Reproduction in Domestic Animals

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Evaluation of Different Cryoprotectant Combinations in Vitrification and Slow Freezing for Ovarian Tissue Preservation in Domestic Cats

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ABSTRACT

Over the past decade, increased hunting and habitat disturbance have significantly impacted the endangered population within the Felidae family. Recognising this, it becomes imperative to implement strategies aimed at mitigating this concerning conservation scenario. For this, female fertility preservation is crucial in this context, and studies concerning this field are still scarce. In the realm of cryopreservation, prevalent methods involve slow freezing (SF) and vitrification (V). This study aimed to evaluate various cryoprotective combinations for V or SF processes applied to domestic cat ovarian tissue. Twenty ovaries from 10 healthy cats were dissected, and cortical regions were sectioned into eight fragments measuring 3 mm^3 each. These fragments were randomly allocated to three different treatment groups for V (V1, V2 and V3) or SF (SF1, SF2 and SF3). Each group employed solutions with varying concentrations of DMSO, EG and either trehalose or sucrose. The assessment included histological evaluation, follicle counting, immunohistochemical analysis of proliferative activity, and ultrastructural examination. The results demonstrated that the V1 protocol—composed of an equilibration solution with 10% DMSO, 10% EG and 0.1 M trehalose, followed by a V solution with 20% DMSO, 20% EG and 0.1 M trehalose—proved most effective. This combination best preserved follicular morphology, reduced degeneration, supported follicle proliferation and maintained favourable ultrastructural integrity compared to other treatments. These findings provide a valuable foundation for improving fertility preservation in domestic cats, with potential applications for endangered felid conservation programs.

1 | Introduction

Over the past decade, hunting and habitat disruption have resulted in a notable rise in the number of endangered species within the Felidae family. The International Union for Conservation of Nature (IUCN) has reported that a significant proportion of wild cat species currently fall under classifications such as at-risk, vulnerable, endangered, or on the brink of

imminent extinction, as delineated in the Red List (IUCN 2023). In consideration of this information, it is imperative to implement measures aimed at reversing or alleviating this alarming conservation predicament.

Preservation strategies have utilised biological resources for biodiversity maintenance, acknowledging the potential risk to the entire ecosystem, resulting from the loss of a species (Praxedes et al. 2018; Tanpradit et al. 2015). Ex-situ conservation methods, such as captive breeding programs, offer viable alternatives for the reintroduction of individuals from threatened species (Conde et al. 2011; McGowan et al. 2017). In this context, domestic cats emerge as an optimal research model for studies on feline reproduction. Owing to their reproductive similarities to wild species, they have proven to be a practical and ethically viable alternative.

In the context of cryopreservation, the predominant methods involve slow freezing (SF) and vitrification (V). These techniques have exhibited significant success across a range of species, yielding favourable outcomes in the preservation of ovarian tissue, restoration of endocrine function, and the achievement of fertility following the transplantation of cryopreserved ovarian tissue (Candelaria and Denicol 2023; Dolmans and Donnez 2021; Hartzler et al. 2023; Lunardi et al. 2013; Nowak et al. 2022; Vilela et al. 2019).

To ensure cell viability and mitigate cellular damage during the cryopreservation process, cryoprotective agents (CPAs) are employed to protect against dehydration injury and control ice crystal formation and growth. These agents establish a protective barrier against potential damage induced by the formation of ice crystals (Benson et al. 2008). CPAs are commonly classified as either intracellular or extracellular, depending on their mode of action within cells. Intracellular agents encompass low-molecular-weight organic compounds, such as ethylene glycol (EG) and dimethyl sulfoxide (DMSO), renowned for their high penetration efficiency, allowing them to traverse the cell membrane effectively (Fickel et al. 2007; Purdy 2006). On the other hand, extracellular agents typically comprise macromolecules or sugars that have limited penetration into the cell but induce cellular dehydration through osmotic effects. This, in turn, reduces penetration of ice crystals into the cell. Commonly used sugars in this category include sucrose, glucose, galactose and trehalose (Çelikkan et al. 2023; Santos et al. 2010; Shaw et al. 2000).

Several studies have investigated reproduction and the influence of different concentrations of cryoprotectants on the cryopreservation of ovarian tissue in wild felids (Andrews et al. 2019; Brito et al. 2018; Crichton et al. 2003; Jewgenow et al. 2011; Lima et al. 2006; Wiedemann et al. 2012, 2013). Tanpradit et al. (2015) demonstrated that both cryoprotectant concentration and cryopreservation methods have discernible effects on diverse parameters, including follicular morphology, follicular and stromal apoptosis, and the incidence of polyovular follicles. However, a notable gap in knowledge concerning optimal protocols for preserving ovarian function in felines is attributed to the intricate and sensitive nature of ovarian tissue in responding to variations in cryopreservation conditions (Wood et al. 1997).

The recent study conducted by Brito et al. (2018) compared two cryoprotectant combinations (20% EG and 10% EG + 10% DMSO) for the cryopreservation of ovarian tissue from female cats. The combination of EG + DMSO and trehalose was effective in preserving the number of normal follicles in solid surface V. These findings align with previous studies that reported the development of early- to advanced-stage follicles after SF of ovarian tissue obtained from female cats using EG (Bosch et al. 2004; Wiedemann et al. 2012, 2013).

The efficacy of ovarian tissue cryopreservation in felids is highly dependent on the strategic composition of cryoprotectant solutions, as these chemical agents play a fundamental role in mitigating cryoinjury during both V and SF protocols. This study aimed to evaluate and compare two established cryopreservation techniques—V and SF—applying three distinct cryoprotectant formulations to domestic cat ovarian tissue. Each protocol incorporated varying concentrations of DMSO and EG, supplemented with either trehalose or sucrose as non-permeating cryoprotectants. These findings not only establish an optimised protocol for domestic cat ovarian tissue banking but also provide critical empirical data that may inform the development of assisted reproduction strategies for endangered felid species, where female gamete preservation represents a crucial component of modern conservation efforts. The results underscore the importance of cryoprotectant selection in maintaining tissue functionality post-preservation while highlighting the need for species-specific optimisation of reproductive biotechnologies.

