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Comparison of *in vitro* Maturation Media on Cattle Oocytes after *in vitro* Embryo Production

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Abstract: The study was conducted to compare differences *in vitro* maturation media and their effect on subsequent *in vitro* embryo production. The study procedures were approved by the agricultural research council animal ethics committee (Ref no: APAEC 2020/05) and the Tshwane university of technology animal research ethics committee (Ref no: AREC 2021/08/004). Follicular fluid containing a suspension of oocytes was retrieved from ovaries through aspiration and slicing techniques. The retrieved oocytes were graded and randomly matured into four media (TCM199, Vitromat Protect, and BO IVM®) groups for 22 h, evaluated for polar body extrusion, fertilized through their respective *in vitro* fertilization media, and evaluated for pronucleus status. Presumptive zygotes were further cultured *in vitro* in their respective media, incubated for either 18 or 96 h, and evaluated for cleavage rates. The data was subjected to an appropriate analysis of variance. Student's Least Significant Differences (t-LSD) was calculated at a 5% significance level to compare means of significant treatment effects. The BO-IVM® (57.4%), TCM 199 (55.6%), and VitroMat Protect® (54.0%) recorded higher results than EGF (26.9%) for polar body formation on oocytes ($P<0.05$). Vitrofert® medium recorded a higher percentage (43.3%) of total fertilization rate when compared to the other media ($P<0.05$). VitroCleave Plus® medium recorded a higher total cleavage rate (43.3%) as compared to the other media ($P<0.05$). In conclusion, TCM 199, BO-IVM®, and VitroMat Protect® media rendered higher results of oocyte polar body extrusion rates compared to the EGF medium. Vitrofert® medium recorded a higher percentage (43.3%) of total fertilization rate when compared to the other media. Higher embryo development (4-8 cells and total cleavage) rates were observed in VitroCleave Plus® medium. Overall, the introduced media (VitroMat Protect®) and its media suite successfully adapted to the laboratory environment in South Africa (SA) and can therefore be adopted for optimizing the *in vitro* Embryo Production (IVEP) of cattle oocytes.

Keywords: Ovaries, Aspiration, Grading, Cumulus Oocytes Complexes

Introduction

An essential component of the *in vitro* embryo production (IVEP) innovation is the number of high-quality oocytes extracted from the ovary. Baldassarre *et al.* (1996); Luciano *et al*. (2021) reported that oocytes for IVEP or *In Vitro* Fertilization (IVF) are extracted via aspiration, slicing, or puncturing techniques from a variety of sources, including the oviducts after ovulation, mature follicles just before ovulation, or immature and atretic follicles generated from slaughterhouse material. Ovaries collected from slaughtered animals are way cheaper and the most adequate source of primary oocytes for the production of embryos by *In Vitro* Production (IVP) procedure (Wang *et al*., 2007; Ratul, 2020).

Although oocytes can be retrieved in relatively large numbers from abattoir ovaries, (Hoque *et al*., 2011) found that they frequently had inferior development potential

when compared to *in vivo* developed or immature oocytes collected after gonadotropin treatment. This poor development potential is linked to the maturation process that limits the suitability of these oocytes for biotechnology research and delays the application of IVP (Cognie *et al*., 2003; Hoque *et al*., 2011). The cumulus cells surrounding the oocytes are vital for oocytes maturation, energy substrates, and messenger molecules for the development of oocytes (Hoque *et al*., 2011). *In Vitro* Maturation (IVM) is characterized by a physiological process that comes first and could be a prerequisite for effective fertilization and embryo development (Lonergan and Fair, 2016). Oocyte maturational (cytoplasmic and nuclear) and developmental (fertilization, pronuclear formation, and cleavage) competencies are influenced by the number of follicular fluids and the amount of the follicle from which the Cumulus-Oocyte Complex (COCs) is extracted (Vatzias and Hagen, 1999). Embryos are at risk from *in vitro* gamete handling since they are in a laboratory setup and can easily be harmed (Nkadimeng *et al*., 2016). Hence, IVM is one of the fundamental steps within the IVP process along with IVF.

Obtaining viable embryos from females who are unable to conceive through conventional methods is the aim of commercial IVF (Faber *et al*., 2003). Furthermore, IVF complements embryo transfer programs in livestock, particularly in females not responding to excessive stimulation, failing to produce transferrable embryos, or exhibiting abnormalities in their reproductive tracts (Faber *et al*., 2003). Therefore, IVF could be a strategy to catalyze embryo production in these females. However, IVF success depends largely on the effective oocyte maturation, sperm choice, sperm capacitation, and IVF medium used. Oocytes and sperm cells interact in a controlled manner during this process, which results in the development of embryos in culture. In livestock animals, fertilization takes place within the oviduct (Coy *et al*., 2012). The developing zygote continues to form while it passes through the oviduct, reaches the uterus, and remains free in the female reproductive tract lumen until implantation.

The IVF procedure allows gamete interaction to occur in culture and avoids the effects of the female regeneration tract, which may be a useful way to think about the physiological requirements and factors for mammalian fertilization and early fetus development. The acrosome of the sperm releases an enzyme during fertilization that starts to tear the oocyte's zona pellucida, allowing a sperm cell to enter and transfer its Deoxyribonucleic Acid (DNA) inside. When a sperm successfully penetrates the zona pellucida, it changes its electrical properties and forms a zygote.

After IVF, the presumptive zygotes are then introduced to culture conditions and procedures. The embryo goes through a series of cell divisions during this phase, starting off as a single cell, dividing into two cells, four cells, and then eight cells. The embryo should be in the blastocyst stage, which has about 100 cells, on the eighth day of culture (Wassarman *et al*., 2001). The IVF could be again a complement to an *In Vitro* Culture (IVC) embryo exchange program and allows for successful IVEP. Thus, IVEP is one of the assisted reproductive biotechnologies necessary for genetic improvement in cattle production and conservation (Baldassarre and Karatzas, 2004).

