Original Article

Dogs Spermatozoa Labelling with ^{99m}Tc-HMPAO

Kaywalee Chatdarong[1*] Eva Axnér[2] Bernt Jones[3] Catharina Linde Forsberg[2]

Abstract

The study was aimed at finding out whether dog sperm could be labelled using ^{99m}Tc-HMPAO and finding the optimum incubation temperature and amount of ^{99m}Tc-HMPAO to label spermatozoa from fresh ejaculates and from frozen-thawed semen. A radiopharmaceutical was prepared by reconstituting 0.5 mg of HMPAO with 5 ml pertechnetate solution containing 100 kBq ^{99m}Tc. Semen samples were obtained from three dogs, pooled and divided into aliquots containing 100x10^6 spermatozoa each. Fresh and frozen-thawed semen samples were used. In experiment 1, the fresh and frozen-thawed spermatozoa were incubated with ^{99m}Tc-HMPAO at 20^{o}C or 37^{o}C for 20 min. In experiment 2, 0.01, 0.1 or 0.5 ml of radiopharmaceutical was used to incubate the spermatozoa at room temperature (20^{o}C) for 20 min. The excess radioactivity was removed by three times washing using centrifugation at 500 x g for 6 min and resuspending with TRIS buffer. The incubation procedure with the radiopharmaceutical did not influence the membrane integrity, but sperm progressive motility was reduced by each washing and most of the spermatozoa maintained only stationary motility. The incubation temperature was not found to have an effect on the labelling efficiency. The highest labelling efficiency, 52%, was obtained with fresh semen incubated with 0.01 ml ^{99m}Tc-HMPAO. Since less than 5% of excess radioactivity was removed from the supernatant by the third washing, it is recommended to do no more than two washings in order to reduce the loss of progressive motility. This study shows that dog spermatozoa can be effectively radiolabelled, which is of value for further studies on sperm transport in the canine female genital tract.

Keywords : Dog, spermatozoa, technetium, HMPAO

[1]**Department of Obstetrics, Gynaecology and Reproduction, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand.**

[2]**Department of Clinical Sciences,** [3]**Department of Biomedical Sciences and Veterinary Public Health, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU), SE-750 07 Uppsala, Sweden.**

[*]**Corresponding author**

บทคัดย่อ

การติดสารกัมมันตภาพรังสีเทคนีเซียมในตัวอสุจิสุนัขด้วยสารเอชเอ็มพีเอโอ

เกวลี ฉัตรดรงค์[1*] เอียวา แอ๊กเนียร์[2] แบรนท์ โจนส์[3] แคทธารีน่า ลินด์ ฟอร์สแบร์[2]

