DOI: 10.31276/VJSTE.65(1).76-80

Influence of fetal calf serum on the production of bovine embryos *in vitro*

Khanh Van Nguyen[1], Thi Kim Yen Pham[1], Thi Au Hoang[1], Quang Minh Luu[2], Doan Lan Pham[3*]

[1]*Key Laboratory of Animal Cell Biotechnology, National Institute of Animal Science, No. 9, Tan Phong Street, Thuy Phuong Ward, Bac Tu Liem District, Hanoi, Vietnam*

[2]*Ministry of Science and Technology, 113 Tran Duy Hung Street, Cau Giay District, Hanoi, Vietnam*

[3]*National Institute of Animal Science, No. 9, Tan Phong Street, Thuy Phuong Ward, Bac Tu Liem District, Hanoi, Vietnam*

Received 21 June 2022; accepted 13 September 2022

Abstract:

The aim of the present study was to evaluate the effect of foetal calf serum (FCS) on the production of bovine embryo *in vitro*. We conducted two experiments: (1) to evaluate the effect of FCS on the production of bovine embryo *in vitro* and (2) to evaluate the effect of duration of FCS supplementation on the production of bovine embryo *in vitro*. In experiment 1, we divided mature bovine oocytes after *in vitro* fertilization into four embryo culture media: CR1aa, CR1aa+FCS, SOFaa, and SOFaa+FCS. Embryos cultured in CR1aa and SOFaa media supplemented with FCS resulted in a higher cleavage and blastocyst formation rates than when cultured in CR1aa and SOFaa without FCS (74.22 and 82.96% vs. 71.6 and 70.62%; 4.11 and 22.31% vs. 16.98 and 34.51%; respectively, $p<0.05$). The percentage of hatching blastocysts was highest when embryos were cultured in SOFaa medium supplemented with FCS (10.54%; $p<0.05$). Embryos cultured in the medium with FCS had a higher the average number of cells/blastocyst than the medium without FCS. The SOFaa+FCS group had a higher average number of cells/embryos than the other groups (94.32; $p<0.05$). In experiment 2, we added FCS to bovine embryo culture *in vitro* at the following times: 0 h, 48 h, and 120 h after fertilization. The rate of oocyte cleavage, blastocyst formation, and hatching blastocyst rate of the 0-h group were higher than that of 48-h and 120-h groups (82.96 vs 70.5 and 70.24%; 34.51 vs 24.21 and 23.18%; 10.5 vs 6.12 and 4.13%; respectively, $p<0.05$). However, there was no difference in the average number of cells/blastocysts between the groups. In conclusion, the addition of FCS to embryo culture media improved the efficiency of bovine embryo production *in vitro* and *in vitro* bovine embryo quality. The appropriate time to add FCS to bovine embryo culture *in vitro* was 0 h after fertilization.

Keywords: **bovine embryos *in vitro*, embryo development, foetal calf serum.**

Classification numbers: **3.4, 3.5**

1. Introduction

The generation of calves derived from embryos *in vitro*, showing that bovine embryo *in vitro* has a vital role in the cattle industry was first reported in [1]. The success of *in vitro* oocyte maturation techniques, *in vitro* fertilization, and *in vitro* embryo culture have shortened and accelerated the rate of genetic improvement in cattle. The process of *in vitro* embryo culture has fundamental differences compared with *in vivo* embryo culture [2]. *In vitro* embryo culture media play an essential role in developing bovine embryos *in vitro* and have a diverse composition, including simple media defined with salts or complex unspecified media supplemented with serum and unknown components [3]. Factors and protein sources have been used to supplement the embryo culture medium to support embryonic development *in vitro* [4].

Current prevalent *in vitro* bovine embryo culture media are CR1aa or SOF with or without the addition of foetal calf serum (FCS) and amino acids. FCS is often added to the embryo culture medium *in vitro* because it contains nutritional factors and substances beneficial to embryo development such as antioxidants, growth factors, and necessary nutrients required for cell growth and development [5]. Serum affects the division and formation of morula, i.e., follicular bovine oocytes after fertilization. Some studies have shown that the presence of serum in bovine embryo culture media supports blastocyst development and hatching as well as increases the total cell number per blastocyst [6]. However, the presence of FCS in bovine embryo culture *in vitro* increased the lipid content of the embryos, which causes adverse effects on embryo freezing. In addition, bovine serum can carry pathogenic viruses that affect the

*Corresponding author: Email: pdlanvn@yahoo.com

formation and development of the foetus after embryo transfer [7]. Therefore, up to now, there is still much controversy surrounding the use of FCS for *in vitro* bovine embryo culture.

