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Cobalamin supplementation during *in vitro* maturation improves developmental competence of sheep oocytes

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ABSTRACT

Pregnancies obtained by Assisted Reproductive Technologies are at higher risk of miscarriage than those obtained naturally. Previously, we reported impaired placental vascular development of *in vitro* produced (IVP) sheep embryos and defective DNA methylation in the placentae of those embryos. One reason behind these observed defects may be an impaired One Carbon Metabolism (OCM) The present study was performed to test the hypothesis that Cobalamin (Vitamin B12, an important OCM co-factor) supplementation during IVM corrects DNA methylation of IVP embryos and, consequently, ameliorates placental vasculogenesis. To this aim, embryos derived from oocytes matured with Cobalamin (B12 group) or without (negative control group, −CTR) were transferred to synchronized recipient sheep. At day 20 of pregnancy, collected embryos were morphologically evaluated while placentae were subjected to qPCR and histological analysis. The positive control group (+CTR) consisted of conceptuses obtained from naturally mated sheep. Results showed an increased fertilization rate in the B12 group vs −CTR (69.56% vs 57.91% respectively, $P = 0.006$) not associated with quantitative improvement in blastocyst and/or implantation rate (44.32% vs 36.67% respectively, $P > 0.05$). Moreover, Cobalamin supplementation during oocyte IVM ameliorated resulting conceptuses quality, in terms of placental vascularization (vessels' maturity and vasculogenetic factors' expression). The expression of DNA methyltransferases (*DNMT1, DNMT3A* and *DNMT3B*) was also improved in placentae from the B12 group. In conclusion, Cobalamin supplementation during oocyte IVM improves IVP embryo quality. These results suggest that Cobalamin should be included in standard IVM media.

1. Introduction

Assisted Reproductive Technologies (ART) have contributed to the births of >1% of children worldwide and these numbers are increasing every year [1,2]. Though the majority of children born by ART are healthy at birth, several studies have demonstrated that *in vitro* embryo production (IVP) may be associated with increased pregnancy complications [3,4] developmental and/or imprinting defects [5–8] and postnatal diseases [9–12]. In our previous work, we have observed that sheep IVP conceptuses showed impaired cardiovascular development, such as delayed placental vasculogenesis and a thinner ventricular wall, associated with cardiac and placental hemorrhages [6,7]. Also, defective DNA methylation machinery, in particular DNMT1 dysfunction and deregulated expression of imprinted genes in placental tissues, has been described [6]. One of the possible causes behind these observed developmental defects may reside in alterations of the One Carbon Metabolism (OCM). This metabolic pathway is responsible for several cellular processes, such as cell proliferation, DNA and protein synthesis, gene expression and methylation of DNA, RNA and protein. Dysfunctions of the OCM may lead to reduced pregnancy success and compromised fetal development [13–18]. In particular, deficiency of one or both OCM cofactors – Folate (Vitamin B9) and Cobalamin (Vitamin B12) – during pregnancy is associated with adverse pregnancy outcomes (*i.e.*, neural tube defects, intrauterine growth retardation, abnormal fetal brain development, impaired cardiovascular development and epigenetic defects) [14,19–21], and, in the long term, to metabolic diseases and impaired cognitive and motor function [21–23] in both human and animal models. Commonly used medium for *in vitro* maturation (Medium 199, M-199), does not

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contain Cobalamin. Based on this, we hypothesized that the supplementaion of the maturation medium with Cobalamin may ameliorate the development of embryos, by improving DNA methylation status. To test our hypothesis, we used sheep *(ovis aries)*, as it is a powerful model to study ART pregnancy [24]. To produce IVP embryos, oocytes were *in vitro* maturated with 200 pM Cobalamin (B12 group). Matured MII oocytes and resulting embryos were evaluated for developmental competence and DNA methyltransferase expression profile. Moreover, vascular development was investigated in placentae at day 20 of pregnancy. Two control groups were created: untreated IVP embryos (−CTR) and naturally mated ones (+CTR). Our data showed that Cobalamin supplementation during IVM enhanced the quality of IVP embryos.

2. Materials and methods

All chemicals, unless otherwise indicated, were obtained from Sigma Aldrich Chemicals Co. (St. Louis, MO, USA).

All animal experiments were performed in accordance with the DPR 27/1/1992 (Italian Animal Protection Regulations) and in conformity with the European Community regulations 86/609.

2.1. In vitro maturation (IVM)

Sheep ovaries were collected from local slaughterhouses and transferred to the laboratory within 1–2 h. Oocytes were aspirated with 21 G needles in the presence of TCM-199 medium (Gibco, Thermo Fisher Scientific, Milan, Italy) containing Hepes and Heparin. Then, all oocytes with an unexpanded cumulus and uniform cytoplasm were divided into two groups: untreated control oocytes (−CTR) and treated oocytes (Group B12). Untreated control oocytes were *in vitro* maturated (IVM) in standard medium (bicarbonate-buffered TCM-199 (Gibco) containing 2 mM glutamine), 0.3 mM sodium pyruvate, 100 μM cysteamine, 10% fetal bovine serum (FBS) (Gibco), 5 μg/mL FSH (Ovagen, ICPbio Reproduction, Auckland, New Zealand), 5 μg/mL LH and 1 μg/mL estradiol. Treated oocytes were *in vitro* matured in standard medium supplemented with 200 pM Cobalamin, a concentration that represents the lower amount requested not to be considered deficient [23,25]. Maturation was conducted in 4-well culture plates (Nunclon, Roskilde, Denmark) containing 0.4 mL of IVM medium and a maximum of 30 oocytes. Maturation condition were 5% CO_2 in humified atmosphere and 39 °C for 24 h.

