Oxidative stress in donor mares for ovum pick-up delays embryonic development

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Abstract

The *in vitro* production of equine embryos via ovum pick-up (OPU) and intracytoplasmic sperm injection (ICSI) has increased rapidly. There is a marked effect of the individual mare on the outcome of OPU-ICSI, but little is known about the influence of the mare's health condition. This study aimed to investigate the potential associations between the concentrations of interleukin-6 (IL-6), reactive oxygen metabolites (d-ROMs), and biological antioxidant potential (BAP) in serum of oocytes' donor mares and the subsequent embryonic development. Just before OPU, a blood sample was collected from 28 Warmblood donor mares, that were subjected to a routine OPU-ICSI program. The serum concentrations of IL-6, d-ROMs, and BAP were assayed photometrically. The maturation, cleavage and blastocyst rate as well as the kinetics of blastocyst development were recorded. The average blastocyst rate was 24.68±5.16 % and the average concentrations of IL-6, d-ROMs, and BAP were 519.59±157.08 pg/mL, 171.30±4.55 carratelli units (UCARR), and 2711.30±4.55 µmol/L, respectively. Serum concentrations of IL-6, d-ROMs, and BAP were not significantly different between mares yielding at least one blastocyst (552.68±235.18 pg/mL, 168.36±5.56 UCARR, and 2524.80±159.55 µmol/L) and mares yielding no blastocysts (468.47±179.99 pg/mL, 175.85±7.89 UCARR, and 2999.50±300.13 µmol/L, respectively). Serum concentrations of d-ROMs were significantly lower in mares with fast growing (at day 7-8 post ICSI; 148.10±8.13 UCARR) compared to those with slow growing blastocysts (≥ day 9 post ICSI; 179.41±4.89 UCARR; P= 0.003). Taken together, the serum concentration of IL-6, d-ROMs, and BAP do not determine the mare's ability to produce blastocysts *in vitro*. Although it may be questioned whether a single sample is representative of the mare's health status, changes in serum metabolites related to oxidative stress at the time of oocyte retrieval were linked to a delayed blastocyst development in a clinical OPU-ICSI outcome.

Keywords: IL-6; Oxidative stress; d-ROMs; BAP; OPU-ICSI; Mares

1. Introduction

Ovum pick-up (OPU) and intracytoplasmic sperm injection (ICSI) are substantially used to produce equine embryos *in vitro* [1-3]. The OPU-ICSI program is multi-advantageous and is as effective as embryo flushing when measured by the number of day 45 pregnant recipients per mare [4]. Regardless the stage of the ovarian cycle, follicular health and season, OPU-ICSI allows the production of a high number of foals, even from old, subfertile [5], and euthanized mares [6]. The success rate of OPU-ICSI is mainly evaluated by the mare's ability to produce a blastocyst and by the rate of (transferable) blastocysts [2], which are repeatable for an individual mare between two consecutive sessions [7].

There are several known mare related factors that can affect the success rate of OPU-ICSI program. Aged mares (> 20 y) have a relatively low number of ovarian follicles [8]. As such, the number of embryos per OPU session declines in old mares, but the mare's age does not have a significant effect on the developmental competence of the oocytes [7, 9]. A second factor which markedly affects the success rate of OPU-ICSI is the mare's breed [2]. The oocytes of Arabian donor mares show significantly lower cleavage and blastocyst rates compared to those of Warmblood mares [10]. Still, maternal age and breed are constant factors, and they cannot explain short-term fluctuations in the success rates of OPU-ICSI for an individual mare. The relationship between maternal health, oocyte quality, and OPU-ICSI outcome has been scarcely investigated in mares. On the one hand, it has been shown that the physiological status (transitional vs. cycling; [7, 11]) and the presence of reproductive disorders [11] are not significantly affecting the blastocyst rate. On the other hand, mares engaged in intense sporting activities [11] and obese mares [12, 13] display a decreased oocyte developmental competence *in vitro*. Nevertheless, more research is needed to study the impact of the mare's health condition on the success rate of OPU-ICSI.