2 | Materials and Methods

2.1 | Animals and Ovarian Tissue Collection

Twenty ovaries were surgically removed from 10 healthy female cats (approximately 1 year old at random stages of oestrous cycle), through elective bilateral ovariohysterectomy (OHE) at a veterinary clinic (*Casa do Gato*, Brasília-DF, Brazil). Only macroscopically normal ovaries without corpus luteum or cysts were deemed eligible for inclusion in the study.

2.2 | Experimental Design

The ovaries were promptly transported to the laboratory in a sterile 0.9% saline solution at 37°C within 1 h. Following the methodology outlined by Gorricho et al. (2018), eight fragments, each measuring 3 mm^3, were extracted from the cortical region of each ovary, resulting in a total of 16 ovarian tissue fragments per cat ($n = 160$ fragments in total). From each ovary, two fragments were immediately fixed in 4% paraformaldehyde for 24 h, serving as the fresh control ($n = 40$ fragments), while a small portion was preserved in Karnovsky's solution for subsequent

transmission electron microscopy (TEM) analysis. The remaining fragments ($n = 120$) were randomly assigned to various experimental groups ($n = 20$ fragments in each group/6 experimental groups). The solutions for the experimental groups were designated as follows:

- Slow freezing 1 (SF1): 1 M DMSO + 1 M EG + 10% fetal bovine serum (FBS) + 0.4% trehalose;
- Slow freezing 2 (SF2): 1.5 M DMSO + 10% FBS + 0.4% sucrose;
- Slow freezing 3 (SF3): 1.5 M DMSO + 10% FBS + 0.4% trehalose;
- Vitrification 1 (V1): equilibration solution: 10% DMSO + 10% EG + 0.1 M trehalose; vitrification solution: 20% DMSO + 20% EG + 0.1 M trehalose;
- Vitrification 2 (V2): equilibration solution: 10% DMSO + 10% EG + 0.1 M sucrose; vitrification solution: 20% DMSO + 20% EG + 0.1 M sucrose;
- Vitrification 3 (V3): equilibration solution: 20% DMSO + 0.1 M trehalose; vitrification solution: 40% DMSO + 0.1 M trehalose.

All solutions were prepared in Minimum Essential Medium (MEM + HEPES). The experimental procedures are visually

