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Augmentation of Temperatures, Media and Cryoprotectants on *in vitro* Fertilization of Pig Oocytes

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Abstract: There are numerous challenges to the large-scale production of pig embryos using *in vitro* procedures. The aim of the present study was to compare the different holding temperatures, *in vitro* maturation media, and permeating cryoprotectants in the *in vitro* fertilization of pig oocytes. Ovaries were obtained from the local slaughterhouse, oocytes were retrieved by means of aspiration and slicing techniques and categorized into grades A, B, and C. Good quality oocytes were stored in an Eppendorf tube containing a holding solution at 5, 18, 24, and 38.5°C for 5, 30, 60, and 120 min, respectively. Oocytes were matured for 44 h and then checked for polar body extrusion. Matured oocytes were exposed to cryoprotectant Toxicity Test (TT1) with 7.5% of DMSO + 7.5% of EG and (TT2) with 15% of DMSO + 15% of EG. Oocytes were then *in vitro* fertilized with fresh semen, incubated for 24 h and the pronucleus was checked. The analysis of variance (ANOVA) was utilized to analyze data using the General Linear Model (GLM) procedure and treatment means were compared with the Least Significance Difference (LSD) test. The results revealed that at 38.5°C, the cumulus oocytes complexes (COCs) expansion rate was significantly ($p<0.05$) higher (75.8±14.0), no damaged cytoplasm and the polar body was higher (25.5±7.6) compared to all other treatments. Oocytes polar body extrusion were (32.8±13.6, 25.8±9.1 and 11.5±6.9) for NCSU-23, NCSU-37 and TCM-199 respectively. There was a significant ($p<0.05$) difference in the total fertilization rate of the control (75.8±17.2) and the combination of DMSO and EG (50.3±21.4). Pig oocytes are very sensitive to lower temperatures after IVM. The NCSU-23 and NCSU-37 media enhance pig oocytes polar body extrusion. The combination of DMSO and EG affected the pronucleus since the cryoprotectants have toxicity when used on pig oocytes after maturation.

Keywords: IVF, Oocytes, Permeating Cryoprotectants, Polar Body, Pronuclear

Introduction

Pigs are believed to represent important experimental models (Fang *et al*., 2022). Recently, strains of pigs have been developed with genetic modifications that mimic various human diseases (Perleberg *et al*., 2018). *In vitro* methods use ovaries from slaughterhouses, which are easily accessible, affordable, and capable of producing vast quantities of oocytes in a short period of time, to support studies for *in vitro* reproductive biotechnology. Although pig ovaries are available at many slaughterhouses, most slaughtered females are pre-pubertal. The most popular techniques for retrieving oocytes from slaughterhouse ovaries are follicular aspiration and ovarian cutting (Marques *et al*., 2015). The oocyte retrieval method, which can affect oocyte quality and maturation, influences whether high-quality blastocysts can be successfully produced *in vitro* (Hammoud and Jebur, 2022). Oocyte quality and developmental capacity are acquired gradually throughout maturation (Silvestri *et al*., 2021).

There are numerous challenges involved in successfully producing *in vitro* pig embryos on a large scale (Absalón-Medina *et al*., 2014). Pig oocytes

maturation takes place over a longer period than in most other species. The basic medium for In Vitro Maturation (IVM) of pig oocytes are North Carolina State University medium-23 (NCSU-23) with 10% Pig Follicular Fluid (PFF), NCSU-37 with 10% PFF and Tissue Culture Medium-199 (TCM-199) with 10% PFF (Nguyen *et al*., 2021). Pig oocytes are known to be sensitive to temperatures below 15°C (Dai *et al*., 2015) because of their large size (Amstislavsky *et al*., 2019) and high intracellular lipid amount that remains primarily stored as droplets inside the cytoplasm (Tharasanit and Thuwanut, 2021). Other germ and somatic cells are less sensitive to temperature than oocytes. According to Lin *et al*. (2011), compared the oocytes of cattle and goats, pig oocytes are more sensitive to low temperatures. In pigs (Somfai *et al*., 2013) and other mammalian species (Kuwayama, 2007), it has been shown that combining different Cryoprotectants (CPAs) produces better results than using a single type CPAs.