Female gametes are vulnerable to oxidative stress [14]. Estimation of the systemic oxidative stress index (OSI), measured by derivatives of reactive oxygen metabolites (d-ROMs) and biological

antioxidant potential (BAP) has been well established in women undergoing *in vitro* fertilization [15, 16]. There is a direct association between serum and follicular fluid values of d-ROMs and OSI in women [17], where higher values in serum were accompanied with abnormal fertilization, while increased values in follicular fluid were associated with diminished embryo quality [18]. In horses, we recently showed that the values of OSI in serum and follicular fluid are correlated too [19].

Pro-inflammatory cytokines play a vital role in maintaining the ovarian physiology during folliculogenesis, oocyte maturation and ovulation [20]. There is a strong association between serum and follicular fluid concentrations of IL-6 both in women [21] and mares [19]. Excess IL-6 has been associated with decreased estradiol synthesis and aromatase activity in granulosa cells of women *in vitro* [22]. Higher IL-6 concentrations inhibited the expression of luteinizing hormone receptor mRNA during the maturation and differentiation of cultured rat granulosa cells [23]. Higher follicular fluid IL-6 values in women were associated with decreased clinical pregnancy rate [24]. In mares, higher concentrations of IL-6 within the preovulatory follicle have been correlated with diminished oocyte quality [25].

The relationship between maternal inflammation or oxidative stress, and the OPU-ICSI outcome has not been previously investigated in mares. We hypothesize that there is an association between the serum concentrations of oxidative stress markers (d-ROMs, BAP, and OSI) and the pro-inflammatory cytokine IL-6 at the time of oocytes retrieval (OPU) and the oocyte developmental competence in mares. Therefore, the objective of the present study was to investigate the associations between the serum concentrations of d-ROMs, BAP, OSI, and IL-6 and the OPU-ICSI outcome in mares.

2. Materials and methods

For this study, no specific samples were acquired from or extra procedures were performed with the mares included in this study as analyses were performed during routine clinical OPU-ICSI services. For this reason, no extra ethical clearance was necessary for the present study.

2.1. Animals

Twenty-eight Warmblood mares, with a body condition score ranged between 3 to 6 [26] and aged 2-23 years old were used between mid-January and mid-March 2022. These mares regularly participated in the OPU-ICSI program at the equine reproduction clinic, Faculty of Veterinary Medicine, Ghent University.

2.2. OPU procedures

Just before conducting the OPU and after blood sampling, a preoperative regime [9] of benzylpenicillin (20000 IU/ kg intramuscular; Penikel®, Kela, Sint Niklaas, Belgium) and flunixin meglumin (1.1 mg/ kg intravenous; Wellicox®, Ceva Santé Animale, Naaldwijk, The Netherlands) was used. During the OPU, detomidine hydrochloride (0.01 mg/ kg intravenous; Domidine®, Eurovet Animal Health BV, Bladel, The Netherlands) and butorphanol tartrate (0.01 mg/ kg intravenous; Dolorex®, MSD Animal Health, Sint-Lambrechts-Woluwe, Belgium) were used for sedation. To subside intestinal contractions, N-butylscopolammonium bromide (0.3 mg/ kg intravenous; Buscopan®, Boehringer Ingelheim, Brussel, Belgium) was injected. Urinary bladder catheterization and epidural anesthesia were not applied. After proper aseptic preparation for the perineal region, the transvaginal transducer (7.5 MhZ linear probe, MyLabOne, Esaote, Genoa, Italy; [9]) equipped by a 12-G double-lumen needle attached via a double way tube system to a prewarmed collection bottle of flushing medium (Equiplus®, Mintube, Tiefenbach, Germany). All visible antral follicles were punctured, aspirated, scraped, and flushed 8 times.

