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Original article

Macromolecular source as dependent on osmotic pressure and water source: effects on bovine in vitro embryo development and quality

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Abstract — This study evaluated the protective effect of protein, as dependent on osmolarity, and the quality of water sources used to prepare embryo culture media. In Experiment 1, two concentrations of NaCl were used to obtain culture media with normal (280 mOSM) and low (245 mOSM) osmolarity, each supplemented with either bovine serum albumin (BSA) or polyvinyl alcohol (PVA). Low osmolarity improved blastocyst rates in the presence of BSA ($P < 0.01$) and tended to do it in medium containing PVA ($P < 0.07$). Furthermore, low osmolarity allowed PVA to increase inner cell mass (ICM) numbers and ICM/total cell rate ($P < 0.05$), while trophectoderm (TE) and total cell counts tended to decrease ($P < 0.08$). In Experiment 2, culture media were prepared with two water sources (Milli-Q and Sigma-W3500-) in combination with BSA or PVA. Both water sources yielded similar embryo development rates, but in the presence of BSA, Milli-Q water produced embryos with increased ICM/total cells rates ($P < 0.05$). On the contrary, Sigma water tended to increase trophectoderm cell counts ($P < 0.08$). In conclusion, the present study showed that low osmolarity is beneficial to embryo development and combinations of macromolecule and osmolarity influence trophectoderm differentiation. Both Milli-Q and Sigma supported embryo development at comparable rates, although in the presence of BSA, blastocysts obtained in the medium prepared with Milli-Q water had superior quality in terms of ICM/total cells rates.

in vitro produced bovine embryo / osmolarity / water quality / protein / cell number

1. INTRODUCTION

Intracellular osmolarity has been suggested to play an important role during in vitro development [1–3], being influenced by the osmolarity of the environment. Li and Foote [4] indicated that the osmolarity of the bovine embryo culture medium

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should be comprised between 250 and 270 mOSM. Mammalian embryo culture media routinely use NaCl for osmolarity adjustment and ionic balance maintenance [5]. A reduced NaCl concentration in the fertilization medium and in the defined culture medium improves, respectively, monospermic fertilization [6] and embryo development [7] in bovines. In contrast, it has been reported that a high concentration of NaCl during in vitro fertilization is beneficial to the development of rat embryos [8]. Although little is known regarding the effects of NaCl concentration in media used to produce bovine embryos in vitro, the sodium ion is involved in a large number of cellular functions. High concentrations of exogenous inorganic salts may cause protein structure changes by increasing the intracellular ion levels [6, 9]. NaCl concentration is not, however, the only substance responsible for the osmotic pressure regulation of culture medium, since amino acids can act as intracellular organic osmolytes [10, 11]. Accordingly, exogenous protein present in the culture medium can be endocyted by the embryo in order to maintain the intracellular amino acid pool [12]. Cells in the brain and kidney, as probably other cells do, accumulate organic osmolytes in their cytoplasm in response to an osmolarity increase [10]. Usually, culture media are added with protein supplements such as fetal calf serum (FCS) or bovine serum albumin (BSA) which can contribute amino acids to the medium [13]. These protein sources can be substituted by commercial replacements [14] or synthetic macromolecules such as polyvinyl-alcohol (PVA), a compound seemingly without biological activity, which allows one to obtain defined culture conditions. Although FCS and BSA can introduce viral contaminants [15], and bacterial endotoxins [16, 17] and show variability among batches [17], protein has an important protective effect against heavy metals and toxic substances, which can be present in water [18] and/or oil used to cover culture medium [19, 20]. The protective effect of BSA could be more important than its nutritive role [21]. Water quality used in bovine embryo culture media strongly affects embryo development [22], and variations in embryo development and pregnancy rates achieved among in vitro fertilization laboratories may be partially attributable to differences in the water quality used to prepare culture media [23]. In vitro fertilization (IVF) laboratories can obtain water for their cultures from tap water purification or commercially available water (tested for cell or embryo culture). To assist in the selection, operation and maintenance of a water purification system, the level of contaminants must be carefully monitored, and a chemically defined embryo production system should be employed to evaluate water quality. Removal of water contaminants is an important goal, since water constitutes the predominant component in any medium formulation. The objectives of this work were to study the protective effect of protein during the bovine embryo culture in vitro as dependent on its ability to compensate changes in osmolarity and water quality in culture medium.