The quantity of high-quality oocytes extracted (grade A and B) from each ovary is critical for IVEP. This technique also contributes to the development of animals with high genetic merit. Oocyte maturation, fertilization with motile, capacitated sperm, and culture to the blastocyst stage are the three stages that make up the IVEP process. Shabankareh *et al*. (2015) reported that IVEP of cattle embryos is associated with distinctive issues such as handling of ovaries prior to oocyte recovery (e.g., time from slaughter to oocyte recovery), oocyte recovery technique (e.g., aspiration or slicing), the size range of follicles from which oocytes were retrieved, quality of oocytes used, quality of an embryo produced, environment and *in vitro* handling. These issues are taken from the reality that, the source of ovaries collected from slaughterhouse animals is not known or rather heterogeneous hence there is variety in oocyte quality.

There have been several IVM, IVF, and IVC protocols created by diverse researchers for cattle IVEP, but because of different reasons such as natural contrasts, sort of breeds used, and laboratory-to-laboratory contrasts, the success of these protocols is not ensured for the productivity of embryos.

Additionally, the International Embryo Transfer Society Data Retrieval committee (IETSDR) indicates that Africa is lacking in terms of regional production of *in vitro* embryos using oocytes from abattoirs. This might be caused by the fact that the procedure of IVEP itself is cost-effective and the heterogeneous state of the abattoir ovaries seems to play a huge role since it cannot be controlled entirely.

Furthermore, several maturation media such as tissue culture medium 199 (TCM 199), Bracket and Oliphant's-*In Vitro* Maturation (BO IVM), and Epidermal Growth Factor (EGF) have been used before in cattle oocytes maturation, however, in an attempt to improve IVM results and options, this study seeks to introduce cattle suitable VitroMat protect® medium that was currently developed in Australia and from our knowledge has not been used in SA for cattle IVEP. This medium is internationally known for its capacity to protect COCs from cellular stress and provide additional support to the oocyte during maturation. It also provides good maturation quality and eliminates the need for the pre-IVM phase.

Embryo production in Africa is lacking. The IVM media also influence these conditions since the *in vivo* environment of oocytes or embryos cannot be entirely mimicked *in vitro* (Sirard, 2017). Therefore, the IVP rate of cattle oocytes globally continues to be below the standard in comparison to *in vivo* counterparts

(Zhang *et al*., 2010). A report from observed a further decrease in the use of hormones during IVM of beef cattle oocytes which led to a decrease in embryo production. Thus the possibility of small antral follicles oocytes growing under the Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), and estradiol basal levels (E_2) is still questioned (Ferré *et al*., 2002; Lekola, 2015). Furthermore, there is ongoing debate regarding the role of gonadotrophins in oocyte maturation, subsequent fertilization, and early development.

Materials and Methods

Ethical Approval and Study Site

The Tshwane University of Technology Ethics Committee (Ref no. AREC2021/08/004) and the agricultural research council ethics committee (Ref no. APAEC 2020/05) both reviewed and approved the experiments. This research was carried out in the Agricultural Research Council (ARC) Irene (Pretoria), animal production, Germplasm Conservation and Reproductive Biotechnologies (GCRB) laboratories. The area is situated on the Highveld at an altitude of 1525 m above sea level in Pretoria, South Africa, at 25°53'59.6" south latitude and 28°12'51.6" east longitude (Webb *et al*., 2004). The experiments were replicated 10 times.

Chemicals and Reagents

Except where otherwise stated, all chemicals and reagents were purchased from Sigma Chemical Co. in St. Louis, Mo, USA.

Ovary Collection in Cattle

From a local slaughterhouse, sixty cattle ovaries from donors with unknown reproductive status were obtained (Springs, Gauteng province) and transported to the laboratory using a thermos flask containing buffered saline water (Adcock Ingram critical care, SA) at 37°C within 2 h after slaughter. The temperature of the saline water in the thermos flask was tested and recorded upon arrival in the laboratory. To remove blood contamination from donor cows that had been slaughtered, ovaries were rinsed with fresh saline water and then sprayed with 75% ethanol absolute (Laboratory consumables and chemicals supply cc, SA).

Oocytes Retrieval by Aspiration Technique

Ovaries were aspirated through a sterile 18-gauge needle (U-LIFE-MEDICAL, SA) attached to a 10 mL syringe (U-LIFE-MEDICAL, SA) to retrieve oocytes. Retrieved follicular fluid was transferred into a 50 mL tube containing modified dulbeccos phosphate buffered saline (mDPBS). This mixture was then transferred into a graded petri dish with the addition of mDPBS. The COCs were selected under an Olympus microscope visualization as previously described by Lekola (2015).

Grading of Cattle Oocytes

Oocytes retrieved were classified as grade A, B, and or C. Oocytes designated as Grade A were those that had numerous full layers of cumulus cells and homogeneous cytoplasm; Oocytes with homogeneous cytoplasm and thin or incomplete layers of cumulus cells were classified as Grade B; those with few or no cumulus cells were classified as Grade C. The total number of oocytes in grades A, B, and C were recorded.

In vitro Maturation of Cumulus Oocytes Complexes

After collection, assessments, and classification of all the oocytes, Grades A and B oocytes were selected and they were washed three times in dishes containing modified medium 199 (M1 99) and three times in dishes containing mDPBS medium. These oocytes were then *in vitro* matured into 500 µL maturation media (1: TCM 199 containing medium 199+10% FBS supplemented with FSH, LH, and E_2 hormones, sodium pyruvate, and antibiotics; 2: BO-IVM® which is serum-free, supplemented with low glucose, gonadotrophic hormones, and gentamycin; 3: VitroMat Protect® containing 4 mg/mL bovine serum albumin and 100 mIU/mL of equivalents of FSH; and 4: EGF) inside 1 four well dish all covered with 250 µL mineral oil. All maturation groups of oocytes were then incubated for 22 h at a temperature of 38.5°C with 5% carbon dioxide (CO_2), 5% oxygen (O_2), and 100% humidity.

Evaluation of Oocytes Polar Body

A portion of the oocytes (n = 20) from each group of IVM medium were taken out of the incubator after 22 h of IVM and vortexed in 200 µL of modified medium 199 (M 199) for 4 min. They were then transferred into a dish with 3 mL of M 199 for polar body extrusion evaluation while the other portion was prepared for IVF. The vortexed oocytes were evaluated with the aid of an Oosight Imaging System (Hamilton Thorne) inverted microscope Olympus 1 × 71 (New York microscope Co, USA) at 20 ×/0.45 Rc_2 magnification. The observations were made in relation to the existence of polar body extrusion between the zona pellucida and the cytoplasmic space of the oocyte.

