AGRICULTURAL ACADEMY
INSTITUTE OF ANIMAL SCIENCES-KOSTINBROD

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IN VITRO METHODS FOR ASSISTED REPRODUCTION IN FARM ANIMALS

ABSTRACT

of a dissertation
for awarding the educational and scientific degree "Doctor"

Field of higher education:
6. Agricultural sciences and veterinary medicine
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Scientific specialty: Breeding of farm animals, biology and biotechnology of reproduction

Academic supervisor: Prof. Dr. Plamen Todorov

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The dissertation work was discussed and proposed for defence by the extended meeting of the Scientific Council of the Genetics, Breeding and Reproduction Department at the Institute of Animal Science - Kostinbrod.

The dissertation comprises 135 pages, 14 tables, 17 figures, and 23 photographs. In the list of cited literature, 179 literary sources in Latin are indicated.

The numbering of the sections, tables, figures and photographs in the Abstract does not correspond to those in the dissertation.

The defence of the dissertation will take place on ……..2024 from ……..hours in the hall ……of the Institute of Animal Science – Kostinbrod.

The materials related to the defence are available from the scientific secretary of IAS – Kostinbrod and on the website of IAS – Kostinbrod https://www.ias.bg/

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1. INTRODUCTION

Assisted reproductive technologies (ART) are used by breeders to improve the overall genetic profile of their herds. Obtaining bovine embryos by *in vitro* methods is important not only as a basic technology for the development of new scientific methods (intracytoplasmic sperm injections (ICSI), gene transfer, cloning by nuclear transfer, sex determination, preimplantation diagnostics, etc.) but in the breeding work.

Some commercial applications of *in vitro* fertilization technology include:

1. Improving the productive and genetic characteristics of the animals
2. Overcoming infertility of valuable highly productive animals
3. Production of transgenic and cloned animals
4. Provide a source of sexed embryos
5. Twin production in beef cattle
6. At the molecular level, the technique is used to elucidate events related to maturation, fertilization of oocytes, and embryo development, these events are difficult to study under natural conditions in living animals.

In vitro embryo production is a three-step process involving *in vitro* oocyte maturation (IVM), *in vitro* fertilization (IVF), and subsequent *in vitro* culture (IVC) of the zygote to the blastocyst stage. Embryo development *in vitro* is strongly influenced by events occurring during the maturation, fertilization, and subsequent development of fertilized oocytes. Improving efficiency and identifying sources of variation between IVF systems or between different laboratories are more important when routinely producing blastocysts from individuals of high genetic merit.

Oocytes used for research are usually obtained from the ovaries of slaughtered cows. In terms of efficiency, approximately 90% of immature oocytes undergo nuclear maturation and progress from prophase I to metaphase II, with approximately 80% undergoing *in vitro* fertilization and dividing at least once to the two-cell stage. However, more than half fail to reach the blastocyst stage, 7 to 8 days later. Thus, the main decline in development occurs during the last part of the process (IVC), and the percentage of blastocysts produced drops sharply. However, there is strong evidence that egg quality is a major factor determining blastocyst production. In many *in vitro* laboratories, matured bovine oocytes and embryos are successfully cryopreserved by vitrification.

Despite the results achieved so far, it is necessary to improve the *in vitro* method for obtaining bovine embryos and to study the influence of vitrification of matured bovine oocytes and embryos on their development.

2. PURPOSE AND OBJECTIVES

The present dissertation work aims to improve the method of obtaining bovine embryos *in vitro* and to study the influence of cryopreservation of oocytes and embryos by vitrification on their development.

The goal thus set implies the realization of the following main tasks:

1. Study the effect of the duration of transportation of the ovaries from the slaughterhouse to the laboratory, on the quality and quantity of the obtained cumulus-oocyte complexes.

2. Establishing the effect of the method for obtaining cumulus-oocyte complexes on the embryos obtained *in vitro*.

3. Study the effect of the functional state of the ovaries, the size, and number of follicles on the results of the *in vitro* obtained embryos.

4. Study the influence of the duration of the *in vitro* maturation of the cumulus-oocyte complexes on the results of the *in vitro* obtained embryos.

5. Investigate the effect of the addition of 5% or 10% FCS in *in vitro* culture with CR1, KSOM, or SOF media.

6. Investigate the effect of cumulus cells (CC) during vitrification of matured bovine oocytes.

7. Study of the effect of applying a simplified method for vitrification of embryos.

3. MATERIAL AND METHODS

The experiments were carried out during the period February 2019 - March 2023 at the *in vitro* laboratory of the Besamungsverein, Neustadt a.d.Aisch, Germany, at the *in vitro* laboratory of the Institute of Animal Sciences - Kostinbrod and at the National Institute for Agriculture and Food Research and Technology (INIA, Department of Animal Reproduction), Madrid, Spain.

3.1. MATERIAL

3.1.1. Equipment - stereo microscope, microscope, CO2 incubator, incubator, centrifuge, pH meter, vortex, electronic balance.
3.1.2. Consumables - Four Well Multidish plates, 35 mm diameter Petri dishes, 92 mm diameter Petri dishes, 6 well plates, 12 well plates, centrifuge tubes, 50 ml, standing, centrifuge tubes, 50 ml, beakers, screw bottles, automatic pipettes, serological pipettes.
3.1.3. Chemicals - Bovine serum albumin (BSA), BME Essential Amino Acids Solution, Heparin, Hyaluronidase type I-S, MEM Non-essential Amino acids Solution, MgCl2, NaCl 0.9%, Pyruvic acid.
3.1.4. Hormones - Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH)
3.1.5. Media for *in vitro* embryo production - All media are from Sigma, Germany
3.1.5.1. Phosphate-buffered saline (PBS) - 4 l bidistilled water, 32 g NaCl, 0.8 g KCl, 4.6 g $Na_2HPO_4*2H_2O$, 0.8 g KH_2PO_4, 0.4 g $CaCl_2$, 0.4 g $MgCl_2*6H_2O$, pH 7.3-7.4

3.1.5.2. Modified Parker's Medium (MPM) Medium for *in vitro* maturation of embryos
3.1.5.3. Sperm TALP - Capacitance medium
3.1.5.4. Fert TALP medium for *in vitro* fertilization
3.1.5.5. Media for *in vitro* culture of embryos - KSOM, CR1, SOF. Before work, the medium together with BME, MEM, and FCS was equilibrated at 39°C at 5%CO_2 for two hours.
3.1.5.6. TSM-199 - Ready medium from Sigma, Germany
3.1.6. Vitrification media

3.2. METHODS

3.2.1. Transport of the ovaries

Morphologically intact ovaries from slaughtered cows were collected from the abattoir. The transport to the laboratory was carried out within two to six hours in a solution of 0.9% NaCl at a temperature of 20 - 40º C. In the laboratory, the ovaries were washed with PBS solution before aspiration or slicing.

3.2.2. Collection and recovery of the cumulus-oocyte complexes (COC)

COCs were obtained by aspiration or by slicing. All follicles 2 - 8 mm in size were aspirated manually using a 5-cc syringe and an 18G needle. Slicing of the ovaries was done with a scalpel. The follicular fluid was collected in 50 ml conical centrifuge tubes, in which about 2 ml of MPM (Modified Parker's Medium) supplemented with 20% fetal calf serum (FCS) was previously placed. After 10 to 15 min, the sediment was aspirated using a Pasteur pipette and placed in a small petri dish containing about 3 ml MPM + 20% FCS. Sediment examination was performed under a stereomicroscope at 40x magnification. COCs with more than three layers of cumulus cells and uniform cytoplasm were left for experiments.

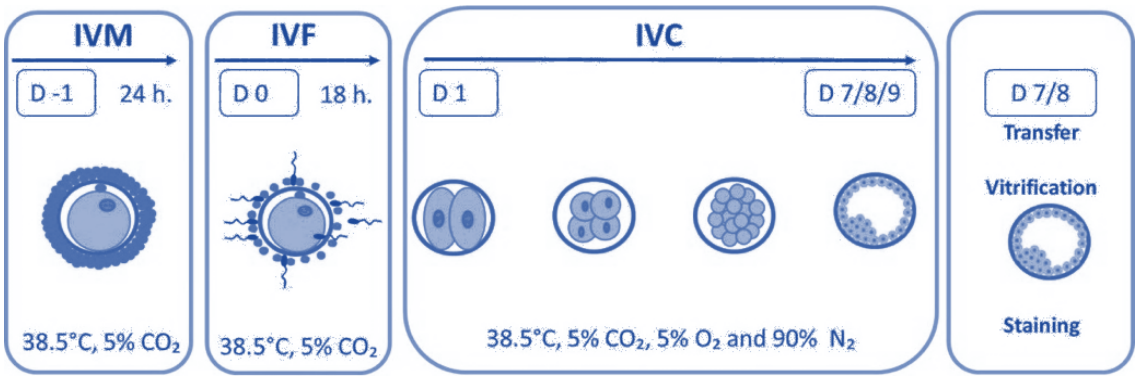


Figure 1: In vitro embryo production model

3.2.3. *In vitro* maturation

The maturation was performed in Modified Parker Medium (MPM) with the addition of 20% decomplemented (56°C, 30 minutes) estrous serum (ECS) and 2 mg/ml follicle-stimulating hormone (FSH).