2. MATERIALS AND METHODS

All products used in the experiments were purchased from Sigma (Barcelona, Spain) unless otherwise indicated.

2.1. Oocyte recovery

The ovaries that were recovered from slaughtered cows were placed in NaCl solution (9 $mg \cdot mL^{-1}$) containing antibiotics (penicillin, 100 $UI \cdot mL^{-1}$ and streptomycin sulphate, 100 $\mu g \cdot mL^{-1}$) and maintained at 30–35 °C until the recovery of cumulus-oocyte complexes (COC). The ovaries were washed twice in distilled water and once in freshly prepared saline. COC were aspirated from 2 to 7 mm visible follicles

through an 18-gauge needle connected to a syringe and recovered in a 50 mL Corning tube. Follicular fluid and COC were placed in an ovum concentrator (Em-Con, Comextrade, Tarragona, Spain) and rinsed 3 times with a holding medium (HM: TCM199 $HNaCO_3$-Invitrogen-, Barcelona, Spain + 25 mM Hepes + BSA 0.4 $g{\cdot}L^{-1}$) supplemented with 2 $UI{\cdot}mL^{-1}$ of heparin.

2.2. In vitro maturation

Only oocytes enclosed in a compact cumulus with an evenly granulated cytoplasm were selected for maturation. The COC were washed 3 times in HM and twice in the maturation medium, which consisted of TCM199 $HNaCO_3$ (2.2 $g{\cdot}L^{-1}$), FCS (10% v/v), FSHp (1 $\mu g{\cdot}mL^{-1}$), LH (5 $\mu g{\cdot}mL^{-1}$), 17 β-estradiol (1 $\mu g{\cdot}mL^{-1}$) and cysteamine (100 μM). Maturation was performed by culturing approximately 50 COC in 500 μL of maturation medium in four-well dishes at 39 ºC in 5% CO_2 in air and high humidity.

2.3. In vitro fertilization

Sperm separation was carried out using a swim-up procedure similar to that reported by Parrish et al. [24]. Briefly, semen from 1 frozen straw of a single bull was thawed in a water bath and added to a polystyrene tube containing 1 mL of pre-equilibrated Sperm–TALP. After 1 h of incubation, approximately 700 μL of the upper layer of supernatant containing the motile sperm was removed. The sperm were centrifuged for 7 min at 200 *g* and the supernatant was aspirated to leave a pellet of approximately 100 μL in volume. Sperm concentration was determined with a haemocytometer. After 22 to 24 h of maturation, the COC were washed 2 times in holding medium and placed in four-well culture dishes containing pre-equilibrated fertilization medium (Fert-TALP) with heparin (10 $\mu g{\cdot}mL^{-1}$, Calbiochem, La Jolla, CA). Spermatozoa were then added at a concentration of 2×10^6 $cells{\cdot}mL^{-1}$ of medium, each well containing 100 COC maximum in 500 μL of medium. In vitro fertilization was accomplished by incubating oocytes and sperm cells together for 18–20 h at 39 ºC in 5% CO_2 and high humidity.

2.4. Embryo culture

Presumptive zygotes were vortexed for 2 min to separate cumulus cells and were washed 3 times in HM and twice in synthetic oviduct fluid (SOF) as modified by Holm et al. [25], before being transferred to dishes containing 400 μL of SOF. Culture medium was prepared under paraffin oil (Uvasol; Merck, Darmstadt, Germany) and equilibrated in an incubator for 2 h before the addition of zygotes. The culture was carried out at 39 ºC, 5% CO_2, 5% O_2 and 90% N_2. Embryo culture was maintained up to Day 9, and embryonic development was evaluated on Days 3, 6, 7, 8 and 9. Total blastocyst rate (including early blastocysts, blastocysts and expanded blastocysts) was recorded on Days 7, 8 and 9, and the expansion rate was evaluated on Day 8 [26].