In Vietnam, research on the production of bovine embryos *in vitro* has been carried out since the 1990s and is currently being continued at the Institute of Biotechnology, Vietnam Academy of Sciences; the Key Laboratory of Animal Cell Biotechnology, National Institute of Animal Sciences, and the Vietnam Academy of Agriculture [8]. Researchers are also trying to find ways to improve and enhance the quality of bovine embryos *in vitro* [3]. However, no reports have evaluated the effect of foetal calf serum (FCS) on the production of bovine embryo *in vitro*. This study was conducted to generate quality commercial *in vitro* bovine embryos for cryopreservation or embryo transfer.

2. Materials and methods

2.1. Collection oocytes and in vitro maturation

Ovaries were collected from female beef cattle at slaughterhouses and carried to the laboratory within 2-3 h in Dulbecco's phosphate buffered saline supplemented with antibiotics. Then antral follicles with 3-8-mm diameter were aspirated with 18 G needles attached to a syringe containing oocyte collection medium (TALP-Hepes + calf serum and antibiotics) and the liquid mixture was placed in 15-ml centrifuge tubes for at least 5 min at 37°C. Cumulus oocytes complexes (COCs) were collected under the stereomicroscope after introducing the sediment into an oocyte collection medium. Bovine oocytes after collection were immediately washed three times in the culture medium, then transferred to culture in 4-well plates (Thermo Fisher Scientific, Waltham, MA, USA) containing the culture medium covered by mineral oil (Sigma, USA) and incubated in a humidified atmosphere of 5% CO_2 in air at 38.5°C (50 oocytes/well) for 22-24 h. The maturation culture medium was TCM 199 medium (Invitrogen Co., Carlsbad, CA, USA) supplemented with 1 g/ml cysteamine, 200 µg/ml FSH (follicle-stimulating hormone), 5% foetal bovine serum (Sigma; USA), and antibiotics.

2.2. In vitro fertilization of bovine oocytes

After 22-24 h of maturation, COCs were fertilized with frozen-thawed bull spermatozoa that had been previously tested for IVF as described by N. Saito, et al. (1995) [9]. Frozen-thawed bull spermatozoa were thawed at 37°C and the sperm were washed and pelleted in BO-IVF medium (IVF Bioscience, USA) by centrifuge at 1800 g for 5 min at room temperature and motile sperm were recovered. Then, sperm were diluted in BO-IVF medium (final density of $5x10^6$ sperm/ml). Before fertilization, matured bovine oocytes were kept or partially removed from the surrounding cumulus cell layer and washed in the BO-IVF medium. Thereafter, the diluted sperm and oocytes were transferred to the insemination droplet containing BO-IVF medium and co-culture for 5 h at 38.5°C, 5% CO_2, 5% O_2, and saturated air humidity.

2.3. In vitro bovine embryo culture

After 5 h of co-culture, cumulus cells were removed by repeated pipetting and then matured oocytes with an extruded first polar body (presumed zygotes) were selected with up to 50 presumed zygotes washed and transferred to 4-well dishes containing IVC medium (CR1aa or SOFaa with or without FCS) according to the experimental design below. The dishes were incubated at 38.5°C in a humidified atmosphere of 5% CO_2, 5% O_2 in air. The CR1aa medium contained albumin serum bovine, sodium pyruvate, and glutamic and amino acids. The SOFaa medium contained sodium pyruvate, lactate, amino acid, albumin serum bovine, and myo inositol.

(1) Experiment 1: To evaluate the effect of FCS on the production of bovine embryo *in vitro*, we divided mature bovine oocytes after *in vitro* fertilization into four embryo culture media: CR1aa, CR1aa supplemented with 2.5% FCS (v/v), SOFaa, and SOFaa supplemented with 2.5% FCS (v/v).

(2) Experiment 2: To evaluate the effect of FCS supplementation duration on the production of bovine embryo *in vitro*, we supplemented with 2.5% FCS (v/v) to bovine embryo culture *in vitro* at the following times: Day 0, Day 2, and Day 5 after fertilization.