Before starting the maturation, the maturation medium was equilibrated for two to three hours at 39°C, 5% CO_2 and maximum air humidity. For the maturation were used four

well multidish plates (Four Well Multidish Plates, Nunc) and 400 µl of medium was placed in each well and covered with 400 µl liquid paraffin. 20 to 40 COC were placed in one well for maturation. Maturation was carried out at 39°C, 5% CO_2 in air with maximum humidity for 18 - 24 hours. After completion of maturation, the oocytes were transferred to fertilization medium.

3.2.4. *In vitro* fertilization

3.2.4.1. Capacitation medium

A modified Tyrode's lactate medium (PARRISH et al., 1985) was used for capacitation, hereafter referred to as Sperm-Talp. The medium was prepared as a stock solution without phenol red, was stored at 4°C and could be used for up to four weeks. On the day of the experiment, 6 mg/ml BSA (Bovine Serum Albumin) and sodium pyruvate were added to the stock solution. The solution was filtered and the pH was adjusted to 7.4. The thus prepared capacitation medium was incubated in a thermostat at 39°C, 5% CO_2 and maximum air humidity.

3.2.4.2. Medium for *in vitro* fertilization

The so-called modified Tyrode's lactate medium hereafter referred to as Fert-Talp, was used for fertilization. It was prepared as a stock solution which, when stored at 4°C, could be used for up to two weeks. On the day of the experiment, 6 mg/ml BSA and sodium pyruvate were added to the stock solution, the pH was adjusted to 7.4, and the solution was filtered. Deep frozen as sterile solutions heparin, epinephrine, and hypotaurine were added to it. 100 µl of this medium was dropped into small petri dishes and covered with 3 ml of liquid paraffin. The prepared medium was equilibrated until use at 39°C, 5% CO2 in air and maximum humidity.

3.2.4.3. Sperm preparation

Two 500 µl straws of deep-frozen sperm from bulls were used for the *in vitro* fertilization for each experiment. The straws were dipped for 8 seconds in 39°C warm water and the seminal fluid was mixed in a pre-warmed centrifuge tube. 1 ml of Sperm-Talp was placed in pre-prepared tubes (1.8 ml. Nunc), 200 µl of seminal fluid was carefully layered with a micropipette, the tubes were closed and incubated for one hour at 39°C. After incubation, 800 µl of the supernatants were pipetted off, collected in a centrifuge tube and centrifuged for 10 min at 100 x G.

3.2.4.4. Fertilization of *in vitro* matured oocytes

The fertilization of the matured oocytes was carried out in small petri dishes. For this purpose, two drops of 100 µ1 of the fertilization medium were dripped into each plate and covered with 3 ml of liquid paraffin. Mature cumulus-expanded oocytes were washed once in fertilization medium and transferred to the drops. From 20 to 40 oocytes were cultured in one micro drop. Sperm at a concentration of ~106 sperm/ml were added to the microdroplets and co-cultured at 39°C, 5% CO_2 in air and maximum humidity. Incubation was carried out for 14 to 24 hours, depending on the start of the experiment.

3.2.5. *In vitro* cultivation

3.2.5.1. Cultivation media and conditions

After fertilization, oocytes were washed once and cultured in three types of media: Synthetic Oviductal Fluid (SOF), Charles Rosenkrans 1 (CR1) or Potassium simplex optimized medium (KSOM) supplemented with 5 or 10% fetal calf serum (FCS) on a monolayer from oviductal epithelial cells or a monolayer of granulosa cells. A fine pipette was used to remove the cumulus cells upon transfer to the culture medium.

For cultivation were used four well plates, each well containing 400 µl of SOF with 5 or 10% non-inactivated FCS. 20 to 40 oocytes were placed in each well, covered with 400 µl of liquid paraffin and incubated at 39°C, 5% CO2 in air and maximum humidity.

3.2.5.2. Embryos evaluation

After 44 to 48 hours of culture, the embryos were carefully separated from their surrounding epithelial cells and were evaluated morphologically. Embryos with intact, evenly separated blastomeres were considered as cleaved and therefore fertilized. After 48 and 140 to 168 hours, embryos were evaluated again under a stereomicroscope.

The embryos were transferred to culture media (CR1, SOF or KSOM) on epithelial cells. Media were changed 24 to 48 hours before embryo placing. Embryos were cultured in these media to the expanded blastocyst stage, without changing the media.

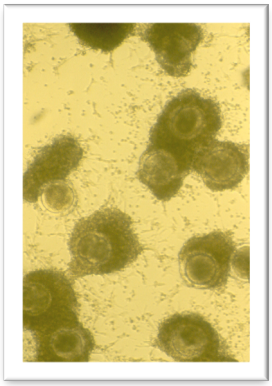


Photo 1: Zygotes and embryos at the 2-cell stage (48th hour)

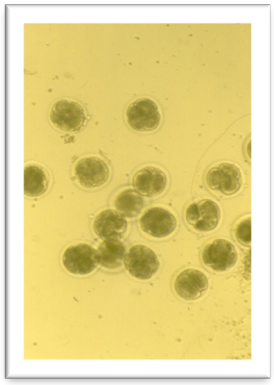


Photo 2: Embryos 4-6 cell stage (72nd hour)

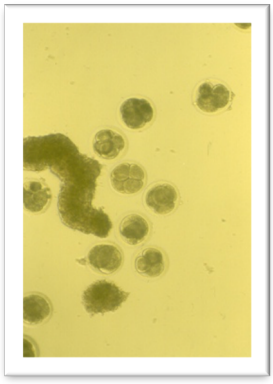


Photo 3: Embryos 8-16 cell stage (96th hour) co-cultured with granulosa cells

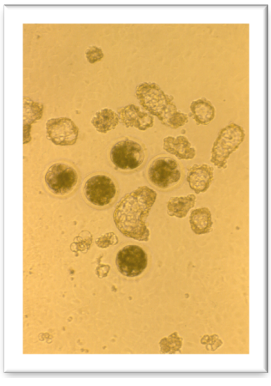


Photo 4: Compacting embryos - morula (120 hours, monolayer of epithelial cells)

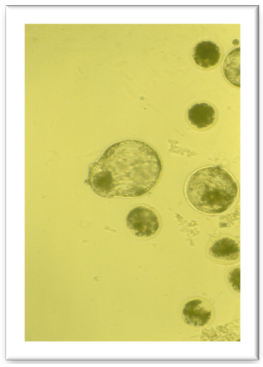


Photo 5: Expanded blastocysts and hatching blastocyst (Day 8-9)

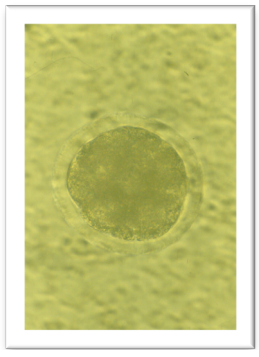


Photo 6: Compact morula on a monolayer of epithelial cells

COC staining

After 22 hours of *in vitro* maturation, COCs were completely stripped with 0.3% hyaluronidase in Ca-Mg-free DPBS and stained by the aceto-orcein method to examine the meiotic status of the oocyte. Oocytes were stained with 1% orcein in 45% acetic acid for 20 min

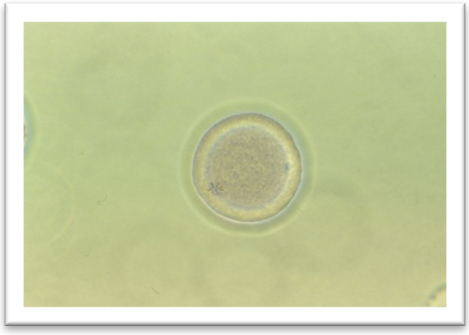


Photo 7: Oocytes in MII stained with aceto-orcein

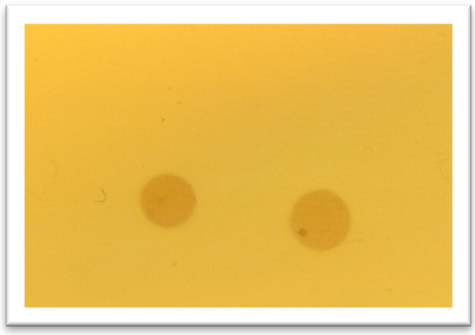


Photo 8: Oocytes in MII stained with aceto-orcein

In addition to the aceto-orcein method, oocytes were also stained with Hoechst 33342 for the stage of nuclear maturation such as germinal vesicle (GV), germinal vesicle breakdown, metaphase I (MI) or metaphase II (MII). For data analysis, oocytes at the GV stage were considered immature and at MII oocytes were considered fully mature, as germinal vesicle breakdown and MI stages were combined and defined as intermediate stages.

