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Abstract

Wharton's jelly (WJ) is fundamental for the well-being of the fetus, binding the umbilical vessels and protecting them from twisting and compression. Gross and microscopic studies have been undertaken on the umbilical cord (UC) of human placentae in both normal and high-risk pregnancies, while they are limited on the equine UC. The aim of this study was to describe microscopically and immunohistochemically the equine UC in normal pregnancies, with particular attention to WJ. Forty-seven healthy mares, with no complications during pregnancy, admitted to the hospital for attending delivery were enrolled. Clinical data was collected at foaling on foal health and placental characteristics. UC samples were collected from three sites (amniotic, allantoic and in the region of vein anastomosis) for histology. The thickness of different layers of arteries and veins and WJ in different UC portions were measured (μm). Wharton's Jelly was weighted (g) and its sections were stained with Masson's trichrome stain, orcein technique and silver impregnation. Immunohistochemistry was undertaken using antibodies raised-against collagen type I, V, VI and fibrillin. Forty-seven UCs, from 19 colt and 28 filly foals, were analyzed for WJ weight and 8/47 UCs were examined histologically. Warton's jelly was only found in the amniotic portion of the UC closest to the foal's abdomen. The weight of WJ (4.0±3.3 g) did not vary between colts and fillies and it was not correlated with any of the clinical or UC parameters measured. The tunica media of arteries and veins was thicker in the amniotic portion of the UC, as described in human UCs in late pregnancy. This finding could be an adaptation to aid in resisting compression because of fetal movements and UC twisting. The umbilical vein was thicker than the umbilical arteries in the tunica media and tunica adventitia in the sections examined throughout the length of the cord. This preliminary study describes gross and histological WJ's structure in the mare. However, further studies are required to better characterize UC's changes throughout pregnancy and in the presence of mare's or fetal disease.

Keywords: equine; umbilical cord; histomorphology; Wharton's jelly; umbilical vessels layers;

1. Introduction

In equine medicine, neonatal risk identification should include a systematic evaluation of fetal membranes, using macroscopic and histopathologic evaluation to recognize placental alterations not observed during pregnancy [1]. For this reason, a complete examination of placenta and umbilical cord (UC), including their histopathological aspects, are important for identifying at-risk neonates and planning appropriate treatments.

The umbilical cord is an essential connection between mother and fetus and it is designed to protect blood flow to the fetus during pregnancy [2]. UC is composed of a vascular component and a gelatinous substance, called Wharton's jelly (WJ). In human medicine, WJ is a well-defined structure, composed of fibroblasts, myofibroblasts, mesenchymal stromal cells and collagen fibers, forming a network of interconnected cavities. The amorphous ground substance of the jelly is mainly made up of hyaluronic acid and glycosaminoglycans dissolved in an aqueous solution of salts, metabolites, and plasma proteins [3-8]. Wharton's jelly plays a trophic, depositing, and mechanical role in tensile and compressive strength of the UC. The thickness and rigidity of WJ influence the contraction or relaxation of the umbilical vessels, acting as a support and preventing excessive distention [4,9-10]. This tissue is fundamental for the well-being of the fetus, maintaining constant bidirectional blood flow between the mother and the fetus during fetal movements, avoiding tension, twisting, compression, and possible node formation [2,11].

During equine pregnancy, UC forms at the beginning of the fetal stage. Indeed, the membranes and associated vessels, that separate the yolk sac and the allantoic sac, combine from day 40 forming the UC, which lengthens until the fetus reaches the floor of the allantoic sac at day 48 of gestation [12]. To date, in equine species the most investigated and known aspects of the UC are the macroscopic appearance also in relation to its association with abortions [13-15] and the isolation of Mesenchymal stromal cells derived from WJ and UC blood [8,16-18]. Several studies described macroscopic characteristics of UC in different breeds [17, 19-23]. Furthermore, the main histological studies were

conducted on normal or abnormal placenta [24-26], while only two studies described some histological aspects of UC [27,28]. On the other hand, the microscopic aspects of human UC have been studied more than in the horse, both in normal and high-risk pregnancies [6, 29,30]. In fact, any disease during pregnancy, which affects both the mother and the fetus, have a great impact on the UC morphological aspect [6, 29-31]. To the authors' knowledge, no studies are present about the quantitative and qualitative presence of WJ in equine species. This preliminary study aimed to describe the equine UC, with particular attention to WJ in normal pregnancy and healthy foals. For the first time in equine species, WJ was described macroscopically, histologically and immunohistochemically.

