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Bovine *in vitro* embryo production protocol: does it really influence embryo cryotolerance?*

Protocolo de produção *in vitro* de embriões bovinos: este realmente influencia a criotolerância dos embriões?

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ABSTRACT

The protocol of *in vitro* production (IVP) of bovine embryos is one of the critical factors determining embryo viability after cryopreservation. In this study were used two differents protocols to produce IVP bovine embryos, with variations in protein source, oocyte/zygote density per media volume, with the aim to determine the *in vitro* and *in vivo* embryo survival after vitrification using hand-pulled glass micropipettes. Expanded blastocysts (D7) were morphologically selected by size (≥180 µm) and osmotic behavior before they were randomly allocated to sub-groups by protocol: non-vitrified embryos (control; C) and vitrified embryos (V). For the evaluation of the *in vitro* survival, control embryos and a group of warmed vitrified embryos were *in vitro*-cultured (IVC) for 72 h. Re-expansion rates of warmed embryos at 24 h of IVC were 94.8% and 93.2% for Protocols 1 and 2, respectively. Hatching rates at 72 h of IVC of embryos from Protocol 1 (C=80% and V=75.8%) tended to be higher (P=0.0561, Chi^2 test) than those from Protocol 2 (C=67.2% and V=59.3%). For the evaluation of *in vivo* survival, 21 vitrified embryos per protocol were singly non-surgically transferred to synchronized recipients (n=42) after the in-straw cryoprotectant dilution, resulting in 4 (19%) pregnancies per group on Day 60 of gestation. In conclusion, despite a lower variation on *in vitro* embryo development between both IVP protocols, the use of different protocols under the same laboratory conditions did not afect the *in vitro* and *in vivo* embryo viability after vitrification into hand-pulled glass micropipettes.

Key words: vitrification, embryo survival, direct transfer.

RESUMO

O protocolo de produção *in vitro* (PIV) de embriões bovinos é um dos fatores críticos na determinação da viabilidade dos embriões após a criopreservação. Neste estudo foram utilizados dois protocolos de PIV com variações na fonte protéica, relação oócito/zigoto por volume de meio e condições de cultivo objetivando determinar a sobrevivência *in vitro* e *in vivo* dos embriões após vitrificação dentro de micropipetas de vidro estiradas à mão. Blastocistos expandidos (D7) oriundos dos dois protocolos foram selecionados morfologicamente pelo tamanho (≥180 µm) e comportamento osmótico antes de serem aleatoriamente distribuídos em dois sub-grupos: embriões não vitrificados (controle; C) e embriões vitrificados (V). Para a avaliação da sobrevivência *in vitro*, os embriões controle e grupos de embriões reaquecidos foram cultivados *in vitro* (CIV) por 72 h. As taxas de re-expansão após 24 h de CIV dos embriões re-aquecidos foram 94,8% e 93,2% para os Protocolos 1 e 2, respectivamente. As taxas de eclosão (72 h) dos embriões do Protocolo 1 (C = 80% e V = 75,8%) tenderam a ser maiores do que as do Protocolo 2 (C = 67,2% e V = 59,3%). Para a avaliação da sobrevivência *in vivo*, após a remoção dos crioprotetores dentro da palheta, 21 embriões vitrificados de cada protocolo foram transferidos por via transcervical para receptoras síncronas (n=42). Aos 60 dias de gestação, foram diagnosticadas por palpação retal 4 (19%) prenhezes em cada grupo. Em conclusão, apesar de uma pequena variação na taxa de desenvolvimento *in vitro* entre os diferentes protocolos, os resultados de sobrevivência *in vitro* e *in vivo* demonstraram que, sob condições laboratoriais similares, os diferentes protocolos não afetaram a viabilidade dos embriões após a vitrificação em micropipetas de vidro estiradas à mão.

Descritores: vitrificação, sobrevivência embrionária, transferência direta.

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INTRODUCTION

Bovine embryos generated by distinct *in vitro* production (IVP) systems can respond differently to cryopreservation approaches [1,5,36]. Consequently, it is difficult to compare data from different laboratories due to the diverse experimental and enviromental conditions. This variability may be determined by a multitude of factors such as water quality [21,22], type of oil overlay [36], oocyte quality [15,28], and culture systems [18,19,27], among others.

The embryo cryotolerance largely depends on the IVP conditions, which is a determinant of embryo quality, but cryosurvival can also be improved by adjusting or improving the cryopreservation procedures. The OPS technology has been proven successful not only for the vitrification of bovine immature oocytes and IVP embryos [37], but also in combination with the in-straw cryoprotectant dilution method [16,34]. In addition, the rapid cooling and warming rates in the OPS system may be accompanied by variations in the vitrification container [3,20]. In this view, the use of glass micropipettes may offer physical advantages over plastic straws. However, methodologies that allow the direct transfer of the embryos after the in-straw cryoprotectant dilution [16,34] have not been tested with glass micropipettes.

