Resveratrol treatment during maturation enhances developmental competence of oocytes after prolonged ovary storage at 4 °C in the domestic cat model

Resveratrol treatment during maturation enhances ~~embryonic development~~ developmental competence of oocytes after prolonged ovary storage at 4°C in the domestic cat model

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Abstract

Resveratrol (Resv; 3,4,5-trihydroxy-trans-stilbene) is a phytoalexin with antioxidant activity that modulates redox homeostasis in oocytes and improves *in vitro* embryo production. Cold storage of cat ovaries for a period longer than 24 h alters oxidative status of oocytes after *in vitro* maturation and reduces their developmental competence. The aim of this study was to evaluate the effect of resveratrol supplementation to the maturation medium on embryo development of oocyte after storage of domestic cat ovaries at 4° C ~~up to 48h~~ for 24 h or 48 h. Cumulus–oocyte complexes (COCs) were recovered from ovaries of domestic queens ~~stored at 4°C for 24 and 48h~~ and cultured in maturation medium supplemented with (+) or without (-) 5 µM resveratrol for 24 h. COCs collected from fresh ovaries were matured *in vitro* (IVM) in standard conditions as control. After IVM, oocytes were *in vitro* fertilized (IVF) and presumptive zygotes cultured for 7 days. Oocyte nuclear maturation, reactive oxygen species (ROS) and glutathione (GSH) levels as well as ~~embryonic~~ cleavage,

blastocyst formation and blastocyst cell number were determined. There were no differences in the maturation rates of oocytes between the control and stored groups, irrespective of resveratrol supplementation. Resveratrol treatment during IVM significantly increased the level of GSH and reduced the level of ROS of oocytes recovered from ovaries stored for 48 h as compared to the ~~not~~ non-treated group (48 h-). The ~~percentage~~ rate of blastocyst formation from oocytes ~~of 48h stored group~~ recovered from ovaries after 48 h storage that underwent IVM ~~matured~~ with resveratrol was higher ($P<0.05$) than that of oocytes matured without resveratrol and similar to that of control oocytes. Resveratrol treatment ~~significantly~~ increased ($P<0.05$) cell number in blastocysts from 24 h+ and 48 h+ groups as compared to their respective counterparts. In conclusion, our results demonstrated that resveratrol supplementation during ~~*in vitro* maturation~~ IVM can reverse the adverse effect of oxidative stress on oocytes, and enhances embryo development after ovary storage at 4° C for 48 h. These results may provide a basis for improving culture conditions and extend the possibility of storage of cat ovaries for more than 24 h thus ensuring ~~a~~ successful *in vitro* embryo production.

1. Introduction

The accelerating decline in wild animal species through the ~~word~~ world is an alarming problem. At present, according to the Red List of Endangered Species of the IUCN, most of the 35 species of wild ~~felids~~ felines are classified as endangered, near threatened or vulnerable [1].

In vitro embryo production technology (IVEP) represents a valuable tool that may help in safeguarding the future of feline endangered species [2-4].

In the case of wild felids ~~wildlife animals~~, the use of IVEP ~~this technique~~ may ~~demand~~ include the collection of ovaries from ~~feline~~ females post mortem or after ~~which died in their natural habitat or zoos or are~~ ovariectomy for medical reasons and subsequent ~~the~~ transport of explanted gonads ~~even for long distance~~ from the collection site to ~~the~~ specialized laboratories.

With ~~In~~ this perspective, several studies ~~have been~~ were carried out to establish the ideal conditions for temporary storage of domestic cat ovaries during transport ~~using the domestic cat as model~~ [5-10]. ~~Based on the obtained results the researchers indicated that the~~ A low temperature (4° C) and a time limit of 24 h ~~are~~ were the most ~~favorable~~ favourable conditions for ~~the~~ preservation of preantral follicle morphology and ~~to maintain~~ for maintenance of the meiotic and cytoplasmic developmental competence of oocytes retrieved from antral follicles [6,7,9,11-13]. Although storing cat ovaries at 4° C for 48 h did not affect the *in vitro* meiotic ability of oocytes, ~~the~~ extension of the storage period beyond 24 h markedly reduced IVEP efficiency [12,13]. Indeed, only oocytes retrieved from ovaries stored at 4° C for a maximum of 24 h ~~could~~ produced blastocysts following *in vitro* fertilization [10,12,13]. ~~and developed into live offspring after transfer into recipients~~ Live offspring have been produced after transfer of embryos derived following recovery of oocytes from ovaries stored for 24 h to 28 h at 4° C [14]. To date, only in the study of Cocchia et al. *in vitro* blastocysts were obtained from cultured oocytes of cat ovaries preserved at 4 °C for up to 72 h by adding superoxide dismutase (SOD) to the transport ~~media~~ medium [10].