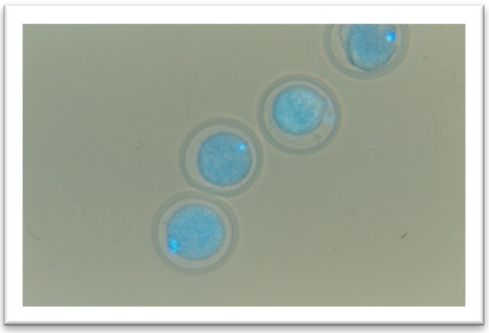


Photo 9: Oocytes in MII stained with Hoechst 33342 (x200)

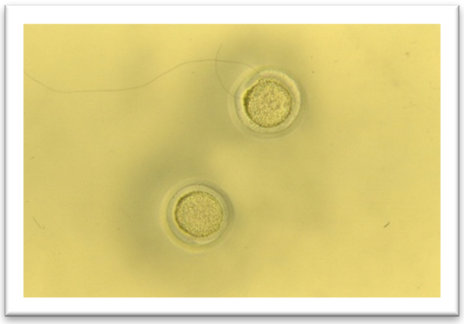


Photo 10: Oocytes in MII stained with Hoechst 33342 (x200)

3.2.6. Vitrification

3.2.6.1. Vitrification and warming procedures

3.2.6.1.1. Media preparation modified from Kuwayama et al. 2007

In vitro matured bovine oocytes were vitrified in manipulation medium TCM199 supplemented with 20% fetal calf serum (FCS). This medium is the base for the preparation of all vitrification media. The vitrification and thawing steps were performed at 38.5 °C, on a heated surface.

Vitrification was performed in two stages: equilibration and vitrification. Oocytes were equilibrated by sequentially transferring them into three drops of 75 µl equilibration medium composed of manipulation medium supplemented with 7.5% ethylene glycol (EG)

and dimethyl sulfoxide (DMSO). After the oocytes regained their initial volume, they were transferred into four consecutive 50 µl drops of vitrification medium composed of manipulation medium with added 15% EG, 15% DMSO, and 0.5 M sucrose. Oocytes were placed in equilibration medium for 10–15 min and vitrification medium for 45–60 s. After freezing and standing for one week in liquid nitrogen (LN2), oocytes were thawed by transferring them to a warming medium composed of handling medium with 1 M sucrose. This was followed by a three-step wash of hyperosmolar sucrose reduced from 1 M to 0.5 M (first wash for 3 min), 0.25 M (second wash for 5 min), and 0 M in manipulation medium (third wash for 5 minutes). Oocytes were washed in manipulation medium three times and then incubated in maturation medium for 2 h to allow them to recover.

At least a day before vitrification, the basic media were prepared: Equilibration medium, vitrification medium, diluent and manipulation medium.

The most commonly used cryodevices for vitrification are straws and cryotop. The Cryotop cryodevice was used in the vitrification experiments of matured bovine oocytes:

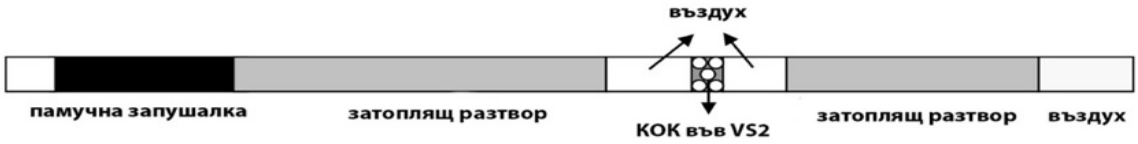


Figure 2: Loading a 0.25 ml straw cryodevice for vitrification of cumulus-oocyte complexes (COCs).



Figure 3: Loading cryodevice Cryotop for vitrification of cumulus-oocyte complexes

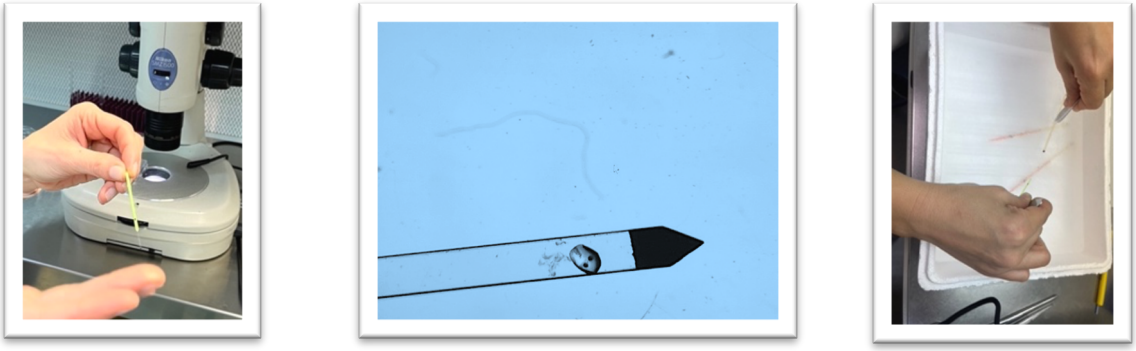


Photo 11, 12, 13: Loading the cryodevice Cryotop

4. RESULTS AND DISCUSSION

The results presented in the dissertation can be conditionally divided into two groups:

The first group of experiments covers the preparation of cumulus oocyte complexes, in vitro maturation, in vitro fertilization and in vitro cultivation.

4.1. Effect of the length of time of transport of ovaries from the slaughterhouse to the laboratory on the quality and quantity of cumulus-oocyte complexes obtained and effect of the temperature of the transport environment

An experiment was conducted to monitor the effect of the length of time for transporting the ovaries from the slaughterhouse to the laboratory on the quality and quantity of the resulting cumulus-oocyte complexes. Ovaries that were obtained from slaughtered cows were divided into three groups, according to the duration of the transportation time, 2, 4 and 6 hours, respectively. In each group there were ten ovaries placed in a solution of 0.9% NaCl at a temperature of 20ºC. In this experiment, COCs were obtained only by slicing the ovaries. An assessment was made according to the criteria for classifying the cumulus-oocyte complexes.

Figure 4 compares the results of the duration of transport time of the ovaries from the slaughterhouse to the laboratory. Very good quality COCs were transported in 2 hours to the laboratory.

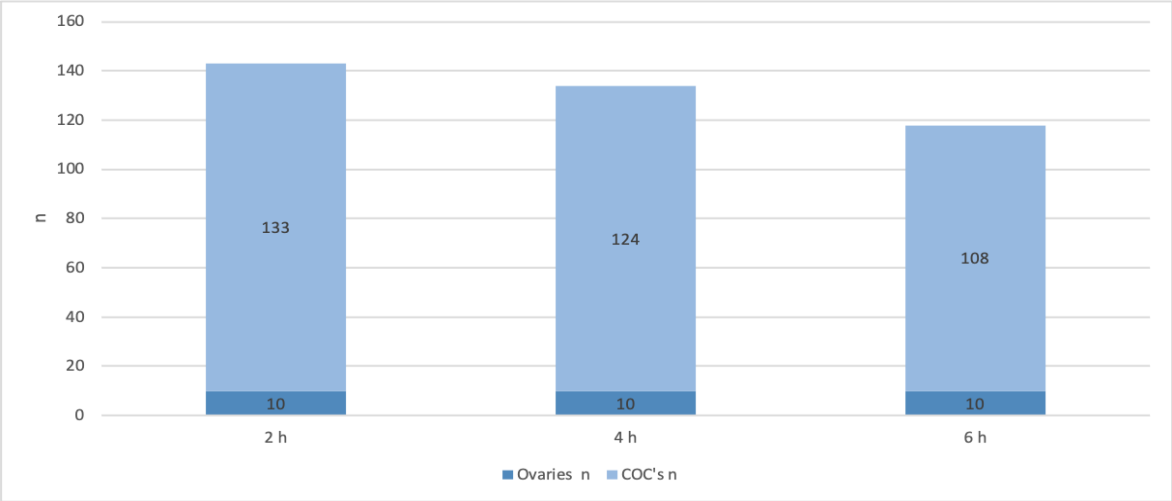


Figure 4: Effect of the duration of transport time of the ovaries from the slaughterhouse to the laboratory

In addition to the duration of transport of the ovaries from the slaughterhouse to the laboratory, the influence of the transport temperature on the COC quality was also investigated. With a duration of transport of the ovaries from the slaughterhouse to the laboratory of 2 hours, three medium temperatures were tested. Any change in temperature affects the quality of COC, and at a temperature of up to 20ºC the quality is very good. At

an environment temperature between 20°C and 30°C, both quality and quantity are good, and at a temperature of 30°C and 40°C, the quality is satisfactory.

