosin in porcine oocytes. However, in the Poniedziałek-Kempny (unpublished data) and Gajda et al. (2015) studies, the obtained DCI in the control group could be disputed, since it was very high (6.59% and 4.35%, respectively), which is exceptional compared to other reports in the literature. Considering no differences were observed in developmental competences between experimental groups in porcine experiments, such a high DCI in the control group could be a result of factors other than T β 4. On the other hand, this could be explained by the species-specific differences in the responses to T β 4 between pigs and cattle.

In conclusion, the findings presented in this report constitute the first analysis of the influence of T β 4 on embryonic development and the quality of obtained bovine blastocysts. Our results suggest that the addition of 0.5 mg T β 4 to the *in vitro* maturation medium significantly increased the number of obtained blastocysts and had no negative impact on blastocyst quality.

References

- Caballero J.N., Gervasi M.G., Veiga M.F., Dalvit G.C., Perez-Martínez S., Cetica P.D., Vazquez-Levin M.H. (2014). Epithelial cadherin is present in bovine oviduct epithelial cells and gametes, and is involved in fertilization-related events. *Theriogenology*, 81: 1189–1206.
- Dubé K.N., Smart N. (2018). Thymosin β 4 and the vasculature: multiple roles in development, repair and protection against disease. *Expert Opin. Biol. Ther.*, 18: 131–139.
- Fan Y., Gong Y., Ghosh P.K., Graham L.M., Fox P.L. (2009). Spatial coordination of actin polymerization and ILK-Akt2 activity during endothelial cell migration. *Dev. Cell*, 16: 661–674.
- Gajda B., Poniedziałek-Kempny K., Rajska I., Smorąg Z. (2015) Effect of thymosin on *in vitro* fertilization and developmental competence and quality of pig embryos. *Anim. Reprod.*, 12: 735.
- Gao F., Zhang J., Wang X., Yang J., Chen D., Huff V., Liu Y.X. (2014). Wt1 functions in ovarian follicle development by regulating granulosa cell differentiation. *Hum. Mol. Genet.*, 23: 333–341.
- Guimarães A.L., Pereira S.A., Diógenes M.N., Dode M.A. (2016). Effect of insulin-transferrin-selenium (ITS) and l-ascorbic acid (AA) during *in vitro* maturation on *in vitro* bovine embryo development. *Zygote*, 24: 890–899.
- Guinobert I., Viltard M., Piquemal D., Elalouf J.M., Marti J., Lelièvre-Pégorier M. (2006). Identification of differentially expressed genes between fetal and adult mouse kidney: candidate gene in kidney development. *Nephron. Physiol.*, 102: 81–91.
- Hall A.K. (1991). Differential expression of thymosin genes in human tumors and in developing human kidney. *Int. J. Cancer*, 48: 672–677.
- Hall A.K., Aten R., Behrman H.R. (1991 a). Differential modulation of thymosin genes in the immature rat ovary by gonadotropins. *Mol. Cell. Endocrinol.*, 79: 37–43.
- Hall A.K., Aten R., Behrman H.R. (1991 b). Thymosin gene expression is modulated by pregnant mare's serum gonadotropin, human chorionic gonadotropin, and prostaglandin F2 alpha in the immature rat ovary. *Endocrinology*, 128: 951–957.
- Hall N.R., O'Grady M.P., Menzies R.A. (1992). Thymic regulation of the hypothalamic-pituitary-gonadal axis. *Int. J. Immunopharmacol.*, 14: 353–359.
- Hinkel R., El-Aouni C., Olson T., Horstkotte J., Mayer S., Müller S., Willhauck M., Spitzweg C., Gildehaus F.J., Münzing W., Hannappel E., Bock-Marquette I., DiMaio J.M., Hatzopoulos A.K., Boekstegers P., Kupatt C. (2008). Thymosin β 4 is an essential paracrine factor of embryonic endothelial progenitor cell-mediated cardioprotection. *Circulation*, 117: 2232–2240.
- Huang H.C., Hu C.H., Tang M.C., Wang W.S., Chen P.M., Su Y. (2007). Thymosin β 4 triggers an epithelial-mesenchymal transition in colorectal carcinoma by upregulating integrin-linked kinase. *Oncogene*, 26: 2781–2790.
- Kim S., Kwon J. (2017). Thymosin β 4 has a major role in dermal burn wound healing that involves actin cytoskeletal remodelling via heat-shock protein 70. *J. Tissue Eng. Regen Med.*, 11: 1262–1273.
- Labas V., Teixeira-Gomes A.P., Bouguereau L., Gargaros A., Spina L., Maresting A., Uzbekova S. (2018). Intact cell MALDI-TOF mass spectrometry on single bovine oocyte and follicular cells combined with top-down proteomics: A novel approach to characterise markers of oocyte maturation. *J. Proteomics*, 175: 56–74.
- Lee J.W., Kim H.S., Moon E.Y. (2019). Thymosin β -4 is a novel regulator for primary cilium formation by nephronophthisis 3 in HeLa human cervical cancer cells. *Sci. Rep.*, 2: 6849.