~~In a previous study~~ Previously, we analyzed the effect of domestic cat ovary storage at 4° C up to 96 h on oxidative status and developmental competence of oocytes after *in vitro* maturation (IVM) and IVF [13]. ~~Our findings proved~~ We found that the extension of storage period beyond 24 h induced a progressive increase of reactive oxygen species (ROS) associated with elevated lipid peroxidation of ~~*in vitro* matured~~ oocytes after IVM and reduced their ability to develop to the blastocyst stage [13]. ~~These~~ Our results suggested that oxidative stress may be one ~~of the~~ factor~~s~~ involved in the progressive decline of the oocyte developmental ability following prolonged cold storage of ovaries.

It is well known that oxidative stress negatively affects oocyte quality [15] and can impair subsequent fertilization [16] and embryo development [17].

Under normal physiological conditions the oocyte possesses an antioxidants defense mechanisms, including both enzymatic and non/enzymatic, ~~antioxidants,~~ which hinders excessive free radical formation [15]. However, intrinsic and extrinsic factors may interfere with oocyte redox equilibrium, leading to ~~the~~ increased production of ROS and/or the reduction of intracellular antioxidant concentrations [18-20].

Resveratrol (Resv; 3,4,5-trihydroxy-trans-stilbene), a phytoalexin produced by ~~different~~ plants, ~~is able to perform~~ has many biological activities, including antioxidant activity [21,22]. An increasing ~~number~~ amount of evidence indicate~~d~~s that resveratrol positively affects oocyte ~~*in vitro* maturation~~ IVM and subsequent embryonic development [23]. Supplementation of culture medium with resveratrol had beneficial effects on *in vitro* embryo production in ~~different~~ domestic species as demonstrated by improved blastocysts development rates, hatching blastocyst rates, and number of cells in blastocysts after IVM/IVF [23]. *In vitro* studies have shown that resveratrol exerted antioxidant action mainly through scavenging ROS and increasing glutathione (GSH) content of ~~the~~ oocytes. Furthermore, resveratrol acting on mitochondrial turnover regulated ROS production at the electron transport chain level [24,25].

Here, we investigated whether the addition of resveratrol to the IVM medium would improve the developmental competence of cat oocytes after ovary storage at 4° C up to 48 h. To this end, we analysed oocytes redox status after IVM (GSH and ROS levels) and, after IVF frequencies of cleavage and blastocyst development and blastocyst quality were examined.

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2. Materials and methods

All chemicals in this study were purchased from Sigma-Aldrich Chemical Company (St. Louis. MO, USA) unless stated otherwise.

2.1. Ovary collection and storage

Ovaries were harvested from 45 domestic queens (*Felis catus*, 8 months to 2 years of age) at random stage of the oestrus cycle. Ovaries were placed in sterile 15 mL tubes containing PBS (Dulbecco's Phosphate Buffered Saline) with penicillin (100 IU/mL) and streptomycin (100 mg/mL) and immediately transported to the laboratory. Ovaries were randomly divided in two groups: fresh ovaries (0h, control group) and ovaries to be stored at 4° C (stored group). For storage, each ovary was transferred under sterile conditions to a tube containing 4 mL of fresh pre-cooled PBS and held at 4° C in a refrigerator for 24 h or 48h.

2.2. Experimental design

Cumulus-oocyte complexes (COCs) recovered from ovaries stored at 4° C for 24 h and 48 h were matured *in vitro* in presence (+) or absence (-) of 5µM resveratrol. COCs collected from fresh ovaries (0h) were matured *in vitro* in standard conditions without resveratrol as the control.

