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Original Research Article

Could assisted reproductive techniques affect equine fetal membranes and neonatal outcome?

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ABSTRACT

Embryo transfer (ET) and intracytoplasmic sperm injection (ICSI) are widely used in equine species, but their effects on fetal adnexa and neonates have not been investigated yet. The aim of this study was to retrospectively evaluate whether pregnancies obtained by ET or ICSI could be associated with the presence of macroscopic alterations of fetal membranes (FM) and umbilical cord (UC) and if the use of these techniques could influence neonatal outcome. Sixty-six light breed mares hospitalized at the Veterinary Teaching Hospital, University of Bologna, for attending delivery were included in the study. Mares were divided into Artificial Insemination (AI; 32/66 mares, 48 %), Embryo Transfer (ET; 12/66 mares, 18.2 %) and Intracytoplasmic Sperm Injection (ICSI; 22/66 mares, 33 %) groups. All the medical reports of mares and their foals were reviewed and data about mare, pregnancy, foaling, fetal membranes, umbilical cord and foal were recorded. The occurrence of dystocia resulted statistically different between AI group and ICSI group ($p = 0.0066$), and between AI group and ET group ($p = 0.044$). Macroscopic examination of FM revealed alterations in 30/66 mares (46 %): 8/32 in AI (25 %), 7/12 in ET (58 %) and 15/22 in ICSI (68 %) with significant lower incidence in AI compared to ET ($p = 0.04$) and ICSI ($p = 0.002$) groups. Alterations reported were chorionic villi hypoplasia, chorioallantois edema, allantois cysts, necrotic areas and greenish-grey concretions. Total length of UC resulted significantly shorter in ICSI group (49 ± 9 cm; $p < 0.03$) compared to AI (60 ± 17 cm) and ET (59 ± 15 cm). However, there were no differences in the incidence of foals' diseases at birth and in foals' survival among groups ($p > 0.05$). The results demonstrate that transfer of *in vivo* or *in vitro* produced embryos may lead to alterations of placental development, as observed in other species, without being associated with a higher incidence of neonatal morbidity and mortality. Further studies about trophoblast development, FM histological evaluation, and placental gene expression should be carried out to clarify the mechanisms underlying the placental alterations.

1. Introduction

Assisted reproductive techniques (ARTs) has been defined as procedures that involve *in vitro* manipulation of oocytes, semen and embryos with the aim of establishing a pregnancy. In equine species, the use of these techniques is relatively recent compared to other domestic species, such as cattle and pigs [1]. These techniques include ovarian stimulation, semen collection and preservation, *in vitro* fertilization, intracytoplasmic sperm injection, embryo preservation and transfer, and cloning procedures, which can induce significant modifications in gametes [2,3] and in embryonic microenvironment [4–6]. The early stages of embryo development are known to be strongly affected by environmental conditions, with long-term effects on fetus, newborn foal and adult health [7–9]. It is therefore essential to evaluate whether the use of ARTs in equine species could affect pregnancy, fetal membranes and umbilical cord, the onset of dystocia and the health of mare and foals as extensively reported in bovine and ovine [10–15].

Although it is known that pregnancies produced by somatic cell nuclear transfer (SCNT) in the mare could present fetal membranes alterations, umbilical cord abnormalities, hydroamnios, hydroallantois, abortion, maladjustment, enlarged umbilical remnant, and angular deformity of the forelimbs [16–21], only few studies have focused on the potential of postnatal consequences of ARTs [22–24].

The aim of this study was to retrospectively evaluate whether the

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transfer of *in vivo* or *in vitro* produced embryos may be associated with the presence of macroscopic alterations of fetal membranes and umbilical cord and whether the use of these techniques could influence pregnancy and the neonatal outcome.

2. Materials and methods

2.1. Population and data collection

All the medical records of mares and their foals obtained by ET, ICSI and artificial insemination (AI) and hospitalized for attending delivery at the Equine Perinatology and Reproduction Unit (Department of Veterinary Medical Sciences, University of Bologna, Italy) from 2014 to 2022 were reviewed. Mares with pregnancy obtained with artificial insemination that gave birth during 2019 were considered the control group. Data collected at admission and during hospitalization were obtained from clinical records and from the hospital veterinary medical information system (FeniceVET®, ZakSoft srl, Bologna, Italy).