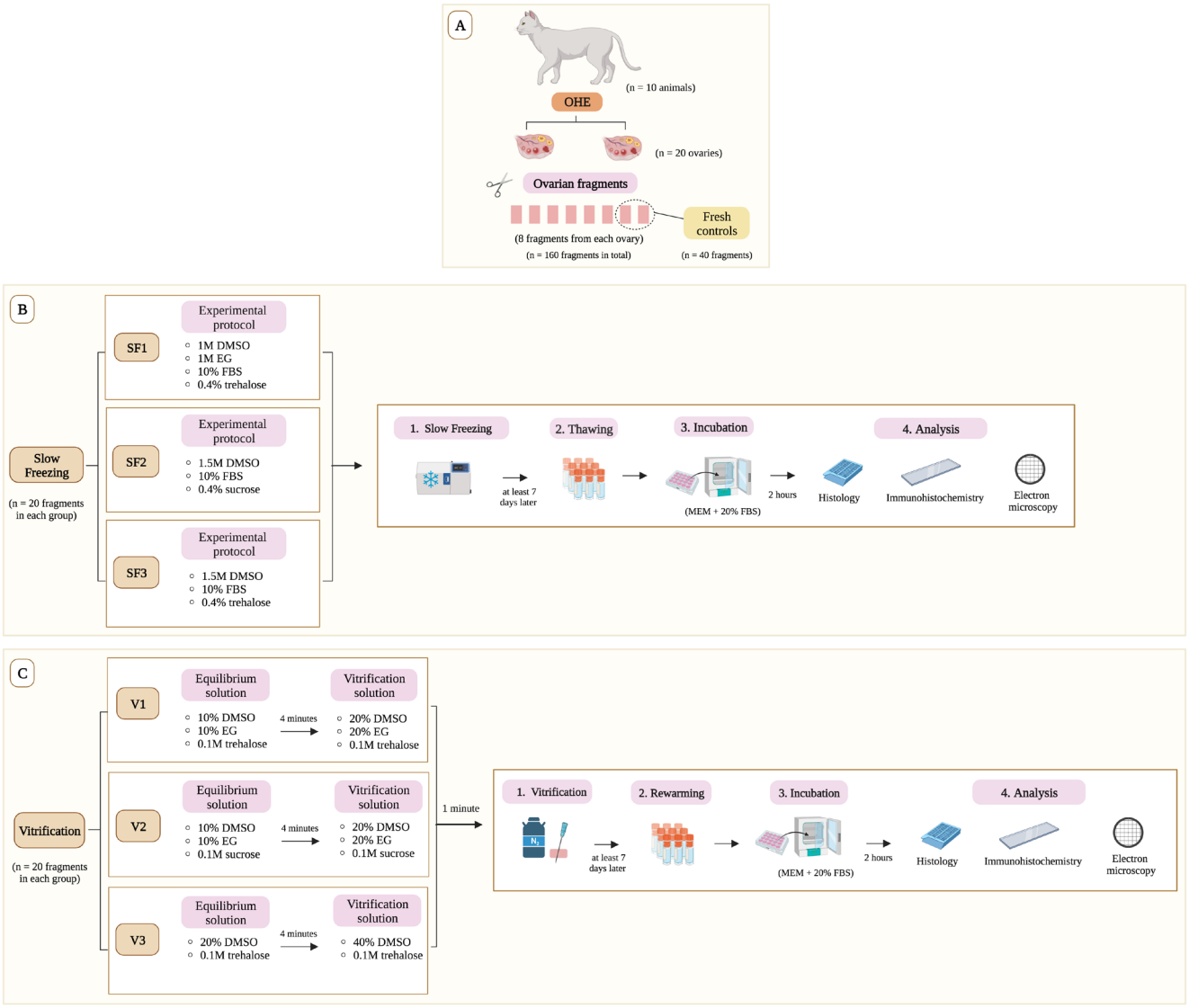

FIGURE 1 | Experimental design. (A) The cortical region of ovaries from 10 healthy cats was each cut into eight 3 mm^3 pieces. Pieces from each ovary were allocated to eight groups and processed as shown in the figure. (B and C). Fragments underwent SF (SF1, SF2 and SF3) and V (V1, V2 and V3) procedures as shown in the image and immediately after were stored in liquid nitrogen (−196°C) for at least 7 days before thawing. After thawing/rewarming, fragments were incubated in MEM + 20% FBS for 2 h and then fixed in 4% paraformaldehyde for histological analysis. SF1 (slow freezing 1): 1 M DMSO + 1 M EG + 10% fetal bovine serum (FBS) + 0.4% trehalose. SF2 (slow freezing 2): 1.5 M DMSO + 10% FBS + 0.4% sucrose. SF3 (slow freezing 3): 1.5 M DMSO + 10% FBS + 0.4% trehalose. V1 (vitrification 1): Equilibration solution: 10% DMSO + 10% EG + 0.1 M trehalose; vitrification solution: 20% DMSO + 20% EG + 0.1 M trehalose. V2 (vitrification 2): Equilibration solution: 10% DMSO + 10% EG + 0.1 M sucrose; vitrification solution: 20% DMSO + 20% EG + 0.1 M sucrose. V3 (vitrification 3): Equilibration solution: 20% DMSO + 0.1 M trehalose; vitrification solution: 40% DMSO + 0.1 M trehalose. DMSO, Dimethyl sulfoxide; EG, Ethylene glycol; FBS, Fetal bovine serum; MEM, Minimum Essential Medium.

depicted in Figure 1, offering a schematic visual representation of the study design.

2.3 | Ovarian Tissue Cryopreservation and Thawing/Rewarming Processes

2.3.1 | SF and Thawing

The cryopreservation of ovarian tissue fragments followed the protocol outlined by Lima et al. (2006). In summary, the fragments were placed in cryovials containing 1 mL of freezing solution (SF1, SF2 or SF3), as previously described, and maintained at 10°C for 10 min. Subsequently, the vials were transferred to a programmable freezer (Cryogen-Neovet, Uberaba, Brazil) and cooled at −2°C/min until reaching −7°C, at which point crystallisation was manually induced (seeding). The cryovials were then further cooled at −0.3°C/min to −35°C. Finally, each cryotube containing one fragment was immersed and stored in a liquid nitrogen tank (−196°C) for at least 7 days.

For the thawing process, ovarian tissue fragments underwent a sequential procedure to remove the cryoprotectants. Initially, they were exposed to ambient room temperature for 1 min, followed by immersion in a water bath set at 37°C for 5 min to ensure complete thawing of the samples. Subsequently, the samples were subjected to three successive 5-min rinses at room temperature in solutions containing MEM and 10% FBS with decreasing concentrations of sucrose or trehalose, according to the experimental group (0.4%, 0.2% and 0%), with progressively decreasing concentrations of cryoprotectants: 0.5 M, 0.25 M and 0 M for the SF1 group, or 0.75 M, 0.375 M and none for the SF2 and SF3 groups.

2.3.2 | V and Rewarming

For the V technique, the cryopreservation of ovarian tissue fragments followed the protocol outlined by Brito et al. (2018). In brief, the fragments underwent a 4-min exposure to the equilibration solution, followed by a 1-min immersion in the V solution, both consistently maintained at 0°C, as previously detailed. The V process was manually executed using a 30G × 1/2 needle (Needle Immersed Technique), rapidly freezing the tissue by direct contact with liquid nitrogen. Subsequently, the fragments were promptly transferred to cryotubes cooled to approximately −196°C, with each needle containing one fragment placed into each cryotube. The cryotubes were then stored for at least 7 days.

Before analysis, the fragments underwent rewarming. Subsequently, each sample remained in the needle and was allocated in the rewarming solutions. Initially, they were transferred to 1.5 mL MEM containing 0.4% sucrose (or trehalose, according to the experimental group) with 20% FBS, and incubated for 5 min. They were then transferred to a solution containing 0.2% sucrose (or trehalose according to the experimental group) in 1.5 mL of MEM supplemented with 20% FBS, and incubated for another 5 min. Finally, the samples were moved to MEM without trehalose or sucrose for 5 min to remove all cryoprotective solutions.

2.3.3 | Incubation After Thawing/Rewarming

After the thawing or rewarming process, the retrieved fragments underwent a 2-h incubation in MEM supplemented with 20% FBS. The incubation took place in an incubator set at 37°C with elevated relative humidity and 5% CO_2, aiming to allow the tissue to return to its normal temperature and metabolism, without intent to promote growth (Borges et al. 2009). Following the incubation, the fragments were promptly fixed in 4% paraformaldehyde for 24 h and subsequently transferred to 70% ethanol for histological analysis.

2.4 | Histological and Immunohistochemical Analyses

2.4.1 | Histological Analysis

After fixation, the ovarian tissue samples underwent dehydration using increasing ethanol solutions. Subsequently, the samples were clarified in three xylene baths and embedded in Paraplast (Sigma-Aldrich, St. Louis, MO, USA). All ovarian tissue was serially sectioned into 5 μm thick slices, with every fifth section subjected to haematoxylin–eosin (HE) staining. The remaining sections were reserved for immunohistochemistry. The slides were examined under a light microscope (Leica DM500, Wetzlar, Germany), and follicles were counted and classified based on their developmental stage (primordial and growing follicles) and integrity, including morphologically normal (MN) or degenerated structures (Lee et al. 2016; Picton 2001; Picton and Gosden 2000). Follicles demonstrating a consistent arrangement of granulosa cells, round oocytes and visible nuclei were classified as MN. In contrast, follicles classified as degenerated exhibited at least two of the following characteristics: disorganised granulosa cells, oocytes entirely or partially detached from the granulosa cells, vacuoles in the cytoplasm and oocytes with pyknotic nuclei (Paulini et al. 2016). Only the structures featuring an oocyte with a discernible nucleus surrounded by granulosa cells were considered in the analysis.

