Sveriges lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine and Animal Science
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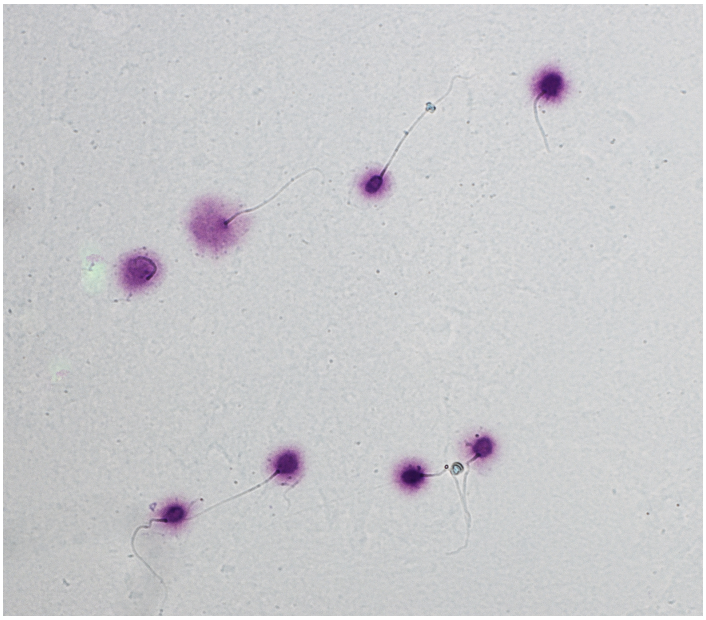

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Halomax® som metod för kromatinfärgning av kattspermier

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SUMMARY

Wild felids all over the world are threatened by extinction. As a part of the preservation task concerning those species, in zoos as well as *in situ* in their wild habitat, the use of assisted reproduction technology (ART) is explored, e. g. artificial insemination (AI) and *in vitro* fertilization (IVF). For practical use there is a need for feasible and reliable methods for collecting, evaluating, storing and eventually using gametes for fertilizing embryos, and hopefully establishing and maintaining pregnancy. It is impractical to use wild felids for extensive research because of the scant numbers of individuals, accessibility etcetera. Therefore the domestic cat frequently serves as a model animal for research. This student work focus on evaluating a certain method for measuring DNA fragmentation in cat spermatozoa, Halomax®. We thawed epididymal sperm from 12 cats, one from corpus epididymidis and one from cauda epididymidis for each cat except one. The measurements were compared to results in a PhD-project, showcasing the DNA fragmentation index (%DFI); Acridine orange using both epi-fluorescence microscope and flow cytometry. It resulted in a significant difference in the DFI between spermatozoa from corpus or cauda using Halomax® but no significant correlations between methods were found. The discrepancy between these methods has been noted in earlier studies and one of the theories states that the methods could be measuring different kinds of strand breaks in the DNA. The difference between epididymal regions has also been seen in previous studies, and is believed to be due to the maturing process of spermatozoa or some kind of phagocytosis of defective spermatozoa.

SAMMANFATTNING

Vilda kattdjur världen över hotas av utrotning. Som ett led i arbetet för att bevara dessa genom avelsprojekt i djurparker samt i det vilda, utförs forskning om möjligheterna att använda artificiell fortplantning, i form av till exempel artificiell insemination (AI) och *in vitro* fertilisering (IVF). För att det ska kunna fungera i praktiken behövs enkla och beprövade metoder för att samla in, utvärdera, förvara och sedan använda könsceller för befruktning och förhoppningsvis fortsatt dräktighet. Det är inte genomförbart att använda de vilda kattdjuren för mer omfattande studier, på grund av det låga individantalet, tillgänglighet och liknande. Därför används tamkatter frekvent som modelldjur vid forskning. Målet med studentarbetet var att utvärdera en metod, Halomax®, för att mäta förekomsten av DNA-fragmentering i tinade spermieprov från bitestikeln från 12 tamkatter och jämföra resultatet med de resultat som fåtts vid andra analyser inom ett doktorandprojekt. Resultaten från Halomax® jämfördes med resultaten från mätningar med epi-fluorescens mikroskop och flödescytometri, i båda fallen färgade med akridin orange. DNA fragmenterings index (DFI) jämfördes även mellan bitestikelhuvudet och -svansen hos samma individ. Resultatet blev en signifikant skillnad mellan bitestikelhuvudet och -svansen med Halomax®, men ingen signifikant korrelation mellan metoderna. Att metoderna inte korrelerar med varandra har tidigare observerats inom andra djurslag och en av teorierna som lagts fram är att metoderna mäter olika typer av DNA-skador. Skillnad i DFI hos spermier från de olika regionerna har även tidigare observerats och tros kunna ha samband med spermieutvecklingen alternativt någon form av fagocytering av defekta spermier i bitestikeln.

