Adaptation of the technology for culturing bovine embryos on commercial media for human embryos

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Abstract. We have studied the efficiency of using commercial media developed for human-assisted reproductive technologies (ART) for bovine embryos. The dynamics of bovine cells and embryos at the stages of maturation, fertilization, and preimplantation development were analyzed on three different media types, including ones particularly designed for bovine ART. Cumulus-oocyte complexes were divided into three groups: group 1 – maturation, fertilization, and culture were performed on media IVF-Bioscience (BO-IVM, BO-IVF, BO-IVC) developed for bovine animals; group 2 – maturation, fertilization, and culture were done on Vitrolife human ART media (G-IVF, G-TL); group 3 – maturation, fertilization, and culture were done on Irvine Scientific (Continuous Single Culture Complete) ART media. Significant distinctions in maturation, cleavage and blastulation indicators were found between embryos cultured on Vitrolife G-series human ART media and embryos processed on IVF-Bioscience bovine cattle media and Irvine Scientific Continuous Single Culture Complete human ART media. The difference in performance between the last two media was less pronounced. The obtained results make it possible to simplify the maturation of oocytes and the cultivation of bovine embryos to achieve reproducible outcomes without variability from batch to batch.

1 Introduction

The need to develop efficient techniques for obtaining and maturing animal oocytes is imposed by the intensification of research in the field of the fundamentals of gametogenesis and the improvement of cellular technologies, such as cloning, transgenesis, including genome editing using CRISPR/Cas9 technology (1-8).

The protocols of fertilization and in vitro culturing of oocytes of livestock developed to date provide a sufficiently high yield of preimplantation embryos at the cleavage stage. Nevertheless, the potential for further development in oocytes matured outside the body is still considerably lower than in oocytes matured *in vivo* (9-11). Enhancing the viability of embryos produced *in vitro* and ensuring a satisfactory birth rate of living descendants is one

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of the main research areas in reproductive technologies. Meanwhile, preimplant human embryos are currently the most studied owing to the widespread use of assisted reproductive technologies (ART) in medicine. The consequence of this was the onset of numerous culture media of industrial production around the world, which were developed considering the needs of human embryos at the stages of cleavage and blastulation. Most commercial culture media for human ART undergo multi-stage testing and quality control procedures. Therefore, they enable to achieve of a high level of embryo blastulation. Regardless, the mass production of media for livestock embryos is limited to one or two manufacturers, and logistics and transport conditions for delivery at the required temperature are not always available. Frequently, in experiments on bovine embryos *in vitro*, proprietary developments of culture media with variable composition are used, which can result in low reproducibility of the results (12). In order to successfully implement currently popular biotechnological programs and to conduct fundamental research on oogenesis and embryogenesis, it is essential to have optimal and available culture media to ensure the full development of early embryos *ex vivo* (13). This study aimed to determine whether the media developed for human ART can support the maturation, fertilization, and further preimplantation development of a bovine embryo at a level comparable to media specifically designed for bovine ART.

2 Materials and methods

2.1 Material sampling

After slaughter, the ovaries of cattle were selected and transported to the laboratory at 38.5°C in controlled temperature conditions for 4-5 hours. Aspiration of follicles from 2 to 15 mm was performed using an 18G needle attached to a 5-10 ml syringe. Aspiration of follicles and further processing of oocytes and embryos were done in sterile conditions of the clean zone in laminar flow units equipped with heated surfaces up to 38.5 °C. The resulting cumulus-oocyte complexes were randomly distributed into three groups: group 1 – maturation, fertilization and culture were performed on media IVF-Bioscience (BO-IVM, BO-IVF, BO-IVC) developed for bovine animals; group 2 – maturation, fertilization and culture were done on Vitrolife human ART media (G-IVF, G-TL); group 3 – maturation, fertilization and culture were done on Irvine Scientific (Continuous Single Culture Complete) ART media.

2.2 Maturation of oocytes

Maturation of oocytes of the first group was carried out for 24 hours in a culture media BO-IVM with Sage oil for tissue cultures at a temperature of 38.5 C, a carbon dioxide level of 6.5 vol.% and oxygen – 5.0 vol.%.

The embryos of the other two groups were matured by adding hormones 0.5 mcg/ml of follicle-stimulating hormone (FSH) and 0.5 mcg/ml of human chorionic gonadotropin (hCG) to the fertilization media: for the second group – in the G-IVF media, for the third group – in Continuous Single Culture Complete. Afterward, embryos were incubated for 24 hours in a culture media covered with Sage oil for tissue cultures at a temperature of 38.5 C, a carbon dioxide level of 6.5 vol.% and oxygen – 5.0 vol.%.

