The effect of swim-up purification and incubation of cells on sperm viability in dogs of different ages

D. Bukowska[1], B. Kempisty[2], J. Sikora[3], M. Jackowska[1], M. Wozna[1], P. Antosik[1], H. Piotrowska[4], J. Budna[5], J.M. Jaskowski[1]

[1]Department of Veterinary, Poznan University of Life Sciences, Poznan, Poland
[2]Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan, Poland
[3]Department of Biology and Environmental Studies, Poznan University of Medical Sciences, Poznan, Poland
[4]Department of Toxicology, Poznan University of Medical Sciences, Poznan, Poland
[5]Department of Clinical Immunology, Poznan University of Life Sciences, Poznan, Poland

ABSTRACT: The influence of selected semen extenders on the motility of frozen-thawed dog spermatozoa has been clearly demonstrated in several studies, although there are no reports indicating the effect of swim-up purification on sperm viability in this species of mammals. Therefore, this study was aimed at investigating phosphatidylserine (PS) externalization and necrosis in sperm after variable lengths of time of *in vitro* incubation after swim-up purification. Dog semen samples were collected from (i) ten dogs aged six months to 1.5 year, (ii) ten dogs aged six to eight years, and (iii) ten dogs aged 11 to 13 years. A flow cytometric method was employed to evaluate dog sperm viability in animals of different age groups after employment of a swim-up (SU) purification technique. After SU spermatozoa were incubated for 15, 30, 45 and 60 min in Sperm-TALP medium. We observed an increase in the number of viable sperm (double negatives) after 15 min of incubation compared to sperm undergoing PS externalization and late necrotic sperms ($P < 0.001$) in each group of dogs. We also found a higher number of early necrotic sperm after 60 min of *in vitro* incubation ($P < 0.001$). The amounts of late necrotic sperm and cells with PS externalization were similar among animals of different age groups. We show for the first time that most viable sperm are recovered after an *in vitro* incubation step of 15 min (control samples in this study) because as the time of incubation increases so does the number of degenerated or damaged cells. The higher number of early necrotic cells after 60 min of *in vitro* incubation may be a special feature of this species and may result from the induction of necrosis in the sperm. This knowledge may be used in future experiments for the preparation of spermatozoa following *in vitro* fertilization in dogs.

Keywords: dogs; sperm; sperm viability; necrosis; flow cytometry

During apoptosis and cellular necrosis, disruption of the asymmetry of membrane phospholipids occurs, which leads to a disturbance of the plasma membrane integrity (Martin et al., 1995). Apoptosis and necrosis are two major forms of cell death. Necrosis affects large number of cells and results in cell swelling and rupture, but apoptosis, termed programmed cell death, affects a single cell. Apoptosis is characterized by several ultrastructural and biochemical changes, including chromatin and cytoplasm aggregation and disruption of plasma membrane symmetry that occur in the early stages of this process. Normally, phosphatidylserine (PS) is present on the inner cytoplasmic leaflet of the plasma membrane. During apoptosis, PS is translocated to the outer leaflet (Martin et al., 1995). It is known that PS translocation is a

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specific marker of disturbances in membrane stability, since there is an increase in the ability of the cell to bind annexin-V in a calcium-dependent manner (Andree et al., 1990). Annexin-V (An-V) and prodidium iodide (PI) are employed as markers of sperm viability, specifically by PI staining for DNA and An-V staining for PS externalization (Karabinus et al., 1991; Lezcano et al., 2004).

It is known that mammalian spermatozoa are characterized by high levels of heterogeneity, especially in morphology, viability and motility (Rodriguez-Martinez et al., 1997). There are several sperm separation techniques which are able to improve sperm quality by separating out those with an increased rate of motility, viability and normal morphological appearance. Sperm separation procedures isolate motile cells from non-motile, and morphologically normal cells from sperm with tail and mid-piece defects, and remove seminal plasma (Centola, 1998; Henkel et al., 2003; Van Kooij et al., 2004). The most important sperm purification procedure includes fractionation of subpopulations using density gradient centrifugation and the use of a self-migration test called the swim-up technique (SU) (Rodriguez-Martinez et al., 1997). The effectiveness of sperm separation procedures can vary widely because different sperm parameters are evaluated (Sakkas and Tomlinson, 2000; Fraczek et al., 2004; Van Kooij et al., 2004). The most important sperm parameters, according to which sperm can be effectively separated after purification, include sperm motility, morphology, concentration, viability, membrane stability, acrosomal status and chromatin integrity. Comparing the SU technique with density-gradient centrifugation results in similar sperm parameters, although SU gives a lower concentration of cells. The SU procedure is a basic sperm manipulation prior to *in vitro* fertilization (Shamsuddin et al., 1993; Rosenkranz and Holzmann, 1997; Sakkas and Tomlinson, 2000).