CONTENT

Introduction

One of the biggest challenges of biologists and veterinarians worldwide is the conservation of wild felids. For the past few decades there has been a focus on artificial reproduction as a tool for preservation of important genetic variation. Some researchers have observed the success in livestock reproduction with the help of assisted reproductive technology (ART), especially artificial insemination (AI), though it cannot be directly translated between species (Wildt *et al.*, 2010). Therein lies the issue, because species specific studies are a condition for progress. The opinion that the development of the technology has slipped away ahead of the practical need has also been raised, but either way there seems to be a value of artificial insemination as a part of conservation in addition to general understanding in species biology and physiology (Wildt *et al.*, 2001). However other researchers have concern about losing the bigger picture while micromanaging the reproduction step (Wildt *et al.*, 2010).

Higher proportion of spermatozoa with abnormal morphology is seen in felids than is usual in other species but there are still no unequivocal definitions of normozoospermia and teratozoospermia in felids. The importance of this ”teratozoospermia” is undefined, since cheetahs with up to 70 % abnormal spermatozoa still sire offspring and cats with a low proportion of normal sperm still produce kittens (Wildt *et al.*, 1983; Axnér and Linde Forsberg, 2007). Fertility is probably determined by the severity of the present abnormalities rather than the total percentage (Pukazhenthi *et al.*, 2001). Even the morphologically normal sperm of tomcats with high percentage abnormal sperm have reduced functional capacity (Penfold *et al.*, 2003). Understanding felid reproduction and development of species specific protocols would not be feasible without the domestic cat as a model (Pukazhenthi *et al.*, 2001).

This study was carried out as a part of a PhD research project with the aim to find out if epididymal sperm from all parts of the epididymidis could be used to perform assisted reproductive technology in wild felids, with the domestic cat as a model animal. The goal in this student work was to evaluate if Halomax® is a sufficiently reliable method to measure the proportion of spermatozoa with DNA fragmentation in a cat sperm sample, and to compare between cauda and corpus epididymidis. There is hope that spermatozoa from the corpus will be sufficiently mature for fertilization and could be used as an addition to caudal sperm. This would give a larger number of spermatozoa from each individual in cases when you only have the testicles, i.e. post castration or from deceased individuals.

Literature

Evaluation of DNA fragmentation in feline sperm

There is a range of different methods for DNA evaluation in sperm. Many of the traditional techniques are time consuming and very expensive, which is the reason for development of new methods with a more clinical applicability. The following described methods are those involved in this study.

Acridine Orange

Acridine orange is a dye used for evaluation with fluorescence microscopy or flow cytometry. Acridine orange (AO) fluorescence green when it intercalates as a monomer into double stranded DNA (ds-DNA) and yellow or red when it binds to single stranded DNA (ss-DNA) as an aggregate (Evenson *et al.*, 1980). Single stranded DNA occurs where there is some kind of strand break.

The technique is to smear the sperm sample onto a glass slide, fixing in Carnoy's solution, then stain with Acridine orange and fix the staining before evaluation with epi-fluorescence microscopy.

Flow cytometry

In flow cytometry the cells are passed one by one through a laser beam. Different cells will reflect the laser in specific ways which is registered in a plot diagram. When applied to sperm DNA fragmentation the use of Acridine orange stain enables differentiation of sperm into those with and without strand breaks (Evenson *et al.*, 1980). The Acridine orange fluorescence green when engaged with ds-DNA and orange or red when engaged with ss-DNA, as mentioned earlier. The flow cytometer then analyzes the fluorescence when sperm cells are passed by the laser and the results are plotted in a diagram, with green on one axis and red/orange/yellow on the other. A higher proportion of spermatozoa with fragmented DNA gives rise to more dispersion in the plot diagram. The benefit with flow cytometry is its ability to analyze thousands of cells per minute. This flow cytometry technique used with specific data software is called sperm chromatin structure assay (SCSA).

Halomax® - Sperm chromatin dispersion test

Sperm chromatin dispersion test is a technique for evaluating DNA quality in spermatozoa, visualizing DNA dispersion in spermatozoa and makes it possible to evaluate with microscopy, light or fluorescence. The Halomax® kit is a variation of the method, meant to be quicker and easier to use in the everyday clinical practice.