The aim of this study was to determine the influence of two IVP protocols on the *in vitro* development and *in vitro* and *in vivo* survival of bovine expanded blastocysts after vitrification into hand-pulled glass micropipettes.

MATERIALS AND METHODS

Source of *cumulus-oocyte complexes*

Bovine ovaries were transported to the laboratory in PBS at 24-32°C upon slaughter. *Cumulus-oocyte complexes* (COCs) isolated by ovary slicing were evaluated under a stereomicroscope and only COCs with evenly granulated cytoplasm and surrounded by a compact and dense cumulus cell layer were used in two distinct IVP protocols.

In vitro Embryo Production Protocols

The IVP systems used in this study were based on established procedures by Rodrigues *et al*. [29] and Vieira *et al.*, [37], for Protocols 1 and 2, respectively, with minor modifications. Distinctions between IVP protocols during the various embryo production steps are depicted in Table 1.

- ***In vitro* maturation (IVM).** COCs were *in vitro*-matured in TCM-199 supplemented with 25.0 mM $NaHCO_3$, 0.2 mM pyruvic acid, 50 µg/mL gentamicin, 0.5 µg/mL FSH, 0.03 IU/mL hCG and heat inactivated estrous serum (Table 1) for 24 h at 39°C and 5% CO_2 in humidified air.
- ***In vitro* fertilization (IVF).** Frozen-thawed spermatozoa were segregated by swim-up in Sperm-TALP [25]. Capacitated sperm cells were added to Fert-TALP medium [25] at 10^6 cells/mL and co-cultured with COCs for 20 h.
- ***In vitro* culture (IVC).** For Protocol 1, presumptive zygotes were cultured in modified SOF medium [6] at 39°C for 168 h, under the conditions described in Table 1. Cleavage rates were recorded on Day 2 of development (IVF = Day 0). For IVC in Protocol 2, presumptive zygotes were incubated in SOFaaci medium [11] at 39°C in 5% CO_2 in humidified air. Cleavage rates were recorded at 24 h of IVC, and at this time, uncleaved structures were removed from the wells and embryos were further cultured, under controlled atmosphere (Table 1) for additional 144h at 39°C.

Table 1. Major distinctions between protocols (Prot.) for bovine *in vitro* embryo production.

IVP Step	Prot.	E_2	Protein source	Structures	Media volume	Media	Oil overlay	O_2 Tension
IVM	1	Yes	10% ECS	15 COC	100 µL	TCM 199	Yes	20%
	2	No	10% EMS	30-40 COC	400 µL		No	20%
IVF	1	-	BSA	15 COC	100 µL	Fert-TALP	Yes	20%
	2	-	BSA	30-40 COC	400 µL		No	20%
IVC	1	-	10% ECS	20 Emb.	80 µL	SOF	Yes	5%/168h
	2	-	5% EMS	30-40 Emb.	400 µL	SOFaaci	Yes	20%/24h 5%/144h

Embryo selection

At the end of the culture period (168 h = Day 7), blastocyst rates were recorded and only embryos reaching the expanded blastocyst stage (≥180 μm diameter) and classified morphologically as grade 1-2 [10] were selected for the experiments. The basic medium (BM) for embryo maintenance, vitrification and warming was PBS supplemented with 10% fetal calf serum (FCS). Embryos from both protocols that did not shrink after exposure to BM for 5 min were randomly allocated either to the non-vitrified control sub-group or to the vitrified sub-group. Embryos identified as controls were maintained in BM on a warm stage at 39°C for approximately 60 min, time required for the vitrification and warming of both vitrification sub-groups. Twelve replicates were carried out to standardize procedures and minimize experimental variations.

Vitrification procedure

Embryos were exposed for 5 to 10 min to BM + 3% ethylene glycol (EG) for the evaluation of their osmotic behavior [13] and to promote an additional cryoprotectant cell influx to attain cellular saturation prior to vitrification [24]. Embryos that did not fully re-expand after shrinkage were discarded from the study. The vitrification procedure was based on methods originally described by Vajta *et al*. [35] using hand-pulled glass micropipettes (GMP), with outer diameters of approximately 0.6 mm, instead of plastic straws. GMPs containing vitrified embryos to be warmed for the *in vitro* development experiment were held vertically in a small plastic tube partially immersed in LN_2, whereas GMPs containing vitrified embryos to be stored for their subsequent transfer to recipients (*in vivo* development experiment) were placed into pre-cooled and labeled 0.5 mL plastic straws [33], and stored in a LN_2 tank.