2.3. In vitro embryo production

The collection of oocytes [9] was carried out under sterile conditions using a laminar air flow equipped with a stereomicroscope (Olympus SZX7®, Olympus Corp., Japan). The whole contents of the collection bottle (follicular fluid, flushing medium, and scrapped follicular cells) were filtrated through a sterile 70 µm filter (Cell strainer®, BD Biosciences, Falcon, Erembodegem, Belgium) and the COCs were recovered from the filtrated contents in medium 199 with Hank's salts (Gibco, Life

Technologies, Merelbeke, Belgium) supplemented with 10% fetal bovine serum (FBS; Gibco). According to the schedule, the recovered COCs were either directly transferred to maturation medium (medium 199 with Earl's salts (Gibco) containing 10% (v/v) FBS (Gibco), 9.4 µg/mL follicle stimulating hormone, and 1.88 g/ml luteinising hormone (Stimufol, Reprobiol, Ouffet, Belgium)) or were kept overnight in a commercial embryo holding medium (Emcare®, Agtech, Zulte, Belgium) at room temperature (~22 °C) prior to maturation. *In vitro* maturation was carried out in groups of 2-18 COCs in 100-500 µl maturation medium under oil (CooperSurgical, Venlo, The Netherlands) at 38.2 °C in 5% CO_2 containing air for 28-32 h. A small piece of straw with frozen semen was thawed in 1 mL G-MOPS (38.2 °C ; Vitrolife, Londerzeel, Belgium) and centrifuged twice (400 × g/ 3 min at room] temperature; ~22 °C). After the first centrifugation, the supernatant was discarded and the pellet was re-suspended in 1 mL G-MOPs. After the second centrifugation, the supernatant was discarded and the pellet was resuspended in 200 µl G-MOPS. Immediately before ICSI, a small amount of the resuspended sperm was added to a 5 µl droplet of 7% polyvinylpyrrolidone (CooperSurgical, Venlo, The Netherlands). Intracytoplasmic sperm injection, *in vitro* culture of presumptive zygotes, and the evaluation of embryonic development were performed until day 13 post ICSI [9].

2.4. Blood collection and laboratory analyses

A single blood sample per mare was collected from the jugular vein into vacutainer tubes with clot activator (BD Vacutainer®, BD-Plymouth, UK). To separate the serum, samples were centrifuged at 2460 × *g* for 20 min at 4 °C. Serum was aliquoted into sterile 2 ml Eppendorf tubes and stored at –80 °C until further biochemical analysis.

Colorimetric kits (Diacron®; Diacron International, Italy) were used to measure the serum concentrations of d-ROMs and BAP, according to the manufacturer's guidelines [19]. A Multiskan GO spectrophotometer (Thermo Fisher Scientific, Finland; at 37° C) was used to estimate the photometric measurements for both kits at 505 nm. The coefficient of variation was 1.72 % for d-ROMs and 2.32 % for BAP. The lowest limit of detection for d-ROMs and BAP was 11 UCARR and

150 µmol/L, respectively. The OSI was determined from the concentrations of d-ROMs and BAP using the formula d-ROMs/BAP × 100 [27].

Serum concentrations of IL-6 were measured spectrophotometrically using an equine IL-6 ELISA kit (Nori®, Genorise Scientific, USA), according to the manufacturer's guidelines. A Multiskan GO spectrophotometer (Thermo Fisher Scientific, Finland; room temperature) was used to determine the optical density at 450 and 540nm, which was followed by a wavelength correction. The average coefficient of variation was 6.49 % and the lowest detection limit was 16 pg/mL.

2.5. Study design

Blood sampling was performed just before OPU. Immediately after OPU, the cumulus-oocyte complexes (COCs) were recovered, matured, and fertilized by ICSI.

At each OPU-ICSI session, (a) the number of aspirated follicles, (b) the number of recovered oocytes, (c) the recovery rate (b/a ×100), (d) the number of mature oocytes, (e) the maturation rate (d/b ×100), (f) the number of cleaved presumptive zygotes, (g) the cleavage rate (f/d ×100), (h) the number of produced blastocysts, (i) the blastocyst rate (h/d ×100), (j) the proportion of the cleaved zygotes that developed to blastocysts (h/f×100), (k) the time of blastocyst formation, and (l) the serum concentrations of d-ROMs, BAP, OSI, and IL-6 were recorded. The mares were divided into different groups according to (1) their ability to produce blastocysts: blastocyst producing (≥1 blastocyst; *n*=17) and non-producing (0 blastocyst; *n*=11) mares, (2) the required time for embryonic development: mares with fast growing (first blastocyst developed at day 7-8 post ICSI; *n*=6) and mares with slow growing (first blastocyst developed at day ≥ 9 post ICSI; *n*=11) embryos, and (3) age: young (≤ 14 y), middle-aged (15-19 y), and old (≥ 20 y) mares [28].

