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Original article

Antioxidant supplementation to medium for *in vitro* embryo production in *Felis catus*

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Abstract

The development of *in vitro* embryo production (IVEP) techniques in *Felis catus* is a fitting model with potential application to the conservation of endangered felid species. To improve the quality of IVEP techniques an appropriate balance of pro- and antioxidants should be provided. Under *in vitro* conditions, high levels of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) mRNA provide a defence mechanism against oxidative stress for embryos. In order to improve the development of cat oocytes, the effects of SOD and CAT supplemented to *in vitro* maturation (IVM) medium and of GPx supplemented to *in vitro* fertilization (IVF) medium on development and embryo production *in vitro* were evaluated. Data showed an increase of 70 and 77 % of cleaved embryo and blastocyst formation, respectively, in the experiment with SOD and CAT addition to IVM medium; in the experiment with GPx addition to IVF medium the number of cleaved embryos doubled and the number of embryos increased by 96 %. Therefore, our results were positive and encourage us to continue studies on cat oocytes evaluating the effects of various dosages and combination of antioxidants.

Key words: assisted reproduction technology, superoxide dismutase, glutathione peroxidase, catalase, domestic cat

Introduction

In vitro embryo production (IVEP) is an emerging and cutting-edge assisted reproductive technology (ART) that can potentially be applied for the conservation of threatened species in danger of extinction. *Felis catus* represents a practical model for the development of IVEP technology in feline species. Accordingly, the potentially beneficial effects generated by the use of pro- and antioxidants in IVEP are of utmost relevance (Cocchia et al. 2010a,b).

Oxidative stress (OS) in spermatozoa strongly affects the success of *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) as well as artificial insemination (AI) (Agarwal et al. 2006). A fine balance between pro- and antioxidant species is required for maintaining spermatozoa functions both in male and female genital tracts. Higher concentrations of spermatozoa are used for IVF than in natural conditions; therefore, the balance between pro- and antioxidant factors requires further evaluation. The determination of pro- and antioxidants in follicular and epididymal

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fluids is helpful to better understand the modification *in vivo* and to optimize *in vitro* maturation (IVM) environment for oocytes (Noblac et al. 2011). Under *in vitro* conditions, the embryo is exposed to potentially damaging of internal and external sources of reactive oxygen species (ROS). Because of the nature of standard *in vitro* culture conditions, the risk of exposure to ROS and OS is much higher than that under *in vivo* conditions (Cocchia et al. 2015a).

During cell metabolism ROS are customary synthesized; normal ROS production is necessary for maintenance of body functions, while excessive production is harmful (Tafuri et al. 2015). The resulting free radicals produce structural and functional alterations by molecular oxidation (Ciani et al. 2018). ROS are counteracted by a complex defense system of enzymatic and non-enzymatic antioxidants (Costantino et al. 2009, Ciani et al. 2015, Esposito et al. 2017). Among the enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are the most important ones of the endogenous antioxidant barrier. They are capable to inactivate the excess concentration of radicals like superoxide anion first, and hydroperoxide after. Enzymatic antioxidants were found in male and female genital tract secretions, including SOD, CAT and GPx (Ciani et al. 2015, Tafuri et al. 2015). SOD, present naturally in seminal plasma, catalyzes the breakdown of the superoxide anion into hydrogen peroxide (Cocchia et al. 2011, Ciani et al. 2015). SOD has been reported to increase the proportion of zygotes that undergo the first cleavage division while improving cleavage past the two-cell stage and blastocyst development (Ochota et al. 2016). Elevated levels of SOD may provide protection from gynecologic diseases, such as pre-eclampsia or diabetes-induced embryopathy (Agarwal and Allamaneni 2004). CAT is another enzymatic antioxidant present in seminal plasma, in the corpus luteum and in oviduct fluid that can neutralize hydrogen peroxide by conversion into oxygen and water (Chi et al. 2008). GPx is present in the mammalian epididymis and in semen and catalyzes the reduction of hydrogen peroxide and organic peroxides in water and alcohol while oxidizing glutathione (Galecka et al. 2008, Pipolo et al. 2018). GPx is also located within the glandular epithelium of the uterine endometrium and in the cumulus cells of females and in the sperm mitochondrial matrix and in seminal plasma, so it can come from the prostate (Formigari et al. 2007, Tafuri et al. 2015).