The cleavage rates were checked on Day 2, the blastocyst rates were checked on Day 7, and the hatching blastocyst rates were checked on Day 8 or Day 9 after *in vitro* fertilization. *In vitro* bovine hatching blastocysts are shown in Fig. 1.

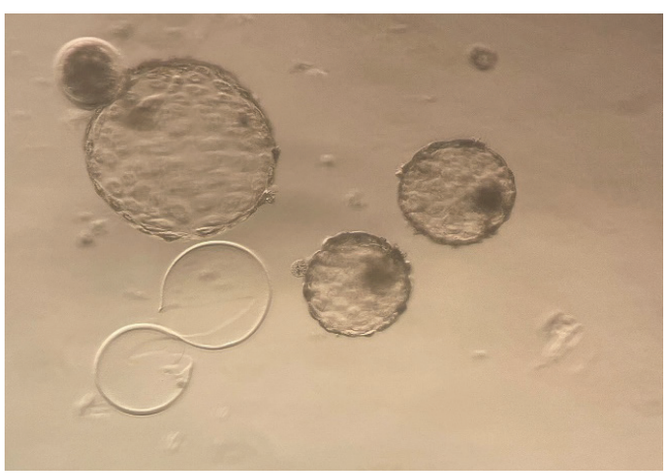

Fig. 1. *In vitro* bovine hatching blastocyst.

2.4. Hoechst 33342 staining method

Oocytes/embryos were washed in PBS solution supplemented with 0.3% PVP. Next, the oocytes/embryos were transferred to the oocytes/embryo staining solution (50 µl Hoechst 33342 stock + 450 µl absolute ethanol) overnight at 4°C (Hoechst 33342 stock: 250 µg Hoechst 33342/ml absolute ethanol). After the oocytes/embryos were stained in staining solution, the oocytes/embryos were transferred to absolute ethanol, and then transferred to a glycerol solution. After being washed in glycerol, the oocytes/embryos were transferred to the slide, one drop per oocyte/embryo, and lined up vertically on the slide. The lamen was placed on the slide and the sample was examined under a fluorescence microscope (Fig. 2).

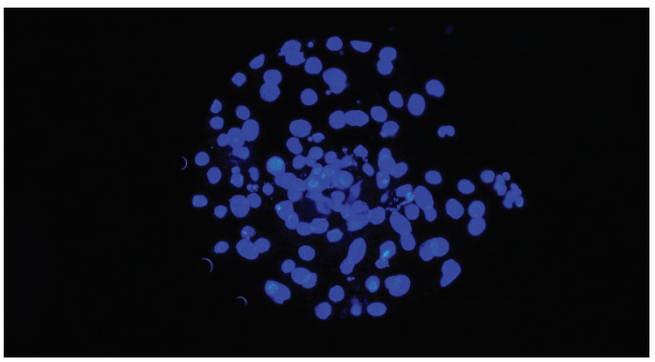

Fig. 2. Bovine blastocysts stained with Hoechst 3332.

2.5. Data analysis

Data were expressed as mean ± SEM values and analysed by t-test with $p<0.05$ defined as the significance level.

3. Results and discussion

3.1. Effect of FCS on the production of bovine embryos in vitro

The success of the production of bovine embryos *in vitro* depends not only on the IVM and IVF media but also on the embryo culture medium. The current basic *in vitro* bovine embryo culture medium is CR1aa or SOFaa with or without serum. To evaluate the effect of FCS on the production of bovine embryos *in vitro*, we divided *in vitro* mature bovine oocytes after *in vitro* fertilization into four different embryo culture media: CR1aa, CR1aa+FCS, SOFaa, and SOFaa+FCS. The effect of FCS on the efficiency of production of bovine embryos *in vitro* was evaluated based on the division rate, blastocyst generation, extruded blastocysts, and the quality of the *in vitro* bovine blastocysts produced (the average number of cells/blastocysts). The results are shown in Table 1.

Table 1. Effect of FCS on *in vitro* bovine embryogenesis.