Halomax® measures the chromatin stability in spermatozoa. The kit itself contains the reagents needed to denature and mount the sample. Stain is not included in the kit, but

the mounted sample can be stained with either fluorescence stain or stain for light microscope. Several studies used Wright's stain for light microscopy (Enciso *et al.*, 2006; García-Macías *et al.*, 2006; Fraser *et al.*, 2010; Vernocchi *et al.*, 2014). The correlation between the results from Halomax® using fluorescence microscopy and light microscopy is good, but fluorescence shows more detail at evaluation (Garcías-Macía *et al.*, 2006).

Other species

The Halomax® kit comes in species specific packages. A cat-specific kit was developed only recently and hence the majority of the studies with this test have been executed in other species than felids. In humans chromatin dispersion test is a validated technique for differentiating between fertile and infertile sperm samples, with high correlation to other methods (Ribas-Maynou *et al.*, 2013). In this study they used another kit, Halosperm®, the human variation of Halomax® from the same manufacturer.

Species specific Halomax® has been used in mice to evaluate differences between epididymal spermatozoa from caput versus cauda (Pérez-Cerezales *et al.*, 2011). They also calculated the correlation between Halomax® and the other available methods in use, i.e. neutral comet assay, terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) and SCSA. There was no detectable difference between the regions when evaluated with Halomax®. The terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) did not show any differences between the regions either, whereas both neutral comet assay and sperm chromatin structure assay (SCSA) showed a significant difference between caput and cauda. Only SCSA and neutral comet assay were correlated to each other. The author presents two theories about this. The first is that Halomax® and TUNEL have a lower sensitivity for damaged DNA. The second states the different methods measure different kinds of damage and therefore show different results according to type of damage.

The non-correlation between SCSA and Halomax® was found in a second study as well (Garcías-Macía *et al.*, 2006). In this study the Halomax® kit also showed a significantly lower incidence of DNA fragmentation in the high fertility group when correlated to pregnancy rates, indicating the kits' relevance for predicting fertility in bulls. Yet another study contradicts the results from the mouse study, finding a correlation between results from neutral comet assay and Halomax® (Fraser *et al.*, 2010) using ejaculated boar semen.

The instructions for Halomax® claims that there are two different types of spermatozoa to look for, one with big halo (dispersed DNA) and one with small halo (non-dispersed DNA). But studies in boar sperm have shown four differentiated types of sperm staining patterns using species specific Halomax®, interpreted as

representing different stages of chromatin dispersion (Enciso *et al.*, 2006; Alkmin *et al.*, 2013), and at least six different stages in the koala (Zee *et al.*, 2009). García-Macías *et al.* (2006), on the other hand, only identified the original two types of sperm when evaluating bull semen.

The cat

As stated earlier, until very recently, there was no commercial Halomax® kit aimed for feline species. To my knowledge, there are no studies evaluating Felis-halomax®. But the use of Halomax® for canines has been tested on domestic cat epididymal sperm samples (Vernocchi *et al.*, 2014). No significant difference between the results derived from Halomax® or TUNEL was detected. This indicates Halomax® could be a good method for evaluating DNA fragmentation in cat spermatozoa. There were only two chromatin patterns discriminated in this study, small and big halo (non-dispersed and dispersed DNA, respectively).

Sperm development in epididymidis

In the average cell the genome is packed around histones, binding it together. During the sperm passage through the epididymis the histones are exchanged with protamines, which means the proportion of histones in spermatozoa are lower in the cauda epididymidis in comparison with the caput (Hingst *et al.*, 1995). High presence of histones in the cauda epididymidis has been linked to incidence of morphological abnormalities. During the epididymal transit the proportion of morphologically abnormal sperm decline, except tail defects, which are amplified slightly (Axnér *et al.*, 1999; Sringam *et al.*, 2011). The spermatozoa contain less ss-DNA and more ds-DNA in the cauda, compared to in the caput (Sringam *et al,*. 2011), and a significant difference in DFI found between region 2 and 3 (both located in the caput) indicating an early process. The highest proportion of mature chromatin structure was found in corpus and cauda compared to the regions located in caput epididymidis, and Sringam *et al.* (2011) found no significant difference between corpus and cauda (region 5 and 6). The theories presented on this subject state either a maturation process during passage, phagocytosis of defect spermatozoa, or a combination of the two processes.