2.4.2 | Immunohistochemical Analysis

The immunohistochemical analysis involved five sections per slide with six repetitions, totalling 30 slides per experimental group. These slides were randomly selected to ensure representative sampling and minimise bias in the analysis. The primary antibody used was anti-Ki-67 (Recombinant Rabbit Monoclonal Antibody [SP6], MA5-14520, Invitrogen), following modifications made to the protocol outlined by Lima et al. (2015). In brief, the slides were deparaffinised in xylene solutions and rehydrated. Antigen retrieval was performed by immersing the sections in a citrate buffer at 97°C for 1 h. Endogenous peroxidase blocking was carried out using solutions from the Leica Novolink Polymer Detection System kit (Newcastle upon Tyne NE 12 8EW, United Kingdom).

The primary antibody was used at a dilution of 1:25 and the incubation period lasted for 2 days. On the second day, the sections

underwent incubation with the secondary antibody (Polyclonal rabbit anti-human, A0082, Dako Denmark A/S). Subsequently, the sections were incubated with diaminobenzidine (DAB—K3468, Dako) and counterstained with haematoxylin. Fresh cat ovarian tissue was used as a positive control. Negative controls consisted of the substitution of non-immune mouse (Ki-67) serum collected in-house, at the same protein concentration as the primary antibody.

In the evaluation of samples treated with Ki-67, a light microscope (Leica DM500, Wetzlar, Germany) was used for follicle counting. Follicles were considered positive if at least one of the granulosa cells exhibited immunostaining with the antibody, following criteria established in a prior study (Paulini et al. 2016).

2.5 | Ultrastructural Analysis

A small sample was obtained from the central cross-section of the fragments from the fresh group, and each treatment was fixed in Karnovsky's solution (2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 4 h at 4°C. Samples were subsequently washed in sodium cacodylate buffer (0.1 M), post-fixed with 2% osmium tetroxide (1:1), 1.8% potassium ferricyanide and contrasted in a block with uranyl acetate in water (0.5%) overnight. Fragments were then rinsed and dehydrated in increasing concentrations of acetone and embedded in Epon resin. Afterward, they were sectioned using a Leica EM UC7 ultramicrotome (Wetzlar, Germany). Semithin sections (1 μm thick) were stained with toluidine blue and examined under light microscopy (Zeiss Axioskop, NY, USA) to detect follicles. Ultrathin sections (70 nm) from each follicle were cut with a diamond knife, mounted on copper grids, examined and photographed using a transmission electron microscope (JEOL, JEM 1011, Japan) to evaluate ovarian follicle ultrastructure. The organelles, basal and plasmatic membranes, together with the oocyte nuclear envelope and granulosa cells, were analysed based on previous studies involving mammalian follicles (Kim et al. 2018). To identify early signs of degeneration that could not be observed at lower magnifications, such as in light microscopy, only follicles that presented normal morphology in semithin sections were evaluated by TEM. The experimental groups that showed the most favourable results in the histological analysis were chosen for this evaluation, and at least three follicles per group were examined.

3 | Statistical Analysis

For the assessment of follicle counting and classification results, the ratio of MN to degenerated follicles was examined. Chi-square tests were employed for both the MN to degenerated follicle ratio and the immunohistochemical test with the Ki-67 antibody. The analysis was conducted using R software (version 4.1.0, Vienna, Austria) and a post hoc analysis was performed using the Fifer Package (version 1.0).

4 | Results

4.1 | Follicle Evaluation

A total of 60,185 follicles were analysed in all ovarian tissue samples, comprising 20,238 (34%) classified as primordial and 39,947 (66%) as growing follicles. Among these, 56,444 (94%) follicles were classified as MN. The numbers and statistical analysis of MN, primordial and growing follicles are shown in Figure 2.

Cryopreserved samples subjected to SF (SF1, SF2 and SF3 experimental groups) demonstrated a significantly reduced number ($p < 0.001$) of MN primordial follicles compared to the fresh control (Figure 2A). Concerning growing follicles, the SF1 experimental group exhibited a significantly reduced number ($p < 0.001$) of MN follicles. However, no significant differences were observed between SF2 and the fresh control (Figure 2B). Notably, the SF3 experimental group showed a higher number of degenerated growing follicles compared to the fresh control (Figure 2B).

Regarding the primordial follicles found in the V groups, the results were diverse. The V1 experimental group showed no significant difference compared to the fresh control, while the V2 experimental group exhibited a significant increase in follicular count, and the V3 experimental group showed a decrease in MN follicles compared to fresh tissue (Figure 2A). Concerning growing follicles, a significant increase ($p < 0.001$) in the follicular count was observed in the V1 experimental group (Figure 2B). In contrast, the V2 group showed no differences, and the V3 group showed a significant reduction in follicular count compared to the fresh control.

In the overall comparison among experimental groups, the total count of MN primordial and growing follicles was lower in the SF groups compared to the V group. The V2 group, utilising EG and sucrose, stood out for its higher count of primordial follicles (Figure 2A). Group V1, employing the combination of cryoprotectants DMSO + EG and trehalose, stood out for the highest count of growing follicles (Figure 2B). Typical ovarian follicle patterns are illustrated in Figure 2C–H.

Furthermore, primary alterations identified in both the cryopreserved groups and the fresh control included oocytes completely detached from the follicle and follicles detached from the stroma. Notably, in this study, SF3 exhibited the highest degree of cytoplasmic and nuclear degeneration among the analysed groups (data not shown).

4.2 | Cell Proliferation

The assessment of the proliferative status within the ovarian cell population was conducted through immunostaining using the Ki-67 monoclonal antibody. In fresh ovarian tissue samples, 64 activated follicles were examined, and 62 (97%) exhibited positive nuclear staining of granulosa cells. In the groups subjected

