1 **MATERNAL AND NEONATAL EVALUATION OF REACTIVE OXIGEN**

2 **METABOLITES (D-ROMS) AND BIOLOGICAL ANTIOXIDANT POTENTIAL (BAP) IN**

3 **THE HORSE**

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17 **Abstract**

18 The aim of the present work was to evaluate Reactive Oxygen Metabolites and biological

19 antioxidant potential in mares and foals in order to study perinatal oxidative status. A total of 60

20 animals were included in the present study. Maternal and foal venous blood samples were collected

21 immediately after delivery along with a sample drawn from one of the umbilical arteries and plasma

22 samples were evaluated for lactatemia, d-ROMs and BAP. T test for unpaired data was applied

23 between mares vs umbilical artery blood vs foals, both for d-ROMs and BAP. Pearson test with

24 two-tailed p-value and a confidence interval of 95% was performed between d-ROMs and BAP, and

25 between d-ROMs and lactatemia, both for mares and foals. Finally, T-test for unpaired data was

26 performed between fillies and colts. T-test showed differences between mares vs their own foals vs

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27 umbilical artery blood, but not foals vs umbilical artery blood, both for d-ROMs and BAP. A

28 positive correlation was found both in mares and foals between BAP and d-ROMs, and in mares

29 between lactatemia and d-ROM. No differences in gender were found in BAP concentration. Our

30 data are in line to previous studies performed in women and cattle.

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1. Introduction

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. [1-5] During pregnancy, delivery and lactation, an overproduction of reactive oxygen species (ROS) may take place due to the increased metabolism, along with the physical effort. [6-8] Several papers confirmed the presence of oxidative stress during pregnancy, [9] parturition, [10-11] post-parturient period in women. [12-14] and also newborn babies are susceptible to oxidative stress due to an imbalance between antioxidant and oxidant-generating systems. [15-26] The placenta seems to have an important role in protecting human fetus from oxidative stress. In fact, many clinical studies reported the protective mechanism against O_2 toxicity in the human feto-placental unit during pregnancy and at time of parturition. [5, 26-28] In veterinary medicine, antioxidative/oxidative status has been studied during pregnancy, lactation, and at delivery both in cows and their newborn calves and ewes [29-34] The aim of the present work was to measure mares and foals concentrations of reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) in order to evaluate maternal and neonatal oxidative status at delivery and to verify the protective role of placenta against fetal oxidative stress.

2. Materials and Methods

2.1 Animals

Two Throughbred, 1 Purebred Arabian, 27 Standardbred trotter, 4 mixed breed mares and their foals, for a total of 68 animals housed at the studfarm "La Piaggia" (Galleno, Pisa, Italy) and admitted at the Department of Veterinary Sciences, University of Pisa from 2012 to 2013 foaling seasons were included in the present study. The mares were hospitalized because the owners requested an attended parturition. Ethical approval and informed written consent were obtained from the owners according to the Ethics Committee on Animal Experimentation of the University of Pisa. Mares and their foals underwent similar management conditions. Mares were included in this study according to the following criteria: 1) pregnancy length > 320 days; [35] 2) unassisted

delivery; 3) mares treated against gastrointestinal parasites and vaccinated against equine influenza, tetanus, and equine herpes virus-1 according to guidelines of the American Association of Equine Practitioners; [36] 4) healthy at physical examination. Attendance at birth was ensured by monitoring mammary development and measuring the concentration of calcium in the mare's colostrum every evening at 6 pm using a commercially avalilable kit (Foal Watch[TM], Chemetrics, Calverton, VA, USA). Mares were constantly supervised 24 hours/day when calcium in the colostrum was over 200 ppm.

Inclusion criteria for foals were: 1) Apgar Score 5 minutes after birth ≥7; [37] 2) IgG ≥ 800 mg/dl at 24 hours of age (Snap Foal IgG test Kit, Idexx, USA); [37] 3) righting reflex present immediately after foaling, suck reflex within 10 minutes, sternal recumbence within 5 minutes, quadrupedal position within 60 minutes and nursing the mare within 120 minutes after birth. [38] Foals were given physical examinations before each blood collection and appeared to be clinically healthy during the all study period.