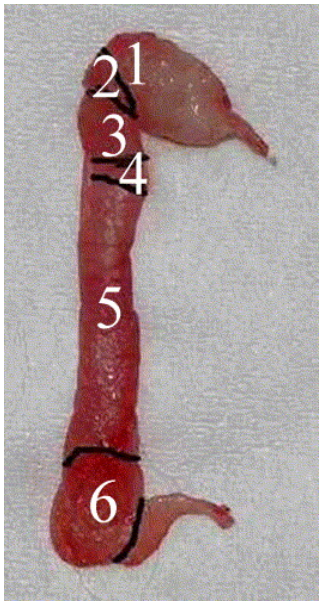

Fig 1. *The regions of the feline epididymis. 1-4 is situated in the caput, 5 correspond to corpus and 6 to cauda. (Axnér, 2006)*

DNA fragmentation, sperm quality and fertility

Semen has different traits, uncompensable and compensable ones. Traits where sperm dosage does not affect the embryogenesis postfertilization, i.e. higher dose does not improve the result (Saacke *et al.*, 2000). DNA fragmentation is considered to be an uncompensable semen trait which means when there is a fault in the DNA the embryo development after fertilization will be affected. Viability and morphology are compensable semen traits, and fertilization is improved with higher sperm dosage, whilst DNA damage leads to loss of the embryo often before maternal recognition of pregnancy even with morpholocally normal sperm with good viability.

A study in human showed significant correlation between morphology and DFI but not strong enough to rule out DNA fragmentation as a cause of infertility in normozoospermic males (Larson-Cook *et al.*, 2003). The same study showed a significant relationship between DFI and implantation rate. They concluded that the threshold value for DFI is when the oocytes capacity for DNA repair is exceeded, and then the embryo development will fail. In cats Vernocchi *et al.* (2014) distinguished a correlation between DFI and morphometry, which is a computerised alternative to subjective morphology evaluation.

High DFI using Halomax® predicts low fertility in bulls (Karoui *et al.*, 2012). When inbreeding increases, the DFI increases.

Earlier it has also been shown that there is a relationship between low fertility in bulls and low resistance against heat denaturation of DNA using flow cytometry (Evenson *et al.*, 1980), and the fertility was inversely correlated to the resistance. This is a measurement of how intact the DNA structure is.

Penfold *et al.* (2003) concluded that morphologically good spermatozoa from three tomcats with high proportion of abnormal sperm had a higher DFI than spermatozoa from three donors with low proportion abnormal spermatozoa (<40%). When used for *in vitro* fertilization (IVF) sperm from cats with low proportion of abnormalities resulted in significantly more fertilizations but the percentage of each group developing to morula was similar. Intracytoplasmic sperm injection did not improve the result for the males with high proportion of abnormal spermatozoa and DFI, but decreased the success for the low DFI group.

Cryopreservation and sperm quality

Cryopreservation is a preservation method for preserving sperm samples (or oocytes) in liquid nitrogen, to prolong the viability. There has been a study showing a high frequency of post-thaw acrosomal defects in both electroejaculated and epididymal sperm from tomcats, which is known to cause decreased fertilization rates (Tebet *et al.*, 2006). A previous study in cats has shown 9-15 % DNA fragmentation in frozen-thawed semen, from ejaculated samples (Vick *et al.*, 2012).

There have been some studies investigating the post-thaw pregnancy rate in cat ejaculated and epididymal sperm (Tsutsui *et al.* 2000b; 2003). They used intrauterine insemination and got a pregnancy rate of 57.1% and 27.3% respectively, even though there was no significant difference between the parameters of motility and semen volume between the groups. As a comparison, the same team has determined a conception rate of 80.0% using fresh ejaculated semen under the same circumstances (Tsutsui *et al.*, 2000a), and Tanaka *et al.* (2000) produced a 77.8% pregnancy rate with intravaginal insemination of fresh semen. Chatdarong *et al.* (2007) explored the noninvasive AI techniques with both fresh and frozen-thawed sperm. Intravaginal insemination with fresh semen resulted in 28.7% pregnancy rate in queens, and 0 % pregnancy rate with frozen-thawed sperm. The pregnancy rate with frozen-thawed sperm was improved when intrauterine insemination (transcervical) was used, 41.7 % of inseminated queens became pregnant.

Materials and methods

Animals

The study included 12 cats subjected to routine castration in the vicinity of Uppsala. Of these cats ten were claimed to be House Cats, one was a Sacred Birman and in one case there was no breed reported. Mean age was 14 months, median 12 months.

Sperm samples

The sperm samples used in this study were frozen earlier in the PhD project. Sperm from two different regions of epididymis, corpus and cauda, were frozen. Sperm recovery was done within 24 hours after the castration. Briefly, the epididymides were dissected free from visible blood vessels and connective tissue. The tissue were cut into small pieces and placed in warm phosphate buffer solution (PBS) in a plastic tube. Then the tubes were incubated in 37.0 °C for 10 minutes. The tissue was removed from the sample. The spermatozoa were checked for motility and progressive motility by using CASA (Computer Assisted Sperm Analyzer, Sperm VisionTM PRISM version 3.5 software, Minitube of America, Verona, United States), and the DNA integrity was evaluated using Acridine orange (Polyscience, Inc. Warrinton, PA, USA) with flow cytometry and epi-fluorescence microscopy.

