Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2026.108

RESEARCH ARTICLE

Restoration of Mitochondrial Homeostasis in Post-Ovulatory Ageing Oocytes Through Elamipretide–Mediated Modulation of SIRT3, Redox Balance, and Bioenergetics

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ARTICLE HISTORY (26-038)

Received: January 18, 2026
Revised: April 15, 2026
Accepted: April 21, 2026
Published online: April 24, 2026

Key words:

ABSTRACT

The study investigated whether the mitochondrial-targeted antioxidant peptide SS-31 (elamipretide) can mitigate post-ovulatory ageing-related mitochondrial dysfunction in bovine oocytes by modulating SIRT3 expression, oxidative stress, ATP content and mitochondrial membrane potential during in vitro maturation. Cumulus–oocyte complexes were collected from bovine ovaries and matured for 12, 36, or 52 hours and treated with SS-31 at 0.3, 0.6, or 0.9 µM, or cultured without antioxidants. SIRT3 expression was quantified by immunofluorescence, reactive oxygen species levels were measured using fluorescent probes, ATP content was evaluated using bioluminescence assays, and mitochondrial membrane potential was evaluated using JC-1 staining. Data were analyzed using two-way analysis of variance (ANOVA) with post hoc testing. SS-31 led to a dose-dependent increase in SIRT3 expression at all time points. ROS levels increased with duration of incubation; however, SS-31 treatment notably reduced oxidative stress, particularly at early and mid stage incubation. The ATP content increased over time, but remained lower in oocytes treated with SS-31, which may reflect alterations in mitochondrial metabolism; however, this requires further investigation. Mitochondrial membrane potential was enhanced by SS-31, with the strongest improvement observed at 0.6 µM after 12 and 36 hours. SS-31 appears to alleviate age-associated mitochondrial deterioration by improving SIRT3 expression, reducing ROS accumulation, and stabilizing mitochondrial membrane potential. In this context, SS-31 appears to support oocyte bioenergetic quality and may represent a promising approach as a tool for improving reproductive outcomes, particularly in cases of age-related fertility decline.

To Cite This Article: Kowalczyk A, García MC, Malagón JAH, Araujo JP, Witkiewicz W, Gruszczyńska J, Samardžija M, Wrzecińska M, Gałęska E, Czerniawska-Piątkowska E and Dobrzański Z, 2026. Restoration of mitochondrial homeostasis in post-ovulatory ageing oocytes through elamipretide–mediated modulation of SIRT3, redox balance, and bioenergetics. Pak Vet J, 46(5): 1209-1218. http://dx.doi.org/10.29261/pakvetj/2026.108

INTRODUCTION

Due to social pressure, increased life expectancy, popularization of effective contraception, and development of assisted reproductive technologies, delayed parenthood has become increasingly common, which emphasises the issue of decreased fertility associated with advanced age. Today, women strive to get pregnant at an advanced age for various reasons (Mills *et al.*, 2011). Several approaches are currently being

explored to improve fertility rates and increase the likelihood of pregnancy in women of advanced age. The decline in reproductive capacity is one of the physiological processes affected by ageing. However, it should be noted that the present study models post-ovulatory ageing under *in vitro* conditions rather than chronological ageing of the organism.

As women age, there is an inevitable decline in both the quantity and quality of ovarian follicles. Traditionally, this reduction in the number has been attributed to apoptosis-driven follicular atresia, while the decreased quality of oocytes is mainly linked to aneuploidy resulting from meiotic errors (Chiaratti, 2021). Mitochondria have gained attention as essential organelles, particularly due to their central roles in energy production and regulation of programmed cell death. These functions are also closely involved in ensuring correct spindle formation and chromosome alignment during meiosis (Tilly and Sinclair, 2013; Pasquariello *et al.*, 2019). Furthermore, mitochondria are the key to numerous cellular processes, especially in meeting the energy demands necessary for oocyte maturation and embryogenesis (Babayev *et al.*, 2016). Effective mitochondrial function is generally related to a higher content of mitochondrial DNA (mtDNA), increased ATP synthesis (although this relationship may vary to some extent depending on cell conditions) (Simsek-Duran *et al.*, 2013), stronger mitochondrial membrane potential (Yao *et al.*, 2018), and active quality control via mitophagy (Peters *et al.*, 2020). Research also indicates a positive connection between robust mitochondrial activity and improved embryo viability and fertility outcomes (May-Panloup *et al.*, 2021). A key factor in the reduced developmental potential of aged oocytes is oxidative stress, which alters mitochondrial function, damages DNA, and disrupts spindle organisation, ultimately lowering oocyte quality (Zhu *et al.*, 2023; Ju *et al.*, 2024). Excessive production of reactive oxygen species (ROS) is recognized as a contributor to cellular ageing, not only in systemic diseases but also within the reproductive system, negatively affecting fertility (Yan *et al.*, 2022). Mitochondrial dysfunction and oxidative stress, as fundamental mechanisms of postovulatory oocyte aging, are remarkably well conserved in mammals (Ahmad *et al.*, 2013). These same consequences of these processes have also been observed in cattle, pigs, and mice, demonstrating the value of animal models for studying the biological basis of cellular ageing (Khan *et al.*, 2016).

SIRT3, a mitochondrial sirtuin protein, plays a critical role in regulating mitochondrial protein acetylation and contributes to maintaining energy balance and responding to nutritional changes. It is known to alleviate oxidative stress by deacetylating and activating superoxide dismutase (SOD) (Tao *et al.*, 2014). Although its precise role in oocyte maturation is still under investigation, disruptions in SIRT3 function may be associated with age-related weakness in oocyte property and ovarian capacity. Understanding the role of granulosa and cumulus cells may pave the way for new therapeutic strategies that aim to improve mitochondrial metabolism and improve fertility outcomes in women undergoing in vitro fertilisation (IVF).