2.3 Fertilization of oocytes and embryo culturing

Cryopreserved bull sperm in 0.5 ml straws were used for in vitro fertilization. The straws were thawed in a water bath with a temperature of 37 °C for 30 seconds. 400 g of sperm was centrifuged in a density gradient using 3 ml 80% Percoll (Irvine Scientific) for 15 minutes. 200g of the sperm precipitate was washed after centrifugation with a buffer media containing 3 IU of heparin for 10 minutes.

Following centrifugation and washing, sperm with concentrations of 1.0–2.0 x10^6 active sperm per 1 ml was introduced into the fertilization media with cumulus-oocyte complexes: for the first group - BO-IVF, for the second group - G-IVF, and for the third group Continuous Single Culture Complete (without the addition of hormones). Then we incubated the resulting material by coating the media with Sage oil for tissue cultures at a temperature of 38.5 ° C, a carbon dioxide level of 6.5 vol.%, and oxygen - 5.0 vol.%.

16-18 hours post-insemination, the cumulus-oocyte complexes were denuded from cumulus cells and sperm cells and then transferred to the culture media: for the first group - BO-IVC, for the second group - G-TL, and the third group - Continuous Single Culture Complete. The embryos were cultured under the same incubation conditions using oil without changing the media for the entire period of development up to the blastocyst stage, which averaged 170-180 hours after fertilization.

The statistical processing was done on the Statistica 10.0 software package by nonparametric analysis methods with the determination of the reliability of differences by the Mann–Whitney U test.

3 Results

The following time parameters were evaluated:

A total of 375 cumulus-oocyte complexes of cattle were studied. The following assessment criteria were used:

- the maturation level was assessed by the number of oocytes with the first polar body after denudation;
- the cleavage embryo level was assessed by the number of embryos that began dividing 48 hours after fertilization;
- the embryo blastulation level is determined by the number of embryos that reached a blastocyst stage (Table 1, Figure 1).

Table 1. Culturing results of bovine embryos in three comparison groups on different media types.

Indicator	Group 1 (IVF-Bioscience)	Group 2 (Vitrolife)	Group 3 (Irvine Scientific)
Number of cumulus-oocyte complexes	$184^{***,(*)}$	$85^{/*/}$	106
Maturation	$128^{***,(**)}$	$20^{/*/}$	67
Cleavage	$111^{***,(*)}$	$10^{/**/}$	56
Blastulation	$41^{***,(*)}$	$0^{/***/}$	19

The significance level of differences between groups 1 and 2: p≤0.05; p**≤0.01; p***≤0.001*
The Significance level of differences between groups 1 and 3: p(*)≤0.05; p(**)≤0.01; p(***)≤0.001
The significance level of differences between groups 2 and 3: p//≤0.05; p/**/≤0.01; p/***/≤0.001*

The number of fertilized oocytes was not assessed, since it is complicated to visualize pronuclei in bovine oocytes due to the large number of lipid granules in the cytoplasm.

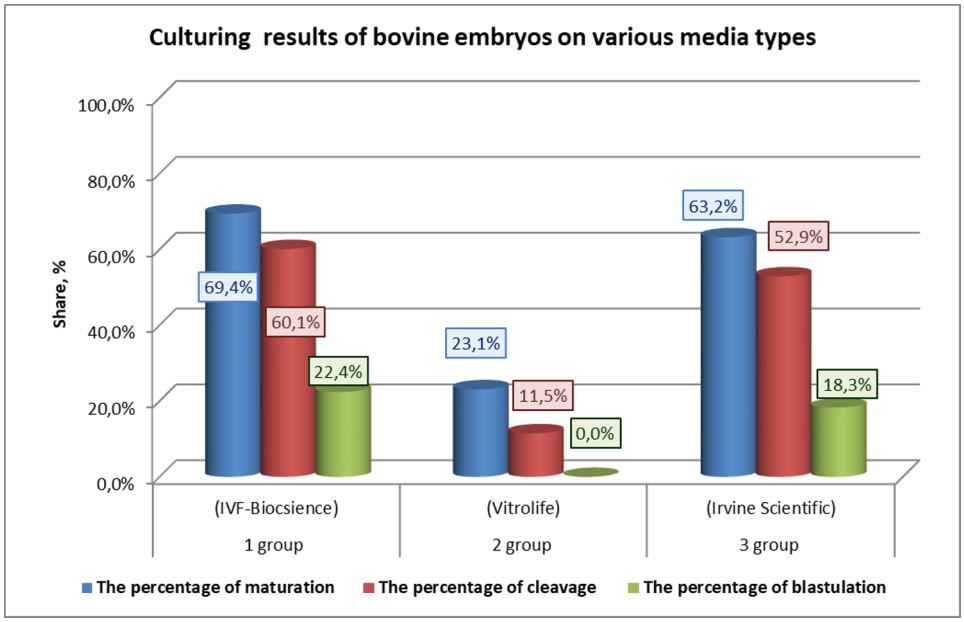

Fig. 1. Bovine embryos have matured, underwent cleavage and reached the blastulation stage on various media types.