Mares hospitalized for attending delivery were admitted at approximately 310 days of gestation and remained under around-the-clock observation until at least 7 days post-partum. The mares were housed in separate wide straw-bedded boxes with night vision cameras, fed hay ad libitum and concentrates twice a day, and were allowed to go to pasture during the day. All mares received a complete physical examination twice a day during hospitalization and a complete blood cell count and blood chemistry at admission. Additionally, transrectal palpation and ultrasonographic examination were performed to evaluate the combined thickness of the uterus and placenta (CTUP), fetal presentation and vitality, and quality of fetal fluids at admission and every ten days until parturition. In case of suspected high-risk pregnancy, a transabdominal ultrasonographic examination was performed to evaluate CTUP and fetal biophysical profile. The reference ranges of CTUP were considered in relation to gestational age, as reported elsewhere [25]. High-risk pregnancy was defined by the occurrence of a history of premature udder development/lactation, increase in combined thickness of the uterus and placenta, purulent/serosanguineous vulvar discharge and/or systemic illness of the mare [26]. All parturitions were attended to promptly intervene in case of dystocia. Dystocia was defined as any impediment to normal delivery due to complications of maternal, fetal and/or fetal membrane origin [27,28].

The following data were recorded for all mares: breed, age, parity, weight at admission (kg), ARTs conception method (AI, ET, ICSI), gestation length (days), type of pregnancy (normal or high-risk), duration of stages II and III (min, h respectively), dystocia resolution procedure (assisted vaginal delivery - AVD, controlled vaginal delivery - CVD, fetotomy or cesarean section) [28], gross fetal membranes alterations [29,30], placental total weight (kg), chorioallantoic weight (kg), fetal membranes/foal weight ratio (%) [31], gross umbilical cord (UC) alterations, total UC length (cm), total umbilical coil number, umbilical coiling index (UCI: umbilical coils divided by umbilical cord length) [32], length and number of coils of the allantoic and amniotic portion of the UC, site of UC insertion, placental vascularization pattern (type 1, type 2, type 3) [33], postpartum complications, outcome (discharge, death or euthanasia).

The following data were collected from all foals at birth: weight (kg), sex, APGAR score [34], temperature (°C), time of onset of suckling reflex (min from birth), time to acquire sternal recumbency (min from birth), standing position (min from birth) [35], first intake of colostrum (min from birth), IgG concentration at 12–24 h of life (via DVM Rapid Test II immunoturbidimetric test, MAI Animal Health, Elmwood, WI), neonatal diseases, outcome (discharged, death or euthanasia). Stillbirth was recorded.

Mares and their respective foals were divided into three groups accordingly to the conception method: AI group, ET group, and ICSI group.

2.2. Statistical analysis

Data were analyzed for normality with the Kolmogorov-Smirnov test. Given the non-normal distribution of data, nonparametric tests were used for statistical analysis. Data were expressed as mean, standard deviation, median, minimum and maximum values. The chi-squared test (χ^2) was used to evaluate the presence of a significant difference between the AI, ET and ICSI Groups for categorical variables. The Kruskall-Wallis test was used to evaluate the presence of a significant difference between the AI, ET and ICSI groups for numerical variables. If a significant difference was found, groups were subsequently compared with the Mann-Whitney test. A $p < 0.05$ was considered statistically significant. All statistical analyses were carried out using SPSS software (IBM® SPSS® Statistics).

3. Results

Sixty-six mares were included in this study and divided into three groups: AI group (32/66 mares; 48 %) as control; ET group (12/66 mares; 18 %); ICSI group (22/66 mares; 33 %).

Regarding the breed, 52/66 mares were Standardbred (78.8 %), 9/66 Italian Saddlebred (13.6 %), 3/66 Quarter Horse (4 %) and 2/66 Thoroughbred (3 %). In the ET group, 8/12 were Standardbred with a fetus of the same breed (67 %), 2/12 Standardbred with Italian Saddlebred fetuses (17 %), 1/12 Standardbred with a Quarter Horse fetus (8 %), and 1/12 Thoroughbred with an Italian Saddlebred fetus (8 %). In the ICSI group, 19/22 were Standardbred mares with the fetus of the same breed (87 %), 1/22 Standardbred with an Italian Saddlebred fetus (5 %), 1/22 Standardbred with a Quarter Horse fetus (5 %), and 1/22 Italian Saddlebred with a fetus of the same breed (5 %).

Data regarding mares, pregnancy, foaling and postpartum are reported in Table 1. Regarding the high-risk pregnancies in AI group, 5/32 mares (16 %) had placental edema, 3/32 (9 %) had suspected placentitis, 1/32 (3 %) had abdominal hernia and 1/32 (3 %) had rupture of the prepubic tendon. Of these mares, 7/10 (70 %) had dystocic delivery. In ET group, 1/12 (8 %) had placental edema and had dystocia.