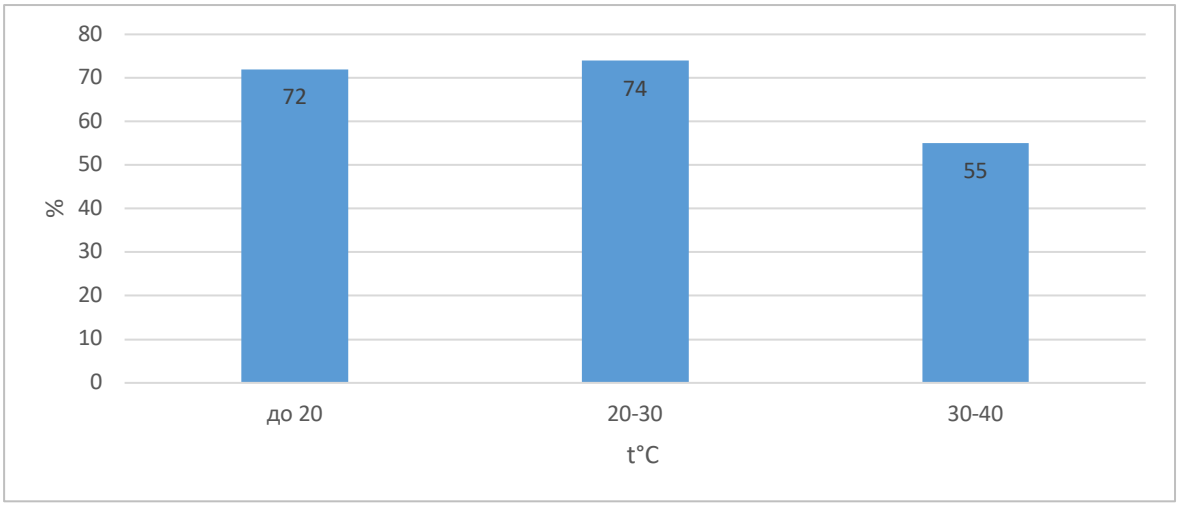


Figure 5: Effect of the temperature of the transport medium of the ovaries from the abattoir to the laboratory on the quality of the obtained oocytes

4.2. Effect of the COC recovery method on their amount

In the conducted experiment to establish the effect of the method for obtaining cumulus-oocyte complexes on their quantity, the results of obtaining oocytes by aspirating, by slicing and a combined method the ovary were compared. The total number of COCs obtained from one ovary by aspiration averaged 10.88 oocytes, 14.46 by slicing and 18.38 by the mixed method (aspiration plus slicing) (Figure 6).

The mixed method produced significantly more COCs ($P<0.05$) compared to aspiration alone or slicing alone, but no significant effect was found between slicing and aspiration plus slicing (P>0, 05). This is probably due to the fact that the incision reaches follicles that are located deep in the cortex and are not visible.

The disadvantage of this method is that a larger amount of blood and cells are obtained in the manipulation medium and the process of isolating quality oocytes is difficult. In addition, the likelihood of mycotic contamination is greater. According to our research, the slicing method produced a higher number of moderate-quality COCs and more embryos, while the aspiration method produced a moderate number of high-quality COCs and a good number of embryos. The functional state of the ovaries and the estrous cycle of the cows were not included in this experiment. Methods of obtaining oocytes do not affect subsequent embryonic development after *in vitro* fertilization at the MII stage.

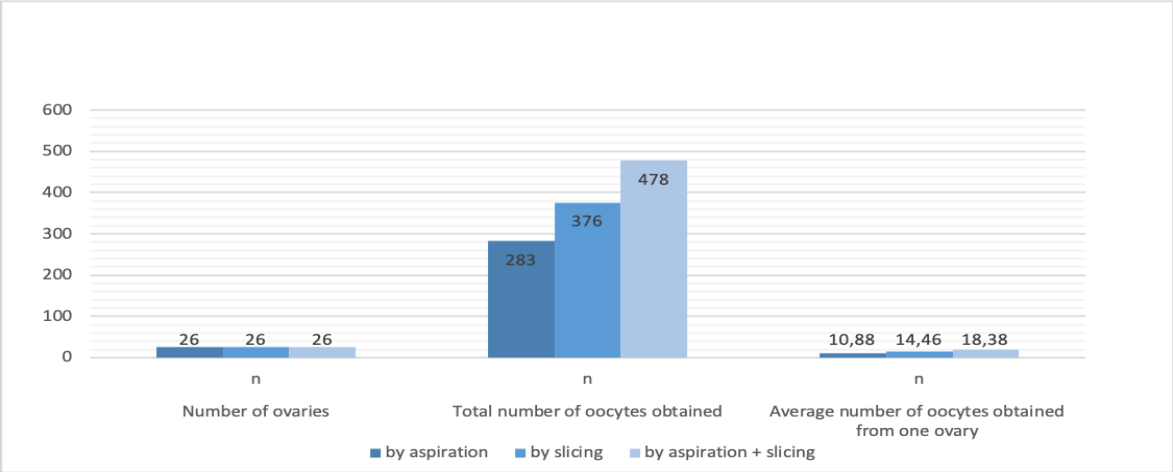


Figure 6: Effect of COC preparation method on the amount of oocytes obtained

4.3. Effect of ovarian functional status and different follicle size on the developmental potential of bovine oocytes.

The experiment investigated the effect of corpus luteum and different follicle size on the developmental potential of bovine oocytes.

Ovaries were obtained from cows with a clinically normal reproductive tract after slaughter. To assess the developmental potential of the oocytes, they were divided into three groups: with corpus luteum (CL+), without corpus luteum (CL-), and control ovaries. After classifying ovaries based on the presence or absence of CL, follicles were divided into three groups according to their diameters—small (SF, 3–6 mm), medium (MF, 6–9 mm), and large (LF, 9–15 mm). In the experiment, 1197 oocytes were included, divided into 7 experimental groups based on their origin (with corpus luteum - SF, with corpus luteum - MF, with corpus luteum - LF and without corpus luteum - SF, without corpus luteum - MF, without corpus luteum - LF and control), and their developmental potential was assessed after the *in vitro* process. In the control, oocytes were unselected based on the presence or absence of CL and follicle size.

Table 1 shows the developmental potential of oocytes obtained from corpus luteum and non-corpus luteum ovaries of different follicle sizes. In this experiment, follicle size had a definite effect on oocyte development, and oocytes from small follicles were considered to have the lowest development rate among the groups.

However, there is heterogeneity in the developmental competence of oocytes originating from different sized ovarian follicles with CL. The percentage of blastocysts obtained from ovaries without CL and medium follicles and without CL and large follicles was greater ($P<0.001$) than that of ovaries with CL and small follicles and ovaries with CL and medium follicles. However, there were no significant differences in blastocyst formation rates between the control group, without CL and small follicles, and with CL and large follicles.

Table 1: Development of bovine oocytes obtained from ovaries with follicles of different sizes, with corpus luteum (CL) or without corpus luteum CL

Groups	Subgroups	Number of oocytes (n)	Divided (n %)		Blastocysts from the total number of oocytes (n %)		Blastocysts from the divided (n %)	
Effect of the corpus luteum	Control	226	186	82,5	83	36,9	77	41,2
	Without CL	438	361	82.5	172	39.4	166	46.1
	With CL	483	354	73.3	157	32.5	137	38.6
Size effect on the follicle	Control	226	186	82.5	83	36.9	77	41.2
	Small follicles 3 - 6 mm	371	290	78.2	118	31.8	102	35.2
	Medium follicles 6 – 9 mm	425	366	86.1	161	37.9	149	40.7
	Large follicles 9 –15 mm	125	102	81.2	53	42.1	48	47.5

(p <0.001)

Table 2 shows whether the presence or absence of a corpus luteum, by interactions with follicle size, had an effect on development to blastocyst (P<0.001)

Table 2: Effect on blastocyst development in presence or absence of corpus luteum by follicle size interactions

Groups	Subgroups	Number of oocytes (n)	Divided (n %)		Blastocysts from the total number of oocytes (n %)		Blastocysts from the divided (n %)	
Effect of CL and size of the follicle	Control	226	186	82.5	83	36.9	77	41.2
	Without CL SF (3 - 6 mm)	185	150	81.3	67	36.2^{ab}	59	39.1^{ab}
	Without CL MF(6 – 9 mm)	202	174	85.9	77	38.1^{a}	75	42.9^{a}
	without CL LF (9 –15 mm)	61	51	83.8	24	39.8^{a}	23	45.9^{a}
	With CL SF (3 - 6 mm)	186	141	75.8	57	30.8^{c}	51	36.2^{b}
	With CL MF (6 – 9 mm)	223	183	82.1	75	33.5^{bc}	70	38.4^{b}
	With CL LF (9 –15 mm)	64	49	76.7	22	34.9^{b}	20	40.6^{ab}

CL - Corpus luteum
a, b, c - Values in the column with different superscripts are significantly different (p < 0.001). (Student's t-test).
without CL – without corpus luteum, with CL – with corpus luteum, SF – small follicles, MF – medium follicles, LF – large follicles

In this experiment, the presence or absence of CL by follicle size interactions had no effect on the extent of expansion and separation between groups.