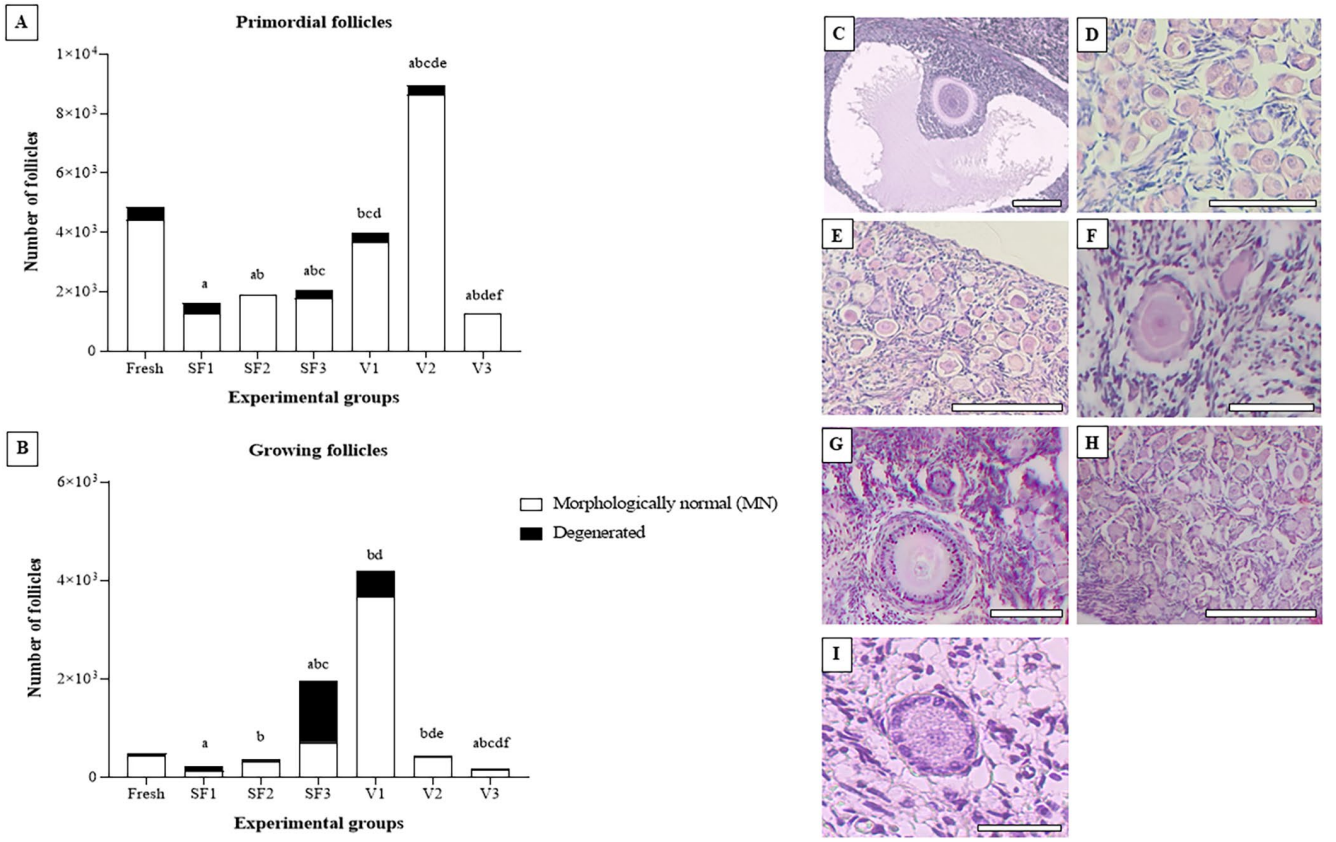

FIGURE 2 | Comparison of the number of morphologically normal (MN) and degenerated primordial and growing follicles in the experimental groups. (A) Number of MN and degenerated primordial follicles. (B) Number of MN and degenerated growing follicles. (C) MN primordial follicles identified in the SF1 group. (D) MN primordial follicles found in the SF2. (E) MN growing follicle found in the SF3 group. (F) MN growing follicle identified in the V1 group. (G) MN primordial follicles found in the V2 group. (H) MN growing follicle identified in the V3 group. All group comparisons were conducted; however, only statistically significant differences ($p < 0.01$) are displayed. SF1 (slow freezing 1): 1 M DMSO + 1 M EG + 10% fetal bovine serum (FBS) + 0.4% trehalose. SF2 (slow freezing 2): 1.5 M DMSO + 10% FBS + 0.4% sucrose. SF3 (slow freezing 3): 1.5 M DMSO + 10% FBS + 0.4% trehalose. V1 (vitrification 1): Equilibration solution: 10% DMSO + 10% EG + 0.1 M trehalose; vitrification solution: 20% DMSO + 20% EG + 0.1 M trehalose. V2 (vitrification 2): Equilibration solution: 10% DMSO + 10% EG + 0.1 M sucrose; vitrification solution: 20% DMSO + 20% EG + 0.1 M sucrose. V3 (vitrification 3): Equilibration solution: 20% DMSO +0.1 M trehalose; vitrification solution: 40% DMSO + 0.1 M trehalose. Bars = 50 μm. a = significant differences compared to Fresh tissue ($p < 0.05$); b = significant differences compared to SF1 ($p < 0.05$); c = significant differences compared to SF2 ($p < 0.05$); d = significant differences compared to SF3 ($p < 0.05$); e = significant differences compared to V1 ($p < 0.05$); and f = significant differences compared to V2 ($p < 0.05$).

to SF, 171 follicles were analysed, with 152 (89%) displaying positive nuclear staining for Ki-67. Finally, in the vitrified samples, 231 follicles were examined, and 193 (83%) exhibited positive staining.

The quantity of activated follicles positive for Ki-67 is illustrated in Figure 3A. Positive follicles were identified as those containing at least one granulosa cell demonstrating Ki-67 positivity, as visualised by brown coloration in Figure 3B–E.

The average proportion of Ki-67-positive follicles was significantly lower ($p < 0.01$) in the V2 group compared to the fresh control. Nevertheless, no significant differences were observed when comparing the proportion of positive and non-positive follicles in each group with the fresh control. All group comparisons were conducted; however, only statistically significant differences ($p < 0.01$) are displayed.

4.3 | Ultrastructure Analysis

In the TEM analysis of fresh samples, we observed the absence of a formed zona pellucida in primordial follicles, as mentioned by several authors previously (Carrijo Jr. et al. 2010; Paulini et al. 2014). Furthermore, granulosa cells displayed MN cell nuclei and mitochondria positioned closely to the nucleus. Overall, the oocytes presented homogeneous and slightly electron-dense cytoplasm, with robust adhesion of granulosa cells (Figure 4).

