Accepted Manuscript

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

Federica Zacchini, Paola Toschi, Grazyna Ewa Ptak

PII: S0093-691X(17)30053-5

DOI: 10.1016/j.theriogenology.2017.01.035

Reference: THE 13990

To appear in: *Theriogenology*

Received Date: 8 November 2016

Revised Date: 30 December 2016

Accepted Date: 19 January 2017

Please cite this article as: Zacchini F, Toschi P, Ptak GE, Cobalamin supplementation during *in vitro* maturation improves developmental competence of sheep oocytes, *Theriogenology* (2017), doi: 10.1016/j.theriogenology.2017.01.035.

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

Federica Zacchini[1], Paola Toschi[2] and Grazyna Ewa Ptak[1,3*]

[1] Institute of Genetics and Animal Breeding, Polish Academy of Sciences, 05-552 Jastrzebiec, 36a Postepu str., Poland

[2] Faculty of Veterinary Medicine, University of Teramo, via Renato Balzarini, 64100 Teramo, Italy

[3] National Research Institute of Animal Production, ul. Krakowska 1, 32-083 Balice n/Krakow, Poland

*Corresponding author: grazyna.ptak@izoo.krakow.pl

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.

Key words: oocyte, Cobalamin, placental vasculogenesis, sheep, DNA methylation

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 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.

85

86 **2.1- In vitro maturation (IVM)**

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

99

100 **2.2- In vitro embryo production**

101 *In vitro* fertilized (IVF) embryos were produced as previously described [26]. Briefly, matured oocytes (-
102 CTR, n=220; B12, n=191) were partially stripped of cumulus cells by repeated pipetting. Frozen semen was
103 rapidly thawed at 37°C and washed twice by centrifugation at 500 g for 5 min in bicarbonate-buffered
104 Synthetic Oviductal Fluid (SOF) with 4 mg/mL BSA. IVF was carried out in 50 µL drops, using 5×10^6
105 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
106 bicarbonate-buffered SOF enriched with 20% (v/v) heat-inactivated oestrous sheep serum, 2.9 mM Ca^{2+}
107 lactate, and 16 µM isoproterenol. Presumptive zygotes were transferred into 20 µL drops of SOF enriched
108 with 1% (v:v) Basal Medium Eagle (BME) essential amino acids, 1% (v:v) Minimum Essential Medium
109 (MEM) non-essential amino acids (Gibco), 1 mM glutamine and 8 mg/mL fatty acid-free BSA (SOFaa-
110 BSA). Zygotes were cultured in a humidified atmosphere of 5% CO_2, 7% O_2, 88% N_2 at 38.5°C, and the
111 medium changed on day 3 (supplemented with glucose) and day 5 (supplemented with 10% FBS charcoal
112 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

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.

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).

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 perfomed as 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 hours 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 4N 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, CA, USA) at room temperature for 2 h, washed in blocking medium and incubated with goat anti-mouse IgG FITC conjugate antibody (dilution 1:200; F9137, Sigma-Aldrich) at room temperature for 1 h. Mounted specimens were analyzed with an epifluorescence microscope.

2.5- Histological analysis

Chorion-allantois tissues were fixed in 4% (w:v) paraformaldehyde and subsequently dehydrated into increasing ethanol solutions for 5 min at each step and then cleared in xylene mixture. Finally, placentae were paraplast embedded. For hematoxylin eosin staining, 5 µm sections were used. Pictures were taken using the Nikon Eclipse E600 microscope. Placental vessels were divided into three different developmental stages as previously described in Fidanza et al. [7]. Briefly, *Stage 1* corresponds to early vasculogenesis (formation of hemangioblastic cell cords), *Stage 2* to tube formation characterized by endothelial cells becoming flattened, additional mesenchymal cells closely apposed to the endothelial tubes and hematopoietic stem cells becoming visible in the capillary lumen, *Stage 3* to late vasculogenesis characterized by well-formed capillaries surrounded bybasal lamina and perivascular cells.

2.6 - Expression analysis

Oocytes: MII oocyte were collected after IVM. After removal of granulosa cells and zona pellucida, oocytes were washed in PBS + 0.4% PVP and pool of 5 MII oocytes were snap frozen and kept at -80° until analysis. mRNA was isolated from oocyte pool ($n \geq 10$/group) using Dynabeads® mRNA DIRECT Kit