Another source of heterogeneity in oocyte developmental competence is the size of the follicle from which the oocyte is derived. Therefore, the present experiment was conducted to evaluate the developmental potential of oocytes obtained from CL and non-CL

ovaries and different follicle sizes. The results showed that the presence or absence of CL, by accounting for interactions with follicle size, had an effect on the percentage of oocyte-derived blastocysts. The percentage of blastocysts derived from oocytes from medium and large ovarian follicles lacking CL was greater than that from oocytes derived from small and medium ovarian follicles having CL. Furthermore, our results showed that there was a significant difference in the percentage of development to blastocysts between oocytes derived from follicles of the same size from ovaries lacking CL and ovaries with CL. The rates of blastocyst formation from oocytes derived from small and medium ovarian follicles with CL have been shown to be lower than those from ovaries that are without CL.

In the present experiment, there was homogeneity in the developmental competence of oocytes originating from small-, medium-, and large-sized ovarian follicles that lack corpus luteum. Follicle size had a positive effect on oocyte developmental competence, with the percentage of blastocysts obtained from ovaries with CL and large follicles being greater than those obtained from ovaries with CL and small follicles. In cattle, goats and sheep, oocytes derived from large follicles are more competent for *in vitro* embryonic development than oocytes derived from small follicles. Our results confirmed these observations, in which oocytes from small follicles were thought to have the lowest developmental rates in the groups.

Furthermore, in the present experiment, all oocytes derived from CL ovaries were not affected by the negative effects of the corpus luteum, and there was heterogeneity in oocyte development due to the effect of follicle size. When the diameter of the follicle increases to approximately 2 mm (small follicles) and the oocytes increase in diameter from 110 to 120 μm, developmental competence is acquired and the majority of oocytes after fertilization continue their embryonic development. Therefore, it can be assumed that the corpus luteum has a negative impact mostly on the growth of small growing follicles rather than on large growing follicles.

On the other hand, the concentration of various biochemical constituents of the follicular fluid may change with advancing follicular development and the presence or absence of CL. Follicular fluid from follicles in non-CL bovine ovaries is more suitable for use in oocyte maturation than that from CL ovaries because their data showed that during aspiration, the metabolite content of follicular fluid from ovaries that did not carrying CL was higher compared to those of ovaries carrying CL. These different biochemical constituents of follicular fluid may be responsible for higher rates of cleavage and reaching blastocysts from non-CL ovaries than those from CL ovaries. In addition, the glucose content of follicular fluid from large follicles was significantly greater compared with that from small and medium follicles in non-CL ovaries. Therefore, the presence or absence of CL through interactions with follicle size has an effect on the oocyte environment and their developmental competence as observed in our experiment. After the formation of the CL, new blood vessels form and so it will receive a greater volume of blood flow than other tissues in the ovary. Indeed, the increase in blood flow to follicular cells in non-CL ovaries leads to an increased supply of gonadotropins and other systemic biochemical and hormonal factors required for follicular development. Oocytes originating from large follicles were not affected by the negative effects of CL as much as those originating from small and medium follicles. Indeed, collection of ovaries categorized based on the presence or absence of CL

and different follicle sizes would be useful in the commercial production of bovine laboratory embryos.

Not only the size of the follicle, but also the diameter of the retrieved oocyte was also positively correlated with the increased quality and *in vitro* developmental competence of the oocytes. Oocytes derived from larger follicles have a larger diameter. Similarly, *in vitro*-derived embryos from oocytes derived from smaller follicles (<6 mm) have lower developmental competence due to incomplete nuclear and cytoplasmic maturation, a greater degree of atresia, and a higher degree of abnormalities at fertilization, lack of production of important proteins that are required for growth competence *in vitro* (SAAD et al., 2019). Therefore, it is plausible to assume that for good oocyte quality and a better *in vitro* result, follicles ≥ 6 mm should be aspirated.

Oocytes derived from < 6 mm follicles have insufficient synthesis of maternal mRNA, which is required for the compaction and differentiation of advanced stage embryos, and a lack of production of essential proteins that are required for *in vitro* growth competence. According to some authors, the mean number of oocytes obtained from ovaries with a follicle size ≥6 mm is up to 3 times higher than that of <6 mm follicles.

Therefore, it is plausible to assume that for good oocyte quality and a better outcome of *in vitro* derived embryos, follicles larger than 6 mm should be aspirated.

4.4. Effect of duration of maturation on cleavage and further development of *in vitro* derived embryos

An experiment was conducted to determine the influence of the duration of maturation on the cleavage and further development of *in vitro*-derived embryos. The resulting COCs were divided into five groups. Maturation was performed in MRM medium supplemented with 20% decomplemented (56°C, 30 minutes) estrous serum (ECS) and 2 mg/ml follicle-stimulating hormone (FSH). Graduation was terminated at different times during two hours (18, 20, 22, 24 and 26 hours) from its beginning.

The matured oocytes were then fertilized in TALP medium and cultured to the blastocyst stage. The obtained results are presented in table 3:

Table 3: Effect of duration of in vitro maturation on bovine oocyte development

Duration of maturation hours	Number of oocytes n	Divided		Morula		Blastocyst	
		n	%	n	%	n	%
18	247	119	48.2[a]	60	50.5	31	26.1[c]
20	270	153	56.7	76	49.7	44	28.8[b]
22	225	143	63.6	74	51.8[a]	41	28.7[b]
24	240	145	60.4[a]	73	50.4[a]	37	25.5[a]
26	269	138	51.3[c]	49	35.5	31	22.4

P <0,05

The highest degree of cleavage was at the matriculation duration of 22 hours - 63.6%, and the lowest at the matriculation of 18 hours - 48.2%. The highest percentage in the morula stage was reached by 51.8% of the divided oocytes matured during 22 hours. Of the separated, the highest percentage of blastocysts, 28.8%, was found in those matured for 20 hours. The most suitable hours for in vitro maturation of oocytes that have reached the blastocyst stage are 20 and 22 hours.

4.5. Effect of different FCS concentrations in three in vitro culture media – CR1, SOF and KSOM

The successful development of in vitro produced (IVP) bovine embryos depends on many factors during the stages of in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC). In in vitro embryo production, the culture medium can affect embryonic development.

Despite the fact that bovine embryos can be cultured in vitro in a medium under the defined conditions, the beneficial effect of fetal calf serum (FCS) affects their development. Embryos cultured without proteins show changes in metabolism, reduced development (THOMPSON et al., 1998) and in cell number (LIM et al., 2007) compared to embryos cultured in media with the presence of proteins.

The requirement for biological components such as FCS or bovine serum albumin (BSA) in IVP is a matter of debate due to their complex and undefined compositions. FCS has been shown to increase membrane permeability and increase the number of embryos that reach the blastocyst stage, as well as the proportion of blastocysts hatched.

Despite the common use of several media for in vitro embryo production, comparisons between supplements of different FCS concentrations in different in vitro culture media have not been systematically tested. This experiment was done to evaluate the effect of different FCS concentrations in three (CR1, KSOM and SOF) bovine embryo culture media.

Increasing the concentration of FCS from 5 to 10% in CR1 medium decreased the rate of separation ($P < 0.05$). However, there were no significant differences in development to blastocysts ($P > 0.05$) and hatched blastocysts ($P > 0.05$) between embryos cultured with 5 or 10% FCS (Table 4).

Table 4: Effect of addition of 5 or 10% fetal calf serum (FCS) in CR1 medium on in vitro development of bovine embryos

FCS (%)	Fertilized oocytes (n)	Divided (n	%)	Blastocyst (n	%)
5	420	350	(83.37) [a]	167	(39.81)
10	306	199	(65.17) [b]	104	(34.23)

With KSOM medium, the degree of separation was similar for zygotes cultured in 5 or 10% FCS ($P > 0.05$). While the proportion of blastocysts was significantly greater ($P < 0.05$) when embryos were cultured in KSOM supplemented with 5% compared with 10%

FCS, hatching rates were not significantly different (P > 0.05) between groups. , as shown in Table 5.

Table 5: Effect of addition of 5 or 10% fetal calf serum (FCS) in KSOM medium on in vitro development of bovine embryos.

FCS (%)	Fertilized oocytes (n)	Divided (n	%)	Blastocyst (n	%)
5	405	296	(72.98)	165	(40.75) [a]
10	278	193	(69.42)	73	(26.26) [b]

Similar results were observed with SOF medium (Table 6). Different concentrations of FCS did not affect the rate of separation (P > 0.05). When the FCS concentration was increased from 5 to 10%, the rate of blastocyst development was significantly reduced (P < 0.05). However, there were no significant differences (P > 0.05) in blastocyst hatching between embryos cultured with different concentrations of FCS.

Table 6: Effect of addition of 5 or 10% fetal calf serum (FCS) in SOF medium on in vitro development of bovine embryos.

