



Article

Cryopreservation and In Vitro Culture of Isolated Porcine Ovarian Follicles

Bence Somoskői * , Dóra Török, Lilla Bordás, József Rátky and Sándor Cseh

Department of Obstetrics and Food Animal Medicine Clinic, University of Veterinary Medicine, H-1078 Budapest, Hungary; torok.dora@univet.hu (D.T.); bordas.lilla@univet.hu (L.B.); ratky.jozsef@univet.hu (J.R.); cseh.sandor@univet.hu (S.C.)

* Correspondence: somoskoi.bence@univet.hu; Tel.: +36-709053501

Simple Summary

The long-term storage of ovary-originated follicles can serve as a promising approach to conserve genetic material and fertility of female animals, even after death. However, since a standardized protocol is lacking, there is a need for a well-working process providing a high survival rate of frozen–thawed samples. The objective of this study was to analyze the effect of two different freezing methods (open and closed vitrification) on porcine follicles, assessing the survival rate and hormonal production during a 10-day-long culture. Although refinement is needed, our data indicate that our culture media is suitable to maintain the hormonal production of these samples. Regarding the freezing system, open vitrification is an appropriate method to preserve the preantral follicles and provides superior survival rates to the closed method.

Abstract

Cryopreservation of preantral follicles (PAFs) is a promising tool for gene conservation and fertility preservation. However, standardized protocols for the cryopreservation and in vitro culture of isolated follicles—particularly in pigs—are still lacking. This study aimed to analyze the survival and developmental potential of porcine PAFs vitrified using two different methods: open pulled straw (OPS) and cryotube (CT). Ovaries of Hungarian Large White sows were collected from a local slaughterhouse and enzymatically digested to isolate preantral follicles. Morphologically normal follicles were assigned to three groups: fresh control, OPS-vitrified, and CT-vitrified. All follicles were cultured for 10 days in FSH-supplemented medium, with growth, survival, and estradiol (E2) production monitored. Survival rate was lower in the CT group (83.3%) than that of the control and OPS (97.4% and 94.4%, respectively). The follicular area was consistently larger in control than in CT and OPS, with no difference between vitrified groups. E2 production varied among treatments: OPS follicles showed lower E2 levels on Day 2, no differences were detected on Day 7, and CT follicles produced less E2 on Day 10. These results indicate that OPS is the more suitable vitrification method for porcine PAFs and that the culture system supports hormone production; however, it may require refinement to provide long-term follicle maintenance.

Keywords: vitrification; isolated follicles; porcine



Academic Editor: Xueming Zhang

Received: 19 December 2025

Revised: 22 January 2026

Accepted: 23 January 2026

Published: 26 January 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and

conditions of the [Creative Commons](#)

[Attribution \(CC BY\)](#) license.

1. Introduction

In the domains of conservation biology and assisted reproduction, there is an increasing necessity to devise effective methodologies for storing and sustaining the reproductive potential of endangered species, as well as those of considerable genetic importance [1,2]. The cryopreservation of preantral follicles (PAFs) has emerged as a promising fertility preservation approach. It is evident that these immature follicles contain oocytes at an early stage of development. Consequently, they exhibit a greater degree of resistance to freezing-induced damage in comparison to fully grown, mature oocytes [3,4].

The substantial surface area to volume ratio of PAFs (particularly in the secondary stage) facilitates enhanced substance exchange, thereby rendering them more resilient to cryopreservation and more appropriate for the preservation of female germplasm. The collection of these samples can be performed on any female animal, irrespective of its age or stage in the estrous cycle, and even on animals that die unexpectedly [5,6]. Furthermore, cryopreserving PAFs provides a large supply of oocytes for assisted reproduction techniques (ARTs), enabling the production of genetically superior animals. These features are all potentially beneficial to cryopreservation, and PAFs are therefore considered an important focus of cryopreservation programs [7].

Vitrification can be performed using two different approaches: open and closed systems [8]. In the case of an open system, the sample comes into direct contact with liquid nitrogen (LN₂), resulting in an extremely high cooling rate (>10,000 °C/min) [9]. However, concerns have been raised about the potential for disease transmission through liquid or vapor-phase nitrogen-mediated infection during storage [10]. In order to circumvent the potential for contamination, closed vitrification protocols have been developed. These protocols facilitate indirect contact between the sample and the liquid nitrogen (LN₂). However, this technique necessitates the sealing of the sample-holding device prior to cooling, which consequently results in a lower cooling rate [8]. To date, there is no standard protocol or even any data on the cryopreservation of porcine isolated follicles.

In the last few years, our research team has analyzed the opportunities of cryopreservation and in vitro culture of isolated PAFs, establishing well-working multispecies protocols [11,12].

It is hypothesized that an open vitrification system could provide an enhanced survival rate quality for the frozen–thawed PAFs, due to the higher cooling rate. The objective of the present study was to make a comparison between the survival and developmental rate of porcine isolated PAFs after vitrification with different methods (closed vs. open system).

2. Materials and Methods

All the methods used in this study are based on Somoskői et al. 2023 [13], with slight modifications.

Figure 1 shows the methods in a summarized way.