After copulation, sperm must traverse the cervical mucus, which is a specific barrier that allows the migration of only those spermatozoa characterized by increased motility and normal morphology. Only the progressively motile cells are able to fertilize an oocyte. Therefore, in our experiment we analyzed motile spermatozoa after swim-up purification.

The aim of the present study was to determine whether PS externalization and necrosis are suitable indicators of viability in dog spermatozoa isolated from animals of various ages using the swim-up technique. We also studied the effect of *in vitro* incubations of varying durations on the levels of PS externalization and necrosis in sperm.

MATERIAL AND METHODS

All media components used in this study were obtained from Sigma-Aldrich (St Louis, MO, USA). The beagle dogs were divided into three groups depending on their age: (1) ten dogs aged six months to 1.5 year, (2) ten dogs aged six to eight years, and (3) ten dogs aged 11 to 13 years. A total 30 of animals were used in this study.

Collection of dog semen and swim-up purification

The semen samples were collected from each dog by an experienced operator using a latex artificial vagina (AV) and manual stimulation. Each collection was finished when prostatic fluid had been flowing long enough for the operator to be fairly certain that a full sperm-rich portion had been obtained. Immediately after collection the semen samples were purified using the swim-up (SU) technique. For SU purification, 100 µl of freshly collected dog semen was placed under a layer of medium (1 ml Sperm-TALP medium supplemented with fatty acid-free bovine serum albumin (6 mg/ml) and sodium pyruvate (1mM)). The semen samples were incubated for 30 min at 38.5 °C in an atmosphere containing 5% CO_2. After this time, the upper 500 µl of medium was collected. The collected spermatozoa were then incubated in Sperm-TALP medium (described above) in separate experiments for 15, 30, 45 and 60 min. Fifteen minutes of *in vitro* incubation was used as a control because this is the length of time required for incubation of spermatozoa with An-V and PI. The sperm were then centrifuged at 250 × g for 5 min. The recovered spermatozoa were used in an An-V/ PI-binding assay. Sperm viability, using PI staining for DNA and An-V staining for PS externalization, was evaluated according to the methods described by Lezcano et al. (2004) and Karabinus et al. (1991).

Annexin-V/PI-binding assay

An Annexin-V-FLUOS Staining Kit (Roche, Cat. No.11 858 777 001) was used to detect the transition

of phospholipids of the spermatozoa plasma membrane according to the manufacturer's instructions. Aliquots of semen were diluted in annexin-V-binding buffer (10mM HEPES/NaOH, pH 7.4, 140mM NaCl, 2.5mM $CaCl_2$), to a concentration of 2×10^6 cells/ml. One hundred µl of sample (corresponding to 2×10^5 spermatozoa/ml) were transferred to 5 ml tubes and supplemented with 5 µl annexin-V-FITC and 5 µl PI (50 µg/ml). The contents of the tubes were mixed and the solutions incubated for 15 min at room temperature in the dark. Prior to flow cytometric analysis, 400 µl of binding buffer was added to each tube.

Flow cytometry

The samples were analyzed using a FACScan flow cytometer (Becton Dickinson) equipped with a 15 mW 488 nm, air-cooled argon ion laser. For each spermatozoon, forward light scatter (FSC), orthogonal light scatter (SSC), annexin-V-FITC (FL1) and PI (FL3) fluorescence, were evaluated using Cellquest software (Becton Dickinsion, San Jose, CA, USA). For the gated spermatozoa, four different kinds of cells were observed. Cell populations were gated as viable spermatozoa (annexin-V–, PI–), cells with translocation of phosphatidylserine (annexin-V+, PI–), late necrotic cells (annexin-V–, PI+), and early necrotic spermatozoa (annexin-V+, PI+).

Statistical analyses

Two-way ANOVA followed by the Tukey post-test were used to compare the results of the number of cells of each type present in the three age groups of dogs, following staining with An-V and PI. Differences were considered to be significant at $P < 0.05$, $P < 0.01$ and $P < 0.001$. The software program GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA) was used for statistical calculations.