2.6. Statistical analyses

The assessment of the normality of data was performed using a Shapiro-Wilk test. The mean values of d-ROMs, BAP, and OSI, but not IL-6, were normally distributed. For the blastocyst producing

mares, Spearman's correlation coefficients between the blastocyst rate, the proportion of cleaved zygotes that developed to blastocysts, and the serum concentrations of d-ROMs, BAP, OSI, and IL-6 were calculated. Differences in serum parameters between groups based on the mare's ability to produce embryos (blastocyst producing vs. non-producing mares) and the onset of embryonic development (fast vs. slow growing blastocysts) were determined using the independent t-test or Mann-Whitney U-test. Differences between groups based on maternal age were explored using Welch one-way ANOVA followed by Games-Howell or Kruskal-Wallis test. The data were analyzed using the statistical package for social science SPSS® (SPSS Inc., version 16.0, Chicago, IL. USA), and a *P*-value <0.05 was considered significant. Data are presented as mean ±SEM.

3. Results

The mean ±SEM values of all the studied parameters are presented in Table 1. There were no significant correlations between the blastocyst rate, the proportion of cleaved zygotes that produced blastocysts, and the serum concentrations of d-ROMs, BAP, OSI, and IL-6.

As shown in Table 2, serum concentrations of BAP were significantly higher in old mares (3544.60±218.07 µmol/L) compared to the young ones (2461.40±133.56 µmol/L). Values of OSI were significantly increased in young mares (7.41±0.52) compared to old ones (4.95±0.24). Serum concentrations of d-ROMs and IL-6 were not significantly different between young, middle-aged, and old mares.

Serum concentrations of d-ROMs, BAP, OSI, and IL-6 were not significantly different between the blastocyst producing and non-producing mares (Table 3). Serum concentrations of d-ROMs (Table 4) were significantly (*P*= 0.003) higher in mares with slow growing blastocysts (179.41±4.89 UCARR) compared to those with fast growing ones (148.10±8.13 UCARR).

4. Discussion

In this study, we found an association between the serum concentrations of oxidative stress markers (d-ROMs, BAP, and OSI) and the pro-inflammatory cytokine IL-6 at the time of OPU and kinetics of embryo development. More specifically, high concentrations of d-ROMs at the time of OPU are linked to delayed embryonic development in mares. This may point out that a disturbance in maternal health related to oxidative stress can affect the OPU-ICSI outcome in mares.

Overall, the OPU-ICSI results in this study were consistent with those reported previously [2, 9]. Serum concentrations of d-ROMs, BAP, and IL-6 were within the previously reported range in Warmblood mares during the non-breeding season [19]. In agreement with literature, ageing had no effect on the serum concentrations of d-ROMs in horses [29]. In our study, the serum concentrations of BAP were significantly higher and the values of OSI were significantly lower in old mares compared to young ones. In humans, the total antioxidant capacity in serum also increases with advancing age, which may be related to diet and daily routine [30]. The effect of age on the serum antioxidant status in horses is not clear. Some studies did not report any significant effect of ageing on the serum total antioxidant status [31] and BAP [29] in horses. On the other hand, Andriichuk et al. [32] found that physical exercise increases the plasma concentrations of thiobarbituric acid reactive substrates, catalase, and glutathione reductase in Warmblood horses. While our study indicates an effect of ageing on oxidative stress, further influence of diet and physical activity remains to be determined.