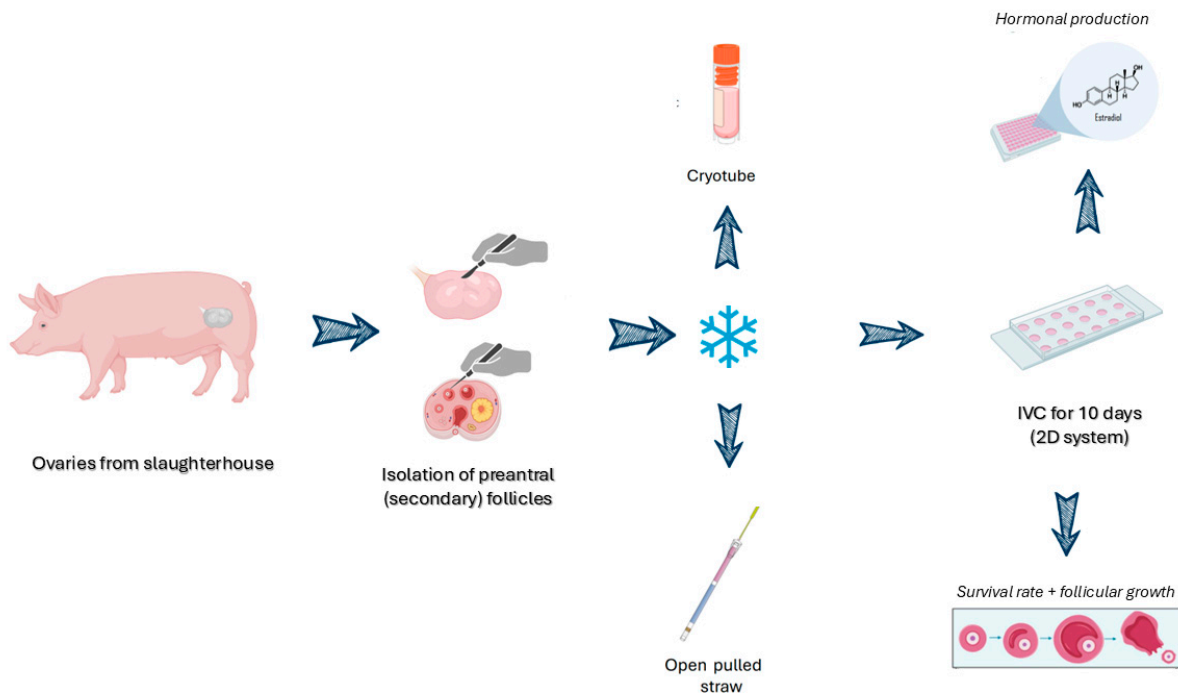


Figure 1. Schematic representation of the methods used in this study. Created with BioRender.com.

2.1. Collection of Ovaries

Ovaries of Hungarian Large White sows were collected from a local slaughterhouse ($n = 5$). After collection, ovaries were placed in sterile 500 mL containers, filled with filtered Dulbecco phosphate-buffered saline (DPBS; Merck KGa, Darmstadt, Germany) + 1% PEN/STREP (100 IU penicillin and 100 mg streptomycin/L; Merck KGaA, Darmstadt, Germany). Samples were stored at room temperature and delivered to the laboratory within 2 h.

2.2. Isolation of Preantral Follicles

The ovarian cortex of each ovary was sliced into approximately 1 mm² pieces with a surgical blade, subsequently placed in a digestive solution (HEPES-modified Medium 199 + 3 mg/mL collagenase [both from Merck KGa, Darmstadt, Germany]), and incubated for 60 min at 37 °C. Thereafter, following the enzymatic digestion, preantral follicles were manually isolated using 28G needles attached to 1 mL syringes. Following the isolation process, morphologically normal secondary follicles (which are characterized by multiple layers of evenly distributed granulosa cells, surrounded by the basal lamina and the absence of abnormal oocyte structure, highly dense granulosa cell area, or damaged basement membrane) were randomly divided into three groups: fresh control, open pulled straw (OPS) vitrified, and cryotube vitrified (CT). The entire procedure was conducted utilizing a stereomicroscope (Olympus SZX7, Olympus Corporation, Tokyo, Japan).

2.3. Vitrification and Thawing

In this research, two distinct vitrification protocols were utilized to preserve morphologically normal, intact PAFs: cryotube (CT) vitrification and OPS vitrification. The following assertion is made with reference to Somoskoi et al., 2023 [13].

The cryotube vitrification process was conducted in accordance with the following protocol. Follicles were subjected to an incubation in an equilibration solution (ES) comprising base medium (HEPES-modified Medium199 + 10% FBS) + 10% ethylene glycol (EG) + 10% DMSO (dimethyl-sulfoxide) + 0.3 M sucrose for a duration of 4 min. Subsequently, the samples were transferred into a vitrification solution (VS), which comprised

base medium, 25% ethylene glycol, 25% dimethyl sulfoxide, and 0.3 M sucrose. The samples were then equilibrated for 45 s. Following equilibration in the VS, the follicles were placed into cryotubes (10 follicles per vial) containing 200 μ L of the VS. The cryotubes were then directly plunged into liquid nitrogen (LN2) and stored for a period of one week. The thawing process was initiated by immersing the cryotubes in a water bath maintained at a temperature of 37 °C for a duration of one minute. Subsequently, the follicles were subjected to a series of three solutions, each characterized by a gradual decrease in sucrose concentration (base medium + 0.3 M; 0.15 M; 0.075 M sucrose). Each step in the process was completed within a time frame of five minutes.

OPS vitrification: PAFs were washed in Holding Medium (HM) (HEPES-modified Medium 199 + 20% FBS), then incubated for 3 min in equilibration solution (ES) (Holding Medium + 7.5% EG + 7.5% DMSO). Following the process of incubation in ES, the follicles were transferred into a vitrification solution (VS) (comprising HEPES-modified Medium 199, 20% fetal bovine serum (FBS), 16.5% EG, 16.5% DMSO, and 1M sucrose) for a period of 30 s. Thereafter, the follicles were collected into OPSs (10 follicles per straw; supplied by VitaVitro Biotech, Shenzhen, China) and plunged into liquid nitrogen (LN2). Subsequent to a 30 s exposure to liquid nitrogen (LN2), the pulled straws containing the follicles were transferred into 0.5 mL containers, ultrasonically sealed, and stored in LN2 for a period of one week. The thawing process involved the removal of OPSs from liquid nitrogen (LN2) and subsequent immersion in a modified HM + 50% sucrose medium (SM) (consisting of HEPES-modified Medium 199, 1 M sucrose, and 20% fetal bovine serum [FBS]). This was followed by a series of three washing steps, during which the sucrose concentration in the medium was systematically reduced. It is imperative to note that all the steps of the vitrification and thawing procedures were carried out at ambient temperature.