(Invitrogen Dynal AS, Oslo, Norway) containing Oligo(dT)25 magnetic beads. The procedure was carried out according to the manufacturer's instructions using a magnetic separator (Dynal MPC-P-12 magnet; Invitrogen). *Placental tissue*: Total RNA from placental tissues (n ≥ 8/ group) was extracted using an SV Total RNA Isolation System (Promega, Milan, Italy) according to the manufacturer's instructions. Total RNA integrity was assessed by a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Samples with an RNA Integrity Number of at least 8.5 were used for subsequent analysis. All samples were reverse-transcribed using GoScript™ Reverse Transcription System (Promega) according to the manufacturer's protocol. The obtained cDNAs, from both oocytes and placentae, were used for gene expression analysis using specific 5'-3' primer pairs designed to anneal at 56/58°C with an amplification efficiency (E) range between 2.1 and 1.9 (Table 1). Real-time PCR was carried out using Sso Advanced Universal SYBR green Supermix (Bio-Rad, Milan, Italy) with a CFX Connect Real-time PCR detection system (Bio-Rad), according to the manufacturer's instructions. Relative gene expression data were calculated using the comparative threshold cycle method (ΔΔCt) with GAPDH, μTUBULIN and SDHA as housekeeping genes.

2.7- Statistical analysis

Statistical analysis was performed using Instat 5 (GraphPAD software for science, San Diego, CA, USA).All data reported are expressed as mean with relative standard error of mean (SEM). Decimal variables were analyzed using a Mann-Whitney test, while variables expressed as percentages were analyzed with a Fisher's Exact test. Only p values <0.05 were considered significant. Immunofluorescence results were obtained using ImageJ software. Primer sets were designed using the Primer 3 tool; the reference stability value was calculated using geNorm; and efficiency values and data analysis of the amplification runs were performed using BioRad software**.**

3. Results

3.1- Cobalamin supplementation during *in vitro* maturation enhances oocyte quality

Qualitative evaluation of maturation rate following Cobalamin supplementation did not reveal any significant differences between B12 and –CTR groups. The fertilization rate following IVF was significantly increased in B12 vs -CTR (176/253 – 69.56% vs. 150/259 – 57.91%, respectively, P = 0.006, Fisher's Exact test) while no differences were observed at the blastocyst rate (78/176 – 44.32% vs. 55/150 – 36.67%, respectively) or hatching rate (Figure 1A). In terms of further development, a higher but not significant implantation rate was detected in B12 (12/20, 72.72%) vs -CTR (8/11, 60%). Differently, implantation rate of both IVP groups was lower than naturally mated +CTR (8/8, 100%) (P =0.008). Moreover, gross morphological evaluation of conceptuses revealed similar development in B12, -CTR and +CTR embryos (Figure 1B, C).

3.2- Cobalamin supplementation improves DNA methylation status of embryos and placentae

qRT-PCR analysis revealed an increased expression of *DNMT1* (P = 0.02) and *DNMT3B* (P = 0.012) in B12 mature (MII) oocytes (Figure 2A). The increased levels of *DNMTs* did not directly affect DNA methylation of MII oocytes, while it led to an increased global methylation in blastocysts derived from B12 oocytes (P = 0.04) (Figure 2B, C). In early placentae, the expression profiles of DNMTs was comparable in B12 and +CTR groups, while that of -CTR was significantly deregulated (P < 0.03) (Figure 3).

3.3- Cobalamin supplementation during IVM ameliorates vasculogenesis in early placentae

We evaluated placental vasculogenesis by assessing the maturity of vessels and the expression profile of a subset of factors regulating vessel formation (*VEGF*, *VEGF2R*, *ANG2*, *TIE2*) (Figure 4). Our data demonstrated a delayed placental vasculogenesis in the -CTR group vs. naturally mated +CTR (stage 2: P = 0.006; stage 3: P = 0.0002) (Figure 4A, B). Interestingly, we observed a rescue of vasculogenesis in B12 placentae; however, the number of stage 3 vessels remained reduced compared to +CTR (P = 0.006) (Figure 4A, B). Moreover, qRT-PCR revealed that the *VEGF*, *VEGF2R*, *ANG2*, *TIE2* expression profile of B12 placentae reflected that of +CTR, while in -CTR placentae *ANG2* (P = 0.04) and *TIE2* (P=0.03) were downregulated (Figure 4C).

4. Discussion

The present study showed that Cobalamin supplementation during the *in vitro* maturation of oocytes improved placental vasculogenesis of resulting sheep embryos. Results demonstrated that the addition of Cobalamin, missing in commercially available IVM medium, rescued some of the defects generally associated with *in vitro* embryo production (*i.e.* hypomethylation, *DNMT* deregulation and impaired vasculogenesis).

Our first finding was an increased fertilization rate following IVF in the B12 group not associated with quantitative improvement in blastocyst and/or implantation rate, as both -CTR and B12 embryos showed reduced implantation ability vs. +CTR. However, B12 embryos showed improved quality, in terms of methylation status and placental vasculogenesis. Our findings support the concept that non-optimal maturation of oocytes is one of the underlying causes of impaired development, as suggested by Jongbloet [27]. In particular, it has been observed that sub-optimal oocyte maturation due to dysfunction of the OCM (*e.g.* MTHFR polymorphism, folate deficiency) may be responsible for reduced embryonic development and poor pregnancy outcome following ART [4,28].