Regarding dystocia, some mares had more than one cause of dystocia in all groups. They included: maternal origin such as uterine inertia, ineffective abdominal contractions and reduced size of the birth canal, fetal origin such as abnormal attitudes, positions and presentations and fetal membranes origin, such as premature placental separation. Details regarding the clinical description of the dystocic parturition and foals and mares' outcome are summarized in Table 2.

Regarding sick foals born from dystocia, in AI group 3 stillborn were born with CVD due to fetal malposition; one stillborn with AVD due to premature placental separation and fetal malposition. One foal experienced AVD due to uterine inertia and had congenital flexural limb deformities. In ET group, 2 foals with perinatal asphyxia syndrome (PAS) and 1 foal with congenital flexural limb deformities were born with AVD due to fetal malposition. In ICSI group, 2 foals with congenital flexural limb deformities and 2 foals with umbilical remnant diseases were born with AVD due to fetal malposition. Additionally, 3 foals with PAS were born with AVD resulting from maternal causes of dystocia.

The chi-square test did not show any difference in the incidence of dystocia between groups ($p > 0.05$). Considering the total population, dystocia occurred in 36/66 mares (54.5 %). Only considering mares with a normal pregnancy (55/66), dystocia occurred in 22/55 mares (40 %): significant differences were found between AI group and ICSI group ($p = 0.0066$), and between AI group and ET group ($p = 0.044$); no significant differences were found between ET group and ICSI group ($p > 0.05$).

Post-partum complications occurred in 24/66 mares (36 %) without differences between groups ($p > 0.05$): 13/24 (54 %) in AI group, 3/24 (13 %) in ET group and 8/24 (33 %) in ICSI group. In all groups, some mares had more than one complication.

Data regarding macroscopic evaluation of the fetal membranes and

Table 1
Age, weight, parity and gestation length and data related to pregnancy, delivery and postpartum in AI, ET and ICSI groups. Data are expressed as mean ± standard deviation, and median (minimum - maximum values). High-risk pregnancy (HRP), normal pregnancy (NP), retained fetal membranes (RFM), vaginal hematoma (VH), puerperal septic metritis (PSM), perineal laceration (PL), vaginal laceration (VL), cervical laceration (CL), constipation (Cons), laminitis (Lam).

Parameter	AI group (N = 32)	ET group (N = 12)	ICSI group (N = 22)
Mare age (y)	11 ± 6 10 (4–24) (n = 32)	10 ± 4 8 (5–17) (n = 12)	5 ± 1 *(p < 0.001) 4 (4–10) (n = 22)
Mare weight (kg)	581 ± 68 580 (484–700) (n = 10)	550 ± 75 540 (450–700) (n = 12)	534 ± 47 545 (414–600) (n = 22)
Mare parity (n)	3.9 ± 2.9[a] 4 (1–12) (n = 31)	1.5 ± 0.7[b]* p < 0.001 1 (1–3) (n = 12)	1 ± 0.2[b]*p < 0.001 1 (1–2) (n = 22)
Gestation length (days)	339 ± 14 338 (299–371) (n = 32)	341 ± 6 341 (333–355) (n = 12)	343 ± 11 341 (326–366) (n = 22)
High-risk pregnancy (n)	10/32 (31 %)*(p = 0.012) (n = 32)	1/12 (8 %) (n = 12)	0/22 (0 %) (n = 22)
Duration stage II (min)	16 ± 11 13 (5–55) (n = 32)	16 ± 11 16 (4–41) (n = 12)	13 ± 6 13 (3–28) (n = 22)
Dystocia (n)	16/32 (50 %) (n = 32)	6/12 (50 %) (n = 12)	14/22 (64 %) (n = 22)
HRP + Dystocia (n)	7/32 (22 %)	1/12 (8 %)	0
NP + Dystocia (n)	3/22 (14 %)[a]	5/11 (42 %)[b]* p = 0.044	14/22 (64 %)[b]* p = 0.0066
Post-partum complications (n)	13/32 (40.6 %) (n = 32)	3/12 (25 %) (n = 12)	8/22 (36.4 %) (n = 22)
Pathological condition (n)	RFM 8/17 (47 %) VH 2/17 (12 %) PSM 2/17 (12 %) PL 2/17 (12 %) VL 1/17 (6 %) Cons 1/17 (6 %) Lam 1/17 (6 %) (n = 17)	RFM 3/6 (50 %) VH 1/6 (17 %) PSM 1/6 (17 %) CL 1/6 (17 %) (n = 6)	RFM 6/10 (60 %) PSM 2/10 (20 %) PL 1/10 (10 %) CL 1/10 (10 %) (n = 10)

The presence of (*) indicates a significant difference between the groups in the row. Different letters in rows indicate significant differences between groups.

