Placental development during early pregnancy in sheep: Effects of assisted reproductive technology on fetal and placental growth[1]

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Application of ART techniques decreased fetal size and cell proliferation in fetal and maternal placenta during early pregnancy. Thus, ART may have specific effects on growth and function of ovine maternal and fetal placenta and fetal tissues through regulation of cell proliferation and tissue growth, and likely other mechanisms.

SUMMARY

Assisted reproductive technologies (**ART**) may have profound effects on placental and fetal development, possibly leading to compromised pregnancy. To determine the effects of ART on the fetal size and cellular proliferation in maternal and fetal placental tissues, pregnancies were achieved through natural breeding (**NAT**), or transfer of embryos generated through in vivo (**NAT-ET**), in vitro fertilization (**IVF**), or in vitro activation (**IVA, clones**). On day 22 of pregnancy, tissues were collected and fetuses were measured. Then, expression of Ki67 (a marker of proliferating cells) was determined using immunohistochemistry followed by image analysis. Fetal length and labeling index (proportion of proliferating cells) in maternal and fetal placenta were less ($P<0.05$) in NAT-ET, IVF and IVF than in NAT. Thus, ART, including simply embryo transfer, may have deleterious effects on growth and function of ovine placental and fetal tissues through regulation of cell proliferation and tissue growth. These data provide a foundation for determining the expression of specific factors regulating placental and fetal tissue growth in pregnancies after ART application. In addition, these data will help us to better understand placental regulatory mechanisms in compromised pregnancies, and to identify strategies for rescuing such pregnancies.

INTRODUCTION

Early pregnancy is a critical period because of the major developmental events that take place, including embryonic organogenesis as well as formation of the placenta, a process known as placentation manifested by enhanced cell proliferation and vascular development (Mossman,

1937, 1987; Green and Winters, 1945; Boshier, 1969; Guillomot et al., 1981; King, 1982; Reynolds et al., 2002, 2006, 2010).

The pattern of placental growth during early pregnancy after natural breeding has been established for sheep (Zheng et al., 1996; Grazul-Bilska et al., 2010, 2011). Comparison of the development of placentas from natural pregnancies and pregnancies achieved by various assisted reproductive technologies (ART), such as after transfer of embryos created through in vitro fertilization (IVF) has demonstrated differences in placental and fetal growth in several species (Barnes, 2000; Cai et al., 2006; Grazul-Bilska et al., 2006; Romundstad et al., 2006; Allen et al., 2008; Collier et al., 2009; Delle Piane et al., 2010; Sellers Lopez et al., 2010; Esh-Broder et al., 2011; Tomic and Tomic, 2011). For early pregnancy in cows, both greater and less crown-rump length of fetuses created in vitro and then transferred compared to fetuses created in vivo has been reported (Bertolini et al., 2002; Farin et al., 2006). However, data concerning fetal and placental growth including cell proliferation in utero-placental tissues during early pregnancy established through ART application are very limited.

Factors influencing fetal and placental growth have a dramatic impact on fetal and neonatal survival and development (Reynolds and Redmer, 2001; Reynolds et al., 2002, 2006, 2010). Recent observations indicate that compromised fetal growth impacts not only the neonatal period but also life-long health and productivity in humans and livestock species (Nathanielsz 2006, Barker 2007).

We hypothesized that growth of maternal and fetal placenta, and fetus will be altered in pregnancies achieved through application of ART compared to natural pregnancies. In addition to our control group which was naturally bred (NAT), we chose three ART methods to establish pregnancies as follows: (i) superovulation induced by multiple injections of follicle stimulating hormone (FSH) combined with natural breeding, embryo flushing from donors and transfer to recipients (NAT-ET), (ii) transfer of embryos obtained through in vitro fertilization (IVF) of oocytes collected after induction of multiple follicular development using FSH, and (iii) transfer of embryos obtained through in vitro activation (IVA; i.e., parthenotes, which are clones containing only maternal genes) of oocytes collected from FSH-treated donors. In the NAT-ET group, embryos were only briefly removed from uterine environment and had maternal and paternal genomes, in IVF group embryos were created on culture dish and possessed both maternal and paternal genomes, but in IVA group embryos created on culture dish had only maternal genome. Parthenogenetic embryos are used to study the role of maternal genome and the effects of a lack of paternal genome on further embryonic development, imprinted genes and other processes in several species (Loi et al., 1998; Xu and Yang, 2001; Krivokharchenko et al., 2003; Kono et al., 2006; Ferrandi et al., 2002; Lagutina et al., 2004; Grazul-Bilska et al., 2008; Maalouf et al., 2008). The aim of this study was to determine fetal growth and cell proliferation in fetal and maternal placenta during early pregnancy in NAT, NAT-ET, IVF and IVA groups in sheep.