The CPAs have an impact on the survival and growth of the preserved oocytes (Mazur *et al*., 2008). For successful oocytes vitrification, the type and concentration of CPAs in the cryopreservation medium should be considered. The use of high concentrations of CPA can increase the toxicity of the oocytes. The survival of vitrified-warmed oocytes is compromised by the vitrification solution volume being increased (Kuwayama, 2007). The most popular penetrating cryoprotectant is Ethylene Glycol (EG), which has a low molecular weight (62.1 g/moL), high permeability to osmotic shock, and low toxicity (De Oliveira *et al*., 2021). Dimethyl Sulfoxide (DMSO) has a high glass-forming potential, increases the permeability of other cryoprotectants, and is often combined with EG (Bartolac *et al*., 2018). Issues have lately been raised over the safety of employing DMSO for vitrification, as solvents may produce toxic substances for the developing embryos (Balaban *et al*., 2008; Yuta *et al*., 2017). Dimethyl Sulfoxide was discovered to enhance intracellular calcium concentrations in mouse oocytes by altering intracellular organelles (Larman *et al*., 2006). The zona reaction occurs when cortical granules in oocytes exocytize, resulting in an increase in intracellular calcium (Suri *et al*., 2023). According to Sensui *et al*. (2023), the zona reaction prevents sperm from entering the perivitelline spaces. Vitrified oocytes capacity to fertilize and develop can therefore be enhanced by suppressing the intracellular calcium surge. The study was conducted to compare the quality and quantity of retrieved oocytes using aspiration and slicing techniques. The survival rate of pig oocytes preserved at 5, 18, and 24°C. *In vitro* maturation media (North Carolina State University-NCSU-23, Tissue Culture Medium-TCM-199 also North Carolina State University-NCSU-37) for cytoplasmic and nuclear maturation. Survivability of matured oocytes after being exposed to 7.5% Dimethyl Sulfoxide (DMSO) + 7.5% Ethylene Glycol (EG) and 15% DMSO + 15% EG on fertilization ability following *In Vitro* Fertilization (IVF).

Materials and Methods

Animal Ethics, Chemicals and Reagents

The Agricultural Research Council ethics committee APIEC (2019/32), as well as the Tshwane University of Technology ethics committee AREC (2021/08/009), examined and authorized the experiment. The chemicals and reagents were acquired from Sigma Chemical Co. in St. Louis, Missouri, USA, unless otherwise noted.

Study Location

The research was carried out in the Germplasm Conservation and Reproductive Biotechnologies laboratory of the Agricultural Research Council-Irene. The area is located in Pretoria, South Africa, at 25° 53′ 59.6″ South latitude and 28º 12′ 51.6″ East longitude, on the Highveld at an elevation of 1525 m above sea level. The weather ranges from hot summer days (17.5-32°C) to mild winter days with very cold nights (1-17°C).

Collection of Pig Ovaries

The ovaries were extracted from the slaughterhouse of unidentified pre-pubertal hybrid gilt breeds. Ovaries collected were stored inside the thermoflask at 38°C, protected with buffered saline solution, and transferred from the slaughterhouse to the laboratory in an hour post-slaughter. To get rid of blood clots and superficial bacterial contamination, extra ovarian tissues were removed, ovaries were washed three times using saline solution, and stored in a sterile beaker containing normal saline until oocytes retrieval.

Experiment I

Experiment I enclosed the oocytes retrieved by slicing and aspiration to isolate Cumulus-Oocyte Complexes (COCs). About 71 ovaries per treatment replicated seven times were collected to determine the quality of oocytes retrieved by aspiration and slicing. The quality of oocytes was visualized by grading COCs based on morphology, good oocytes (grade A), fair oocytes (grade B), and poor oocytes (grade C). Oocyte number was visualized by calculating good, fair, and poor oocytes from both retrieving techniques. Oocytes with many complete layers of cumulus cells and uniform cytoplasm were graded as good, oocytes with thin or incomplete layers of cumulus cells and uniform cytoplasm as fair, and oocytes with few or no cumulus cells as poor. Thereafter ovary slicing was the selected method to perform other objectives.

Oocytes Retrieval by Aspiration

During aspiration, COCs were aspirated from all the visible follicles measuring >2-6 mm diameter using a

sterile 18-gauge hypodermic needle attached to a 10 mL disposable syringe containing 0.5-1 mL of aspiration medium. In a sterile 50 mL tube containing modified Dulbecco's Phosphate Buffered Saline (mDPBS) follicular fluid has been allowed to settle for approximately 15-20 min at 37°C. After 10-15 min, the supernatant was discarded and the pellet was suspended in an oocyte washing medium and screened for oocyte in a 90 mm petri dish.