2. Materials and Methods

2.1 Animals and data collection

Healthy mares with normal pregnancies hospitalized at the Perinatology and Reproduction Unit of the Equine Clinical Service, Department of Veterinary Medical Sciences (DIMEVET), University of Bologna, during four foaling seasons (2016-2019), were included in the study. Mares were housed in wide straw bedding boxes and fed with hay ad libitum and concentrates twice a day. During the day, the mares were allowed to go to pasture. Information about mares' age, breed and parity have been recorded at admission. At foaling, the following data were registered: gestational length, fetal membranes' weight, fetal membranes alterations (allantochorion, allantoamnion and UC). Macroscopic evaluation of UCs was performed as previously described by our research group [17]. The coils were counted before the rupture of the UC, while the UC length was measured after its rupture and the expulsion of the placenta, using a centimeter ruler. A coil is a 360-degree spiral course of umbilical vessels. The Umbilical Coiling Index (UCI), the ratio between total coils and total UC length, was then determined [17].

Furthermore, within 5 min after birth, the APGAR score [32] was calculated and the foal's weight and gender were recorded. Only healthy mares with normal pregnancy, eutocic delivery and healthy foal were enrolled. Foals were classified as healthy when they had an Apgar score ≥ 9 [32], a normal

clinical evaluation during hospitalization, including a complete blood count and serum chemistry at birth, and an IgG serum concentration > 800 mg/dL at 12-24 h of life.

2.2 UC's sampling

All the UCs were collected entirely at foaling immediately after spontaneous breaking. The weight of WJ was obtained from 39/47 UCs at the Animal Reproduction and Biotechnology Laboratory of the Equine Clinical Service (DIMEVET, University of Bologna) and the microscopic evaluation was obtained from 8/47 UCs at the Laboratory of Normal Veterinary Anatomy (DIMEVET, University of Bologna).

2.3 Wharton's jelly weight

Samples for WJ weight were stored in D-PBS (Dulbecco's phosphatase-buffered solution) containing penicillin (100 IU/mL) and streptomycin (100 mg/mL), at 4°C for at the latest 12h. Wharton's Jelly was isolated by cutting it with sterile surgical forceps and scissors, and weighed with an electronic balance.

2.4 Microscopic description and measurement of different layers

Samples for UC microscopic evaluation were stored in PBS (Phosphate Buffer Solution), containing 0.1% (v/v) sodium-azide and 30% (v/v) sucrose solution, at 4°C until analysis. Ten sections of 15 μm in three different portions for each UCs (amniotic portion, allantoic portion and at the site of vein anastomosis and in particular at the division of the two umbilical veins) (Figure 1) were obtained by cryostat and mounted in gelatin-coated glass slides. Umbilical cord's sections were stained with Masson's trichrome stain. The thickness of different layers of umbilical arteries and veins and WJ were measured (μm) in each UC's portion using image analysis software (ImageJ, processing and analysis in Java, Version 1.6). In particular, the values of different layers of the largest and smallest vessel were recorded for both arteries and veins (see Table S1 in the Supplementary Material) and averages were calculated.

For an accurate WJ analysis, a section was also stained with orcein technique to identify elastic fibers and with silver impregnation technique to identify reticular fibers.

2.5 Immunohistochemical analysis

The immunohistochemical analysis was conducted, giving particular attention to WJ. After three washes for 10 mins in PBS, sections were incubated with 1% H_2O_2 in PBS for 30 mins at room temperature (RT) for eliminating endogenous peroxidase activity. Sections were then rinsed in PBS three times for 10 mins and incubated in PBS plus 10% (v/v) normal goat serum (Colorado Serum, Denver, CO, #CS 0922) and 0.5% Triton X-100 (Merck, Darmstadt) for 2 hours at RT.

