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Different *in vitro* culture systems affect the birth weight of lambs from vitrified ovine embryos

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Summary

It has been reported that different *in vitro* culture systems affect the birth weight of lambs. The aim of this study was to test body weight and lambing rate of lambs born from five different *in vitro* culture systems after vitrification. Oocytes of Sarda sheep were matured in TCM-199 plus 0.4% bovine serum albumin (BSA) using systems: (i) 4 mg/ml fatty acid-free BSA (BSA4); (ii) 8 mg/ml fatty acid-free BSA (BSA8); (iii) BSA8–hyaluronan (BSA8–HA); (iv) BSA8–charcoal-stripped FBS (BSA8–CH); or (v) with 10% fetal bovine serum (FBS; serum) and fertilized with fresh semen. The presumptive zygotes were cultured up to the blastocyst stage with BSA8, BSA8-HA, BSA8-CH or serum or BSA4. In the third and fifth days of culture 5% charcoal-stripped FBS was added into BSA8-CH and serum, while 8 mg/ml or 4 mg/ml fatty acid-free BSA was added as BSA8, BSA8-HA and BSA4 respectively; 6 mg/ml HA was added to BSA8-HA. In total, 240 vitrified blastocysts were transferred into synchronized ewes. The lambing rate was not significant different between BSA groups or between serum groups (BSA8-CH and serum), while serum groups showed significant lower values when compared with BSA groups. Only BSA8 groups produced heavy lambs (≥4.5 kg) with a significant difference between BSA4 and BSA8 groups ($P < 0.05$).

Keywords: Birth weight, *In vitro* produced blastocyst, Sheep, Vitrification

Introduction

For *in vitro* production (IVP), technology has been improved significantly in cattle and sheep, but the quality of the embryos produced is impaired in comparison with their *in vivo* counterparts (Niemann *et al.*, 2002).

The influence of various *in vitro* procedures on embryo survival and the production of normal offspring has been investigated in different species (Kazuhiro *et al.*, 2002; Lazzari *et al.*, 2002; Dattena *et al.*, 2007). The birth of unusually large lambs has been reported to be probably due to an inappropriate environment in which the embryo is stored for a relatively long time or to extensive manipulation in the laboratory. Several factors give rise to the heavy calf or lamb and they include asynchronous transfer, progesterone treatment (Kleeman *et al.*, 1994), nuclear cloning of embryos (Willadsen *et al.*, 1991), and *in vitro* culture (Holm *et al.*, 1996; Walker *et al.*, 1996).

Several authors have reported an increase in birth weight of lambs following transfer of cultured embryos (Walker *et al.*, 1992; Thompson *et al.*, 1995; Lazzari *et al.*, 2002; Dattena *et al.*, 2007). Our laboratory had reported previously normal birth weights of Sarda lambs born from blastocysts produced *in vitro* and cultured with the addition of 5% charcoal-stripped FBS added only on the third and fifth days of culture (Ptak *et al.*, 1999).

It is well known that a considerable proportion of the offspring born from transfer of IVP embryos is affected by large offspring syndrome (LOS). This syndrome is characterized by a variety of pathologies, including abnormal phenotypes, placental and organ defects, increased gestation length, elevated abortion and increased perinatal death, but the most important

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feature is a significant increase in birth weight (Walker *et al.*, 1996; Sinclair *et al.*, 1998; Young *et al.*, 1998; Niemann & Wrenzycki, 2000; Lazzari *et al.*, 2002).

In this paper we have considered five groups of embryos produced *in vitro* in different culture media, vitrified and transferred into recipient ewes. The groups are indicated as BSA4, BSA8, BSA8-HA, BSA8-CH and serum. The rationale of these five groups was the following: in previous paper Dattena *et al.* (2007) showed that bovine serum albumin (BSA; 8 mg/ml) gives heavy lambs, for this reason we decided to halve the dose from 8 to 4 mg/ml. In previous studies other authors have reported that the use of hyaluronan (HA) improves embryo quality in general (Furnus *et al.*, 1998; Kano *et al.*, 1998; Lane *et al.*, 2003; Dattena *et al.*, 2007), for this reason we used BSA with HA during the culture period. The BSA8-CH group follows the protocol used traditionally in our laboratory (Accardo *et al.*, 2004) and replaced the first protocol used in our laboratory (Ptak *et al.*, 1999) represented by the serum group.

The aim of the present study was to compare body weight and lambing rate of lambs born from five different *in vitro* culture systems used in the last 10 years in our laboratories and to investigate if different culture systems can lead to differences in body birth weight and in lambing rate.

Materials and methods

Except when otherwise indicated, all chemicals were obtained from Sigma-Aldrich.

Collection of ovaries and oocytes

Ovaries of adult Sarda sheep were obtained from local slaughterhouses and transported to the laboratory within 2–3 h in saline solution at a temperature of ∼35–37°C. Oocytes were collected by cutting the ovaries. Only the follicular oocytes covered by at least two layers of granulosa cells and an even cytoplasm were selected for *in vitro* maturation.