2.4. *In Vitro Culture (IVC)*

Fresh and frozen/thawed PAFs were cultured individually in 100 μ L drops of culture medium, using 18-well culture dishes (ibiTreat: #1.5 polymer coverslip, ibidi GmbH, Grafelfing, Germany). The culture medium is composed of Opti MEM (obtained from Thermo Fisher Scientific Inc., Waltham, MA, USA), which has been supplemented with 5% fetal bovine serum (FBS) (obtained from Thermo Fisher Scientific Inc., Waltham, MA, USA), 1% yeast extract (obtained from ITS-G, 100X; also from Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.5% antibiotic-antimycotic solution (obtained from Thermo Fisher Scientific Inc., Waltham, MA, USA), and 100 mIU/mL rFSH (obtained from R&D Systems Inc., Minneapolis, MN, USA). The follicles were then cultured for a period of 10 days at a temperature of 38.5 °C, with the presence of 6.5% CO₂ (Figure 2). Half of the medium was changed on Days 2, 7, and 10, at which time samples were collected from the culture media for further analysis. It has twofold higher concentrations of FSH on each changing day (dynamic culture). The rate of normal and abnormal follicles was subjected to analysis through the calculation of the rate of follicles exhibiting atresia (irregular shape and dark, dense cell material) by Day 10.

In vitro growth (area change) of follicles was examined during the IVC period. The measurement was conducted on follicles that were deemed to be viable, as evidenced by the absence of atresia. Concurrently with the medium change, images were captured of each sample. The evaluation of growth was conducted by measuring the follicular area (in mm²) and subsequently analyzing the data using ImageJ (version 1.54j, NIH, Bethesda, MD, USA) with the designated “Area Measurement” function.

Furthermore, the production of estradiol (17 β -estradiol; E2) from each follicle was evaluated. The aspirated culture medium was collected in Eppendorf tubes and stored at a temperature of –20 °C until measurement. The concentration of estradiol in the samples

was measured with an ELISA assay (DE2693, Demeditec Diagnostics, Kiel, Germany; analytical sensitivity 10.6 pg/mL; intra-assay CV < 5%; inter-assay CVs were 14.9% and 6.9% for low and high controls, respectively).

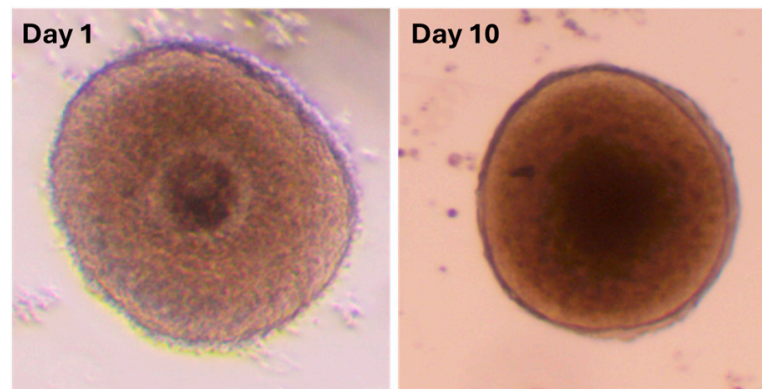


Figure 2. Representative images of Day 1 and Day 10 follicles, respectively.

2.5. Statistical Analysis

Data analysis was performed with version 4.1.1 of R (R Foundation for Statistical Computing, Vienna, Austria).

Prior to the statistical analysis, the normality of the data was analyzed with the Shapiro–Wilk test. Given that the data did not demonstrate a normal distribution, non-parametric methods were employed. Consequently, in the context of growth and hormonal production, the median was utilized as opposed to the mean. An analysis was conducted to ascertain the differences in in vitro growth and estradiol production among the various treatments on Days 2, 5, and 10. To this end, the Kruskal–Wallis test was employed, with the post hoc Dunn test (with the Benjamini–Hochberg adjustment method) utilized for the purpose of pairwise comparison of treatments. A comparative analysis of the growth and estradiol production data within groups was conducted utilizing a Friedman test, followed by a post hoc Wilcoxon rank sum test for multiple comparisons. The differences in the in vitro survival rate among the various treatments were analyzed using a chi-squared test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Survival Rate

The survival rate of follicles throughout the 10-day-long in vitro culture period was lower for the CT group (83.3% [15/18]) than for the control and OPS groups (97.4% [75/77] and 94.4% [17/18], respectively).

3.2. Follicular Growth

During the 10-day-long culture period, higher follicular area was found in the control group (0.49 mm^2 on D2; 0.33 mm^2 on D7; 0.27 mm^2 on D10) than in the CT (0.29 mm^2 on D2 [$p < 0.001$]; 0.18 mm^2 on D7 [$p < 0.001$]; 0.19 mm^2 on D10 [$p < 0.05$]) and OPS (0.29 mm^2 on D2 [$p < 0.001$]; 0.19 mm^2 on D7 [$p < 0.001$]; 0.18 mm^2 on D10 [$p < 0.001$]) groups (ranges can be seen in the graph). However, a comparison of cryopreserved groups (OPS and CT) did not show any differences on D2, D7, and D10 (Figure 3).

Analysis of area change within groups showed that follicles decreased their area day by day, except for the CT follicles on Day 10, for which the area did not change from Day 7 (Table 1).