2.5. Differential cell counts

Day 8 expanded and hatched blastocysts were fixed and stained for differential cell counting as described by Van Soom et al. [27]. Briefly, expanded blastocysts were incubated in PBS (Invitrogen, Barcelona, Spain) + 5% pronase for 1 min and in acid Tyrode solution for 1 min to remove the zonae pellucida. Both hatched and zona-free expanded blastocysts were incubated in trinitrobenzenesulfonic acid and in a rabbit antiserum (antiDNP-BSA) solution. Then, the blastocysts were incubated in guinea-pig complement serum for 30 min at 39 ºC. Embryos were subsequently washed in TCM199 Hepes + 10 $\mu L{\cdot}mL^{-1}$ propidium iodide. The samples were fixed in ethanol and incubated in bisbenzimide (Hoescht 33342; 10 $\mu L{\cdot}mL^{-1}$ ethanol). Finally, mounting

on a glass slide allowed evaluation under a fluorescence microscope at 400× and with an excitation filter of 330–385 nm and barrier filter of 420 nm. Trophectoderm cells (TE) fluoresce red and inner cell mass (ICM) appear blue. After counting both populations of cells, the ICM/total cells rate was used as a criterion for embryo quality [28].

2.6. Experiment 1

Two concentrations of NaCl (107.6 mM and 88 mM) were used to obtain culture media with normal (275–285 mOSM) and low (240–250 mOSM) osmotic pressure, respectively. Both media were supplemented with either an embryo tested BSA Fraction V (A3311) (4 $mg \cdot mL^{-1}$) or PVA (3 $mg \cdot mL^{-1}$) [25]. Osmotic pressure measurements were performed with a vapor pressure osmometer (Vapro™, Wescor Inc., Utah, USA). Embryo development (10 replicates) and differential cell counts (6 replicates) were analyzed.

2.7. Experiment 2

The effect of preparing culture media with two sources of water (ultrapure Milli-Q water [M], and commercially available Sigma water [S]) (W3500) in combination with the addition of two macromolecules, BSA or PVA, was analyzed. The parameters studied were embryo development (10 replicates) and differential cell counts (6 replicates).

2.8. Statistical analysis

Data from the embryo development were considered as categorical variables, and therefore previously analyzed using the Categorical Data Modeling procedure (CATMOD) [29]. In this analysis only those effects showing a significant influence on dependent variables (treatment and replicate) were used to fit a linear model. Subsequently, variables within each experiment were analyzed fitting the General Linear Model (GLM) procedure of SAS [29]. Development data are referred to as the frequency percentages of matured oocytes, while blastocyst cell counts are absolute values. Least square means and the corresponding standard errors were estimated for all fixed effects showing a significant F value. As indicated in the tables, the Duncan multiple-range test was performed on these main-effect means.

3. RESULTS

3.1. Experiment 1

No differences were found for the cleavage percentage, with values comprised between 82.7 ± 1.9 (culture under normal osmotic pressure + BSA) and 87.0 ± 1.9 (culture under low osmotic pressure + PVA). As shown in Table I, low osmolarity improved blastocyst rates on Days 7, 8 and 9 in the presence of BSA and tended to do it in the presence of PVA ($P < 0.07$). Under normal osmotic pressure, Day 7 blastocyst rates were improved in the presence of BSA. As compared to BSA, PVA under low osmolarity increased ICM numbers and ICM/total cell rate while TE and total cell counts tended to decrease (Tab. II) ($P < 0.08$). None of these effects could be observed under normal osmolarity. On the contrary, the protein in the medium with normal osmolarity gave blastocysts with a higher ICM/total cells rate than protein in the low osmotic pressure medium.

3.2. Experiment 2

Differences on developmental rates were not found between Milli-Q and Sigma water, but BSA improved development when added to the SOF medium made of both Milli-Q and Sigma water (Tab. III). In the

Table I. In vitro embryo development under normal (275–285 mOSM; N) and low (240–250 mOSM; L) osmolarity in SOF medium supplemented with BSA or PVA as a macromolecular (MCM) source.