Warming and in straw cryoprotectant dilution procedures

The warming procedure was based on the methods originally described by Isachenko *et al*. [12], using a warming solution (WS) composed of BM + 0.26 M sucrose. Following warming and pre-dilution [12], the contents of the GMPs were transferred into a plastic straw loaded with WS. For the *in vitro* development experiment, each straw loaded with a single embryo was held horizontally for 10 min at RT; then, embryos were expelled into a dish containing BM. Subsequently, vitrified and the non-vitrified control embryos were transferred to 100 mL drops of SOFaaci supplemented with 5% (v/v) of EMS, under oil, for additional 72 h of IVC for the determination of re-expansion and hatching rates. For the *in vivo* development experiment, each straw loaded with a single embryo was held horizontally for 5 min, at RT, prior to the direct non-surgical single embryo transfer (ET) to synchronous (± 24 h) recipient females. Pregnancy diagnosis was performed on Day 60 of gestation (53 days after ET) by rectal palpation.

Statistical analysis

Re-expansion, hatching and pregnancies rates were analyzed by the Chi-square test. Probability of $P<0.05$ was considered statistically significant.

RESULTS

After twelve replications, cleavage rates were different (P=0.0002) between protocols. However, the number of expanded blastocysts on D7 was similar between groups (Table 2). Re-expansion rates of vitrified embryos did not differ (P>0.05) between protocols, but hatching rates of both vitrified and non-vitrified control embryos from Protocol 1 had a trend (P=0.0561) to be higher than embryos from Protocol 2 (Table 3). Embryo transfers after in-straw cryoprotectant dilution resulted in 4 (19%) pregnancies per experimental group at 60 Days of gestation.

Table 2. Comparative embryo yield efficiency between two IVP protocols (12 replications).

Protocol	IVC n	Cleavage n (%)	Embryos D7 n (%)	Expanded blastocysts n (%)
1	2155	1491[a] (69.2)	523[a] (24.7)	253[a] (11.7)
2	2605	1931[b] (74.1)	583[a] (22.4)	279[a] (10.7)

[a,b]Columns without common superscripts differ (P<0.05).

Table 3. *In vitro* developmental rates of vitrified and non-vitrified (control) embryos produced by two different IVP protocols.

Protocol	Re-expansion at 24h		Hatching at 72h	
	vitrified	control	vitrified	control
1	55/58 (94.8%)	-	44/58[A] (75.8%)	48/60[A] (80.0%)
2	55/59 (93.2%)	-	35/59[B] (59.3%)	43/64[B] (67.2%)

[A,B]Columns without common superscripts differ (P<0.1).

DISCUSSION

The worldwide increase in the number of research groups adopting IVP technologies, along with the rise in its commercial applications, have tremendously diversified IVP procedures among different laboratories. Consequently, embryo production efficiencies between IVP protocols, or even laboratories, are very difficult to reconcile. In this study, embryo developmental rates were relatively similar between IVP protocols, despite differences regarding presence or absence of oil underlay and/or steroid hormone during IVM, or protein source, oocyte/embryo density, and media volume during the IVP steps.

The medium droplet method used in Protocol 1 was first introduced by Gwatkin [9] for mammalian embryo culture and still remains very popular for the IVP of mammalian embryos. Nevertheless, it is known that the use of oil overlay determines a high contacting surface/volume ratio with the culture medium, resulting in an interaction, and potential exchange, of lipophilic substances (e.g., estradiol, progesterone) between both fluids, which may cause changes in medium composition [36], affecting embryo development. In this view, our results corroborated with Xu *et al*. [38], in which the absence of oil overlay during bovine IVM showed better cleavage rates than the maturation in medium under oil (Table 2). Such differences could well be attributed to better IVM conditions under Protocol 2 (no oil overlay).