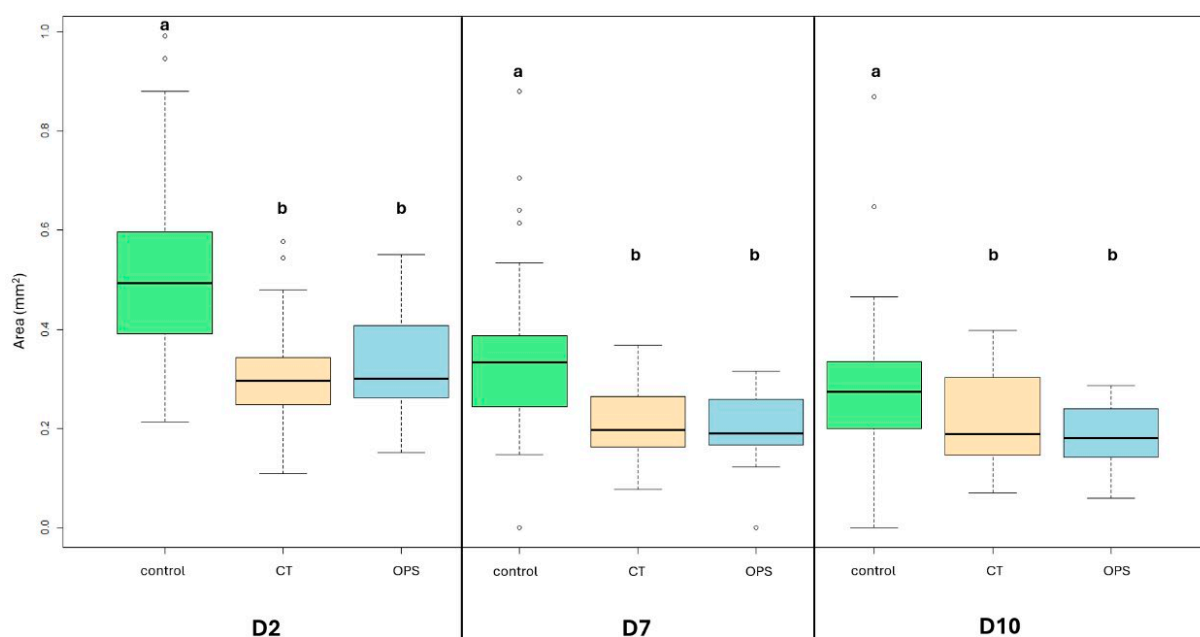


Figure 3. Changes in porcine ovarian follicle median area in fresh and vitrified groups, during the 10-day-long IVC period. (a,b) $p < 0.05$; differences among groups were analyzed with Kruskal–Wallis test followed by post hoc Dunn test (with Benjamini/Hochberg adjustment method) for pairwise comparison. Control = non-vitrified control group; CT = cryotube vitrification; OPS = open pulled straw vitrification.

Table 1. Changes in median follicular area within the different treatment groups. p -values in *italics* indicate significant difference ($p < 0.05$). Friedman test with post hoc Wilcoxon rank sum test for pairwise comparison. Control = non-vitrified control group; CT = cryotube vitrification; OPS = open pulled straw vitrification.

Treatment	Interval	<i>p</i> Value	Size Change
Control	D2–7	<i>$p < 0.001$</i>	decreasing
	D7–10	<i>$p < 0.001$</i>	decreasing
	D2–10	<i>$p < 0.001$</i>	decreasing
CT	D2–7	<i>$p < 0.001$</i>	decreasing
	D7–10	<i>$p = 0.8$</i>	unchanged
	D2–10	<i>$p < 0.001$</i>	decreasing
OPS	D2–7	<i>$p < 0.001$</i>	decreasing
	D7–10	<i>$p < 0.05$</i>	decreasing
	D2–10	<i>$p < 0.001$</i>	decreasing

3.3. Estradiol Production

Differences were detected in the median E2 concentrations among the different treatments during the 10-day-long IVC period, except on Day 7. On Day 2, the median hormonal level of OPS follicles was lower compared to fresh and CT groups (15.34 pg/mL vs. 22 pg/mL [$p < 0.05$] and 21.85 pg/mL [$p < 0.05$], respectively), while control and CT follicles produced similar levels of estradiol. On Day 7, differences in hormonal production were not found (27.8 pg/mL, 34.5 pg/mL, and 32 pg/mL in control, CT, and OPS groups, respectively). On Day 10, the follicles of the CT group produced estradiol in a lower concentration than that of the control and OPS groups (28.66 pg/mL vs. 34.8 pg/mL [$p < 0.001$] and 34.8 pg/mL [$p < 0.05$], respectively). Ranges can be seen in the graph (Figure 4).

