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Comparison of four different permitting and combination of two best cryoprotectants on freezing Nguni sperm evaluated with the aid of computer aided sperm analysis

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ABSTRACT

Cryopreservation has been reported to damage approximately 40–50% of viable sperm in bull semen. The present study was undertaken to assess the cryo-effectiveness of glycerol (GLY), ethylene glycol (EG), dimethyl sulfoxide (DMSO) and propylene glycol (PND) as cryoprotectant during the cryopreservation of Nguni bull semen. Semen was collected from 18 Nguni bulls and evaluated macroscopically and microscopically for sperm parameters. Thereafter, the semen samples were diluted with egg-yolk citrate extender supplemented with either 12% GLY or DMSO or EG or PND cryoprotectant. Semen samples were loaded into straws and placed into a controlled rate programmable freezer and stored in a liquid nitrogen tank. Following semen thawing, artificial insemination (AI) was done on synchronized Nguni cows. The in vitro fertilization (IVF) was conducted on cow's oocytes to test the fertilizing ability. Data was analyzed with the aid of ANOVA. A significant difference ($p < 0.05$) was recorded between fresh total sperm motility rate (94.7 ± 2.6%) and frozen-thawed sperm total motility rate with GLY (77.8 ± 11.0%), EG (20.4 ± 10.1%), DMSO (15.7 ± 11.9%) and PND (11.2 ± 11.3%). Interestingly, a positive correlation between total sperm motility and pregnancy rate ($r = 0.42$) was recorded. However, a negative correlation of Nguni sperm parameters with IVF ($r = -0.53$) was obtained. The freezing-thawing process did reduce the Nguni sperm total sperm motility percentage.

1. Introduction

The Nguni cattle breed is indigenous to Southern Africa. It is a sub-type of the African Sanga cattle, associated with the pastoralist cattle culture of black people in Africa. It is a hardy cattle breed, uniquely adapted to the South African environment and regarded as inferior breed in the past. The Nguni gene pool was nearly being extinct, because of the dilution through replacement and cross-breeding with exotic stock. The scientific community has long indicated that the bull is the major profit driver in a beef cattle enterprise [1]. A bull with poor sperm motility rate generally indicates a limitation to accomplish fertilization of cow ova. Moreover, the use of traditional sperm testing methods is challenging and continues to show poor reproducibility between AI centers and research laboratories.

The computer aided sperm analysis (CASA) system is a recent improved tool to analyze and validate bull fertility. The CASA has been demonstrated to be a useful tool to assess sperm fertility parameters such as motility rates of various sperm traits [2]. Artificial insemination is regarded as one of the assisted reproductive techniques (ART's) to distribute the genetic material of livestock [3]. These ART's, together with the recent improved tools (CASA) to analyze and validate bull fertility, can also be used to conserve and distribute genetic material. It is, however, also a challenge to

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distribute semen in the communal cattle-farming systems and also to maintain a live bull in the communal sector, due to ownership issues. Therefore, it is vital to freeze the bull semen and distribute it around AI centers. Consequently, it is important to find a suitable cryoprotectant and its concentration to maintain high quality semen post-thawing to quantify the sperm fertility of the Nguni bull. Regardless of the methods used to analyze sperm, there are many factors, which play a role in the successful cryopreservation of semen such as reactive oxygen species, type of extender, concentration of cryoprotectant, freezing methods, equilibration times and the temperatures used [4]. The cryopreservation process is known to damage sperm cell morphology, resulting in a loss of approximately 40–50% of viable sperm and leading to a lower fertility rate [5]. In order to improve the efficiency of cryopreservation of bull semen, an understanding about basic essential cryobiological properties of these cells is needed. This includes the sperm's response to the addition of permeating cryoprotective agents. The level and type of cryoprotectants in semen diluents also have an effect on the sperm survival rate [6].

Previous studies have indicated that the use of a 12% GLY concentration resulted in acceptable frozen-thawed sperm motility rates in bovine semen [7]. However, there is still limited information on the semen cryopreservation of South African Nguni bulls and the relationship with fertility rate. The GLY cryoprotectant is traditionally used for semen preservation [8] [9]; because it acts directly on the sperm plasma membrane. Yet better results have been also recorded with the use of EG, when preserving human and bovine semen [10]. This may be due to its low molecular weight [11] and a greater membrane permeability than e.g. propanediol (PND) (76.10 g/molar) and DMSO (78.13 g/molar). According to Novok [12] DMSO is used as an important cryoprotectant to preserve organs, tissues and cells, without suspensions. Although there are different types of cryoprotectants such as e.g. DMSO and EG Ref. [13] that provide a better protection to bull semen during cryopreservation, GLY is generally the most preferred cryoprotectant during the cryopreservation of bull semen. As it has been indicated that EG permeates the sperm plasma membrane faster than PND and DMSO, results in damage to the sperm during equilibration and cryopreservation. Semen cryopreservation contributes to the expansion of reproductive techniques, such as AI and IVF [14]. In addition, cryopreservation of Nguni bull sperm and the correlation with pregnancy rate remains an area of interest in South Africa for evaluating the Nguni bull reproductive efficiency.