Thereafter, sections were incubated for 48 h at 4°C with rabbit anti-bovine collagen type I polyclonal antibody (dilution 1:80; Chemicon, Temecula, CA-USA, batch NG1804950), rabbit anti-human collagen type V polyclonal antibody (dilution 1:40; Chemicon, Temecula, CA-USA, batch 0604027824), rabbit anti-collagen type VI polyclonal antibody (dilution 1:10-1:40; Chemicon, Temecula, CA-USA, batch NG 1833210) and anti-fibrillin clone 689 purified mouse monoclonal antibody (dilution 1:200; Chemicon, Temecula, CA-USA, batch NRG1758239). The chosen antibodies were previously used and validated in the equine species [33]. The primary antibody was diluted in a solution (1.8% NaCl in 0.01 M PBS containing 0.1% sodium azide) containing 1% normal goat serum and 0.5% Triton X-100.

After three washes in PBS, the sections were incubated in goat biotinylated anti- rabbit 10µg/ml (Vector Laboratories, Burlingame, CA, USA, BA-1000) or goat biotinylated anti- mouse 10µg/ml (Vector Laboratories, Burlingame, CA, USA, BA-9200) for 2 hours at RT. The secondary antibody was diluted in PBS plus 1% v/v normal goat serum and 0.5% v/v Triton X-100.

The sections were transferred to avidin–biotin complex (ABC kit Vectastain, PK-6100, Vector Laboratories, Burlingame, CA) for 30 mins and the immunoperoxidase reaction was developed by 3,30-diaminobenzidine (DAB kit, SK-4100, Vector Laboratories, Burlingame, CA). Slides were dried overnight, dehydrated in ethanol, cleared in xylene, and coverslipped with Entellan (Merck, Darmstaldt, Germany). All the incubations were performed in a humid chamber. Masson trichrome

147 staining was also performed to better highlight the fibroblasts.
148 Negative controls were produced by replacing the primary antibodies with PBS.
149 All sections were observed under a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany).
150 Images were recorded by a Polaroid DMC digital photocamera (Polaroid Corporation, Cambrige,
151 MA, USA) and DMC 2 software.
152 In WJ, the density of fibroblast cells was calculated as the number of cells/mm^2 in each section sep-
153 arately. Fibroblast cells were counted at 20× magnification in 10 fields of view in each specimen.
154 Only cells with an evident nucleus were counted.
155
156 *2.6 Statistical analysis*
157 Data were analyzed for normality using a Shapiro-Wilk test. Since distribution was normal, data were
158 expressed as mean ± standard deviation.
159 Student T-test was used for evaluating the difference between WJ's weight and foal's gender.
160 Pearson's test was performed to compare WJ weight and clinical data (mare's age and parity, gesta-
161 tional length, foal's weight, fetal membranes' weight) and UC characteristics (total length, total coils,
162 UCI, amniotic and allantoic length and coils).
163 Student T-test was used for evaluating the difference between arteries and veins in different vessels'
164 layers (amniotic epithelium, tunica intima, media and adventitia) in three different UC portions (am-
165 niotic, allantoic portion and at anastomosis site).
166 One-way ANOVA was used for evaluating the three different UC portions (amniotic, allantoic por-
167 tion and at anastomosis site) for each vessels layer (amniotic epithelium, tunica intima, media and
168 adventitia, total thickness) of arteries and veins.
169 All analyses were carried out using the software IBM SPSS Statistics 23 (IBM Corporation, Milan,
170 Italy). Significance was assessed for $P<0.05$.
171
172 **3. Results**

Forty-seven mares (37 Standardbred, 7 Italian Saddlehorse, 2 Arabian and 1 Quarter Horse) were included in the study. Recorded data are reported in Table 1. Fetal membranes alterations were not present. Thirty-nine UCs were analyzed for WJ's weight, while 8/47 UCs were analyzed for microscopic description, measurement of different layers, immunohistochemical analysis and fibroblast cells count.

In all the collected UCs, WJ was found only in the intramniotic portion, close to the foal, while it was absent in the allantoic portion (Figure 1). Foals were 19 males and 28 females and WJ's weight was 4.0±3.1 g and 4.1±3.4 g respectively. No differences were found between WJ's weight and foal's sex. The amount of WJ (g) was not correlated with any of the clinical and umbilical registered data.