In addition, the use of larger numbers of oocytes [15] or medium volume during IVM may also be beneficial to maturation. However, despite the higher cleavage rate observed in Protocol 2, blastocyst rates were not different between IVP protocols. This effect could be associated with the embryo incubation in 5% CO_2 in air (20% O_2) during the first 24 h of IVC, which is in accordance with results by others [40]. This biphasic gas atmosphere procedure was used in Protocol 2 to avoid the excessive manipulation of the dish prior to cleavage rate evaluation and removal of uncleaved structures from the wells [30]. Yuan *et al.* [40] demonstrated that the IVC at 20% O_2 for 72 h compromised the subsequent zygote development, even if the O_2 tension were lowered to 5% afterwards. In Protocol 1, the entire IVC period occurred under a low O_2 tension (5%), and in a higher embryo density [26] and serum concentration (10% vs. 5%) than Protocol 2. More serum in the medium provides more glucose levels and stimulates blastocysts development by accelerating the process of blastulation [17,31,39]. Despite these potential embryotrophic effects in Protocol 1, the use of EMS in Protocol 2 could also confer similar blastocyst yields, probably induced by embryotrophic factors (e.g., polyamine mitogenic factor) present in the equine serum [7]. The similar blastocyst rates observed between both IVP protocols showed compensatory differences during the distinct stages of the IVP process. These data are in accordance with the observation that embryo production is also dependent on the origin and quality of the oocytes [15,28], in parallel to the IVP protocol.

During embryo selection by morphology and diameter, some embryos shrank upon exposure to PBS + FCS, despite medium isosmolarity. This osmotic behavior is thought to be an adjustment of the embryonic metabolic pathways to the abrupt change in substrate composition [8]. In this study, embryos that shrank in PBS or in a 3% EG solution were discarded; only embryos capable of activating osmotic control mechanisms necessary to tolerate the cryopreservation process [13] were selected. Interestingly, in some replications of this study, part of the embryos in the control group did not hatch after IVC, despite the selection of early blastulation and higher diameter criteria to improve embryo selection [23]. The lower hatching rate in non-vitrified embryos appeared to characterize metabolic alterations not identified or detectable during the selection process.

In the vitrified groups, embryos from Protocol 1 had a trend for a better hatching rate ($P=0.0561$, Chi^2 test). However, this trend observed in the *in vitro* survival study was not confirmed in the *in vivo* studies after the transfer of embryos to synchronous recipients ($P>0.05$). These results agree with Donnay *et al*. [4], i.e., that no correlation exists between hatching and pregnancy rates. The lack of correlation between *in vitro* and *in vivo* development could also be attributed to the influence of the IVC conditions in the hatching rates post-warming [27].

If *in vitro*-produced bovine embryos are to be fully utilized in practice, the warming procedure must be simple and efficient enough to allow embryos to be handled and transferred in a fashion as simple as artificial insemination with thawed semen. In this study, embryos were successfully vitrified employing hand-pulled glass micropipettes (GMP) instead of hand-pulled plastic straws [35]. The use of a tube with a larger ope-

ning for the warming of vitrified samples [12] enhances the warming rate and facilitates the process when compared with the use of 0.25 mL straws [16,34]. In addition, the dilution of the cryoprotectant agents within plastic straws allowed embryos to be prepared, effectively and promptly, for direct transfer without the need of a stereomicroscope, which represents a considerable improvement and simplification for the direct transfer of bovine embryos vitrified into GMP or OPS.

The vitrification procedure usually results in cellular and subcellular damage, most of which are gradually restored over a 24 h period [32]. A reduction in the inner cell mass and trophectodermal cell number in vitrified embryos [14] might be one of the possible causes of placentary alterations, which may be associated with lower gestation rates after *in vitro* embryo manipulations [2]. In this study, pregnancy rates on Day 60 for both protocols were lower than those reported by Lewis *et al*. [16]. However, those authors transferred two embryos per recipient, and performed the pregnancy diagnoses 40 days after transfer, which should confer higher pregnancy rates than the data reported in this study. The transfer of a single embryo per recipient and the pregnancy diagnosis on Day 60 were chosen for practical and economical reasons, since the highest rates of embryonic and fetal losses in IVP embryos are known to occur between Days 30 and 44 of pregnancy [2]. After this period, pregnancy rates will reflect more closely the effectiveness of each IVP protocol. This fact highlights the importance of a late gestation diagnosis, which is of economical importance to production *per se*, since losses after Day 60 of pregnancy do not appear to be higher in IVP pregnancies than controls [2].

CONCLUSIONS

Major variations in IVP protocols in this study included oil overlay and/or estradiol supplementation, protein source, oocyte/zygote density, medium volume, and culture atmosphere. Such differences in IVP protocols, under the same laboratorial conditions, did not affect embryo development to the blastocyst stage, the cryotolerance of expanded blastocysts, and pregnancy rates of vitrified embryos following transfer to synchronous recipients.

Further work is required to determine the effect of additional steps in different protocols on embryo yield, quality and cryotolerance. However, judging from these *in vitro* and *in vivo* experiments, the vitrification of embryos in hand-pulled glass micropipettes, combined with alternative warming and in-straw cryoprotectant dilution procedures, was proven effective for the direct transfer of IVP bovine blastocysts under field conditions, which has an important role in the practical implications for IVP research and its commercial application.

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