Although oxidative stress markers in serum did not determine the mare's ability to produce embryos, mares with slow growing blastocysts showed significantly higher serum concentrations of d-ROMs. In a previous study, we found that serum and follicular fluid values of OSI (d-ROMs/BAP × 100) are directly correlated in Warmblood mares [19]. Speed of embryo development affects both pregnancy and foaling rates in mares. Ducheyne et al. [33] found that fast growing embryos (formed before day 9 post ICSI) yield significantly higher pregnancy rates compared to the slow growing ones

(formed after day 9 post ICSI). Foaling rate was significantly higher for day 7 and day 8 embryos (71.7% and 53.3%) compared to day 9 and day 10 embryos (38.5% and 25%; [34]). In mammals, the oocyte developmental competence is linked to maternal health [35]. Maternal oxidative stress is increasing the concentrations of reactive oxygen species (ROS) in oocytes, which reduces their viability [36, 37]. Oocytes with higher levels of ROS show delayed two-cell, four-cell, and blastocyst development in mice [36, 38]. Oxidative stress in serum and follicular fluid significantly decreases the clinical pregnancy rate in women [15, 39]. Higher values of d-ROMs in follicular fluid of women have been associated with abnormal fertilization and production of bad quality embryos [18]. Here, we hypothesize that the higher serum concentrations of d-ROMs may be reflected by increased d-ROMs in the follicular fluid, which may affect oocytes quality, resulting in delayed embryo development. More studies are necessary to further explore the effect of oxidative stress and antioxidants on the oocyte developmental competence in horses.

In this study, serum concentrations of IL-6 neither affected the mare's ability to produce embryos nor the blastocyst rate. There is a positive association between serum and follicular fluid concentrations of IL-6 in mares [19, 25]. In the follicle, IL-6 is responsible for extracellular matrix formation and stabilization, which regulates cumulus cells expansion and increases the oocyte competence [40]. Several studies have been conducted, but there is no conclusive answer regarding the role of IL-6 in oocytes and subsequent embryos. Higher concentrations of IL-6 in FF can either increase [41] or decrease [24] the pregnancy outcome in women. Supplementation of culture media with IL-6 improved fetal development of IVF-embryos in cows [42] and supported embryonic compaction, blastulation and hatching in mice [43]. The expression of IL-6 and IL-6 signal transducer genes in granulosa cells was upregulated with advancing maternal age in mares [28]. In women with infertility, there was a downregulation in the expression of IL-6 signal transducer and IL-6 receptor genes in granulosa cells of older patients [44]. However, maternal age did not affect the concentrations of IL-6 in mares' serum

(this study) and women's FF [45]. Therefore, it seems that the role of IL-6 in oocytes and embryos is species-specific and dose-dependent.

5. Conclusions

In conclusion, mares with higher serum concentrations of d-ROMs at the time of oocyte retrieval (OPU) show a delayed embryonic development. More studies should be conducted to investigate underlying mechanisms and potential therapy by antioxidants supplementation during the *in vitro* maturation of the oocytes collected from mares with oxidative stress. The measured ranges of d-ROMs, BAP, and IL-6 concentrations in serum at the time of OPU cannot be used to predict the mare's ability to produce embryos *in vitro*.

CRediT authorship contribution statement

Conceptualization: MH, KS, JG; Animals work: JG, MP, SP, IG, EVB; Lab work: MH, DAV, TDC, KS; Data curation: MH, DAV; Original draft writing: MH; Editing, Review, and Supervision: JL, KS, AVS; Providing fund: JL, JG, KS.

Conflict of interest

The authors declare no competing interests.

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References

[1] Galli C, Duchi R, Colleoni S, Lagutina I, Lazzari G. Ovum pick up, intracytoplasmic sperm injection and somatic cell nuclear transfer in cattle, buffalo and horses: from the research laboratory to clinical practice. Theriogenology 2014; 81(1): 138-51. https://doi.org/10.1016/j.theriogenology.2013.09.008

[2] Lazzari G, Colleoni S, Crotti G, Turini P, Fiorini G, Barandalla M, et al. Laboratory production of equine embryos. J Equine Vet Sci 2020; 89: 103097. https://doi.org/10.1016/j.jevs.2020.103097

[3] Stout TAE. Clinical application of in vitro embryo production in the horse. J Equine Vet Sci 2020; 89: 103011. https://doi.org/10.1016/j.jevs.2020.103011

[4] Cuervo-Arango J, Claes AN, Stout TA. A retrospective comparison of the efficiency of different assisted reproductive techniques in the horse, emphasizing the impact of maternal age. Theriogenology 2019a; 132: 36-44. https://doi.org/10.1016/j.theriogenology.2019.04.010