The *in vitro* model of the ART does not exactly reflect the *in vivo* conditions of oviducts, but has a greater concentration of radicals that must be counteracted in order to avoid their deleterious action on biological macromolecules and therefore the embryo production and its survival. The aim of our research, therefore, was to test the effects of supplementation of the oocyte IVM medium with SOD and CAT and the IVF medium with GPx on *in vitro* developmental competence and embryo production rate in *Felis catus*.

Materials and Methods

Chemicals and experimental design

Chemical reagents were acquired from Sigma-Aldrich (Milan, Italy) unless specified different. For the present study ovaries at various phases of the estrous cycle from domestic cats (*Felis catus*, 1-8 years old) were obtained after ovariohysterectomies carried out in the Veterinary Hospital (OVUD), following the procedures according to the Ethical Committee of our University. To determine the efficacy of antioxidant supplementation in IVM and IVF medium experiments were performed.

To assess the frequency of maturation of oocytes and blastocyst formation, SOD and CAT were added to IVM medium (metaphase II = MII and polar body formation) and the aceto-orcein staining was carried out to estimate the maturation phase of the oocytes. Oocytes were stained in a drop of lacto-aceto-orcein dye for 15 min, and washed in 45 % acetic acid. The stained oocytes were covered with a cover glass to get squash preparations by tapping on the cover glass. The squash preparation were examined under light microscope.

To evaluate the frequency of the maturation of oocytes, cumulus-oocyte complexes (COCs; n=315) were collected and splitted randomly into the experimental (n=156) or control (n=159) groups. COCs were cultured as previously described (Pope 2014, Cocchia et al. 2015b). Briefly, IVM was performed using maturation medium (SOFaaBSA) with or without SOD (25 IU/mL) and CAT (50 IU/mL) for 24 h at 38.5°C in 5% CO_2. Then, to determine the chromatin status, COCs were stained with aceto-orcein; only oocytes with an evident metaphase and polar body were classed like meiotically mature (MII).

To evaluate the blastocyst formation, COCs (n = 489) were collected from fresh excised ovaries. The COCs were splitted randomly into experimental (n=243) and control (n=246) groups and grown in maturation medium (SOFaaBSA) supplemented with or without SOD (25 IU/mL) and CAT (50 IU/mL). Then, oocytes were fertilized *in vitro* in SOFaaBSA in CO_2 (5%) for 18 h at 38.5°C with fresh epididymal spermatozoa. Following IVF, zygotes were cultured in SOFaaBSA with different molecules in CO_2 (5%), N_2 (5%) and O_2 (90%) for 8 days at 38.5°C.