SS-31 (Elamipretide) is a peptide that has a dimethyltyrosine residue, which allows it to scavenge oxygen radicals and inhibit the oxidation of linoleic acid and low-density lipoproteins, making it a new targeted mitochondrial antioxidant (Calkins *et al.*, 2011). SS-31 (d-Arg-dimethylTyr-Lys-Phe-NH_2; ~639.8 Da) can not only undergo cellular uptake but also be transported through cells, thus rapidly being absorbed and exerting its functions after administration (Cerrato *et al.*, 2015). Elamipretide has been thoroughly characterised in preclinical animal studies due to its capacity to ameliorate mitochondrial dysfunction and its prospective therapeutic relevance in cardiovascular, renal, and neurodegenerative pathologies (Sabbah *et al.*, 2016; Ding *et al.*, 2021). To date, there is little evidence to support the efficacy of SS-31 peptide therapy for reproductive disorders.

The rationale behind using the bovine model system was the established success of the model in reproductive biology studies, as well as the similarity between bovine and human oocytes, with regard to the mitochondrial content, metabolism, and susceptibility to oxidative stress. Another benefit of using bovine oocytes is the provision of an in vitro system to study post-ovulatory ageing.

This study provides new insights into a comprehensive analysis of the action of SS-31 on cell ageing processes. We hypothesized that SS-31 supplementation would mitigate post-ovulatory ageing–associated mitochondrial dysfunction in bovine oocytes by modulating SIRT3 expression, reducing ROS levels, and improving mitochondrial membrane potential.

MATERIALS AND METHODS

The study used a bovine *in vitro* model: Taking into account the fact that these studies only cover veterinary procedures and biological material was collected postmortem from a local slaughterhouse, it was not essential to acquire the consent of the local animal ethics committee for this objective.

Cumulus–Oocyte Complexes Collection and Maturation: The ovarian material was obtained from 16 cows between 15 and 22 months of age at a local slaughterhouse and transported to the laboratory within two hours. The samples were transported in Hank's Balanced Salt Solution (Gibco-Invitrogen, Thermo Fisher Scientific, USA) containing 0.05 mg/ml gentamicin (G1397, Sigma Aldrich) and 0.15% bovine serum albumin (BSA), and maintained at 36.7 °C. Cumulus–oocyte complexes (COCs) were isolated from the ovaries and matured in vitro following the methodology outlined previously (Pioltine *et al.*, 2021). The maturation was carried out at 38.3°C over periods of 12, 36, and 52 hours. After 24 hours of culture, selected COCs from both control and heat-stressed groups were processed. For the induction of heat stress, the COCs were cultured at 41.0 ° C for 2 hours under 5% CO_2 conditions, after which they were returned to standard maturation conditions (38.3°C) (Lonergan *et al.*, 2003). Following treatment, the COCs were removed from their cumulus cells by vortexing in a 0.3% hyaluronidase solution.

SS-31 Treatment: For each experimental trial, selected cumulus–oocyte complexes (COCs) were randomly assigned to one of four treatment groups. These were

cultured in 50.00µL OMM drops on culture plates, with each drop containing 10 oocytes. The groups included an untreated control (without antioxidant) and three treatment groups supplemented with varying concentrations of mitochondrial-targeted antioxidant SS-31 (0.3, 0.6, and 0.9 µM/L). SS-31 was added at the start of IVM (0 h). The SS-31 compound (MTP-131; Bendavia) was obtained from MyBioSource, Inc. (USA).

Immunofluorescence Staining: The oocytes that had been stripped of their surrounding cells were fixed in a 4% paraformaldehyde solution prepared in PBS for 40 minutes at room temperature. Following fixation, they were rinsed twice with PBS containing 1% bovine serum albumin (BSA). Permeabilization was performed by incubating the oocytes in PBS with 0.5% Triton X-100 for 40 minutes at room temperature (Hao *et al.*, 2009). The samples were then blocked for nonspecific binding using a blocking buffer made of PBS with 5% BSA and 0.5% Triton X-100 for another 40 minutes at the same temperature. Next, the oocytes were incubated for one hour at 37 °C with a mouse-derived anti-alpha-tubulin antibody (Sigma-Aldrich, T6199, Merck, Darmstadt, Germany) diluted 1:500. Subsequently, two washes were performed using a labelling buffer containing 1% BSA and 0.1% Triton X-100, which was also used for all subsequent washing steps. To detect SIRT3 expression, the oocytes were then incubated overnight at room temperature with a rabbit anti-SIRT3 primary antibody (Abcam, ab189860, Cambridge, UK; 1:50 dilution). Following two washes, secondary antibodies were applied: anti-mouse Alexa 647 (Thermo Fisher Scientific, A-21235, USA; 1:250 dilution) and anti-rabbit FITC (Thermo Fisher Scientific, 31635, USA; 1:250 dilution). Nuclear staining was performed with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, 9542, Merck, Darmstadt, Germany) at a dilution of 1:100 for 10 minutes at room temperature. Finally, the stained oocytes were mounted in 10 µL of Fluoromount medium on glass slides, covered with a coverslip, and examined under a fluorescence microscope (Levenhuk MED PRO 600 Fluo, USA).

Mitochondrial Staining: To verify the location of SIRT3 within mitochondria, a co-staining procedure was performed using MitoTracker™ Red CMXRos (Invitrogen, Thermo Fisher Scientific, USA) as previously described (Acton *et al.*, 2004; Zhang *et al.*, 2011), a dye that labels active mitochondria. The oocytes were incubated with a 100 nM concentration of MitoTrackerTM Red CMXRos diluted in TCM199 medium for 40 minutes at 38.5°C under conditions of 5% CO2. After incubation, the oocytes were rinsed twice with PBS and subsequently fixed in 4% paraformaldehyde at room temperature for 15 minutes. Fixed samples were then processed using the previously described immunofluorescence staining protocol (Zhang *et al.*, 2015).