Groups			5–8 cells	% Blastocysts[f]			
Osm[d]	MCM	N[e]	Day 3	Day 7	Day 8	Day 9	Day 8 Expanded
N	BSA	208	59.0 ± 2.5[x]	16.3 ± 2.7[xa]	21.1 ± 2.5[x]	22.4 ± 2.6[x]	14.1 ± 2.6
N	PVA	205	61.1 ± 2.5[a]	8.1 ± 2.7[xb]	14.6 ± 2.5[x]	15.8 ± 2.6[x]	8.4 ± 2.6[x]
L	BSA	206	70.0 ± 2.5[yb]	27.5 ± 2.7[y]	32.3 ± 2.5[y]	32.8 ± 2.6[y]	19.8 ± 2.6[y]
L	PVA	205	66.0 ± 2.5	13.6 ± 2.7[x]	17.8 ± 2.5[x]	17.8 ± 2.6[x]	8.8 ± 2.6[x]

[d] Osmotic pressure; [e] Number of oocytes in culture; [f] Blastocyst rate (includes early blastocysts, blastocysts and expanded blastocysts); Replicates: 10. Data are LS mean percentages of cultured oocytes ± SE. Different superscripts in columns show significant differences: [a,b] $P < 0.05$; [x,y] $P < 0.01$.

Table II. Differential cell counts in Day 8 fully expanded and hatched blastocysts cultured in SOF medium under normal (275–285 mOSM; N) and low (240–250 mOSM; L) osmolarity, and containing BSA or PVA as macromolecular (MCM) source.

Groups			Number of cells			
[c]Osm	MCM	N[d]	ICM[e]	TE[f]	Total	% ICM/Total
N	BSA	27	35.7 ± 3.1[a]	76.1 ± 7.4[x]	108.8 ± 7.2	30.4 ± 3.0[a]
N	PVA	16	26.3 ± 3.6	81.4 ± 8.8[a]	106.2 ± 8.5	24.0 ± 3.6
L	BSA	14	24.9 ± 4.0[b]	110.0 ± 9.7[by]	133.1 ± 9.4	20.1 ± 4.0[b]
L	PVA	11	36.0 ± 4.9[a]	73.0 ± 12.1[x]	109.0 ± 11.7	33.4 ± 5.0[a]

[c] Osmotic pressure; [d] Number of embryos; [e] Inner cell mass; [f] Trophectoderm; Replicates: 6. Data are LS mean number of cells ± SE. Different superscripts in columns show significant differences: [a,b] $P < 0.05$; [x,y] $P < 0.01$.

Table III. In vitro embryo development in SOF medium prepared with Milli-Q (M) or Sigma (S) water and supplemented with BSA or PVA as macromolecular (MCM) source.

Groups			5–8 cells	% Blastocysts[e]			
Water	MCM	N[d]	Day 3	Day 7	Day 8	Day 9	Day 8 Expanded
M	BSA	206	60.0 ± 2.3	16.9 ± 2.1[a]	25.3 ± 2.6[ax]	25.7 ± 2.7[ax]	13.5 ± 2.2
M	PVA	204	58.5 ± 2.3	8.2 ± 2.1[b]	12.2 ± 2.6[cy]	13.5 ± 2.7[cy]	8.5 ± 2.2
S	BSA	208	59.0 ± 2.3	16.3 ± 2.1[a]	21.1 ± 2.6[ab]	22.4 ± 2.7[ab]	14.1 ± 2.2
S	PVA	205	61.1 ± 2.3	8.16 ± 2.1[b]	14.6 ± 2.6[bcy]	15.8 ± 2.7[bc]	8.4 ± 2.2

[d] Number of oocytes in culture; [e] Blastocyst rate (includes early blastocysts, blastocysts and expanded blastocysts); Replicates: 10. Data are LS mean percentages ± SE. Different superscripts in columns show significant differences: [a,b,c] $P < 0.05$; [x,y] $P < 0.01$.

Table IV. Differential cell counts in Day 8 fully expanded and hatched blastocysts produced in SOF medium prepared with Milli-Q (M) or Sigma (S) water and supplemented with BSA or PVA as macromolecular (MCM) source.