The concentration of resveratrol ~~has been~~ was chosen based on preliminary studies (data not shown) where we tested three different concentrations ~~of resveratrol~~ (1, 2.5 and 5 µM). A dose of 5 µM was found as ~~the ideal~~ most effective to reduce ROS levels of ~~*in vitro* matured~~ IVM cat oocytes following ovary storage at 4° C for 24 h or 48 h.

Experiment 1 was performed to evaluate the effect of resveratrol supplementation during IVM of cat oocytes retrieved from stored ovaries on oocyte nuclear maturation and intracellular levels of GSH and ROS.

In Experiment 2, COCs recovered from control and stored ovaries underwent IVM/IVF. During or after embryo culture, incidence of cleavage, frequency of blastocyst formation and blastocyst cell number were evaluated.

2.3. Oocyte recovery and IVM

Ovaries from fresh and stored groups were sliced with a scalpel blade to release the COCs. Then, COCs were collected in sterile Petri dishes in dissection medium (DM; 25 mM Hepes-buffered TCM 199) supplemented with 0.1% (wt/vol) polyvinyl alcohol (PVA) and antibiotics. Only COCs with darkly pigmented ooplasm and completely surrounded by at least one layer of cumulus cells were selected. For IVM COCs were cultured in groups of 25 to 35 in 650 µL of IVM medium (TCM 199 supplemented 0.36 mM pyruvate, 2 mM glutamine, 2.2 mM calcium lactate, 1.2 mM

cysteine, 4 mg/mL BSA and FSH (1 IU⁄mL) and LH (1 IU⁄mL), in four-well Petri dishes in a humidified atmosphere of 5% CO_2, at 38.5°C. ~~for 24h.~~

2.4. Assessment of oocyte nuclear maturation

After IVM for 24 h, groups of COCs were completely denuded of granulosa cells via gentle pipetting with a fine bore glass pipette in DM, stained with Hoechst 33342 (10µg/mL) in 1:1 (v/v) glycerol/PBS solution, placed on a slide and overlaid with a coverslip supported by four droplets of Vaseline®. The nuclear configuration was examined under an epifluorescence microscope (Olympus IX 70, Italy) and oocytes were classified as metaphase-II (MII) based on the presence of a metaphase plate and first polar body.

2.5 Measurement of intracellular ROS and GSH levels

Groups of MII oocytes (30 to 35 /~~each~~ group) were selected under a stereomicroscope (Olympus SZ-PT) ~~on the basis of the~~ for the presence of the first polar body and sampled for measurement of intracellular ROS and GSH levels. Briefly, 2′7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA; Molecular Probes Inc., Eugene, OR, USA) and 4-chloromethyl-6,8-difluoro-7-hydroxycoumarin (CellTracker Blue CMF_2HC Molecular Probes) [13] were used to detect intracellular ROS as green fluorescence and GSH levels as blue fluorescence. Oocytes from each treatment group were incubated in the dark for 30 min, in DPBS-PVA containing 10µM H_2DCF-DA and 10µM CellTracker Blue. After incubation, the oocytes were washed with DPBS-PVA; each of the oocyte~~s~~ was placed in a 50 µL droplet of DPBS and observed using an epifluorescence microscope (Olympus IX 70) with UV filters (460 nm for ROS and 370 nm for GSH). Oocytes were positioned in the plane of focus, and the area of measurement was adapted to the size of the oocyte. Microscope adjustments and photomultiplier settings were kept constant for all experiments. Data on emission intensity/oocyte were reduced by compensation for the background fluorescence. The fluorescent images were saved as graphic files in TIFF format. Intensities of fluorescence were analyzed using Image J software (version 1.40; National Institute of health, Bethesda, MD) and normalized to those of control oocytes.

2.6 IVF and embryo culture

After IVM, oocytes were ~~fertilized~~ co-incubated with frozen-thawed epididymal spermatozoa (1×10^6 motile sperm~~atozoa~~/mL) in synthetic oviductal fluid (SOF) supplemented with ~~4mg/mL~~ 6mg/mL BSA, 100 IU/mL penicillin, 50 µg/mL gentamicin, for 22h at 38.5 °C in a humidified atmosphere of 5% CO_2 in air [26].