Oocytes Retrieval by Slicing

During the slicing, the ovaries were cut into thin slices using the standard method described by Henning *et al*. (2019). The follicular fluid was then transferred into a 50 mL tube and placed in the water bath for 10-15 min to allow the pellet to settle. Using a Pasteur pipette, the supernatant was removed deprived of disrupting the pellet.

Grading of Pig Oocytes

Three plates (Falcon 1008) contained three mL of mDPBS, whereas the other 3 (Falcon 1008) had three mL containing medium 199 (M-199) supplemented with 10% fetal bovine serum. The oocytes were inspected under a microscope (Olympus BX71, Philippines) and then rinsed three times in a 35 mm Petri plate (Falcon 1008) filled with three mL containing mDPBS. Using a pipette, the oocytes were washed three times using M-199. After washing they were then graded according to Pawshe *et al*. (1996). Grade C as well as B oocytes were thrown away and the grade A oocytes chosen on behalf of holding, *in vitro* maturation also *in vitro* fertilization or cryoprotectants toxicity test.

Experiment II

Experiment II enclosed the feasibility of preserving porcine oocytes without freezing. A total of 200 oocytes per treatment were preserved in an Eppendorf tube containing ViGRO™ Holding Plus medium, replicated eight times. Pig Cumulus-Oocyte Complexes (COCs) were preserved in ViGRO™ Holding Plus at 5, 18 and 24°C for 5, 30, 60 and 120 min. After preservation, oocytes were washed three times in M-199 and thereafter exposed to IVM medium (NCSU-23). Oocytes were evaluated for morphology based on cytoplasm damage and cytoplasm and nuclear maturation. The purpose of this objective was to evaluate short-term oocyte preservation and long-distance transportation, which not only avoids toxicity and cryoinjuries but also allows synchronous maturation.

Preserving of Oocytes at Different Temperatures

Good quality oocytes were stored in an Eppendorf tube containing holding solution (ViGRO™ Holding Plus) at 5, 18, and 24°C for 5, 30, 60, and 120 min. At 38.5°C oocytes were not exposed to holding solution at different holding times, it was regarded as a control treatment.

Experiment III

Experiment III enclosed the evaluation of the ability of the three different maturation media to mature good-quality oocytes retrieved from slicing. A total of 203 oocytes per treatment, replicated five times were matured using North Carolina State University-23 (NCSU-23) with glucose and taurine, North Carolina State University-37 (NCSU-37) with glucose and taurine and Tissue Culture Medium-199 (TCM-199) with bovine serum albumin, epidermal growth factor, and PFF. Most of the IVM studies evaluated the use of maturation media with hormones for 44 h incubation. Other studies incubate oocytes in IVM media for 22 h without hormones and for 22 h with hormones. The study evaluated the pig oocyte maturation media incubated for 44 h without hormones. After 44 h of maturation polar bodies' status was evaluated. NCSU-23 was the selected medium to further other objectives.

Pig Oocytes in vitro Maturation

Good quality pig oocytes were matured into a Nunc 4-well multi dish each containing 500-µL of NCSU-23, NCSU-37, and TCM-199. The amount of 250-µL mineral oil was used in the media before incubation. The oocytes were incubated at 38.5°C with 5% CO_2 for 44 h (Zhang *et al*., 2019). Eppendorf tubes were used to store mature oocytes containing 200-µL pre-warmed M-199 during vortexing (Lin *et al*., 2015). Oocytes were placed in a petri dish with three mL of M-199 after vortexing to evaluate polar body development on oocytes for each treatment.

Sperm Preparation and Motility

Using the hand-gloved technique, boar semen was collected. To separate the sperm-rich fraction from the gel fraction, a thermoflask full of warm (37°C) water and a glass beaker with a gauze filter were used. The ejaculate was taken to the laboratory for its macroscopic and microscopic evaluation within 30 min post-collection. The initial sperm macroscopic characteristics (semen volume, pH, and sperm concentration) were assessed. A 250 mL beaker was used to measure the volume of the semen and an Oakton pH meter was used to determine the pH. A spermacue® (mini tub, Germany) was used to measure the concentration. A swim-up technique was used to analyze the rates of motility. To do this, 10-µL containing semen was combined with 200-µL containing Beltsville Thawing Solution (BTS), and 5-µL of the mixture was placed on a warmed microscope slide (76×26×1 mm-Wadmar-Knittel, Germany) and covered with a cover slip (22×22 mm-Wadmar-Knittel, Germany). The mixture was then analyzed using a microscope (Nikon, Eclipse) equipped with a Sperm Class Analyzer® (SCA®) warm plate, adjusted to 37°C. Sperm Class Analyzer® (SCA®) technology was used to evaluate the characteristics of sperm motility.