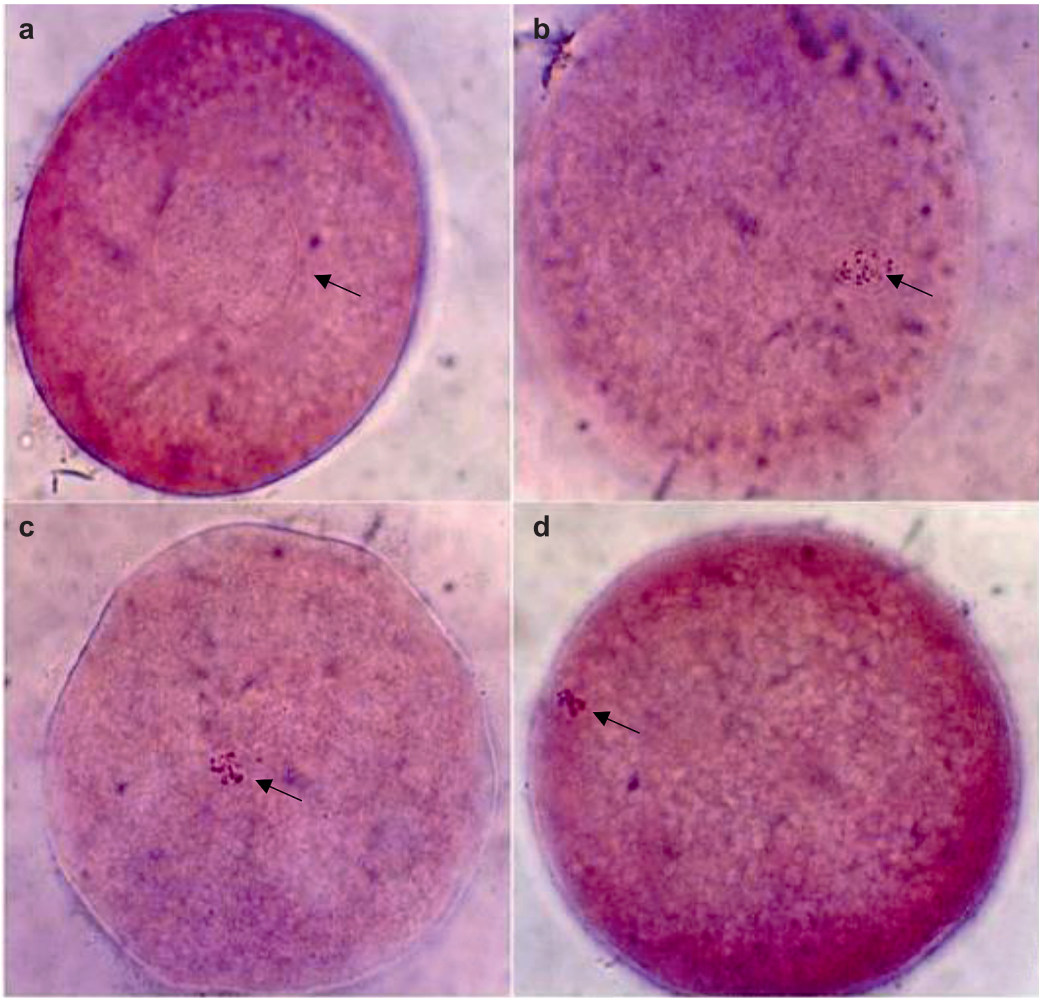

Fig. 1. Oocytes stained with aceto-orcein to visualize the nuclear structures (400X magnification). Nuclear morphology was classified as germinal vesicle intact (GV; a), germinal vesicle breakdown (GVBD; b), prometaphase I (PM; c), metaphase (d).

In order to evaluate the efficacy of GPx supplementation in IVF medium, COCs (n=576) were harvested from fresh ovaries and grown for 24 h in maturation medium (SOFaaBSA). They were then splitted into experimental (COCs n=303) and control (COCs n=273) groups and fertilized *in vitro* in SOFaaBSA medium with or without GPx (25 IU/mL) with epididymal fresh spermatozoa. After 18 h, the zygotes were grown with different molecules in SOFaaBSA. The percentage of oocytes that underwent cleavage/total number of oocytes that underwent IVF x 100 represents the value of cleavage rate. For comparative purposes, from both total number of IVF COCs and cleaved embryos, the blastocyst rate on day 8 was calculated. *In vitro* development competence was determined as the number of blastocysts produced compared to the total number of embryos.

Oocyte collection and COCs IVM

The experimental procedure was described by Cocchia (2010a). Briefly, the ovaries were stored in Dulbecco's PBS with kanamycin (75 mg/mL) until oocyte harvesting at room temperature (RT). Within 3 h of excision, ovaries were chopped with a scalpel in a Petri dish with HEPES synthetic oviductal fluid (HSOF). Oocytes (grade I and II) were selected, washed in HSOF and splitted into the experimental groups (Luvoni 2006).

COCs (25-50 oocytes/mL) were cultured in SOFaaBSA supplemented with amino acids and BSA (6 mg/mL) with porcine follicle-stimulating and luteinizing hormones (0.1 IU; pFSH-LH; Pluset, Laboratorios Calier, Barcelona, Spain); EGF (25 ng/mL); insulin-transferrin-sodium selenite (25 µL/mL) and L-cysteine (1.2 mmol/L) in CO_2 (5%) at 38.5°C. The oocytes were evaluated with a stereomicroscope after 24 h to assess the viability to remove those with cytoplasmic degeneration.