PROCEDURES

Animals and Tissue Collection. The NDSU Institutional Animal Care and Use Committee approved all animal procedures in this study. Estrus was synchronized for adult ewes (n=30;

crossbred Western Range, primarily Rambouillet, Targhee, and Columbia) using a CIDR device (MWI, Boise, ID) implanted for 14 days during breeding season. 24 h after CIDR removal, NAT ewes (n=8) were exposed to a fertile ram and naturally bred, but for NAT-ET (n=7), IVF (n=8) and IVA (n=7) groups estrus was checked twice daily using a vasectomized ram. 5%, 86% and 7% of ewes expressed estrus 24, 36 and 48 h after CIDR removal, respectively. Starting on day 13 of the estrous cycle, ewes from NAT-ET group were treated twice daily with FSH for 3 days but ewes from IVF and IVA groups were treated with FSH for 2 days (Stenbak et al., 2001; Grazul-Bilska et al., 2003, 2006; Borowczyk et al., 2006). On day 15 of the estrous cycle, ewes from NAT-ET group were exposed to a fertile ram for 24-48 h, but for IVF and IVA groups, ovaries were collected, oocytes isolated, matured, and then fertilized or activated in vitro as described in detail before (Grazul-Bilska et al., 2003, 2006, 2008; Borowczyk et al., 2006). Briefly, cumulus oocyte complexes (COC) were isolated from follicles ≥3 mmm; the average number of collected COC/sheep was 19.3±1.6. For IVF and IVA procedures, oocytes (up to 30 oocytes/0.5 ml in 4-well Nunc culture dish) were incubated overnight in maturation media (TCM199; Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS), ovine FSH [5 µg/mL; oFSH-RP-1; NIAMDD-NIH, Bethesda, MD, USA], ovine LH [5 µg/mL; oLH-26; NIADDK-NIH], estradiol -17β [1 µg/mL; Sigma], glutamine [2 mM; Sigma], sodium pyruvate [0.25 mM; Sigma], epidermal growth factor [10 ng/mL; Sigma,] and penicillin/streptomycin [100 units/mL penicillin and 100 µg/mL streptomycin; Gibco, Grand Island, NY, USA]). After denuding oocytes from cumulus cells, half of oocytes from each sheep was used for IVF and another half for IVA. For IVF, oocytes were cultured in fertilization media in the presence of capacitated frozen-thawed sperm (0.5-1 x 10^6 sperm/ml) for 24 h followed by incubation in culture media till embryo transfer (ET; see below). For IVA, oocytes were incubated for 5 min in TCM199 media containing 2% FBS and ionomycin (2.5 µM; Sigma) followed by 3 h incubation with 6-dimethylaminopurine (DMAP; 2 mM; Sigma). In vitro activated oocytes were then transferred to culture media and incubated till ET (see below).

For NAT-ET group, on day 5 post-mating, embryos were flushed, evaluated under the stereomicroscope, and then transferred to synchronized recipients (3 embryos/recipient). For IVF and IVA groups, in vitro generated embryos were transferred on day 5 after fertilization or activation to synchronized recipient ewes (3 embryos/recipient) as described by Grazul-Bilska et al. (2003, 2006). On day 22 after mating, fertilization or activation utero-placental tissues were collected. For histology/immunohistochemistry, specimen pins were inserted completely through the uterus and FM at the level of the external intercornual bifurcation to maintain specimen morphology; cross sections of the entire gravid uterus (approximately 0.5-cm thick) were obtained using a Stadie-Riggs microtome knife followed by immersion in formalin or Carnoy's solution and embedding in paraffin. Fetuses were separated from fetal membranes and crown-rump length of each fetus was measured. We choose day 22 for tissue collection, since in our previous experiments, we have demonstrated that on days 20-22, the major changes in cell proliferation, vascularization and expression of angiogenic factors appeared in fetal and maternal placenta for pregnancies achieved through natural breeding (Grazul-Bilska et al., 2010, 2011), and also placentation is already initiated (Igwebuike, 2009).