Groups			Number of cells			
Water	MCM	N[c]	ICM[d]	TE[e]	Total	% ICM/Total
M	BSA	15	39.2 ± 3.9	64.3 ± 8.8	102.3 ± 9.3	37.2 ± 3.5[a]
M	PVA	10	30.2 ± 5.0	73.7 ± 10.9	101.7 ± 11.5	28.5 ± 4.3
S	BSA	25	29.2 ± 3.4	93.8 ± 7.5	122.2 ± 7.9	24.6 ± 2.9[b]
S	PVA	18	28.4 ± 3.7	79.6 ± 8.5	107.8 ± 8.9	26.9 ± 3.3

[c] Number of embryos; [d] Inner cell mass; [e] Trophectoderm; Replicates: 6. Data are LS means number ± SE. Different superscripts in columns show significant differences: [a,b] $P < 0.05$.

presence of BSA, Milli-Q water gave rise to embryos with an increased ICM/total cell rate while Sigma water tended to increase trophectoderm cell proliferation (Tab. IV) ($P < 0.08$).

4. DISCUSSION

Our study showed that reducing osmotic pressure from 280 to 245 mOSM is beneficial for embryo development in the presence of both protein and PVA (defined medium). Both the osmolarity and macromolecular source influenced trophectoderm cell proliferation. Furthermore, Milli-Q and Sigma water allowed to obtain blastocysts at comparable rates, although Milli-Q water containing BSA gave embryos with higher quality in terms of the ICM/total cells percentage. The analysis of distribution of ICM and TE cells by differential staining has been used as a technique to evaluate embryo quality in several species [30, 31]. In bovine embryos, poor morphology is associated with hatched blastocysts with low ICM-cell numbers [31].

Reduced NaCl concentration in a completely defined medium has been reported to be beneficial for the development of bovine pre-implantation embryos in vitro [7]. High osmolarity and high NaCl concentration are detrimental to mouse embryos [11, 32], and porcine embryos develop at higher rates when lower levels of NaCl are added to the culture media [33]. The NaCl concentration, as well as osmolarity, showed a direct effect on rabbit embryo development, with the zygotes being much more sensitive to these effects than two-cell embryos [4]. The manner in which increased Na^+ concentration adversely affects embryo development is unclear. The sodium ion is known to be involved in a large number of physiological, biochemical and morphological aspects of mammalian cell function [34]. Sodium and chloride ions derived from NaCl critically affect a variety of metabolic aspects, and a high concentration of exogenous inorganic salts may raise the intracellular concentrations of these ions up to the level that causes conformational changes of protein structure and disruption of many cellular processes [9]. Embryo culture in medium containing low concentrations of NaCl results in enhanced mRNA synthesis and stability [35], in contrast to the inhibition of protein synthesis observed after increasing intracellular sodium [36].

Although embryos seem to have a considerable ability to adjust to variations in osmotic pressure, several components present in the culture media help embryos to adapt to the variations in osmotic pressure. The presence of protein (FCS, BSA) in the culture medium improves embryo

development in contrast to simple media under defined conditions [11, 37–44]. With the addition of amino acids, BSA can replace FCS in embryo culture without compromising blastocyst rates [45]. The embryo endocytosis of BSA provides amino acids to the intracellular medium [13], which can act as organic osmolytes, allowing culture at higher osmolarities. Glycine is the most abundant amino acid in oviduct fluid and protects the mammalian embryo against osmotic stress [10, 11, 46]. In addition, betaine and glutamine can protect against deleterious effects of high concentrations of NaCl [1, 2], and inositol provides substantial protection to one-cell and two-cell rabbit embryos against osmotic variations [25, 47]. These effects seem to be due to the maintenance of an appropriate K+/Na+ intracellular ratio which is reflected in a greater relative rate of protein synthesis [11, 36, 47]. Our experimental design does not allow us to distinguish between the effects of osmolarity and NaCl concentration. Consequently, the adjustment of the osmotic pressure with other osmolytes as sorbitol should be considered in order to analyze NaCl concentration and osmotic pressure as single effects [5]. Sorbitol and taurine have the ability to reduce the detrimental effects of high NaCl concentration in maturation media for porcine oocytes by an increase of the oocyte intracellular glutathione levels and an enhanced male pronuclear formation after sperm penetration [48].