[5] Claes A, Stout TAE. Success rate in a clinical equine in vitro embryo production program. Theriogenology 2022; 187: 215-18. https://doi.org/10.1016/j.theriogenology.2022.04.019

[6] Hinrichs K, Choi YH, Norris JD, Love LB, Bedford-Guaus SJ, Hartman DL, et al. Evaluation of foal production following intracytoplasmic sperm injection and blastocyst culture of oocytes from ovaries collected immediately before euthanasia or after death of mares under field conditions. J Am Vet Med Assoc 2012; 241(8): 1070-4. https://doi.org/10.2460/javma.241.8.1070

[7] Cuervo-Arango J, Claes AN, Stout TA. Mare and stallion effects on blastocyst production in a commercial equine ovum pick-up–intracytoplasmic sperm injection program. Reprod Fertil Dev 2019b; 31(12): 1894-1903. https://doi.org/10.1071/rd19201

265 [8] Claes A, Ball BA, Scoggin KE, Roser JF, Woodward EM, Davolli GM, et al. The influence of
266 age, antral follicle count and diestrous ovulations on estrous cycle characteristics of
267 mares. Theriogenology 2017; 97: 34-40. https://doi.org/10.1016/j.theriogenology.2017.04.019

268 [9] Papas M, Govaere J, Peere S, Gerits I, Van de Velde M, Angel-Velez D, et al. Anti-Müllerian
269 hormone and OPU-ICSI outcome in the mare. animals 2021; 11(7): 2004.
270 https://doi.org/10.3390/ani11072004

271 [10] Galli C, Colleoni S, Duchi R, Lazzari G. Male factors affecting the success of equine in vitro
272 embryo production by ovum pickup-intracytoplasmic sperm injection in a clinical setting. J Equine
273 Vet Sci 2016; 43: S6-S10. https://doi.org/10.1016/j.jevs.2016.05.014

274 [11] Colleoni S, Barbacini S, Necchi D, Duchi R, Lazzari G, Galli C. Application of ovum pick-up,
275 intracytoplasmic sperm injection and embryo culture in equine practice. In: Proceedings of the 53rd
276 Annual Convention of the American Association of Equine Practioners. 2007; 554-9.
277 https://www.ivis.org/library/aaep/aaep-annual-convention-orlando-2007/application-of-ovum-pick-
278 up-intracytoplasmic-sperm-injection-and-embryo-culture-equine-practice

279 [12] Sessions-Bresnahan DR, Schauer KL, Heuberger AL, Carnevale EM. Effect of obesity on the
280 preovulatory follicle and lipid fingerprint of equine oocytes. Biol Reprod 2016; 94(1): 1-12.
281 https://doi.org/10.1095/biolreprod.115.130187

282 [13] Sessions-Bresnahan DR, Heuberger AL, Carnevale EM. Obesity in mares promotes uterine
283 inflammation and alters embryo lipid fingerprints and homeostasis. Biol Reprod 2018; 99(4): 761-72.
284 https://doi.org/10.1093/biolre/ioy107

285 [14] Aitken RJ. Impact of oxidative stress on male and female germ cells: implications for
286 fertility. Reproduction 2020; 159(4): R189-R201. https://doi.org/10.1530/REP-19-0452

[15] Di Rosa A, Albani E, Morenghi E, Iommiello VM, Levi Setti PE. A new method to assess oxidative stress in ART cycles. Gynecol Endocrinol 2016; 32(3): 210-2. https://doi.org/10.3109/09513590.2015.1110134

[16] Luti S, Fiaschi T, Magherini F, Modesti PA, Piomboni P, Semplici B.et al. Follicular microenvironment: Oxidative stress and adiponectin correlated with steroids hormones in women undergoing in vitro fertilization. Mol Reprod Dev 2021; 88(2): 175-84. https://doi.org/10.1002/mrd.23447

[17] Nakazawa R, Matsuyama T, Shiota A. Noninvasive embryo evaluation method combining time-lapse images with biomarkers in follicular fluid and serum. Jpn IVF Soc J 2021; 1: 10-8. http://doi.org/10.50850/00000334