Immunohistochemistry. Immunohistochemical procedures were described previously (Grazul-Bilska et al. 2010, 2011). Briefly, paraffin-embedded uterine tissues containing FM were sectioned at 4 µm and mounted onto slides. Sections were rinsed several times in PBS

containing Triton-X100 (0.3%, v/v) and then were treated for 20 min with blocking buffer [PBS containing normal horse serum (2%, vol/vol)] followed by incubation with specific primary antibody for Ki67 (a marker of proliferating cells; 1:500; mouse monoclonal; Vector Laboratories, Burlingame, CA, USA) overnight at 4˚ C. Primary antibodies were detected by using secondary anti-mouse antibody coupled to peroxidase (ImPress Kit; Vector Laboratories). Then, the sections stained with Ki67 were counterstained with nuclear fast red (Sigma, St. Lois, MO, USA). Control sections were incubated with normal mouse IgG (4 μg/mL) in place of primary antibody.

Image analysis. For each tissue section, images were taken at 400x magnification, using an Eclipse E600 Nikon microscope and digital camera for 5-10 randomly chosen fields (0.025 mm^2 per field) from maternal placenta containing caruncle (CAR), inter-CAR (ICAR) and fetal placenta (FM), separately. To determine labeling index (LI) in maternal and fetal placenta an image analysis system (Image-Pro Plus, Media Cybernetics, Inc., Bethesda , MD, USA) was used as described previously (Grazul-Bilska et al. 2010, 2011). The LI was calculated as the percentage (%) of proliferating Ki67-positive cells out of the total number of cells in CAR, ICAR and FM tissue area.

Statistical Analysis. Data were analyzed using the general linear models (GLM) procedure of SAS and presented as means ± SEM with the main effect of pregnancy type (SAS Institute 2010). When the F-test was significant ($P<0.05$), differences between specific means were evaluated by using the least significant differences test (Kirk 1982).

RESULTS

The length of the fetus was the greatest ($P<0.0001$) in NAT group, less in NAT-ET, and least in IVF and IVA groups (Fig. 1A). In IVF and IVF groups, length of fetus was approximately 2-fold less than in NAT group (Fig. 1A).

Marker of proliferating cells, Ki67 protein was detected in nuclei of fetal and maternal placenta in all groups (Fig. 2). Labeling index was greater ($P<0.001$) in fetal placenta than in maternal placenta in all groups. In NAT group, LI was 24.5±2.9% and 3.5±0.3% in fetal and maternal placenta, respectively. Labeling index in CAR and ICAR of maternal placenta was similar; therefore data were combined for these two uterine compartments within each group. In maternal placenta, LI was less ($P<0.001$) in NAT-ET group and least in IVF and IVA groups compared to NAT, and in fetal placenta, LI was less ($P<0.001$) in NAT-ET and IVF groups and least in IVA group compared to NAT (Fig. 1B).

DISCUSSION

Application of ART may have no effects or some negative effects on placental and fetal development or pregnancy outcome in several species including humans, mice, sheep or cows. Compared to in vivo natural fertilization, IVF has been demonstrated to affect embryonic and fetal development, placentation and implantation, placental function and growth, duration of gestation, embryonic loss/survival, appearance of some pathologies, birth weight and others in several species (Barnes, 2000; Bertolini et al., 2002; Cai et al., 2006; Farin et al., 2006; Grazul-

Bilska et al., 2006; Romundstad et al., 2006; Allen et al., 2008; Collier et al., 2009; Delle Piane et al., 2010; Sellers Lopez et al., 2010; Esh-Broder et al., 2011; Tomic and Tomic, 2011).

In the present experiment, combination of induction of superovulation with natural breeding and ET (our NET-ET group) decreased fetal size by 15%, but application of IVF or IVA decreased fetal size by more than 50% during early pregnancy. For cows, shorter crown-rump length of fetuses created in vitro compared to fetuses created in vivo has been reported for early pregnancy (Bertolini et al., 2002). On the other hand, Farin et al. (2006) reported that length of bovine embryos produced in vitro almost doubled compared to embryos produced in vivo during early pregnancy; this could lead to large offspring syndrome. Thus, conditions created during superovulation combined with natural breeding and embryo transfer, in vitro fertilization or activation and early embryonic development may have negative effects on fetal growth during early pregnancy.