Evaluation of the nuclear stage of maturation

After IVM oocytes, denuded in hyaluronidase (0.2% w/v) and placed in KCl solution (0.7% w/v)

Table 1. Evaluation of the effect of SOD and CAT supplementation to *in vitro* maturation (IVM) medium on oocytes maturation.

	N. COCs IVM (mean ± SD)	MII (mean ± SD) (%)
Control group	16.24±3.42	13.68 ± 2.13 (81)
Experimental group	18.27±1.43	13.76 ±3.54 (79)

COCs: cumulus-oocyte complexes

Table 2. Evaluation of the efficacy of SOD and CAT supplementation to *in vitro* maturation (IVM) medium on embryo cleavage and blastocyst formation.

	N. COCs IVC (mean ± SD)	Cleaved embryo (mean ± SD) (%)	N. Blastocyst (mean ± SD) (%)
Control group	18.15±2.11	6.34± 2.68 (33) *A	3.18 ±0.65 (16) *A
Experimental group	16.88±3.26	11.25±3.08 (65) *B	5.41±1.04 (32) *B

* $p<0.05$ A *vs* B columns
COCs: cumulus-oocyte complexes;
IVC: *in vitro* culture

for 3-5 min at RT, were placed on a microslide and fixed overnight in fixative solution (acetic acid/ethanol, 1/3, w/v) (Hewitt et al. 1998). Then, nuclear structures were visualized with aceto-orcein (2% orcein, 45% acetic acid) staining and using phase contrast microscopy (400X). Nuclear morphology was assorted as follows: germinal vesicle intact (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), MII and undetermined nuclear status.

Sperm collection, IVF and IVC

The experimental procedure was described by Cocchia (2015b). Briefly, matured COCs were subjected to IVF with fresh spermatozoa collected from epididymis of domestic cats after orchiectomy at the OVUD. The testicular-epididymal tissue, within 3 h of removal, was placed in Dulbecco's PBS and carried to our laboratory. The caudal portion of each epididymis was minced with a scalpel to release the spermatozoa. Following incubation, the epididymal tissue was eliminated, the medium collected and centrifuged. The pellet concentration was assessed by phase-contrast microscopy and resuspended in fresh IVF medium to a concentration of 10 x 10^6 sperm/mL. After incubation, motile sperms were chosen, the supernatant was collected and supplemented with penicillamine-hypotaurine--epinephrine (20 mg/mL) and heparin (10 mg/mL).

For IVF, COCs (30-40) were placed in IVF medium and cultured with sperm, then cumulus cells were removed. The denuded oocytes were observed and degenerated ones were discarded. The zygotes were grown in SOFaaBSA (16 mg/mL); cleavage was established after 24 h of *in vitro* culture (IVC), and embryos were cultured in medium with fetal bovine serum (10%) until day 8, every 4 days the medium was renovated (Ciani et al. 2008).

Statistical analysis

All data, expressed as mean ± standard deviation (SD), were analyzed with JMP 8.0.2, SAS Institute Inc., USA. Normality was tested with a Shapiro-Wilk's W test. As not all data were normally distributed, non-parametrical tests were used. The Kruskal-Wallis one-way analysis of variance by rank was applied to compare developmental data in experimental and control groups. Statistical significance was fixed at $p\leq0.05$.

Results

Evaluation of the effect of SOD and CAT supplementation in IVM medium on oocytes maturation

From each domestic cat 20-30 COCs were collected, of which only those identified as grade I and II COCs were tested. The efficacy of antioxidants supplementation in IVM medium was assessed by aceto-orcein staining (Fig. 1). To visualize the nuclear structures aceto-orcein staining was used. Nuclear morphology was identified as follows: germinal vesicle intact (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), MII and undetermined nuclear status.

The results of the effect of SOD and CAT supplementation to IVM medium in each group are shown in Table 1. SOD and CAT in IVM medium were not effective in improving oocyte maturation, in fact maturation rate did not show any significant difference between control and experimental groups (Table 1).

Table 3. Evaluation of the efficacy of GPx supplementation to *in vitro* fertilization (IVF) medium on embryo cleavage and blastocyst formation.