Experiment IV

Experiment IV enclosed the evaluation of the survivability of matured pig oocytes after exposure to a holding solution containing mDPBS with 20% FBS and a 15% combination of DMSO and EG (7.5% each) for 3 min. Pig oocytes were exposed to a vitrification solution containing mDPBS with 20% FBS and a 30% combination of DMSO and EG (15% each) for 30 sec. Other matured oocytes were not exposed to cryoprotectant toxicity. A total of 210 oocytes per treatment, replicated ten times were used for the study. The combination of EG and DMSO is chemically hazardous, hence the quest for a less toxic and more effective cryoprotectant is worth researching. Post-exposed oocytes were washed three times in M-199, not cryopreserved, and fertilized with fresh semen to evaluate the pronuclear status and the effect of cryoprotectant toxicity.

Oocytes Toxicity Test

A toxicity test was conducted to evaluate the effect of permeating cryoprotectants on the viability of MII-oocytes. After the maturation procedure, a cryoprotectant toxicity test was performed under six solution tests namely base, rinsing, holding, vitrification, warming, and rehydration solution test. The base medium for the dilution of cryoprotectant was mDPBS with 20% FBS and the warming solution was 0.15 M sucrose in mDPBS with 20% FBS. The oocytes were introduced into a base solution for 1 min, rinsed for 1 min, holding solution (15% EG and DMSO) for 3 min, vitrification solution (30% EG and DMSO) for 30 sec, warming solution for 30 sec and rehydration solution for 1 min. Untreated oocytes from a control group were cultured in mDPBS that had 20% FBS. At room temperature, all manipulations were carried out in 100 mm plastic petri dishes (Greiner, Solingen, Germany). Oocytes at the Metaphase II (MII) stage were equilibrated in DMSO and EG for the toxicity test. The MII oocytes underwent fertilization right after dilution. After the treatments, fertilization rates were determined.

Pig Oocytes in vitro Fertilization

Fresh semen was centrifuged at 2000 rpm at 37°C for 2 min, a Pasteur pipette was used to remove the supernatant and an additional 8 mL of modified Tris Buffered medium (mTBM) was added. After the second centrifugation, the supernatant was extracted. *In vitro* fertilization media was then used to dilute the sperm pellet. The Sperm Class Analyzer® (SCA®), a computer-assisted sperm analysis system, was used to evaluate the sperm motility and velocity characteristics before IVF. A 5-µL fresh sperm sample had been placed into a pre-warmed microscope slide with a cover slip. Slides were then set onto the SCA® system for motility assessment, oocytes were washed five times in pre-prepared dishes (Falcon 1008) using 100-µL droplets of mTBM covered with 3 mL mineral oil. Oocytes were placed in a dish containing 50-µL in each seven drops of mTBM and 3 mL mineral oil. Each 50-µL drop of oocytes added 50-µL of semen/sperm for a total capacity of 100-µL. The IVF dish was incubated for 24 h in the incubator at 38.5°C with 5% CO_2.

Evaluation of Pig Oocytes Maturation

After maturation, oocytes were denuded of cumulus cells by vortexing and evaluated with the aid of an Oosight imaging system connected to an inverted research microscope. Oocytes were analyzed for maturity after 44 h of incubation, those with a Germinal Vesicle (GV) or in metaphase I were considered immature and those in MII with the first polar body as mature. Cytoplasm damage was evaluated by visualizing parameters such as cracked cytoplasm, darkish in color, and lyse and cytoplasm shrinkage.

Evaluation of Pig Oocytes in vitro Fertilization

For observation of post-fertilization pronucleus, oocytes were denuded of cumulus cells by vortexing and mounted on glass slides. We used Vaseline and clear nail polish to keep the coverslip in contact with the oocytes without extensive pressure. Hoechst 33342 was used at a concentration of 10 g/mL to stain oocytes. Oocytes with one Pronucleus (PN) were classified as activated, those with two pronuclei as monospermy, and those with more than two decondensed sperm heads or two pronuclei as polyspermy. Oocytes in the first polar body, MII, were assumed to be unfertilized.