[18] Terao H, Wada-Hiraike O, Nagumo A, Kunitomi C, Azhary JM, Harada M, et al. Role of oxidative stress in follicular fluid on embryos of patients undergoing assisted reproductive technology treatment. J Obstet Gynaecol Res 2019; 45(9): 1884-91. https://doi.org/10.1111/jog.14040

[19] Hedia M, Leroy LMRJ, Govaere J, Van Soom A, Smits K. Lipid metabolites, interleukin-6 and oxidative stress markers in follicular fluid and their association with serum concentrations in mares. Vet Res Commun 2023. https://doi.org/10.1007/s11259-023-10122-0

[20] Field SL, Dasgupta T, Cummings M, Orsi NM. Cytokines in ovarian folliculogenesis, oocyte maturation and luteinisation. Mol Reprod Dev 2014; 81(4): 284-314. https://doi.org/10.1002/mrd.22285

[21] Chen CD, Chen HF, Lu HF, Chen SU, Ho HN, Yang YS. Value of serum and follicular fluid cytokine profile in the prediction of moderate to severe ovarian hyperstimulation syndrome. Hum Reprod 2000; 15(5): 1037-42. https://doi.org/10.1093/humrep/15.5.1037

[22] Deura I, Harada T, Taniguchi F, Iwabe T, Izawa M, Terakawa N. Reduction of estrogen production by interleukin-6 in a human granulosa tumor cell line may have implications for endometriosis-associated infertility. Fertil Steril 2005; 83(4): 1086-92. https://doi.org/10.1016/j.fertnstert.2004.12.014

[23] Tamura K, Kawaguchi T, Kogo H. Interleukin-6 inhibits the expression of luteinizing hormone receptor mRNA during the maturation of cultured rat granulosa cells. J Endocrinol 2001; 170(1): 121-7. https://doi.org/10.1677/joe.0.1700121

[24] Altun T, Jindal S, Greenseid K, Shu J, Pal L. Low follicular fluid IL-6 levels in IVF patients are associated with increased likelihood of clinical pregnancy. J Assist Reprod Genet 2011; 28(3): 245-51. https://doi.org/10.1007/s10815-010-9502-8

[25] Sessions-Bresnahan DR, Carnevale EM. The effect of equine metabolic syndrome on the ovarian follicular environment. J Anim Sci 2014; 92(4): 1485-94. https://doi.org/10.2527/jas.2013-7275

[26] Henneke DR, Potter GD, Kreider JL, Yeates BF. Relationship between condition score, physical measurements and body fat percentage in mares. Equine Vet J 1983; 15(4): 371-2. https://doi.org/10.1111/j.2042-3306.1983.tb01826.x

[27] Po E, Williams C, Muscatello G, Celi P. Assessment of oxidative stress biomarkers in exhaled breath condensate and blood of Thoroughbred foals. Vet J 2013; 196(2): 269-71. https://doi.org/10.1016/j.tvjl.2012.08.018

[28] Sessions-Bresnahan DR, Carnevale EM. Age-associated changes in granulosa cell transcript abundance in equine preovulatory follicles. Reprod Fertil Dev 2015; 27(6): 906-13. https://doi.org/10.1071/RD14467

[29] Mochizuki M, Minowa F, Ishimoto C, Gin A, Ishioka K, Okubo K. The effect of aging on biochemical markers in equine serum. J Equine Vet Sci 2016; 42: 1-6. https://doi.org/10.1016/j.jevs.2016.03.011

[30] Limberaki E, Eleftheriou P, Vagdatli E, Kostoglou V, Petrou C. Serum antioxidant status among young, middle-aged and elderly people before and after antioxidant rich diet. Hippokratia 2012; 16(2): 118-23. https://pubmed.ncbi.nlm.nih.gov/23935266

[31] Gorecka R, Sitarska E, Kluciński W. Antioxidant parameters of horses according to age, sex, breed and environment. Polish J Vet Sci 2002; 5(4): 209-16. https://pubmed.ncbi.nlm.nih.gov/12512552/

[32] Andriichuk A, Tkachenko H, Tkachova I. Oxidative stress biomarkers and erythrocytes hemolysis in well-trained equine athletes before and after exercise. J Equine Vet Sci 2016; 36: 32-43. https://doi.org/10.1016/j.jevs.2015.09.011