	N. COCs IVC (mean ± SD)	Cleaved embryo (mean ± SD) (%)	N. Blastocyst (mean ± SD) (%)
Control group	17.65 ± 4.28	5.27 ± 1.48 (29) *A	2.36 ± 0.92 (11) *A
Experimental group	18.14 ± 2.65	10.63 ± 2.15 (61) *B	4.63 ± 0.80 (28) *B

* $p<0.05$ A *vs* B columns
COCs: cumulus-oocyte complexes;
IVC: *in vitro* culture

Evaluation of the efficacy of SOD and CAT supplementation in IVM medium on embryo cleavage and blastocyst formation

From each domestic cat 20-30 COCs were collected, of which only those classified as grade I and II were tested. The results of SOD and CAT supplementation on IVM medium in each group are reported in Table 2. The frequencies of cleavage and blastocyst development were greater in experimental group in respect to the control ($p<0.05$).

Evaluation of the efficacy of GPx supplementation in IVF medium on embryo cleavage and blastocyst formation

From each domestic cat 20-35 COCs were collected, of which only those classified as grade I and II COCs were tested. In this set of experiments developmental competence, as number of blastocysts producted and embryos, was evaluated. The efficacy of GPx supplementation to IVF medium in each group are shown in Table 3. Data showed that the frequency of cleavage was significantly increased in GPx supplementation group with respect to control group ($p<0.05$). Moreover, blastocyst formation was higher in experimental group compared to the control group ($p<0.05$).

Discussion

The effects of SOD and CAT supplementation to the IVM medium on oocyte maturation, cleavage and blastocyst production were assessed in our study; also the effects of addition to the IVF medium of GPx were studied by assessing the incidence of cleavage and blastocyst development. Overall, our data demonstrated a beneficial effect of addition of antioxidants to IVM and to IVF media on blastocyst growth of cat oocytes.

Endogenous and exogenous factors influence the ART outcome (Agarwal et al. 2006), one of which is OS that occurs when there is a discrepancy between ROS and antioxidant ability. ROS are involved in several physiological mechanisms including many reproductive processes (oocyte maturation, fertilization promotion, embryo development and gestation). After fertilization, embryos can produce ROS (Guerin et al. 2001, Pero et al. 2017). Goto (1993) demonstrated that ROS production was higher in IVC in respect to *in vivo* mouse embryos. In the clinical ART laboratory, the risk of embryonic environmental exposure to ROS and OS is much greater than *in vivo* (Gough et al. 2011, Li et al. 2019). Moreover, internal stresses further increase the load of ROS that require counter-measures for control. Many studies report that OS is responsible to accumulation of harmful metabolites that lead to follicular atresia in mammals (Devine et al. 2012). A physiological ROS amount in follicular fluid indicates a healthy developing oocyte. Conversely, a ROS increase leads to the modification of biomacromolecules damaging cells and consequently occurrence of OS. During the culture and development phases *in vitro*, gametes and embryos are subjected to a high amount of ROS due to deficiency of enzymatic antioxidants naturally occurring in pregnancy (Zhang et al. 2006, Takahashi 2012).

In our study, antioxidant enzymes were added to culture medium (IVM or IVF) in order to evaluated if they are able to preserve embryos from damage induced by OS. We previously demonstrated the beneficial effect of adding SOD to the transport medium of domestic cat ovaries (Cocchia et al. 2015b). We also demonstrated that the addition of antioxidants in the extender on refrigerated semen of fertile and infertile dogs has led to an improvement in reproductive performance (Del Prete et al. 2018a). Furthermore, an increase in the sperm concentration and the quality of chilled semen was also achieved by supplementing the stallion diet with natural antioxidants (Del Prete et al. 2018b). The results showed that supplementation of IVM medium with SOD and CAT did not improve oocyte maturation rate, while it did enhance advancement to the blastocyst phase.

In vitro embryo development is highly affected by culture environment, thus in our study the purpose of adding antioxidant enzymes to IVM or IVF media was to improve *in vitro* conditions and decrease the detrimental effects of OS. The efficacy of SOD and CAT addition in medium for cat oocyte IVM

has not been previously reported. Recently, a study demonstrated that the supplementation *in vitro* with SOD and taurine in IVM and IVC media increased blastocyst development from poor-quality cat oocytes (Ochota et al. 2016).