Correct DNA methylation during the pre-implantation period is fundamental for ensuring appropriate embryonic development, resulting in healthy offspring [29,30]. Dysfunction of the DNA methylation machinery, leading to impaired DNA methylation and/or deregulated expression of imprinted genes, has been described in both pre- and post-implantation embryos following ART [6,31-33] and in case of defective OCM pathway [18,34]. Nevertheless, the underlying mechanisms responsible for the epigenetic defects have not been understood. The dysregulation of key players involved in DNA methylation is one reasonable explanation. The main enzymes involved in the establishment and maintenance of DNA methylation are the DNA methyltransferases DNMT1, DNMT3A and DNMT3B [35]. The impaired expression and/or activity of DNMTs in ART embryos and placentae has been previously reported [6, 36]. Here we showed that Cobalamin supplementation leads to an increased expression of *DNMT1* and *DNMT3B* in MII oocytes. It suggests that Cobalamin positively contributed to the acquisition of epigenetic competence during oocyte maturation. This speculation was further confirmed by the correction of the hypomethylated status in blastocyst stage embryos derived from B12 oocytes. Our data revealed a comparable expression of *DNMT3A* in MII oocytes in both the B12 and -CTR groups. We could hypothesize that *in vitro* culture did not affect *DNMT3A* expression or that our findings could have been influenced by experimental limitation. In fact, it is

widely described that DNMT3A has several isoforms differently expressed during development. For example, Hara et al. [37] reported that DNMT3A2, but not DNMT3A, is mainly expressed in growing murine oocytes, so it may be possible that we evaluated *DNMT3A*, as it was the only sequence available in our model (sheep). To confirm that the expression profile of *DNMTs* as well as the global methylation in the B12 group reflects what occurs *in vivo*, the best control should be oocytes and blastocysts produced *in vivo*. However, obtaining oocytes and early embryos in sheep, or in other mono/bi-ovulatory large animals, requires very high numbers of animals and involves technical difficulties, therefore is not feasible.

Abnormal expression of *DNMTs* and/or hypomethylation have been described following ART protocols [6,32,34].. Our data confirmed the dysregulation of *DNMTs* in IVP placentae, as previously described by us [6] and others [38-39] and, of relevance, we found that *DNMTs'* expression profile in B12 tissues reflected that of *in vivo* +CTR. Taken together, these results demonstrated that Cobalamin supplementation during sheep oocytes' *in vitro* maturation corrected the defective methylation status of IVP embryos.

Another key event in the establishment of healthy pregnancy is placentation – the formation of a functional interface between the fetus and the mother in order to ensure maternal-fetal exchange of oxygen, nutrient and waste products [40-42]. Once placentation has started, the vascular network begins to develop extensively [43]. This process is regulated by the expression of vasculogenetic and angiogenetic factors involved in the recruitment of hemangioblast, their organization into tubes and/or further remodeling and differentiation to create mature vessels [43,44]. At the molecular level, vasculogenesis is regulated by Vascular Endothelial Growth Factor (VEGF), Angiopoietin (ANG) and their receptors (respectively, VEGFR and Tie2). Perturbation of VEGF/VEGFR and/or ANG/Tie2 systems has been proposed as one of the reasons behind defective placental vascularization in ART [7,39] and/or compromised pregnancy (*i.e.* IUGR, pre-eclampsia) [45-47]. Our data showed delayed vasculogenesis associated with deregulated expression of *ANG2* and *Tie2*, but not *VEGF* nor its receptor *VEGF2R*, in -CTR placentae. Interestingly, the expression profile of all studied factors in B12 placentae reflected that of naturally mated ones (+CTR) and the impaired vasculogenesis was partially rescued in B12 placentae, as reduced percentage of vessels at late vasculogenesis (stage 3) was observed. The proper expression of the VEGF system as well as the presence of hemangioblast and immature vessels indicated a correct initiation of vasculogenesis in both -CTR and B12

tissues. On the contrary, the improved expression of *ANG2/Tie2* and vessel maturity in B12 placentae led us to speculate that Cobalamin may rescue the delayed vascularization in IVP tissues, through improvement of vasculogenetic factors' expression. This enhanced vascularization can be directly associated with the improvement of epigenetic machinery described above. We can speculate that Cobalamin supplementation can lead to an increased uptake of methyl donors during oocyte maturation, thus contributing to epigenetic modification during pre-implantation development and to feto-placental development in the peri-implantation period.

