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Improved quality of porcine embryos cultured with hyaluronan due to the modification of the mitochondrial membrane potential and reactive oxygen species level

Marek Romek [a, *], Barbara Gajda [b], Ewa Krzysztofowicz [a], Marcin Kucia [a], Agnieszka Uzarowska [a], Zdzislaw Smorag [b]

[a] Department of Cell Biology and Imaging, Institute of Zoology, Jagiellonian University, Gronostajowa 9, 30-387 Krakow, Poland
[b] Department of Animal Reproduction Biotechnology, National Research Institute of Animal Production, 32-083 Balice/Krakow, Poland

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ABSTRACT

Although considerable progress has been made in pig embryo culture systems, the developmental competence and quality of the produced embryos are still lower than their *in vivo*-derived counterparts. Because hyaluronan (HA) regulates various cellular processes and possesses antioxidant properties, this glycosaminoglycan seems to be a promising supplement in culture media. However, until now, its beneficial influence on *in vitro* pig embryo development has been debatable. Hence, we aimed to investigate the effect of 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL concentrations of HA on the developmental potential and quality of cultured porcine embryos. We found that 1 mg/mL HA supplementation significantly increased the obtained percentages of cleaved embryos to ~95%, morulae to ~87% and blastocysts to ~77%. At 0.5 mg/mL and 1 mg/mL HA concentrations, we observed a significantly improved blastocyst quality, expressed as the total number of cells per blastocyst, number of cells in the inner cell mass, number of TUNEL-positive nuclei per blastocyst, the TUNEL index and the blastocyst diameter. Because the inner mitochondrial membrane potential ($\Delta\Psi m$) and reactive oxygen species (ROS) level are important for proper embryo development, for the first time, we measured these two parameters in cultured embryos at various HA concentrations and during their development up to the expanded blastocyst stage. For blastocysts cultured with 1 mg/mL HA, the $\Delta\Psi m$ and ROS level were ~1.6 and 2.7 times lower, respectively, than those of the control blastocysts. Both $\Delta\Psi m$ and the ROS level were increased in parallel during *in vitro* embryo development with and without HA, but this increase was less pronounced in the presence of HA. Hence, our quantitative data unequivocally show that supplementation of NCSU-23 culture medium with 1 mg/mL HA improves the developmental potential and quality of pig embryos. This effect results from a significant decrease in the ROS level induced by the HA-dependent $\Delta\Psi m$ reduction.

1. Introduction

Culture conditions, including media composition, are the main factors affecting the yield and quality of pig blastocysts obtained *in vitro*. Several media, such as North Carolina State University (NCSU)-23 [1,2] or NCSU-37 media [3], Beltsville embryo culture (BECM)-3 [4] and porcine zygote medium (PZM) [5], are readily available for the successful culture of porcine embryos to the blastocyst stage [6]. In our work, we have been using NCSU-23 medium because it provides the most suitable environment for porcine embryo culture regarding a high percentage of cleaved embryos, obtained morulae and blastocysts, as well as a relatively high total cell number [7,8]. Due to a better understanding of embryo metabolism, in addition to salts and proteins, culture media contain defined levels of different energy sources: glucose, calcium, lactate and pyruvate. However, such cultured pig embryos still possess lower developmental competence, expressed as a percentage of cleaved embryos, and fewer morulae and blastocysts

* Corresponding author.
E-mail addresses: marek.romek@uj.edu.pl (M. Romek), barbara.gajda@izoo.krakow.pl (B. Gajda), e.krzysztofowiczromek@upcpoczta.pl (E. Krzysztofowicz), marcin.kucia@onet.pl (M. Kucia), agnieszka.uzarowska@doctoral.uj.edu.pl (A. Uzarowska), zdzislaw.smorag@izoo.krakow.pl (Z. Smorag).

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than their *in vivo* developed counterparts [6]. Moreover, it was demonstrated that *in vitro* culture reduced embryo quality by lowering the total cell number in blastocysts [7], number of the cells in the inner cell mass [8], diameter of produced blastocysts [9,10] and increased the frequency of apoptosis [11]. The transfer of blastocysts with such parameters into the reproductive tract of recipient gilts resulted in a lower percentage of pregnancies and smaller litter sizes [6,11].

It is also possible to improve the developmental competence and quality of cultured porcine embryos using different supplements, e.g., vitamin E [12] and L-ascorbic acid [13], as well as different antioxidants, such as resveratrol [14], flavonoids [15] and β-mercaptoethanol [16]. Another promising supplement is hyaluronan (HA), a polysaccharide that belongs to the glycosaminoglycan family. It is found in the extracellular matrix of most animal tissues, as well as in the uterine, oviductal and follicular fluids in mice, pigs, cattle and humans [17]. For pigs, the physiological concentration of HA in these fluids ranges from 0.04 to 1.83 mg/mL [18]. It was also shown that for bovine oocytes and embryos, the addition of HA into maturation and culture media improved their developmental potential [19,20] and quality [21,22]. Furthermore, culture with HA improved bovine embryo development after freezing [21]. Regarding pigs, HA promoted the viability of oocytes during *in vitro* maturation [23], but experimental results published to date on the influence of HA on cultured embryo development have been contradictory [24,25]. Nonetheless, we expect that HA supplementation indeed exerts a beneficial effect on pig embryo survival rate and blastocyst quality because hyaluronan regulates various cellular functions such as signaling, gene expression, differentiation, and the cell cycle [26]. Hyaluronan increases cell survival [27] and reduces lipid peroxidation, as well as the incidence of apoptosis during *in vitro* bovine embryo development [20]. Moreover, HA directly influences cell bioenergetics [27], and in porcine oocytes and embryos, it raises the activity of superoxide dismutase, catalase and glutathione peroxidase [28]. Therefore, we hypothesize that hyaluronan may also modify the value of the inner mitochondrial membrane potential ($\Delta\Psi m$), a key factor in cellular energy metabolism, and the level of reactive oxygen species (ROS).