Furthermore, we added GPx to IVF medium because, during IVF, sperm cells produce great amounts of ROS (Galecka et al. 2008). Our results showed that the rate of embryo and blastocyst formation was increased in the experimental in respect to control group.

We showed the positive effect of SOD and CAT addition to cat oocyte IVM medium and GPx in the IVF medium on *in vitro* feline oocyte formation. Although further studies are required to better understand the role of SOD, CAT and GPx, the data obtained encourage us to examine different concentrations of the same and/or different antioxidants, at various stages of oocyte maturation and embryo production *in vitro* and to evaluate possible mechanisms of action.

References

Agarwal A, Allamaneni SS **(2004)** Role of free radicals in female reproductive diseases and assisted reproduction. Reprod Biomed Online 9: 338-347.

Agarwal A, Said TM, Bedaiwy MA, Banerjee J, Alvarez JG **(2006)** Oxidative stress in an assisted reproductive techniques setting. Fertil Steril 86: 503-512.

Chi HJ, Kim JH, Ryu CS, Lee JY, Park JS, Chung DY, Choi SY, Kim MH, Chun EK, Roh SI **(2008)** Protective effect of antioxidant supplementation in sperm-preparation medium against oxidative stress in human spermatozoa. Hum Reprod 23: 1023-1028.

Ciani F, Cocchia N, Rizzo M, Ponzio P, Tortora G, Avallone L, Lorizio R **(2008)** Sex determining of cat embryo and some feline species. Zygote 16: 169-177.

Ciani F, Cocchia N, d'Angelo D, Tafuri S (**2015**) Influence of ROS on ovarian functions. In: Wu B (ed) New discoveries in embryology. IntechOpen, London, UK, pp 41-73.

Ciani F, Tafuri S, Troiano A, Cimmino A, Fioretto BS, Guarino AM, Pollice A, Vivo M, Evidente A, Carotenuto D, Calabrò V **(2018)** Anti-proliferative and pro-apoptotic effects of Uncaria tomentosa aqueous extract in squamous carcinoma cells. J Ethnopharmacol 211: 285-294.

Cocchia N, Ciani F, Russo M, El Rass R, Rosapane I, Avallone L, Tortora G, Lorizio R **(2010a)** Immature cat oocyte vitrification in open pulled straws (OPSs) using a cryoprotectant mixture. Cryobiology 60: 229-234.

Cocchia N, Ciani F, El-Rass R, Russo M, Borzacchiello G, Esposito V, Montagnaro S, Avallone L, Tortora G, Lorizio R **(2010b)** Cryopreservation of feline epididymal spermatozoa from dead and alive animals and its use in assisted reproduction. Zygote 18: 1-8.

Cocchia N, Pasolini MP, Mancini R, Petrazzuolo O, Cristofaro I, Rosapane I, Sica A, Tortora G, Lorizio R, Paraggio G, Mancini A **(2011)** Effect of SOD (superoxide dismutase) protein supplementation in semen extenders on motility, viability, acrosome status and ERK (extracellular signal-regulated kinase) protein phosphorylation of chilled stallion spermatozoa. Theriogenology 75: 1201-1210.

Cocchia N, Tafuri S, Abbondante L, Meomartino L, Esposito L and Ciani F (**2015a**) Assisted reproductive technologies in safeguard of feline endangered species. In: Wu B (ed) New discoveries in embryology. IntechOpen, London, UK, pp 199-229.

Cocchia N, Corteggio A, Altamura G, Tafuri S, Rea S, Rosapane I, Sica A, Landolfi F, Ciani F **(2015b)** The effects of superoxide dismutase addition to the transport medium on cumulus-oocyte complex apoptosis and IVF outcome in cats (*Felis catus*). Reprod Biol 15: 56-64.

Costantino M, Giuberti G, Caraglia A, Caraglia M, Lombardi A, Misso G, Abbruzzese A, Ciani F, Lampa E **(2009)** Possible antioxidant role of SPA therapy with chlorine–sulphur–bicarbonate mineral water. Amino Acids 36: 161-165.