Table 2
Dystocia's causes and complications among groups. (#) express the total number of causes/complications (more than one cause in some mares). Post-partum complications (Ppc), retained fetal membranes (RFM), vaginal hematoma (VH), puerperal septic metritis (PSM), vaginal laceration (VL), cervical laceration (CL).

Group	Cause of dystocia#	Dystocia resolution	Foal's condition	Mares developed Ppc after dystocia (N)	Ppc#
AI group (N = 16)	Maternal 7/22 (32 %) Fetal 11/22 (50 %) Fetal membranes 4/22 (18 %) (n = 22)#	AVD: 13/16 (81 %) CVD: 3/16 (19 %)	Healthy 11/16 (69 %) Sick 1/16 (6 %) Stillborn 4/16 (25 %)	N = 8/16 (50 %)	RFM 6/10 (60 %) PSM 2/10 (20 %) VL 1/10 (10 %) VH 1/10 (10 %) (n = 10)#
ET group (N = 6)	Maternal 2/8 (25 %) Fetal 5/8 (62.5 %) Fetal membranes 1/8(12.5 %) (n = 8)#	AVD 4/6 (66 %) CVD: 2/6 (33 %)	Healthy 3/6 (50 %) Sick 3/6 (50 %	N = 1/6 (17 %)	RFM 1/4 (25 %) PSM 1/4 (25 %) CL 1/4 (25 %) VH 1/4 (25 %) (n = 4)#
ICSI group (N = 14)	Maternal 10/14 (71 %) Fetal 4/14 (29 %) (n = 14)#	AVD 14/14 (100 %)	Healthy 7/14 (50 %) Sick 7/14 (50 %)	N = 8/14 (57 %)	RFM 6/9 (66 %) PSM 2/9 (22 %) CL 1/9 (11 %) (n = 9)#

UC are reported in Table 3. In all groups, fetal membranes alterations were more than one. In AI group chorionic villi hypoplasia (4/32; 13 %) and chorioallantois edema (4/32; 13 %) were the most frequent alterations, followed by alterations of amniotic membrane caused by premature expulsion of meconium (2/32; 6 %) and mucopurulent exudate on the surface of fetal membranes (1/32; 3 %). In ET group, chorionic villi hypoplasia was the most frequent alteration (6/12; 50 %), followed by cystic neoformations on the allantoic side (2/12; 17 %) and chorioallantois edema (1/12; 8 %). In ICSI group, chorionic villi hypoplasia was the most frequent alteration (10/22; 46 %), followed by cystic neoformations on the allantoic side (2/22; 9 %), areas of necrosis (1/22; 5 %) and greyish-green concretions (1/22; 5 %). The incidence of macroscopic alterations in fetal membranes were lower in AI group as compared to ET ($p = 0.04$) and ICSI ($p = 0.002$) groups. On the contrary, no differences were observed between ET and ICSI groups ($p > 0.05$).

The most represented vascularization pattern was type 1 in all groups.

No differences were found in the incidence of macroscopic alterations of the umbilical cord between groups ($p > 0.05$). In AI group, multiple cystic formations were the only detected alteration (5/32; 16 %). In ET group, multiple cystic formations were the most frequent alteration (2/12; 17 %), followed by vascular anomalies (1/12; 8 %). In the latter group, one umbilical cord had more than one alteration. In ICSI group, multiple cystic formations were the only detected alteration (3/22; 14 %). The most frequent alterations regarding fetal membranes and umbilical cord are reported in Fig. 1.

Data regarding foals' clinical examination at birth, IgG concentration at 12–24 h and neonatal diseases are reported in Table 4. There were no differences ($p > 0.05$) for APGAR score, time to sternal recumbency, suckling reflex and first intake of colostrum between groups.

Several sick foals were born from high-risk pregnancies (1/10, 10 % in AI group; 1/4, 25 % in ET group): some of them were born from dystocia (1/10, 10 % in AI group; 3/4, 75%in ET group; 7/7, 100 % in ICSI group), and some of them had gross alterations of fetal membranes or umbilical cords (3/10, 30 % in AI group; 3/4, 75 % in ET group; 5/7, 71 % in ICSI group). All stillborn foals (4/32 foals,13 %) belonged to AI group and were born from high-risk pregnancies and dystocia; half (50 %) of them also presented gross alterations of fetal membranes or umbilical cord. The chi-square test did not show difference in the incidence of sick foals between groups ($p > 0.05$).

All the mares of the three groups were discharged. Overall, excluding the 4 stillbirth, 61/62 foals were discharged (98 %), and 1/66 was euthanized (2 %) belonging to the AI group.

