

The effect of elevated temperatures on the development of cattle embryos cocultured with bovine oviduct epithelial cells (BOECs) and a KSOMaa medium*

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The aim of this study was to compare the effect of elevated temperatures on the development of cattle embryos cocultured with bovine oviduct epithelial cells (BOECs) and cultured in a KSOMaa medium. Zygotes obtained *in vitro* were cocultured with BOECs and cultured in KSOMaa (potassium simplex optimised medium modified by amino acids) at a control temperature (38.5°C), and elevated temperatures (40.5°C and 41°C) for 168 hours. At 38.5°C there was no significant difference in embryo development between BOEC and KSOMaa treatments. At 40.5°C embryos cocultured with BOECs developed significantly better compared to the KSOMaa system, as observed at the cleavage rate (73.45±2.45 vs 66.75±1.34%), minimum 8 cell rate (20.36±1.42 vs 16.78±1.48%) and the blastocyst rate (3.13±3.25 vs 0%), (P<0.001). At 41°C there were significant differences in the cleavage rate (62.25±1.72 vs 56.31±3.47%) and the minimum 8 cell rate (13.06±1.97 vs 9.64±2.14%) in the BOEC coculture system compared to the KSOMaa system (P<0.001), at no differences in the blastocyst rate. In embryos cocultured with BOECs, the *hsp70* mRNA expression was higher than in embryos cultured in KSOMaa: 38.5°C-1.42±0.05 vs 0.77±0.04; 40.5°C-3.82±0.04 vs 1.92±0.24; 41°C-6.74±0.20 vs 2.14±0.13, (P<0.001). Also, the HSP70 protein level was higher in embryos cocultured with BOECs than those cultured in KSOMaa: 38.5°C-0.98±0.01 vs 0.17±0.02; 40.5°C-1.96±0.01 vs 0.82±0.01; 41°C-2.45±0.01 vs 1.30±0.01, (P<0.001). This data indicates that bovine embryos cultured at elevated temperatures in the presence of BOECs develop better than embryos cultured in KSOMaa and also that BOECs stimulate embryos to switch on their defence mechanisms to a significantly higher level than in embryos cultured in KSOMaa.

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For many years both *in vivo* and *in vitro* research has been conducted to examine the effect of heat stress on fertility of animals including cattle [Hansen 2007, 2012, Rynkowska 2011]. Among other things such research has focused on the influence of heat stress on cattle embryo development. Previously, the negative impact of elevated temperatures on *in vitro* development of cattle embryos has been demonstrated based on various culture periods (from several to around 15-20 hours), and on cultures in different types of synthetic media such as CR1, KSOM and SOF at elevated temperatures [Edwards and Hansen 1997, Rivera and Hansen 2001, Rivera *et al.* 2004]. These studies have shown that elevated temperatures may lead to various dysfunctions in embryo development, such as changes in their metabolism, nuclear and cytoplasmic disorders, incorrect maternal-embryonic transition (MET), deficient activation of the embryonic genome and incorrect gene expression.

In contrast to the previous studies, in which embryos were cultured in synthetic media to analyse the negative effect of elevated temperatures on cattle development, in the present study a novel model mimicking *in vivo* conditions was used. Cattle embryos were cocultured with bovine oviduct epithelial cells (BOECs) at elevated temperatures. This system reflects, with a high probability, the relationship between the embryo and the mother [Besenfelder *et al.* 2012, Duszewska *et al.* 2012, Ulbrich *et al.* 2010]. BOECs originate from an oviduct, which is the organ that *in vivo* provides the optimum environment for the development of early embryos, but also for such processes as gamete and embryo transport and fertilization of oocytes [Besenfelder *et al.* 2012].