Medium	Cleaved	Blastocysts	Hatching blastocysts	The average number of cells/blastocysts
CR1aa	71.64^{a}±1.92 (175/245)	4.11^{a}±2.12 (10/245)	0	71.5^{a}±1.98
SOFaa	70.62^{a}±1.76 (184/261)	22.31^{b}±2.09 (58/261)	2.15^{a}±1.98 (5/261)	80.93^{b}±2.31
CR1aa + FCS	74.22^{b}±2.01 (181/244)	16.98^{b}±1.58 (41/244)	4.59^{a}±2.03 (11/244)	81.35^{b}±2.15
SOFaa + FCS	82.96^{c}±1.35 (222/268)	34.51^{c}±1.76 (92/268)	10.54^{b}±1.74 (28/268)	94.32^{c}±2.01

Note: Values in the same column with different letters are significantly different ($p<0.05$).

Table 1 shows that embryo culture in CR1aa and SOFaa medium supplemented with FCS gave higher division and blastocyst formation rates when cultured in CR1aa and SOFaa without serum supplementation (74.22% and SOFaa, respectively, 82.96 vs 71.64 and 70.62%; 4.11 and 22.31% vs 16.98 and 34.51%; $p<0.05$).

To date, there is no consensus among the results of studies on the effects of FCS during *in vitro* bovine embryo culture. Our research results are similar to those of S.B. Sena-Netto, et al. (2020) [10], still, there were differences from previous studies [7, 11]. According to S.B. Sena-Netto, et al. (2020) [10], the presence of FCS in the embryo culture medium increased the blastocyst formation rate. According to B.H. Choi, et al. (2019) [7], the division rate when growing bovine embryos *in vitro* in the FCS medium was higher than in the serum-free medium. However, the blastocyst formation rate of the group with FCS was lower than that without FCS. Meanwhile, A.P. Gandhi, et al. (2000) [11] did not find a difference in blastocyst formation rate when growing bovine embryos *in vitro* in media with FCS or without FCS; however, blastocysts derived from SOFaa medium had the highest average number of cells/blastocysts. The difference between experimental results may be due to the origin of bovine oocytes used in the study, experimental conditions, and embryo culture environment.

In vitro embryo culture produces differences from living organisms, especially in ruminants [2]. The *in vitro* embryo culture medium plays an essential role in developing *in vitro* embryos. *In vitro* embryo culture media have a diverse composition, including simple saline solutions or indeterminate complex media supplemented with serum [3]. The presence of FCS in IVC media was proposed because the serum contained components that provide nutrients to the embryo and inactivate toxic agents [7]. According to A. Abdel-Wahab, et al. (2018) [12], the supplementation of FCS into the embryo culture medium is very important, because FCS

contains many hormones, vitamins, transport proteins, and growth factors that optimize oocyte maturation. FCS contains potent antioxidant activities which assist in chelating free radicals and protecting oocytes from oxidative stress conditions. In addition, the presence of FCS in the embryo culture medium reduced hardening of the zona pellucida, thereby improving the efficiency of embryo production and embryo quality *in vitro*.

Blastocyst quality is essential to confirm the pregnancy rate after embryo transfer [13]. The percentage of hatching blastocysts and the average number of cells/blastocysts were considered to some extent as a measure of embryo quality. In this study, the rate of hatching blastocysts was highest when *in vitro* bovine embryos were grown in SOFaa medium supplemented with FCS (10.54%; $p<0.05$). Embryos were produced in medium with FCS had a higher the average number of cells/blastocysts than the medium without FCS. The SOFaa+FCS group had a higher average number of cells/embryos than the others (94 vs 32; $p<0.05$). The percentage of hatching blastocysts and the average number of cells/blastocysts in the SOFaa+FCS group were higher than those reported by A.P. Gandhi, et al. (2000) [11]. According to A.P. Gandhi, et al. (2000) [11], the percentage of hatching blastocysts and the average number of cells/blastocysts of the SOFaa+FCS group reached 9.5% and 92.1%, respectively, meanwhile, according to our research, they reached 10.54% and 94.32%. This difference may be because we used TCM199 medium for maturation, while A.P. Gandhi, et al. (2000) [11] used SOFaa. In addition, the percentage of hatching blastocysts and the average number of cells/blastocysts depend on the quality of oocytes and the quality of sperm used by each laboratory.

Despite the addition of FCS, *in vitro* culture of bovine embryos in SOFaa medium supplemented with serum resulted in a higher rate of blastocyst formation than in CR1aa medium supplemented with FCS (34.51% vs 98%; $p<0.05$). SOFaa is a common medium used for *in vitro* bovine embryo culture. This medium contains the same ingredients as bovine oviduct fluid such as hormones, growth factors, and some amino acids. The presence of these factors in the embryo culture medium increases the ability of embryos to develop before transfer [14].