การศึกษานี้มีวัตถุประสงค์เพื่อทดสอบความเป็นไปได้ในการติดสารกัมมันตภาพรังสีเทคนีเซียมในตัวอสุจิสุนัขด้วยสารเอชเอ็มพีเอโอ และหาอุณหภูมิและปริมาณที่เหมาะสมของสารกัมมันตภาพรังสีที่ใช้ติดตัวอสุจิสุนัข เตรียมเวชภัณฑ์กัมมันตภาพรังสีโดยละลายสารเอชเอ็มพีเอโอ 0.5 มก. ด้วยเปอร์เทคนีเตท 5 มล. (ขนาดกัมมันตภาพรังสีเทคนีเซี่ยม 100 กิโลเบคเคอเรล) เก็บตัวอย่างน้ำเชื้อจากสุนัข 3 ตัว นำมารวมกันแล้วจึงแบ่งให้น้ำเชื้อแต่ละส่วนมีตัวอสุจิประมาณ 100 ล้านตัว ใช้น้ำเชื้อสดและน้ำเชื้อแช่แข็งที่ละลายแล้วเป็นตัวอย่างศึกษา การทดลองที่ 1 ทำการติดเวชภัณฑ์กัมมันตภาพรังสีเทคนีเซียมที่ 20 หรือ 37 °ซ. เป็นเวลา 20 นาที การทดลองที่ 2 ทำการติดตัวอสุจิสุนัขด้วยเวชภัณฑ์กัมมันตภาพรังสีปริมาณ 0.01 0.1 หรือ 0.5 มล. ที่ อุณหภูมิห้อง (20 °ซ.) เป็นเวลา 20 นาที ล้างสารกัมมันตภาพรังสีส่วนเกินออกโดยใช้การปั่นที่ความเร็ว 500 เท่า นาน 6 นาที แล้วละลายตะกอนใหม่โดยใช้สารละลายทริสบัฟเฟอร์ การติดเวชภัณฑ์กัมมันตภาพรังสีไม่มีผลต่อการเคลื่อนไหวและความแข็งแรงของผนังหุ้มเซลล์อสุจิ แต่การเคลื่อนที่ไปข้างหน้าเปลี่ยนเป็นการเคลื่อนไหวอยู่กับที่หลังการปั่นแต่ละครั้ง อุณหภูมิต่างกันไม่มีผลต่อประสิทธิภาพการติดสารกัมมันตภาพรังสี ตัวอสุจิจากน้ำเชื้อสดมีอัตราการติดสารกัมมันตรังสีสูงสุด คือ ร้อยละ 52 ในสารกัมมันตภาพรังสีและสารเอชเอ็มพีเอโอ 0.01 มล. เนื่องจากพบสารกัมมันตภาพรังสีส่วนเกินเพียงร้อยละ 5 หลังการล้าง 3 ครั้ง จึงแนะนำว่าไม่ควรล้างสารกัมมันตภาพรังสีด้วยการปั่นเกิน 2 ครั้ง เพราะจะทำให้การเคลื่อนที่ไปข้างหน้าของตัวอสุจิเสียไป การศึกษานี้แสดงให้เห็นว่าตัวอสุจิสุนัขสามารถติดเวชภัณฑ์กัมมันตภาพรังสีได้ อันจะเป็นประโยชน์ในการทดลองเรื่องการเคลื่อนย้ายตัวอสุจิในทางเดินระบบสืบพันธุ์สุนัขเพศเมีย โดยการตรวจดูด้วยกล้องรังสีแกมม่าต่อไป

คำสำคัญ : สุนัข ตัวอสุจิ เทคนีเซี่ยม สารเอชเอ็มพีเอโอ

[1]ภาควิชาสูติศาสตร์ เธนุเวชวิทยาและวิทยาการสืบพันธุ์ คณะสัตวแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย กรุงเทพฯ 10330
[2]ภาควิชาคลินิคัลไซนซ์, [3]ภาควิชาไบโอเมดิคัลไซนซ์ มหาวิทยาลัยสวีดิชอะกริคัลเจอร์รัลไซนซ์ เมืองอุ๊ปซอล่า ประเทศสวีเดน
[*]ผู้รับผิดชอบบทความ

Introduction

There are relatively few studies on sperm transport in the canine female genital tract. Labelling of dog spermatozoa with a radiopharmaceutical is a primary step for the study of *in vivo* sperm migration. It is desirable to find suitable conditions for labelling without inflicting significant damage to spermatozoa. In nuclear medicine, ^{99m}Tc-HMPAO (hexamethylpropylene amine oxime) is widely used for brain scintigraphy and leukocyte labelling. Currently, this substance has been applied for spermatozoa labelling in studies of sperm transport within the female genital tract. Spermatozoa from bulls (Balogh et al., 1995), men (Ozgür et al., 1997), rabbits (Balogh et al., 1995; Bockish, 1993) and stallions (Balogh et al., 1995; Katila et al., 2000) have been successfully labelled with ^{99m}Tc-HMPAO. Monitoring of sperm migration *in vivo* using radiolabelled spermatozoa and scintigraphy gives a real time image of sperm transport and muscular contractions in the female genital tract. It is a non-invasive technique, suitable for repeated studies over time in the intact animal.

Earlier studies have been carried out to find the proper radionuclide and conditions for labelling of spermatozoa (Kung and Blau, 1980; Lorton et al., 1980), with the objective to find a stable radiolabelling technique with high efficiency, causing minimum harm to sperm motility and viability. Labelling efficiency of 15% has been obtained with $200x10^6$ of bovine spermatozoa/ml (spz/ml)

using ^{131}I. With ^{111}In-oxine and ^{67}Ga-oxine, 80% and 40% labelling efficiency was achieved with 1000x10^6 and 100x10^6 spz/ml, respectively (Lorton et al., 1980). ^{99m}Tc as pertechnetate was reported to be superior to other radionuclides for labelling as it did not impair motility of spermatozoa from bulls, rams and boars, and had a binding of 70-90%. However, it was found that 4% of ^{99m}Tc was lost from bovine spermatozoa with each washing and that 50% of the ^{99m}Tc was lost from the spermatozoa after 2 cm migration through bovine cervical mucus (Lorton et al., 1980). Therefore, it is desirable to find conditions for labelling spermatozoa, which result in a more stable product. It is assumed that the initially lipophilic ^{99m}Tc-HMPAO molecule can enter the cell membrane and then assume a structural isomeric transformation in the cytoplasm by which it becomes hydrophilic and thus it is no longer capable of penetrating out of the cell (Kung and Blau 1980).