Except for BOECs, cattle embryos were cultured in a synthetic medium – KSOMaa (potassium simplex optimized medium modified by amino acids) [Sagirkaya *et al.* 2006], because in this medium cattle embryo development is similar to that in the presence of BOECs. KSOMaa was also used to study the effect of increased temperatures on early embryo development [Rivera *et al.* 2004]. In both systems embryos were cultured for 168 hours at elevated temperatures (40.5°C and 41°C). This approach was based on some observations suggesting that in cattle the rectal temperature may reach or exceed 41°C in summer [Rivera and Hansen 2001] and the fact that these temperatures were used in other studies [Edwards and Hansen 1997, Rivera and Hansen, 2001, Rivera *et al.* 2004]. The ability of the embryo to survive at elevated temperatures depends on various factors, such as race, genotype, sex, the stage of embryo development as well as cellular defence mechanisms, which to a certain degree provide resistance to higher temperatures [Hernández-Cerán *et al.* 2004; Hansen 2007, Ferreir *et al.* 2011, Paula- Lopes 2013, Sakatani 2013]. The main factor in the induction of embryo thermotolerance is a heat shock protein 70 (HSP70), which plays an important role in embryogenesis by protecting embryos against the effects of heat stress [Luft J and Dix 1999, Collier *et al.* 2008].

The objective of this study was to determine the effect of elevated temperatures (40.5°C and 41°C) on the development of cattle embryos cocultured with bovine oviduct epithelial cells (BOECs) and cultured in a synthetic medium – KSOMaa,

based on the evaluation of cattle embryo development and the analysis of *hsp70* gene expression at both mRNA and protein levels in embryos.

Material and methods

Material collection

Bovine oviducts and ovaries were collected *post mortem* from slaughtered cattle. Ovaries were placed in PBS containing 0.2 mg/mL streptomycin and 250 IU/mL penicillin. Oviducts were obtained from cattle in phase I of the ovarian cycle based on ovarian morphology described by Ireland *et al.* [1980]. Selected oviducts were placed in HANKS medium without Ca^{2+} and Mg^{2+} , supplemented with antibiotics (50 µg/mL gentamicin 100 IU penicillin and 50 µg/mL streptomycin). Ovaries and oviducts were transported to the laboratory at 30°C in less than 2 hours.

Embryo sources

Immature cumulus-oocyte complexes (imCOCs) were aspirated from ovarian follicles of 2-6 mm in diameter using a vacuum pump and they were washed two times in TCM 199 HEPES without NaHCO_3 medium, supplemented with 10% (vol/vol) FBS (GIBCO, Scotland) and 50 µg/mL gentamicin, pH 7.4. ImCOCs were placed in groups of 20 in 500 µl of the oocyte maturation medium [TCM 199 with HEPES containing (vol/vol) 10% FBS (GIBCO, Scotland), and 0.02 IU NIH-pFSH/mL, 1 µg/mL β -oestradiol and 0.2 mM sodium pyruvate (MERCK, France), 50 µg/mL gentamicin, pH 7.4] and cultured for 24 hours at 38.5°C in a humidified atmosphere with 5% CO_2 .

Frozen semen was thawed at 37°C, centrifuged (200xg) for 10 minutes and washed in 2 mL Sp-TALP containing 6 mg/mL BSA fraction V and 50 µg/mL gentamicin, pH 7.4. Spermatozoa underwent capacitation by the swim-up method in 1mL Sp-TALP. *In vitro* fertilization was performed in Fert-TALP containing 6 mg/mL BSA fraction FAF, 0.2 mM sodium pyruvate (MERCK, France), PHE – 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine, 50 µg/ml gentamicin and 2 µg/mL heparin; pH 7.4. Oocytes and spermatozoa were coincubated at 38.5°C in a humidified atmosphere with 5% CO_2 . After 18 hours putative zygotes were denuded of cumulus cells by vortexing in a 1.5mL Eppendorf tube at 1600 rpm/min. for 3 min.

BOEC preparation

BOECs were isolated mechanically from oviducts according to the procedure described by Rottmayer *et al.* [2006]. BOECs were washed three times in TCM 199 HEPES without NaHCO_3 , supplemented with 10% (vol/vol) bovine foetal serum (FBS) (GIBCO, Scotland) and 50 µg/mL gentamicin, pH 7.4. Then BOECs were washed twice in the cell culture medium (CM): TCM 199 HEPES supplemented with 10% (vol/vol) FBS and 50 µg/mL gentamicin, 7.4. Oviduct epithelial cells were cultured for 48 hours to form BOEC aggregates at 38.5°C in a humidified atmosphere with 5% CO_2 . Next the BOEC aggregates were selected and washed three times in CM.