FCS (%)	Fertilized oocytes (n)	Divided (n	%)	Blastocyst (n	%)
5	421	351	(83.37)	226	(53.68) [a]
10	320	257	(80.46)	90	(28.15) [b]

Reduction to 5% FCS during in vitro culture resulted in a higher percentage of blastocysts in KSOM and SOF medium. This increase in blastocysts when FCS concentration was reduced in SOF culture medium has been described previously (ASSUMPÇÃO et al., 2002). The decrease in the percentage of blastocysts in medium supplemented with higher concentrations of FCS may be due to morphological changes provoked by FCS. Several studies have shown lipid accumulation in ruminant embryos cultured in serum-supplemented media (REIS et al., 2003). Morula and blastocysts developing in BSA-supplemented TCM199 have a large number of high-density lipid droplets suggestive of unsaturated fatty acid content, while those cultured in BSA-free medium have large vesicles resembling lysosomes but with less lipid droplets similar to *in vivo* developed morula and blastocysts.

Peroxidation of these lipids can cause structural damage affecting membrane function and permeability especially in mitochondrial membranes and lead to irreversible loss of functions such as respiration, oxidative phosphorylation, ion transport (KOWALTOWSKI and VERCESE, 1999) and altered embryonic metabolism.

The increase in the number of blastocysts when cultured in KSOM and SOF supplemented with 5% FCS may be due to less accumulation of lipid droplets and less changes in mitochondria. This leads to slight changes in energy metabolism, providing better energy utilization to achieve higher blastocyst levels compared to embryos cultured in 10% FCS medium.

Using CR1 medium, there was no significant difference in the rate of development to blastocysts when cultured with 5% or 10% FCS. This is likely due to a higher concentration of pyruvate in the CR1 medium (2.0 M) compared to SOF (0.30 mM), KSOM (0.5 mM) and the physiological concentration in the oviduct (0.5 mM). Apparently, the amount of pyruvate added to the medium does not affect embryonic metabolism. However, among the FCS-induced mitochondrial changes, there was a significant presence of a large number of immature mitochondria, similar to early embryonic stages. ATP production and glucose consumption are low in the immature mitochondria of early embryos.

Thus, the addition of 10% FCS to the culture medium may have provoked the increase of immature mitochondria in later-stage embryos and the consumption of more pyruvate than embryos with mature mitochondria.

Another suggestion as to how pyruvate concentration may have reduced the damage provoked by elevated serum concentration in CR1 medium compared to the other mediums could be the ability of pyruvate to inhibit apoptosis induced by oxidative stress. Free radicals produced by serum in the embryonic in vitro medium reduce cell number and regulate mitochondrial-mediated apoptosis. In addition, the serum induced an increase in the apoptosis rate and a decrease in the number of blastocyst cells in in vitro produced bovine embryos. Pyruvate acts as an antioxidant in cells, inhibiting reactive oxygen species (ROS) through changes in cellular state. Pyruvate has been shown to have the ability to eliminate hydrogen peroxides (H2O2) and other ROS (BASSENGE et al., 2001).

As a result of the experiments carried out, it was found that the reduction of FCS concentrations in the media KSOM and SOF were beneficial for the in vitro cultivation, resulting in a higher percentage of blastocysts.

The second group of experiments involved cryopreservation by vitrification of immature oocytes and vitrification of embryos.

4.6. Effect of cumulus cells on vitrification of matured bovine oocytes

Despite the great progress made, vitrification of oocytes and embryos remains a challenge because after thawing their survival and development are compromised. In order to implement the best vitrification protocol, one must minimize the damage that oocytes and embryos suffer during the process and achieve a higher rate of survival and development.

Experiments were conducted to develop and optimize the strategy for vitrification of matured bovine oocytes and embryos. To realize this general goal, it was necessary to determine the effect of cumulus cells on the vitrification of matured bovine oocytes.

Vitrification and thawing

In vitro matured bovine oocytes were vitrified with some modifications as described by KUWAYAMA et al. (2005). The manipulation medium (CM) used was TCM199 supplemented with 20% fetal calf serum (FCS). This medium is the base for the preparation of all vitrification media. The vitrification and thawing steps were performed at 38.5 °C, on a heated surface.

Vitrification was performed in two stages: equilibration and vitrification. Oocytes were equilibrated by sequentially transferring them into three drops of 75 μl equilibration

medium (CE) composed of manipulation medium supplemented with 7.5% ethylene glycol (EG) and 7.5% dimethyl sulfoxide (DMSO). After oocytes recovered their initial volume, they were transferred into four consecutive 50 μl drops of vitrification medium (VC) composed of manipulation medium (MM) supplemented with 15% EG, 15% DMSO, and 0.5M sucrose. Oocytes were placed in the equilibration medium for 10–15 min and in the vitrification medium for 45–60 s. After freezing and standing for one week in liquid nitrogen (LN2), oocytes were thawed by transferring them to a warming medium composed of handling medium with 1 M sucrose. This was followed by a three-step wash of hyperosmolar sucrose reduced from 1 M to 0.5 M (first wash for 3 min), 0.25 M (second wash for 5 min), and 0 M in manipulation medium (third wash for 5 minutes). Oocytes were washed in manipulation medium three times and then incubated in maturation medium for 2 h to allow them to recover.

In vitro fertilization and in vitro cultivation

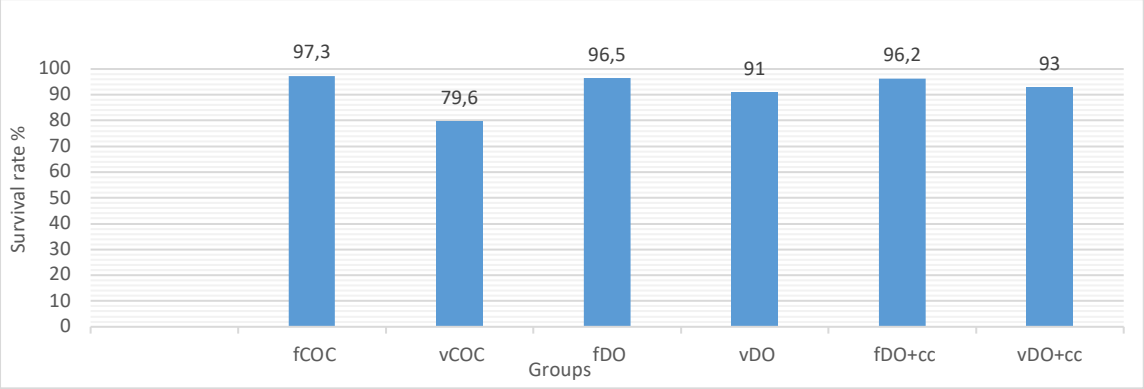
Fresh oocytes (non-vitrified) and vitrified oocytes were fertilized under identical conditions with frozen/thawed bull semen. A final sperm concentration of 1x106 sperm/ml was adjusted with Tyrode's albumin – lactate – pyruvate (TALP) fertilization medium consisting of bicarbonate-buffered Tyrode's solution supplemented with bovine serum albumin (BSA) (6 mg/ml) and heparin (25 mg/ml). At 21 hours after in vitro fertilization, the cumulus cells of presumptive zygotes were removed, washed and cultured in groups of 25 in 50 μl drops of Synthetic oviductal fluid medium (SOF) supplemented with 0.4% bovine serum albumin. Cultivation was performed in an incubator at 39°C in 5% CO2 air at maximum humidity. Cleavage rates were determined 48 h post-fertilization, and development to the blastocyst stage on day 8.

In the experiment, matured bovine oocytes (n=1200) were randomly divided into two groups - fresh and vitrified. Fresh oocytes were also divided into three groups: fresh cumulus-oocyte complexes (fCOC), fresh naked (denuded) oocytes (fDO) and fresh naked oocytes, with added cumulus cells at the time of fertilization (fDO+cc). Oocytes in the vitrified group were also divided into three groups after warming: vitrified cumulus-oocyte complexes (vCOC), vitrified denuded oocytes (vDO), and vitrified denuded oocytes with added cumulus cells at fertilization (vDO+cc). Intact CCs that were added to the vitrified oocytes at the time of fertilization were obtained one day before thawing and were cultured under the same conditions as the rest of the oocytes. CCs were removed by pipetting after fertilization and before assessment of survival, cleavage and reaching the blastocyst stage.

The survival rate of the fresh oocyte groups was very close to 100%, with no significant differences between groups. However, the survival rate of vDO in groups (91.0%) and vDO+cc (93%) was higher than vCOC (79.6%), $p<0.05$.

Vitrified COCs had significantly lower survival rates (79.6%) compared to their fresh counterparts 97.3% respectively, ($p<0.05$).