Matured Oocytes Preparation for Fertilization

A total of five drops of 100 µL pre-warmed Bracket and Oliphant (BO-IVF®) medium topped with 3 mL of mineral oil were used to wash matured oocytes from TCM 199 and EGF media 5 times (Sigma Aldrich®, USA). Then 20 oocytes were added to 50 µL of brand-new BO IVF drop. The matured oocytes were washed three times in 100 µL of BO-IVF® medium for BO IVM®$_C$ media (IVF

Bioscience, UK). These oocytes were then added to the micro drops used for fertilization and covered in 3 mL of mineral oil. Matured oocytes were washed twice in 380 µL of VitroFert® medium that had been pre-warmed and coated with 250 µL of mineral oil for the VitroMat Protect® medium. Twenty oocytes were added to 200 µL of fresh VitroFert® medium supplemented with 20 µL of heparin and coated with 250 µL of mineral oil.

Thawing of Frozen Semen Straw and Capacitation of the Sperm

Frozen thawed bull sperm of known fertility status, purchased at ABS company was used for IVF. The frozen straws with a volume of 0.25 mL were thawed in the air for 10 sec before being immersed in 37°C water for 1 min. The contents of the straw were collected into a 15 mL falcon® tube after the straw had been dried and cut on both of the sealed ends (Nest Biotechnology Co, China). Before fertilization, 5 µL of the semen was placed on a heated microscope slide (Labocare, UK) to assess the sperm's motility with the help of a technique known as the Sperm Class Analyzer®.

The frozen-thawed sperm was combined with 6 mL of pre-warmed BO wash medium containing caffeine for BO IVF_T and BO IVF_{EGF}. At 1500 rpm, the mixture was centrifuged at 37°C for 8 min. Following centrifugation, a pellet was formed at the bottom of the falcon tube. The top portion was carefully removed with the serological pipette so as not to disturb the sperm pellet. The pellet was then combined with the same quantity of BO wash media and the mixture was centrifuged once more at the same rate. The pellet was left at the bottom of the tube after centrifugation and the supernatant was discarded. The final sperm pellet, which contained 265×10^6/mL of sperm, was diluted with BO wash media based on the number of drops containing oocytes and the sperm concentration.

For VitroFert® medium, the frozen-thawed sperm was diluted with 2 mL pre-warmed BO wash® medium. The mixture was centrifuged at 37°C for 8 min at 1500 rpm. A pellet formed at the bottom of the tube after centrifugation and the top portion was gently removed using the serological pipette without disturbing the sperm pellet. The pellet was infused with the same quantity of BO wash® media and the mixture underwent a second centrifugation at the same rate. The pellet was left at the bottom of the tube after the supernatant was removed during centrifugation. The final sperm pellet, which had a concentration of 5×10^6/mL, was diluted with BO wash® media based on the number of drops containing oocytes and the sperm cell concentration.

Sperms were selected for BO $IVF^{®}_{C}$ using BO Semenprep medium® (IVF Bioscience, UK). After being diluted with 4 mL of prewarmed Semenprep® in a 15 mL tube, the contents of the frozen, thawed straw were centrifuged at 328 × g for 5 min. Fresh warmed BO Semenprep® was added and the mixture was then resuspended and centrifuged. Following centrifugation, the supernatant was withdrawn until 350-700 µL of sperm suspension remained in a 4 mL tube. After the second centrifugation, the supernatant was once more removed, leaving a volume of 350-700 µL for the sperm suspension. The pellet in this volume, which had a 2.0×10^6/mL concentration, was then diluted with Semenprep®, suspended, and used for IVF.

In vitro Fertilization of Matured Oocytes

The BO $IVF^{®}_{C}$ (IVF Bioscience, UK), VitroFert® medium (ART Lab Solutions, Australia), BO IVF_T, and BO IVF_{EGF} media (Agricultural Research Council, SA) were used for IVF. Matured oocytes were fertilized according to their individual IVF drops with frozen-thawed sperm and incubated for 6 h to evaluate the pronucleus, while the remaining oocytes were incubated for 18 h at 38.5°C, 5 CO_2, 5 O_2 and 100% humidity to perform IVC. Following IVF for 6 h, the presumptive zygotes were freed of cumulus cells by vortexing and prepared for pronucleus status staining.

Evaluation of Pronucleus in Presumptive Zygotes

Preparation of Hoechst 33342 Stain

An amount of 25 mg (0.025 g) of Hoechst 33342 (Sigma B226) was diluted in 2.5 mL pure water (CRITI CARE, SA) to make-up stock 1 of the Hoechst solution. The concentration of this solution was 10 mg/mL. This solution was covered with aluminum foil and stored at 4°C. Stock 2 of the Hoechst solution was prepared by adding 8 mL Dulbecco's Phosphate Buffered Saline (DPBS), 2 mL glycerol, and 20 µL of stock 1 inside a 10 mL tube on the day of use.

Preparation of Presumptive Zygotes for Staining

Presumptive zygotes were removed from fertilization drops six hours after insemination in accordance with each IVF group's protocol and cumulus cells were eliminated by vortexing in 200 µL of pre-warmed modified M199 media for 1 min and 30 sec (Fig. 3A). The next step was to prepare the presumptive zygotes for staining with a Hoechst solution by washing them in modified M 199.

Staining and Mounting the Presumptive Zygotes

Presumptive zygotes from all the media were washed three times in modified M199 according to their respective groups, following the method of (Bohlooli *et al*., 2015). Four drops of Vaseline were made in a square shape around a microscopic slide. Presumptive zygotes were then transferred into a microscopic glass slide. A minimal volume (2-10 µL) of stock two Hoechst solution was added on the sides of the coverslip until the whole area under the slip was covered. This was done carefully not to wash away the presumptive zygotes from the slide. A

cover slip (Labocare, UK) was put over the microscopic slide and gently squeezed until it touched the presumptive zygotes drop. The ends of the microscopic coverslip were quickly sealed using colorless nail polish. The slides were allowed to dry for 2 h in a dark compartment before evaluating the pronucleus under the inverted Olympus 1X71 microscope (New York microscope Co, USA) at 20 x/0.45 Rc_2 magnification with a UV filter.