Experimental design

Zygotes with normal morphology were randomly assigned to one of the two culture systems. In system I embryos were cocultured with BOECs, while in system II they were cultured in KSOMaa. In both systems embryos were cultured at the control temperature (38.5°C) and experimental temperatures: 40.5°C and 41°C for 168 hours in a humidified atmosphere with 5% CO₂. In system I the total number of zygotes cocultured with BOECs was 1582 (38.5°C-593; 40.5°C-579; 41°C-410), in each experiment from 15 to 20 zygotes were cocultured with 20 BOEC aggregates in 40 µl droplets of TCM 199 HEPES containing 10% (vol/vol) FBS and 50 µg/mL gentamicin, pH 7.4, overlaid with mineral oil. In system II the total number of zygotes cultured in KSOMaa was 1393 (38.5°C -437; 40.5°C -552; 41°C-404), and in each experiment from 15 to 20 zygotes were cultured in KSOMaa medium (CHEMICON, USA) containing 6mg/mL BSA, overlaid mineral oil.

At every 48 hours half of the media was removed and replenished with fresh media. In both system embryo development was evaluated after 48 (the percentage of cleaved embryos), 96 (the percentage of minimum 8-cell embryos) and 168 (the percentage of blastocysts) hours post fertilization (hpf).

After 168 hours embryos from systems I and II were frozen at -70°C for subsequent analysis of *hsp70* mRNA and protein expression.

Analysis of *hsp70* mRNA in cattle embryos cocultured with BOECs and in KSOMaa

The *hsp70* mRNA level was measured by Real-time quantitative PCR. 347 bovine embryos cocultured with BOECs and 350 embryos cultured in KSOMaa at control (38.5°C) and experimental temperatures (40.5°C and 41°C) were used for analyses of the *hsp70* mRNA level. Two micrograms of total DNase-treated RNA were used for reverse transcription using random hexamers from an RNeasy® Micro kit (QIAGEN, Germany). Real-time quantitative RNA analysis was performed in a Light Cycler (Roche Diagnostics, Mannheim, Germany) using the respective pairs of oligonucleotide primers: *hsp70F*: 3' ACTT GGAA GTAA ACAG AAAC GGGT GAAA AA 5'; *hsp70R*: 5' AAGG TGCT GGAC AAGT GCCA GGAG GTGA TT 3', and *s18F*: 5' GAG GAT GAG GTG GAA CGT GT 3', *s18R*: 5' CCT GTG GTC TTG GTG TGG CT 3'. Amplified products were detected using SYBR Green I (Roche Diagnostics). Next the PCR products from each primer pair were subjected to melting curve analysis and agarose gel electrophoresis. The Light Cycler 3.5 Software was used for data analysis. Expression was normalized relative to that of the control transcript encoding *18S* rRNA selected among other reference using the NormFinder software (www.mdl.dk).

Analysis of HSP 70 protein levels in cattle embryos cocultured with BOECs and in KSOMaa

Western blot analysis was performed to detect HSP70. Forty µg of total embryo protein extract from embryos cocultured with BOECs and in KSOMaa were used. Protein extracts from both variants were obtained using a Tris/NP40 lysis buffer (0.1 M Tris,