In the analyses conducted on follicles in the V2 group, ultrastructural changes were observed, including detachment between the oocyte and the zona pellucida. Nevertheless, nuclei containing nucleoli and heterochromatin were identified. In Figure 5A, the oocyte exhibited homogeneous and lightly electron-dense cytoplasm, with minimal adherence of granulosa cells. In Figure 5C, the cytoplasm of the sample was not homogeneous; however,

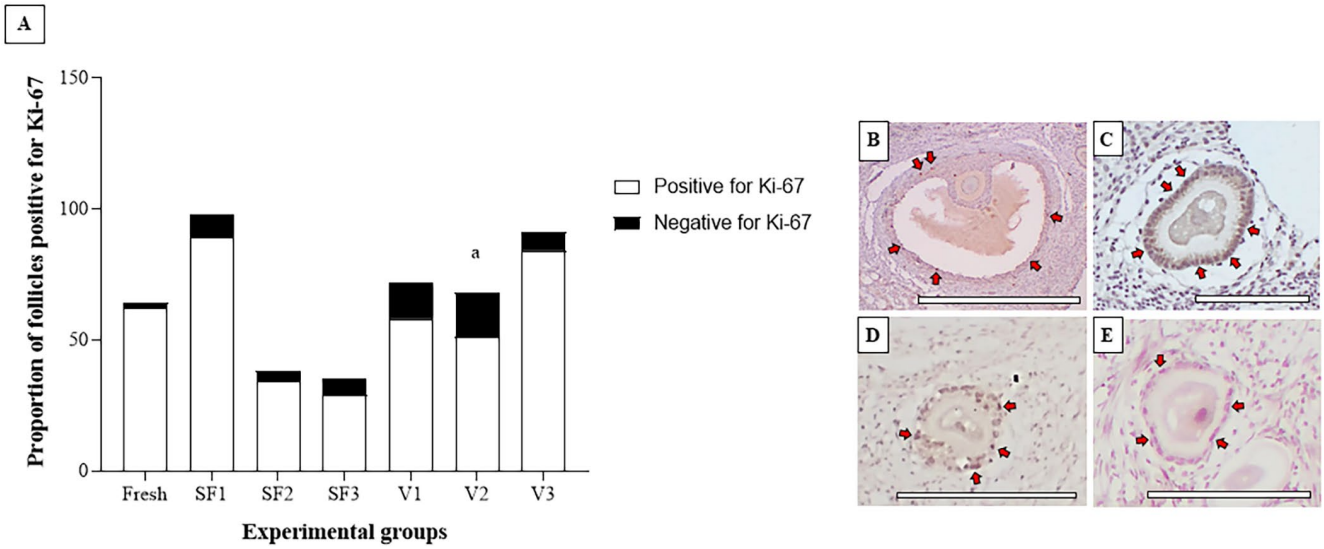

FIGURE 3 | Proportion of activated follicles positive for Ki-67 across all groups. The graphical representation reflects the statistical analysis of the number of Ki-67 positive follicles in both the fresh control and cryopreservation groups. (A) Follicle from Fresh group. (B) Follicle from SF1 group. (C) Follicle from V1 group. (D) Follicle from V2 group. The arrows highlighted in the illustration delineate granulosa cells expressing Ki-67. Bars = 20 μm. * = significant differences compared to Fresh tissue ($p < 0.01$).

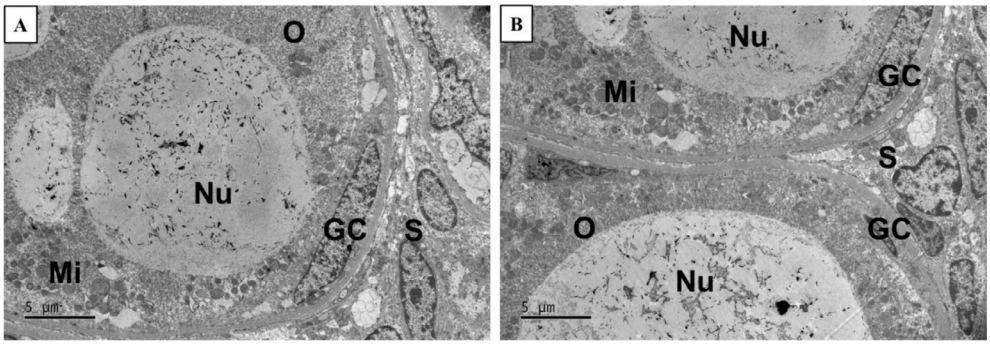

FIGURE 4 | Electron micrograph of primordial follicles in fresh cat ovarian tissue. (A, B) Oocyte (O) displaying nucleus and well-structured cytoplasm, and rounded mitochondria (Mi). GC = granulosa cells. Nu, Nucleus; S, Stroma region. Bars: 5 μm.

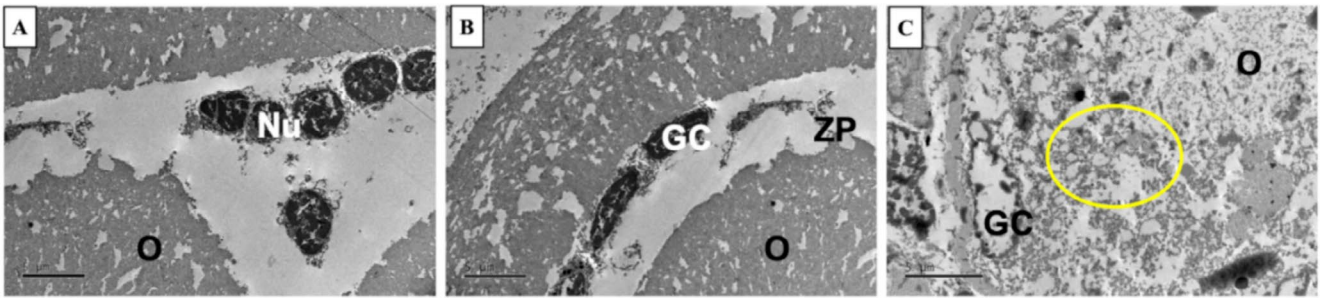

FIGURE 5 | Micrograph of pre-antral follicles from the V2 group. (A, B) The presence of the zona pellucida (ZP) enveloping the oocyte (O). Follicle exhibits detachment of the oocyte from the zona pellucida, and the granulosa cells feature rounded nuclei (Nu). (C) The highlighted yellow circle delineates an unrecognised cytoplasmic degeneration in granulosa cells (GC)/oocyte (O). V2 (vitrification 2): Equilibration solution: 10% DMSO + 10% EG + 0.1 M sucrose; vitrification solution: 20% DMSO + 20% EG + 0.1 M sucrose. Bars = 5 μm.

nuclei of the oocyte and strong adherence to granulosa cells were still observable.