Preparation of Presumptive Zygotes for in vitro Culture Procedure

The remaining presumptive zygotes were taken out of the incubator after 18 h. For the culture of presumptive zygotes in BO-IVC® medium (IVF Bioscience, UK), cumulus cells were removed by vortexing the presumptive zygotes for 2 min in the same solution. For the other media (SOF-BSA, VitroCleave Plus®), presumptive zygotes were removed from fertilization drops and cumulus cells were removed by vortexing in 200 µL of pre-warmed modified M199 medium for 1 min 30 sec.

In vitro Culture of the Presumptive Zygotes

Presumptive zygotes from both BO IVF_T and BO IVF_{EGF} media were washed five times in 5 drops of 100 µL pre-warmed SOF medium supplemented with BSA (SOF-BSA) and transferred into 50 µL of SOF-BSA medium covered with mineral oil. The culture of presumptive zygotes from BO $IVF^{®}_C$ to BO IVC® was carried out in NUNC dishes, which contained 100 µL BO-IVC® medium drops under mineral oil. The presumptive zygotes were washed 2 times in 100 µL pre-warmed BO IVC® medium drops. These were then transferred into fresh 100 µL IVC drops of the same medium covered with mineral oil. Presumptive zygotes from VitroFert® medium were washed 2 times in 380 µL of pre-warmed VitroCleave PLUS® medium and transferred into 100 µL of VitroCleave PLUS® drop covered with mineral oil. Presumptive zygotes from both groups of SOF-BSA and BO IVC® media were cultured for 48 h while presumptive zygotes from VitroCleave PLUS® medium were cultured for 96 h in a modular chamber containing 5% O_2 and 5% CO_2 mixed gas added for a minute. The sperm-exposed presumptive zygotes from groups of SOF-BSA and BO IVC® media were examined for earlier stages of cleavage after 48 h of culture (day two of IVC) and at 96 h for VitroCleave PLUS ® medium.

Statistical Analysis

SAS was used to do an adequate Analysis of Variance (ANOVA) on the data (1999). To check for deviations from normality, the standardized residuals were put through the Shapiro Wilk's -test (Shapiro and Wilk, 1965). Outliers were eliminated until the distribution was normal or symmetrical in cases where significant deviation from normality was seen and was caused by skewness. To compare the means of significant treatment effect means, Student's t-LSDs (Least Significant Differences) were determined at a 5% significance level (Snedecor and Cochran, 1967). The results were put through a 1:1 Frequency table, and a ChiSquare (χ^2) test was run to ensure that the scores were distributed equally. The R × C frequency tables with contingencies were used to test for associations between treatments (Snedecor and Cochran, 1967). The Pearson correlation coefficient was used to calculate the co-efficiency of correlation between the parameters. The statistical program SAS version 9.4 was used to carry out the aforementioned analysis (SAS, 1999).

Results

Maturation rates (PB extrusion) of oocytes matured from different media are presented in Table 1. The BO-IVM® (57.4%), TCM 199 (55.6%), and VitroMat Protect® (54.0%) media recorded higher percentages of polar body extrusion Fig. 1 than EGF medium (26.9%; $P<0.05$).

This study further evaluated the pronuclear formation of oocytes fertilized in different IVF media (BO IVF_T, BO $IVF^{®}_C$, VitroFert®, and BO IVF_{EGF}) as per their respective IVM media are presented in Table 2. The highest percentage of total fertilization rate was recorded on VitroFert® medium (43.3%) as compared to BO IVF_T (23.4%), BO IVF_C (13.3%), and BO IVF_{EGF} (21.7%; $P<0.05$). Nevertheless, there was no difference recorded in the percentage of presumptive zygotes with 1 PN amongst all the IVF media (Fig. 2A and D). VitroFert® medium had less percentage (56.7%) of presumptive zygotes with 0PN fertilization when compared to the other media ($P<0.05$). Noteworthy, Vitrofert® medium recorded a higher percentage (25.0%) of normal fertilization (2PN %) rate as shown in (Fig. 2B) when compared to the other media ($P<0.05$). There was no difference observed in the percentages of polyspermy for all media (Fig. 2C).

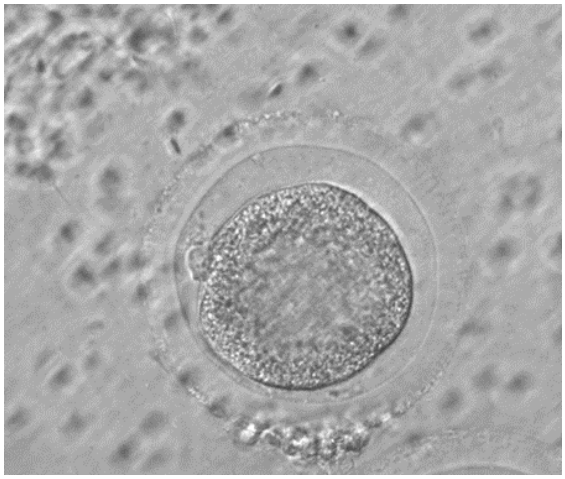

Fig. 1: Oocyte with polar body extrusion post 22 h of *in vitro* maturation

The results of embryo development following IVC of presumptive zygotes in different media (SOF BSA_T, BO $IVC^{®}_C$, VitroCleave PLUS®, SOF BSA_{EGF}) are presented in Table 3. A higher percentage of the presumptive zygote that did not cleave (1 cell) was observed in SOF BSA_T medium (50.0%) as compared to SOF BSA_{EGF} (26.6%; P<0.05). Moreover, a similar trend was observed for the percentage of presumptive zygotes that cleaved to 2 cell stage represented by Fig. 3B and there was no difference for all the media. VitroCleave PLUS® medium recorded a higher percentage (15.0%) of presumptive zygotes that cleaved to 4 cell stage (P<0.05) as compared to BO $IVC^{®}_C$ (0.0%) and SOF BSA_{EGF} (0.0%). Despite that, no difference was observed for the percentage of the presumptive zygote in SOF BSA_T (10.0%). A percentage of presumptive zygotes that reached 8 cell stage was observed in VitroCleave PLUS® medium (6.6%) though, not different from the other media. There was a difference in the percentage of lysed presumptive zygotes recorded on SOF BSA_{EGF} medium (53.3%) as compared to SOF BSA_T (3.3%), VitroCleave PLUS® medium (3.3%) and BO $IVC^{®}_C$ medium (20.0%; P<0.05). Overall, the VitroCleave PLUS® medium recorded a higher total cleavage rate (43.3%) as compared to the other media (P<0.05).