pH 7.5; 0.2% NP40; Complete Protease Inhibitor Cocktail Tablets, Roche, Switzerland; 2mM PMSF) and they were resolved by electrophoresis on 10% SDS/PAGE. Separated proteins were electroblotted on the PVDF membrane (Immobilon-P Membrane). The membranes were initially blocked by gentle agitation in TBST [Tris buffered saline (50 mM Tris/HCl, pH 7.6, and 150 mM NaCl) with 0.15% Tween 20] containing 5% fat-free skimmed milk powder for 1 hour at room temperature (20°C), followed by overnight incubation at 4°C with goat polyclonal primary antibody raised against bovine HSP70 HSP70 in a 1:1000 dilution (sc-1060, Santa Cruz Biotechnology, USA). Actin, the loading control for embryo extracts, was detected using an anti-human goat polyclonal antibody in a 1:2000 dilution (sc1615; Santa Cruz Biotechnology). Then membranes were washed three times and incubated with the HRP-conjugated donkey anti-goat secondary antibody in a 1:20000 dilution (sc-2020, Santa Cruz Biotechnology) for 1 hour at room temperature. (sc-1060, Santa Cruz Biotechnology, USA). Immunoreactive bands were detected using the ECL Plus Western blotting detection system (ECL Immobilion™ Western Chemiluminescent HRP Substrate, MILLIPORE Corporation, USA) using the Amersham Hyperfilm™ ECL (GE Healthcare Limited, UK). Reactive bands were quantified relative to actin using a Molecular Imager with the Quantity One software (Bio-Rad). Most reagents and media used were obtained from Sigma-Aldrich (Poznań, Poland), unless otherwise indicated.

Statistical analysis

Development rates at the different stages were determined based on the number of zygotes and analyses by one-way ANOVA as well as multiple pair-wise comparisons using Tukey's test Statgraphics 5.0 Centurion (USA). Differences at $P < 0.001$ were considered significant. The effects of temperature on *hsp70* mRNA and the HSP70 protein expression in embryos were determined using REST, which was run in Microsoft Excel and the pair-wise T-test.

Results and discussion

At the control temperature of 38.5°C (Fig. 1A) there were no significant differences between system I – BOECs and system II - KSOMaa in cleavage rates (48 hpf), minimum 8 cell rates (96 hpf) and blastocyst rates (168 hpf). At the elevated temperature of 40.5°C (Fig. 1B) the cleavage rate, minimum 8-cell rate and the blastocyst rate were significantly higher in system I – BOECs than in system II - KSOMaa ($P < 0.001$). After 168h coculture with BOECs 17 blastocysts (3.14%) were obtained, while no blastocysts were detected after culture in KSOMaa. At the higher experimental temperature of 41°C (Fig. 1C) the cleavage rate and minimum 8-cell rate were significantly higher in system I – BOECs than in system II – KSOMaa, ($P < 0.001$). After 168 hours embryo development was arrested in both systems.

In comparison with the control temperature of 38°C both elevated temperatures (40.5 and 41°C) caused a negative effect on embryo development in both culture

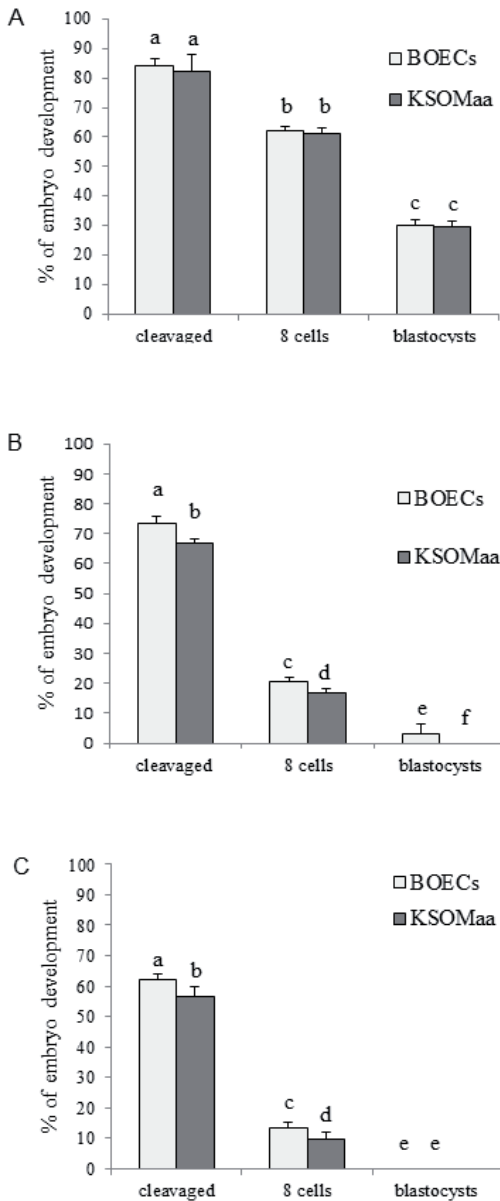


Fig. 1. Analysis of development of cattle embryos cocultured with BOECs (system I) and cultured in KSOMaa (system II) at various temperatures: 38.5°C (A), 40.5°C (B) and 41°C (C). Data presented means±SD. Bars with different letters within a graph denote a statistical difference at $P < 0.001$.

