Modern Agricultural Science and Technology, ISSN 2375-9402, USA
November 2019, Volume 5, No. 5-6, pp. 15-20
Doi: 10.15341/mast(2375-9402)/03.05.2019/002
Academic Star Publishing Company, 2019
www.academicstar.us

Culture Medium and Oocyte Quality on Bovine Oocyte Maturation and in Vitro Fertilization

I. Covelo, Marcos Alejandro Puente, and C. M. Tartaglione

Laboratory of Animal Reproduction, School of Agricultural Sciences, National University of Lomas de Zamora, Argentina

Abstract: The complete maturation of the oocyte is the result of the interaction of gonadotropins, steroids and follicular signals. Fertilization anomalies in oocytes matured and fertilized in vitro are probably due to insufficiencies in cytoplasmic maturation. Therefore, the appropriate culture medium together with its interaction with oocyte quality is a promising combination for successful in vitro fertilization and subsequent embryo collection. The objective of the present work was to evaluate a protocol of maturation and a protocol of in vitro fertilization of *category 1 oocytes* (cytoplasmic and nuclear) obtained by puncture and aspiration, recovered from 305 cow and heifer slaughterhouse ovaries. A total of 1206 oocytes were collected. After maturation, 922 mature oocytes were obtained, of which 76.4% had extrusion of the first polar corpuscle and 82.3% expansion of the cumulus, and subsequently fertilized, obtaining a fertilization rate of 73.6%. The correct choice of oocytes and the procedures of in vitro maturation and fertilization using the protocols described in this trial gave repeatable and satisfactory results.

Key words: embryos, FIV, maturation, oocyte

1. Introduction

The results of in vitro production of embryos have been progressing significantly as knowledge about production, transfer and/or cryopreservation requirements, and effect of supplementation of defined culture media for each stage of the system advanced.

The process of in vitro production of bovine embryos can be divided into three fundamental steps, which, independently of the protocol used, are the following: Oocyte obtainment and maturation, fertilization of mature oocytes and embryo culture. These three steps comprise a complex series of physiological processes, which are not all known with accuracy, but in which each of them is concatenated with the next and determines the success of the subsequent stage.

Corresponding author: Marcos Alejandro Puente, Zootechnicist Engineer; research areas/interests: animal reproduction. E-mail: ingmarcospuente@gmail.com.

The maturation of oocytes implies the resumption of meiosis, its progression towards metaphase II (nuclear maturation) and a series of morphological, functional and biochemical events, necessary for fertilization and subsequent embryonic development [1]. The complete maturation of the oocyte (nuclear and cytoplasmic) is based on a complex interaction of gonadotropins, steroids and follicular signals. In addition, the anomalies in the fertilization of oocytes matured and fertilized in vitro are probably due to insufficiencies in cytoplasmic maturation [2].

Therefore, positive or negative oocyte in vitro maturation (IVM) lead to the success or failure of the entire system. In this stage, approximately 80% of the immature oocytes cultured reach metaphase II and expel the first polar corpuscle between 16 and 24 h after maturation.

Ball et al. (1984) [3] report that the final maturation of the ovoplasm must occur simultaneously with the nuclear maturation of the oocyte. In the same way, it has been demonstrated that the cumulus cells that

surround the immature oocyte fulfill a central role not only in nuclear maturation but also in the cytoplasm [4, 5].

Then, in the second step, in vitro fertilization (IVF), approximately 70-80% are fertilized and begin to divide, at least, until the 2-4 cell stage. In the third step, only 30-35% reach the blastocyst stage in the culture for 6-7 days. This step is not only the longest in the embryo production system but also the least efficient [6, 7].

Therefore, the appropriate culture medium and its interaction with oocyte quality are a promising binomial for the success of the next stage, which entails in vitro fertilization and subsequent obtainment of embryos [3, 8-10].

The objective of the present work was to evaluate a protocol of maturation and a protocol of in vitro fertilization of *category 1 oocytes* (cytoplasmic and nuclear) obtained by puncture and aspiration, recovered from ovaries of cows and heifers in a slaughterhouse.