RESULTS

Using flow cytometry to assess the staining of cells with An-V and PI in the three age groups of dogs, we found an increased number of viable (An-V–/PI–) spermatozoa in each of the groups after 15, 30, 45 and 60 min of *in vitro* cell incubation compared to spermatozoa with PS externalization (An-V+/PI–) and late necrotic cells (An-V–/PI+), $P<0.001$, (Figures 1 and 2). After different incubation times, we also observed a higher number of early necrotic spermatozoa (An-V+/PI+), as compared to An-V+/PI– and An-V–/PI+ spermatozoa, in each of the groups ($P < 0.001$). An increased number of viable cells was seen in dogs aged 0.5 to one to five years and six to eight years, when comparing to the group of dogs aged 11 to 13 years, however the difference was not statistically significant. Moreover, we observed a higher number of early necrotic spermatozoa after 60 min, compared to 15 min of *in vitro* incubation in all three groups of dogs ($P < 0.01$). The number of cells characterized by PS externalization and late necrotic cells after 15, 30 and 45 of *in vitro* incubation were at similar levels in dogs aged 0.5 to 1.5 years. However, we found increased numbers of necrotic spermatozoa after 60 min of incubation ($P < 0.05$) in this group, (Figures 1 and 2). The numbers of these cells in the two other groups of dogs were similar. In summary, in all three groups of dogs we observed a tendency toward an increased number of viable cells after 15 min of *in vitro* incubation and toward a higher number of early necrotic spermatozoa after 60 min of *in vitro* incubation.

DISCUSSION

For several years, the assessment canine semen was based on conventional light microscopic evaluation to determine, in particular, sperm morphology and mobility. The limitations of this method include the analyst's subjectivity and the limited number of cells analyzed. There were also no methods that included an assessment of the sperm's fertilizing potential and viability. In the last decade, several new techniques have been introduced for the evaluation of the sperm's molecular and reproductive potential, including fluorescent staining assays for the assessment of plasma membrane integrity, capacitation, and acrosome status (Rijsselaere et al., 2005).

Freezing techniques and the evaluation of fertilizing potential of frozen-thawed dog spermatozoa have been intensively investigated in several studies (Zavos et al., 1992; Martins-Bessa et al., 2006; Schafer-Somi et al., 2006, 2007; Cardoso et al., 2007). However, determining the viability of sperm