systems. Our results confirm earlier studies [Edwards and Hansen, 1997, Rivera and Hansen 2001, Rivera *et al.* 2004, Siva *et al.* 2013]. In most cases cattle embryos did not reach the crucial 8 cell stage, meaning that the embryos did not reach the stage, at which maternal-embryonic transition (MET) takes place [Vigneault *et al.* 2004].

However, it should be noted that at the elevated temperature of 40.5°C, 3.14% of the embryos cocultured with BOECs for 168 hours reached the blastocyst stage. This indicates that BOECs stimulate the embryos to protect them from the effects of the elevated temperature.

BOECs may be involved in the defensive mechanisms of the embryo against the effects of elevated temperatures, mainly based on the heat shock 70 protein (HSP70). At a control temperature of 38.5°C (Fig. 2) the level of *hsp70* gene expression was significantly higher in embryos cocultured with BOECs when compared to embryos cultured in KSOMaa ($P<0.001$). Moreover, at elevated temperatures (40.5°C, 41°C) the level of *hsp70* mRNA expression in embryos cocultured with BOECs (Fig. 2) was significantly higher compared to embryos cultured in KSOMaa ($P<0.001$).

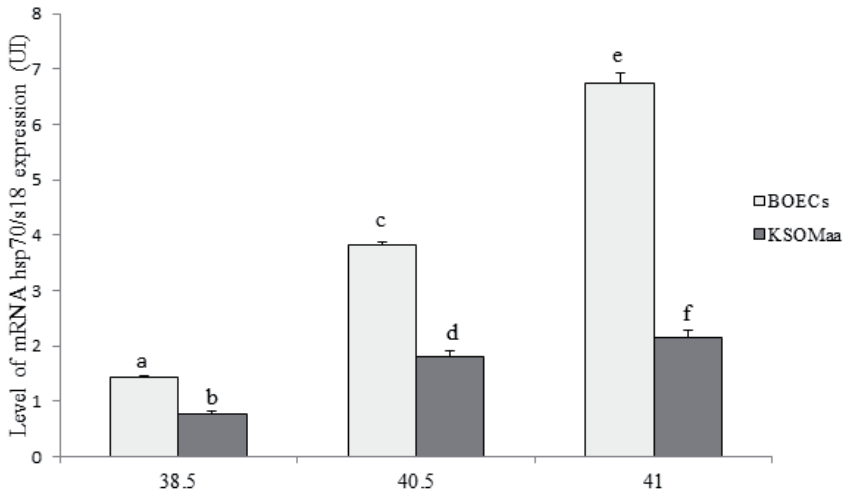


Fig. 2. Analysis of *hsp70* mRNA expression in embryos cocultured with BOECs (system I) and cultured in KSOMaa (system II) at various temperatures. Data presented means \pm SD. Bars with different letters within a graph denote a statistical difference at $P<0.001$.

The lowest HSP70 level in embryos was observed at the control temperature (38.5°C) in both culture systems (Fig. 3A). However, as it was demonstrated by Western blot quantification (Fig. 3 B) at the control temperature of 38.5°C the level of HSP70 was significantly higher in embryos cocultured with BOECs when compared to embryos cultured in KSOMaa ($P<0.001$). Analogously, the level of HSP70 in embryos cocultured with BOECs (Fig. 3B) was significantly higher at elevated temperatures (40.5°C, 41°C) in comparison to embryos cultured in KSOMaa ($P<0.001$).