2.2. Sample collection and handling

Immediately after delivery, maternal and foal venous blood samples were drawn by the jugular vein and collected into lithium-heparinized test tubes (FL Medical, Padua, Italy). Simultaneously a heparinized blood sample was drawn from one of the umbilical arteries from each umbilical cord. Heparinised samples were centrifuged at 3000 g for 10 minutes, as recommended by the manufacturer, plasma was frozen at -18 °C and then analysed in a single batch.

2.3. Biochemical analysis

All plasma samples were evaluated for plasma lactate (Accutrend Lactate®, Micralab srl, MI), plasma reactive oxygen metabolites concentrations by d-ROMs test (Diacron srl, Grosseto, Italy) and biological antioxidant potential by BAP test (Diacron srl, Grosseto, Italy). D-ROMs and BAP tests were carried out by a spectrophotometer (Slim, SEAC, Florence, Italy). The stability of the d-ROMs and BAP tests on stored horse blood was previously evaluated. [39]

91   2.4 Statistical analysis

92   The maternal, umbilical artery blood and neonatal d-ROMs and BAP concentrations, and lactatemia

93   were expressed as mean±standard deviation (X±SD). Normal distribution of data was evaluated by

94   Komolgorov-Smirnov test. Data showed a Gaussian distribution. T test for unpaired data was

95   applied to verify differences between mares and their own umbilical artery blood and foals. Pearson

96   test with two-tailed p-value and a confidence interval of 95% was performed to evaluate correlation

97   between d-ROMs and BAP, and between d-ROMs and lactatemia, both for mares and foals. Finally,

98   T-test for unpaired data was performed between fillies (n=16) vs colts (n=18) to verify differences

99   in antioxidant status related to gender. Results were considered to be statistically significant when

100 $p<0.05$ (Statgraphics plus, USA).

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102 **3. Results**

103 d-ROMs and BAP concentrations in mares, umbilical artery blood and foals are reported in Table 1.

104 Lactatemia in mares and foals was 2.96±1.15 mmol/L and 4.01±1.71 mmol/L, respectively. T-test

105 for unpaired data showed statistical differences between mares vs their own foals (p<0.0001), mares

106 vs umbilical artery blood (p=0.005), but not foals vs umbilical artery blood (p=0.3) for d-ROMs.

107 BAP concentration was statistically different between mares vs foals (p=0.04) and mares vs

108 umbilical artery blood (p=0.02), but not between foals vs umbilical artery blood (p=0.7). Pearson

109 test showed a positive correlation in mares between BAP and d-ROMs ($r^2= 0.644$, p=0.0001) and

110 between lactatemia and d-ROMs ($r^2=0.561$, p<0.001), while in foals correlation was found only

111 between d-ROMs and BAP ($r^2=0.653$, p<0.01). T-test performed between fillies and colts did not

112 show statistical differences for d-ROMs and BAP concentrations related to gender (p=0.3).

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114 **4. Discussions and conclusions**

115 Oxidative stress is defined as an imbalance between oxidants and antioxidants in which the oxidant

116 activity exceeds the neutralizing capability of antioxidants, resulting in cellular injury and activation

117 of pathologic pathways. [2] This work investigates antioxidative/oxidative profile of mares and their

118 own foals measuring the d-ROMs and BAP concentrations.