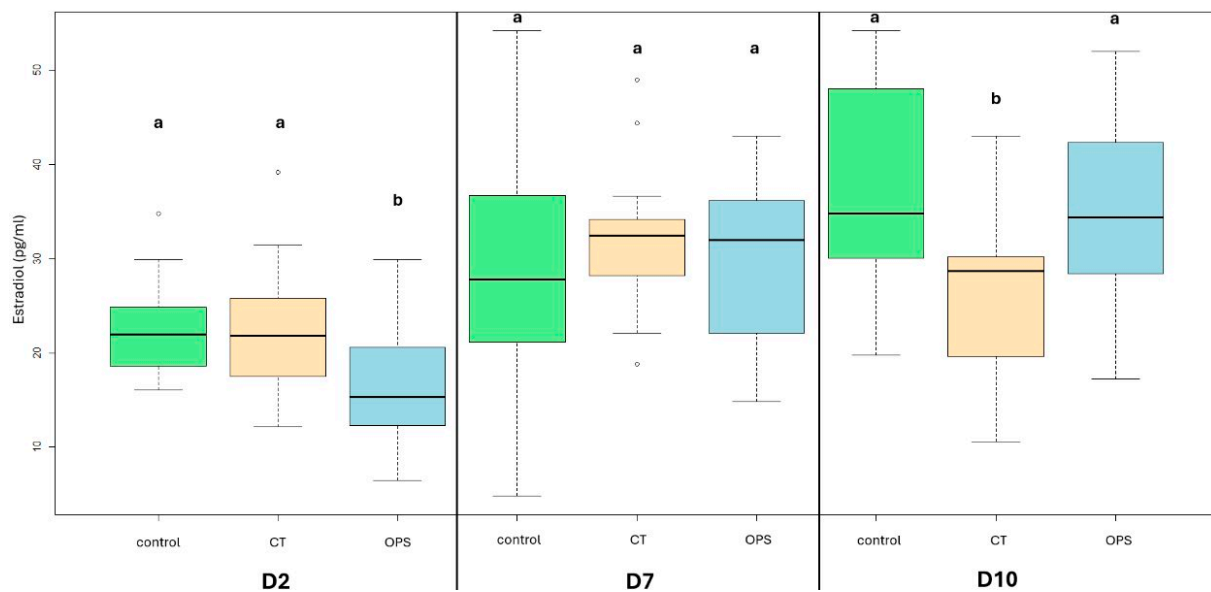


Figure 4. Changes in hormonal production (median estradiol concentration) in fresh and vitrified porcine ovarian preantral follicles, during the 10-day-long IVC period. (a,b) $p < 0.05$; differences among groups were analyzed with Kruskal–Wallis test with post hoc Dunn test (with Benjamini/Hochberg adjustment method) for pairwise comparison. Control = non-vitrified control group; CT = Cryotube vitrification; OPS = open pulled straw vitrification.

The alterations in estradiol production across the various treatment groups were also subjected to analysis. As demonstrated in Table 2, follicles of fresh control and OPS demonstrated a higher level of estradiol production on Day 10 than on Day 2. In contrast, the CT group exhibited no significant change in estradiol production over the course of the experiment.

Table 2. Changes in estradiol production within the different treatment groups. p -values in *italics* indicate significant difference ($p < 0.05$). Friedman test with post hoc Wilcoxon rank sum test for pairwise comparison. Control = non-vitrified control group; CT = cryotube vitrification; OPS = open pulled straw vitrification.

Treatment	Interval	<i>p</i> Value	Size Change
Control	D2–7	$p = 0.8$	unchanged
	D7–10	$p < 0.001$	increasing
	D2–10	$p < 0.001$	increasing
CT	D2–7	$p < 0.001$	increasing
	D7–10	$p = 0.06$	unchanged
	D2–10	$p = 0.18$	unchanged
OPS	D2–7	$p < 0.001$	increasing
	D7–10	$p = 0.13$	unchanged
	D2–10	$p < 0.001$	increasing

4. Discussion

In the last decade, the status of the indigenous pig breeds has attracted more and more attention [14]. In Europe and the Caucasus region, the demographic status of local pig populations is alarming. Currently, 14 breeds are classified as being at critical risk of extinction, 5 as critical-maintained, 24 as endangered, 11 as endangered-maintained, and

6 as vulnerable [15]. From a biological perspective, indigenous breeds represent a unique genetic reservoir. They are well adapted to their local environments, which is essential for the sustainability and resilience of pig production systems. In terms of long-term food security, these breeds exhibit adaptation capacity to changing environmental conditions and disease resistance [16]. A recent and unforeseen threat to the European indigenous pig population has emerged in the form of African Swine Fever (ASF), which entered the EU from the east, primarily affecting the wild boar population. The disease is currently present in fourteen Member States. Once a farm is contaminated, all animals have to be slaughtered [17]. If an indigenous breed population became infected, such total culling could result in a severe bottleneck effect, or the irreplaceable disappearance of a whole breed [18]. Therefore, in addition to in situ preservation strategies, the establishment of ex situ gene banks is critically important. In such a situation, storage of preantral follicles could be a valuable tool for gene preservation of the female side.

There are only a few studies dealing with the cryopreservation of porcine ovarian follicles, and most of these analyze the freezing injury to tissue-embedded follicles. In a study by Moniruzzaman et al. [19], tissue slices from 15-day-old neonatal piglets were vitrified and the viability of the primordial follicles was tested using xenografting [19]. This study employed a vitrification method similar to ours, involving the supplementation of the equilibration and vitrification media with 7.5% EG + 7.5% DMSO and 15% EG + 15% DMSO, respectively. The slices were cryopreserved using a Cryotop, a commonly used open vitrification system. After thawing, the slices were xenografted into mice and analyzed for viability and morphology after two months. They found that frozen-thawed follicles had developed; however, they did not reach the antral stage. In a recent paper, Hwang et al. [20] vitrified ovarian tissue slices using the modified Kitazato Cryotissue method (a modification of Cryotop) and analyzed the morphology of oocytes and the rate of apoptotic granulosa cells in fresh, vitrified, and frozen control samples (i.e., fresh samples directly plunged into liquid nitrogen). They found that vitrification caused a higher rate of abnormal oocytes than fresh samples (25.8% vs. 6.6%), but direct freezing of samples without equilibration resulted in an even higher rate (67.7%) of abnormal oocytes. Follicles contained only TUNEL-negative cells in both the fresh and vitrified groups. Borges et al. assessed the effect of slow freezing with different cryoprotectants on porcine ovarian tissue by storing the samples in a cryotube [21]. The cooling rate was similar to embryo-based protocols (seeding on -7°C , then $-0.3^{\circ}\text{C}/\text{min}$) and the morphology of follicles was histologically analyzed soon after thawing. They found that the highest normal morphology can be reached if DMSO or EG was used as cryoprotectants ($67 \pm 4.8\%$ and $81.8 \pm 1.4\%$, respectively), instead of 1,2-propanediol ($56 \pm 10\%$) or glycerol (0%) (normal morphology in fresh samples was $97.7 \pm 1.2\%$). Similar results were found by Han et al., who analyzed the transport of different cryoprotectants in tissue slices [22]. Ethylene glycol and DMSO showed the best results compared to GLY and PG, based on spectroscopic and thermal approaches. In another research, Gabriel et al. vitrified porcine ovarian slices in cryotubes [23]. They applied EG as the only intracellular cryoprotectant (15 and 20% in equilibration and vitrification medium, respectively) and analyzed the effect of sucrose supplementation on primordial and primary follicles. They found that all of the sucrose supplementation decreased the normal rate in a dose-dependent manner (the highest rate was 65.28% in 0 M sucrose vs. 75% in fresh samples), showing the higher sensitivity of primary follicles than that of primordial ones. Since isolated follicles contain a relatively small number of cells, including special oocytes, supplementing the vitrification medium with sucrose is essential in order to provide the high osmolarity, which allows the exchange of intracellular water and ethylene glycol. This kind of mixture provides a high survival rate in the OPS (94.4%) and a relatively high rate in the CT (83.3%) group.