Table 3
Macroscopic characteristics of fetal membranes and umbilical cord and fetal membranes/foal weight ratio (%) in AI, ET and ICSI Groups. Data are expressed as mean ± standard deviation, and median (minimum - maximum values).

	AI Group (N = 32)	ET Group (N = 12)	ICSI Group (N = 22)
Fetal membranes weight (kg)	5.3 ± 1.1 5.4 (3.2–7.7) (n = 32)	5.2 ± 1.1 5.3 (3.9–6.7) (n = 12)	4.8 ± 0.9 4.8 (3.1–6) (n = 22)
Chorioallantoic membrane weight (kg)	3.5 ± 0.8 3.3 (2.2–5.9) (n = 32)	3.5 ± 0.9 3.5 (2.3–5.2) (n = 12)	3.4 ± 0.7 3.3 (2.4–4.8) (n = 22)
Vascular pattern	16/19 type I (84 %) 3/19 type II (16 %) (n = 19)	4/6 type I (67 %) 1/6 type II (17 %) 1/6 type III (17 %) (n = 6)	6/8 type I (75 %) 2/8 type III (25 %) (n = 8)
Fetal membrane alterations (n)	8/32 (25 %) (n = 32)	7/12 (58 %) * (n = 12)	15/22 (68 %) * (p = 0.002) (n = 22)
Fetal membranes/ foal weight ratio (%)	11 ± 2 11 (7–17) (n = 32)	11 ± 2 11 (8–17) (n = 12)	11 ± 1 11 (8–14) (n = 22)
Total umbilical cord length (cm)	60 ± 17 58 (30–103) (n = 32)	59 ± 15 55 (43–85) (n = 11)	49 ± 9 *(p < 0.03) 50 (34–65) (n = 21)
Allantoic umbilical cord length (cm)	31 ± 11 29 (10–54) (n = 32)	31 ± 12 30 (15–55) (n = 11)	23 ± 7 23 (12–37) (n = 21)
Amniotic umbilical cord length (cm)	30 ± 10 30 (13–60) (n = 31)	28 ± 10 26 (20–55) (n = 11)	26 ± 8 25 (16–39) (n = 21)
Total umbilical cord coils (n)	6 ± 2 5 (3–10) (n = 32)	5 ± 2 5 (2–9) (n = 11)	5 ± 1 5 (2–9) (n = 22)
Allantoic umbilical cord coils (n)	3 ± 1 3 (1–8) (n = 32)	3 ± 1 3 (1–3) (n = 11)	2 ± 1 2 (1–6) (n = 22)
Amniotic umbilical cord coils (n)	3 ± 1 3 (1–6) (n = 31)	2 ± 1 2 (1–6) (n = 11)	2 ± 1 3 (1–4) (n = 22)
Umbilical coiling index (UCI)	0.10 ± 0.03 0.10 (0.06–0.19) (n = 32)	0.09 ± 0.02 0.08 (0.05–0.14) (n = 11)	0.09 ± 0.02 0.09 (0.06–0.15) (n = 21)
Umbilical cord insertion	4/30 base of pregnant horn (13 %) 5/30 base of non-pregnant horn (17 %) 21/30 between two horns (70 %) (n = 30)	2/10 base of pregnant horn (20 %) 8/10 between two horns (80 %) (n = 10)	8/19 base of pregnant horn (42 %) 11/19 between two horns (58 %) (n = 19)
Umbilical cord alterations (n)	5/32 (16 %) (n = 32)	2/12 (17 %) (n = 12)	3/22 (14 %) (n = 22)

The presence of (*) indicates a significant difference between the groups in the row.

4. Discussion

This study aimed to evaluate if pregnancies achieved by *in vivo*- or *in vitro*-produced embryos may be associated with the presence of macroscopic fetal membranes and umbilical cord alterations. In addition, it was evaluated if the use of these techniques may affect the course of pregnancy and the occurrence of neonatal diseases.

In the present study, most of the recipients included were Standardbred mares in both ET and ICSI groups. However, other morphologically similar breeds have been used and due to morphology and size similarity, no abnormalities were found in neonatal growth, as reported by Valenzuela et al. [24]. Conversely, many abnormalities have been observed when embryos and mare's breed were not similar [36–41]. In the equine species, the development of these anomalies depends on the type of placentation, since the nutritional supply to the fetus, which depends on the contact surface between the placenta and the endometrium, is determined by uterus and mare's size [39]. Fetal development is therefore correlated with the size of the recipient, the weight of the placenta, the gross placental area, and the microcotyledonary density [37,41]. If a recipient is smaller than the breed of the embryo, intrauterine growth retardation (IUGR) may occur, as it happens in other conditions that interfere with functional placental area, such as in twin pregnancies, placentitis, or in case of severe atrophy of chorionic villi [42].