119 In this study, the d-ROMs amount was higher in maternal blood than in umbilical artery blood and

120 foals' blood at birth. In women the concentration of lipoperoxides increases as pregnancy advances

121 and the highest concentrations are observed during delivery. [5] Moreover, the concentration of

122 oxidative agents is higher in maternal blood than in cord blood at birth. [40] In our study, the

123 concentration of d-ROMs has not been determined during pregnancy, but our results regarding the

124 differences in d-ROMs concentration between mares and umbilical artery blood/foals at birth are in

125 line to previous studies performed in women. [5, 40] These differences could support the hypothesis

126 of the positive role of the placenta in preventing the passage of oxidative agents from the mare to

127 the foal at the time of parturition, as suggested in humans. [5, 27] The prevention could be due to a

128 higher placenta secretion of oxidative agents at the maternal side compared to the foetal side, as

129 suggested for women. [41-42]

130 In the present study, also BAP concentration in the mare is higher than in the umbilical artery blood

131 and in the foal. Newborn babies are at high risk for oxidative stress at birth and are very susceptible

132 to oxidative damage by Reactive Oxigen Substances (ROS) because the extrauterine environment is

133 richer in oxygen than the intrauterine environment. During delivery, in fact, the foetus is transferred

134 from an intrauterine hypoxic environment with 20–25 mmHg oxygen tension (paO$_2$: 20–25 mmHg)

135 to an extrauterine normoxic environment with approximately 100 mmHg paO$_2$. [43] The increase in

136 oxygen tension induces the production of ROS [44-45] that is also exacerbated by the low

137 efficiency of the natural antioxidant system in newborns. [5, 21, 46] Inami et al. [47] reported lower

138 antioxidative activities and higher concentration of thiobarbituric acid-reactive substances in serum

139 of newborn calves as their dams. Our results on BAP values are in line with literature; the lower

140 BAP concentration in foals than in mares obtained in this study may be caused to a lower

141 antioxidative activity in newborn foals due to immature defence systems, as already reported for

142 babies and cows. [31, 47]

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143   Castillo et al. [29] studied antioxidative/oxidative status of plasma in cows from 10 weeks before

144   parturition until 8 weeks post-partum. The authors observed an increase in antioxidant status up to 1

145   week postpartum and then a decrease till 8 weeks postpartum associated to a parallel peroxidative

146   damage trend. Our results reported a positive correlation (r= 0.644 p<0.05) between BAP

147   antioxidants and oxidative parameters in the mare, similarly to what happens in cattle, suggesting

148   an effective reaction of the antioxidant system in healthy mares during critical period, such as

149   parturition and lactation. [29, 31, 48] The missing correlation between d-ROMs and BAP amounts

150   in foals could be explained by the lower antioxidant activity in newborn in comparison with adult

151   horses. [31, 47]

152   Lista et al. [5] reported the influence of gender on oxidative/antioxidative status. The authors

153   observed that females were less susceptible to oxidative stress because they showed higher values

154   of total antioxidative system as compared to males. To the authors' knowledge there are no data on

155   this item in veterinary medicine. Our results did not show differences on BAP concentration

156   between fillies and colts, thus in foals the antioxidative activity did not seem to be influenced by

157   gender.

158   The most common cause of an increase in blood lactate concentration in horses is a decreased tissue

159   perfusion and oxygen delivery with subsequent anaerobic metabolism. In adult horses, decreased

160   tissue perfusion is most often due to hypovolemia, but inappropriate vascular tone during severe

161   sepsis may also contribute to hyperlactatemia. In septic equine neonates, hypovolemia,

162   inappropriate vascular tone, and decreased cardiac output have been implicated as causes of

163   hyperlactatemia. [49-51] Hypoxia-reperfusion and inflammation are among the major mechanisms

164   of induction of oxidative stress. [52] Besides, lactate induces the production of mitocondrial

165   reactive oxigen species (ROS). [53] Our results show a positive correlation between lactatemia and

166   d-ROMs in mares at parturition, suggesting that the rise of d-ROMs at delivery may be due to the

167   increase of blood lactate.

168   Our results are in line with literature in humans and other animal species, confirming the

169   importance of a balanced oxidative status in mares at delivery.

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171 **References**

172 **1.** Agarval A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod
173 Biol Endocrinol, 2005; 3(28):1-21.

