REPRODUCTIVE BIOLOGY

SHORT COMMUNICATION

The use of a hypo-osmotic swelling (HOS) test on sperm of the pig (*Sus scrofa domesticus*), emu (*Dromaius novaehollandiae*), Asian elephant (*Elephas maximus*), hamadryas baboon (*Papio hamadryas hamadryas*), and central rock rat (*Zyzomys pedunculatus*)

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SUMMARY

A hypo-osmotic swelling test using TALP-HEPES medium over a range of 50 to 300 mOsm/kg was applied to sperm from domestic and endangered species. Maximal responses of curling of the sperm tails were seen over a range of osmolalities for epididymal sperm from the pig (100 mOsm/kg), hamadryas baboon (range 50-125 mOsm/kg), and central rock rat (range 50-100 mOsm/kg), and the ejaculated sperm from the emu (50 mOsm/kg) and the Asian elephant (range 75-150 mOsm/kg). A solution of TALP-HEPES medium at 100 mOsm/kg would be suitable to obtain the maximal response in this range of mammals tested, though it would need to be diluted to at

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least 50 mOsm/kg when testing the viability of the emu sperm. *Reproductive Biology 2009* ***9*** *2: 181-187.*
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INTRODUCTION

First described for use with human sperm [5], the hypo-osmotic swelling (HOS) test enables the identification of sperm with functionally intact membranes and is one of a range of tests commonly used to determine sperm viability. The osmotic stress caused by the chosen hypo-osmotic medium must be sufficient to affect an influx of water into the cell to result in an increase in volume and hence curling of the tail, but to prevent lysis of the sperm membrane. The test has been applied to a range of domestic and non-domestic mammals from the bull [16] to the giant panda [15], and birds such as the turkey [3] and emu [9]. The HOS test is an ideal method being quick, simple, and requiring minimal equipment, and has been shown to correlate well with supravital staining [17] and be a good predictor of fertility [14]. The aims of the present study were therefore to describe the application of a shortened HOS test to assess sperm in a range of domestic and threatened non-domestic species, and determine the osmolality required to obtain the maximal response in terms of the curling of sperm tails.

MATERIALS AND METHODS

The HOS test used was a modification of a shortened protocol described by others [14] in which sperm are incubated in a hypo-osmotic solution for only 5 min compared to e.g. 45 min. The present study used TALP-HEPES medium [13] with 15 mM sodium bicarbonate as the test solution; a stock without BSA was made and the medium adjusted with ultrapure water to give final osmolalities of 50, 75, 100, 125, 150, 200, 250 and 300 mOsm/kg confirmed with a cryoscopic osmometer (Osmomat 030; Gonotec GmbH, Germany). Aliquots were stored frozen at -20°C until required. The test was

performed by mixing 50 µl of sperm sample with 0.45 ml hypo-osmotic medium, and incubating for 5 min at 37°C. A 0.2 ml aliquot of the mixture was placed on a microscope slide warmed to 37°C, covered with a 22 mm×22 mm coverslip, and a minimum of 100 spermatozoa were examined using phase contrast microscopy at 400×magnification. The number of sperm with curled tails (viable) and non-curled tails (non-viable) present was recorded. The objective was to identify the osmolality that gave the maximal response for any given sample to determine the optimal osmolality for that individual or group. The response of the sperm at the different osmolalities for each sample or group of samples was compared with the χ^2-test using the actual numbers of sperm counted, initially by a 8×2 contingency table and then by individual 2×2 contingency tables using the Bonferroni inequality in the calculation of probability [4]. The range of osmolalities giving responses that were not different from the peak value were identified. Differences were considered significant if $p<0.05$.

Post-mortem epididymal sperm was collected for testing in the range of osmolalities given above in four pigs, one hamadryas baboon (*Papio hamadryas hamadryas*), and one central rock rat (*Zyzomys pedunculatus*). This was done by removing tissue from the caudal region of both epididymides and placing in TALP-HEPES containing 3 mg/ml bovine serum albumin and 15 mM sodium bicarbonate. The tissue was gently incised and squeezed to release the sperm, the medium aspirated and placed in a conical tube and maintained at 37°C for a maximum of 1 hour before use. Semen was collected using previously described techniques; an artificial cloaca was used with three male emus (*Dromaius novaehollandiae*; [8]) whilst rectal massage was used for one male Asian elephant (*Elephas maximus*; [18]).