Epididymal samples from corpus and cauda had been used for freezing. The samples were mixed with an egg yolk containing extender and frozen in straws in liquid nitrogen, according to routine methods developed at SLU (Swedish University of Agricultural Sciences).

Sperm chromatin dispersion test – Halomax®

The instructions included in the kit by the manufacturer were followed. In short the sperm samples were thawed in 37.0°C water bath for 30 seconds then placed in 1.5 ml tubes and kept at 37.0°C. The agarose setting gel was then melted in 100°C water bath for 5 minutes, after that it was placed in a 37.0°C water bath to equilibrate the temperature. Sperm samples from 4 individuals were prepared at the same time. Aliquots of 25 µl from each sperm sample were mixed with agarose in tubes. The slide was placed on a heated stage before application of samples, to facilitate application of all 4 samples before the gel set. The cell suspension was placed on a pre-warmed slide and all samples were duplicated in 2 wells each. A coverslip was mounted on the slides. After this step the slide was placed in horizontal position on a cooled metal plate and put into the fridge at 4°C for 5 minutes, in some cases this time had to be extended to enable sufficient setting of the agarose.

Then the slide was taken out of the fridge and the coverslip carefully removed. The slide was put on an elevated surface in a Petri dish and was covered with the lysis solution for 5 minutes. Thereafter the slide was washed with distilled water for 5 minutes, and at last dried with first 70% and second 100% ethanol for 2 minutes respectively.

Sample staining

One sperm sample was used to test different alternatives for staining. As mentioned earlier, other studies have used Wright staining for evaluating DNA fragmentation index (DFI) with Halomax®. We tried both SYBR-14 and Giemsa stain for fluorescence and light microscope respectively. SYBR-14 turned out to be hard to evaluate compared to Giemsa and light microscope. So we decided to use Giemsa staining for this study. The samples were stained with a 1:1 Giemsa:PBS solution, which was freshly prepared every day.

Staining time

In the earlier work on cat sperm, the samples were stained with Wrights solution for 35 minutes (Vernocchi *et al.*, 2014), therefore this time was used as a starting point. When it was tried, the staining was insufficient. Therefore staining time was prolonged to 60 minutes which resulted in a better staining. During the progress of the work the staining time was changed a couple of times due to inconsistency in staining intensity and background stain disturbing the evaluations. In the end the majority of samples were stained around 45 minutes.

At least 300 spermatozoa per sample were counted, and DNA fragmentation index (DFI) was calculated as the percentage fragmented spermatozoa of the number of counted spermatozoa.

Other analyses

Straws from all samples had been evaluated post-thaw previously after Acridine orange staining with both flow cytometry and counting manually in an epi-fluoresence microscope.

Statistics

The proportion of spermatozoa with fragmented DNA in the corpus and cauda epididymidis was compared with a paired t-test after checking normal distribution of differences with the Ryan-Joiner's test. Pearson's correlations between the different methods were calculated after checking normal distribution of the data. Spearman's rank correlation was used to evaluate the relation between fragmented DNA evaluated

with Halomax® in the corpus and in the cauda epididymidis. All calculations were performed in Minitab® 16.2.4.0 (2013 Minitab Inc).