In the present study, no difference was found between foal's weight at birth in the ET and ICSI groups compared to AI group. This is in agreement with literature, since in the equine species, unlike in bovine and ovine species, the production of *in vivo* and *in vitro* embryos is not associated with the development of Large Offspring Syndrome (LOS). In the equine species, excessive fetal growth is extremely rare, as the size of the mare and placenta influences fetal size [37,41]. In cattle and sheep, LOS can occur *in vitro* embryo production (IVEP) [43,44] and more frequently in SCNT [45]. It can occur in late gestation and involves a variety of fetal, placental and neonatal developmental defects [46]. Numerous studies have shown that abnormal fetal and placental development in ruminants is due to the presence of serum in the culture medium and the use of co-cultures [44,47]. Notwithstanding, during *in vitro* maturation of equine oocytes 10 % calf serum is utilized, while on day 6 after ICSI it is replaced with 10 % of a mixture (1:1) of fetal calf serum and serum replacement [48].

The high incidence of high-risk pregnancies in the present study in the AI group may be due to the fact that AI mares are predominantly chosen for their genetic value and sports performance, while recipient mares were younger, with parity ranging from 1 to 3 and less likely to develop a high-risk pregnancy [28]. In this study, a very high incidence of dystocia (54.5 %) was detected, even considering only mares with a normal pregnancy and admitted exclusively for attending delivery (40 %). This high percentage is partially related to the fact that this study was conducted in an equine hospital facility where mares with high-risk pregnancy and fetal posterior presentation were referred, as reported in a previous retrospective study about dystocia [28]. In the literature, dystocia's rates are decidedly lower, ranging from 2.7 % to 16 % in Thoroughbred, Standardbred and Quarter Horse [28,49,50]. The higher incidence of dystocia was observed in the ICSI group where, on one hand, there were no high-risk pregnancies, but on the other hand the maternal causes of dystocia were the most prevalent. This can be explained by the fact that all ICSI mares that had dystocia were primiparous, and dystocia is more common in primiparous mares than in multiparous ones [28,49,50]. The same observation can be done for the mares in the ET group.

In the post-partum period, the most frequent complications in mares were retention of fetal membranes (RFM) and reproductive tract trauma, which were more frequent following dystocia. According to literature, the incidence of RFM increases of about 50 % after dystocia [51], while obstetric manipulation led to increased injuries [52] and infections of the genital tract [53]. Despite the high incidence of dystocia, the ET and ICSI groups had a lower incidence of postpartum complications compared to the AI group, probably because in the last group there were older and multiparous mares.

Macroscopic alterations of fetal membranes were more prevalent in ET and ICSI groups, as preliminary observed by Lanci et al. [23]. However, in other studies, these alterations were not observed in IVEP pregnancies [22,24], but were present in SCNT ones, where placental alterations were very common and have been observed in apparently healthy foals [20,21]. In SCNT derived pregnancies, chorionallantoic edema, hypoplasia, atrophy and necrosis of villi, areas of placental hemorrhage and necrosis, placentitis, enlarged allantoic vessels with thrombi and signs of vasculopathy have been observed [16–18,20].

At macroscopic observation of the umbilical cord, UC length was shorter in ICSI group. The possible consequences of a reduced UC length