In vitro *maturation (IVM)*

Oocytes were washed in HEPES-buffered TCM199 (H-TCM199) supplemented with 0.4% bovine serum albumin (BSA). The medium used for maturation was bicarbonate-buffered TCM199 that contained 2 mM glutamine, 100 μM cysteamine, 0.3 mM sodium pyruvate, 1 μg/ml estradiol-17β, 0.1 i.u./ml follicle stimulating hormone (r-FSH) and 0.1 i.u./ml luteinizing hormone (LH) supplemented with 0.4% BSA (BSA4, BSA8, BSA8-HA and BSA8-CH groups) or with 10% fetal bovine serum (FBS; serum group). The oocytes were incubated in 400 μl of medium in 4-well dishes (Nunc, Nunclon Denmark), each well contained 20–30 oocytes covered with mineral oil. *In vitro* maturation (IVM) conditions were 5% CO_2 in humidified air at 39°C for 24 h.

In vitro *fertilization (IVF)*

After maturation, the oocytes were partially denuded of granulosa cells with 300 i.u./ml of hyaluronidase in H-TCM199. Fresh semen from a Sarda breed ram of proven fertility was collected and kept at room temperature for up to 2 h and then washed in synthetic oviduct fluid (SOF) supplemented with 0.4% BSA. The fertilization medium consisted of SOF (Tervit *et al.*, 1972) enriched with 20% heat inactivated estrous sheep serum. A maximum of 10–15 oocytes per drop were fertilized in 50 μl with 1×10^6 sperm/ml at 39°C and 5% CO_2 in humidified air.

In vitro culture (IVC)

On the day after fertilization, presumptive zygotes were washed in SOF to remove spermatozoa debris. The presumptive zygotes were allocated to 20-μl culture drops (5–6 embryos/drop) and cultured for 6–7 days until blastocyst stage in medium that consisted of SOF supplemented with 1% basal Eagle's medium (BME), 1% minimum essential medium (MEM), 1 mM glutamine and 8 mg/ml fatty acid-free BSA (BSAfaf), for BSA8, BSA8 + HA, BSA8-CH and serum groups, or 4 mg/ml fatty acid-free BSA for the BSA4 group. On the third and fifth days of culture (day 0: day of fertilization) 5% of charcoal-stripped FBS was added to the medium of the BSA8-CH and serum groups, whilst 8 or 4 mg/ml fattyacid-free BSA was added in BSA8 and BSA8-HA or BSA4 groups respectively and 6 mg/ml HA was added to the BSA8-HA group. Embryos were incubated in humidified air that consisted of 7% O_2, 5% CO_2, 88% N_2 and at 39°C (see Table 1).

Vitrification and warming of embryos

Dulbecco's phosphate-buffered saline (PBS) supplemented with 0.3 mM Na pyruvate and 20% FBS was used as the basic vitrification solution. Blastocysts were exposed at room temperature to 10% ethylene glycol (EG) and 10% dimethyl sulphoxide (DMSO) for 5 min, then to 20% EG, 20% DMSO, and 0.5 M sucrose for <30 s. Blastocysts were loaded into open pulled straws (OPS) and immediately plunged into liquid N_2. The straws were warmed in a water bath at 37°C and the contents expelled into a Petri dish, mixed in 0.5 M sucrose for 3–5 min and moved into a drop of H-TCM199 and 20% FBS for the transfer to recipient ewes, in accordance with Dattena *et al.* (2004).

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Table 1 Groups of culture media with different supplements of BSA and serum

Culture groups	IVM	IVC Day 1	IVC Days 3–5
BSA4 (Mara, 2009)	0.4% BSA	4 mg/ml BSA_{faf}	4 mg/ml BSA_{faf}
BSA8 (Dattena, 2007)	0.4% BSA	8 mg/ml BSA_{faf}	8 mg/ml BSA_{faf}
BSA8-HA (Dattena, 2007)	0.4% BSA	8 mg/ml BSA_{faf}	8 mg/ml BSA_{faf} + 6 mg/ml HA
BSA8-CH (Accardo, 2004)	0.4% BSA	8 mg/ml BSA_{faf}	5% Charcoal-stripped FBS
Serum (Ptak, 1999)	10% FBS	8 mg/ml BSA_{faf}	5% Charcoal-stripped FBS

BSA, bovine serum albumin; CH, charcoal stripped; FBS, fetal bovine serum; HA, hyaluronan.

Embryo transfer

Only embryos of high quality (Q1) were utilized for embryo transfer. In total, 240 *in vitro* produced embryos (Q1) were used; 240 blastocysts (48 from the BSA4 group, 51 from the BSA8 group, 50 from the BSA8-HA group, 46 from the BSA-CH group and 45 from the serum group) were transferred in pairs into synchronized sheep by inguinal mini-laparatomy with the help of a Tom-cat.