The number of cells of the blastocyst and the ICM/total cells rate is considered to be a potential indicator of embryo quality [31]. The mean cell numbers of blastocysts recovered in vivo decreases in parallel to embryo quality [49], and IVP embryos have fewer cells than their in vivo counterparts [50, 51]. A minimum number of ICM cells are required to obtain a pregnancy after embryo transfer, and culture conditions influence the cell allocation in different species [27, 31]. Some factors in the culture media, such as growth factors present in the serum, can modify the distribution of the embryonic cells in favor of the TE [48], which can lead to pregnancy abnormalities encountered after transfer of in vitro cultured bovine [49–51] and ovine [52] embryos.

In the present study, low osmotic pressure led to increased TE cell differentiation in the presence of BSA and high ICM proportions in the presence of PVA showing that combinations of macromolecule and osmolarity influence trophectoderm cell differentiation.

Water quality and storage period seriously affect bovine embryo development in protein-free medium [22]. In our laboratory, pre-treated water obtained by reverse osmosis and electrodeionization from an Elix system is deionized, ultrafiltered (5 KDa), UVA-rays treated and sterile filtered (0.22 μm) through a Milli-Q system (Gradient A-10). The pyrogenic endotoxin level is lower than 0.03 $EU \cdot mL^{-1}$ and the electrical conductivity is lower than 0.055 $\mu S \cdot cm^{-1}$. This water was immediately used after production, to prevent negative effects of storage. The Sigma water, as reported by the supplier, is deionized and distilled. No data on electrical conductivity were provided, and the endotoxin level was ≤ 1.0 $EU \cdot mL^{-1}$, compatible with embryo development. High endotoxin levels seem to have an adverse effect on embryonic development and subsequent pregnancy rates in humans [23].

Many laboratories have used the sperm motility bioassay (SMS) to check the quality of water used in the preparation of media for gamete and embryo culture. The presence of protein in the culture medium would reduce or eliminate the ability of the SMS test to detect impurities in water [18]. Consequently, in our study the percentage and quality of blastocysts obtained (measured as ICM/total cells rate) were used to evaluate the quality of two types of water for preparing culture media with BSA or PVA.

Both Milli-Q and Sigma water gave similar blastocyst rates, which were improved

when BSA was added to the medium. In fact, the addition of BSA to the culture medium prevents the toxic effect of metal ions, which can activate superoxides to produce free oxygen radicals, highly toxic for the embryo [56]. A higher BSA concentration in the CZB medium, as well as the addition of EDTA, showed a protective effect against toxic components present in the silicone oil [19]. The toxic effects derived from the silicone oil when used in the microdrop culture system [19, 20] were reduced by using paraffin oil [20]. In our work we used the same batch of paraffin oil during the whole experimental procedure [25].

Commercial BSA preparations, such as that used in the present work, reflect a significant contamination with citrate. Besides its stimulating effect on fatty acid synthesis, citrate in the culture medium may act as a chelator of metal ions together with BSA itself [57].

When BSA was added to the culture medium, Milli-Q water gave blastocysts with an ICM/total cells rate higher than Sigma water, which produced embryos with increased trophectoderm cell proliferation ($P < 0.08$). These results show a beneficial interaction between Milli-Q water and BSA in culture, producing embryos with a higher ICM/total cells rate, and consequently better quality [45]. It should, however, be considered that BSA can introduce differences between batches [17, 21], so we decided to use the same batch of BSA throughout the experiment.

The present study showed that Milli-Q and Sigma water are similar in terms of embryo production under defined conditions. However, when BSA was present, the blastocysts obtained in the medium prepared with Milli-Q water exhibited superior quality measured as ICM/total cells rate. Low osmotic pressure is beneficial to embryo development, and combinations of macromolecule and osmolarity influence trophectoderm differentiation.

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