Table 1: Effect of different maturation media on *in vitro* maturation rate of cattle oocytes

IVM media	No of COCs matured	Oocytes PB %
TCM 199	200	55.6±05.9[a]
BO-IVM®	200	57.4±06.9[a]
VitroMat Protect®	200	54.0±05.1[a]
EGF	200	26.9±10.9[b]

[a,b]Means with different letters within columns differ significantly (P<0.05)

TCM 199 = Tissue Culture Medium, BO-IVM = Brackett and Oliphants-*in vitro* maturation, EGF = Epidermal Growth Factor, PB = POLAR BODY

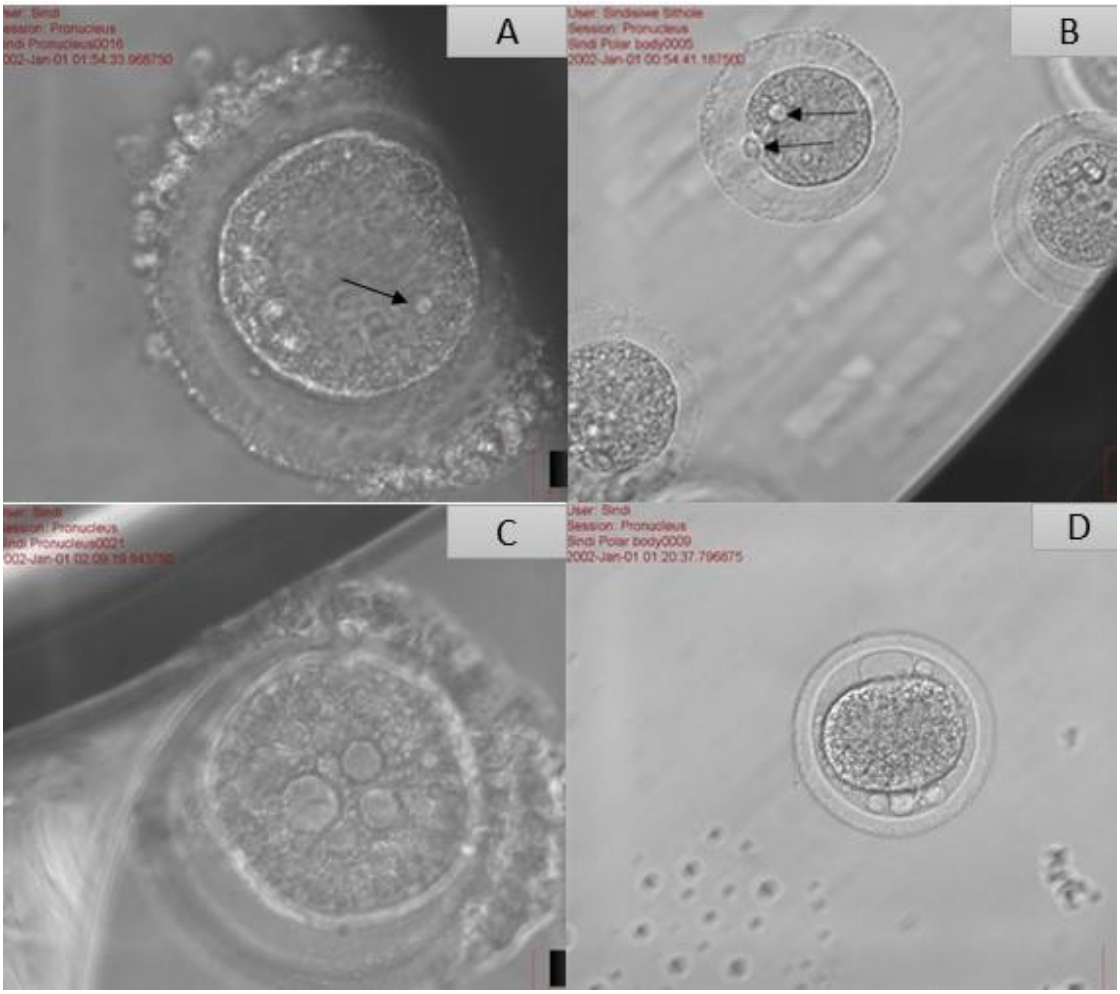

Fig. 2: (A) 1PN extrusion, (B) 2PN extrusion, (C) >2PN extrusion with different sizes and (D) >2PB extrusion as a sign of successful fertilization and completion of meiosis

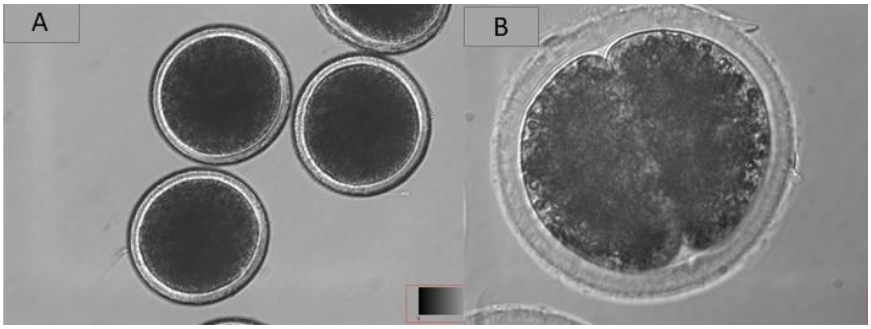

Fig. 3: (A) Presumptive zygote with 1 cell; and (B) Presumptive zygote with 2 cell

Table 2: Effect of different *in vitro* fertilization media on fertilization rate of cattle oocytes