2. Material and Methods.

2.1 Ovary Collection Method

The culture medium was composed of 0.9 percent sodium chloride and 5 ml of antibiotic (Penicillin and Streptomycin).

2.2 Oocyte Aspiration Method

Modified phosphate buffered saline (PBS) was the aspiration medium used.

2.3 Oocyte Obtainment

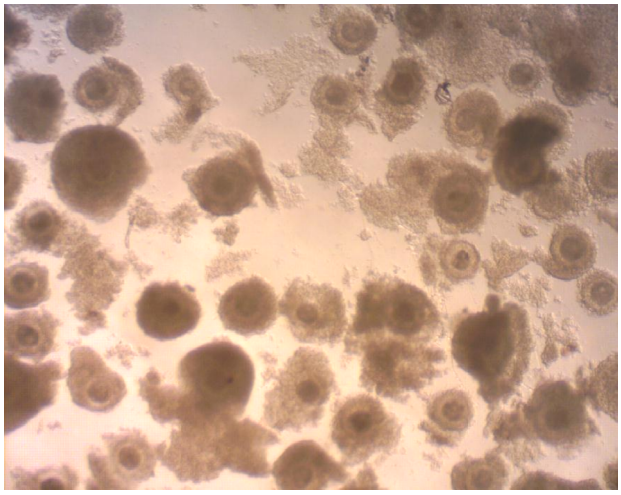
A total of 305 ovaries were obtained in different days conforming a total of 24 repetitions. In situ, the ovaries of the female reproductive tract were sectioned and placed in a thermos containing sterile saline and antibiotics (100 IU/ml penicillin, 100 mg/ml streptomycin) at 36°C. Then, they were transported to the laboratory within 30 minutes after the animals were slaughtered.

Oocyte aspiration was performed with a 5 or 10 ml syringe and an 18 G sterile hypodermic needle. Then the follicular fluid was stored in a 15 ml Falcon tube in a thermostatic bath at 34°C and, after a few minutes awaiting their decantation, they were placed in Petri dishes for observation.

The oocytes were selected using a magnifying glass by evaluating their general appearance, cytoplasm and the cumulus cells that surround them.

Those oocytes that were completely surrounded by 3 or more compact layers of cumulus cells and present homogenous ovoplasms were classified as suitable and are selected for in vitro maturation. On the contrary, those that were surrounded by less than 3 strata of cumulus cells, non-compact cumulus and had heterogeneous or pyknotic ovoplasms were classified as unsuitable.

At the end of the culture period, the degree of in vitro maturation was determined in a total 922 oocytes taken at random.



2.4 Maturation Medium

Cumulus-oocyte complexes (COC) were recovered by aspiration from follicles 3 to 6 mm in diameter. After washing three times in TCM199, 10 intact COC, with several dense layers of cumulus cells, were selected for “in vitro” maturation. Maturation was performed in TCM199 supplemented with 10% (v/v) fetal calf serum, 10 µg FSH/mL, 1 µg LH/mL and 1 µg

estradiol/mL (Sigma Chemical Co.) for 24 h at 39°C in a humidified atmosphere of 5% CO_2 in air.

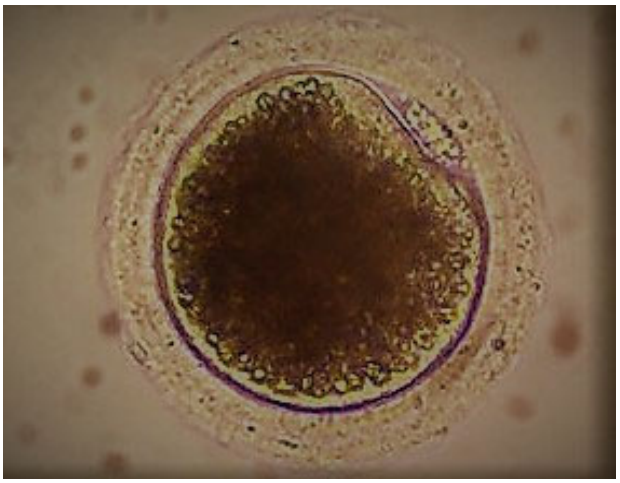
The medium was adjusted to pH 7.4 and an osmolarity of 295 +/- 5 mOs/L. Sera and growth factors were incorporated into the media on the in vitro culture day. The media were balanced by placing them in the incubator to obtain the temperature, humidity and saturation of CO_2, for at least two hours.