RESULTS AND DISCUSSION

The proportion of curled tails of the pig epididymal sperm (fig. 1A) was affected by the osmolality of the medium ($\chi^2=717.49$, df=7, $p<0.001$), being maximal at 100 mOsm/kg and significantly higher than that seen in the medium at either 75 mOsm/kg ($p<0.001$) or 125 mOsm/kg ($p<0.001$). The

optimal osmolality here compares favorably with a longer protocol using 100 mOsm/kg [6], but was higher than that used in a short protocol using a solution of 75 mOsm/kg [14]. The sperm from the hamadryas baboon (fig. 1A) showed >40% of the sperm with curled tails at all osmolalities tested even at 300 mOsm/kg which is unusual. TALP-HEPES medium has been used to suspend sperm from this species before without problems [12], and so the cause of this remains unclear. Nevertheless, osmolality did affect the proportion with curled tails (χ^2=226.00, df=7, $p<0.001$), with the maximal response being similar in the range 50-125 mOsm/kg, thereafter being significantly lower ($p<0.001$). The central rock rat sperm (fig. 1A) was also affected overall (χ^2=139.12, df=7, $p<0.001$), and had maximal rates of sperm response between 50 and 100 mOsm/kg which declined significantly thereafter ($p<0.05$).

Ejaculated sperm from the Asian elephant was affected overall (χ^2=416.08, df=7, $p<0.001$) and had the maximal response in the range 75-150 mOsm/kg (fig. 1B), declining significantly ($p<0.001$) thereafter. The emu sperm (fig. 1B) showed overall differences (χ^2=1718.12, df=7, $p<0.001$), but had a very different profile with the maximal response of 70% at 50 mOsm/kg, being significantly higher ($p<0.001$) than that at 75 mOsm/kg and beyond. An earlier study of emu sperm using Dulbecco's Modified Essential Eagles medium showed an optimal osmolality of 34 mOsm/kg [9] which is lower than the lowest solution used in the current study. The effect of hypoosmotic medium on bird spermatozoa has been investigated previously albeit in the context of cryopreservation [1], and there appears to be marked differences between species. Exposure to a NaCl solution at 50 mOsm/kg for 10 minutes caused the majority of sperm from the Peregrine falcon to die in contrast to sperm of three eagle species, which were far more tolerant. In the same study, >80% turkey sperm died when exposed to saline at 50 mOsm/kg. Paradoxically the HOS test has been successfully applied to sperm of the turkey where the maximal swelling of sperm tails occurred in water having an osmolality of only 19 mOsm/kg [3]. The results of the present study support the view that avian sperm is less prone to curling in the hyposmotic environment than mammalian sperm.