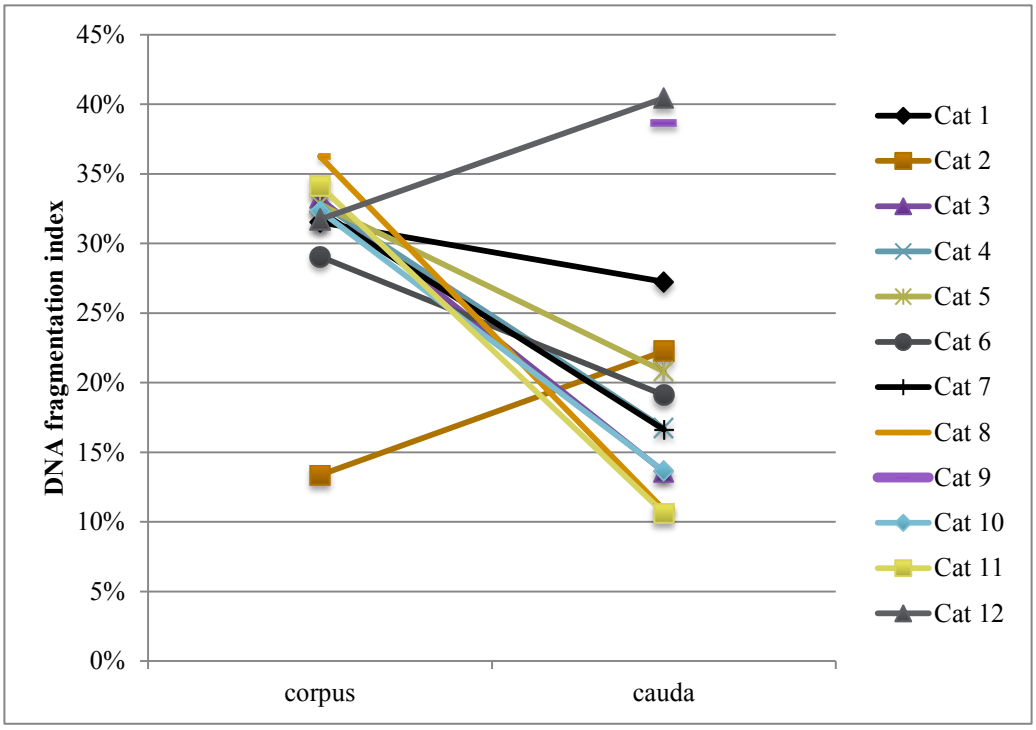

Fig 2. *Showing the change of each individual's DFI between regions.*

Results

DNA fragmentation index was calculated for both corpus and cauda for all 12 cats, except cat 9. Most cats had a lower DFI in cauda than in corpus (Figure 2).

With Halomax® there was a significant difference between DFI between the corpus and cauda epididymidis using paired t-test and 95 % confidence interval. The mean values and standard error of mean for DFI in cauda were 20.88 % ± 2.87 and 30.88 % ± 1.83 for corpus. Figure 3 shows the sample distribution in the two regions.

There was no significant difference between corpus and cauda either in flow cytometry analyses or with epi-fluorescence microscopy. Mean value and standard error of mean for DFI of the flow cytometry was 1.79 % ± 0.26 for corpus and 1.27 % ± 0.17 for cauda. The results from epi-fluorescence were 19.63 % ± 3.27 and 15.71 % ± 2.87 for

corpus and cauda respectively (see figure 5). There was no significant difference between the regions with either of these methods.

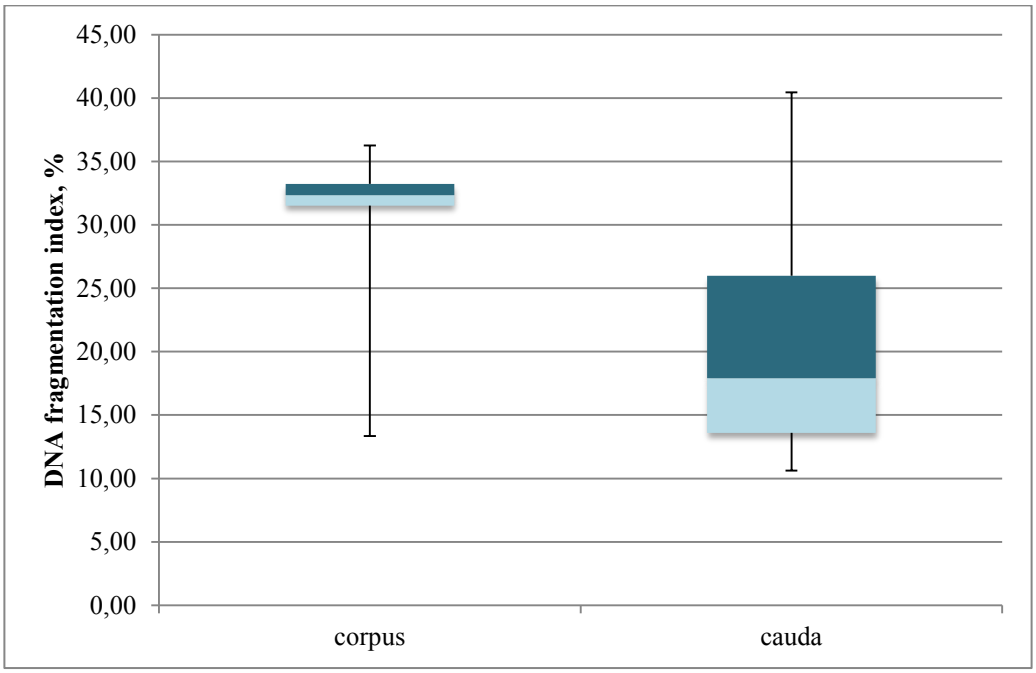

Fig 3. *Box-and-whiskers plot of DFI in corpus and cauda epididymidis using Halomax®.*

We found no significant correlation between the results from Halomax® and the results from Acridine orange using epi-fluorescent microscopy or flow cytometry, with Pearson correlation. There was a significant correlation between the regions stained with Halomax®, calculated using Spearman's correlation ($r=-0.772$, $p = 0.005$).