Presumptive zygotes were washed and cultured in 650 µL of SOF containing 4 mg/mL BSA, 100 IU/mL penicillin and 1% MEM non-Essential amino acids. On Day 3 after IVF (Day = 0) ~~the~~ embryos were transferred to SOF with 10% fetal calf serum (FCS) and 2% MEM essential amino acids, and cultured at 38.5° C in a humidified atmosphere of 5% CO_2 in air for an additional 4 days [26]. ~~The embryos that cleaved and reached the blastocyst stage~~ Cleavage and blastocyst development were evaluated morphologically on Day 2 and on Day 7 after IVF, respectively.

2.7. Assessment of blastocyst cell number

Analysis of blastocyst cell number was performed by differential staining of the inner cell mass (ICM) and trophectoderm (TE) cell compartments [27]. To differentially stain ICM and TE nuclei, blastocysts derived from control and stored groups were exposed to 1% Triton X-100 in 20 mM HEPES-buffered TCM 199 containing 30 mg/mL propidium iodide (PI) for 35 to 40 s. Then, blastocysts were ~~then~~ transferred into ice-cold ethanol between 2 to 5 s. Finally, blastocysts were incubated in medium with 50% (v/v) glycerol and ethanol containing 0.1 mg/mL bis-benzimide (Hoechst 33342) for 5 min. After staining, ~~the~~ blastocysts were ~~directly~~ mounted into a small droplet of glycerol on a glass slide and examined under epifluorescence microscope (Olympus IX70). A digital image of each embryo was taken, and the numbers of TE (red) and ICM (blue) nuclei were counted.

2.8. Statistical Analysis

All statistical analysis was performed using STATA\IC 11.28 (StataCorp LP,USA). Data of nuclear maturation, cleavage and development to blastocyst stage were analyzed by chi-square test. Data (mean ± SEM) of blastocyst cell number, intracellular ROS and GSH levels were analyzed by one-way univariate analysis of variance (ANOVA) using Bonferroni's as post hoc test.

The level of statistical significance was set at $P < 0.05$. All ~~the~~ experiments were replicated ≥ ~~at least for~~ three times.

3. Results

3.1. Experiment 1

3.1.1. Effect of Resveratrol on oocyte nuclear maturation and intracellular GSH and ROS levels

Treatment with resveratrol did not ~~significantly~~ affect the maturation rate of the oocytes of 24 h+ (41/66, 62.1%) and 48 h+ (48/77, 62.3%) stored groups compared to their respective counterpart (24 h-:38/63, 60.3%; 48 h-:41/69, 59.4%) and control (52/85, 61.2%).

The levels of GSH of oocytes after IVM (Fig.1 A, A') were similar between control (1±0.26 pixels/oocytes) and stored groups (24 h-:1.2±0.18, 48 h-:0.9±0.27 pixels/oocytes). Treatment with resveratrol ~~significantly~~ increased (P< 0.05) GSH level of oocytes from ovaries stored for 24 h and 48 h as compared to the control group (24 h+:1.6±0.26, 48 h+:1.5±0.13 and control:1±0.26 pixels/oocytes). ~~Significantly,~~ Higher levels of GSH were recorded in the 48 h+ group vs. ~~respect to its counterpart~~ the 48 h- group.

Levels of ROS were higher (P< 0.05) in oocytes of the 48 h- (1.3±0.11 pixels/oocytes) group, as compared to those found in the 24 h- and control groups (0.93±0.24 and 1±0.07 pixels/oocytes, respectively).

Resveratrol supplementation decreased ~~significantly~~ intracellular ROS levels in both the 24 h+ (0.5±0.08 pixels/oocytes) and 48 h+ (0.9±0.0.09 pixels/oocytes) groups as compared with their respective counterparts (P< 0.05; Fig.1 B, B').