The objectives of the present study were to find out whether dog sperm could be labelled using ^{99m}Tc-HMPAO and to find the optimum incubation temperature and amount of ^{99m}Tc-HMPAO to label spermatozoa from fresh ejaculates and from frozen-thawed semen.

Materials and Methods

Semen preparation

Semen samples were collected by digital manipulation from 3 privately owned dogs of different breeds (1 Briard, 1 German Shepherd Dog and 1 crossbreed), with ages ranging between 4 and 6 years. The pre-sperm and sperm-rich fractions were collected in a calibrated plastic vial. Each ejaculate was pooled and subsequently analyzed to determine its volume, sperm concentration, motility and membrane integrity. The percentage of motile spermatozoa was estimated by subjective microscopic examination at a magnification x 200 using a phase contrast microscope. The sperm concentration was determined using a photometer (SpermaCue, Minitüb, Tiefenbach, Germany). Sperm membrane integrity was assessed by epifluorescence microscopy using the dual staining carboxyfluorescein diacetate and propidium iodide (CFDA-PI) as described by Rota et al. (1995). For each sample, 200 cells were counted and classified as having an intact plasma membrane (stained green with CFDA and unstained with PI), or having a damaged plasma membrane (the nucleus stained red with PI). The pooled semen was centrifuged at 700 x g for 8 min., seminal plasma discarded and the sperm pellet diluted with a TRIS diluent (TRIS buffer), containing 2.4 g TRIS, 1.4 g citric acid, 0.8 g glucose, 0.06 g Na-benzylpenicillin and 0.1 g streptomycin sulphate solubilised in 100 ml of distilled water (8), to a final estimated sperm concentration of 100 x 10^6 spz/ml. Frozen semen samples were prepared from the same dogs, with freezing procedures as described by Rota et al. (1997) and with a concentration of 100 x 10^6 spz/straw. The thawing was done by placing the straws in a water bath at 70°C for 8 sec. Thereafter, each straw was emptied in 1 ml of TRIS buffer at 37°C, pooled and left at this temperature for 5 min. before assessment of motility and membrane integrity.

Radiopharmaceutical preparation

A labelling radiopharmaceutical solution was prepared by reconstituting 0.5 mg of HMPAO (Ceretec, Nycomed Amersham, Buckinghamshire, UK) with 5 ml pertechnetate solution containing 100 kBq ^{99m}Tc according to the manufacturer's instruction. The ^{99m}Tc was prepared fresh each time from a generator (Medigen-Tc (Mo-99), MAP Medical Technologies Oy, Tikkakoski, Finland).

Radiolabelling of spermatozoa

The diluted fresh and frozen-thawed semen samples were divided into 1 ml aliquots containing approximately 100 x 10^6 of spermatozoa, and were then centrifuged at 500 x g for 6 min. The supernatants were removed and the sperm pellets were resuspended in ^{99m}Tc-HMPAO at three different concentrations and

were incubated at two different temperatures.

Experiment 1. Effect of incubation temperature

The fresh, and frozen-thawed sperm pellets were resuspended with 0.1 ml (2 kBq) or 0.5 ml (10 kBq) ^{99m}Tc-HMPAO and incubated at room temperature (20°C) or in a water-bath at 37°C for 20 min. Fresh and frozen-thawed sperm pellets resuspended in 1 ml TRIS buffer were used as controls for each incubating temperature. Each treatment was made in duplicate.

Experiment 2. Effect of amount of radiopharmaceutical

The fresh, and frozen-thawed sperm pellets were resuspended with 0.01 ml (0.2 kBq), 0.1 ml (2 kBq) or 0.5 ml (10 kBq) ^{99m}Tc-HMPAO and incubated at room temperature (20°C) for 20 min. Fresh and frozen-thawed sperm pellets resuspended with 1 ml Tris buffer were used as controls. Two replicates were made for each treatment.