		Pronucleus status%				
IVF media	No of IVF oocytes	0PN	1PN	2PN	>2PN	Total fertilization rate
BO IVF_T	60	76.7±15.3[a]	11.6±12.60	10.0±00.0[b]	1.7±02.90	23.3±15.3[b]
BO $IVF^{®}_C$	60	86.7±07.6[a]	6.7±02.90	6.7±07.6[b]	0.0±00.00	13.3±07.6[b]
VitroFert®	60	56.7±05.8[b]	10.0±05.00	25.0±10.0[a]	8.3±02.90	43.3±05.8[a]
BO IVF_{EGF}	60	78.3±07.6[a]	6.7±07.60	6.7±07.6[b]	8.3±10.40	21.7±07.6[b]

[a,b]Means with different letters within columns differ significantly (P<0.05)
IVF = *in vitro* fertilization; PN = Pronucleus; BO IVF_T = Brackett and Oliphants *in vitro* fertilization$_{TCM199}$; BO IVF_C = Brackett and Oliphants *in vitro* fertilization Commercial; and BO IVF_{EGF} = Brackett and Oliphants *in vitro* fertilization

Table 3: Effect of *in vitro* culture media on embryo development of cattle oocytes

		IVC % - Day 2					
IVF media	No of IVF oocytes	Lysed	1 cell	2 cell	4 cell	8 cell	Total cleavage rate
SOF BSA_T	60	3.3±5.7[c]	50.0±10.0[a]	13.3±05.7	10.0±10.0[ab]	0.0±00.0	23.3±5.7[b]
BO $IVC^{®}_C$	60	20.0±0.0[b]	30.0±10.0[b]	18.3±07.6	0.0±00.0[b]	0.0±00.0	18.3±7.6[bc]
VitroCleave PLUS®	60	3.3±5.7[c]	10.0±10.0[c]	21.6±12.5	15.0±08.6[a]	6.6±11.5	43.3±5.7[a]
SOF BSA_{EGF}	60	53.4±5.7[a]	26.6±05.7[bc]	10.0±00.0	0.0±00.0[b]	0.0±00.0	10.0±0.0[c]

[a,b,c]Means with different letters within columns differ significantly (P<0.05)
SOF BSA_T = Synthetic oviduct fluid bovine serum albumin$_{TCM}$ 199, BO $IVC^{®}_C$ = Brackett and Olifants *in vitro* culture commercial, SOF BSA_{EGF} = Synthetic oviduct fluid bovine serum albumin$_{EGF}$

Discussion

Four different IVM media (TCM 199, BO IVM®, VitroMat Protect®, and EGF) were used in this study to determine their effect on cattle oocyte PB extrusion. These media have the ability to support *in vitro* embryonic development. The PB extrusion of oocytes in TCM 199 (55.6%), BO IVM® (57.4%), and VitroMat Protect® (54.0%) media did not differ. Kim *et al*. (1990) reported similar results where a maturation rate of 55% on cattle oocytes cultured in TCM 199 was reported. However, these results differed from that observed by Singh *et al*. (2015) who reported a maturation rate of 75.5% in indigenous zebu cattle using TCM 199 medium for the maturation of oocytes. Furthermore, (Rahman *et al*., 2018) reported a 74.5% maturation rate on the oocytes of zebu cows using TCM 199 medium. These results were higher than the ones in the present study perhaps because the TCM 199 medium used in (Rahman *et al*., 2018) study was supplemented with bovine serum albumin while the one that was used in the present study was supplemented with fetal bovine serum. The bovine serum albumin is known to contain low lipid content while fetal bovine serum contains high lipid content resulting in higher lipid accumulation in oocytes (Del Collado *et al*., 2016). The IVM causes changes in mitochondrial and lipid dynamics, which may have negative effects on oocyte development rates and embryo lipid accumulation (Del Collado *et al*., 2016). Noteworthy results from the present study were higher than that of (Fernández Reyes *et al*., 2007) where the maturation rate of cattle oocytes ranged from 22 to 45%.

The polar body extrusion rate of oocytes matured in TCM 199 in the present study was 55.6%. The TCM 199 medium has been widely used for the maturation of oocytes in other species and the results obtained vary (Wang *et al*., 2007; Shirazi *et al*., 2012; Hoque *et al*., 2011; Lekola, 2015). Karami Shabankareh *et al*. (2011) obtained a maturation rate of 75.1% in ovine oocytes using TCM

199 medium. Fan *et al*. (2017) reported a 52.0% maturation rate in goats using TCM 199 media. This gives evidence that TCM 199 medium can be used in almost all the species for IVM of oocytes.

The BO IVM® medium obtained a polar body extrusion rate of 57.4% in the present study. The BO IVM® medium has been widely used by many studies (Arslan *et al*., 2019; Borş *et al*., 2021; An *et al*., 2019; Steele *et al*., 2020) in cattle oocytes for embryo production; however, the IVM rate data was not published. The results obtained from this present study for maturation of oocytes in EGF medium were 26.9%, of which this rate was lower compared to the other media (TCM 199, BO IVM®, and VitroMat Protect®), however, it enhanced cumulus expansion leading to extrusion of the first polar body in cattle COCs. This lesser effect of EGF on cattle oocyte polar body extrusion rate might be owing to the dissimilarities in the composition of the medium (not supplemented with hormones and other known beneficial constituents e.g., serum) and may also be associated with the fact that recombinant human EGF was used. To our knowledge, there is not much information reported on the use of EGF for IVM rate in cattle oocytes independently, except for supplementation to an IVM medium in different concentrations (Harper and Brackett, 1993).

In many species' oocytes, EGF is known to trigger the resumption of meiosis. Additionally, the EGF effect on oocyte maturation may be caused by the oocyte connection with cumulus cells being disrupted or by the generation of a positive maturational signal. Pyoos *et al*. (2018) reported a polar body extrusion rate of 72.5% in pigs using EGF media. However, the EGF used by Pyoos *et al*. (2018) was supplemented with bovine serum albumin, whereas in this present study, EGF was made of a serum-free and hormone-free medium. To carefully consider the relationship between growth factors and the influence of nuclear maturation, cytoplasmic maturation, and cumulus expansion on the formation of the polar body while effectively excluding the influence of unidentified serum factors, the composition of EGF used in this study was developed. In as much as this present study has no quantitative or qualitative measures of COCs expansion made, EGF has been known to successfully expand COCs during IVM as the first sign of a successful maturation rate. This entails that the effect of EGF may be, in part, independent of cumulus cells.

