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DEVELOPMENT OF RABBIT PREIMPLANTATION EMBRYOS UNDER THERMAL STRESS *IN VITRO*

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ABSTRACT

The aim of our study was to examine the influence of short-term (6h) hyperthermia (41.5 °C and 42.5 °C) on the developmental capacity of preimplantation rabbit embryos *in vitro*. Rabbit embryos were isolated from superovulated and inseminated rabbit females after slaughter by flushing from oviducts of 19 hpc. The embryos were cultured in k-DMEM medium with the addition of 10 % fetal calf serum. In each experiment morula stage embryos were divided to hyperthermia (HT) and control group (C). Embryos from HT group were cultured at 41.5 °C or 42.5 °C for 6 hours and then post-cultured at 37.5 °C for 20 h. Control embryos were cultured at 37.5 °C. Then the embryos were evaluated for developmental stages and parts of the embryos were analyzed for the detection of Hsp 70 proteins. It was observed that rabbit embryos were able to resist against hyperthermia at 41.5 °C and the development to the blastocyst stage was not negatively influenced. These embryos showed the presence of protective Hsp 70 proteins as a reaction on thermal stress. Oppositely, hyperthermia at 42.5 °C significantly altered embryo development, which resulted in complete developmental arrest of embryos at lower developmental stages, whilst not a single embryo developed to expanded or hatching blastocyst stage. The presence of Hsp 70 proteins in these embryos was not confirmed. These results demonstrate a threshold of thermotolerance of preimplantation rabbit embryos to thermal stress *in vitro* and support hypothesis about protective role of Hsp proteins in embryo thermotolerance.

Key words: rabbit embryos, hyperthermia, Hsp 70

INTRODUCTION

Preimplantation embryos are highly sensitive to stress conditions. The embryos may be negatively influenced by environmental changes, which can induce developmental disorders. This susceptibility is probably caused by inability of embryos to adapt to stress factors, especially during the transition from maternal to embryonic control (Ju et al., 1999). The exposure to thermal stress is a proper model for testing the effects of stress conditions on preimplantation embryos and their response to stress action. Consequences of thermal stress depend on the duration of thermal exposure, developmental stage and animal species of the embryo. Developmental disorders in pig embryos occurred after short-term (10-60 min) incubation at 43 – 45.5 °C, while at 42 °C the embryos had even higher diameter and cell number than at control temperature (Kojima et al., 1996). Sakatani et al. (2004) reported that the rate of bovine blastocysts was decreased after exposure of early stage embryos to heat-shock at 41 °C, but it did not change, when the morula stage embryos were exposed to thermal stress.

Ealy et al. (1995) noted that development of two-cell bovine embryos to 16-cell stage or more cell stage was not damaged following heat shock at 40 °C, while at 41 °C for 3 h the cleavage was declined, but it was not observed in case of morula stage embryos. Therefore, it is evident that the resistance of preimplantation embryos to stress conditions is changed during development. Elevation of thermotolerance is associated with several mechanisms at cellular level. One of such mechanisms of stress response is assumed to be a production of heat-shock proteins (Hsp) by embryonal cells (Mirkes et al., 1999; Paula-Lopes a Hansen, 2002). Similar events were

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observed in rabbit embryos. The exposure of rabbit females to thermal stress in the time of oocyte maturation declined the rate of embryos at higher developmental stages (Cheng et al., 1999). Wolfenson and Blum (1988) reported that rabbit embryos are more sensitive to thermal stress at earlier stage compared to 6th day of development.

In our study we tested effect of elevated temperatures (41.5 °C or 42.5 °C) on developmental capacity of rabbit embryos *in vitro* in relation to the production of Hsp 70 proteins. We used morula stage embryos, which is characteristic by the presence of adaptation mechanism against stress factors.