Lambing

At day 146 of pregnancy, ewes were allocated indoor to monitor the onset of lambing. The lambs were weighed and identified within 6 h of birth.

Table 2 Lambing rates and heavy lamb percentage of embryos produced *in vitro* after culture with different supplements, vitrified and transferred into recipient ewes

Culture medium	No. blastocysts vitrified transferred	Lambs born/transferred embryos (%)	Heavy lambs (%)
BSA4	48	21/48 (43.7)^{a}	0/21 (0)^{a}
BSA8	51	24/51 (47.0)^{a}	5/24 (20.8)^{b,a}
BSA8-HA	50	22/50 (44.0)^{a}	4/22 (18.2)^{b,a}
BSA8-CH	46	10/46 (21.7)^{b,c,b,a}	0/10 (0)^{a}
Serum	45	11/45 (24.4)^{a,b,b,a}	0/11 (0)^{a}

Values in the same column with different letters differ: $^{a,b}P < 0.05$, $^{a,c}P < 0.001$.
BSA, bovine serum albumin; CH, charcoal stripped; HA, hyaluronan.

Results

Lambing rate

When lambing rate was compared within groups statistical difference was found between the BSA groups and the BSA-CH or serum groups (BSA4 and BSA8-HA versus BSA8-CH $P < 0.05$, BSA8 versus BSA8-CH $P < 0.001$, BSA8 and BSA8-HA versus serum $P < 0.05$). There was no significant difference between the BSA groups or between the BSA8-CH and serum groups (see Table 2).

Birth weight

The body weight of Sardinian lambs was considered heavy when it was ≥ 4.5 kg. Thus heavy body weight was found for lambs in BSA8 (5/24, 20.8%) and BSA8-HA (4/22, 18.2%), but only between BSA4 and BSA8 groups was there a significant difference (BSA4 versus BSA8 and BSA8-HA $P < 0.05$). Whilst no heavy lambs were produced in the BSA4, BSA8-CH and serum groups (Table 2).

Discussion

The results presented in this study demonstrated that the use of different supplements can affect lamb body weight. Although it was not the aim of this work to report the embryo cryotolerance, and thus the lambing rates of the vitrified IVP embryos, it has been reported for completeness.

In BSA8-CH and serum groups, the lambing rate was lower when compared with the BSA groups with significant differences between groups BSA4 and BSA8-CH, BSA8 and BSA8-CH, BSA8-HA and BSA8-CH, BSA8 and BSA8-HA versus serum ($P < 0.05$, $P < 0.001$, $P < 0.05$, $P < 0.05$ and $P < 0.05$ respectively). Thus, embryos produced with BSA instead of serum demonstrated a higher cryotolerance. These data are similar to those reported by Dattena *et al.* (2007) and Mara *et al.* (2009) in sheep and by Lazzari *et al.* (2002) in cows.

In our experience, the addition of HA improved neither development to the blastocyst stage nor pregnancy and lambing rates in vitrified embryos when compared with BSA8 (Dattena *et al.*, 2007). When compared with the BSA8-CH and serum groups, the lambing rate in the BSA8-HA group was significantly improved, although the increase was less than that of the BSA groups. These results are difficult to compare with previous studies because most of the authors did not transfer the embryos. These results are in agreement with other studies in which an enhanced lambing rate was reported when this macromolecule

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was used to substitute serum (Lane *et al.*, 2003; Dattena *et al.*, 2007; Mara *et al.*, 2009).

As reported by previous authors, *in vitro* culture (Walker *et al.*, 1992; Holm *et al.*, 1996) and vitrification of embryos (Young *et al.*, 1998; Sinclair *et al.*, 1999) may result in fetuses that are heavier than usual. In this study there were significant differences among the groups analyzed in term of birth weight, in both the BSA8 and BSA8-HA groups in which heavy lambs were born.

Similarly, Lazzari *et al.* (2002), Dattena *et al.* (2007) and Mara *et al.* (2009) reported the presence of heavy born animal from blastocysts produced *in vitro* with BSA. In contrast, other studies reported reduction of large offspring when BSA was added in place of serum during culture (Thompson *et al.*, 1995; van Wagtendonk-de Leeuw *et al.*, 2000). These conflicting findings underscore that incidence of heavy lambs varies greatly and that these conditions are difficult to reproduce (Young *et al.*, 1998). In addition, serum or BSA doses, number of batches and the culture system used varied greatly from laboratory to laboratory, making data difficult to compare.

Thus, in order to reduce the heavy lamb syndrome, keeping good cryotolerance, we decided to halve the dose of BSA from 8 mg/ml to 4 mg/ml. It has been reported by Lazzari *et al.* (2002) that doses of BSA between 8 mg/ml and 16 mg/ml are considered high. Indeed, in this study when 4 mg/ml BSA treatment was used no heavy lambs were born.

In conclusion, the different culture media used in this study affected the body weight and the quality of embryos in term of cryotolerance.

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