The development of an immature oocyte inside the ovarian follicle to an oocyte that is matured is subjected to numerous controlling factors, such as maturation environment, type of medium, and additives (Gil *et al*., 2010; Park *et al*., 2005). According to Lorenzo *et al*. (1994), cumulus oophorus development in cattle oocytes occurs in reaction to an ever-changing milieu of gonadotrophins, growth factors, steroids, factors secreted by the oocyte, and other unknown molecules. These combinations could be contributing to maturational changes that happen in the oocyte, mediated by intracellular messengers such as cyclic adenosine monophosphate, calmodulin, or diacyglycerol (Lorenzo *et al*., 1994).

The IVM environment has a major influence on the oocyte's ability to acquire the potential to develop to the blastocyst stage. Of the components added to the media to improve development, proteins and serum appear to have the most influence on embryo development, morphology, and metabolism (Carolan *et al*., 1995; Thompson, 1997). An overall reason that might lead to such results may be that polar body formation occurs after the COCs expand from the zona pellucida. The matured oocyte reaches the metaphase II stage (Jamnongjit and Hammes, 2005), during which the evaluation of the polar body is performed by vortexing which leads to the damage or loss of cells.

In vitro maturation of oocytes is affected by several external and internal factors. The primary determinant may be the source of oocytes: The mammalian oocyte serves as the basis for a variety of contemporary reproductive technologies, including IVF, the preservation of genetic diversity, the production of high-value products through transgenesis and cell treatments using the stem cell approach (Motlik *et al*., 2000). An abundant source of oocytes can meet the growing demand for mature oocytes (Motlik *et al*., 2000). The current study was conducted using oocytes collected from slaughterhouse animals of which this source serves as the traditional source of oocytes for IVP. Since most laboratories utilize oocytes taken from a slaughterhouse, other effects can be linked to the reproductive condition of the female, the effect of the breeding season of the female, and the effect of the post-slaughter ovarian storage period, which may negatively affect oocyte quality.

Nevertheless, in an attempt to optimize Germplasm and Reproduction Biotechnology IVM laboratory conditions in SA, this study introduced the VitroMat Protect® maturation medium together with its IVF and IVC media suite. VitroMat Protect® medium is a medium that was developed in Australia and from our knowledge has not been used in SA for cattle IVEP. Therefore, this study is the first attempt to provide evidence on this medium in terms of IVM, IVF, and IVC of cattle oocytes in SA. This medium is internationally known for its ability to provide additional support to the oocyte during maturation and protects the COCs against cellular stress; it provides high maturation quality and no pre-IVM phase is required.

Huong and Andrew (2017) have internationally used this medium and its media suite in cattle oocytes (IVM, IVF, and IVC, data not published). VitroMat Protect® media contains BSA of which the addition of BSA protein in IVM media has been shown to contain steroids such as eostradiol at levels that are adequate for desirable cytoplasmic and nuclear maturation, though its use as a supplement is disconcerting as it may contain indeterminate contaminants (Mingoti *et al*., 2002).

The fertilization process is made up of several post-fusion events such as the release of oocyte metaphase arrest, extrusion of cortical granules and a second polar body, and the transformation of sperm nucleus and oocyte chromosomes into male and female pronuclei. These changes are initiated by Ca21 oscillations in the oocytes after sperm-oocyte fusion. This current research work was undertaken to determine the fertilizable ability of matured oocytes in 4 different IVF media (BO IVF_T, BO $IVF^{®}_C$, VitroFert®, and BO IVF_{EGF}) as per their respective IVM media on cattle oocytes. The fertilization rate (pronucleus formation) results from this study observed a difference in the total fertilization rate and presumptive zygotes with 2 PN in all the media.

It is well established that the IVM of the oocyte is divided into nuclear and cytoplasmic processes. Nuclear maturation involves the resumption of meiosis and progression to the metaphase-II stage. Cytoplasmic maturation encompasses a variety of cellular processes that must be completed for the oocytes to be fertilized and developed into a normal embryo and offspring (Eppig, 1996). The BO IVF_T (76.7%), BO $IVF^{®}_C$ (86.7%), and BO IVF_{EGF} (78.3%) recorded the highest percentage of 0PN% presumptive zygote rate as compared to VitroFert® medium (56.7%) when the same bull ejaculate was used. In such observations as the presently observed results, it is assumed that fertilization was not successful. What happens is that the sperm does not show up as a pronucleus inside the oocyte and in the absence of sperm, the oocyte does not complete meiosis. This present study recorded no difference in presumptive zygotes with 1 PN amongst all the IVF media. Generally, a small number of oocytes usually about 5% of all oocytes inseminated will show some number other than 0PN or 2PN. Oocytes with 1PN are considered activated, however, are abnormally fertilized. This lack of pronuclei indicates that fertilization did not succeed and the presence of just a single polar body confirms this finding.

Moreover, the VitroFert® medium recorded a higher (25.0%) percentage of normal fertilization (2PN %) rate when compared to the other media. Oocytes go through two unique stages of meiosis. The first stage is completed just before a retrieval process and renders the oocyte ready for sperm infusion. A polar body is a tiny structure that the oocyte uses to discard some of its DNA. The presence of the first polar body indicates that the oocyte is matured and ready for fertilization. A second polar body is produced at the second stage of meiosis, however, an oocyte can only reach this point if a sperm penetrates it (both polar bodies are visible). The sperm head enlarges and develops into a structure known as a pronucleus once it has entered the oocyte. Because it only contains half the DNA of a typical animal nucleus, the term "pronucleus" is used. In response, the oocyte undergoes meiosis, releasing the second polar body and forming another pronucleus. The two pronuclei are easily noticeable in the center of the oocyte and the oocyte currently possesses the appropriate amount of DNA to produce a new individual [(half from the male (sperm) and a half from the female (oocyte)]. The presence of 2 polar bodies confirms that the oocyte has fertilized normally.