3.2. Experiment 2

3.2.1. Effect of resveratrol on embryo development and blastocyst quality

~~The~~ Cleavage and development after IVF of oocytes across treatments are shown in Table 1. The percentage of oocytes that progressed to the first cleavage stage was similar among groups. The ~~rate of~~ number of blastocysts that developed relative to the total number of ~~cleaved~~ embryos (developmental competence) and to the total number of oocytes (blastocyst yield) ~~was~~ were lower ($P<0.05$) in the 48 h- group compared to values in the control, 24 h-, 24 h+ groups. Resveratrol treatment ~~significantly~~ increased ($P<0.05$) blastocyst development ~~of~~ in the 48 h+ group at rates comparable to development in the control, 24 h- and 24 h+ groups and higher than in the 48 h- group ~~respective counterpart~~.

The mean number of total, ICM and TE cells/blastocyst were ~~significantly~~ lower ($P<0.05$) after ovary storage for 48 h as compared to the control and 24 h stored groups. Resveratrol treatment ~~significantly~~ increased ($P<0.05$) blastocyst cell number in both 24 h and 48 h groups as compared to their respective counterparts. These values are similar to those of fresh control group (Table 2 and Fig. 2).

Table 1

Effect of Resveratrol treatment during IVM on cleavage and blastocyst development of cat oocytes retrieved from ovaries stored at 4° C for 24 h or 48 h.

Storage time (h)	Resv 5µM	Oocytes fertilized, n	Cleaved, n (%)	Blastocysts, n (% of oocytes)	Blastocysts, n (% of embryos)
0 (Control)		82	36 (43.9)	22(26.83)[a]	22 (61.11)[a]
24	-	79	32 (40.51)	18 (27.78)[a]	18 (56.25)[a]
	+	77	32 (41.56)	19 (24.67)[a]	19 (59.37)[a]
48	-	84	31 (36.90)	4 (4.76)[b]	4 (12.90)[b]
	+	74	28 (37.84)	14 (18.92)[a]	14 (50.00)[a]

Values in the same column with different superscript (a and b) letters differ significantly (P<0.05).

Table 2

Effect of resveratrol treatment during IVM on total, inner cell mass (ICM) and trophoectoderm (TE) cell number of blastocysts developed from cat oocytes retrieved from ovaries stored at 4° C for 24 h or 48 h

Storage time (h)	Resv 5 µM	Blastocysts, n	Cell number (mean ± SEM) TOTAL	ICM	TE
0 (Control)		22	186.4 ± 7.8^{ab}	43.1 ± 2.5^{ab}	143.3 ± 6.8^{ab}
24	-	18	170.5 ± 10.8^{a}	39.4 ± 4^{a}	131.1 ± 8.3^{a}
	+	19	231.1 ± 15.2^{b}	57.7 ± 3.2^{b}	173.4 ± 11.2^{b}
48	-	4	84.8 ± 16.7^{c}	16.3 ± 1.5^{c}	68.5 ± 3.5^{c}
	+	14	146.8 ± 10.1^{a}	36.3 ± 3.7^{a}	110.5 ± 8.2^{a}

Different superscripts (a, b and c) among storage times indicate $P<0.05$.

4. Discussion

The possibility of using oocytes recovered from ~~temporary stored~~ ovaries after short-term cold storage for *in vitro* embryo production may ~~contribute to~~ support the preservation of wildlife and endangered species. The present study represents a novel contribution to the improvement of the IVEP technique using cat oocytes recovered from ~~cold stored~~ ovaries stored up to 48 h at 4° C.

In this research, we explored the potential beneficial effect of resveratrol addition to the IVM medium on embryonic development, after IVF of domestic cat oocytes recovered from ovaries stored at 4° C for 24 h or 48 h.

The rationale for the resveratrol treatment derived from the results of our previous study which had suggested the involvement of oxidative stress in the decline of developmental competence of cat oocytes following ovary storage at 4° C for ~~more than~~ > 24 h [13]. Indeed, after IVM, cat oocytes showed an increase of ROS levels at 48 h of ovary storage and ~~associated with a~~ reduced *in vitro* development to the blastocyst stage [13] compared to those of oocytes collected from fresh ovaries.

The main findings of the research demonstrated that the addition of 5 µM resveratrol to IVM medium of cat oocytes: *i)* increased GSH levels and reduced ROS levels of IVM oocytes after recovery from ovaries stored for 24 h or 48 h, *ii)* improved ~~the~~ embryo development to the blastocyst stage after IVF of oocytes retrieved from ovaries stored for 48 h and *iii)* enhanced the mean number of cells / blastocyst in both 24 h and 48 h stored groups.