In summary, we described that the addition of one cofactor, Cobalamin, positively affected sheep oocytes' *in vitro* maturation and, consequently, IVP embryo development in terms of DNA methylation and placental vasculogenesis. However, some considerations should be taken in mind when looking at the present work. First, only one factor (Cobalamin) was added to the IVM medium.Further studies should investigate whether other compounds are missing in the IVC system. Second, we focused our attention on the peri-implantation period, as it is a critical window for the proper establishment of pregnancy. The evaluation of additional time points and/or pregnancy outcomes would further confirm our results. Third, in light of our findings, therapeutic treatment based on vitamins B should be suggested not only to pregnant women, but also to those planning a pregnancy.

Acknowledgements:

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme [grant agreement No 692185 - ERAofART] and by the EU Programme FP7-KBBE-2012.1.3-04 [grant agreement no.312097 - FECUND] to GEP. This study was also partially financed by the IGAB PAS project [S.III.1.3]. The authors are participating in the COST action FA 1201 'Epiconcept' Epigenetic and Periconception Environment.

The authors declare no conflicts of interest. The contents of this publication are the sole responsibility of the Institute of Genetics and Animal Breeding of the Polish Academy of Sciences and the University of Teramo and do not necessarily reflect the opinion of the European Union.

306

307 **References**

308 [1] Sunderam S, Kissin DM, Crawford SB, Folger SG, Jamieson DJ & Barfield WD. Assisted reproductive
309 technology surveillance - United States, 2011. MMWR Surveillance Summaries. 2014; 63(10):1-28.
310 [2] Kupka MS, Ferraretti AP, de Mouzon J, Erb K, D'Hooghe T, Castilla JA et al. Assisted reproductive
311 technology in Europe, 2010: results generated from European registers by ESHRE. Hum Reprod 2014; 29
312 (10):2099-113.
313 [3] Hansen M, Kurinczuk JJ, Milne E, de Klerk N & Bower C. Assisted reproductive technology and birth
314 defects: a systematic review and meta-analysis. Hum Reprod Update 2013;19(4):330-53.
315 [4] Bloise E, Feuer SK and Rinaudo A. Comparative intrauterine development and placental function of Art
316 concepti: implication for human reproductive medicine and animal breeding. Hum Reprod Update 2014;
317 20(6):822-39.

318 [5] Källén B, Finnström O, Lindam A, Nilsson E, Nygren KG & Otterblad PO. Congenital malformations in
319 infants born after in vitro fertilization in Sweden. Birth Defects Res A 2010;88(3):137-43.

320 [6] Ptak GE, D'Agostino A, Toschi P, Fidanza A, Zacchini F, Czernik M et al. Postimplantational mortality
321 of in vitro produced embryos is associated with DNA methyltransferase 1 (DNMT1) dysfunction in placenta.
322 Hum Reprod 2013; 28:298-305.
323 [7] Fidanza A, Toschi P, Zacchini F, Czernik M, Palmieri C, Scapolo P, et al. Impaired placental
324 vasculogenesis compromises the growth of sheep embryos developed in vitro. Biol Reprod 2014; 91(1):21 1-
325 7.
326 [8] Hiura H, Okae H, Chiba H, Miyauchi N, Sato F, Sato A et al. Imprinting methylation errors in ART.
327 RMB 2014;13(4):193-202.
328 [9] Barker DJ & Carl PM . Fetal undernutrition and disease in later life. Rev Reprod 1997;2:105–112.

329 [10] Fernandez-Gonzalez R, Moreira P, Bilbao A, Jimenez A, Perez-Crespo M, Ramirez MA et al. Long-
330 term effect of in vitro culture of mouse embryos with serum on mRNA expression of imprinting genes.
331 development. and behavior. PNAS. 2004; 101:5880–5885.

[11] Ceelen M, van Weissenbruch MM, Vermeiden JPW, van Leeuwen FE & Delemarre-van de Waal HA. Cardiometabolic differences in children born after in vitro fertilization: follow-up study. J Clin Endocr Metab 2008; 93:1682–1688.

[12] Sampino S, Zacchini F, Swiergiel AH, Modlinski AJ, Loi P & Ptak GE. Effects of blastomere biopsy on postnatalgrowth and behavior in mice. Hum Reprod 2014; 29(9):1875–1883.

[13] Nelen WL, Blom HJ, Steegers EA, den Heijer M, Thomas CM & Eskes TK. Homocysteine and folate levels as risk factors for recurrent early pregnancy loss. Obstet Gynecol 2000; 95:519–524.

[14] Deng L, Elmore CL, Lawrance AK, Matthews RG & Rozen R. Methionine synthase reductase deficiency results in adverse reproductive outcomes and congenital heart defects in mice. Mol Genet Metab 2008; 94(3):336–342.

[15] Molloy AM, Kirke PN, Troendle JF, Burke H, Sutton M, Brody LC et al. Maternal vitamin B12 status and risk of neural tube defects in a population with high neural tube defect prevalence and no folic Acid fortification. Pediatrics 2009;123(3):917-923.