This study further recorded no difference in the percentages of polyspermy presumptive zygotes in all media. More than two pronuclei typically indicate that more than one sperm was successful in entering the oocyte. If this occurs, each sperm that entered will have its nucleus in addition to the oocyte's nucleus. For instance, an oocyte penetrated by three sperm will have four pronuclei. Oocytes with more than two pronuclei are abnormal because the oocyte has three distinct defense systems that prohibit penetration by more than one sperm. Similar to these results, (Nedambale *et al*., 2006) reported a higher incidence of polyspermic fertilization in cattle using BO IVF, in both the 6 (7%) and 18 h (11%). This present study further recorded a high total fertilization rate on VitroFert® medium (43.3%) as compared to BO IVF_T (23.4%), BO $IVF^{®}_C$ (13.3%), and BO IVF_{EGF} (21.7%).

The specific purpose of the IVF media has been to produce favorable conditions for capacitated sperm to fertilize mature oocytes since the beginning. Additionally, the IVF medium must be essential for producing conditions that fulfill the appropriate physiological requirements for the metabolic activities of both sperm and oocytes during IVF incubation, as well as for separating frozen-thawed motile sperm from their cryoprotectants (Rahman *et al*., 2020). These conditions are provided by the female reproductive system *in vivo*, however *in vitro* IVF medium must imitate them (Adeoya-Osiguwa and Fraser, 2002). The fertilization rates further depend on oocyte quality, sufficiency, and efficacy of the media and optimization of incubation conditions. But for IVEP in animals, the IVF media, the health and quantity of motile sperm, the sperm capacitation agent, and the length of the sperm-oocyte co-incubation are all thought to be crucial variables. In an attempt to improve IVC rates in our laboratory, this study further evaluated IVC of presumptive zygotes after embryo development in cattle oocytes using different media (SOF BSA_T, SOF BSA_{EGF}, Vitro Cleave PLUS®, and BO $IVC^{®}_C$). The percentage of presumptive zygotes reaching 2 cell stage from this present study ranged from 10.0-21.6% in all the media while 4 cells ranged from 0-15.0% with VitroCleave PLUS® media at 15.0%. A development to 8 cell stage was only observed in VitroCleave PLUS® medium (6.6%) however, not different from the other IVC media. Moreover, a total high cleavage rate was observed in VitroCleave PLUS® media (43.3%). The source of the semen, the medium used to culture the presumptive zygotes, and the ability of the oocytes to adapt to the media's developmental

competency are some of the variables that may have an impact on the findings of this study. This present study recorded a 2-4 cell rate ranging from 10.0-13.3%, and a total cleavage rate ranging from 10.0-23.3% using SOF BSA medium. Contrarily to these results specifically where SOF BSA medium is concerned, Nkadimeng *et al*. (2016) reported a 2-4 cell percentage development of 65.0, 39.2% for 8 cells, and a total cleavage rate of 72.0% using SOF BSA medium at an incubation temperature of 39°C. The current results in embryo development may also be attributed to the fact that denuded presumptive zygotes using a vortexing mechanism after fertilization were used, of which the denuding of cumulus cells using this mechanism damages the cytoplasm. However, the effect of media supplements cannot be avoided where embryo development is concerned. Mammalian oocytes' meiotic regulation can be influenced or even controlled by the components of maturation media and culture circumstances (Kito and Bavister, 1997).

The design and preparation of IVC media where assisted reproduction of cattle is used has helped in supporting the development of cattle oocytes and embryos. Research conducted on IVC media has aimed to match the fundamental elements that are present in the oviduct and uterus of female cattle by supplementing them with protein and growth factors (Do *et al*., 2016). Currently, there is a considerable deviation in the components of IVC media used in cattle-assisted reproduction. Henceforth, major innovations are required to not only achieve a greater success rate of IVP and to enhance the cryotolerance of cattle IVF-derived embryos but also to provide a defined IVC medium that may be recognized as a point of reference across the cattle breeding industry.

Numerous additives, including serums, follicular fluid, hormones, epidermal growth factors, antioxidants, caffeine, L-carnitine, and retinoic acid, have been used successfully during IVM to enhance the results of IVP in various species, including cattle (Moawad *et al*., 2020).

In addition to adding nutritional value, several protein sources like serum and bovine serum albumin have been mixed in culture media. The serum also serves a variety of other support roles for oocyte maturation and embryo development (Moawad *et al*., 2020). For instance, it helps to avoid the hardening of the zona pellucida, which could harm fertilization (Downs *et al*., 1986). By limiting the action of proteolytic enzymes rising from the premature release of cortical granules, fetuin, an important glycoprotein component of FCS, can help to prevent the hardening of zona pellucida and thus improve fertilization outcomes (Schroeder *et al*., 1990). Additionally, the serum has antioxidant benefits that work by lessening the formation of superoxide (Moawad *et al*., 2020). Additionally, albumin, which is essential for controlling the osmolality of the media, is assessed in the serum as a source of protein (Thompson, 2000).

Conclusion

The TCM 199, BO IVM®, and VitroMat Protect® media rendered higher results of oocyte polar body extrusion rates as compared to the EGF medium. VitroFert® medium recorded the highest total fertilization rate of presumptive zygotes as opposed to the other media. This entails that the Vitro Fert® medium can be used as an alternative medium for successful fertilization in cattle oocytes. The highest embryo development (4-8 cell and total cleavage) rates were observed in VitroCleave Plus® media. Overall, the introduced media (Vitro Mat Protect®) and its media suite successfully adapted to our laboratory environment in SA and can therefore be adopted for optimizing the IVEP of cattle oocytes.

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Author's Contributions

Sindisiwe Mbali Sithole: Conception and designed, acquisition of data, analysis and interpretation of data, drafted the article and reviewed, gave final approval of the version to be submitted and any revised version.

Masindi Lottus Mphaphathi, Tshimangadzo Lucky Nedambale: Conception and designed, supervision, reviewed the article, gave final approval of the version to be submitted and any revised version.

Maleke Dimpho Sebopela: Acquisition and analysis of data, reviewed the article, gave final approval of the version to be submitted and any revised version.

Ethics

The experiments were evaluated and approved by the agricultural research council (Ref no: APAEC 2020/05) and Tshwane university of technology ethics committee (Ref no: AREC 2021/08/004).

Conflict of Interest

All authors have reviewed and approved this final version of the manuscript. There is no competing interest in our submission.

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