When evaluating the samples stained with Giemsa in the Halomax® kit in light microscope we could identify five different patterns of chromatin dispersion (some of them represented in figure 4). There were spermatozoa with no halo, a small halo or a bigger but condensed halo, all three types had normal sized heads. There were also spermatozoa with big, visually dispersed halo with a small or absent head. The last type of pattern was spermatozoa with big "bubbly" head and varying degrees of dispersed chromatin. The first three types, with normal head size, were counted as normal, while the others were counted as having dispersed chromatin. A criterion to be counted as a spermatozoon was the presence of a tail. In some samples there were detached tails without a sperm head and those were not counted.

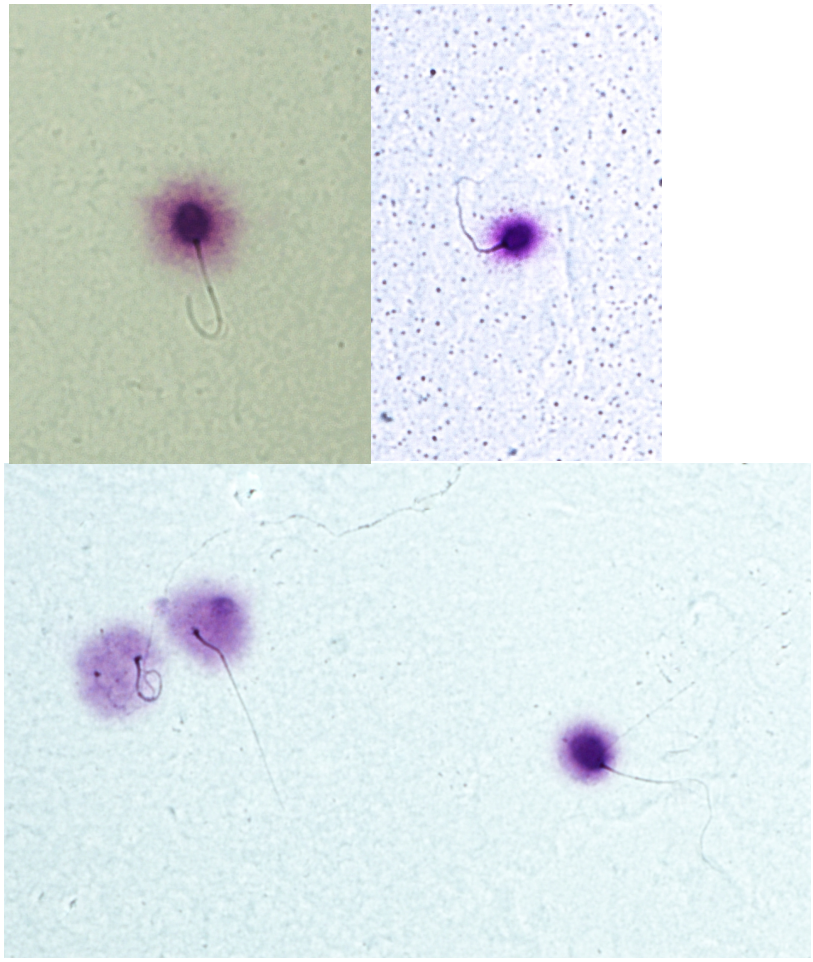

Fig 4. *Upper left, spermatozoon with normal head and halo. Upper right, spermatozoon with normal head and small halo. Lower, two spermatozoa with dispersed chromatin on the left and one normal spermatozoon at the right.*