A growing number of evidence confirmed the beneficial effect of supplementing culture medium with resveratrol on *in vitro* embryo production in ~~different~~ domestic species as demonstrated by improved rate of blastocyst development and more cells/blastocyst after IVM/IVF [23]. To date, no studies have been performed to evaluate the influence of resveratrol treatment on IVEP of cat oocytes.

Species-specific differences in the oocyte maturation and embryonic development with different resveratrol concentrations have been described [23]. The overall results indicate that supplementation of culture medium with low concentration of resveratrol (0.5- 2 µM) enhances the quality of oocytes and leads to an improved embryo development [28-30] whereas high concentration of resveratrol (≥ 10 µM) decreases nuclear maturation [29,31], and blastocyst formation [30]. In pigs, IVM with 10 µM resveratrol reduced the ability of the oocytes to reach the MII stage through the down-regulation of the PCNA and POU5F1 genes [29]. Resveratrol treatment of 20 and 40 µM resveratrol decreased nuclear maturation *in vitro* in bovine oocytes by binding to arylhydrocarbon receptors [31].

Recent studies provided new evidences that resveratrol treatment may be ~~also considered~~ ~~a~~ useful ~~approach to~~ for protecting ~~the~~ developmental competence of oocytes under sub-optimal conditions, such as cryopreservation [32], heat stress [20] and ~~oocyte~~ exposure to toxic substances [33].

Comizzoli et al. reported the protective effect of 1.0 mmol/L resveratrol exposure of immature cat oocytes before vitrification on their ability to reach MII, undergo cleavage, and develop to the 8–16 cells stage after IVF [34].

The first part of our study was aimed to explore the effect of resveratrol supplementation to IVM medium on meiotic competence and redox status of oocytes following cold storage of ovaries. No ~~significant~~ difference in the percentage of oocytes reaching the MII stage was found after resveratrol treatment as compared to control oocytes~~, an observation~~ which is consistent with the results reported in goats [28,35] and pigs [29]. Conversely, in bovine, IVM with resveratrol (1µM) increased the rate of nuclear maturation of oocytes from adult animals [30] whereas it had no effect on ~~that of~~ prepubertal females ~~ones~~ [36]. In addition, the same resveratrol concentration (1µM) promoted nuclear maturation both in human and ~~mice~~ mouse aged oocytes [37].

Although no effect on nuclear maturation ~~has been~~ was observed, we found that ~~the~~ treatment with resveratrol was effective for reducing ROS and increasing GSH content in IVM oocytes in both the 24 h and 48h cold storage groups.

Resveratrol is known to protect cells from oxidative stress by direct ROS scavenging and activating endogenous antioxidants [25,26]. Furthermore, resveratrol treatment during IVM enhanced intracellular GSH levels ~~of the oocyte~~ in oocytes [28-30,35]. Reduced glutathione is the main non enzymatic defence system against free radicals in oocytes and embryos [17]. Indeed, glutathione ~~giving a~~ provides electron and ~~converting~~ converts it into its oxidized form (GSSG) ~~acts~~ acting as a cofactor of antioxidant enzymes [38]. A high GSH content in mature oocytes is also considered a marker of cytoplasmic maturity and it is positively related with embryonic development [39-41]. In cattle [30], pigs [29] and goats [28,35], resveratrol addition to the IVM medium increased GSH and decreased ROS levels promoting oocyte cytoplasmic maturation, as indicated by ~~the achievement of~~ higher blastocyst rate after IVM/IVF.

In agreement with these findings, our results demonstrated that, by modulating the redox homeostasis of ~~the~~ oocytes during maturation, resveratrol improved the blastocyst formation rate and number of cells in blastocysts from oocytes undergoing IVM/IVF after recovery from ovaries stored at 4° C for 48 h.

To the best of our knowledge, this investigation is the first to report blastocyst development after in oocytes recovered from ovaries stored at 4° C for 48 h at a rate similar to that of fresh oocytes. Further studies are needed to validate the survival and the quality of *in vitro* produced embryos by assessing their *in vivo* developmental competence.