174 2. Soffler C. Oxidative stress. Vet Clin Equine Pract, 2007; 23:135-57.

175 3. Perrone S, Negro S, Tataranno ML, Buonocore G. Oxidative stress and antioxidant
176 strategies in newborns. J Matern Fetal Neonatal Med, 2010; 23(S3):63-5.

177 4. Howlader ZH, Perveen S, Tamanna S, Khan TA, Begum F. Oxidative Stress and
178 Antioxidant Status in Neonates Born to Pre-eclamptic Mother. J Trop Pediatr, 2009;
179 55(6):363-7.

180 5. Lista G, Castoldi F, Compagnoni G, Maggioni C, Cornelissen G, Halberg F. Neonatal and
181 maternal concentrations of hydroxil radical and total antioxidant: protective role of placenta
182 against fetal oxidative stress. Neuroendocrinol Lett, 2010; 31(3):319-24.

183 6. Wisdom SJ, Wilson R, McKillpo JH, Walker JJ. Antioxidant systems in normal pregnancy
184 and in pregnancy-induced hypertension. Am J Obst Gynecol, 1991;165:1701-4.

185 7. Miller JK, Brzezinska-Slebodzinska E, Madsen FC. Oxidative stress, antioxidants and
186 animal function. J Dairy Sci, 1993; 76: 2812-23.

187 8. Jain Sk, Wise R, Bocchini JJ Jr. Vitamin E and Vitamin E-quinome levels in red blood cells
188 and plasma of newborn infants and their mothers. J Am Coll Nutr, 1996; 15:44-8.

189 9. Myatt L, Cui X. Oxidative stress in the placenta. Histochem Cell Biol, 2004; 122:369-82.

190 10. Fainaru O, Falmog B, Pinchuk I, Kupferminc MJ, Lichtenberg D, Many A. Active labour is
191 associated with increased oxidisibility of serum lipids ex vivo. BJOG, 2002; 109:938-41.

192 11. Mocatta TJ, Winterbourn CC, Inder TE, Darlow BA. The effect of gestational age and
193 labour on markers of lipid and protein oxidation in cord plasma. Free Radic Res, 2004;
194 38:185-91.

195 12. Kankofer M. Antioxidative defence mechanisms against reactive oxigen species in bovine
196 retained and not-retained placenta: activity of glutathione peroxidase, glutathione-

197   transferase, catalase and superoxide dismutase. Placenta, 2001; 22:466-72.

198   13. Wall PD, Pressman EK, Woods JR Jr. Preterm premature rupture of the membranes and
199   antioxidants: the free radical connection. J Perinat Med, 2002; 30:447-57.

200   14. Pressman EK, Cavanaugh JL, Mingione M, Norkus EP, Woods JR. Effects of maternal
201   antioxidant supplementation on maternal and fetal antioxidant levels: a randomized, double-
202   blind study. Am J Obst Gynecol, 2003; 189:1720-25.

203   15. Saugstad OD. Oxygen toxicity in the neonatal period. Acta Paediatr, 1990; 83: 692-5.

204   16. Kelly FJ. Free radicals disorders of preterm infants. British Med Bull, 1993; 49:668-78.

205   17. Pitkanen OM, Hallman M, Andersson S. Correlation of free oxygen radial-induced lipid
206   peroxidation with outcome in very- low-birthweight infants. J Pediatr, 1993; 116:760-4.

207   18. Supnet MC, David-Cu R, Walther FJ. Plasma xanthine oxidase activity and lipid
208   hydroperoxide levels in preterm infants. Pediatr Res; 1994; 36:283-7.

209   19. Varsila E, Pitkanen OM, Hallman M, Andersson S. Immaturity-dependent free radical
210   activity in premature infants. Pediatr Res, 1994; 36:55-9.

211   20. Rice-Evans CA, Diplock AT, Symons MCR. Techniques in free radical research.
212   Amsterdam: Elsevier, The Netherdands; 1991.