Image Analysis: Oocytes labeled through immunofluorescence were examined using ImageJ software (version 1.51j, Maryland, USA). The expression levels of sirtuins were determined by calculating the mean fluorescence intensity (MFI) for each oocyte, using arbitrary units. The ooplasm was designated as the region of interest for this analysis. All fluorescence images were captured under uniform exposure conditions and identical intensity settings. Negative control samples, prepared without the addition of primary antibodies, were processed and imaged under the same parameters to ensure consistency (Bolte *et al.*, 2006; Wang *et al.*, 2007).

Detection of Reactive oxygen species Levels in Oocytes: Reactive oxygen species (ROS) levels were assessed following the protocol established by Yao *et al.*, (2018). Cumulus–oocyte complexes (COCs) at the germinal vesicle stage were cultured at 38.3°C for durations of 12, 36, and 52 hours. After 12 hours of incubation, oocytes were treated with 0.3% hyaluronidase to detach cumulus cells, and the zona pellucida was enzymatically removed using 0.5% pronase. The prepared oocytes were then subjected to ROS detection.

To evaluate mitochondrial ROS levels, oocytes were incubated with 60 µM dihydrofluorescein diacetate (Sigma-Aldrich, USA) in HEPES-TL buffer containing 1% polyvinylpyrrolidone (HEPES-PVP). To detect cytoplasmic ROS, 37 µM of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen, Thermo Fisher Scientific, USA) diluted in HEPES-PVP was used. Positive controls included treatment with 200 µM hydrogen peroxide (ACS certified; Thermo Fisher Scientific, USA) and 100 µM tert-butyl hydroperoxide (tert-BOOH; Fluka/Sigma-Aldrich) in the same buffer (Khan *et al.*, 2015).

Fluorescence image analysis was performed using a Nikon Eclipse TE300 microscope (Nikon Instruments, USA) equipped with a DAPI filter set (excitation: 330–380 nm). Image analysis was performed using NIS-Elements BR software, version 3.0 (Nikon).

Measurement of ATP Content: Each oocyte was preserved individually at -80 °C in 50 µL of an ATP extraction buffer containing the following components: 99.0 mM NaCl, 3.1 mM KCl, 0.35 mM NaH_2PO_4, 21.6 mM sodium lactate, 10.0 mM HEPES, 2.0 mM $CaCl_2$, 1.1 mM $MgCl_2$, 25.0 mM $NaHCO_3$, 1.0 mM sodium pyruvate, 0.1 mg/mL gentamicin, and 6.3 mg/mL BSA (Stojkovic *et al.*, 2001). To measure ATP levels in individual oocytes, the Bioluminescent Somatic Cell Assay Kit (FL-ASC, Sigma-Aldrich, Canada) was used according to the manufacturer's protocol. A standard curve consisting of six concentration points (ranging from 40 pmol to 125 pmol) was prepared by running standard samples at both the beginning and the end of each assay. Internal quality control samples—representing high, medium, and low ATP levels—were prepared from three denuded oocytes through serial dilution, following the method previously described (Dadarwal *et al.*, 2017). The ATP concentration in each oocyte was then calculated using a linear regression equation derived from the standard curve.

Oocyte Mitochondrial Activity: Mitochondrial function in oocytes was evaluated using the JC-1 dye (T3168, Molecular Probes), which selectively stains mitochondria based on their membrane potential. The oocytes were treated with a 1.8 µM solution of JC-1 prepared in TCM199 medium (excluding HEPES), enriched with 0.3 mM sodium pyruvate and 4 µl/ml gentamycin, and incubated for 40 minutes at 39 °C under high humidity.

Following incubation, the oocytes were rinsed twice with PBS, placed on microscope slides, covered with coverslips, and examined using a fluorescence microscope (Levenhuk MED PRO 600 Fluo, USA). Fluorescence emissions were detected at 546/12 nm for red and 486/20 nm for green signals (Wilding *et al.*, 2001). Mitochondrial activity was assessed by measuring green fluorescence intensity within the oocyte cytoplasm, with regions of interest defined using ImageJ software. All samples were analyzed under consistent exposure and imageing conditions.

Statistical Analyses: Statistical analyses were performed using the Statistical Analysis System software package (SAS 9.2; SAS Institute Inc., Cary, NC, USA), and probabilities ≤0.05 were considered significant, whereas P values >0.05 to ≤0.10 were considered tendencies. Mean and standard deviation (SD) were calculated. To evaluate the effects of antioxidant concentration and incubation time on protein levels, data were analysed using a two-way ANOVA followed by post hoc comparisons with the Least Significant Difference (LSD) test. Differences with a high level of significance ($P < 0.01$) are marked by capital letters and double asterisks (**; A), whereas single asterisks (*) and lowercase letters (a, b) denote statistically significant differences at $P < 0.05$. Each experiment was performed in six independent replicates.

RESULTS

SIRT3 analysis: In the study carried out, a decrease in mean fluorescent intensity (MFI) was observed in the samples tested during the experiment (12, 36, and 52 hours) (Fig. 2). The MFI values correspond to the fluorescence signal intensity of SIRT3 immunolabeling (FITC channel) measured within the ooplasm of each oocyte. This parameter reflects the relative expression level of the SIRT3 protein, quantified using ImageJ software (Schneider *et al.*, 2012) under consistent imaging and exposure conditions. This decrease was highly statistically significant (P<0.01). The highest MFI was recorded after 12 hours of incubation in the group treated with the highest antioxidant concentration of 0.9 µM, reaching a value of 131.632%. In the group with 0.6 µM antioxidant concentration, MFI was 131.526%, while in the group with the lowest concentration (0.3 µM), the MFI reached 124.158%. In the control group (0 µM), the MFI was 118.684%. The lowest mean fluorescent intensity was observed after the longest incubation time, i.e., 52 hours. In each incubation group, MFI increased along with the concentration of the applied antioxidant (Fig. 2). Moreover, statistically significant differences between groups within the same time class were also detected (Fig. 2). Highly significant statistical differences (P<0.01) were found between the control group without antioxidant (0.0 µM) and the other groups (0.3, 0.6, and 0.9 µM), marked with the capital letter on the graph. Significant differences (P<0.05) were also found between the 0.3 µM group and the 0.6 and 0.9 µM groups, marked with letters. However, no statistically significant differences were detected between the 0.6 and 0.9 µM groups after 12 and 36 hours of incubation. In the group incubated for 52 hours, statistically significant differences were observed at the P<0.05 level of P <0.05 and marked with the letter a.