Washing procedures

After incubation, TRIS buffer was added to a final volume of 1 ml. Washings were performed by centrifugation at 500 x g for 6 min to remove radioactivity outside the spermatozoa. The spermatozoa were washed three times and resuspended in TRIS buffer. Sperm motility and the percentage of intact membrane integrity were assessed after the second and the third washings of the pellet. The radioactivity of the second and the third supernatants was measured using a gamma counter (COBRA mod 5005, Packard Instrument Comp., Meriden, CT, USA). The amount of radioactivity removed was calculated as the percentage of total activity used. The labelling efficiency was evaluated as activity bound to the spermatozoa after the third washing.

Statistical analysis

Data on percentage of labelling efficiency were analysed using the general linear model (GLM, SAS, Cary, North Carolina, USA). In experiment 1, the statistical model included the effect of amount of ^{99m}Tc-HMPAO, replication nested within amount of ^{99m}Tc-HMPAO, temperatures and replication nested within temperatures. The effect of amount of ^{99m}Tc-HMPAO and temperature were also tested by assigning replication within amount of ^{99m}Tc-HMPAO and replication within temperature as random errors, respectively. In experiment 2, the statistical model included the effect of the type of semen, amount of ^{99m}Tc-HMPAO, and interaction between amount of ^{99m}Tc-HMPAO and type of semen. Least square means were obtained from each class of the treatments. Level of significance was set at $p \leq 0.05$.

Results

After the washing procedures, the motility of most of the spermatozoa both in control and treatment groups changed from progressive to a stationary motility, characterised by that the sperm head was sticking to the glass slide while the tail was beating. Only few progressively motile spermatozoa were observed. Therefore, after the washings, we report on the percentage of spermatozoa having stationary motility and not on progressive motility.

Experiment 1. Effect of incubation temperature

The fresh semen showed after pooling an initial sperm motility of 80%; and 84% of spermatozoa had intact membranes. Corresponding figures for the frozen-thawed semen after pooling was 70% and 61%, respectively. Mean percentage of spermatozoa with stationary motility and mean percentage of intact plasma membrane integrity is shown for the fresh semen samples in Table 1 and for the frozen-thawed semen samples in Table 2. The percentage of the spermatozoa with stationary motility after washing was similar for both incubation temperatures (20°C and 37°C) in both fresh and frozen-thawed semen.

The efficiencies of labelling of the fresh and the frozen-thawed spermatozoa are presented in Figure 1.

Table 1. Mean stationary motility (%) and intact plasma membrane integrity (%) of dog spermatozoa (spz) in fresh semen incubated for 20 min with 0.1 or 0.5 ml ^{99m}Tc-HMPAO at 20°C or 37°C, after the second, and the third washing.

Incubation temperature	After 2nd washing		After 3rd washing	
	% spz with stationary motility	% spz with intact membrane	% spz with stationary motility	% spz with intact membrane
20°C				
Control	65	77	25	73
0.1 ml ^{99m}Tc-HMPAO	65	82	35	77
0.5 ml ^{99m}Tc-HMPAO	28	77	18	78
37°C				
Control	45	83	25	72
0.1 ml ^{99m}Tc-HMPAO	40	84	23	80
0.5 ml ^{99m}Tc-HMPAO	18	80	18	77

Table 2. Mean stationary motility (%) and intact plasma membrane integrity (%) of dog spermatozoa (spz) in frozen-thawed semen incubated for 20 min with 0.1 or 0.5 ml ^{99m}Tc-HMPAO at 20°C or 37°C, after the second, and the third washing

Incubation temperature	After 2nd washing		After 3rd washing	
	% spz with stationary motility	% spz with intact membrane	% spz with stationary motility	% spz with intact membrane
20°C				
Control	58	49	30	45
0.1 ml ^{99m}Tc-HMPAO	50	45	25	41
0.5 ml ^{99m}Tc-HMPAO	55	43	30	38
37°C				
Control	55	41	50	35
0.1 ml ^{99m}Tc-HMPAO	30	44	30	34
0.5 ml ^{99m}Tc-HMPAO	45	46	15	42