The in vitro culture of the oocytes selected as suitable was performed by placing 10 oocytes in 100 µl microdroplets. The microdroplets were prepared in sterile 60 mm disposable culture plates and covered with 6 ml of mineral oil. Next, the plates were placed in a culture chamber with humidified atmosphere at 39°C and 5% CO_2 in air for 24 hours.

2.5 Maturation Evaluation

At the end of the culture period, the degree of in vitro maturation was determined in a sample of oocytes taken randomly. From the oocyte sample, the degree of expansion of the cumulus cells was evaluated. Subsequently, these cells were removed with the addition of hyalurodinase and vortexed for 10 minutes. Next, the denuded oocytes were fixed in ethanol: acetic acid (3:1 v/v) and stained with 1% aceto-orcein for microscopic evaluation at x400 (Zeiss model Axiovert 135) of the first polar corpuscle and metaphase II.

Those oocytes that presented the first polar corpuscle and were in metaphase II stage, were considered matured.



2.6 Fertilization in vitro

At the end of the maduration period, the oocyte were inseminated using the technique of *in Vitro* fertilization described by Marquant-Le Guienne et al. (1990). Briefly, frozen-thawed sperm, separated by the swim up technique and capacited with heparin 0.02 mg/ml, were used for inseminatión at a concentration of 0.5×10^6 spermatozoa in 50 µl drops. fertilization medium under heavy mineral oil.

3. Results

The results of total oocytes, oocytes with extrusion of the first polar corpuscle, oocytes with expansion of cumulus, and fertilization rate are shown in Table 1. It is demonstrated that the percentage of mature oocytes (extrusion of the first polar corpuscle) through the protocol used is on average 76.4%. Semen from 6 different bulls was used, obtaining an average fertilization rate of 73.6% (4 repetitions per bull).

Table 1 Total oocytes, maturation rate and fertilization rate.

Bull	Total oocytes	Oocytes with extrusion of the first polar corpuscle	Oocytes with expansion of cumulus	Fertilization rate
A	199	149 (74.9%)	168 (84.4%)	74.0%
B	229	195 (85.1%)	205 85.1%	75.3%
C	208	171 (82,2%)	191 (85.5%)	74.0%
D	223	169 (75.8%)	179 (80.2%)	75.0%
E	198	125 (63.2%)	154 (77.7%)	68.3%
F	149	113 (75.8%)	121 (81.2%)	75.0%

4. Discussion

Mammals contain follicles that are in different stages of development, of which only a small proportion will be used during the animal's reproductive life. Therefore, the collection of oocytes from ovaries of donor females or ovaries obtained from cows that went to slaughter allow to recover and profit from non-ovulatory follicles, which under physiological conditions could turn into atretic follicles. The most economical and common way to obtain oocytes is from slaughterhouse ovaries. Accordingly,

there is robust knowledge of those factors that affect the collection of ovaries.

For example, temperatures below 30 °C during ovary storage produce losses in the transcription and injury of the cytoplasmic organelles, which will be important mediators in early embryonic development. This time is equally important in the development. Ovaries can remain in saline at temperatures between 30°C and 38°C for 8 hours without affecting the quality of the oocytes.

The relationship obtained between suitable and unsuitable oocytes per ovary is directly related to three variables involved: ovary collection and transport, methods and instruments used to obtain the oocytes and the correct criteria followed for their selection. Accordingly, the adequate training of the operator and the quality and origin of the original material (ovaries) are important when evaluating results [11].