Statistical Analysis

Analysis of Variance (ANOVA) was utilized to analyze data using the General Linear Model (GLM) procedure. The Fisher's protected t-test for Least Significant Differences (LSD) was used to compare treatment means and a Shapiro-Wilk's test was employed to check for variations from normality (Shapiro and Wilk, 1965). Treatment means were compared using a 5% significance threshold and the least significant differences were considered at ($p<0.05$). SAS version 9.4 (SAS, 1999) was used for data analysis.

Results

Table 1 represents aspiration and slicing techniques for the quality and quantity of pig oocytes. The morphology of the quality of the oocytes was graded as A, B, and C. There was a significant ($p<0.05$) difference between the aspiration and slicing methods on grade A and grade C oocytes. There were no significant differences between the aspiration and slicing methods on grade B oocytes.

Table 1: Comparison of aspiration and slicing technique on quality and quantity of pig oocytes

		No. of oocytes classification (grades)			
Methods	No. of ovaries	A	B	C	Total
Aspiration	71	51.0±14.1[b]	37.9 ±14.2	48.9±18.1[b]	137.8±31.3[b]
Slicing	71	103.7±74.4[a]	49.4±28.6	72.0±26.5[a]	225.1±111.7[a]

[a, b] Values in the same column with different superscripts differ significantly ($p<0.05$)
A: Good quality oocytes, B: Fair oocytes and C: Poor oocytes

Table 2: Comparison of different holding temperatures and holding times on pig oocytes' developmental rate following *in vitro* maturation

Temperatures	Time/duration	No. of oocytes IVM	COCs expanded	Damaged cytoplasm	Oocytes with polar body status
38.5°C	0 min	200	75.8±14.0[a]	0[c]	25.5±7.6[a]
5°C	5 Min	200	58.6±14.9[bc]	7.1±7.1[ab]	11.5±4.2[bcd]
18°C		200	62.1±18.2[ab]	5.8±4.9[ab]	15.3±6.3[b]
24°C		200	53.2±18.1[bcde]	5.8±5.8[ab]	15.0±10.9[b]
5°C	30 Min	200	57.6±14.3[bcd]	4.9±4.7[ab]	10.5±4.7[bcd]
18°C		200	59.7±17.5[b]	5.8±4.4[ab]	10.8±5.2[bcd]
24°C		200	55.9±21.3[bcd]	4.4±3.9[abc]	12.2±6.3[bcd]
5°C	60 Min	200	53.8±15.0[bcde]	5.0±5.2[ab]	9.2±5.2[bcd]
18°C		200	45.2±16.4[cde]	7.3±6.6[ab]	7.6±6.6[cd]
24°C		200	54.4±15.9[bcde]	8.1±5.4[a]	14.5±7.3[b]
5°C	120 Min	200	41.8±8.2[e]	5.6±4.4[ab]	6.2±5.2[d]
18°C		200	52.1±8.9[bcde]	5.8±6.1[ab]	12.1±7.1[bcd]
24°C		200	43.9±11.8[de]	7.0±5.5[ab]	12.4±7.9[bc]

[a-e] Values in the same column with different superscripts differ significantly ($p<0.05$). COCs: Cumulus Oocytes Complexes and IVM: *In vitro* maturation

Table 3: Comparison of different *in vitro* maturation media on the polar body development of pig oocytes

Maturation media	Oocytes No.	Oocytes with polar body development (%)
NCSU-23	203	32.8±13.6[a]
NCSU-37	199	25.8±9.1[ab]
TCM-199	203	11.5±6.9[b]

[a, b] Values in the same column with different superscripts differ significantly ($p<0.05$). NCSU-23: North Carolina State University-23, TCM-199: Tissue Culture Medium-199 and NCSU-37: North Carolina State University-37

Table 4: Effects of permeating cryoprotectants on fertilization rate of pig oocytes

Treatment	No. of IVF oocytes	0PN (%)	1PN (%)	2PN (%)	>2PN (%)	Total fertilization rate (%)
Control	210	24.2±17.2[b]	30.4±20.5	15.8±11.6	29.6±21.5	75.8±17.2[a]
DMSO and EG	210	50.7±1.6[a]	20.0±17.6	11.4±7.8	18.0±19.7	50.3±21.4[b]

[a, b] Values in the same column with different superscripts differ significantly ($p<0.05$). DMSO: Dimethyl sulfoxide, IVF: *In vitro* Fertilization, EG: Ethylene Glycol, 0PN: Non-Pronuclear, 1PN: 1-Pronuclei, 2PN: 2-Pronucleus and >2PN: Poly-Pronucleus

Table 2 represents pig oocytes stored at 5, 18, and 24°C for (5, 30, 60, and 120 min) and 38.5°C for 0 min. The cytoplasm damage was evaluated by visualizing cracked cytoplasm, darkish in color, and lyse and cytoplasm shrinkage with polar body extrusion in all groups after IVM. The results of the survival rate of oocytes stored at different holding temperatures and times showed that during 38.5°C, the cumulus Oocytes Complexes (COCs) expansion rate was considerably ($p<0.05$) high no damaged cytoplasm was seen and the polar body (Fig. 1) was higher compared to all other treatments.