Table 3. Percentage of stationary motility and intact plasma membrane integrity of dog spermatozoa (spz) from fresh and frozen-thawed semen incubated with 0.01, 0.1 or 0.5 ml ^{99m}Tc-HMPAO at 20°C, after three times washing

HMPAO (ml)	Fresh semen		Frozen-thawed semen	
	% spz with stationary motility	% spz with intact membrane integrity	% spz with stationary motility	% spz with intact membrane integrity
Control	30	79	20	29
0.01	35	80	0	23
0.1	10	77	10	28
0.5	35	79	5	24

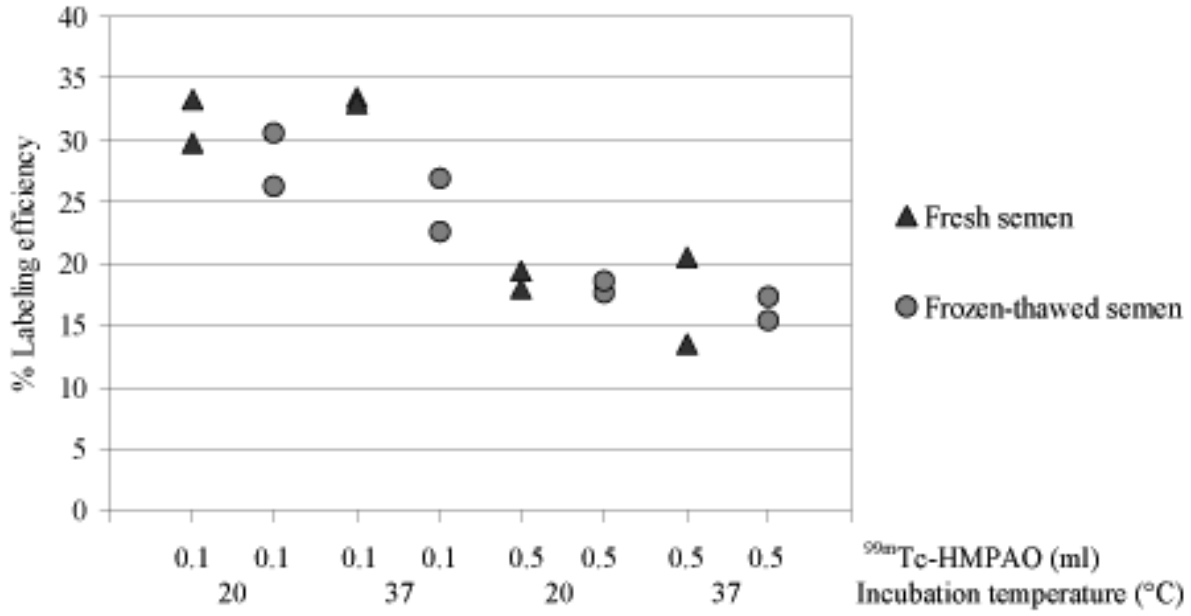

Figure 1. Labelling efficiency of dog spermatozoa from fresh and frozen-thawed semen incubated with 0.1 or 0.5 ml ^{99m}Tc-HMPAO at 20°C or 37°C for 20 min.