Del Prete C, Ciani F, Tafuri S, Pasolini MP, Della Valle G, Palumbo V, Abbondante L, Calamo A, Barbato V, Gualtieri R, Talevi R, Cocchia N **(2018a)** Effect of superoxide dismutase, catalase, and glutathione peroxidase supplementation in the extender on chilled semen of fertile and hypofertile dogs. J Vet Sci 19: 667-675.

Del Prete C, Tafuri S, Ciani F, Pasolini MP, Ciotola F, Albarella S, Carotenuto D, Peretti V, Cocchia N **(2018b)** Influences of dietary supplementation with *Lepidium meyenii* (Maca) on stallion sperm production and on preservation of sperm quality during storage at 5°C. Andrology 6: 351-361.

Devine PJ, Perreault SD, Luderer U **(2012)** Roles of reactive oxygen species and antioxidants in ovarian toxicity. Biol Reprod 86: 27.

Esposito L, Auletta L, Ciani F, Pelagalli A, Pasolini MP, Lamagna B, Piscopo N, Amici A **(2017)** Hair cortisol levels in captive brown hare (*Lepus europaeus*): potential effect of sex, age, and breeding technology. Eur J Wildl Res 63: 62.

Formigari A, Irato P, Santon A **(2007)** Zinc antioxidant systems and metallothionein in metal mediated-apoptosis: biochemical and cytochemical aspects. Comp Biochem Physiol C Toxicol Pharmacol 146: 443-459.

Galecka E, Jacewicz R, Mrowicka M, Florkowski A, Galecki P **(2008)** Antioxidative enzymes-structure, properties, functions. Pol Merkur Lekarski 25: 266-268.

Goto Y, Noda Y, Mori T, Nakano M **(1993)** Increased generation of reactive oxygen species in embryos cultured *in vitro*. Free Radic Biol Med 15: 69-75.

Gough DR, Cotter TG **(2011)** Hydrogen peroxide: a Jekyll and Hyde signalling molecule. Cell Death Dis 2: 213.

Guérin P, El Mouatassim S, Ménézo Y **(2001)** Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update 7: 175-189.

Hewitt DA, Watson PF, England GC **(1998)** Nuclear staining and culture requirements for *in vitro* maturation of domestic bitch oocytes. Theriogenology 49: 1083-1101.

Li F, Cui L, Yu D, Hao H, Liu Y, Zhao X, Pang Y, Zhu H, Du W **(2019)** Exogenous glutathione improves intracellular glutathione synthesis via the γ-glutamyl cycle in bovine zygotes and cleavage embryos. J Cell Physiol 234: 7384-7394.

Luvoni GC **(2006)** Gamete cryopreservation in the domestic cat. Theriogenology 66: 101-111.

Ochota M, Pasieka A, Nizanski W **(2016)** Superoxide dismutase and taurine supplementation improves *in vitro* blastocyst yield from poor-quality feline oocytes. Theriogenology 85: 922-927.

Pero ME, Lombardi P, Longobardi V, Boccia L, Vassalotti G, Zicarelli L, Ciani F, Gasparrini B **(2017)** Influence of γ-glutamyltransferase and alkaline phosphatase activity on *in vitro* fertilization of bovine frozen/thawed semen. Ital J Anim Sci 16: 390-392.

Pipolo S, Puglisi R, Mularoni V, Esposito V, Fuso A, Lucarelli M, Fiorenza MT, Mangia F, Boitani C (2018) Involvement of sperm acetylated histones and the nuclear isoform of glutathione peroxidase 4 in fertilization. J Cell Physiol 233: 3093-3104.

Pope CE **(2014)** Aspects of in vivo oocyte production, blastocyst development, and embryo transfer in the cat. Theriogenology 81: 126-137.

Tafuri S, Ciani F, Iorio EL, Esposito L, Cocchia N (**2015**) Reactive oxygen species (ROS) and male fertility. In: Wu B (ed) New discoveries in embryology. IntechOpen, London, UK, pp 19-40.

Takahashi M **(2012)** Oxidative stress and redox regulation on *in vitro* development of mammalian embryos. J Reprod Dev 58: 1-9.

Zhang M, Hong H, Zhou B, Jin S, Wang C, Fu M, Wang S, Xia G **(2006)** The expression of atrial natriuretic peptide in the oviduct and its functions in pig spermatozoa. J Endocrinol 189: 493-507.