[16] Yajnik CS & Deshmukh US. Fetal programming: maternal nutrition and role of one-carbon metabolism. Rev Endocr Metab Disord 2012;13(2):121-127.

[17] Momb J, Lewandowski JP, Bryant JD, Fitch R, Surman DR, Vokes SA et al. Deletion of Mthfd1l causes embryonic lethality and neural tube and craniofacial defects in mice. PNAS 2013;110(2):549-554.

[18] Steegers-Theunissen RPM, Twigt J, Pestinger V & Sinclair KD. The periconceptional period, reproduction and long-term health of offspring: the importance of one-carbon metabolism. Hum Reprod Update 2013;19(6):640–655.

[19] Gadhok AK, Sinha M, Khunteta R, Vardey SK, Upadhyaya C, Sharma TK et al.. Serum homocysteine level and its association with folic acid and vitamin B12 in the third trimester of pregnancies complicated with intrauterine growth restriction. Clin Lab 2011;57:933–938.

[20] Ikeda S, Koyama H, Sugimoto M & Kume S. Roles of one-carbon metabolism in preimplantation period--effects on short-term development and long-term programming J Reprod Dev 2012;58 (1):38-43.

[21] Rush EC, Katre P & Yajnik CS. Vitamin B12: one carbon metabolism, fetal growth and programming for chronic disease. Eur J Clin Nutr 2014;68(1):2-7

[22] Dror DK & Allen LH. Effect of vitamin B12 deficiency on neurodevelopment in infants: current knowledge and possible mechanisms. Nutr Rev 2008;66(5):250-255

[23] Pepper MR & Black MM. B12 in fetal development. Seminars in Cell and Developmental Biology 2011;22(6):619-623

[24] Barry JS & Anthony RV. The pregnant sheep as a model for human pregnancy. Theriogenology 2008; 69:55–67.

[25] Institute of Medicine, Food and Nutrition Board. Dietary reference intakes: thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington DC: National Academy Press 1998

[26] Zacchini F, Czernik M, Iuso D, Toschi P, Di Egidio F, Scapolo PA, Loi P, Ptak G.Efficient production and cellular characterization of sheep androgenetic embryos" Cell Rep 2011; 13(6):495-502.

[27] Jongbloet PH. Non-optimal maturation of oocyte, maternal MTHFR polymorphisms, periconceptional folate, and decrease of congenital heart defects, Eur Heart J 2007;28(16):2043.

[28] Farin PW, Piedrahita JA & Farin CE. Errors in development of fetuses and placentas from in vitro-produced bovine embryos. Theriogenology 2006;65:178-191

[29] Dupont C, Armant DR& Brenner CA. Epigenetics: definition, mechanisms and clinical perspective. Semin Reprod Med 2009;27(5):351-357.

[30] Le Bouc Y, Rossignol S, Azzi S, Steunou V, Netchine I & Gicquel C. Epigenetics, genomic imprinting and assisted reproductive technology. Annales d'Endocrinologie (Paris) 2010;71(3):237-238.

[31] Laprise SL. Implications of epigenetics and genomic imprinting in assisted reproductive technologies. Mol Reprod Dev 2009;76(11):1006-1018.

[32] Market-Velker BA, Fernandes AD & Mann MR. Side-by-side comparison of five commercial media systems in a mouse model: suboptimal in vitro culture interferes with imprint maintenance. Biol Reprod 2010;6:938–950.

[33] El Hajj N & Haaf T. Epigenetic disturbances in in vitro cultured gametes and embryos: implications for human assisted reproduction. Fertil Steril 2013;99(3):632-641.

[34] Zhang B, Denomme MM, White CR, Leung K, Lee MB, Greene ND et al. Both the folate cycle and betaine-homocysteine methyltransferase contribute methyl groups for DNA methylation in mouse blastocysts. FASEB J2014;29(3):1069-79 .

[35] Hirasawa R, Chiba H, Kaneda M, Tajima S, Li E, Jaenisch R et al. Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. Gene Dev 2008;22:1607–1616.

[36] Petrussa L, Van de Velde H & De Rycke M. Dynamic regulation of DNA methyltransferases in human oocytes and preimplantation embryos after assisted reproductive technologies. Molecular Hum Reprod 2014;20(9):861-874.

[37] Hara S, Takano T, Fujikawa T, Yamada M, Wakai T, Kono T et al. Forced expression of DNA methyltransferases during oocyte growth accelerates the establishment of methylation imprints but not functional genomic imprinting. Hum Mol Genet 2014;23(14):3853-3864

[38] Grazul-Bilska AT, Johnson ML, Borowicz PP, Baranko L, Redmer DA & Reynolds LP. Placental development during early pregnancy in sheep: effects of embryo origin on fetal and placental growth and global methylation. Theriogenology 2013;79(1):94-102.