In this study the expression of *hsp70* on mRNA and protein levels in embryos was observed at the control temperature in both culture systems, which is consistent with earlier reports showing that HSP70 proteins are synthesized constitutively under physiological conditions [Lanneau *et al.* 2008]. In cattle embryos the expression of the *hsp70* gene is observed already in the two-cell stage embryos, while the expression of

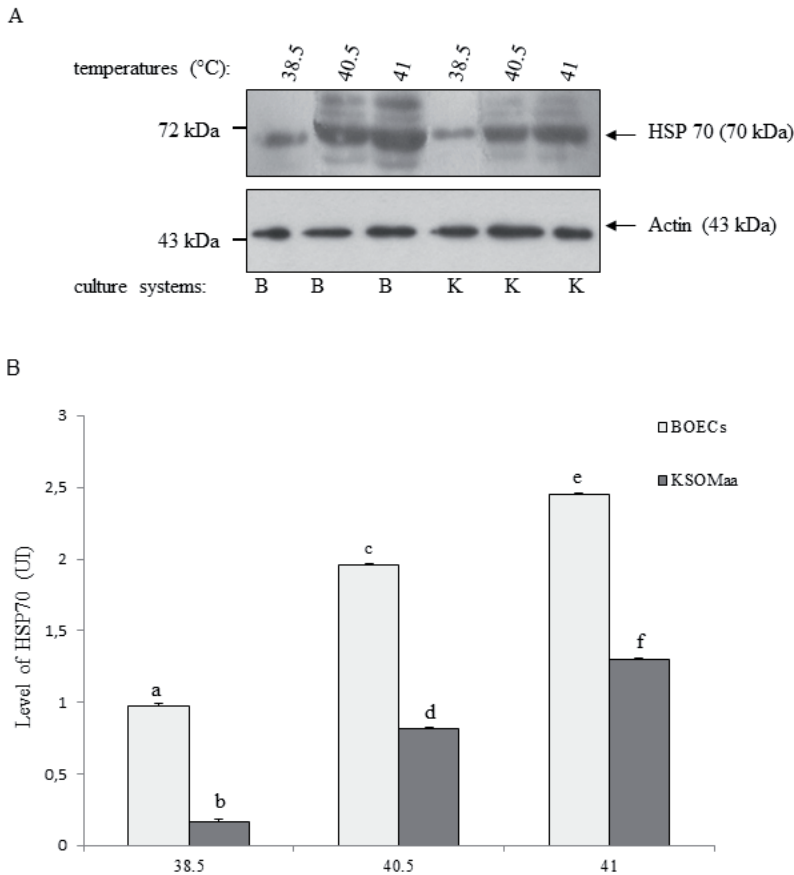


Fig. 3. Representative data of Western blotting analyses are shown (A) - expression of HSP70 in embryos: B-cocultured with BOECs (system I) and K-cultured in KSOMaa (system II) at various temperatures. Quantitation of Western blot analysis of embryos is shown (B). Data presented means \pm SD. Bars with different letters within a graph denote a statistical difference at $P < 0.001$.

most other genes begins only in the 8-16 cell stage embryo [Cordova *et al.* 2014]. In this study the expression of *hsp70* on mRNA and protein levels was higher in embryos cocultured with BOECs (system I) than in embryos cultured in KSOMaa (system II), which is consistent with an earlier report showing that the level of its expression depends on the culture system [Sagirkaya *et al.* 2006]. These results indicate that BOECs stimulate embryos to switch on their defence mechanism based on the HSP70 protein to a significantly higher level than in embryos cultured in KSOMaa. The HSP70 protein promotes cell protection against heat damage, preventing protein denaturation and blocking apoptosis [Collier *et al.* 2008].

In conclusion, at elevated temperatures (40.5°C and 41°C) cattle embryos cocultured with BOECs developed better than embryos cultured in the synthetic medium (KSOMaa).

At the elevated temperature of 40.5°C a low percentage of embryos cocultured with BOECs for 168 hours reached the blastocyst stage. In embryos cocultured with BOECs the *hsp70* mRNA expression increased along with the elevation of temperature. However, in embryos cultured with KSOMaa the *hsp70* mRNA expression increased at elevated temperatures when compared to the control, but it was comparable both at 40.5°C and 41°C. In turn, the level of the HSP70 protein increased along with the elevation of temperature compared to the control in both culture systems, while it was higher in embryos cocultured with BOECs than in those cultured in KSOMaa.

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