3.1 Microscopic description and measurement of different layers

Eight/47 UCs have been microscopically evaluated. All the vessels showed the three typical layers: tunica intima, tunica media and tunica adventitia. The endothelium of tunica intima was regular and well developed, having a single layer of cells (Figure 2a) resting on a basement membrane. The subendothelial layer was composed of connective tissue with some smooth muscle fibers between the collagen fibers (Figure 2a, 2b). The tunica media was thicker than tunica intima and it had multiple concentric smooth muscle and few collagen fibers (Figure 2b, 2c). The tunica adventitia was the thickest layer and the collagen fibers progressively increased going towards the periphery, while smooth muscle fibers decreased (Figure 2c, 2d). A perivascular tissue, composed of dense collagen fibers arranged concentrically, surrounded the tunica adventitia (Figure 2f, 3a). This tight tissue was composed of dense and well-organized connective tissue. On the contrary, WJ was loose connective tissue: collagen fibers were arranged to create a loose reticular texture (Figure 2d, 4, 5). Other cells observed in WJ were fibroblasts (Figure 4e) and white blood cells (Figure 5). Finally, the UC was externally surrounded by amniotic membrane (Figure 2d, 3a), composed by two thin layers: amniotic epithelium and subamnion, an amniotic connective tissue with collagen fibers and fibroblasts (Figure 3a insert).

198 The orcein staining showed the absence of elastic fibers in the WJ. They were less concentrated with
199 an uneven pattern around the vessels (Figure 6a). Finally, the silver impregnation staining revealed a
200 dense network of reticular fibers in the entire section of the cord (Figure 6b).
201 The mean measurements of the tunica intima, media and adventitia of the umbilical arteries and veins
202 are reported in Table 2 and 3, respectively. The measurement of the tunica intima, media and adven-
203 titia of the largest and smallest umbilical vessels are reported in Table S1 in Supplementary materials.
204 The differences between arteries and veins in each umbilical portion are reported in Figure 7.
205 Mean number of fibroblasts counted in the WJ were 57±9 cells/mm^2 and the mean thickness of WJ
206 was 649±474 μm.
207
208 *3.2 Immunohistochemical analysis of WJ*
209 The immunohistochemical analysis revealed the presence of fibroblast cells positive for antibodies
210 anti-type I, V and VI collagen and anti-fibrillin (Figure 8).
211
212 **4. Discussion**
213 In the present study, the microscopic features of UCs collected at delivery of healthy foals born after
214 normal pregnancies were investigated. The histological description focused on the complete analysis
215 of WJ, a still poorly known tissue in the equine species. The present study describes, equine WJ for
216 the first time, focusing on its amount in healthy newborn foals and its histological aspects, such as
217 thickness and fibroblasts concentration.
218 In women, physiological variations in WJ amount can occur in normal pregnancies: WJ decreases
219 significantly with advancing gestation and its water content is significantly lower in term than in
220 preterm neonates, with a progressive reduction from 30 weeks' gestation to term [11, 34-38]. Further-
221 more, a significant positive correlation between WJ weight and both birth weight and placental weight
222 has been reported [39], with a significant negative correlation observed between the WJ density and
223 both birth weight and placental weight [39]. Moreover, it has also been reported that male fetuses

have a higher amount of WJ than females, but the density remains the same [40]. Biochemical and morphological alterations of WJ may cause some prenatal diseases [29, 35, 41-43] and a quantitative variation of WJ seems to be associated with pathological conditions such as hypertensive disorders [29], gestational diabetes [44], foetal distress [40], and foetal growth restriction [35,37,45]. In women with preeclampsia, WJ has more glycosaminoglycans and type III collagen, whereas hyaluronic acid is reduced. Because hyaluronic acid is highly hydrophilic, the amount in the WJ is particularly important for the UC's mechanical properties and macroscopic appearance [29,42,46]. Furthermore, the total absence of WJ has been associated with foetal death [47]. The quantitative decrease of WJ could determine UC torsion, compression or stretching that would adversely affect foetal blood flow [2,48]. Contrary to what expected, in the present study the amount of WJ at term was not correlated with the mare's age and other investigated clinical and UC's parameters. The average age of the mares included in the present study is 11 years, age at which they are in full reproductive activity. It would be interesting to make a comparison between young and old mares, with particular attention to old maidens. Regarding gestational age, the mean included in the present study is within the normal range and, since this is very wide, it could be difficult to find a significant correlation. Based on these results, it would be interesting to compare them with umbilical cords of healthy animals born at different gestational ages. Umbilical cord parameters have not been found to vary with the amount of WJ during normal pregnancy, but it is likely that other parameters come into play. On the other hand, in human medicine, research on this topic has been in progress for more than 40 years, while in equine medicine it is just starting.