Analysing follicles from the V1 group treated with DMSO + EG + trehalose (Figure 6) revealed some undesirable structural changes. The main alteration observed was the detachment of the oocyte from the granulosa cells (Figure 6A). Despite this, the MN cell nucleus was found.

In the SF2 group, the cytoplasm exhibited signs of degeneration. Figure 7A depicts the rupture of the plasma membrane at various points. In Figure 7B, the oocyte's cytoplasm was

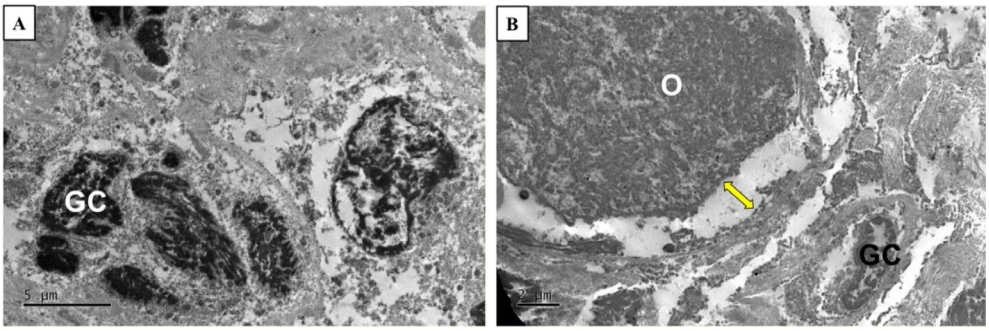

FIGURE 6 | Micrography of pre-antral follicles from the V1 group. (A) Granulosa cell (GC). Bar = 5 µm. (B) Follicle showing detachment of the oocyte (O) from the granulosa cells (GC), marked with a yellow arrow. Bar = 2 µm. V1 (vitrification 1): Equilibration solution: 10% DMSO + 10% EG + 0.1 M trehalose; vitrification solution: 20% DMSO + 20% EG + 0.1 M trehalose.

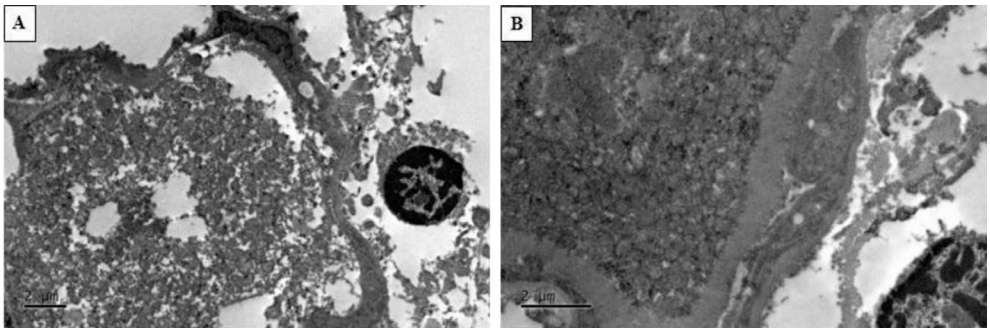

FIGURE 7 | Electron micrograph of the stromal region of the SF2 group. (A) Degenerated follicle. (B) Follicle with numerous organelle degenerations. SF2 (slow freezing 2): 1.5 M DMSO + 10% FBS + 0.4% sucrose. Bars = 2 µm.

homogeneous, and it was impossible to observe nuclei with nucleoli and heterochromatin.

5 | Discussion

Irrespective of the cryopreservation method utilised, histological analysis revealed a significantly lower number of primordial follicles compared to the fresh control group, with the exception of the V2 group, which exhibited a higher count for primordial follicles, as also reported by Çelikkan et al. (2023). The V1 group showed higher counts for growing follicles. Cryoinjuries acknowledged in the literature encompass physical stress, the formation of intracellular ice crystals and subsequent ultrastructural damage (Comizzoli and Holt 2014; Praxedes et al. 2018; Youm et al. 2014). Nevertheless, specimens subjected to SF (SF1, SF2 and SF3) demonstrated a significantly lower follicular count compared to V (V1, V2 and V3). Previous studies have documented the adverse impact of cryopreservation on ovarian stromal cells in both human (Dath et al. 2010; Keros et al. 2009) and feline (Alkali et al. 2024; Bosch et al. 2004) contexts. According to the literature, both SF and V are associated with cryogenic damage (Behl et al. 2023).

Our investigation revealed that the group subjected to V with EG + DMSO + trehalose (V1 group) exhibited a significantly higher count of primordial and growing MN follicles compared to the SF groups, indicating promising outcomes in follicular population preservation. Cryopreservation can lead to several alterations in ovarian stroma, including increased collagen deposition, stromal cell apoptosis, and disruption of the extracellular matrix (Leonel et al. 2019; Vilela et al. 2019). Specifically, SF has been associated with greater stromal degeneration, characterised by increased fibrosis, edema and vacuolization (Leonel et al. 2018; Silva et al. 2024). These changes can adversely affect the ovarian microenvironment, impacting follicular survival and function. Our findings are consistent with these observations, as we noted a higher degree of stromal degeneration in the SF groups compared to the V group.

It is acknowledged that the type and concentration of cryoprotectants can markedly influence the success of V. In a study by Brito et al. (2018), employing the same concentrations of DMSO and EG as utilised in our study (equilibrium solution: 10% DMSO + 10% EG + 0.1 M trehalose; V solution: 20% DMSO + 20% EG + 0.1 M trehalose), a survival rate of 38%–45% in primordial and growing follicles was achieved post-vitrification. These findings align with a prior study in cattle, where more than 80% preservation of growing follicles was attained using DMSO and EG in conjunction with sucrose (equilibrium solution: 10% DMSO + 10% EG + 0.25 M sucrose; V solution: 20% DMSO + 20% EG + 0.25 M sucrose) (Shahsavari et al. 2020).