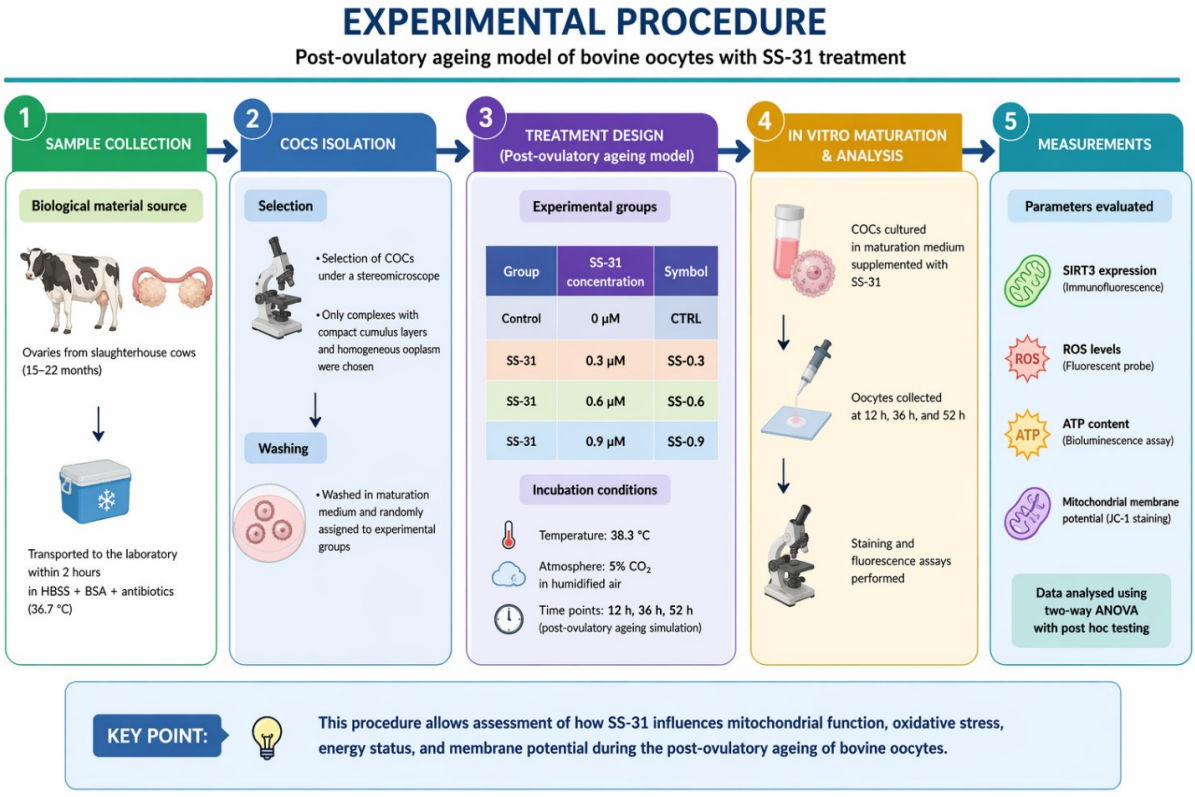

Fig. 1: Methodological procedure diagram [This diagram was developed using Canva (Australia; Canva Pty Ltd), incorporating graphical elements obtained from BioRender (Canada; BioRender Inc.), with additional support provided by AI tools.

ROS levels in oocytes: During the study, the level of reactive oxygen species (ROS) was also analyzed, with the results presented in Figure 3 (Fig. 3). ROS levels were found to increase over the course of incubation (Fig. 3). The highest ROS values were recorded after 52 hours, especially in the control group. The group without the addition of an antioxidant showed the highest ROS levels among all incubation time classes. Specifically, for the 12-hour incubation, the ROS level in the control group was a 1.202 fluorescent ratio, and for the 36-hour incubation, it was a 2.175 fluorescent ratio, and for the 52-hour incubation, it reached a 3.890 fluorescent ratio. Additionally, the ROS levels significantly increased over time at the P<0.01 level (Fig. 3). Statistically significant differences in ROS levels were also observed between the antioxidant concentration groups (0, 0.3, 0.6, and 0.9 µM) at the P<0.05 level, except for the values of 1.597 and 1.709 fluorescent ratio for the concentrations of 0.6 and 0.9 µM, respectively, during the 36-hour incubation (Fig. 3). For the 12-hour incubation, differences were detected between all antioxidant concentration groups, as well as in the 52-hour incubation group. In the 36-hour incubation group , statistically significant differences at the P<0.05 level were found between the control group (0 µM) and the group with the lowest applied antioxidant concentration (0.3 µM) (Fig. 3). Within this 36-hour incubation class, the highest ROS level was recorded in the control group (2.175 fluorescent ratio), while among the antioxidant-treated groups, the highest ROS level was observed in the 0.9 µM group (1.708 fluorescent ratio).

