2 MATERIALS AND METHODS

2.1 Model system and justification of the model

It is important to be able to successfully freeze sperm from the African buffalo. It allows the storage of genetics of valuable animals e.g. from animals with exceptionally large horns, and it allows the transport of such genetics to any place in the world where it can be used at any time. An aliquot of frozen sperm as well as smears or blood samples can be taken and examined for the presence of certain diseases to ensure that no diseases are transmitted with the genetic material. It is

easier to obtain epididymal sperm during culls or hunts than to obtain semen by electro-ejaculation.

2.2 Experimental procedures

2.2.1 Collection and processing of epididymides and spermatozoa

Epididymides were collected from 11 African buffalo bulls culled during a disease eradication program in Huhluwe/Umfolozi National Park in September 2001. This program was performed under the responsibility of the KwaZulu Natal Nature Conservation Service. Only adult bulls with 3 or more pairs of permanent lower incisors (about 3 to 3.5 years of age) were used in the trial.

The testes and epididymides from each bull were collected trough a scrotal incision within 10 minutes after culling. The vas deferens, the cauda epididymidis and part of the corpus epididymidis were dissected free from the testis. The epididymal duct was then dissected free and cut at the site in the tail of the epididymis where the tubular diameter becomes distinctly larger distally. A blunted 23 or 25 G needle connected to a 10 ml syringe was then inserted into the vas deferens and the sperm flushed with air in a retrograde direction.

The harvested sperm of both epididymides of the same bull were mixed and split into two aliquots, each of which was poured into a 15 ml plastic tube, containing 13 ml of the two different cryodiluents. The two semen extenders used were Triladyl™ and AndroMed® (both Minitüb, Germany). The glycerol concentrations of these media are 6.6% and 6.8% respectively. The concentration of the sperm/diluent mixture was determined with a haemocytometer and was between 100 and 150 spermatocytes per ml.

The sperm/diluent mixture was mixed and the two 15 ml tubes were put into a 500 ml water bottle containing water at a temperature of 20°C. The water bottle was placed into a Styrofoam box, containing water and ice cubes in such a relationship that the temperature of the water was 4°C, resulting in a cooling rate of 0.25°C/min. The samples were then transported to a laboratory.

The original difference was attributed to handling mistakes and the semen with poorer motility was discarded.

2.2.5.2 Longevity

The longevity of the sperm was assessed by evaluating the total and progressive motility of the equilibrated sperm. Sperm samples, which were kept in a water bath at 37°C, were examined at 0 (t0), 1 (t1) and 2 (2t) hours after thawing.

2.2.5.3 Acrosomal integrity

The acrosomal integrity was evaluated after a storage time of 5 months. The content of a thawed 0.25 ml straw was emptied in an Eppendorf tube. The sperm diluent mixture was washed twice with 200 µL of PBS by centrifugation at 600 G for 5 min. The supernatant was removed and the pellet re-suspended with 200 µL of PBS. 50 µL of IGEPAL CA-630 (Sigma-Aldrich LTD, Atlasville, South Africa, cat no: D1626) and 50 µL of EthD-1 (Ethidium Homodimer, Laboratory Specialist Services LTD, Clareich, South Africa, cat no: E-1149) were added and incubated for 5 min at 37°C to permeabilize the sperm membrane and to stain the spermatozoa respectively. Then 5 µL of 1mg/ml ssDNA (Deoxyribonucleic acid sodium from Salmon, Sigma-Aldrich LTD, Atlasville, South Africa, cat no: D1626) were added to compete for the unbound EthD-1 and it was further incubated for 1-2 min. Spermatozoa were fixed by addition of 50 µL of 4 % Paraformaldehyde and 1 % Glutaraldehyde in PBS and kept in the dark for 1 min. Then the solution was washed once with 200 µL of PBS by centrifugation at 600 G for 5 min. The supernatant was removed and the pellet re-suspended with 50 µL of PBS. 50 µL (1:1) of FITC-PNA (Lectin from Arachis Hypogaea*Fluorescein, Sigma-Aldrich LTD, Atlasville, South Africa, cat no: L7381) were added and incubated for 10 min at 37°C. It was again washed once with 200 µL of PBS by centrifugation at 600 G for 5 min, the supernatant removed, re-suspended with 25-100 µL of PBS and kept in the dark.

Equal amounts of 2 µL spermsuspension and antifade (Slowfade Antifade Kit, Laboratory Specialist Services LTD, Clareich, South Africa, cat no: S-2828) were put on a microscopic slide and covered with a cover slip. The samples were examined under the epifluorescence microscope (BH2-RFCA; Olympus, Tokyo, Japan) at a magnification of x 400-1000.

2.2.2 Evaluation of fresh sperm

Before further processing the motility was assessed from each solution by means of eyeball assessment using a phase contrast microscope. Percentage of progressive and aberrant motile sperm cells were recorded.

2.2.3 Freezing of sperm

After 2 hours five 0.25 ml French straws were loaded from each of the two sperm dilutions and placed 4 cm above liquid nitrogen for ten minutes before being plunged (ET2). They were then stored in goblets in a liquid nitrogen container until thawing.

The 15 ml tubes with the remaining sperm dilutions were further kept in water bottles in a water bath at 4°C.

The procedure of packing and freezing straws was repeated 8 times after every hour (ET 3-9)

2.2.4 Thawing of sperm

After a storage time of between 4 and 37 days the straws were thawed in water at a temperature of 37°C for at least 30 seconds. The contents of two straws of the same treatment were emptied into two different pre-heated (37°C) 3 ml Perspex-tubes and stored in a water-bath at 37°C.

2.2.5 Evaluation of the post-thaw sperm quality

2.2.5.1 Motility

Twenty five micro litres of the thawed sperm were further diluted with 100 µl of the corresponding diluent. The motility of the sperm from both thawed straws was determined by eyeball assessment using a phase contrast microscope. In three cases the motility differed by 10% or more (10%, 10% and 15% respectively). From these three batches a third straw was thawed and the motility evaluated. The motility was in all cases similar to the motility of the better one of the first two straws thawed.

Spermatozoa were classified according to their appearance as acrosome intact, acrosome damaged or as they had lost their acrosome. One hundred spermatozoa of each sample were evaluated and the results expressed as percentage.

2.2.6 Statistical analysis

2.2.6.1 Equilibration times

A "Two Way Repeated Measures ANOVA" was used to test for differences between the eight different equilibration times separately for the use of AndroMed® or Trilady™. Total and progressive motility were compared separately at any time of their measurement. In the case that normality failed a "Friedman Repeated Measures Analysis of Variance on Ranks"-Test was used.

2.2.6.2 Media and Longevity

To compare the two different media when the equilibration time was the same for both, a paired t-test was used. In the case that normality failed a "Wilcoxon Signed Rank Test" was used. After it became evident that equilibration time does not influence sperm motility the mean values for all equilibration times were used for each buffalo and motilities before freezing, immediately, one and two hours after thawing were compared amongst each other by a "Repeated Measure One Way ANOVA". To identify different groups the Tukey test was used.