Ovarian follicles can be isolated using two approaches: mechanical, which involves the use of a >28 G needle, and enzymatic. In a recent paper, Le et al. [24] assessed the effect of crude (IA and IV) and purified (Liberase TM and DH) collagenases on in vitro growth and oocyte quality during the isolation step. The authors cultured the isolated follicles in an agarose 3D culture system until Day 18. Interestingly, they found that a higher collagenase concentration provided better follicle quality (92.5% G1 at 1 mg/mL and 13.26% at 0.05 mg/mL, respectively), the opposite effect to that observed for oocytes, which deteriorate in quality at higher enzyme concentrations. Therefore, the authors hypothesize that there is an optimal concentration that provides a high rate of good-quality oocytes and satisfactory-quality follicles. They found the optimal concentration of crude collagenase to be 0.1 mg/mL, with 0.07 W/mL of the purified form. In a study by Campos et al. [25], the authors developed a protocol for isolating preantral follicles in collared peccaries. Collagenase IV (0.5 mg/mL) was used for digestion, and this was compared to the mechanical method. The results demonstrated that the enzymatic method provided a higher number of preantral follicles (primordial, primary, and secondary: 961.7 ± 132.9) than the mechanical method (434.3 ± 88.9 , $p < 0.05$). Analysis of viability after 24 h of culture showed that the enzymatic method preserved viability at a higher rate ($98.7 \pm 0.6\%$ vs. $89.2 \pm 1.6\%$ for the mechanical method, respectively). In our study, we isolated the PAFs using collagenase IV by incubating the samples in a solution of 3 mg/mL for 60 min. This method was based on that of Durant et al. (1998) [26], and was also used in our previous studies on canine follicles [12]. This type of digestion yielded high-quality follicles (>80% viable cells) in all examined treatments and is considered an appropriate technique for pig breeding.

Despite the many attempts that have been made to establish well-working protocols for PAF IVC, standardized methods are lacking, and results are inconsistent, especially in the case of pigs. However, all of these methods are based on the supplementation of a common basic medium with a hormone. In a study by Mao et al. [27], the authors investigated the granulosa cell proliferation (follicular growth) when they were cultured in the presence or absence of different concentrations of IGF1 (0–100 ng/mL) and EGF (10 ng/mL) in serum-containing or serum-free medium for 8 days. All of the media contained FSH. Data show that follicles exhibited a dose-dependent response to IGF-1, showing the highest proliferation rate when 100 ng/mL IGF-1 was added to the medium. However, if the medium did not contain serum, follicular growth showed a low rate, irrespective of whether EGF and/or IGF-I were present. In a paper by Rocha et al. [28], secondary follicles were cultured for 4 days. They analyzed the effect of FSH and FSH + AMH supplementation. There was no effect of AMH on growth rate, normal morphology rate, and estradiol production, which means that adding hormones to the culture medium besides FSH is unnecessary and makes the whole procedure more expensive.

During the in vitro culture period, investigating the function of the samples with a non-invasive method, as well as carrying out a morphological assessment, is crucial. One suitable method for achieving this goal is to measure the estradiol production of each follicle. In the papers by Wu et al. [29,30], isolated follicles were cultured for four days in an FSH-supplemented medium. They observed a continuous increase in estradiol levels over the short culture period. Tasaki et al. [31] examined the effect of E2 on the formation of antrum in porcine oocyte-granulosa complexes isolated from preantral and early antral follicles. The samples were cultured for 14 days, and the production of estradiol was measured in the culture media. Furthermore, an estradiol receptor inhibitor was added. Follicles that formed an antrum produced significantly more estrogen than those that did not (8.5 ± 1.2 ng/mL vs. 3.5 ± 0.7 ng/mL, respectively). However, in our study, we did not find such an obvious correlation between hormonal production and growing rate (follicular

area). Furthermore, in that study, if an estradiol receptor antagonist was added, there was no antrum formation, which occurred only if the E2 supplementation was elevated to 1 ug/mL. These data show that E2 is a key factor in follicle development, especially in antrum formation. Therefore, measuring E2 levels from the culture medium is a viable tool to non-invasively analyze the metabolism of PAFs.

Besides the genetic- and fertility-preservation aspect, the cryopreservation and IVC of PAFs could be a useful tool for gene editing (GE) studies in the biotechnology industry. Recent advances in biotechnology are offering techniques that optimize the efficiency of GE animal production; therefore, a substantially higher chance of a satisfactory outcome can be anticipated. In this process, PAF could act as an almost limitless source for recipient oocytes in somatic cell nuclear transfer (SCNT) and advanced techniques, such as CRISPR/Cas9 [32].

Our culture system for porcine PAFs is based on the experiences of our previous studies on mouse and canine samples [11–13]. These findings suggest that our in-house medium can preserve the hormone-producing ability of porcine PAFs; however, it cannot provide the environment for normal growth. Although we found a high 10-day survival rate for fresh and frozen–thawed PAFs, the culture system requires slight refinement to provide suitable conditions for long-term culture. In conclusion, our results suggest that an open vitrification system (such as OPS) is a suitable tool for preserving porcine PAFs, maintaining their functionality more effectively than cryotube freezing.