ATP content: An increase in ATP content was observed across all incubation periods (12, 36, and 52 hours). These differences were highly statistically significant (P<0.01) (Fig. 4). In each of the analyzed classes, the highest ATP levels were recorded in the control groups (0 µM), with values of 1.311 pmol for 12-hour incubation, 1.634 pmol for 36-hour incubation, and 1.921 pmol after 52 hours. Conversely, the lowest observed ATP levels were found in the group with the highest applied antioxidant concentration, i.e., 0.9 µM (1.062, 1.161, and 1.766 pmol, respectively) (Fig. 4). During the study, statistically significant differences at the P<0.05 level were also detected within the antioxidant concentration groups (0, 0.3, 0.6, and 0.9 µM).

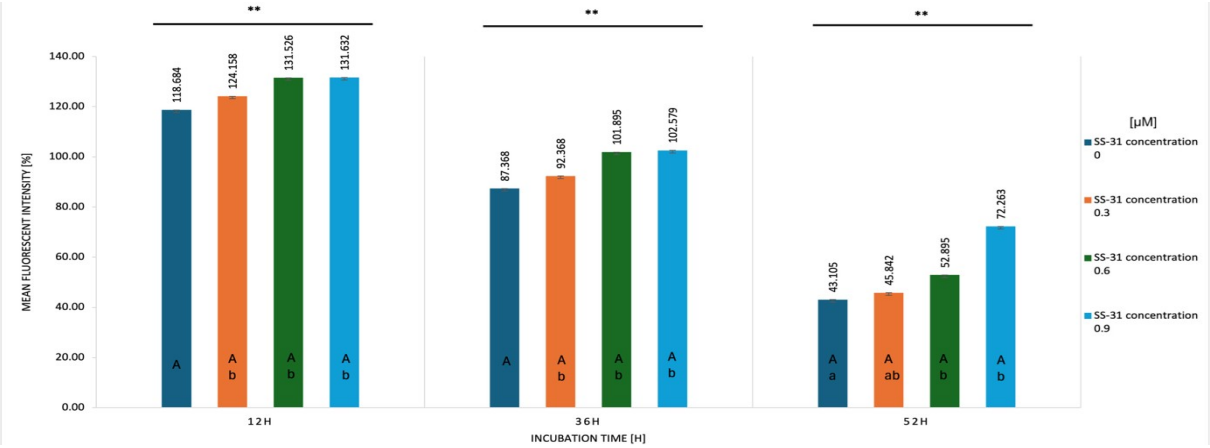

Fig. 2: Dynamics of SIRT3 changes (MFI [%]) in oocytes depending on incubation and antioxidant concentration. ** – indicates highly significant differences between incubation groups, at P<0.01. Markings a, b – indicate significant differences between the selected group of antioxidant concentration and SIRT levels at P<0.05. A – indicates highly significant differences between each group of antioxidant concentration and SIRT levels at P<0.01. Data represent mean ± SD of six independent biological replicates (n = 6), with 10 oocytes analysed per treatment group and incubation time in each replicate (total = 60 oocytes per condition).

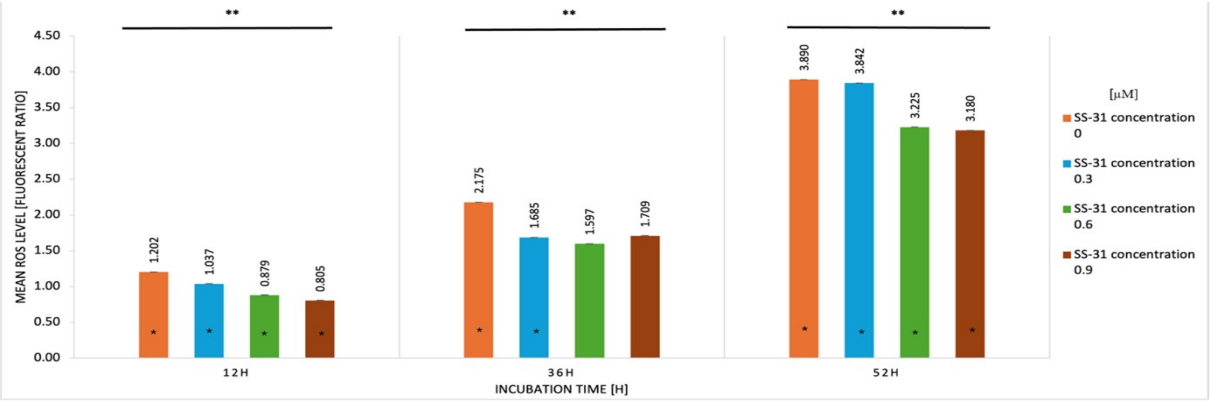

Fig. 3: Changes in ROS level [fluorescent ratio] in oocytes depending on incubation and antioxidant concentration. ** - indicate highly significant differences between incubation groups, at P<0.01. * - indicate significant differences between each group of antioxidant concentration and ROS levels at P<0.05. Data represent mean ± SD from six independent biological replicates (n = 6), with 10 oocytes analyzed per treatment group and incubation time in each replicate (total = 60 oocytes per condition).

Mitochondrial activity: In the case of the analyzed parameter, which is mitochondrial activity, statistically significant differences (P<0.05) were shown in the groups of concentrations 0.0 in relation to 0.3, 0.6, and 0.9 µM in the classes for 12h and 36h incubation. In the 12h incubation class, no statistically significant differences were detected between the samples with different antioxidant values used, similarly to the 52-hour incubation class. Highly statistically significant differences were noted for the 36h incubation class between the tested concentrations of 0, 0.3, 0.6, and 0.9 µM (Fig. 5). After 12h and 36h incubation, the highest values of mitochondrial activity were noted in the group with the applied concentration of 0.6 µM – 55.339 and 55.925 JC1 fluorescence intensity/UA. In the 52-h incubation class, the highest MMT value was recorded for the concentration of 0.3 µM (50.917) (Fig. 5).