2. Materials and methods

2.1. Animals, management and treatments

The Nguni bulls (n = 18), aged between four to seven years were used for semen collection. Nguni cows aged four to six years were used for oestrous synchronization and AI. Body weights (kg) of the bulls and cows were recorded and the scrotal circumference (cm) of the bulls measured at the onset of the trials. All cattle were maintained on natural pasture and water was provided ad libitum. The experimental procedures were approved by Agricultural Research Council ethics committee (APIEC04/14).

2.2. Semen collection

Semen was collected twice per week for three weeks from Nguni bulls with the aid of electro ejaculator into a 15 mL Falcon® tube during the natural breeding season. Immediately after collection, the semen samples were kept in a thermo flask containing warm water at a temperature of 37° C and transported for further analyses.

2.3. Semen collection and evaluation

Semen volume (mL) was measured immediately after each collection by reading volume values on the 15 mL graduated Falcon® tube (352099, USA) and recorded while sperm concentration was determined using the spectrophotometer (JENWAY® 6310). Then the readings on the spectrophotometer was recorded and expressed as (×109 sperm/mL). Sperm motility rates were evaluated using the CASA system. Sperm motility rates were evaluated using the swim up method. Briefly, 10 μL of semen was added to 500 μL Brackett and Oliphant medium. Thereafter, 5 μL of the mixture was pipetted on a microscope slide and covered with a warm glass slide (~76 × 26 × 1 mm, Germany) and placed with a warmed cover slip (22 × 22 mm, Germany) over the microscope-warm plate (Omron®) adjusted at 37 °C. The sperm motility rate was evaluated before freezing and after thawing by Sperm Class Analyzer® system (Microptic, Spain) at the magnification of X 10 (Nikon, China). The sperm morphology was recorded after staining the semen samples with SYBR-14/propidium iodide stain on a microscope slide. The stained slide was then placed on a microscope table for evaluation. During evaluation, a drop of immersion oil was poured on the stained slide, fluorescent microscope (Olympus, Corporation BX 51FT, Tokyo, Japan) was used at X 100 magnification to count 200 sperm per stained slide and results were recorded. Live sperm will fluoresce green, while the dead sperm fluoresced red in colour.

2.4. Semen dilution, freezing and thawing

Raw semen samples were diluted (1:1) with the egg yolk citrate extender, without cryoprotectant (fraction A) and stored at 5 °C for 2 h. Thereafter, a second (fraction B) dilution (1:2) with egg yolk citrate extender supplemented with 12% of GLY or EG or DMSO or PND or the combination of two best cryoprotectants was further equilibrated for an additional period of 2 h at 5 °C. After 2 h of equilibration with fraction B, semen samples were loaded into 0.5 mL polyvinyl chloride straws, sealed and placed horizontally into a controlled programmable freezer. Semen was then frozen using a controlled programmable freezer from 5 to −5 °C at 0.008 °C min and from −4 to − 130 °C at 6 °C min. When the target temperature of −130 °C was reached, the semen straws samples were placed into liquid nitrogen tank (−196 °C) for storage until thawing. After 30 days of storage semen straws were thawed in a water bath at 37 °C for 1 min. Thawed semen samples were evaluated for sperm motility rates traits using the CASA.

2.5. Oestrous synchronization, artificial insemination and pregnancy diagnosis of Nguni cows

For AI, matured Nguni cows were synchronized before. The selected cows were injected with Estradiol® together with the insertion of controlled internal drug release dispensers (CIDR®) (Phamacia & Upjohn, Auckland, New Zealand), for eight days. On the day of CIDR® removal, prostaglandin (PGF2α) was also injected. Fixed time artificial insemination with 0.5 mL frozen thawed semen was performed at 48 and 60 h after the withdrawal of the CIDR®. Pregnancy diagnosis was performed after three months following AI. A rectal palpation together with the ultrasound scanner (Ibex, Colorado, USA) was used to determine pregnancy rate.

2.6. In vitro embryo production

Heterogeneous cattle ovaries were collected at a local abattoir, immediately after slaughter and transported to the laboratory in a saline solution at 37 °C. Ovaries were aspirated to retrieve oocytes

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