Author Contributions: Conceptualization, B.S. and S.C.; Methodology, B.S.; Validation, D.T.; For mal analysis, L.B.; Investigation, B.S., D.T. and L.B.; Resources, J.R.; Data curation, B.S., D.T. and L.B.; Writing—original draft, B.S. and S.C.; Writing—review & editing, J.R. and S.C.; Visualization, B.S.; Project administration, L.B.; Funding acquisition, B.S. and S.C. All authors have read and agreed to the published version of the manuscript.

Funding: Project no. 134887 has been implemented with the support provided by the Ministry of Innovation and Technology of Hungary from the National Research, Development and Innovation Fund, financed under the FK_20 funding scheme. This study was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (BO/00399/24/4). Project no. TKP2020-NKA-01 has been implemented with support provided by the National Research, Development and Innovation Fund of Hungary, financed under the Tématerületi Kiválósági Program 2020 (2020-4.1.1-TKP2020) funding scheme. This study was supported by the strategic research fund of the University of Veterinary Medicine Budapest (Grant No. SRF-001). This study was supported by the scientific research fund of the University of Veterinary Medicine Budapest (Grant No. SRF-002).

Institutional Review Board Statement: Not Applicable.

Informed Consent Statement: Not Applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

PAF	Preantral follicle
LN2	Liquid nitrogen
ART	Assisted reproductive techniques
DPBS	Dulbecco phosphate-buffered saline
CT	Cryotube
OPS	Open pulled straw
ES	Equilibration medium

VS	Vitrification medium
HM	Holding medium
SM	Sucrose medium
FBS	Fetal bovine serum
IVC	In vitro culture

References

1. Pukazhenthi, B.; Comizzoli, P.; Travis, A.J.; Wildt, D.E. Applications of emerging technologies to the study and conservation of threatened and endangered species. *Reprod. Fertil. Dev.* **2005**, *18*, 77–90. [\[CrossRef\]](#)
2. Herrick, J.R. Assisted reproductive technologies for endangered species conservation: Developing sophisticated protocols with limited access to animals with unique reproductive mechanisms. *Biol. Reprod.* **2019**, *100*, 1158–1170. [\[CrossRef\]](#)
3. Abedelahi, A.; Rezaei-Tavirani, M.; Mohammadnejad, D. Fertility preservation among the cancer patients by ovarian tissue cryopreservation, transplantation, and follicular development. *Iran. J. Cancer Prev.* **2013**, *6*, 123–132.
4. Kagawa, N.; Silber, S.; Kuwayama, M. Successful vitrification of bovine and human ovarian tissue. *Reprod. Biomed. Online* **2009**, *18*, 568–577. [\[CrossRef\]](#)
5. Shaw, J.M.; Oranratnachai, A.; Trounson, A.O. Fundamental cryobiology of mammalian oocytes and ovarian tissue. *Theriogenology* **2000**, *53*, 59–72. [\[CrossRef\]](#)
6. Amorim, C.A.; Jacobs, S.; Devireddy, R.V.; Van Langendonck, A.; Vanacker, J.; Jaeger, J.; Luyckx, V.; Donnez, J.; Dolmans, M.-M. Successful vitrification and autografting of baboon (*Papio anubis*) ovarian tissue. *Hum. Reprod.* **2013**, *28*, 2146–2156. [\[CrossRef\]](#) [\[PubMed\]](#)
7. Gupta, P.S.P.; Kaushik, K.; Johnson, P.; Krishna, K.; Nandi, S.; Mondal, S.; Kumar Tej, J.N.; Somoskoi, B.; Cseh, S. Effect of different vitrification protocols on post thaw viability and gene expression of ovine preantral follicles. *Theriogenology* **2022**, *178*, 1–7. [\[CrossRef\]](#)
8. Papatheodorou, A.; Vanderzwalmen, P.; Panagiotidis, Y.; Prapas, N.; Zikopoulos, K.; Georgiou, I.; Prapas, Y. Open versus closed oocyte vitrification system: A prospective randomized sibling-oocyte study. *Reprod. Biomed. Online* **2013**, *26*, 595–602. [\[CrossRef\]](#) [\[PubMed\]](#)
9. Shaw, J.M.; Jones, G.M. Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos. *Hum. Reprod. Update* **2003**, *9*, 583–605. [\[CrossRef\]](#) [\[PubMed\]](#)
10. Bielanski, A.; Vajta, G. Risk of contamination of germplasm during cryopreservation and cryobanking in IVF units. *Hum. Reprod.* **2009**, *24*, 2457–2467. [\[CrossRef\]](#)
11. Bordás, L.; Somoskői, B.; Török, D.; Vincze, B.N.; Cseh, S. Post-thaw viability of mouse preantral follicles after cryopreservation with cryotube freezing and OPS vitrification procedures. *Reprod. Biol.* **2023**, *23*, 100752. [\[CrossRef\]](#)
12. Somoskői, B.; Török, D.; Lacalandra, G.M.; Bordás, L.; Cseh, S. In vitro culture of canine preantral follicles after slow freezing. *Anim. Reprod. Sci.* **2025**, *278*, 107860. [\[CrossRef\]](#)
13. Somoskői, B.; Bordás, L.; Uno, F.; Kispál, D.; Müller, L.; Török, D.; Cseh, S. Effects of different cryopreservation methods on canine isolated preantral follicles. *Anim. Reprod. Sci.* **2023**, *258*, 107361. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Muñoz, M.; Bozzi, R.; García-Casco, J.; Núñez, Y.; Ribani, A.; Franci, O.; García, F.; Škrlep, M.; Schiavo, G.; Bovo, S.; et al. Genomic diversity, linkage disequilibrium and selection signatures in European local pig breeds assessed with a high density SNP chip. *Sci. Rep.* **2019**, *9*, 13546. [\[CrossRef\]](#)
15. FAO. Domestic Animal Diversity Information System (DAD-IS). Risk Status of Animal Genetic Resources. 2025. Available online: <https://www.fao.org/dad-is/risk-status-of-animal-genetic-resources/en/> (accessed on 13 January 2026).
16. Abbott, A. An inside look at the first pig biobank. *Nature* **2015**, *519*, 397–398. [\[CrossRef\]](#)
17. European Food Safety Authority; Ståhl, K.; Boklund, A.E.; Podgórski, T.; Vergne, T.; Aminalragia-Giamini, R.; Abrahantes, J.C.; Papaleo, S.; Mur, L. Epidemiological analysis of African swine fever in the European Union during 2024. *EFSA J.* **2025**, *23*, e9436. [\[CrossRef\]](#) [\[PubMed\]](#)
18. Zorc, M.; Škorput, D.; Gvozdanović, K.; Margeta, P.; Karolyi, D.; Luković, Z.; Salajpal, K.; Savić, R.; Muñoz, M.; Bovo, S.; et al. Genetic diversity and population structure of six autochthonous pig breeds from Croatia, Serbia, and Slovenia. *Genet. Sel. Evol.* **2022**, *54*, 30. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Moniruzzaman, M.; Bao, R.-M.; Taketsuru, H.; Miyano, T. Development of vitrified porcine primordial follicles in xenografts. *Theriogenology* **2009**, *72*, 280–288. [\[CrossRef\]](#)
20. Hwang, I.-S. Fertility preservation in pig using ovarian tissues by vitrification method. *J. Anim. Reprod. Biotechnol.* **2022**, *37*, 106–112. [\[CrossRef\]](#)