3.2. Effect of time of bovine serum supplementation on the production bovine embryo in vitro

In this study, to evaluate the effect of FCS supplementation time on *in vitro* bovine embryogenesis efficiency, we added FCS to the embryo culture medium at the following time points: 0 h, 48 h, and 120 h after fertilization. The results are shown in Table 2.

Table 2. Effect of time of FCS supplementation on the production bovine embryo *in vitro*.

FCS supplementation time	Cleaved	Blastocysts	Hatching blastocysts	The average number of cells/ blastocysts
0 h	(222/268) 82.96^{a}±1.35	(92/268) 34.51^{a}±1.76	(28/268) 10.54^{a}±1.74	94.97±1.89
48 h	(194/276) 70.45^{b}±1.68	(66/276) 24.21^{b}±1.42	(16/276) 6.12^{b}±1.33	93.18±1.16
120 h	(183/261) 70.24^{b}±2.01	(60/261) 23.18^{b}±1.89	(10/261) 4.13^{b}±2.69	92.89±1.76

Note: Values in the same column with different letters are significantly different ($p<0.05$).

Table 2 shows that the percentage of oocytes dividing, blastocyst formation rate, and hatching rate of the 0-h group was higher than that of 48-h and 120-h groups (82.96% vs 70.45% and 70.24%; 34.51% vs 24.21% and 23.18%; 10.54% vs 6.12% and 4.13%; $p<0.05$, respectively). However, there was no difference in the average number of cells/blastocysts between the groups (Table 2). Our results differed from those of A. Abdel-Wahab, et al. (2018) [12]. According to A. Abdel-Wahab, et al. (2018) [12], there was no difference in division rate when adding FCS at 20 h or 42 h post-insemination.

This study suggests that the duration of FCS supplementation affected the efficiency of bovine embryo production *in vitro*. The addition of FCS at 0 h after fertilization improved the efficiency of the production of bovine embryo *in vitro*. According to S.B. Sena-Netto, et al. (2020) [10] and E. Gómez, et al. (2008) [15], the addition of FCS to the embryo culture medium at 0 h after fertilization will accelerate the morula development stage. Although the FCS's role in embryonic development is not entirely understood, it is related to growth factors, amino acids, or FCS's antioxidant capacity in the embryo culture medium. These factors help increase blastocyst generation rate. The quality of embryos generated from culture medium with FCS is better than without FCS [10].

In our study, the addition of FCS at 48 h or 120 h after fertilization reduced blastocyst formation rate. According to S.B. Sena-Netto, et al. (2020) [10], when cultured in embryo culture with only albumin serum bovine, there might be a lack of factors that stimulate embryo development thereby allowing the post-fertilization oocytes to develop to the blastocyst stage less often. FCS supplementation to embryo culture medium improved cell proliferation and supported dilation [16]. Besides, the presence of FCS in the embryo culture medium reduces cell apoptosis. Apoptosis plays a vital role in embryonic development, acting as a quality control measure by eliminating abnormal, damaged, or dormant cells [10].

Although FCS supports the development of bovine embryos, in this study, we only added FCS to the bovine embryo culture medium *in vitro* at a concentration of 2.5% (v/v). According to M.J. Sudano, et al. (2011) [17], the presence of FCS in embryo culture increased the lipid content of bovine embryos *in vitro*, which was not beneficial for *in vitro* cryopreservation of bovine embryos. Therefore, reducing FCS concentration to 2.5% (v/v) in *in vitro* bovine embryo culture is necessary to maintain quality *in vitro* bovine blastocyst production.

In this study, in order to improve the efficiency of bovine embryo production *in vitro* and *in vitro* bovine embryo quality, bovine embryos were cultured and incubated at 5% CO_2. Regulating the CO_2 level during embryo culture helps maintain the right amount of pH inside the incubator (pH=7.2-7.4). It is known that bovine embryos are sensitive to pH as changes in pH can affect cell metabolism and the quality of bovine embryos *in vitro*.

4. Conclusions

The addition of FCS to embryo culture media improved the efficiency of bovine embryo production *in vitro* and *in vitro* bovine embryo quality. The appropriate time to add FCS to bovine embryo culture *in vitro* was 0 h after fertilization.