Cell proliferation in maternal and fetal placenta was decreased by application of ART in our study. Although the LI was approximately 10-fold lower in maternal than fetal placenta, the pattern of changes of LI was very similar in both placental compartments. Placental cell proliferation in pregnancies affected/compromised by application of ART or environmental factors (e.g., maternal nutrition, age or others) has received limited attention. However, decreased LI was observed in placenta of adolescent overnourished ewes, which were also characterized by impaired fetal and placental growth during mid to late gestation (Lea et al., 2005; Redmer et al., 2009). In pregnancy compromised by diabetes, both increased and decreased cell proliferation was observed in placenta in rats (Caluwaerts et al., 2000; Zorn et al., 2011). For diabetic mice, decreased cell proliferation in myometrium during early pregnancy was reported (Favaro et al., 2010). On the other hand, cell proliferation was similar in diabetic and healthy human term placenta (Burleigh et al., 2004). Furthermore, several studies demonstrated high cell proliferation rates in utero-placental tissues during early pregnancy achieved through natural fertilization in humans (Korgum et al., 2006; Kar et al., 2007), sheep (Zheng et al., 1996), cows (Boos et al., 2006; Facciotti et al., 2009), rats (Correia-de-Silva et al., 2004) and monkeys (Blankenship and King, 1994; Wei et al., 2005). Thus, high cell proliferation observed in maternal and fetal placenta in natural pregnancy is decreased during early pregnancy after ART application or compromised by other factors in several species. This likely contributes to impaired fetal and placental growth, and offspring outcome.

In the present study, we have evaluated fetal and maternal placental growth on day 22 of pregnancy only. Therefore, we cannot exclude that the differences in placental growth among investigated pregnancy types may decrease due to possible compensatory mechanisms, or alternatively may increase as pregnancy progresses. Thus, future studies should evaluate placental growth during later stages of pregnancy.

Tissue growth including cell proliferation is regulated by growth and other regulatory factors in placenta and other tissues (Zheng et al., 1996; Reynolds et al., 2006, 2010; Grazul-Bilska et al., 2010, 2011). Since we have observed reduced expression of several growth factors known to regulate placental function including fibroblast growth factor (FGF) 2, FGF receptor, placental growth factor and others in maternal or fetal placenta after application of ART during early pregnancy (Johnson et al., 2011), we hypothesize that application of ART decreased expression

of regulatory factors which in turn contributed to reduced cellular proliferation and fetal size. However, the role and expression of factors controlling tissue growth and cell proliferation in placental function requires further investigation.

In summary, application of ART techniques decreased fetal size and cell proliferation in fetal and maternal placenta during early pregnancy. Thus, ART may have specific effects on growth and function of ovine maternal and fetal placenta and fetal tissues through regulation of cell proliferation and tissue growth, and likely other mechanisms. These data provide a foundation for determining the expression of specific factors regulating placental and embryonic tissue growth in pregnancies after ART application. In addition, these data will help us to better understand placental regulatory mechanisms in compromised pregnancies, and to identify strategies for rescuing such pregnancies.

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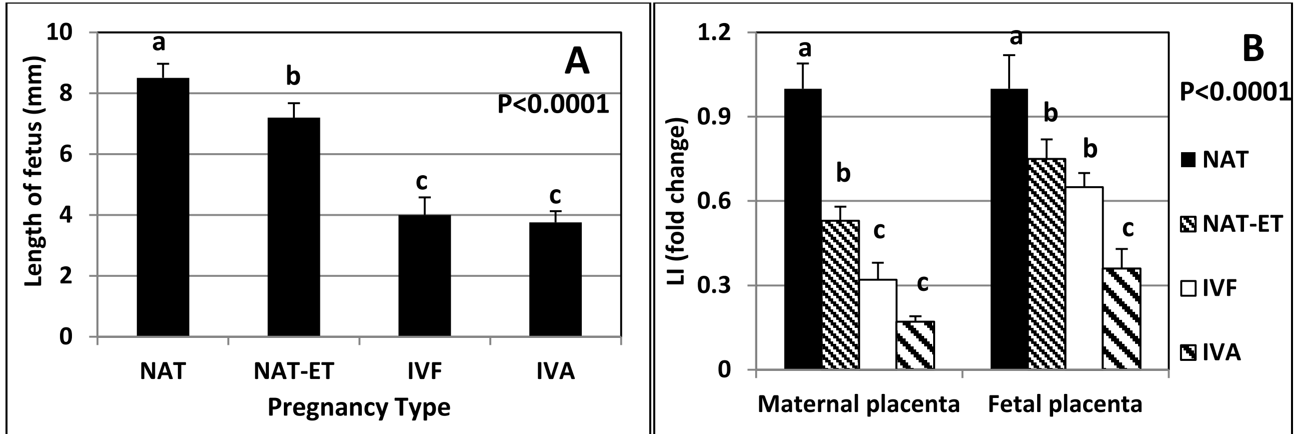

Figure. 1. The length of fetus (A) and labeling index (LI) in maternal and fetal placenta (B) in NAT, NAT-ET, IVF and IVA groups. Values ± SEM with different superscripts (a, b, c) differ within measurement. For LI, data are expressed as fold change compared to NAT control arbitrary set as 1. In NAT group, LI was and 3.5±0.3% and 24.5±2.9% in maternal and fetal placenta, respectively.