Table 3 represent the oocytes incubated for 44 h in three different maturation media to evaluate the nuclear maturation (polar body extrusion) Fig. 1. There was a significant ($p<0.05$) difference between TCM-199, NCSU-23, and NCSU-37 media on oocytes' polar body development. The nuclear maturation rate was significantly ($p<0.05$) higher in NCSU-23 and NCSU-37 than in TCM-199.

Table 4 represents the pig oocytes that were matured for 44 h, after maturation oocytes then underwent the toxicity test after maturation, and then fertilized to test the toxicity and survival of the oocytes exposed to 7.5% DMSO + 7.5% EG and 15% DMSO + 15% EG. The results indicate that exposing oocytes to cryoprotectants after maturation induces subsequent alterations in the total fertilization rate to be lower and 0PN to be high. The pronuclear of *in vitro* fertilized pig oocytes are shown in Fig. 2. DMSO and EG did significantly affect the *in vitro* fertilization rate which is exposed by the formation of pronuclear.

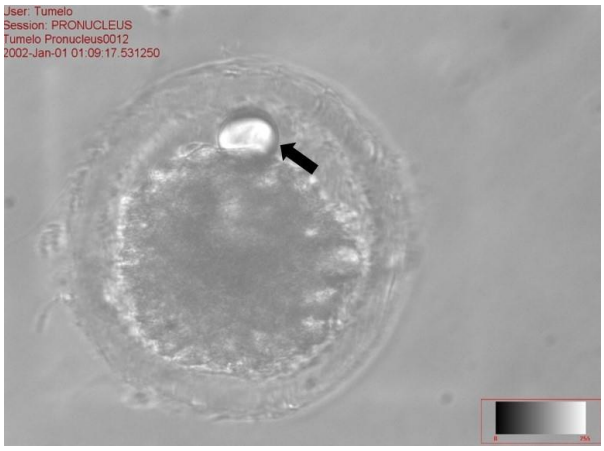

Fig. 1: *In vitro* matured pig oocyte with first polar body indicated with a black arrow

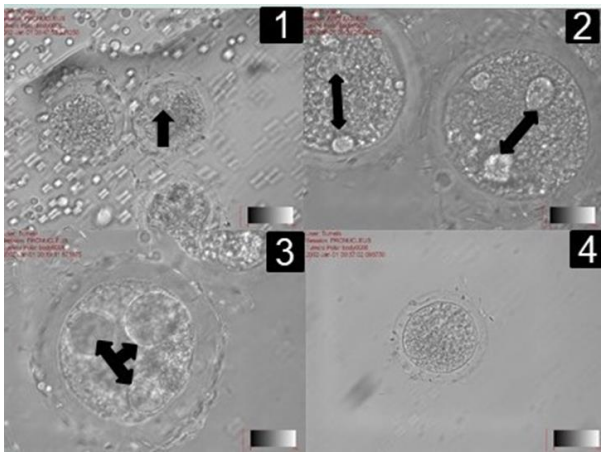

Fig. 2: Oocytes with the arrows showing 1: 1 PN; 2: 2 PN; 3: >2 PN and 4: none PN following *in vitro* fertilization

Discussion

In this study, a mean of 362.9 oocytes was used, but 38% were collected by aspiration and 62% by slicing method. According to Honneysett *et al*. (2021), they recorded similar results for aspiration (28.4%) which was lower, and slicing (71.6%) which was higher in collected oocytes. According to studies (Zeinoaldini *et al*., 2013), the slicing method was able to retrieve more oocytes with cumulus than the aspiration method. The slicing method can minimize mechanical damage during harvesting as compared to aspiration methods. According to Sayid (2021), ovary slicing yielded a greater percentage of oocytes of grade A compared to follicle aspiration. It has been established that follicular aspiration is less effective than whole-ovary slicing. According to Marques *et al*. (2015) data, the slicing technique yielded more oocytes than the aspiration technique. This is consistent with Aryan *et al*. (2023) found that oocytes with more than three COC layers had higher maturation rates and chances of developing into an embryo than oocytes with one or two denuded or partially denuded layers. The oocytes recovery technique clearly has advantages due to its effectiveness, oocytes quality, and oocytes quantity. The slicing technique is thus the most popular for obtaining oocytes of high quality and quantity from pig ovaries.