213   21. Saugstad OD. Mechanisms of tissue injury by oxygen radicals: implications for neonatal
214   disease. Acta Paediatr, 1996; 85:1-5.

215   22. Schmidt H, Grune T, Muller R, Siems WG, Wauer RR. Increased levels of lipid
216   peroxidation products malondialdehyde and 4-hydroxynonenal after perinatal hypoxia.
217   Pediatr Res, 1996; 40:15-20.

218   23. Wiedemann M, Kontush A, Finckh B, Hellwege HH, Kphlshutter A. Neonatal blood plasma
219   is less susceptible to oxidation than in adult plasma owing to its higher content of bilirubin
220   and lower content of oxidable fatty acids. Pediatr Res, 2003; 53:843-49.

221   24. Zhao J, Liu XJ, Ma JW, Zheng RL. DNA damage in heathy neonate. Early Hum Dev, 2004;
222   77:89-98.

223 25. Saugstad OD. Oxidative stress in the newborn – a 30-year perspective. Biol Neonate, 2005;
224   88:228-36.

225 26. Gitto E, Pellegrino S, Gitto P, Barberi I, Reiter RJ. Oxidative stress of the newborn in the
226   pre- and postnatal period and the clinical utility of melatonin, J Pineal Res, 2009; 46(2):128-
227   39.

228 27. Qanungo S, Sen A, Mukherjea M. Antioxidant status and lipid peroxidation in human feto-
229   placental unit. Clin Chim Acta, 1999; 285(1-2):1-12.

230 28. Arikan S, Konukoglu D, Arikan C, Akcay T, Davas I. Lipid peroxidation and antioxidant
231   status in maternal and cord blood. Gynecol Obstet Invest, 2001; 51(3):145-9.

232 29. Castillo C, Hernandez J, Bravo A, Alonso ML, Pereira V, Benedino JL. Oxidative status
233   during late pregnancy and early lactation in dairy cows. Vet J, 2005; 169:286-92.

234 30. Gaal T, Ribiczeyne-Szabo P, Stadler K, Jakus J, Reiczigel J, Kover P, Mezes M, Sumeghy
235   L. Free radicals, lipid peroxidation and the antioxidant system in the blood of cows and
236   newborn calves around calving. Comp Biochem Physiol B Biochem Mol Biol, 2006;
237   143:391-6.

238 31. Albera E, Kankofer M. The Comparison of Antioxidative⁄Oxidative Profile in Blood,
239   Colostrum and Milk of Early Post-partum Cows and Their Newborns. Reprod Dom Anim,
240   2009; 46:763-9.

241 32. Mutinati M, Piccinno M, Roncetti M, Campanile D, Rizzo A, Sciorsci R. Oxidative stress during
242   pregnancy in the sheep. Reprod Domest Anim, 2013; 48: 353-7.

243 33. Rizzo A, Mutinati M, Spedicato M, Minoia G, Trisolini C, Jirillo F, Sciorsci RL. First demonstration
244   of an increased serum level of reactive oxygen species during the peripartal period in the ewes.
245   Immunopharmacol Immunotoxicol, 2008; 30: 741-6.

246 34. Rizzo A, Ceci E, Pantaleo M, Mutinati M, Spedicato M, Minoia G, Sciorsci RL. Evaluation of blood
247   and milk oxidative status during early postpartum of dairy cows. Animal, 2013; 7: 118-23.

248 35. Lester GD. Prematurity, Dysmaturity and assessment of maturity. Part I. The Foal: problems
249   of the Immediate Postpartum Period. In: McKinnon AO, Squires EL, Vaala WE Varner DD,

250   editors. Equine Reproduction. 2nd ed. New York: Blackwell Publishing Ltd; 2011, p.121-7.

251   36. http://www.aaep.org/images/files/AdultVaccinationChartFinal022412.pdf).

252   37. Vaala WE. Perinatology. Vet Clin North Am Eq Pract, 1994; 10: 237-65.