MATERIAL AND METHODS

Isolation and culture of embryos

Female New Zealand White rabbits, kept on the local farm, were treated with PMSG (Werfaser, Alvetra und WERFFT, Vienna, Austria) *i.m*. at 20 IU/kg live weight, 72 hours before mating. Immediately prior to mating, the females were injected *i.m*. with hCG (Werfachor, Alvetra und WERFFT) at 40 IU/kg live weight, afterwards the females were mated with a male of proven fertility belonging to the same breed. At 19 to 20 hours *post coitum* (hpc) the pronuclear stage eggs were flushed from the oviducts of slaughtered animals with PBS (Gibco, Auckland, New Zealand). The flushed eggs were evaluated morphologically and eggs with two pronuclei, two polar bodies and compact cytoplasm were selected for experiments. The eggs were placed into 4-well dishes (Nunc, Roskilde, Denmark), containing 600 µl of k-DMEM medium (Gibco) supplemented with 10% FCS, and cultured in 5% CO_2 at 37.5 °C, up to 72 hours post coitum (hpc). Afterwards, the embryos which reached morula stage were selected, transferred to a drop of fresh culture medium, randomly divided into two groups: control (at 37.5 °C) and hyperthermic (41.5 °C or 42.5 °C) group and incubated for 6 hours. Then the embryos from hyperthermic group were transferred to standard temperature (37.5 °C) and incubated overnight (16-18 h). Following incubation the embryos were examined for the development (blastocyst rate).

Western-blotting for the detection of Hsp 70 proteins

For the detection of Hsp 70 proteins Western-blotting of embryo lyzates was used. The embryos (at least 25 per group) were put in SDS sample buffer with 2-mercaptoethanol (ICN Biomedicals Inc., Ohio, USA) and lyzed by repeated pipetting and 3-time freezing-thawing procedure. Before electrophoresis, embryo lyzates were heated to 95 °C with vigorous shaking for 5 min and cooled to room temperature.

Samples of embryo lysates were electrophoresed in 4% (stacking gel) and 10% (resolving gel) polyacrylamide at 200 V constant voltages according to Laemmli (1970). The protein fractions from the gel were transferred onto nitrocellulose membrane Porablot NCP (Macherey-Nagel, Duren, Germany) by wet transfer in transfer buffer (25 mM Tris, 192 mM glycine, 20% metanol, pH 8.3) using Mini Trans-Blot electrophoretic Transfer Cell (Bio-Rad) for 1 hour. Endogenous peroxidase in samples was quenched by incubation in 3% H_2O_2 for 15 min. Non-specific binding of antiserum was blocked by incubation in 5% BSA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in TTBS (20 nM Tris-base, 137 nM NaCl, 0.1% Tween-20, pH 7.5). Blocked membranes were probed with mouse anti-HSP70 monoclonal antibody (Chemicon International Inc., Temecula, CA, USA) at 1: 500 dilution for 1 hour. Membrane was then incubated with secondary horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Upstate, Temecula, CA, USA) at 1: 1000 dilution for 1 hour. Positive reactions on the membrane were visualized using VisualizerTM Western Blot Detection Kit (Upstate) and ECL Hyper-film (Amersham Life Science, Little Chalfont, UK). The molecular weights of fractions were evaluated using Chemiluminescent BlueRanger (Pierce, Rockford, IL, USA) – pre-stained peroxidase-labelled protein molecular weight marker mix (17.1–207 kDa).

Statistics

The effect of different temperatures on developmental stages of embryos was evaluated by Chi-square test.

Table 1: The influence of hyperthermia *in vitro* on the development of rabbit preimplantation embryos

Group	Number of embryos	Developmental stages, n (%)					
		Arrested	Morula	EBl	XBl	HBl	XBl+HBl
Control	221	15 (6.8)	26 (11.8)	62 (28.1)	51 (23.1)	67 (30.2)	118 (53.3)
HT (41,5 °C)	196	14 (7.1)	27 (13.8)	42 (21.4)	42 (21.4)	71 (36.3)	113 (57.7)
HT (42,5 °C)	29	3 (10.3)	2 (6.9)	24 (82.8) +	0 (0) +	0 (0) +	0 (0) +

EBl – early blastocyst, XBl – expanded blastocyst, HBl – hatching or hatched blastocyst
+ - significant differences ($p < 0.05$) compared with control (37.5 °C)

RESULTS AND DISCUSSION

Influence of heat shock on embryo development

Hyperthermia conditions at 41.5 °C did not influence progress of embryos to higher developmental stages (expanded and hatching blastocysts). Rate of higher stage embryos in hyperthermic group (57.7 %) did not significantly differ from control group (53.3 %). Oppositely, following elevation of the temperature up to 42.5 °C, we observed complete developmental arrest at lower developmental stages (Table 1). None of the embryos developed to expanded or hatching blastocyst stage, the development was terminated mostly at the early blastocyst stage.