Recent studies have been addressed to improve transport and culture medium conditions with the aim to enhance *in vitro* embryo production after cold storage of cat ovaries. The study of Luu et al. [42]

found that the addition of relaxin to ~~the *in vitro* maturation~~ IVM medium ameliorated the blastocyst yield after cat ovary~~ies~~ storage at 4° C for 24 h. Moreover, superoxide dismutase (SOD) supplementation to ~~the~~ transport medium of cat ovaries positively affected oocyte quality, reduced cellular apoptosis and supported ~~the~~ development to the blastocyst stage after 48 h and 72 h storage (7% and 4% blastocysts yield, respectively) [10].

Our rate~~s~~ of blastocyst development (18.9%) in the 48 h stored group was higher than those obtained by Cocchia et al. ~~with the addition of~~ after adding SOD during ovary storage [10].

In the present study, the beneficial effect of resveratrol ~~is~~ was also underlined by its ability to improve blastocyst quality. In fact, we found higher total blastocyst cell numbers, including higher numbers of both ICM and TE cells, in the 24 h and 48 h resveratrol treated groups. Blastocyst cell numbers is considered an important marker of embryo quality. A high number of cells ~~has been~~ was associated with a greater probability of blastocyst implantation after embryo transfer and the birth of viable offspring [27,43-45].

The beneficial effect of resveratrol supplementation to IVM medium on embryo quality was reported also in bovine [30] and porcine [29]. ~~In this regard,~~ The down-regulation of pro-apoptotic and anti-proliferative genes has been identified in pig oocytes as a possible mechanism of action underlying the positive effect of resveratrol on embryo quality [29,46].

Collectively, our results demonstrated that resveratrol supplementation during ~~*in vitro* maturation~~ IVM can reverse the adverse effect of oxidative stress on oocytes. ~~This~~ The beneficial effect was reflected ~~on~~ in improvement of ~~the~~ *in vitro* embryo production when using oocytes retrieved from ovaries stored for 48 h at 4° C.

These findings provide a novel approach for extending the possibility of ovary collection and storage, especially for ~~feline wild species which die in geographic areas far away from specialized ARTs laboratories~~ wild felids located in geographic areas isolated from specialized assisted reproductive technology laboratories.

Declarations

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ARP, AF and LB designed the study, analyzed data and wrote the paper. ARP, AF, ES and LB, conducted experimental procedures of the study. ARP, AF, LF, MTP and LB collaborated in analyzing data and drafting the paper. SP and MTZ performed ovariectomies. LB supervised experiments and performed critical data analysis. All authors read and approved the paper.

Ethics approval and consent to participate

No experimental animals have been used in this work. The samples (ovarian tissues) were collected during routine ovariectomies at the Veterinary Teaching Hospital of the University, following the basic criteria of good veterinary surgery practices.

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Figure Legends

Figure 1. Effect of resveratrol treatment during IVM on A) intracellular GSH and B**)** ROS levels of *in vitro* matured cat oocytes retrieved from ovaries stored at 4° C for 24 h or 48 h. Epifluorescence photomicrographs of MII oocytes that were stained with A') CellTracker Blue to determine the level of GSH and B'). with 2′7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) to detect ROS. Different superscripts among storage times indicate P<0.05. Scale bar = 50µm.

Figure 2. Representative epifluorescence photomicrographs of blastocysts developed from cat oocytes retrieved from ovaries stored at 4° C for 24 h or 48 h, after differential staining. Inner cell mass stained in blue with Hoechst 3342 and thophectoderm stained in red with propidium iodide. Scale bar =100µm.

Table Legends

Table 1. Effect of Resveratrol treatment during IVM on cleavage and blastocyst development of cat oocytes retrieved from ovaries stored at 4° C for 24 h or 48 h.
Values in the same column with different superscript (a and b) letters differ ~~significantly~~ (P<0.05).

Table 2. Effect of resveratrol treatment during IVM on total, inner cell mass (ICM) and trophoectoderm (TE) cell number of blastocysts developed from cat oocytes retrieved from ovaries stored at 4° C for 24 h or 48 h
Different superscripts (a, b and c) among storage times indicate P< 0.05.

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