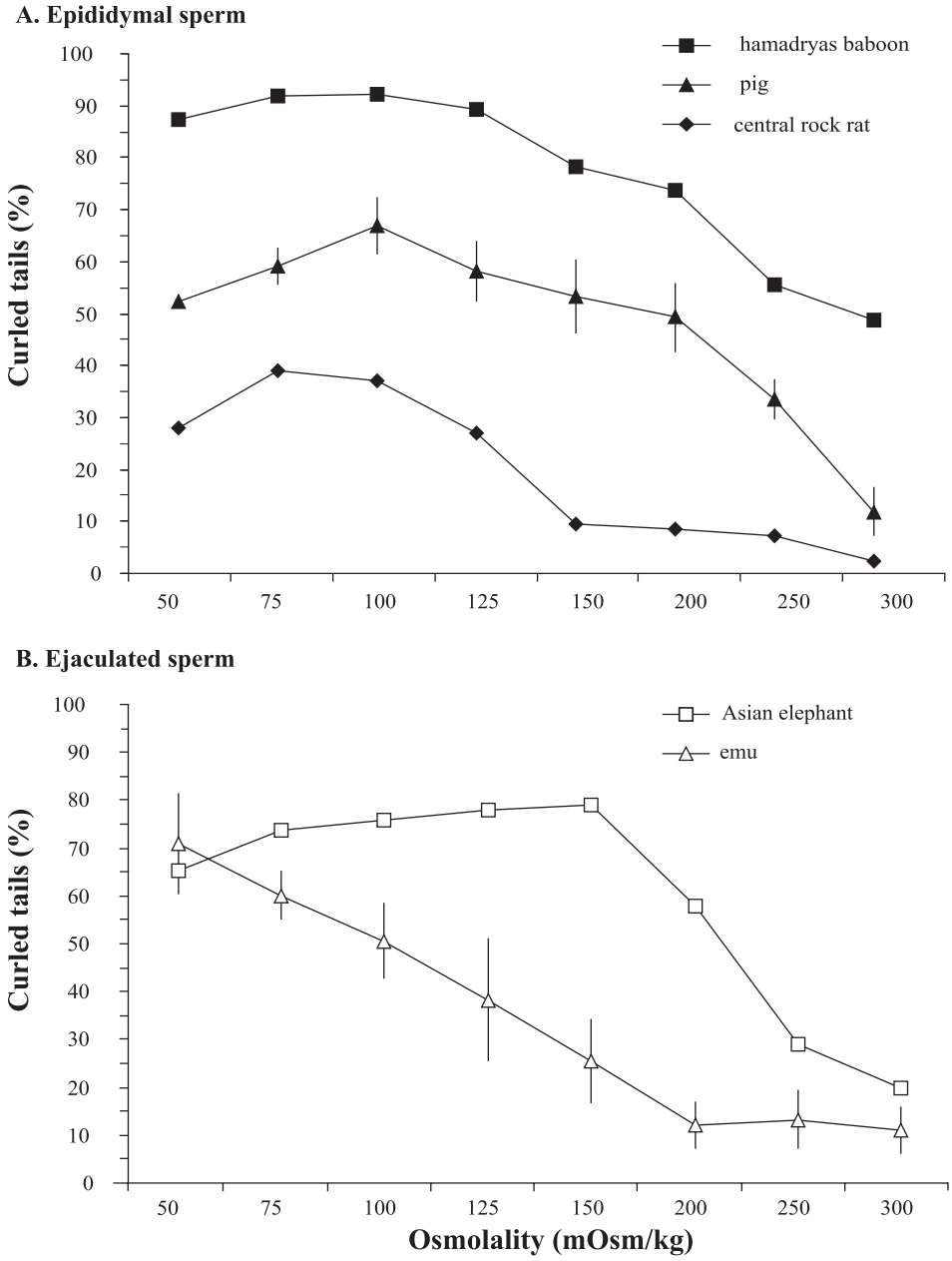

Figure 1. The effect of medium osmolality in the hypo-osmotic swelling test upon the proportion of sperm with curled tails from epididymal sperm (A) of four boars (mean±SEM), one hamadryas baboon and one central rock rat, and ejaculated sperm (B) of three emus (mean±SEM) and one Asian elephant.

The present report describes a modified HOS test protocol based on a protocol used for ejaculated pig sperm [14]. The current modification differs mainly from the original protocol by using HEPES-buffered TALP tissue culture medium rather than Beltsville Thawing Solution (BTS). The only reason for this modification was to make a homologous matrix for existing work being undertaken on epididymal sperm, and to avoid the need for a further medium that was not in common use within our laboratory. The composition of the medium can affect the performance of the test with different sugars and electrolytes clearly resulting in different optimal osmolalities for human [5] and equine [11] sperm. Epididymal sperm from the pig has been used as an experimental model in our laboratory for a number of studies including the evaluation of equipment such as the Sperm Quality Analyzer IIB [10], simply because it is obtained conveniently from a local abattoir. Similarly, on-going reproduction studies on the emu at the local university [7] ensures a supply of ejaculated sperm. However, breeding programmes and post-mortem samples at the Zoo provide a limited opportunity to examine sperm from individual animals of endangered species [2]. Whilst a sample from only one animal is of limited value in drawing firm conclusions, the scarcity of such samples means that they should not be ignored and the maximum amount of information should be gathered wherever possible.