The combination of DMSO and EG (equilibrium solution: 7.5% DMSO + 7.5% EG and V solution: 15% EG + 15% DMSO) with a 0.5 M sucrose adjunct was employed by Moutthan and Comizzoli (2016) in the V of feline ovarian tissue, resulting in a 67.5% incidence of MN growing follicles.

Antecedent research suggests that a cryoprotective solution featuring two intracellular cryoprotectants, alongside

non-permeable antifreeze proteins, exhibits diminished toxicity compared to a solution relying solely on a singular intracellular cryoprotectant (Youm et al. 2014). This phenomenon results from the synergistic effect of distinct cryoprotectant properties, reducing the requisite individual concentrations needed to achieve their properties. This, in turn, mitigates the specific toxicity associated with each antifreeze protein (Wusteman et al. 2004). Conversely, the use of diverse cryoprotectant agents may induce alterations in the pH and osmolality of ovarian tissue, potentially jeopardising cell viability during both the cryopreservation and thawing/rewarming processes. Therefore, maintaining hyperosmolarity during cryopreservation becomes imperative to facilitate interactions and stabilisation of cell membranes, serving as a buffer against the deleterious effects of elevated concentrations of electrolytes in dehydrated cells (Çelikkan et al. 2023; Kim et al. 2018). Trehalose, in this context, emerges as a non-permeable cryoprotectant that has shown favourable outcomes in the V of feline ovarian tissue (Brito et al. 2018).

Our investigation revealed that the use of trehalose resulted in improved preservation of growing (MN) follicles compared to sucrose. This observation can be attributed to the physicochemical properties of trehalose, which mitigate the formation of ice crystals due to its higher glass transition temperature. The elevated glass transition temperature serves as a protective barrier, shielding cells from damage induced by freezing (Olgenblum et al. 2020). These findings align with other studies, such as those conducted by Brito et al. (2018), where feline ovarian tissue vitrified with trehalose demonstrated the presence of well-preserved primordial and secondary follicles. Additionally, these results are consistent with a previous investigation by Leonel et al. (2018), which utilised the same combination in SF and achieved a 70% incidence of growing follicles.

Despite the notable improvement in follicular morphology observed in the study by Leonel et al. (2018), the assessment of follicular ultrastructure revealed a deleterious impact associated with the combination of DMSO and EG, leading to ultrastructural alterations in the analysed follicles. According to the authors, the combination of two cryoprotective agents resulted in lower concentrations required to induce a protective effect. However, despite the potential advantages of utilising lower concentrations, reducing the concentration may increase the risk of ice crystal formation, thereby corroborating the observed deleterious effects. Consequently, it becomes evident that the cryopreservation of feline ovarian tissue requires a minimum concentration of each cryoprotectant to ensure effectiveness.

In our ultrastructural investigations, we observed that in the V1 group, the cell nucleus exhibited normalcy, the nucleoli were well-structured, and the mitochondria were evenly distributed in the granulosa cells. Given the pivotal role of mitochondria in energy generation, metabolic activation, and the regulation of cell survival (Carrijo Jr. et al. 2010; Nasrabadi et al. 2015; Rho et al. 2002), the presence of these organelles is paramount for cryopreservation analysis. Conversely, SF manifested alterations in the stromal region and fibroblasts, particularly in the central areas of the cytoplasm. These changes could potentially be attributed to dehydration or inadequate concentrations of cryoprotectants, leading to ice formation.

Concerning the Ki-67 immunohistochemical outcomes, the lowest count of positive follicles was identified in Group V2 in comparison to the fresh tissue. However, no statistically significant differences were discerned in the number of proliferative follicles across the other groups. This observation indicates that regardless of the combination of cryoprotectants used, there is evidence of cell proliferation and viability in all groups. A recent investigation by Hassan et al. (2023) further expounded that the viability and morphology of follicles in cryopreserved feline ovarian tissue can be influenced by the size of the fragment (1.5–3 mm^3), with smaller fragments demonstrating a higher proportion of viable follicles.

Derived from these findings, one may infer that the combination of DMSO + EG + trehalose using V method holds promise in alleviating the adverse effects of cryopreservation on the ultrastructure of ovarian follicles. It is postulated that trehalose imparts its advantageous effects by serving as an osmoprotective agent, thereby mitigating osmotic stress on cells during cryopreservation procedures. Consequently, this strategic utilisation of cryoprotectants appears to be a viable method for maintaining the structural integrity of feline ovarian follicles.

6 | Conclusion

The cryopreservation protocol developed in this study effectively maintained the structural integrity and proliferative capacity of both primordial and developing follicles while significantly reducing degenerative changes. Ultrastructural analysis through electron microscopy confirmed the superiority of V over SF, as evidenced by preserved nuclear morphology, intact nucleoli and properly distributed mitochondria in granulosa cells. The synergistic combination of DMSO, EG and trehalose proved more effective than individual cryoprotectants, with the Vitrification 1 protocol (10% DMSO; 10% EG; 0.1 M trehalose equilibration solution followed by 20% DMSO; 20% EG; 0.1 M trehalose vitrification solution) demonstrating optimal preservation outcomes for feline ovarian tissue. These findings establish a robust foundation for optimising cryopreservation techniques in feline species, with important implications for conservation strategies targeting threatened felids. Nevertheless, additional research is required to comprehensively assess post-thaw follicular competence through evaluation of cell death pathways, in vitro culture systems and transplantation studies. Such investigations will be crucial for validating the long-term functional preservation of cryopreserved ovarian tissue and its potential application in assisted reproductive technologies for endangered feline species.

Author Contributions

This study was initiated during the COVID-19 pandemic. Given its scientific importance, the research was completed in the post-pandemic period through collaborative efforts, including additional contributions for data analysis. Consequently, the extended list of co-authors reflects this collective work. F.P. and C.M.L.: study design. R.B.R.; A.Q.R.; B.A.A.; T.B.P.; J.K.O.S.; M.V.R.C.C.; J.L.S.B.; Y.B.F.; I.G.M.S.; P.M.Q.B. and F.P.: experimental procedures. R.B.R.; S.N.B.; J.T.G.; A.Q.R. and F.P.: analyses and interpretation of results. R.B.R.; A.Q.R.; T.B.P.; and F.P.: manuscript preparation and revision.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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