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Low levels of apoptotic-like changes in fresh and cryopreserved feline spermatozoa collected from the urethra and epididymis

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ABSTRACT

The aim of this study was to examine apoptotic markers in fresh and frozen-thawed feline spermatozoa collected via urethral catheterization and epididymal slicing. Caspase activation, DNA fragmentation, and phosphatidylserine externalization were evaluated using flow cytometry in sperm cells from both sources before and after cryopreservation. The study revealed no differences between urethral and epididymal spermatozoa, both in fresh and frozen-thawed samples. The level of apoptotic changes in sperm cells in fresh feline semen was low: 0.8 ± 0.8% of live urethral and 0.4 ± 0.4% of live epididymal spermatozoa showed active caspases; 1.6 ± 0.9% and 2.1 ± 1.9%, respectively, showed DNA fragmentation; and 0.3 ± 0.2% and 1.0 ± 1.3%, respectively, showed phosphatidylserine externalization. In both types of sperm cells, cryopreservation did not induce a significant increase in caspase activation (urethral: from 3.9 ± 3.2% to 7.5 ± 5.0%; epididymal: from 4.7 ± 2.9% to 11.7 ± 8.5%). In urethral spermatozoa, phosphatidylserine externalization in live cells was significantly ($P < 0.05$) increased after thawing (from 0.3 ± 0.2% to 2.7 ± 2.5%). This increase was not noted for epididymal spermatozoa (from 1.0 ± 1.3% to 1.7 ± 1.3%). No significant changes in DNA fragmentation were observed (2.1 ± 0.8% and 1.7 ± 1.0%). In conclusion, both urethral and epididymal feline spermatozoa showed equally low levels of apoptotic-like changes. Hence, apoptotic alterations seem to play only a minor role, if any, in urethral and epididymal feline spermatozoa. The deterioration of sperm quality after freezing and thawing is more likely connected with direct damage to the cells than to activation of apoptotic processes.

1. Introduction

Apoptosis is a well-known process of programmed cell death, which involves a series of cellular step-by-step events resulting in characteristic changes in cell function and structure. Apart from morphological changes, which may not be observed in some kinds of cells (like sperm cells [1]), many other biochemical features are considered markers of apoptosis. These most commonly include the activation of caspases, translocation of phosphatidylserine from the inner to the outer layer of the cell membrane, loss of the mitochondrial membrane potential, changes in the permeability of the plasma membrane, and oligonucleosomal DNA fragmentation. Although apoptosis is important for the development and homeostasis of every organism and plays an important role in spermatogenesis [2], its significance in mature spermatozoa is still poorly understood.

Markers of apoptosis have been detected in mature spermatozoa after ejaculation in many species, including

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humans [3], cattle [4], pigs [5,6], horses [7], dogs [8], silver foxes [9], and alpacas [10], to name a few. Although it is unclear whether mature sperm cells can undergo apoptosis or whether they only exhibit apoptotic-like changes [11,12], apoptotic markers appeared to be sensitive indicators of sperm quality [3,13–15], fertilizing ability [16], and oxidative stress [13,15].

Increased levels of some apoptotic markers were found in spermatozoa after cryopreservation [4,17,18]. Based on these findings, apoptosis was proposed to be one of the possible mechanisms involved in sperm cryoinjury [19]. Regardless of whether cryopreservation induces true apoptosis or apoptotic-like changes [12], some of these markers were found to be useful in the prediction of postthaw sperm quality [20,21].

Collection and cryopreservation of cat semen has special interest, as the domestic cat can be used as a model animal for endangered wild cats [12]. However, our knowledge concerning the quality of spermatozoa from different types of cat semen and their changes during cryopreservation is still poorly documented. To date, apoptotic markers have been studied only in fresh and chilled epididymal spermatozoa [22–26]. Only one parameter—the sperm mitochondrial potential [27,28]—has been evaluated in cryopreserved feline spermatozoa. A wide, comparative study of apoptotic changes in sperm cells from feline urethral vs. epididymal semen before and after cryopreservation has not been carried out. Moreover, to the authors' knowledge, active caspases have not been studied in fresh or cryopreserved feline spermatozoa. Because caspases are proteins that are involved only in the apoptosis cascade and are not activated during other metabolic processes, their evaluation can provide valuable information about apoptosis in feline spermatozoa.