[39] Sui L, An L, Tan K, Wang Z, Wang S, Miao K et al. Dynamic proteomic profiles of in vivo- and in vitro-produced mouse postimplantation extraembryonic tissues and placentas. Biol Reprod 2014;91(6): 155

[40] Huppertz B & Peeters LLH . Vascular biology in implantation and placentation. Angiogenesis 2010; 8: 157-167

[41] Reynolds LP, Caton JS, Redmer DA, Grazul Bilska AT, Vonnahme KA, Borowicz PP et al. Evidence for altered placental blood flow and vascularity in compromised pregnancy. J Physiol 2006; 572 (1):51-58

[42] Thornburg KL, O'Tierney PF & Louey S. Review: the placenta is a programming agent for cardiovascular disease. Placenta 2010;24:54–59.

[43] Demir R, Seval Y & Huppertz B. Vasculogenesis and angiogenesis in the early human placenta. Acta Histochem2007; 109:257-265

[44] Charnock-Jones DS, Kaufmann P & Mayhewc TM. Aspects of Human Fetoplacental Vasculogenesis and Angiogenesis. I. Molecular Regulation. Placenta 2004; 25:103–113.

413 [45] Regnault TRH, Galan HL, Parker TA & Anthony RV. Placental development in normal and
414 compromised pregnancies – a review. Placenta 2012;23(16):S119-S129
415 [46] Hagen AS, Orbus RJ, Wilkening RB, Regnault TR & Anthony RV. Placental expression of
416 angiopoietin-1, angiopoietin-2 and tie-2 during placental development in an ovine model of placental
417 insufficiency-fetal growth restriction. Pediatr Res 2005;58(6):1228-1232
418 [47] Dubova EA, Pavlov KA, Lyapin VM, Shchyogolev AI & Sukhikh GT. Vascular endothelial growth
419 factor and its receptors in the placental villi of pregnant patients with pre-eclampsia. B Exp Biol Med.
420 2013;154(6):792-795.
421

422 **Figure Legend**

423 **Table 1: Sequences of primer pairs used for qPCR**

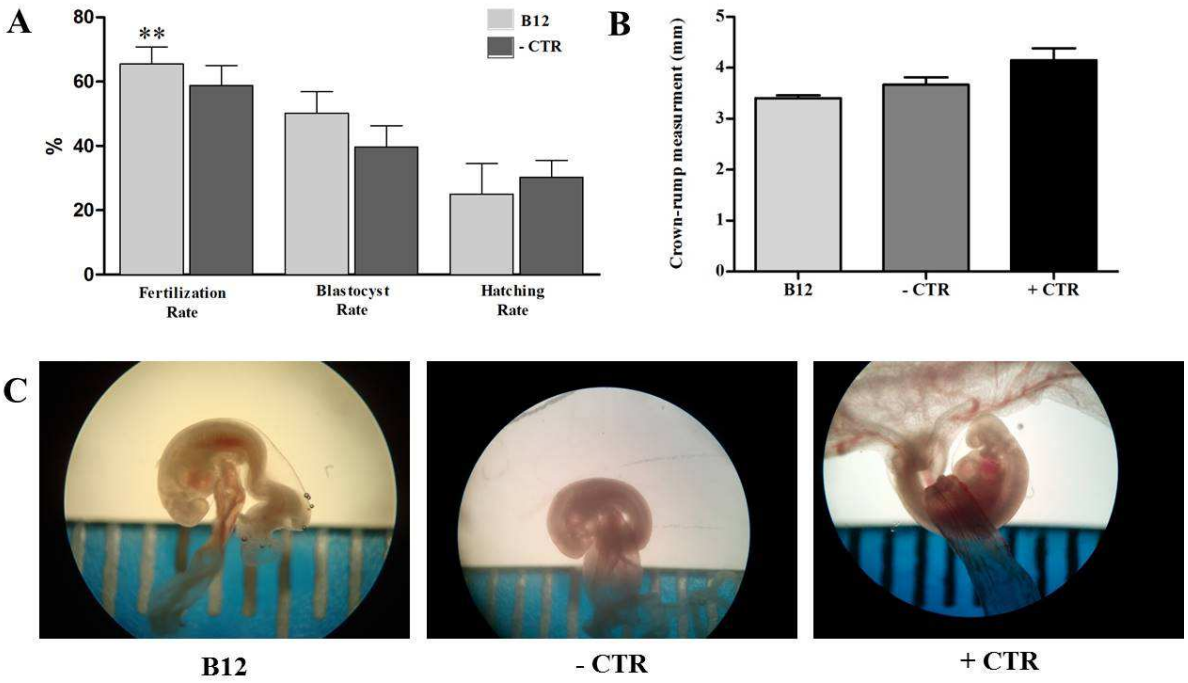
424 **Figure 1: Development of sheep embryos untill day 20 of pregnancy**. A) The fertilization rate in the B12
425 group is significantly increased, while further development up to the blastocyst stage appears similar (**
426 denotes $P < 0.05$, Fisher's Exact test). B) Crown-rump measurement revealed similar sizes of conceptuses
427 from B12, -CTR and +CTR groups. C) Similar developmental stage of B12, -CTR and +CTR conceptuses at
428 day 20 of pregnancy: in all embryos, optical lenses, 2-3 pharyngeal arches and closed anterior neurophores
429 were detectable.