Figure 7: Oocyte survival rates



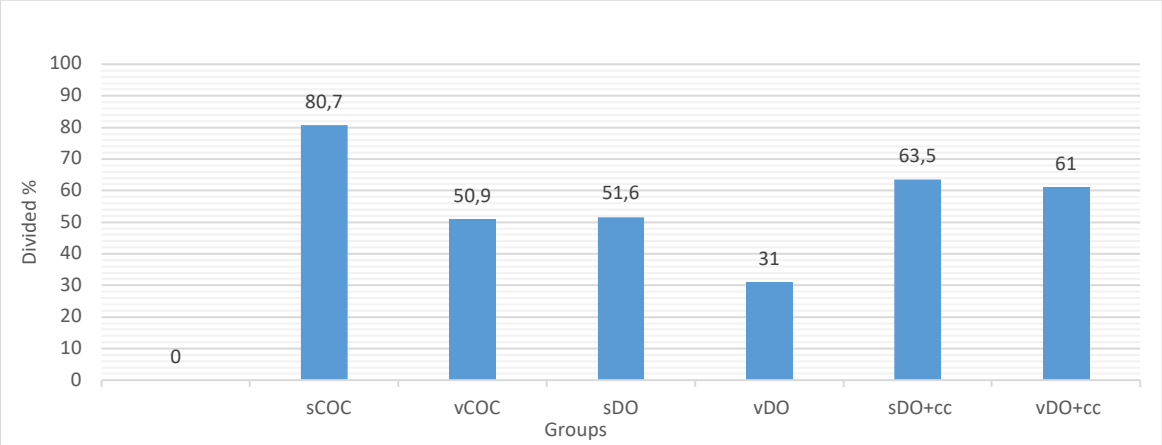
P<0,05;

vCOC – vitrified cumulus-oocyte complexes; vDO – vitrified denuded oocytes; vDO+cc – vitrified denuded oocytes with cumulus cells; fCOC – fresh cumulus-oocyte complexes; fDO – fresh denuded oocytes; fDO+cc – fresh denuded oocytes with cumulus cells

Removal of CC after maturation significantly reduced the number of divided oocytes ($p<0.05$). However, the increase in separation rate was greater with fDO+cc compared to fDO (63.5% vs. 51.6% respectively, $p<0.05$). The same effect was observed among the vitrified groups ($p<0.05$). (Figure 8)

Addition of CC to vitrified denuded oocytes (vDO+kk) resulted in division rates comparable to those of vitrified cumulus-oocyte complexes (61.0% vs. 50.9%, respectively). The division rate of vCOC (50.9%) and vitrified denuded oocytes (vDO) (31.0%) was significantly lower than fresh COC (fCOC) (80.7%) and fresh denuded oocytes (fDO) (51.6%), $p<0.05$

Figure 8: Development rate after fertilization

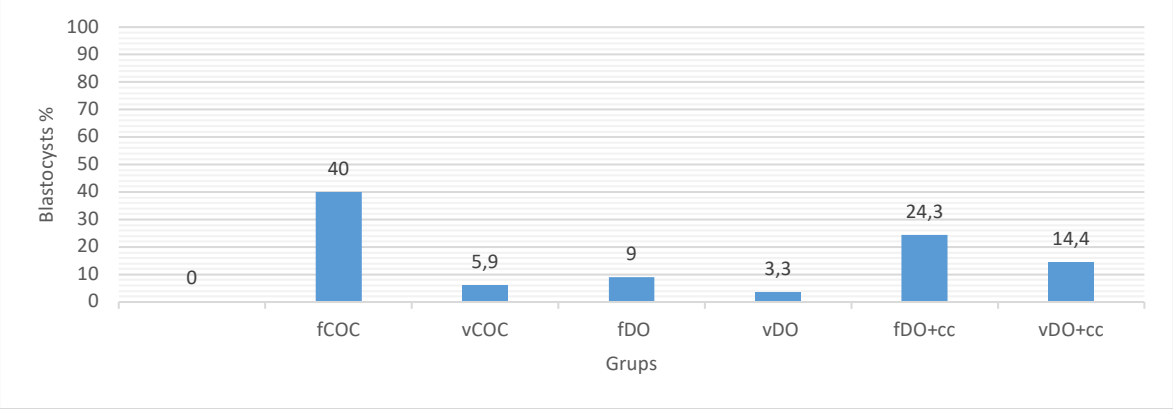


P<0,05

The odds of developing to blastocysts were higher in fresh cumulus-oocyte complexes (fCOC) (40.0%) and fresh denuded oocytes with added cumulus cells (fDO+cc) (24.3%) compared to vitrified cumulus - oocyte complexes (vCOC) (5.9%) and vitrified denuded oocytes with added cumulus cells (vDO+cc) (14.4%), $p<0.05$. Among the vitrified groups, we observed that removal of cumulus cells had no significant effect on blastocyst development of vitrified denuded oocytes (vDOs) (3.3%) compared to vitrified cumulus-

oocyte complexes (vCOCs) (5.9%), but the percentage of development to blastocysts in vitrified denuded oocytes with added cumulus cells (vDO+cc) was significantly higher compared to vitrified denuded oocytes (vDO) (14.4% vs. 3.3% respectively, p<0.05) .

Figure 9: Development of fertilized oocytes to the blastocyst stage

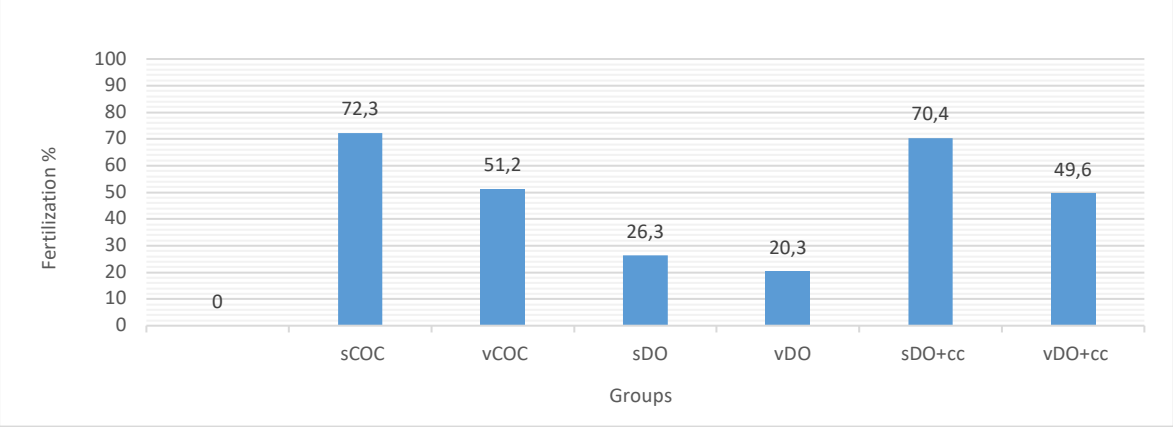


P<0,05

Removal of the cumulus cells before fertilization had a negative effect on the rate of development (Figure 10).

In fresh oocytes, the fertilization rate of fresh denudated oocytes (fDO) (26.3%) was significantly lower compared to fresh cumulus-oocyte complexes (fCOC) (72.3%) p<0.05. The addition of intact fresh cumulus cells to fresh denudated oocytes (fDO) could affect the fertilization rate to the extent that it was not significantly different from fCOC (72.3% vs. 70.4%, respectively). The same effect was observed in vitrified oocytes.
Fertilization rate was significantly higher for vCOC compared to vDO (51.2% vs. 20.3% respectively, p<0.05) and the situation could be reversed when vDO were supplemented with intact CCs (vDO+cc , 49.6%).

Figure 10: Fertilization rate



P<0,05

The effect of cumulus cells during vitrification has been studied by many authors over the years, but conflicting results have been obtained in several species. In humans, a beneficial effect of cumulus cells on the survival of matured oocytes was initially observed (KUWAYAMA et al. 2005), but PAUL et al., (2018) reported no differences in the survival of cumulus oocyte complexes compared to denuded oocytes. In horses, the presence of cumulus cells was found to be beneficial during vitrification of mature oocytes (ZHANG et al. 2020), while GASPARRINI et al., 2007 reported that they played a detrimental role during vitrification of buffalo oocytes. On the other hand, no differences in survival rates were found between cumulus-oocyte complexes and denuded sheep oocytes (ZHANG et al. 2009). Conflicting results have been reported in cattle. CHINEN et al., (2020) observed no effect when cumulus cells were present during vitrification of bovine oocytes. Similarly, ZHANG et al., (2020) observed no effect on the survival of mature bovine oocytes when they were vitrified with cumulus cells. Our observations are in line with recent research. It has been suggested that cumulus cells may impede the diffusion of water and cryoprotectant, leading to inadequate cellular protection. It should be noted that denuded oocytes are easier to manipulate when Cryotop is used during vitrification (TASHIMA et al., 2017). In addition, the amount of fluid around these oocytes is less compared to the cumulus-oocyte complexes. A smaller amount of fluid around the oocyte leads to a higher rate of cooling and rewarming (ARAV, 2014, SINHA et al., 2014), which may be positive for the survival of denuded oocytes compared to cumulus-oocyte complexes, (SOMFAI and Hirao, 2021).