4 Discussion

In this study, we have provided data that will assist in simplifying the maturation of oocytes and the cultivation of bovine embryos to obtain reproducible results without batch-to-batch variability.

According to our results, the difference in the maturation, cleavage and blastulation levels was less pronounced between bovine oocytes developing on specialized media IVF-Bioscience (69.4%, 60.1%, and 22.4%, respectively) designed for cattle, and on Irvine Scientific Continuous Single Culture Complete human ART media (63.2%, 52.9%, respectively). However, there is a decrease in the efficiency of Irvine Scientific media. Probably, this is due to the fact that the mechanisms of oogenesis and fertilization in all mammals are similar, but not completely identical. There were considerable differences in assessed parameters when cultured on Vitrolife G-series human ART media (23.1%, 11.5% and 0%, respectively). While commercial companies do not fully disclose the composition of ART media, it can be assumed that in the Vitrolife G-IVF fertilization media, the fertilization process is prevented by the presence of higher glucose content in comparison with the Continuous Single Culture Complete media. It is known that in the presence of glucose, there is acidification of bovine semen due to glycolysis, which blocks heparin-induced capacitation (14). This glucose effect can be eliminated by adding compounds to increase intracellular cAMP, such as 8-bromo-cAMP, isobutylmethylxanthine (IBMX) or prolonged cultivation with sperm for the metabolism of all glucose present in the media (15). When using Vitrolife G-IVF media for fertilization of human oocytes, such a reduction of sperm fertilization ability is not identified, since this media is broadly used in human IVF programs with good results. Probably, this is due to differences in the mechanisms of capacitation of human and bovine sperm. In this manner, it is known that the sperm of some mammalian species (e.g., human, mouse, rat) can be capacitated in vitro in normal media for embryo culture. However, other species require additional substances: heparin for cattle, epinephrine and taurine for hamster sperm (16). Such variations can be

explained by differences in the reproductive tracts of females of different mammalian species.

The good efficiency of using the Irvine Scientific Continuous Single Culture Complete media is probably also due to the fact that it is a universal medium that supports oocyte fertilization and zygote development without modification of the media from the fertilization day to the day of blastocyst formation. The maturation of bovine oocytes was conducted in the same media with the addition of hormones. Otherwise, a separate specialized media was used for each phase of embryo development in vitro: maturation, fertilization and culture to a blastocyst stage. The principles according to which culture media require modifications are developed and are based on the *in vivo* data about the stages of development, the conditions change according to each stage. Therefore, when cultured in vitro, the embryo is also exposed to 3-4 different media during development. Meanwhile, the effect of transferring a developing embryo between different culture media is unknown. Since different media may have various concentrations of ions and energy substrates, the embryo requires a energy and time to adapt. The adaptation of the embryo to the changing culture media may cause a decreased development potential. The Irvine Scientific Continuous Single Culture Complete media is designed according to the "embryo-free choice/single culture medium" paradigm. It means that it has all the necessary components to support the development of the embryo.

We did not compare the commercial media for ART with TSM199 media, which is often used in research for the maturation of bovine oocytes in vitro. Originally, this media was designed for culturing fibroblasts of chicken embryos. Thus, to support the development of preimplantation bovine embryos, TSM199 media requires supplements not only of hormones for oocyte maturation (FSH, LH/hCG, sometimes estradiol), but also fetal bovine serum (FBS), sodium pyruvate, an antibiotic (often gentamicin); sometimes antioxidants are added. This causes the final composition of the culture media to be highly variable and quite different from the initial composition (17). Additionally, TCM199 contains all 20 essential and non-essential amino acids. It is known that some amino acids are essential for the development of preimplantation embryos. Cysteine, for example, increases the content of glutathione and is important for the redox state of oocytes (18). Other amino acids can inhibit the development of the embryo (phenylalanine, valine, isoleucine, tyrosine, tryptophan, and arginine) (19).

The obtained results indicate that bovine oocytes can mature, fertilize and be cultured in some commercial media for human embryos.

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