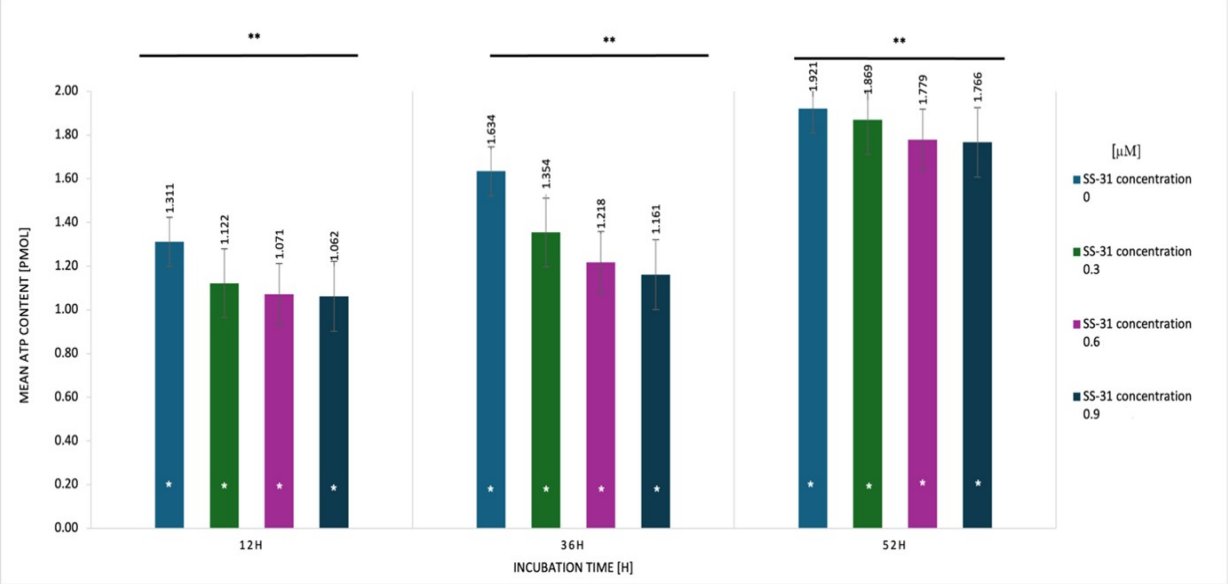

Fig. 4: Changes in ATP content [pmol] in oocytes depending on incubation and antioxidant concentration. * - indicate significant differences between incubation groups, at P<0.05. ** - indicate highly significant differences between each group of antioxidant concentration and MMT levels at P<0.01. Data represent mean ± SD from six independent biological replicates (n = 6), with 10 oocytes analyzed per treatment group and incubation time in each replicate (total = 60 oocytes per condition).

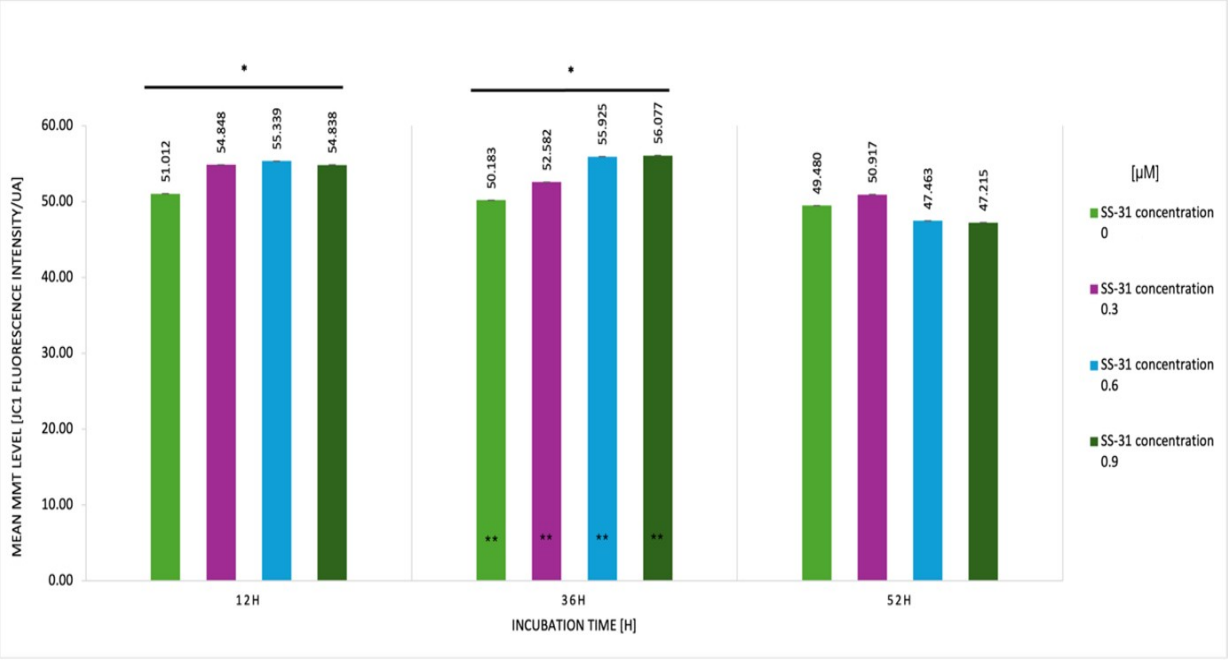

Fig. 5: Changes in MMT level [JC-1 fluorescence intensity/UA] in oocytes depending on incubation and antioxidant concentration. * - indicate significant differences between incubation groups, at P<0.05. ** - indicate highly significant differences between each group of antioxidant concentration and MMT levels at P<0.01. Data represent mean ± SD from six independent biological replicates (n = 6), with 10 oocytes analyzed per treatment group and incubation time in each replicate (total = 60 oocytes per condition).