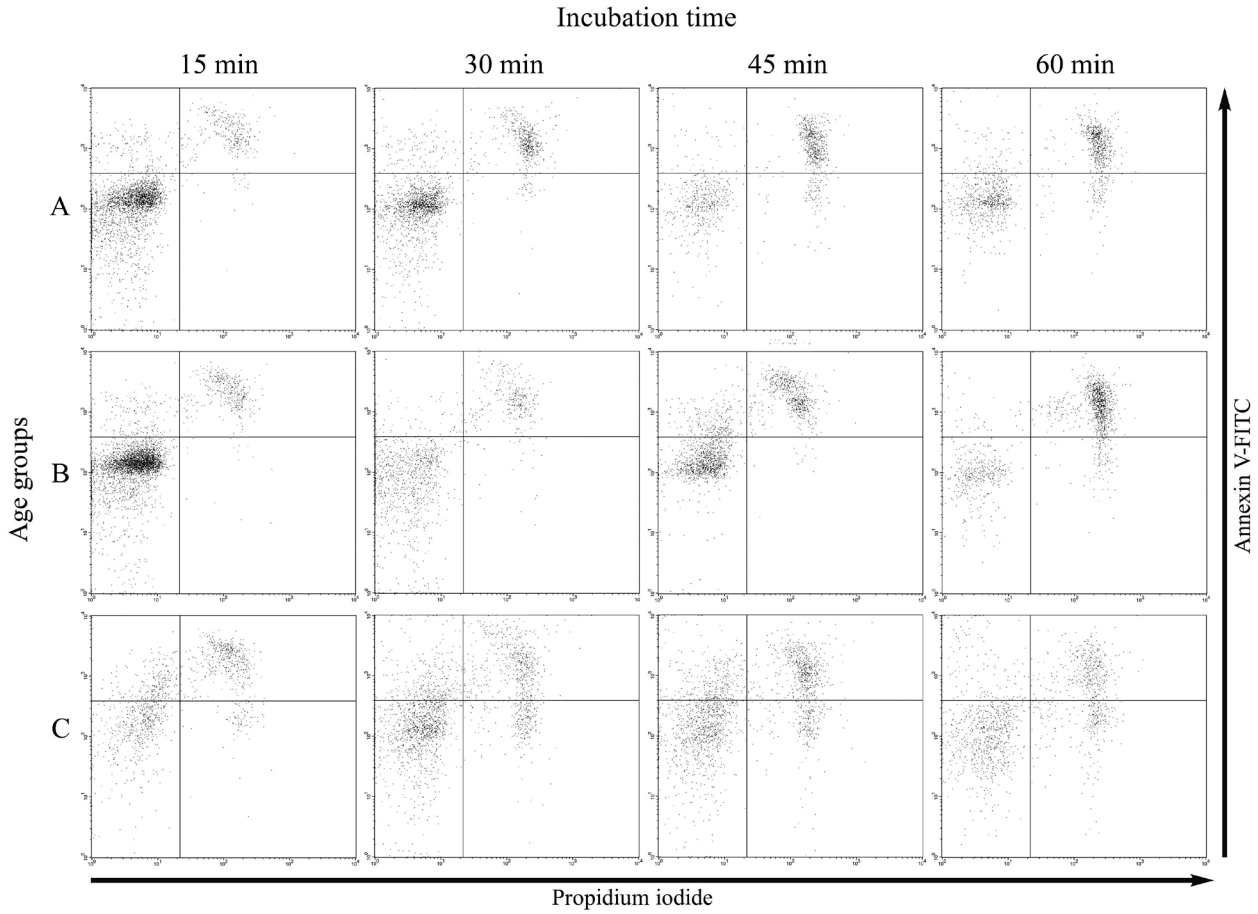

Figure 1. Flow cytometric analyses of sperm viability using Annexin-V (An-V) and propidium iodide (PI) in three different age groups of dogs (A = 0.5–1.5 years), (B = 6–8 years), (C = 11–13 years) after different lengths of time of *in vitro* incubation

The aliquots of semen were diluted in annexin-V-binding buffer to a concentration of 2×10^6 cells/ml. The 100 µl sample (corresponding to 2×10^5 spermatozoa/ml) was transferred to 5 ml tubes and supplemented with 5 µl annexin-V-FITC and 5 µl PI (50 µg/ml). The samples were analyzed by FACScan flow cytometer (Becton Dickinson) equipped with a 15 mW 488 nm, air-cooled argon ion laser. Evaluation determined the percentage of viable spermatozoa (annexin-V–, PI–), cells with translocation of phosphatidylserine, (annexin-V+, PI–), necrotic cells (annexin-V–, PI+), and early necrotic spermatozoa (annexin-V+, PI+)

of dogs of different ages has not been the subject of focus. The present study is the first, to our knowledge, to investigate the effect of age on levels of viable/necrotic spermatozoa in dogs. Moreover, for the first time, the role of the duration of *in vitro* incubation of fresh canine ejaculates on the number of viable/necrotic cells has been determined.

Propidium iodide (PI) was recently used to determine the viability of sperm of several species, including humans (Karabinus et al., 1991). Annexin-V-FITC is a known marker of PS externalization. Espinoza et al. (2009) recently used An-V to assess sperm PS externalization in human spermatozoa in relation to other sperm parameters, including motility, viability and morphology. They observed no differences in PS externalization between the group of fertile and infertile patients. Moreover, they did not find a correlation between sperm PS externalization and other sperm viability parameters. Thus, An-V staining as a marker of PS externalization is not a good marker in the assessment of important sperm viability parameters. In our study we demonstrated that there is no correlation between the number of cells with PS externalization and the age of the dogs or the length of time of *in vitro* incubation of the sperm. However, double staining with An-V and PI (An-V+/PI+) as a marker of early necrosis significantly correlated with 60 min of *in vitro* incubation. Thus, double staining may be a marker of the induction of necro-