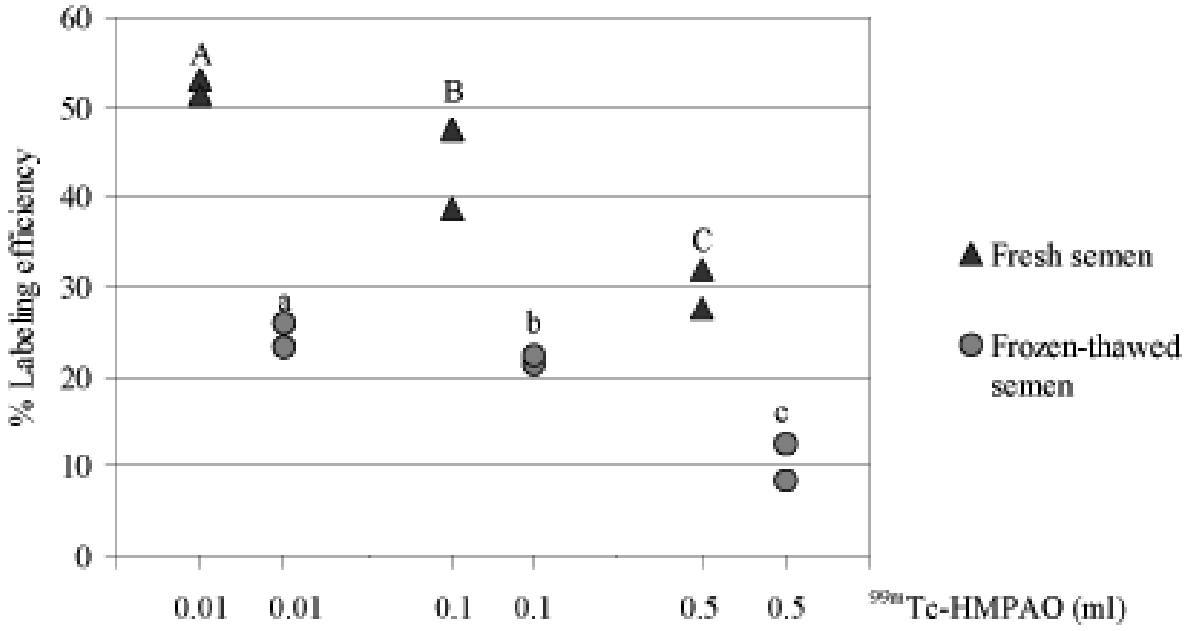

Figure 2. Mean labelling efficiency in fresh and frozen-thawed semen labelled with different amounts of ^{99m}Tc-HMPAO and incubated at 20°C for 20 min. Significant differences are indicated by: AB, bc ($p< 0.05$); BC, ac ($p< 0.01$); AC ($p< 0.001$).

The incubation temperature had no effect on the labelling efficiency, neither in fresh (p>0.05) nor in frozen-thawed spermatozoa (p>0.05). Nor had the amount of labelling substance any effect on the radiolabel of fresh (p>0.05), or frozen-thawed (p>0.05) spermatozoa. However, there was a tendency both in fresh and frozen-thawed semen that the labelling efficiency was higher when 0.1 ml than 0.5 ml radiopharmaceutical was used for the incubation. With the second washing, 9.5±2.3% and 11.7±3.1% of added radioactivity was removed in the supernatant from fresh and frozen-thawed spermatozoa, respectively, whereas only 1.7±0.3% and 3.4±0.5% of added radioactivity was found in the supernatant from fresh and frozen-thawed spermatozoa after the third washing.

Experiment 2. Effect of amount of radiopharmaceutical

In the pooled fresh semen, the initial progressive motility was 90%; and 85% of the spermatozoa had an intact plasma membrane. Corresponding figures for the frozen semen, which was pooled post thawing were 70% and 63%, respectively. The effects on motility and membrane integrity after incubation with radiopharmaceutical and washings are shown in Table 3. There were no effects of the different amounts of radiopharmaceutical used on the percentage of spermatozoa with stationary motility or on the percentage of intact plasma membrane integrity compared to the controls.

The labelling efficiency using 0.01, 0.1 and 0.5 ml ^{99m}Tc-HMPAO at 20^{o}C for 20 min is shown in Figure 2. The highest labelling efficiency, 52%, was obtained from fresh spermatozoa incubated with 0.01 ml ^{99m}Tc-HMPAO. The labelling efficiency for frozen-thawed spermatozoa during the same conditions was 25%. Incubating with 0.01 ml (p<0.01) and 0.1 ml (p<0.05) radiopharmaceutical resulted in a significantly higher labelling of the spermatozoa than with 0.5 ml. There was no significant difference between the spermatozoa labelled with 0.1 and 0.01 ml radiopharmaceutical (p>0.05). After the second washing, only 6.2±2.1% and 13.5±2.1% of added radioactivity was found in the supernatant of fresh and frozen-thawed spermatozoa, respectively. After the third washing the corresponding figures were 2.1±0.3% and 3.4±0.9%, respectively.

Discussion

This study demonstrates that dog spermatozoa can be labelled with ^{99m}Tc-HMPAO. The highest labelling efficiency, 52%, was obtained using 0.01 ml of ^{99m}Tc-HMPAO, and it was similar to what has earlier been reported for horses (57-72%) (Katila et al., 2000), humans (40.9%) (Ozgür et al., 1997) and rabbits (52%) (Bockish, 1993). The number of spermatozoa has been shown to influence the binding of the radiopharmaceutical in semen from boars, bulls and rams (Lorton et al., 1980). In these species optimal labelling efficiency (70%-90% binding) was obtained using 37 to 370 MBq 99mTcO_4^- per $1000x10^6$ spermatozoa (Lorton et al., 1980).This influence of sperm numbers on labelling efficiency needs to be further studied in the dog.