Western-blotting for the detection of Hsp 70 proteins

Using Western-blotting of embryo lysates we confirmed the presence of inducible fraction of Hsp70 proteins in embryos exposed to 41.5 °C as well of constitutive fraction of Hsp70 in embryos cultured in normal conditions (control). The presence of Hsp70 inducible fraction in embryos exposed to 42.5 °C was not revealed.

In our work we studied effect of two elevated temperatures on developmental capacity of rabbit embryos *in vitro*. We used morula stage embryos, which is characteristic by the presence of adaptation mechanism against stress factors. Our results indicate that the temperature 41.5 °C did not alter embryo development. These data correspond to observation of thermotolerance event in morula of other animal species, for instance in bovine (Edwards and Hansen, 1997; Ealy et al., 1995), mouse (Arechiga a Hansen, 1998) and rabbit (Wolfenson and Blum, 1988).

The progress of preimplantation embryos up to expanded or hatching blastocyst stage is a clear indicator of maintaining of high developmental capacity and viability in hyperthermic conditions. Tolerance against elevated temperature is probably associated with increased production of Hsp 70 proteins. In contrast, at 42.5 °C the embryo development to higher preimplantation stages was found to decrease and all embryos were arrested at early blastocyst stage. These results demonstrate a threshold of thermotolerance of preimplantation rabbit embryos to short hyperthermic exposure *in vitro*. As the presence of Hsp 70 proteins in embryos exposed to 42.5 °C was not revealed, we assume that the cause of decline in embryo development may be a destruction of mechanism of proteosynthesis. These results support the hypothesis about protective role of Hsp proteins in embryo thermotolerance.

REFERENCES

ARECHIGA, C. F. - HANSEN, P. J. 1998. Response of preimplantation murine embryos to heat shock as modified by developmental stage and glutathione status. In : *In Vitro Cellular and Developmental Biology.* vol. 34, 1998, p. 655-659.

EALY, A. D. - HOWELL, J. L. - MONTEROSSO, V. H. - ARECHIGA, C. F. - HANSEN, P. J. 1995. Developmental changes in sensivity of bovine embryos to heat shock and use of antioxidants as thermoprotectants. In : *Journal of Animal Scienace*. vol. 73, 1995, p. 1401-1407.

EDWARDS, J. L. - HANSEN, P. J. 1997. Differential Responses of Bovine Oocytes and Preimplantation Embryos to Heat Shock. In : *Molecular Reproduction and Development.* vol. 46, 1997, p. 138-145.

CHENG, H. - DOOLEY, M. P. - HOPKINS, S. M. - ANDERSON, L. L. - YIBCHOK-ANUN, S. - HSU, W. H. 1999. Development of rabbit embryos during a 96-h period of in vitro culture after superovulatory treatment under conditions of elevated ambient temperature. In : *Animal Reproduction Science*. vol. 56, 1999, p. 279-290.

KOJIMA, T.- UDAGAWA, K.- ONISHI, A.- IWAHASHI, H.- KOMATSU, Y. 1996. Effect of heat stress on development in vitro and in vivo and on synthesis of heat shock proteins in porcine embryos. In : *Molecular Reproduction and Development.* vol. 43, 1996, p.452-457.

LAEMMLI, U. K. 1970. Cleavage and structure of proteins during the assembly of the head of bacteriophage T4. In : *Nature*. vol. 227, 1970, p. 680-685.

MIRKES, P. E. – DOGGETT, B. 1999. Accumulation of heat shock protein 72 (hsp 72) in postimplantation rat embryos after exposure to various periods of hyperthermia (40 degrees – 43 degrees C) in vitro: evidence that heat shock protein 72 is a biomarker of heat induced embryotoxicity. In : *Teratology* vol. 46, 1999, p. 301-309.

PAULA-LOPES, F. F. – HANSEN, P. J. 2002. Heat shock-Induced Apoptosis in Preimplantation Bovine Embryos Is a Developmentaly Regulated Phenomenon. In : *Biology of Reproduction*. vol. 66, 2002, p. 1169-1177.

SAKATANI, M. - KOBAYASHI, S. - TAKAHASHI, M. 2004. Effects of heat shock on in vitro development and intracellular oxidative state of bovine preimplantation embryos. In : *Molecular Reproduction and Development.* vol. 67, 2004, p. 77-82.

WOLFENSON, D. – BLUM, O. 1988. Embryonic development, conception rate, ovarian function and structure in pregnant rabbits heat-stressed before or during implantation. In : *Animal Reproduction Science*. vol.17, 1988, p. 259-270.

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