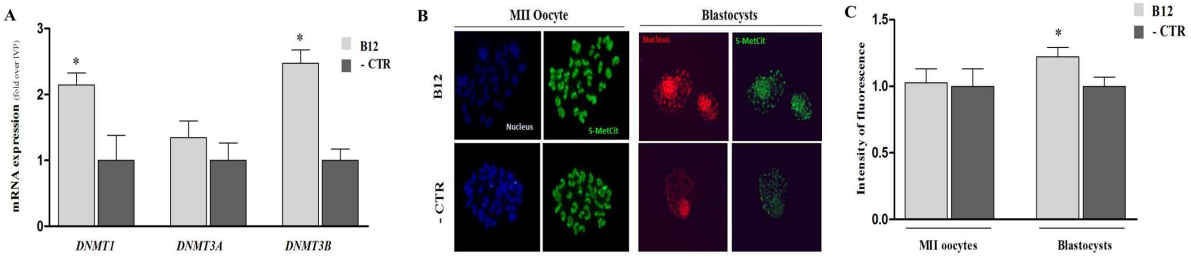
430 **Figure 2: IVM with Cobalamin enhances genome-wide methylation of sheep oocytes.** A) Increased
431 mRNA expression of *DNMT1* and *DNMT3B* in mature oocytes following IVM with Cobalamin. (* denotes P
432 = 0.02; Mann-Whitney test). B-C) Immunostaining anti-5-methylcytidine on MII oocytes and derived
433 blastocysts revealed an increased genome-wide methylation level in blastocyst stage embryos following IVM
434 with Cobalamin. Green indicates 5-methylcytidine; Red/Blue is nuclear counterstaining. Fluorescence
435 intensity was evaluated by ImageJ software. (* denotes P = 0.04; Mann-Whitney test).

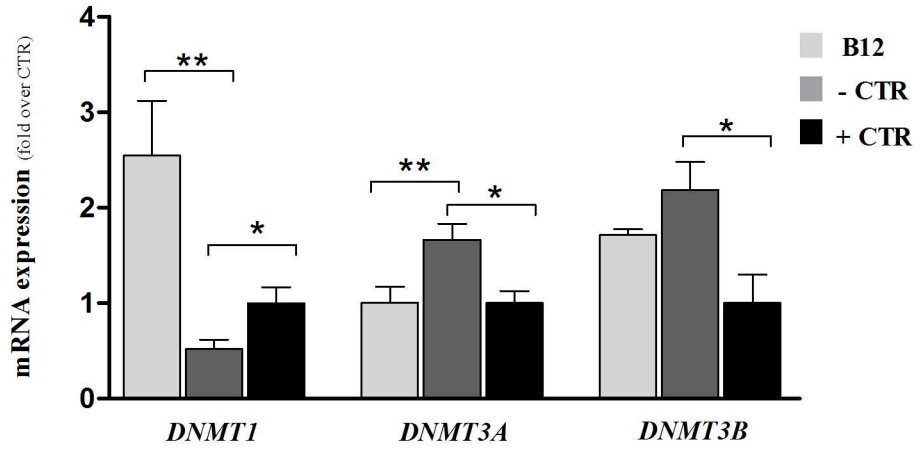
436 **Figure 3: Improved *DNMTs*' expression in placentae from B12 group.** The *DNMTs'* expression profile
437 of B12 placentae is comparable to that of +CTR, while it is deregulated in -CTR. (* denotes $P < 0.03$ in
438 +CTR, a denotes $P < 0.04$ in -CTR; Mann-Whitney test).