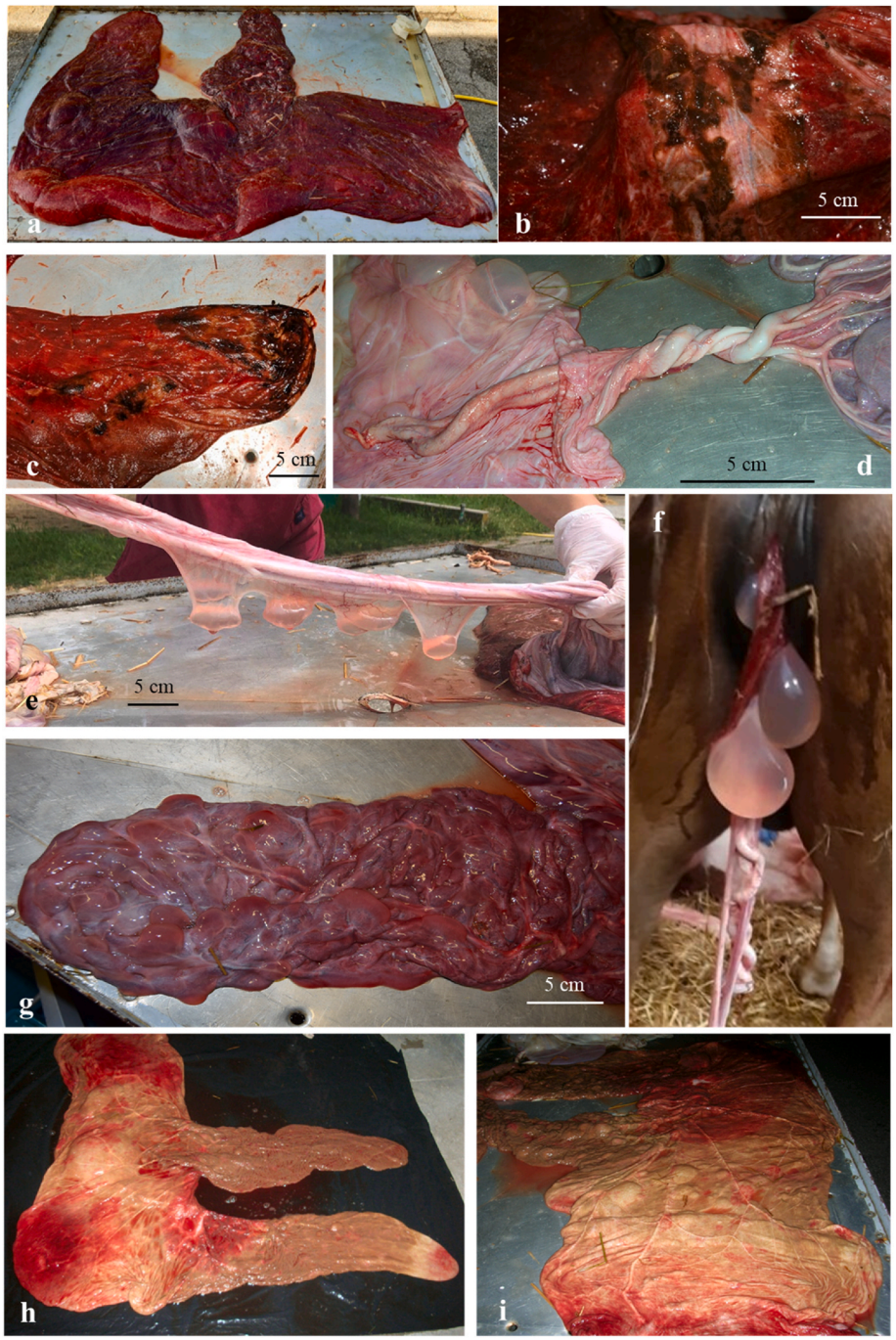

Fig. 1. Chorioallantois edema (a); chorionic areas of necrosis (b–c); a short umbilical cord (d); multiple umbilical cord cysts (e–f); allantois cysts (g); chorionic villi hypoplasia (h–i).

in horses are still unknown. However, Whitehead et al. [33] have shown that it was not associated with abortion or other placental alterations, whereas in humans a reduced cord length is known to be associated with reduced bone mineralization in the newborn due to limitations in fetal movements during intrauterine life [54,55]. Macroscopic alterations of the UC are very frequent in equine pregnancies derived from SCNT and include dilated umbilical vessels with thrombi and signs of vasculopathy, excessive length, thickening and coiling of the cord and the presence of cystic formations [15−19−21].

Unlike in cattle, where some authors report a higher frequency of male calves compared to females in pregnancies obtained from IVEP and SCNT [12,43,56,57], while others have observed a similar proportion [15,58,59], in this study the foal's sex had a similar distribution.

Although significant differences in the presence of macroscopic alterations of fetal membranes were detected in the ET and ICSI groups, these did not result in a higher frequency of sick foals at birth. In fact, in the ET and ICSI groups there are no stillbirth foals, whereas they were 12.5 % in the AI group, involving foals born from high-risk pregnancies and dystocic delivery; only about half of them also showed macroscopic alterations of fetal membranes. These observations are in agreement with the literature for foals obtained by ET and ICSI, indicating no adverse effects from ARTs on neonatal health in the perinatal period [22,60]. Conversely, SCNT pregnancies often result in abortions, perinatal death, or birth of sick foals, which may present neonatal maladjustment syndrome, dysmaturity, pneumonia, umbilical cord swelling, omphalitis, umbilical hernias, blood clots in the urinary bladder, angular

Table 4
Foals' sex, weight, clinical findings and serum IgG concentration in AI, ET and ICSI groups. Data are expressed as mean ± standard deviation, and median (minimum - maximum values). Neonatal encephalopathy (NE), umbilical remnant diseases (URD), congenital flexural limb deformities (CFLD), prematurity (Prem), meconium impaction (MecImp), congenital inguinal hernia (CIH), perinatal asphyxia syndrome (PAS).