In vitro maturation systems must ensure that the resulting oocyte normally completes the first reductional division and is capable of being fertilized, giving rise to a competent zygote that can continue its development after transfer. Leibfried et al. (1989), Auclair et al. (2013) [12, 13] simplify it to three main aspects in oocyte maturation that should be considered during their cultivation: nuclear maturation, ability to be fertilized and ability to continue their development. Oocytes are generally obtained from antral follicles larger than 2 mm and smaller than 6-7 mm in diameter. Oocytes from smaller follicles would lack meiotic competence in culture [14, 15] being incapable of completing nuclear maturation, as oocytes in very early stages are unable to experience the rupture of the germinative vesicle after their isolation from the granulosa cells. On the other hand, oocytes of larger follicles are often found in processes of atresia. Probably, this is the reason why the highest percentages of fertilization and development are obtained from follicle oocytes of between 2 and 7 mm, as shown by the works of Pavlok et al. (1992) [2].

An appropriate selection of oocytes to mature in vitro is usually based on choosing those with homogenous non-granular or polarized cytoplasm and with intact cumulus cells surrounding the gamete, as they represent the highest percentages of maturation [12, 16-19]. The culture conditions during in vitro maturation play a crucial role in the fertilization rates and in the acquisition of the embryos subsequent development capacity. Numerous studies have attempted to improve the culture environment in order to imitate the final events of oogenesis that occur during the periovulatory period in the dominant follicles. On the other hand, the removal of all the cells of the crown and of the cumulus, prior to incubation, is related to a later decrease in the meiotic restart [20].

This is due to fact that the denuded oocytes of the cumulus cells are unable to respond to LH and FSH, as these cells mediate the effect of gonadotropins [3, 11, 21, 22]. Therefore, the positive effect of the addition of epidermal growth factors (EGF) in oocyte maturation media has been demonstrated [23]. Traditionally, albumin-supplemented complex buffered media, serums and/or hormones (FSH, LH and 17 (3-estradiol) have been used to mature bovine oocytes. However, bovine oocytes re-initiate meiosis in a wide variety of media, ranging from a simple saline solution [12] to more complex media. Even so, the latter ones are preferred as it has been found that the medium used affects not only the subsequent capacity of fertilization, but also the embryonic development [24-27]. Within these means, medium 199 (TCM199) has been more widely used, as better subsequent in vitro development and maturity rates have been obtained, compared to other media [25, 28, 29].

It is established that the culture medium used for the in vitro maturation of oocytes significantly influences in vitro fertilization rates [30-32]. Until now, a wide variety of culture media has been used to mature in vitro bovine oocytes. Of all the media used, the TCM-199 medium is the most frequently implemented. This medium can be supplemented with SFB or

heat-treated cow serum (SVC) for the oocyte in vitro maturation. Sirard et al. (1988) [20] report that in vitro maturation can be 90%, when selected oocytes are cultured in the presence of SFB and hormones (FSH, LH, progesterone and estradiol) as a supplement [33].

Weimer et al. (1991) and Gardon (1999) [34, 35] report that high in vitro maturation rates can be obtained (91.3% and 93.8% respectively) by combining TCM-199 medium supplemented with SFB and hormones. The results show that, in spite of having a high percentage of oocytes with extrusion of the first polar corpuscle (76.4%), oocytes with expansion of the cumulus (82.3%), and fertilization rate (73.6%), they are lower than those obtained by the authors mentioned, who used the same medium (TCM 199), in the presence of SFB and hormones (FSH, LH and estradiol) as a supplement.

Ball et al. (1984), Del Campo (1993), Sirard M., (2011) and Schoenfelder et al (2003) [3, 36, 37] report that the final maturation of the ovoplasm must occur simultaneously with the nuclear maturation of the oocyte. Likewise, the expansion of the cumulus cells of the oocytes matured in vitro reflects the ability of the cells to resume the meiotic process. It can be concluded that the correct choice of oocytes and the procedures of maturation and in vitro fertilization, using the protocols described in this trial, gave repeatable and satisfactory results.

Subsequent studies should be done to evaluate the in vitro maturation of oocytes of cows and heifers separately given the incidence and competition that the age of the females may reach.

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