The pig oocytes survivability rates stored at 5, 18, 24, and 38.5°C holding temperatures were observed, the outcomes also show that oocytes are not protected from the harm caused by the holding solution. Depending on the holding temperature and the holding period, preserved oocytes demonstrated significantly slower maturation rates than the control, as shown in Table 3. The viability of the oocytes was specifically dependent on the holding temperature. Pig oocytes maturation rates drastically decreased at 5°C. After leaving the body, pig ovaries can experience autolysis and degeneration (Andrade *et al*., 2001). Since they are unable to stay fresh during prolonged preservation at physiological temperatures. According to the findings of our study, maturation rates gradually decreased when stored at 5°C for 120 min or more. We selected the holding temperatures based on their practical usefulness and frequency of use. An ordinary refrigerator can be used to reach the 5°C cryogenic storage temperature. In an incubator, sperm can be stored at a temperature of 18°C (Wiebke *et al*., 2022). With air conditioning, a comfortable room temperature of 24°C is simple to achieve. Although coldness is common, pig oocytes are extremely sensitive to temperature (Colombo *et al*., 2023; Kalita *et al*., 2018). The oocytes in the DPBS were moved from 35-38.5°C to a storage temperature of 5°C (refrigerator) and the cold shock effect may have further reduced the maturation rate significantly. Additionally, when the preservation time was 120 min or longer at 5°C, a large number of oocytes were dead. Thus, the maturation rate gradually decreased after 120 min or more of storage at 5°C. At a holding temperature of 5°C, the rate of injured oocytes due to coldness was much lower than at 18°C. At a holding temperature of 24°C, a high maturation rate of up to 120 min could be maintained. Pig oocytes displayed varying degrees of membrane damage and a reduction in the oolemma's membrane potential after preservation, which was partly caused by the high lipid content in pig oocytes (Colombo *et al*., 2023). According to Suzuki *et al*. (1996), preservation caused chromosomal abnormalities such as disorganized metaphase plates and multipolar spindles in oocytes. Although studies on the impact of temperature variations on oocytes maturation in some mammals have been published (Kalita *et al*., 2018) and stated that no comprehensive study has looked at the impact of preservation on pig oocytes maturation.

The subsequent nuclear and cytoplasmic reorganization of GV stage oocytes can be adversely affected by low temperatures (Kalita *et al*., 2018) and

even damage actin mitochondria (Shi *et al*., 2007) and microtubules, including those that make up the meiotic spindle (Kalita *et al*., 2018). The possibility of saving females genetic material after a sudden death in the wild, at a zoo, or following a surgical ovariohysterectomy may occasionally call for the transportation of ovaries over great distances from the site of retrieval to the ART facilities. As a result, establishing protocols for fertility preservation requires careful consideration of the storage conditions (temperature, duration, and medium) for explanted ovaries during transportation. Oocytes can be kept at a temperature of 24°C for longer than 120 min.

The impact of three different IVM media on the nuclear maturation rates of pig oocytes was studied. (NCSU-23, TCM-199, NCSU-37). Gil *et al*. (2010) discovered that the most commonly utilized medium for porcine IVM was NCSU-37, TCM-199, and NCSU-23 which is consistent with the current study. The purpose of this study was to compare the polar body status of three maturation media without the addition of hormones or substances. The reasons for the variation in maturation rate between studies could be due to variations in the base medium, which is usually supplemented with small quantities of additional substances that have appeared to be beneficial for oocytes maturation in most of the studies. Tissue Culture Medium-199 was supplemented with bovine serum albumin, epidermal growth factor, and glucose with a lower nuclear maturation. Pyoos *et al*. (2019) found that North Carolina State University-37 performed better than North Carolina State University-23 in an IVM. Pig oocytes maturation has been demonstrated to benefit from the NCSU-23 and NCSU-37. According to Pyoos *et al*. (2019), the results of polar body extrusion of NCSU-23 was 72.7% and NCSU-37 was 81.9%. This might be the result of adding hormones and Pig Follicular Fluid (PFF) to the NCSU-23 which was supplemented with Follicular Stimulating Hormone (FSH) and Luteinizing Hormone (LH) and NCSU-37 which was supplemented with dbcAMP stock solution, Pregnant Mare Serum Gonadotropin (PMSG) and human Chronic Gonadotropin (hCG). Electrolytes, hormones, amino acids, and growth factors are just a few of the substances found in the follicular fluid that may help with cumulus cell growth and nuclear maturation (Hood *et al*., 2022). Cyclic adenosine monophosphate is temporarily increased by the hormones FSH and LH, while EGF peptides are stimulated. Cytoplasmic maturation happens more gradually than nuclear maturation, which can be triggered mechanically by removing the oocytes from the follicle (Soares *et al*., 2020). Hence, to gain a clearer understanding, further studies are required to interpret the effect of higher levels of cytoplasmic lipids and fatty acid quantity in immature pig oocytes has been linked to the pig oocytes' increased sensitivity in *in vitro* maturation.