253   38. Stoneham SJ. Assessing the newborn foal. In: Paradis MR, editor. Equine Neonatal
254   Medicine: a case-based app roach, Amsterdam: Elsevier Inc, 2006; p. 1-11.

255   39. Celi P, Sullivan M, Evans D. The stability of the reactive oxygen metabolites (d-ROMs) and
256   biological antioxidant potential (BAP) tests on stored horse blood. Vet J, 2010; 183:217-8.

257   40. Rogers MS, Mongelli M, Tsang KH, Wang CC. Fetal and maternal levels of lipid peroxides
258   in term pregnancies. Acta Obstet Gynecol Scand, 1999; 78:120-4.

259   41. Walsh SW, Wang Y. Secretion of lipid peroxides by human placenta. Am J Obstet Gynecol,
260   1993; 169(6):1462-6.

261   42. Walsh SW, Wang Y, Jesse R. Placental reproduction of lipid peroxides, thromboxane and
262   prostacyclin in pre-eclampsia. Hipertens Pregnancy, 1995; 15: 101-11.

263   43. Gitto E, Pellegrino S, D'Arrigo S, Barberi I, Reiter RJ. Oxidative stress in resuscitation and in
264   ventilation of newborns. Eur Respir J, 2009; 34: 1461-9.

265   44. Shoji H, Koletzko B. Oxidative stress and antioxidant protection in the perinatal period. Curr Opin
266   Clin Nutr Metab Care, 2007; 10: 324-8.

267   45. Rizzo A, Roscino MT, Binetti F, Sciorsci RL. Roles of reactive oxygen species in female
268   reproduction. Reprod Domest Anim, 2012; 47: 344-52.

269   46. Buonocore G, Perrone S, Longini M, Terzuoli L, Bracci R. Total Hydroperoxide and
270   advanced oxidation protein products in preterm hypoxic babies. Ped Res, 2000; 47(2): 221-
271   4.

272   47. Inanami O, Shiga A, Okada K, Sato R, Miyake YL, Kuwabara M. Lipid peroxides and
273   antioxidants in serum of neonatal calves. Am J Vet Res, 1999; 60:452-7.

274   48. Castillo C, Hernandez J, Pereira V, Sotillo J, Alonso ML, Benedino JL. Plasma
275   malondialdehyde (MDA) and total antioxidant status (TAS) during lactation in dairy cows.
276   Res Vet Sci, 2006; 80:133-9.

277   49. Corley KTT, Donaldson LL, Furr MO. Arterial lactate concentration, hospital survival,

278   sepsis and SIRS in critically ill neonatal foals. Equine Vet J, 2005; 37(1):53-9.

279   50. Wotman K, Wilkins PA, Palmer JE, Boston RC. Association of blood lactate concentration

280   and outcome in foals. J Vet Intern Med 2009; 23(3):598-605.

281   51. Tennent-Brown. Interpreting lactate measurement in critically ill horses: diagnosis,

282   treatment, and prognosis. Comp Continu Educ Vet, 2012; 34(1):1-6.

283   52. Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M. Free radicals, metals and

284   antioxidants in oxidative stress-induced cancer. Chem Biol Interact; 2006; 160(1):1-40.

285   53. Nalos M, McLean AS, Huang S. Rivisiting lactate in critical illness. In Vincent JL, editor.

286   Annual Update in Intensive Care and Emergency, Berlin: Springer-Verlag Heidelberg;

287   2013, p. 413-23.

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	Maternal blood	Cord blood	Foal blood
d-ROMs (U.Carr.)	188.5 ± 62.4^a	142.9 ± 60.0^b	127.4 ± 45.6^b
BAP µmol/l	2064.0 ± 459.7^c	1780.0 ± 479.3^d	1821.0 ± 451^d

290 Table 1. d-ROMs and BAP concentrations in mares, umbilical cord and foals expressed as
291 mean±standard deviation. Within row, different superscripts denote a significant difference (a≠b;
292 c≠d) (p<0.05).