21. Borges, E.N.; Silva, R.C.; Futino, D.O.; Rocha-Junior, C.M.C.; Amorim, C.A.; B  o, S.N.; Lucci, C.M. Cryopreservation of swine ovarian tissue: Effect of different cryoprotectants on the structural preservation of preantral follicle oocytes. *Cryobiology* **2009**, *59*, 195–200. [[CrossRef](#)]
22. Han, J.; Sydykov, B.; Yang, H.; Sieme, H.; Oldenhof, H.; Wolkers, W.F. Spectroscopic monitoring of transport processes during loading of ovarian tissue with cryoprotective solutions. *Sci. Rep.* **2019**, *9*, 15577. [[CrossRef](#)]
23. Gabriel, P.R.; Torres, P.; Fratto, M.C.; Cisale, H.; Claver, J.A.; Lombardo, D.M.; Fischman, M.L. Effects of different sucrose concentrations on vitrified porcine preantral follicles: Qualitative and quantitative analysis. *Cryobiology* **2017**, *76*, 1–7. [[CrossRef](#)]
24. Le, B.A.M.; Nguyen, L.B.L.; Nguyen, P.T.; Vo, H.N.L.; Tran, N.S.T.; Tran, B.N.; Nguyen, N.T.V.; Lam, C.T.; Nguyen, N.-T.; Nguyen, V.T.; et al. Enzymatic isolation of porcine preantral follicles impairs oocyte viability and long-term in vitro growth. *J. Reprod. Dev.* **2025**, *71*, 124–136. [[CrossRef](#)] [[PubMed](#)]
25. Campos, L.B.; Silva, A.M.d.; Moreira, S.S.J.; Santos, C.S.; Apolin  rio, C.A.d.C.; Saraiva, M.V.A.; Oliveira, M.F.d.; Silva, A.R. Establishment of a protocol for the isolation of ovarian preantral follicles derived from collared peccaries (*Pecari tajacu*). *Zygote* **2019**, *27*, 315–320. [[CrossRef](#)] [[PubMed](#)]
26. Durrant, B.S.; Pratt, N.C.; Russ, K.D.; Bolamba, D. Isolation and characterization of canine advanced preantral and early antral follicles. *Theriogenology* **1998**, *49*, 917–932. [[CrossRef](#)] [[PubMed](#)]
27. Mao, J.; Smith, M.F.; Rucker, E.B.; Wu, G.M.; McCauley, T.C.; Cantley, T.C.; Prather, R.S.; Didion, B.A.; Day, B.N. Effect of epidermal growth factor and insulin-like growth factor I on porcine preantral follicular growth, antrum formation, and stimulation of granulosa cell proliferation and suppression of apoptosis in vitro. *J. Anim. Sci.* **2004**, *82*, 1967–1975. [[CrossRef](#)]
28. Rocha, R.M.P.; Rubessa, M.; Lima, L.F.D.; Silva, A.F.B.D.; Winters, R.; Polkoff, K.; Milner, D.; Campello, C.C.; Figueiredo, J.R.D.; Wheeler, M. Effect of base media, FSH and anti-M  llerian hormone (AMH) alone or in combination on the growth of pig preantral follicles in vitro. *Res. Soc. Dev.* **2021**, *10*, e53101522488. [[CrossRef](#)]
29. Wu, J.; Carrell, D.T.; Wilcox, A.L. Development of In Vitro-Matured Oocytes from Porcine Preantral Follicles Following Intracytoplasmic Sperm Injection. *Biol. Reprod.* **2001**, *65*, 1579–1585. [[CrossRef](#)]
30. Wu, J.; Emery, B.R.; Carrell, D.T. In Vitro Growth, Maturation, Fertilization, and Embryonic Development of Oocytes from Porcine Preantral Follicles. *Biol. Reprod.* **2001**, *64*, 375–381. [[CrossRef](#)]
31. Tasaki, H.; Iwata, H.; Sato, D.; Monji, Y.; Kuwayama, T. Estradiol has a major role in antrum formation of porcine preantral follicles cultured in vitro. *Theriogenology* **2013**, *79*, 809–814. [[CrossRef](#)]
32. Tu, C.-F.; Peng, S.-H.; Chuang, C.; Wong, C.-H.; Yang, T.-S. Invited Review—Reproductive technologies needed for the generation of precise gene-edited pigs in the pathways from laboratory to farm. *Anim. Biosci.* **2022**, *36*, 339–349. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.