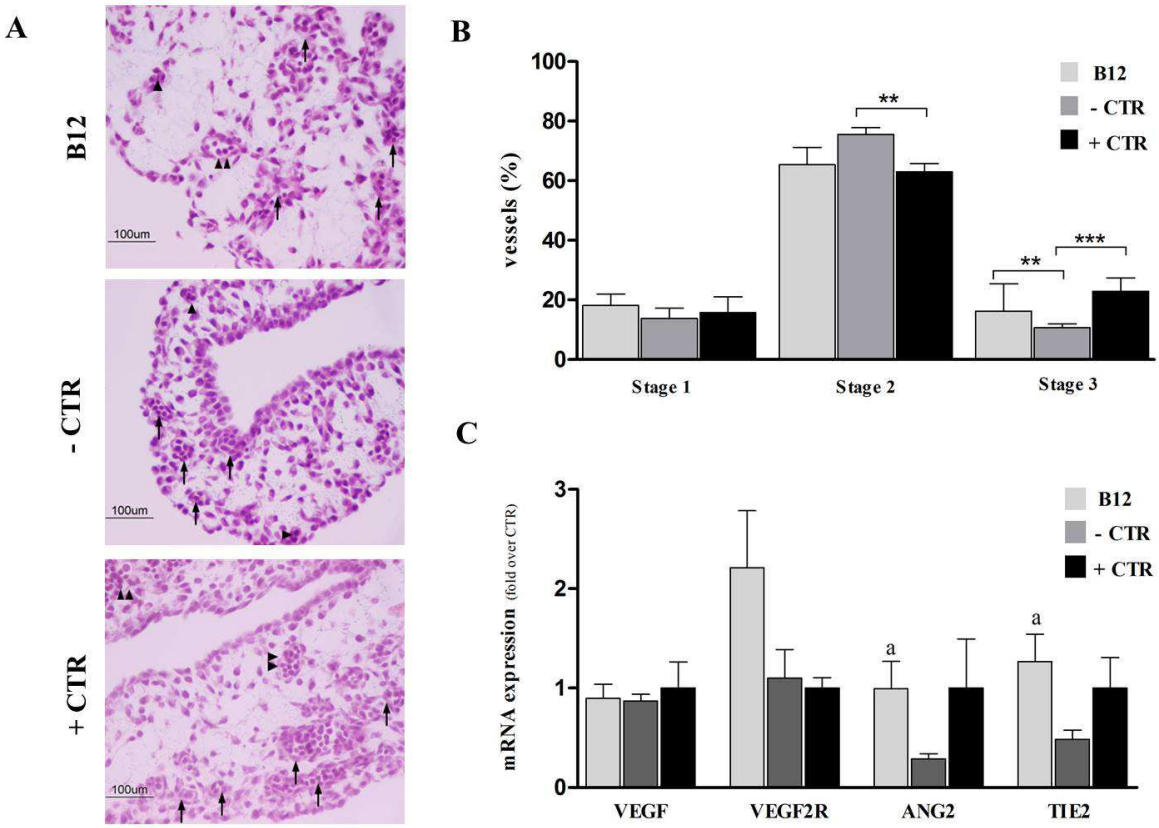
Figure 4: Normal vasculogenesis in placentae from B12 group. A) Hematoxylin eosin staining of 20 days placental tissues. Vessels have been classified as Stage *1*: formation of hemangioblastic cords (arrowhead); *Stage 2*: tube formation (arrow); *Stage 3*: well-formed capillaries surrounded by basal lamina and perivascular cells (double arrowheads). B) The evaluation of vessel maturity demonstrated delayed vasculogenesis in -CTR placentae vs. +CTR (** denotes P = 0.006, *** denotes P = 0.0002). The rescue of delayed vasculogenesis was noticed in the B12 group; only stage 3 vessels' percentage remained reduced compared to +CTR (P = 0.006; Fisher's Exact test.) C) Comparable expression profiles of vasculogenetic and angiogenetic factors in B12 and +CTR placentae. Note the increased expression of *ANG2* and *TIE2* in B12 placenta compared to -CTR. (a denotes P <0.04 vs. IVP, Mann-Whitney test).

Gene targeted	Sequence (5'-3')		Accession Number
	Forward	Reverse	
DNMT1	aagtcaaaccaaagaacc	ttctcatcagagacttgtgg	NM_ 001009473
DNMT3A	aaccttcctggtatgaacagg	ttcagtgcaccataagatgtcc	XM_001252215.2
DNMT3B	ttccagcagataagttggtggc	aacatgggcttcagctgatcc	NM_181813
VEGF	tttctgctctcttgggtgcattgg	atctgcatggtgatgttgaactcc	NM_001025110
VEGF2R	aactgtacggcaagaactgagc	aacgtgctgttcttcttgg	AF513909
TIE-2	ttaccaggtggacatctttgc	ttgggccattctcctttgg	AY288926
ANG2	atagaaatagggaccaacc	ttcttatcttgcagtttgc	AY881029









Highlights

1. Cobalamin supplementation during in vitro maturation (IVM) enhances ovine oocyte quality
2. Cobalamin supplementation improves DNA methyltransferases expression in IVP placentae
3. Cobalamin supplementation during IVM ameliorates vasculogenesis in early placentae