In pre-implantation mammalian embryos, mitochondria produce ATP and ROS molecules and participate in signal transduction, apoptosis, Ca^{2+} maintenance and redox homeostasis [29]. The inner mitochondrial membrane potential is involved in regulating these vital functions, especially ROS and ATP production. It was previously shown that the pattern of changes in $\Delta\Psi m$ during the development of mammalian embryos, from the zygote up to the blastocyst stage, is species specific [30]. This pattern is related to the metabolic alterations of pyruvate, glutamine, glucose in the Krebs cycle, fatty acids in β-oxidation and glycolysis [31,32]. Therefore, its measurement is important to monitor proper pre-implantation embryo development [29]. Furthermore, most ROS molecules are derived from the mitochondrial respiratory complexes, and the rate of their production correlates well with the value of $\Delta\Psi m$ [33]. Reactive oxygen species molecules are important signaling molecules involved in oocyte maturation, blastocyst formation and implantation [34]. However, a high ROS level, especially that of hydrogen peroxide, causes lipid peroxidation in biological membranes and influences RNA transcription, protein synthesis, an embryo cell block and apoptosis [35]. Consequently, a high ROS level is detrimental to embryo development by lowering the quality of the resulting blastocysts and decreasing the blastocyst development rate [36]. Hence, ROS level downregulation by embryo culture systems may improve the pig *in vitro* development.

The principal goal of this study was to decisively determine whether exogenous hyaluronan improves the developmental competence of pig embryos cultured *in vitro*. Furthermore, we aimed to determine whether HA supplementation increases the quality of produced blastocysts. To this end, we examined the influence of HA at three different concentrations in NCSU-23 media on the percentage of cleaved embryos, morulae and blastocysts, as well as on the quality of the blastocysts, determined by the total number of cells per blastocyst, the number of cells in the inner cell mass, the number of TUNEL-positive nuclei per blastocyst, the TUNEL index and the blastocyst diameter. Thus, we found the most effective concentration of HA for pig embryo culture. Because the inner mitochondrial membrane potential and reactive oxygen species level are vital to embryo development, for the first time, we measured these two parameters in pig blastocysts cultured at various HA concentrations and during pre-implantation embryo development from the zygote until the expanded blastocyst stage.

2. Materials and methods

Unless otherwise stated, all chemicals were supplied by Sigma Chemical Company (St. Louis, MO, USA). The experimental procedures used in this study were carried out in accordance with the European Directive 2010/63/EU and approved by the I Local Ethics Committee, Krakow, Poland, decision no. 159/2012.

Zygotes were obtained from six-month-old Polish Large White gilts at approximately 90–110 kg body weight. The donor gilts were superovulated by i.m. injection of 1500 IU pregnant mare serum gonadotropin (PMSG, Serogonadotropin, Biowet, Poland) followed by 1000 IU of human chorionic gonadotropin (hCG, Chorulon, Biomed, Poland) administered 72 h later. At the onset of estrus, i.e., 24 h after hCG injection, the gilts were artificially inseminated (AI) with the standard dose of semen [37]; Then, 24 h after AI, under general anesthesia, the zygotes were surgically collected after flushing the oviducts with phosphate-buffered saline (PBS) supplemented with 4 mg/mL bovine serum albumin (BSA) at 30 °C [38]. Only zygotes were found, and cleaved embryos were absent. The recovered zygotes were examined morphologically, and only those graded as excellent were chosen for further embryo culture [37].

In the first experiment, we analyzed the influence of HA (Croma Pharma, Korneuburg, Austria) supplementation in the culture medium at three different concentrations (0.25 mg/mL, 0.5 mg/mL, and 1 mg/mL) on the developmental potential and quality of pig embryos. No HA was added in the control group. For this, *in vivo*-derived zygotes were cultured up to the expanded blastocyst stage, and the percentages of obtained cleaved embryos, morulae and expanded blastocysts were counted. Next, for each analyzed group of expanded blastocysts, the following parameters were measured: total number of cells per blastocyst, number of cells in the inner cell mass, number of TUNEL-positive nuclei per blastocyst, TUNEL index, blastocyst diameter, mitochondrial membrane potential and ROS level. Three applied concentrations of HA were chosen on the basis of our preliminary experiments (not shown) and are within the physiological range in porcine oviductal fluid [18]. Hyaluronan supplied by Croma Pharma and used in the present study was derived from a strain of *Streptococcus zooepidemicus*. This bacterial glycosaminoglycan polysaccharide is a high grade and does not show variation in molecular weight. Zygotes were randomly assigned to all experimental and control groups. Usually, replicates were run in parallel for all analyzed groups, however, to yield more accurate and reliable summary statistics in our experimental work, especially for the control and 1 mg/mL groups, additional replicates were made.

In the second experiment, we determined the effect of 1 mg/mL HA supplementation in the culture medium on the values of the mitochondrial membrane potential and ROS level for defined stages of pre-implantation pig development. Embryos at the 2- to 4-cell,