Accordingly to the only one study that had briefly described the WJ characteristics in 6 Thoroughbred foals [28], in the present study, reactive fibroblasts containing I, IV and V collagen, and fibrillin surrounded by a dense network of reticular fibers were described, as in the human umbilical cord [49]. Fibroblasts and myofibroblasts are the main cellular component of human WJ, contributing to its elasticity through the synthesis of collagen fibers, which affects the regulation of blood flow through the UC [4]. The same would also appear to be true for the horse. Differently from human [2],

250 in the equine UC, elastic fibers are less concentrated with an uneven pattern around the vessels. Fi-
251 nally, in the equine species, as well as in human [2,49], a dense network of reticular fibers pervades
252 the entire tissue.
253 For the first time, the vessel layers in each portion of the UC were compared for both arteries and
254 veins and the different thicknesses between artery and vein were also observed. These original results
255 cannot be compared with any other study, since Kumar et al. [28] did not report a statistical analysis.
256 As for human vessels, also the equine tunica media of arteries and veins resulted to be thicker in the
257 amniotic portion s as pregnancy advances [50,51]. This because the amniotic portion, closely related
258 to the fetus, is affected by its movements and usceptible to excessive twisting of the UC, apart from
259 a partial occlusion of blood flow. For this reason, the tunica media may be thicker to better resist any
260 compression caused by the fetus. The first detectable equine fetal limb movements occur from day
261 46, when the fetus changes presentation, position and location with great vigor in the first trimester
262 of pregnancy, even about 5 times/hour [52]. It is likely that the extensive fetal activity and mobility
263 in this species during early pregnancy play a role in fetal development of muscle and nerve coordi-
264 nation. The amniotic and the allantoic portion of the UC could become coiled as early as day 68 of
265 gestation [53,54]. The UC coils are attributable to changes in fetal presentation, and it seems that a
266 coil in the allantoic portion of the UC represents a presentation change of the fetal-amniotic unit, and
267 a coil in the amniotic portion represents a presentation change of the fetus within the amnion [54].
268 The umbilical coiling makes the UC a structure both flexible and strong and provides resistance to
269 external forces which could compromise blood flow [17]. The umbilical coiling could be an evolu-
270 tionary purpose whereby cord shortening would decrease the distance between the fetus and the uter-
271 ine attachment in a caudal horn when fetal mobility and changes in presentation have reduced [54].
272 Probably, the shortening of this distance, the presence of the WJ, and maybe even the increased thick-
273 ness of the tunica media in the UC vessels of the amniotic portion could reduce entanglements be-
274 tween the UC and the active and long fetal hindlimbs.

The umbilical vein was found to have greater thickness than the umbilical artery in almost all layers in the three sections of the UC, in agreement with what reported in human medicine [31]. The reason for this difference can only be assumed and some hypotheses can be made, such as the larger caliber of the umbilical vein compared with the umbilical artery, the anastomosis of the two umbilical veins, or the greater resistance. Therefore, even in the equine species, umbilical veins could have a greater thickness because, by carrying oxygenated blood, they actually act as arteries.

4.1 Conclusion

Histological characteristics of WJ, its physiological amount in healthy animals and, particularly, for the first time in the equine species, the measurements of umbilical vessel layers at different locations were described. Contrary to authors' expectations, no correlations were found with the examined parameters in the studied population. Further studies enrolling animals with more variable parameters (such as age, gestational age and UC's parameters) could help to better characterize qualitative and quantitative differences throughout the entire pregnancy and, subsequently, to compare physiological and high-risk pregnancies.

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