- Lucas C.G., Remião M.H., Komninou E.R., Domingues W.B., Haas C., Leon P.M., Campos V.F., Ourique A., Guterres S.S., Pohlmann A.R., Basso A.C., Seixas F.K., Beck R.C., Collares T. (2015). Tretinoin-loaded lipid-core nanocapsules decrease reactive oxygen species levels and improve bovine embryonic development during *in vitro* oocyte maturation. *Reprod. Toxicol.*, 58: 131–139.
- Namgoong S., Kim N.H. (2016). Roles of actin binding proteins in mammalian oocyte maturation and beyond. *Cell Cycle*, 15: 1830–1843.
- Nemolato S., Messina I., Cabras T., Manconi B., Inzitari R., Fanali C., Vento G., Tirone C., Romagnoli C., Riva A., Fanni D., Di Felice E., Faa G., Castagnola M. (2009). Thymosin beta (4) and beta (10) levels in preterm newborn oral cavity and foetal salivary glands evidence a switch of secretion during foetal development. *PLoS One*, 4: e5109.
- Nemolato S., Cabras T., Fanari M.U., Cau F., Fanni D., Gerosa C., Manconi B., Messina I., Castagnola M., Faa G. (2010). Immunoreactivity of thymosin beta 4 in human foetal and adult genitourinary tract. *Eur. J. Histochem.*, 54: e43.
- Opiela J., Romanek J., Lipiński D., Smorąg Z. (2014). Effect of hyaluronan on developmental competence and quality of oocytes and obtained blastocysts from *in vitro* maturation of bovine oocytes. *Biomed. Res. Int.*, 519189.
- Salhab M., Papillier P., Perreau C., Guyader-Joly C., Dupont J., Mermillod P., Uzbekova S. (2010). Thymosins β -4 and β -10 are expressed in bovine ovarian follicles and upregulated in cumulus cells during meiotic maturation. *Reprod. Fert. Develop.*, 22: 1206–1221.
- Salviano M.B., Collares F.J., Becker B.S., Rodrigues B.A., Rodrigues J.L. (2016). Bovine non-competent oocytes (BCB-) negatively impact the capacity of competent (BCB+) oocytes to undergo *in vitro* maturation, fertilisation and embryonic development. *Zygote*, 24: 245–251.
- Skowronek O., Zdebska N., Opiela J. (2016). The impact of thymosin β 4 on bovine oocytes meiotic maturation and DNA fragmentation. *Proc. 4th Winter Workshop of the Society for Biology of Reproduction*, Zakopane, Poland, 3–5.02.2016, p.36.
- Takezawa Y., Yoshida K., Miyado K., Sato M., Nakamura A., Kawano N., Sakakibara K., Kondo T., Harada Y., Ohnami N., Kanai S., Miyado M., Saito H., Takahashi Y., Akutsu H., Umezawa A. (2011). β -catenin is a molecular switch that regulates transition of cell-cell adhesion to fusion. *Sci. Rep.*, 1: 68.
- Tripurani S.K., Lee K.B., Wang L., Wee G., Smith G.W., Lee Y.S., Latham K.E., Yao J. (2011). A novel functional role for the oocyte-specific transcription factor newborn ovary homeobox (NOBOX) during early embryonic development in cattle. *Endocrinology*, 152: 1013–1023.
- Wang W.S., Chen P.M., Hsiao H.L., Wang H.S., Liang W.Y., Su Y. (2004). Overexpression of the thymosin beta-4 gene is associated with increased invasion of SW480 colon carcinoma cells and the distant metastasis of human colorectal carcinoma. *Oncogene*, 23: 6666–6671.
- Warzych E., Pers-Kamezczyk E., Krzywak A., Dudzińska S., Lechniak D. (2013). Apoptotic index within cumulus cells is a questionable marker of meiotic competence of bovine oocytes matured *in vitro*. *Reprod. Biol.*, 13: 82–87.
- Wierchoś-Hilczek A. (2017). Thymosin β 4 structure – multiple biological functions and potential therapeutic applications. *Wiad. Zoot.*, LV: 30–36.
- Wooldridge L.K., Ealy A.D. (2019). Interleukin-6 increases inner cell mass numbers in bovine embryos. *BMC Dev. Biol.*, 19: 2.
- Yan H., Wen J., Zhang T., Zheng W., He M., Huang K., Guo Q., Chen Q., Yang Y., Deng G., Xu J., Wei Z., Zhang H., Xia G., Wang C. (2019). Oocyte-derived E-cadherin acts as a multiple functional factor maintaining the primordial follicle pool in mice. *Cell Death Dis.*, 10: 160.
- Zhao Y., Qiu F., Xu S., Yu L., Fu G. (2011). Thymosin beta 4 activates integrin-linked kinase and decreases endothelial progenitor cells apoptosis under serum deprivation. *J. Cell. Physiol.*, 226: 2798–2806.

Received: 19 XII 2019

Accepted: 3 VII 2020