DISCUSSION

The purpose of this study was to examine how the new mitochondrial-targeted antioxidant SS-31 affected oxidative stress, energy production, and mitochondrial functions in bovine oocytes under conditions that cause cellular ageing. In this context, the results provide additional insight into the role of SS-31's ability to prevent mitochondrial dysfunction linked to oocyte ageing. The experimental model simulates post-ovulatory ageing under carefully regulated in vitro maturation (IVM) conditions. Cellular and mitochondrial alterations similar to those observed in aged female oocytes were induced by prolonged incubation. Thus, while the "ageing" being studied in this work relates to time-induced changes in the mitochondrial function of oocytes in culture, not chronological age of the donor, it should still be understood in terms of reproductive ageing, as mentioned in the Introduction. Thus, the term “ageing” mentioned throughout this paper pertains to post-ovulatory ageing, unless indicated differently.

The progressive decrease in mean fluorescence intensity (MFI) observed over time in all experimental groups is consistent with the known decline in mitochondrial function during cellular ageing (Salmón *et al.*, 2023). During the study, supplementation with SS-31 at all tested concentrations (0.3, 0.6, and 0.9 μM) appeared to attenuate this decrease, which may indicate, at least in part, a protective effect of SS-31 on SIRT3 activity. SIRT3 protein is expressed mainly in mitochondria (Zhou *et al.*, 2022), and it is the antagonist of ageing processes in human mesenchymal stem cells (Diao *et al.*, 2021). In addition, SIRT3 reduces oxidative stress in mitochondria by uptake of ROS (Wu *et al.*, 2025).

SS-31 interacts with cytochrome c and cardiolipin to promote electron transport and mitochondrial functions, and it can accumulate primarily in the inner mitochondrial membrane (Li *et al.*, 2024; Shan *et al.*, 2023). Changes in cardiolipin may cause the inner mitochondrial membrane to fold differently, which may affect the ability of the electron transport chain (ETC) ability to produce ATP. Cell ageing is associated with cardiolipin peroxidation (Szeto, 2014; Dudek, 2019). Elamipretide reduces oxidative stress by eliminating free radicals (Suo *et al.*, 2022). By increasing the activity of mitochondrial complexes, SS-31 has been shown to act on mitochondria and enhance their function (Chatfield *et al.*, 2019). Numerous studies have confirmed SS-31's broad antioxidant effect. (Wu *et al.*, 2015, 2016, 2017; Zhou *et al.*, 2023; Ye *et al.*, 2024). However, according to currently available studies, there is no information on the use of SS-31 on oocytes.

Reactive oxygen species (ROS) analysis revealed a notable, time-dependent rise in oocytes. In contrast to the untreated control group, SS-31 treatment steadily lowered ROS accumulation, particularly at earlier incubation times. This may indicate that SS-31 enhances the antioxidant capacity of oocytes, most likely due to its ability to scavenge oxygen radicals. Studies on subarachnoid haemorrhage (Zhou *et al.*, 2023), the capacity of SS-31 to prevent airway inflammation (Yang *et al.*, 2021), diabetic kidney disease (Hou *et al.*, 2024), and other conditions have shown this.

The control groups showed higher ATP content; in contrast, SS-31–treated oocytes exhibited lower levels, which was unexpected. An increase in mitochondrial membrane potential was observed following SS-31 treatment, especially at 0.6 μM after 12 and 36 hours of incubation. This observation is consistent with the hypothesis that SS-31 may strengthen mitochondrial quality by preserving membrane polarization, which is essential for ATP synthesis and oocyte maturation. However, after a longer incubation period (52 hours), no discernible improvement was found, indicating that the beneficial effects of SS-31 might only be present in the early or intermediate stages of oocyte ageing. According to current findings, SS-31 may reduce oxidative damage and mitochondrial dysfunction linked to oocyte ageing. Although the ATP content of the control group was higher than that of the oocytes treated with SS-31, this observation does not mean that mitochondrial functions have been improved, as the metabolic state of the mitochondria was not directly assessed in this experiment. In contrast, it can suggest a compensatory activation of ATP production due to inefficiency in mitochondrial function. To maintain basal energy levels, dysfunctional mitochondria in ageing oocytes frequently show increased electron leakage, elevated ROS production, and overactivation of oxidative phosphorylation. This process may contribute to accelerated mitochondrial damage and increased energy inefficiency. The stabilization of the cardiolipin molecule and reduced ROS formation by SS-31 likely increased electron transport efficiency and minimized the energy need to produce excessive ATP. Interestingly, as a result, steady-state ATP levels decreased slightly, not due to impaired synthesis but due to improved metabolic efficiency and reduced energy wastage.

Evidence from mouse studies also points to a protective role of this antioxidant in maintaining mitochondrial function, reducing ROS production, and supporting ATP levels and membrane potential. In a study conducted on 56 male mice aged 15 months, the effect of SS-31 on the protective functions of mitochondria against the action of isoflurane was analyzed (Wu *et al.*, 2016). For this purpose, the animals were divided into four groups: control and three tested: one with the addition of SS-31, the second with isoflurane, and the third with isoflurane and SS-31. Each group contained 14 individuals. Isoflurane was administered to the animals as an anesthetic, of which even 1.5% administered for 2 hours can result in impaired cognitive functions in adult mice. SS-31 was administered intraperitoneally in the amount of 0.4 ml/kg. In order to analyze mitochondrial functions, electron transport chains were analyzed in the hippocampus of mice, where it was shown that individuals exposed to isoflurane showed lower activity of ETC complex I, while SS-31 administration led to improvement of the activity of this complex. Analyzing the levels of ATP and ROS, the authors showed that the group of mice treated with isoflurane showed lower ATP levels and higher ROS compared to the control group. SS-31 treatment led to the reversal of the effects of isoflurane and thus of mitochondrial functions (Wu *et al.*, 2016).

The results of this study underscore the multifaceted benefits of SS-31 as a mitochondria-targeted therapeutic, particularly in the context of oocyte ageing. The effect of the peptide in reducing oxidative stress, maintaining mitochondrial membrane potential, and regulating mitochondrial protein expression is undoubtedly essential in improving oocyte quality.