2.2. In vitro embryo production

In vitro fertilized (IVF) embryos were produced as previously described [26]. Briefly, matured oocytes (−CTR, n = 220; B12, n = 191) were partially stripped of cumulus cells by repeated pipetting. Frozen semen was rapidly thawed at 37 °C and washed twice by centrifugation at 500*g* for 5 min in bicarbonate-buffered Synthetic Oviductal Fluid (SOF) with 4 mg/mL BSA. IVF was carried out in 50 μL drops, using 5×10^6 cells/mL and a maximum of 15 oocytes per drop, at 38.5 °C in 5% CO_2 for 20 h. The IVF medium was bicarbonate-buffered SOF enriched with 20% (v/v) heat-inactivated oestrous sheep serum, 2.9 mM Ca^{2+} lactate, and 16 μM isoproterenol. Presumptive zygotes were transferred into 20 μL drops of SOF enriched with 1% (v:v) Basal Medium Eagle (BME) essential amino acids, 1% (v:v) Minimum Essential Medium (MEM) non-essential amino acids (Gibco), 1 mM glutamine and 8 mg/mL fatty acid-free BSA (SOFaa-BSA). Zygotes were cultured in a humidified atmosphere of 5% CO_2, 7% O_2, 88% N_2 at 38.5 °C, and the medium changed on day 3 (supplemented with glucose) and day 5 (supplemented with 10% FBS charcoal stripped). Maturation was assessed by evaluation of cumulus expansion and the extrusion of first polar body. Fertilization rate (number of 2 cells embryos/total number of MII oocytes) was assessed on day 1 and blastocyst formation was recorded on day 7.

2.3. Animal treatment, embryo transfer and sample recovery

2.3.1. Animal treatment and care

Sardinian ewes (n = 25) obtained from local breeders were housed in the authorized experimental farm from the Istituto Zooprofilattico Abruzzo, Loc. Gattia, Italy, fed and kept under the best sheep housing standards. The synchronization of sheep was achieved with Crono-gest sponges of 25 mg (Intervet, Milan, Italy). After 12 days Crono-gest sponges were removed and estrous were monitored for 48 h. Six days after estrous, embryo transfer was performed. Ewes (n = 20) were fasted for 24 h before surgery and then were pre-anestetized with 1 mL IM Acethyl Promazine (Prequillan, Fatro, Ozzano dell'Emilia, Italy) and anesthetized with sodium thiopental (10 mg/kg BW, Penthotal Sodium, Intervet Srl, Milano, Italy). These treatments alleviate level of suffering to minimum. After surgery animals were kept in warm and dry place, isolated from animals until recovery. Post-operatory suffering alleviation was induced by flumixin meglumine (Zoetis, Rome, Italy), given IM, and antibiotic treatment consisted of intramuscular injection of ampicillin (0.02 g/kg, Amplital Vet, Ceva SpA, Agrate Brianza, Italy) every 24 h for 3 days.

2.3.2. Embryo transfer

Twenty females were randomly divided into two groups and were used as recipients of *in vitro* produced embryos. Both −CTR and B12 blastocysts (2–4 per ewe) were surgically transferred to the recipient ewes 6 days after oestrus. Five sheep were naturally mated (+CTR).

2.3.3. Sample recovery

Fetuses and placentae were recovered by para-median laparotomy at 20 days of gestation. Once collected in Petri dishes (90 mm) with warm Ca^{2+}/Mg^{2+} PBS containing 0.005% (w: v) heparin, fetuses were observed under the stereomicroscope to assess their vitality by the presence of heartbeat. Early placental tissues (chorion-allantois) were snap frozen in liquid nitrogen and stored for subsequent analysis and/or fixed for histological evaluation.

2.4. Immuno staining for 5-methylcytidine (5-MeC)

Immunostaining has been performed as previously described [26] with minor modification. MII oocytes and blastocyst stage embryos (day 7) were treated with Pronase/Tyrode's Acid Solution to remove zona. To obtain MII plates, oocytes were exposed to hypotonic solution (0.8% w/v sodium citrate) for 3 min, followed by treatment in 75 mM KCl solution for 3 min at 37 °C. Then, they were pre-fixed in a solution of 75 mM KCl:methanol:acetic acid 3:2:1 v/v for 15 min at −20 °C and then fixed overnight in methanol:acetic acid 3:1 v/v at −20 °C. Subsequently, MII oocytes were spread on slide and MII plates were UV irradiated at 4 °C for 11 h before staining. Blastocysts were washed in PBS/PVP 0.4%, fixed in 4% paraformaldehyde for 15 min and permeabilized in 0.1% Triton X100 for 30 min. They were washed again and hydrolyzed in 4 N HCL for 10 min, neutralized in 100 mM Tris/HCl (pH 8.5) for 15 min, washed in PBS + 0.4% PVP (5 min for 3 times). Both MII plates and embryos were treated with blocking solution (PBS + 1% BSA + 0.05% Tween 20) at 4 °C overnight. They were then incubated with mouse anti-5-methylcytidine antibody (dilution 1:50; sc-56615, Santa Cruz Biotechnology, Santa Cruz,