CRediT author statement

Khanh Van Nguyen: Conceptualization, Methodology, Formal analysis, Writing, Review, Editing; Thi Kim Yen Pham: Data analyst; Thi Au Hoang: Data analyst; Quang Minh Luu: Review, Data analyst; Doan Lan Pham: Conceptualisation, Methodology, Formal analysis, Writing, Review, Editing.

ACKNOWLEDGEMENTS

The projects carried out the study: "Study on the influence of cumulus cells and embryo culture environment on the results of in vitro bovine embryogenesis". The authors sincerely appreciated funding to support regular operations of the Key Laboratory of Animal Cell Biotechnology, National Institute of Animal Science, Hanoi, Vietnam.

COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

[1] B.G. Brackett, et al. (1982), "Normal development following *in vitro* fertilization in the cow", *Biol. Reprod*, **27**, pp.147-158.

[2] P. Lonergan, et al. (2003), "Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage *in vitro* or *in vivo*", *Biol. Reprod.*, **69**, pp.1424-1431.

[3] M. Lane, D.K. Gardner (2007), "Embryo culture medium: Which is the best?", *Best Pract. Res. Clin. Obstet. Gynaecol.*, **21**, pp.83-100.

[4] T.C. Sovernigo, et al. (2017), "Effects of supplementation of medium with different antioxidants during *in vitro* maturation of bovine oocytes on subsequent embryo production". *Reproduction Domest. Animal*, **52**, pp.561-569.

[5] M. Murakami, et al. (2011), "Development and subsequent cryotolerance of domestic cat embryos cultured in serum-free and serum-containing media", *Cryobiology*, **63**, pp.170-174.

[6] E. Wydooghe, et al. (2014) "Replacing serum in culture medium with albumin and insulin, transferrin and selenium is the key to successful bovine embryo development in individual culture", *Reproduction Fertility and Development*, **26**, pp.717-724.

[7] B.H. Choi, et al. (2019), "Effect of serum and serum free media on the developmental competence of OPU derived bovine IVP embryo", *J. Anim. Reprod. Biotechnol*, **34**, pp.305-310.

[8] D.T.K. Lanh, et al. (2021), "Improvement of the efficiency of bovine *in vitro* fertilization embryo production", *Vietnam J, Agri. Sci.*, **19(1)**, pp.25-32 (in Vietnamese).

[9] N. Saito, et al. (1995), *Manual of Bovine Embryo Transfer*, Japan Livestock Technology Association, pp.332-340.

[10] S.B. Sena-Netto, et al. (2020), "The replacement of foetal bovine serum with bovine serum albumin during oocyte maturation and embryo culture does not improve blastocyst quality after slow freezing cryopreservation", *Biopreserv. Biobank*, **18**, pp.171-179.

[11] A.P. Gandhi, et al. (2000), "A single medium supports development of bovine embryos throughout maturation, fertilization and culture". *Hum. Reprod.*, **15**, pp.395-401.

[12] A. Abdel-Wahab, et al. (2018), "The impact of using newborn bovine serum as foetal calf serum substitute in the *in vitro* bovine embryos production system", *Porto Biomed. J.*, **3(2)**, DOI: 10.1016/j.pbj.0000000000000003.

[13] D. Rizos, et al. (2003), "Bovine embryo culture in the presence or absence of serum: Implications for blastocyst development, cryotolerance, and messenger RNA expression", *Biol. Reprod.*, **68**, pp.236-243.

[14] J. Block, et al. (2011), "Improving post-transfer survival of bovine embryos produced *in vitro*: Actions of insulin-like growth factor-1, colony stimulating factor-2 and hyaluronan", *Theriogenology*, **76**, pp.1602-1609.

[15] E. Gómez, et al. (2008), "Serum free embryo culture medium improves *in vitro* survival of bovine blastocysts to vitrification", *Theriogenology*, **69**, pp.1013-1021.

[16] J.A. Neira, et al. (2010), "Effect of the association of IGF-I, IGF-II, bFGF, TGF-β1, GM-CSF, and LIF on the development of bovine embryos produced *in vitro*", *Theriogenology*, **73**, pp.595-604.

[17] M.J. Sudano, et al. (2011), "Lipid content and apoptosis of *in vitro*-produced bovine embryos as determinants of susceptibility to vitrification", *Theriogenology*, **75**, pp.1211-1220.