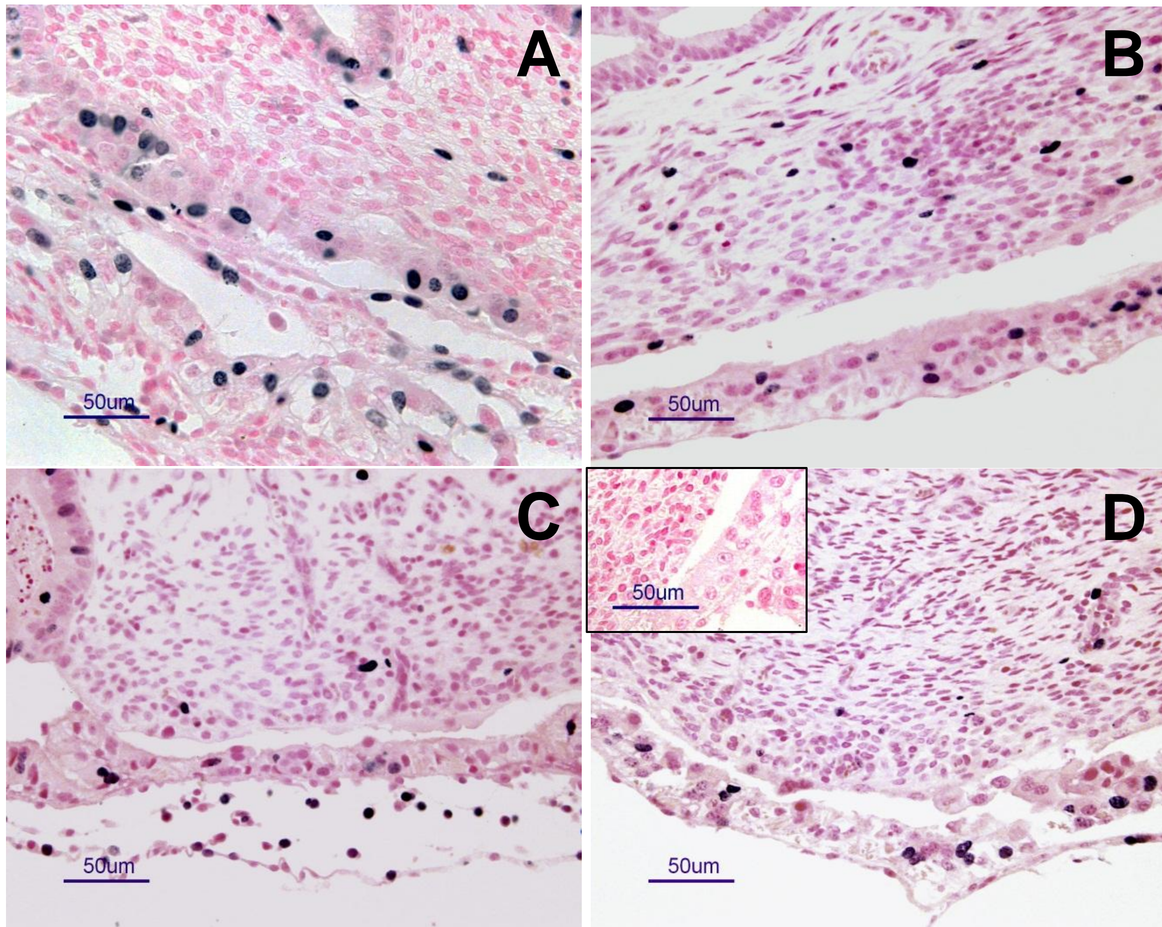

Figure. 2. Representative photomicrographs of immunohistochemical staining for Ki67 in maternal and fetal placenta in in NAT (A), NAT-ET (B), IVF (C) and IVA (D) groups. Dark color represents positive staining and pink color (nuclear fast red staining) indicates unlabeled cell nuclei. Note nuclear staining of Ki-67 in fetal placenta (FM) and endometrium (E, maternal placenta). In inset (D), note a lack of positive staining in the control sections in which mouse IgG was used in place of the primary antibody