Toxicity of cryoprotectant on matured pig oocytes after exposure to Dimethyl Sulfoxide (7.5% DMSO) + ethylene glycol (7.5% EG) and 15% DMSO +15% EG affected the total fertilization rate. These combinations significantly affect the *in vitro* fertilization rate which is exposed by the formation of the pronuclear stage. The success time post-warm survival is influenced by the temperature at which oocytes are exposed to cryoprotectants (Liang *et al*., 2012). A vitrification protocol described by Kuwayama *et al*. (2005), a prolonged equilibration duration was used to allow more cryoprotectants to penetrate, as well as room-temperature vitrification solutions to reduce the harmful effects of warming to 37°C. By the 1-pronuclear, 2-pronucleus, and poly-pronucleus development stages, the total fertilization rate in the DMSO and EG-treated group was 50.3% lower than that of the untreated group (75.8%).

According to Yang *et al*. (2012), buffalo oocytes were less likely to survive vitrification in 35% EG alone than in a combination of 20% EG and 20% DMSO. Cuello *et al*. (2008), on the other hand, found that post-warm survival rates for *in-vivo* derived pig blastocysts vitrified in 40% EG and 17% EG +17% DMSO were comparable. However, in the 7.5% DMSO +7.5% EG and 15% DMSO +15% EG supplemented group, significant developmental inhibition was observed from the fertilization, non-pronuclear; 1-pronucleus, 2-pronuclear or poly-pronucleus were formed. The results showed that pronucleus development is inhibited when mature oocytes are exposed to cryoprotectants before IVF. To increase survival rates, the nature and concentration of the cryoprotectants, as well as the temperature of the vitrification solution at the time of cell exposure, must be considered, and the amount of time the cells spend in the final cryoprotectants before being suspended in LN_2 (García-Martínez *et al*., 2021). To avoid any expected toxic shock caused by being subjected to higher concentrations of cryoprotectants in the final vitrification solution, oocytes may be exposed to cryoprotectants for a shorter period of time, or they may be pre-equilibrated in a vitrification solution with a lower concentration of permeating cryoprotectants (Fahy and Wowk, 2021). It is still unknown when it is best to suspend oocytes in a warming solution. Exposing oocytes post-maturation to the combination of EG and DMSO prevents osmotic shock, facilitating oocyte dehydration and reducing oocytes damage. The concentration of 15 and 30% DMSO and EG can be used to cryoprotect the oocytes before preservation.

Conclusion

When compared to the aspiration technique, the slicing technique for oocyte retrieval yielded more and higher quality oocytes. Given the maturation rate, a storage temperature of 24°C for a 120 min period would be ideal. Better maturation rates can be achieved by using NCSU-37 and NCSU-23 pig oocytes IVM media. The combination of

DMSO and EG affected the pronucleus since the cryoprotectant has toxicity when used on pig oocytes after maturation. Further research is necessary concerning pig oocyte storage to determine its practicality when used in conjunction with *in vitro* maturation and the different combinations of permeating cryoprotectant toxicity and different concentrations on matured pig oocytes.

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Author's Contributions

Tumelo Thelma Maduwa and Masindi Lottus Mphaphathi: Conception, design, data collection, analysis, interpretation, drafted and reviewed of the article.

Tshimangadzo Lucky Nedambale: Conception, designed and Review of the article.

Ethics

The Agricultural Research Council ethics committee (APAEC 2019/32) and the Tshwane University of Technology ethics committee AREC (2021/08/009) both provided ethical clearance.

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