[33] Ducheyne KD, Rizzo M, Cuervo-Arango J, Claes A, Daels PF, Stout TA, et al. In vitro production of horse embryos predisposes to micronucleus formation, whereas time to blastocyst formation affects likelihood of pregnancy. Reprod Fertil Dev 2019; 31(12): 1830-9. https://doi.org/10.1071/rd19227

[34] Lewis N, Canesin H, Choi YH, Foss R, Felix M, Rader K, et al. Equine in vitro produced blastocysts: relationship of embryo morphology, stage and speed of development to foaling rate. Reprod Fertil Dev 2023; 35(4): 338-51. https://doi.org/10.1071/rd22224

[35] Deluao JC, Winstanley Y, Robker RL, Pacella-Ince L, Gonzalez MB, McPherson NO. Oxidative stress and reproductive function: Reactive oxygen species in the mammalian pre-implantation embryo. Reproduction 2022; 164(6): F95-108. https://doi.org/10.1530/REP-22-0121

[36] Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchen MR, et al. Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. PloS one 2010; 5(4): e10074. https://doi.org/10.1371/journal.pone.0010074

[37] Wu LL, Russell DL, Wong SL, Chen M, Tsai TS, St John JC, et al. Mitochondrial dysfunction in oocytes of obese mothers: transmission to offspring and reversal by pharmacological endoplasmic reticulum stress inhibitors. Development 2015; 142(4): 681-91. https://doi.org/10.1242/dev.114850

[38] Han L, Wang H, Li L, Li X, Ge J, Reiter RJ, et al. Melatonin protects against maternal obesity-associated oxidative stress and meiotic defects in oocytes via the SIRT 3-SOD 2-dependent pathway. J Pineal Res 2017; 63(3): e12431. https://doi.org/10.1111/jpi.12431

[39] Bedaiwy MA, Elnashar SA, Goldberg JM, Sharma R, Mascha EJ, Arrigain S, et al. Effect of follicular fluid oxidative stress parameters on intracytoplasmic sperm injection outcome. Gynecol Endocrinol 2012; 28(1): 51-5. https://doi.org/10.3109/09513590.2011.579652

[40] Liu Z, de Matos DG, Fan HY, Shimada M, Palmer S, Richards JS. Interleukin-6: an autocrine regulator of the mouse cumulus cell-oocyte complex expansion process. Endocrinology 2009; 150(7): 3360-8. https://doi.org/10.1210/en.2008-1532

[41] Gavrilovic AZ, Cekovic JM, Parandilovic AZ, Nikolov AB, Sazdanovic PS, Velickovic AM, et al. IL-6 of follicular fluid and outcome of in vitro fertilization. Medicine 2022; 101(29): e29624. https://doi.org/10.1097%2FMD.0000000000029624

[42] Seekford ZK, Wooldridge LK, Dias NW, Timlin CL, Sales ÁF, Speckhart SL,et al. Interleukin-6 supplementation improves post-transfer embryonic and fetal development of in vitro-produced bovine embryos. Theriogenology 2021; 170: 15-22. https://doi.org/10.1016/j.theriogenology.2021.04.004

376 [43] Desai N, Scarrow M, Lawson J, Kinzer D, Goldfarb J. Evaluation of the effect of interleukin-6
377 and human extracellular matrix on embryonic development. Hum Reprod 1999; 14(6): 1588-92.
378 https://doi.org/10.1093/humrep/14.6.1588

379 [44] Hurwitz JM, Jindal S, Greenseid K, Berger D, Brooks A, Santoro N, et al. Reproductive aging is
380 associated with altered gene expression in human luteinized granulosa cells. Reprod Sci 2010; 17:
381 56-67. https://doi.org/10.1177/1933719109348028

382 [45] Piccinni MP, Vicenti R, Logiodice F, Fabbri R, Kullolli O, Pallecchi M, et al. Description of the
383 follicular fluid cytokine and hormone profiles in human physiological natural cycles. J Clin
384 Endocrinol Metab 2021; 106(2): e721-38. https://doi.org/10.1210/clinem/dgaa880