In the present study incubation temperature was not shown to have an effect on dog sperm labelling, whereas for stallion spermatozoa, incubation at 20^{o}C resulted in higher labelling efficiency than did incubation at 37^{o}C (Katila et al., 2000). The incubation procedure with radiopharmaceutical was detrimental to the sperm progressive motility but did not alter the membrane integrity in dog spermatozoa. In the stallion (Katila et al., 2000) repeated centrifugation decreased the motility in dog spermatozoa, resulting in a high percentage of spermatozoa showing only stationary motility. Different protocols for processing of dog semen use centrifugation speeds of between 300 x g and 1000 x g for 3 to 10 min (Rota et al., 1995; Rota et al., 1997; Rijsselaere et al., 2002). Rijsselaere et al. (2002) found significantly more dead and moribund spermatozoa after 2-3 days of cold storage in dog semen that was centrifuged at 1620 x g and 2880 x g for 5 min, than when centrifuged at 180 x g or 750 x g. The centrifugation speed and time used for each washing in the present study should thus not be

detrimental to the fresh spermatozoa, but it is likely that the frozen-thawed dog spermatozoa are less resistant to centrifugation (Catharina Linde Forsberg, unpublished results). Furthermore, the effects of repeated centrifugation of dog spermatozoa have not been well documented. Less than 5% of added radioactivity was found in the supernatant after the third centrifugation, thus two washings should likely be sufficient to remove unbound radioactivity and would cause less damage to the spermatozoa than three washings.

The results of this study show that dog spermatozoa can be labelled with ^{99m}Tc-HMPAO, and it is likely that such radiolabelled spermatozoa can be used in studies of in vivo sperm transport in the canine female genital tract if the progressive motility can be recovered by improving the radiolabelling procedures.

Acknowledgements

The authors thank Mieth Berger, Department of Biomedical Sciences and Veterinary Public Health, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, for technical assistance with the radiopharmaceutical preparation. The study was supported by the Swedish Foundation for International Cooperation in Research and Higher Education (STINT).

References

Balogh, L., Szasz, F., Zoldag, L., Huszenicza, G., Toth, L., Dabasi, G. and Janoki, G. 1995. *In vitro* and *in vivo* motility studies of ^{99m}Tc HM-PAO labelled sperm cells. Acta Vet. Hung. 43: 63-77.

Bockish, A. 1993. Sperm cell dynamics in the female rabbit genital tract after insemination monitored by radiolabeled spermatozoa. J. Nucl. Med. 34: 1134-1139.

Katila, T., Sankari, S. and Mäkelä, O. 2000. Transport of spermatozoa in the reproductive tracts of mares. J. Reprod. Fertil. Suppl. 56: 571-578.

Kung, H. and Blau, M. 1980. Regional intracellular pH shift: a proposed new mechanism for radiopharmaceutical uptake in brain and other tissues. J. Nucl. Med. 21: 147-152.

Ozgür, K., Yildiz, A., üner, M., Erkilic, M., Trak, B. and Erman, O. 1997. Radionuclide hystero-salpingography with radiolabeled spermatozoa. Fertil. Steril. 67: 751-755.

Lorton, S.P., Gatley, S.J., Lieberman, L.M. and First, N.L. 1980. Radiolabelling of mammalian spermatozoa and their use to monitor sperm transport in females. Am. J. Obstetr. Gynecol. 136: 732-736.

Rijsselaere, T., Van Soom, A., Maes, D. and de Kruif, A. 2002. Effect of centrifugation on in vitro survival of fresh diluted canine spermatozoa. Theriogenology. 57: 1669-1681.

Rota, A., Ström, B. and Linde-Forsberg, C. 1995. Effects of seminal plasma and three extenders on canine semen stored at 4^{o}C. Theriogenology. 44: 885-900.

Rota, A., Ström, B., Linde-Forsberg, C. and Rodriguez-Martinez, H. 1997. Effects of Equex STM Paste on viability of frozen-thawed dog spermatozoa during *in vitro* incubation at 38^{o}C. Theriogenology. 47: 1093-1101.