Removal of cumulus cells shortly before in vitro fertilization (IVF) is known to strongly reduce the fertilization rate in cattle (DUJÍČKOVÁ et al., 2021). During fertilization, the cumulus cells attract, capture and select the spermatozoa. They are important in inducing sperm capacitation, the acrosome reaction and penetration and in preventing hardening of the zona pellucida. In the experiment conducted, removal of cumulus cells after *in vitro* maturation reduced the rate of development, cleavage and reaching the blastocyst stage. To solve this problem, intact cumulus cells were added to the denuded oocytes (DO) at the time of fertilization. In this way, denuded oocytes can regain their ability to develop (TASHIMA, et al. 2017). ZHANG et al., (2020) showed that the cleavage rate of vitrified buffalo oocytes increased when denuded oocytes were supplemented with intact cumulus cells, but not during development to blastocysts.
Another way to resolve the negative effect of cumulus cells during vitrification and to allow a positive influence during fertilization involves a partial reduction of cumulus cells before vitrification (DUJÍČKOVÁ et al. 2021).

Our data show that it is advisable to remove at least some of the cumulus cells before vitrification of matured bovine oocytes. Denuded oocytes undergo vitrification at a higher rate than cumulus-oocyte complexes, although their fertilization and subsequent embryo development are compromised due to the lack of cumulus cells. Addition of intact cumulus cells can restore the situation, providing denuded oocytes with similar rates of fertilization and embryo development as vitrified cumulus-oocyte complexes.

4.7. Vitrification of bovine embryos

An experiment was conducted to improve the vitrification method of bovine embryos using a safe, simple and successful technique.

Current vitrification protocols, although successful, have drawbacks that make them difficult to use effectively. Problems include the use of dimethyl sulfoxide (DMSO), a relatively toxic cryoprotectant, micro-sized containers that are often open to contamination and difficult to handle, and short exposure times that are technically difficult and leave no room for error. The method, called S3 vitrification, uses a large, sterile, sealable container and facilitates manipulation. Bovine embryos were vitrified by this method by being incubated in two ethylene glycol solutions at 23°C for 5 minutes each. They were then transferred to a vitrifying solution of glycerol and ethylene glycol (EG) for two minutes, during which time they were loaded into a sterile 0.25 ml paillette that was heat-sealed and vitrified. Sequins were thawed for 5 seconds in air, then for 10 seconds in water at 30°C. The results obtained during vitrification of morula and blastocysts using the S3 method were compared.

After 24 hours of culture, the embryos studied were scored for blastocoel reexpansion. The results obtained showed that in thawed intact morula the percentage was higher (90.8%), while in blastocysts it was 86.5%. A greater percentage of the blastocyst group reached the expanded blastocyst stage, 73.1%. Embryos selected for vitrification were blastocysts, expanded blastocysts, or hatched blastocysts. The S3 technique provides high survival rates of over 90% in bovine embryos.

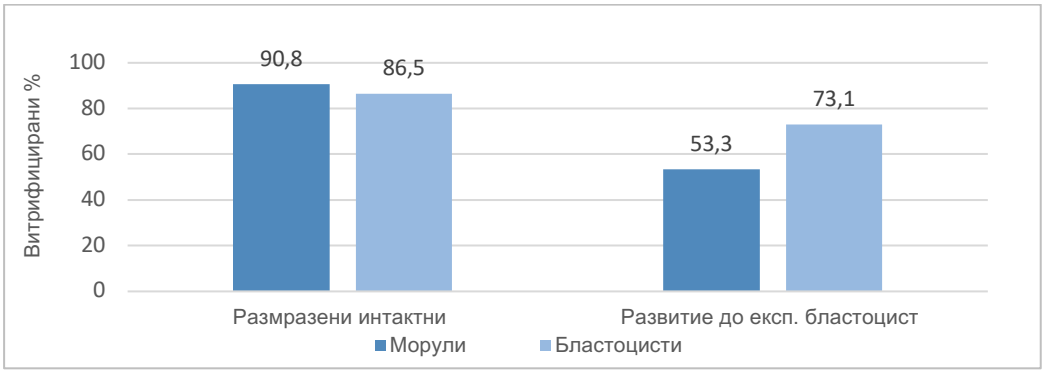


Figure 10: The results obtained in the vitrification of morula and blastocysts by method S3

This new technique, called S3 vitrification, is different, based on the basic principles of cryopreservation. A relatively large container and a sterile 0.25 ml pail can be loaded and sealed easily without rushing the procedure. Furthermore, although a longer exposure period (12 min before cooling) was used than the main methods, there was very little evidence of toxicity.

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Bovine embryos vitrified using the S3 method do not require artificially induced blastocoel collapse prior to freezing to obtain high embryo survival rates. Because the blastocoel is composed primarily of water, ice crystals will form when it freezes, presenting a potentially lethal problem during vitrification. This has led to the development of artificial reduction or collapse of the blastocoel prior to vitrification.

During vitrification and thawing, in most cases the blastocoel does not completely collapse and remains about half its original size. After thawing and removal of the cryoprotectant, the embryo can be easily graded and transferred immediately without the need for culture until re-expansion occurs to confirm its survival. This is different from many of the conventional vitrification procedures in which the embryo shrinks or collapses during thawing and removal of the cryoprotectant (VANDERZWALMEN et al., 2003). It is difficult to evaluate an embryo or determine whether it has survived after blastocoel collapse, and culturing for several hours or overnight before transfer (KUWAYAMA et al., 2005a; 2005b; LIEBERMANN et al., 2006) to visualize re-expand and determine if the embryo and blastomeres are intact. The majority of embryos that are cultured overnight recover fully and continue to expand and sometimes hatch. Avoiding complete collapse of the embryo during thawing and cryoprotectant removal is another advantage of S3 vitrification, as it allows immediate verification of survival, making it easier to make decisions about how many embryos should be thawed without waiting re-expand.

These results demonstrate that embryos can be vitrified using a simple, easy-to-use protocol, in a relatively large, sterile, sealable container without the need for DMSO and in a manner that allows sufficient time for equilibration and loading without requiring to rush during the procedure.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

5.1.1. The best results were obtained when the ovaries were transported from the slaughterhouse to the laboratory for two hours at an ambient temperature of 20°C to 30°C.

5.1.2. The method of obtaining COCs by aspiration is preferable because there is no contamination, but the number of oocytes obtained is between 10 and 11. In the method of obtaining COCs by cutting, there is a risk of mycotic contamination, but 32% more oocytes are obtained.

5.1.3. The size of the follicle has a positive effect on
oocyte development competence. The blastocysts obtained from ovaries without corpus luteum from SF and GF were about 5% larger than those from ovaries with corpus luteum.

5.1.4. When COCs are obtained from ovaries with a follicle diameter of 6 mm to 10 mm, the results are best. The number of follicles affects the results of the obtained in vitro embryos. Ovaries with more than 15 follicles yield about 30% blastocysts.

5.1.5. A duration of 20 to 24 hours of in vitro maturation of oocytes gives the best results.

5.1.6. The addition of 5% fetal calf serum (FCS) to the SOF culture medium resulted in the highest percentage of blastocysts. Decreasing concentrations of FCS in KSOM and SOF media are beneficial for in vitro cultivation. Using CR1 medium, no significant differences were observed in the rate of development to blastocysts when cultured with 5% or 10% FCS.

5.1.7. The survival rates of vitrified denuded oocytes (vDO) in groups (91.0%) and vitrified denuded oocytes with cumulus cells (vDO+cc) (93%) were higher than vitrified COCs (79.6%). Removal of cumulus cells after maturation significantly reduced the number of divided oocytes, but the percentage of development to blastocysts in (vDO+cc) was significantly higher compared to vDO (14.4% vs. 3.3%, respectively).

5.1.8. An advantage of the S3 vitrification method is the avoidance of complete collapse of the embryo during thawing and removal of the cryoprotectant.

5.2. RECOMMENDATIONS

5.2.1. We recommend that cumulus oocyte complexes be obtained from ovaries with a follicle diameter greater than 6 mm.

5.2.2. The best results are obtained when the in vitro maturation lasts for 22-24 hours.

5.2.3. The S3 embryo vitrification method is suitable for practice because it is simple and easy to use, without the need for DMSO. We recommend using it for embryo transfer.

6. CONTRIBUTIONS

6.1. Original scientific contributions

6.1.1. Through comparative studies of different defined nutrient media, the conditions for in vitro cultivation have been optimized. Cultivation in SOF medium supplemented with 5% FCS was found to reliably increase the percentage of blastocysts obtained.

6.1.2. The survival rate of vitrified denuded oocytes cultured in the presence of cumulus cells (vDO+cc) was significantly higher.

6.1.3. A simplified system was developed to package the embryos prior to vitrification using standard sequins.

6.2. Contributions of a scientific and applied nature

6.2.1. The method for obtaining embryos from cattle in vitro, through modifications of established schemes, has been improved.

6.2.2. New data have been obtained on the influence of follicle size on embryo quality.

6.2.3. The advantage of the method of obtaining oocytes by puncturing the follicles has been established.

6.2.4. The most optimal duration of in vitro maturation has been confirmed.

7. PUBLICATIONS IN CONNECTION WITH THE DISSERTATION

1. Tsvetelina Todorova, 2023, Effect of cumulus cells on vitrification of matured bovine oocytes, Bulgarian Journal of Animal Husbandry, LX, 1, 4-12, Web of Science - CABI, EBSCO