The aim of this study was to examine apoptotic markers–caspase activation, DNA fragmentation, and phosphatidylserine externalization–in sperm cells in fresh and frozen-thawed feline semen collected via urethral catheterization in comparison with semen obtained through epididymal slicing.

2. Materials and methods

2.1. Animals

Twenty five privately owned domestic shorthair male cats, aged between 7 months and 7 years were included in the study. All tomcats were clinically healthy and underwent routine castration at the Department of Reproduction and Clinic of Farm Animals of the Wroclaw University of Environmental and Life Sciences (Poland). All procedures were performed with the consent of the Second Local Ethical Committee in Wroclaw.

2.2. Semen collection

Tomcats presented for routine orchiectomy were anesthetized using medetomidine hydrochloride intramuscularly at 100 μg/1 kg of body weight (Sedator 1.0 mg/mL, Novartis, Poland), and urethral semen (CT) was collected after 10 minutes by urethral catheterization, as previously described by Zambelli et al. [29]. In summary, an ordinary tomcat urinary catheter with a shortened tip to acquire an open-ended device was inserted into the urethra for approximately 9 cm (depending on the size of the cat). After several seconds, the catheter was removed from the urethra and the semen sample that was sucked into the catheter by capillary forces was placed into an Eppendorf tube containing 200 μL of prewarmed semen extender Tris Buffer (3.02%, wt/vol) Tris (Sigma–Aldrich, Poland), 1.35% (wt/vol) citric acid (Sigma–Aldrich, Poland), 1.25% (wt/vol) fructose (Sigma–Aldrich, Poland), in double-distilled water; pH 6.5). Ketamine intramuscularly at 5 mg/1 kg of body weight (VetKetam 100 mg/mL, VetAgro, Poland) was administered immediately after the urethral semen collection and orchiectomy was performed. An injection of meloxicam subcutaneous at 0.3 mg/1 kg of body weight (Metacam 5 mg/mL, Boehringer Ingelheim Vetmedica, Germany) was given before the anesthesia to reduce postoperative pain. After orchiectomy, cats were given a mixture of benzathine benzylpenicillin 100,000 IU/mL, procaine benzylpenicillin 100,000 IU/mL, and dihydrostreptomycin sulfate 200 mg/mL intramuscularly at 1 mL/10 kg of body weight (Shotapen L.A., Virbac, France).

Within 5 to 10 minutes after removal of the gonads, epididymal spermatozoa (EP) were collected by epididymal slicing [30]. The caudae epididymides were dissected from the testes and cleaned from the connective tissue and visible blood vessels. The caudae epididymides were then placed into 1 mL of prewarmed semen extender in a glass Petri dish and minced using a scalpel blade. After a 10-minute incubation, the epididymidal tissues were removed, and a suspension of spermatozoa was filtered (CellTrics 30 μm, Partec) into an Eppendorf tube.

2.3. Semen cryopreservation and thawing

Sperm cryopreservation was carried out according to Niżański [30]. After centrifugation at ×620*g* for 5 minutes, the supernatant was removed. The sperm pellet was resuspended at room temperature in a freezing extender containing Tris buffer supplemented with 20% (vol/vol) egg yolk, 6% glycerol (vol/vol), and 1% Equex paste (vol/vol) (Minitube Germany). The final concentration of spermatozoa was adjusted to 40×10^6 cells/mL. After resuspension, the sperm samples were cooled to 5 °C within 1 hour (cooling rate 0.25 °C/minute) [31], equilibrated at 5 °C for 1.5 hours, loaded into precooled 0.25 mL straws and held 5 cm above the surface of liquid nitrogen for 10 minutes. Then, the straws were plunged into the liquid nitrogen and kept at −196 °C. For postcryopreservation assessment, two straws per cat per group were thawed by immersion in a 37 °C water bath for 30 seconds.

2.4. Sperm evaluation

Spermatozoa from both types of semen were subjected to the same assessment procedures. In frozen-thawed samples, the same sperm parameters were assessed as in fresh samples. The assessment of sperm samples was done immediately after collection (fresh semen) or thawing (cryopreserved semen).