Parameter	AI group (N = 32)	ET group (N = 12)	ICSI group (N = 22)
Foal sex (M: male; F: female)	20 M (63 %) 12 F (38 %) (n = 32)	7 M (58 %) 5 F (42 %) (n = 12)	13 M (59 %) 9 F (41 %) (n = 22)
Foal weight (kg)	48 ± 7 49 (28–59) (n = 32)	46 ± 6 45 (38–59) (n = 12)	44 ± 4 44 (38–54) (n = 22)
APGAR score	9 ± 1 9 (8–10) (n = 28)	9 ± 1 9 (7–10) (n = 12)	9 ± 1 9 (7–10) (n = 22)
Rectal temperature (°C)	37.6 ± 0.4 37.6 (36.4–38.2) (n = 28)	37.6 ± 0.3 37.7 (37.1–38.2) (n = 12)	37.4 ± 0.3 37.5 (36.7–38) (n = 22)
Time to sternal recumbency (min)	4 ± 3 4 (0–12) (n = 28)	4 ± 3 4 (0–10) (n = 11)	4 ± 4 3 (0–15) (n = 22)
Time to standing position (min)	67 ± 26 60 (26–150) (n = 28)	89 ± 47 82 (47–205) (n = 11)	72 ± 18 67 (18–111) (n = 22)
Time to suckling reflex (min)	42 ± 29 29 (10–104) (n = 28)	46 ± 49 38 (5–180) (n = 11)	41 ± 48 25 (2–255) (n = 22)
Time to first intake of colostrum (min)	76 ± 26 73 (30–120) (n = 28)	91 ± 35 90 (31–142) (n = 12)	109 ± 49 106 (41–267) (n = 22)
IgG (mg/dL)	1265 ± 608 1157 (396–3681) (n = 27)	1658 ± 505 1854 (777–2315) (n = 11)	1684 ± 632 1600 (700–3000) (n = 22)
Neonatal diseases (n)	18/28 healthy (64 %) 10/28 sick (36 %) (n = 28)	8/12 healthy (67 %) 4/12 sick (33 %) (n = 12)	15/22 healthy (68 %) 7/22 sick (32 %) (n = 22)
Pathological condition (n)	NE 3/10 (30 %) URD 2/10 (20 %) CFLD 2/10 (20 %) Prem 1/10 (10 %) MecImp 1/10 (10 %) CIH 1/10 (10 %) (n = 10)	PAS 2/4 (50 %) URD 1/4 (25 %) CFLD 1/4 (25 %) (n = 4)	PAS 3/7 (43 %) URD 3/7 (43 %) CFLD 2/7 (29 %) MecImp 1/7 (14 %) (n = 7)

deviations and flexural limb deformities, incomplete calcification of carpal bones, multiple rib fractures, and brachygnathism [18,20,21].

5. Conclusions

In the equine species, the production of *in vivo* and *in vitro* embryos may result in a higher frequency of dystocic delivery and fetal membranes alterations. Although these findings, ARTs do not seem to be associated with a higher incidence of neonatal morbidity and mortality; it is well known that the type of parturition and placental insufficiency can affect fetal development and foal's health. Even though they are not considered high-risk foals, like those obtained with SCNT, it would be recommendable a closely monitoring during the perinatal period to promptly detect any problems and appropriately intervene. Despite alterations resulting from the use of ARTs are well-documented in ruminants and they are mainly related to embryonic culture techniques, in horses the most significant alterations are observed in cloning techniques. It would be interesting to further understand the possible consequences resulting from the production of *in vivo* and *in vitro* embryos in the equine species investigating the hormonal parturition pathway of the recipient mare, and histologically evaluating fetal membranes and placental gene expression, in order to highlight the possible mechanisms responsible for placental abnormalities.

CRediT authorship contribution statement

Aliai Lanci: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Francesca Perina:** Writing – review & editing, Visualization, Data curation. **Sabrina Armani:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation. **Barbara Merlo:** Writing – review & editing, Visualization, Formal analysis, Conceptualization. **Eleonora Iacono:** Writing – review & editing, Visualization, Conceptualization. **Carolina Castagnetti:** Writing – review & editing, Visualization, Data curation, Conceptualization. **Jole Mariella:** Writing – review & editing, Visualization, Supervision, Resources, Methodology, Formal analysis, Data curation, Conceptualization.

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