REFERENCES

1. Blanco JM, Gee G, Wildt DE, Donoghue AM **2000** Species variation in osmotic, cryoprotectant, and cooling rate tolerance in poultry, eagle, and peregrine falcon spermatozoa. *Biology of Reproduction* **63** 1164-1171.
2. Breed WG, Leigh CM, Robertson H, Mantellato L, Lambert C, Jequier A, Matson P **2007** Interspecific variation of sperm morphology in the Australian rodent genus *Zyzomys*. *Acta Zoologica (Stockholm)* **88** 257-263.
3. Donoghue AM, Garner DI, Donoghue DJ, Johnson LA **1996** Assessment of the membrane integrity of fresh and stored turkey spermatozoa using a combination of hypo-osmotic stress, fluorescent staining and flow cytometry. *Theriogenology* **46** 153-163.
4. Glantz SA **1992** *Primer of Biostatistics.* McGraw-Hill Inc, New York.
5. Jeyendran RS, Van der Ven HH, Perez-Palaez M, Crabo BG, Zaneveld LJ **1984** Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility* **70** 219-228.

6. Lechniak D, Kedzierski A, Stanislawski D **2002** The use of HOS test to evaluate membrane functionality of boar sperm capacitated *in vitro*. *Reproduction in Domestic Animals* **37** 379-380.
7. Malecki IA, Martin GB **2002** Fertility of male and female emus (*Dromaius novaehollandiae*) as determined by spermatozoa trapped in eggs. *Reproduction, Fertility and Development* **14** 495-502.
8. Malecki I, Martin G, Lindsay D **1997** Semen production by the emu (*Dromaius novaehollandiae*).1. Methods for collection of semen. *Poultry Science* **76** 615-621.
9. Malecki IA, Reed CE, Martin GB **2005** Hypo-osmotic swelling test for measuring integrity of the emu sperm membrane. *Avian and Poultry Biology Reviews* **16** 189.
10. Matson PL, Webb S, Kappelle W, Clarke B **2007** The benefits and limitations of the Sperm Quality Analyzer IIB in assessing porcine sperm motility. *Archives of Andrology Journal of Reproductive Systems* **53** 359-365.
11. Neild D, Chaves G, Flores M, Mora N, Beconi M, Aguero A **1999** Hypoosmotic test in equine spermatozoa. *Theriogenology* **51** 721-727.
12. O'Brien JK, Stojanov T, Heffernan SJ, Hollinshead FK, Vogelnest L, Maxwell WMC, Evans G **2005** Flow cytometric sorting of non-human primate sperm nuclei. *Theriogenology* **63** 246-259.
13. Parrish JJ, Susko-Parrish JL, First NL **1985** Effect of heparin and chrondroitin sulfate on the acrosome reaction and fertility of bovine sperm in vitro. *Theriogenology* **24** 537-549.
14 Perez-Llano B, Lorenzo JL, Yenes A, Trejo A, Garcia-Casado P **2001** A short hyposmotic swelling test for the prediction of boar sperm fertility. *Theriogenology* **56** 387-398.
15. Perez-Garnelo SS, Garde J, Pintado B, Borque C, Talavera C, Delclaux M, Lopez M, De La Fuente Martinez J **2004** Characteristics and in vitro fertilizing ability of Giant Panda (*Ailuropoda melanoleuca*) frozen-thawed epididymal spermatozoa obtained 4 hours postmortem: a case report. *Zoo Biology* **23** 279-285.
16. Revell SG, Mrode RA **1994** An osmotic resistance test for bovine semen. *Animal Reproduction Science* **36** 77-86.
17. Samardzija M, Dobranic T, Kruslin S, Cergolj M, Karadjole M, Prvanovic N, Grizelj J **2008** The use of the hypoosmotic swelling test and supravital staining in evaluation of sperm quality in boars. *Veterinarski Arhiv* **78** 279-287.
18. Schmitt D, Hildebrandt T **1998** Manual collection and characterization of semen from Asian elephants (*Elephas maximus*). *Animal Reproduction Science* **53** 309-314.