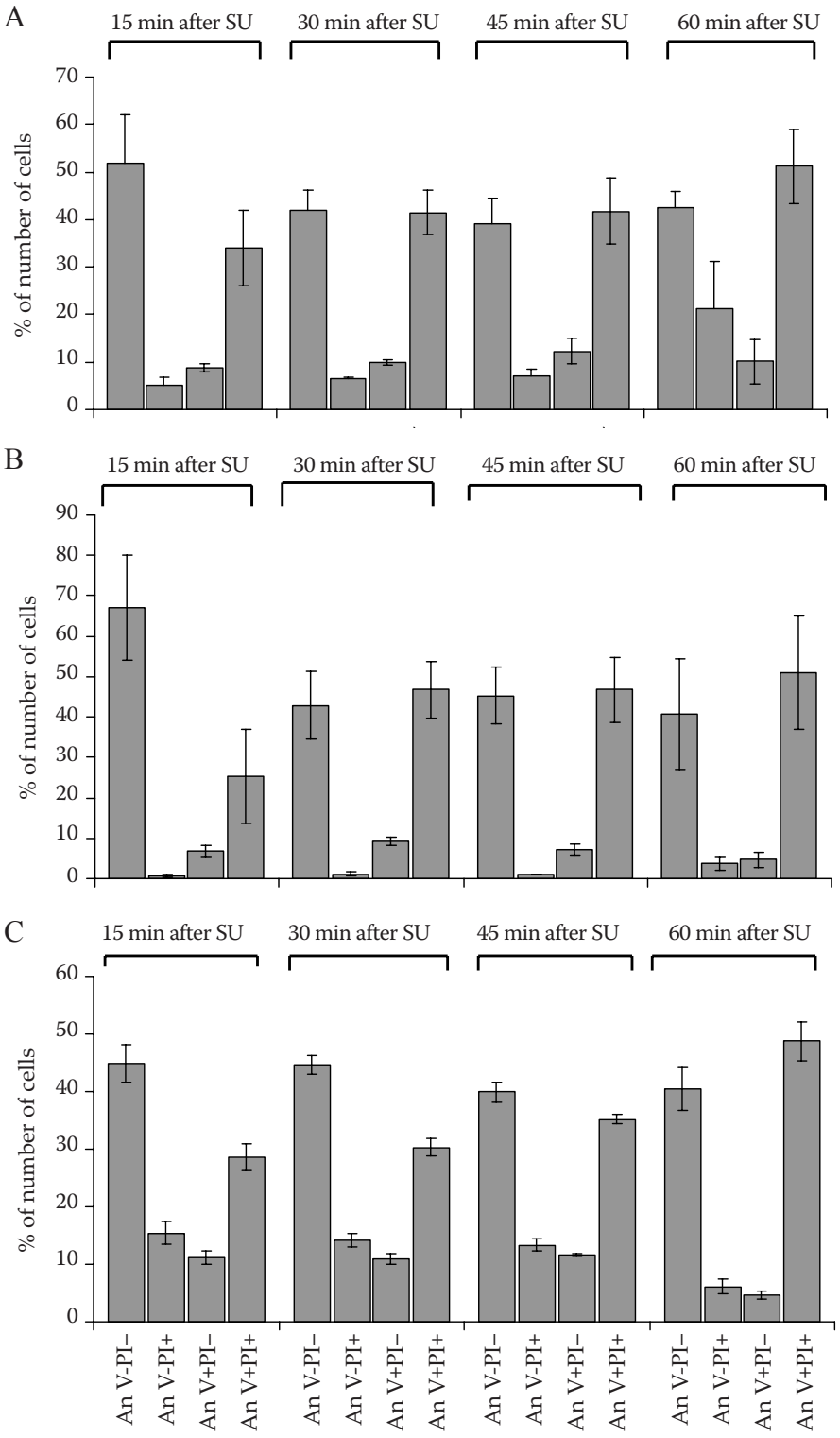

Figure 2. Percent of the numbers of cells after staining with Annexin-V (An-V) and propidium iodide (PI) following swim-up (SU) purification

Spermatozoa were isolated from three age groups of dogs (A = 0.5–1.5 years), (B = 6–8 years B), (C = 11–13 years) by an experienced operator using a latex artificial vagina (AV) and manual stimulation. After collection, the ejaculates were purified by using swim-up technique (SU) and then incubated in Sperm-TALP medium in separate experiments for 15, 30, 45 and 60 min. After incubation, the purified spermatozoa were stained with An-V and PI and evaluated by flow cytometry. Results were repeated in triplicate and are presented as mean ± SEM

sis in dog spermatozoa after longer durations of *in vitro* incubation of these cells. However, there are also data indicating a direct correlation between An-V staining and sperm parameters (Aziz and Agarwal, 2008; Hoogendijk et al., 2008). In similar studies, Chaveiro et al. (2007) determined the number of bull sperm with PS translocation (An-V+) or without PS translocation (An-V–) and enhanced the percentage of motile sperm after SU purification. Similar to the results of the present study, they found that *in vitro* incubation of spermatozoa after SU may have an adverse effect on sperm viability as well as on the stability of the sperm plasma membrane.

The An-V/PI binding assay allows the identification of four different populations of spermatozoa: live unstained cells (An-V–, PI–), cells with externalization of PS (An-V+, PI–), early necrotic cells (An-V+, PI+), and late necrotic cells (An-V–, PI+). We found the highest number of viable spermatozoa in the control group (15 min of *in vitro* incubation). The number of viable sperm decreased as the length of time of *in vitro* incubation increased. Surprisingly, the number of cells with PS externalization and the number of late necrotic sperm were similar, independent of the length of incubation. Thus, the length of *in vitro* incubation does not seem to be an inducer of these processes in sperm.

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Corresponding Author:

Bartosz Kempisty, Poznan University of Medical Sciences, Department of Histology and Embryology, 6 Swiecickiego St., 60-781 Poznan, Poland
Tel. Fax + 48 61 8546515, Fax + 48 61 8546510, E-mail: etok@op.pl