One of the most compelling observations was the dose-dependent upregulation of SIRT3, a mitochondrial deacetylase known to be involved in mitochondrial resilience, redox homeostasis, and overall cellular longevity. Increased SIRT3 levels, particularly at 0.6 and 0.9 μM, indicate that SS-31 can help increase mitochondrial repair mechanisms and thus prevent degeneration that occurs due to oocyte ageing.

Furthermore, SS-31 effectively lowered ROS levels, confirming its antioxidant potential. This reduction in oxidative damage likely contributed to the preservation of mitochondrial structure and function, as reflected in the sustained mitochondrial membrane potential observed in treated groups. These results are significant, considering that high ROS levels are one of the primary causes of meiotic errors and compromised embryo development in aged oocytes.

Interestingly, although ATP content decreased slightly with SS-31 treatment, this may reflect a metabolic shift towards improved mitochondrial efficiency rather than dysfunction. The preservation of mitochondrial membrane polarization, despite lower ATP levels, indicates that SS-31 may promote a more stable and less energetically wasteful mitochondrial state under stress.

Taken together, the results appear to indicate, at least in part, that SS-31 confers cytoprotective effects on oocytes exposed to ageing-related stress, and its actions may be particularly beneficial during the early and intermediate stages of in vitro maturation. From a translational perspective, this highlights the potential of SS-31 as an adjunct in assisted reproductive technologies (ART), especially for patients of advanced maternal age, where mitochondrial dysfunction is a critical barrier to successful fertilization and embryo development.

This study demonstrated the multifaceted benefits of the mitochondria-specific peptide SS-31 in counteracting ageing-induced mitochondrial dysfunction in bovine oocytes. SS-31 stabilized the mitochondrial architecture and improved mitochondrial respiratory performance through selective localization to the inner mitochondrial membrane and binding to cardiolipin. This experiment confirmed that SS-31 treatment significantly increased SIRT3 expression, indicating activation of mitochondrial immune pathways that support redox balance and protein deacetylation. Furthermore, the tested peptide significantly reduced reactive oxygen species (ROS), confirming its potent antioxidant properties and ability to mitigate oxidative stress, a key factor in oocyte ageing. The slight reduction in the ATP content could suggest a change in mitochondrial metabolic activity; however, this hypothesis is still tentative since no metabolic studies have been conducted. Importantly, the mitochondrial membrane potential improved, particularly at 0.6 μM, suggesting that SS-31 preserved membrane polarity and overall organelle integrity. From this, it is evident that SS-31 is an effective means of enhancing mitochondrial function, alleviating oxidative stress, and facilitating cellular stability, therefore, it is a powerful approach towards ensuring biological quality in oocytes through the ageing process.

Limitations and Future Directions

Although this study provides new information on the protective effects of SS-31 on mitochondrial function and oxidative stress in aged bovine oocytes, certain limitations must be acknowledged. First, our analyses focused primarily on mitochondrial activity, SIRT3 expression, ROS levels, and ATP content as indicators of oocyte quality. Although these parameters are fundamental to understanding cellular mechanisms of ageing, we did not assess the direct consequences of SS-31 treatment on oocyte maturation rates, fertilization competence, embryo development, or subsequent embryonic quality. These results are essential for determining the true biological and clinical relevance of SS-31 in improving reproductive potential.

Therefore, future studies should extend the present findings by incorporating in vitro fertilization assays, embryo culture, and developmental competence analyses to establish whether the observed improvements in mitochondrial function translate into enhanced reproductive success. Such experiments would not only validate the role in supporting oocyte viability but also provide a more comprehensive framework for its potential application in assisted reproductive technologies (ART).

In addition, implementing more rigorous imageing archiving strategies will allow the inclusion of representative fluorescence images alongside quantitative data in future investigations, thus improving transparency and reproducibility. By addressing these limitations, subsequent studies can more definitively assess the therapeutic value of SS-31 in counteracting age-related reproductive decline.

Conclusions: This is among the first comprehensive studies to assess SS-31 in the context of reproductive ageing, providing an innovative direction for future fertility-enhancing therapies. The data obtained suggest that SS-31 represents a promising therapeutic agent for improving mitochondrial function and reducing oxidative stress in ageing oocytes. Further research is needed to better understand how this compound may influence conception rates, embryo quality, and long-term developmental outcomes, particularly in relation to assisted reproductive technologies (ART) in women of advanced maternal age. The study positions SS-31 as a candidate for therapeutic development not only in reproductive health but also in broader contexts of mitochondrial dysfunction and age-related diseases. Future studies should aim to validate these findings in human models and explore whether SS-31 enhances not only oocyte quality but also fertilization rates, embryo viability, and pregnancy outcomes. The inclusion of SS-31 in clinical ART protocols may represent a novel strategy to overcome mitochondrial impairments that limit reproductive success in older women.

Authors statement: All listed authors (AK, MCG, JHAM, JPA, WW, JG, MS, MW, EG, EC-P, and ZD)

made substantial contributions to this work AK and MCG were responsible for the study design; MW, AK, WW, ZD, and JHAM developed the methodology; EG, EC-P, MCG, and JG conducted the experiments; EC-P performed data analysis; and MW, AK, JHAM, MS, and EC-P contributed to manuscript preparation.

Conflicts of Interest Statement: The authors declare no conflict of interest.

Funding: This research was funded in part by the National Science Centre, Poland [Grant number: 2025/09/X/NZ3/00003]. For the purpose of Open Access, the author has applied a CC-BY public copyright licence to any Author Accepted Manuscript (AAM) version arising from this submission. The APC/BPC is financed/co-financed by West Pomeranian University of Technology in Szczecin. This research was carried out as a part of the activities of the leading research group – Animal Science for Future (ASc4Future).

Ethical Statement: The experiment has been performed as part of routine activities during the current semen production in the reproductive station and did not require the approval of the ethics committee.

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