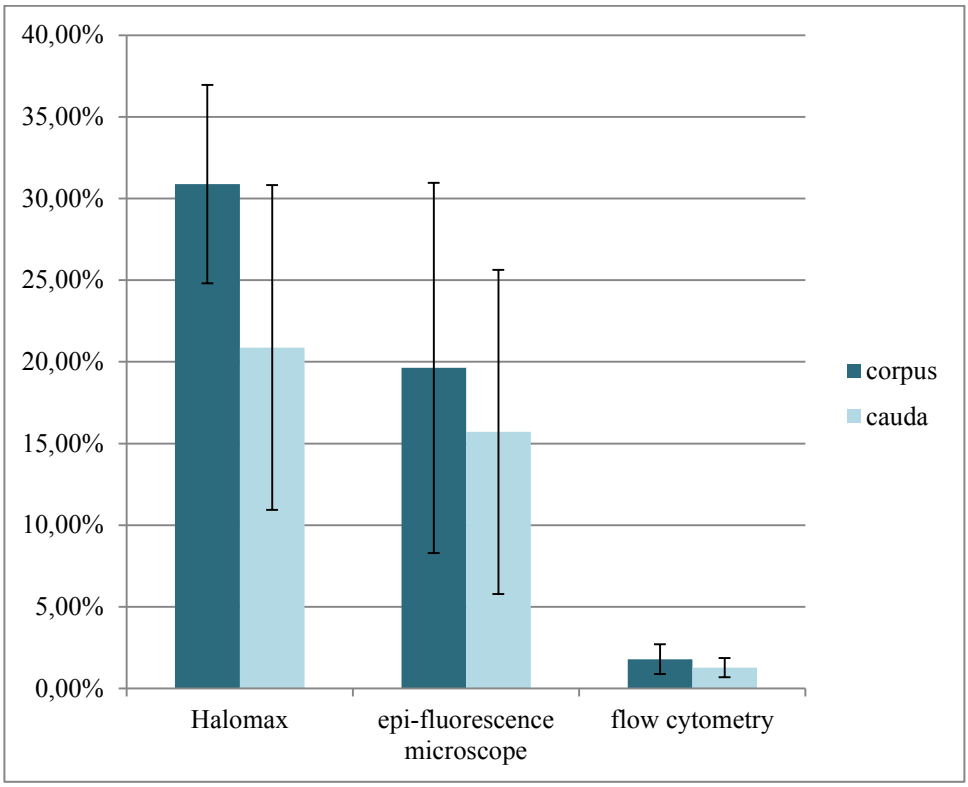

Fig 5. *DNA fragmentation index (DFI) in the different regions, results from the three methods including standard deviations.*

Discussion

The results in this study showed higher sperm DFI than earlier studies in cats, but lower than for the "teratozoospermic" males in Penfold *et al.* (2003). The DFI measured in cat spermatozoa with Halomax® in our study did not correlate to the two other used methods for measuring DFI. The significant difference in DFI between corpus and cauda has not been shown in cats before, although Hingst *et al.* (1995) showed a tendency for a difference between spermatozoa from corpus and cauda but no significant difference. When comparing sperm from caput and cauda epididymidis Hingst *et al.* (1995) observed a significant difference between in the DFI. It could point towards a maturation process early in the epididymidis, in compliance with the results from Sringam *et al.* (2011). In a mouse study some methods showed significant difference between sperm from caput and cauda, not using corpus in the testing (Pérez-Cerezales *et al.*, 2011). If the maturation process of the DNA structure takes place early, mainly in the caput, there would be a chance of corpus spermatozoa working in ART. The results from Acridine orange and flow cytometry point towards this in our study, but with Halomax® there was a significant difference in DFI.

The difference between DNA fragmentations in different regions of the epididymis in male cats has been noticed in earlier studies (Hingst *et al.*, 1995; Sringam *et al.*, 2011). Sringam *et al.* (2011) investigated frozen-thawed sperm from different regions of the epididymis with flow cytometry using the Acridine orange dye. They found a significant difference between spermatozoa from the proximal part of caput (region 1) and distal part of caput epididymidis (region 3), 72.9% and 94.4% sperm with intact DNA, respectively. In corpus (region 5) and cauda (region 6) the same measurements were 99.5% and 99.2% respectively, and no significant difference. A concern was the difficulty to exactly distinguish between regions macroscopically and therefore risking spermatozoa from other regions being included.

Hingst *et al.* (1995) concluded that there was a significant difference in chromatin stability between caput and cauda in cats, 51.1% and 86.5% respectively. Evaluation was made using Acridine orange. The exact number for corpus is not displayed in the article but a figure shows a value around 77%. Presumably there was no significant difference between corpus and cauda. The experiment used fresh spermatozoa.

Other previous studies on epididymal spermatozoa have shown DNA fragmentation results similar to those concluded by Hingst *et al.* (1995). Vernocchi *et al.* (2014) used epididymal spermatozoa from the whole epididymis and got a lower DFI than others, 4.34 % ± 0.93. Whereas Mota and Ramalho-Santos (2006) observed 13 % ± 12 DFI also using the whole epididymidis from tomcats. They used Diff-Quick, which is a method for chromatin staining in sperm, as is Halomax®. Thuwanut *et al.* (2008) obtained the result 13.3 % DFI using only the cauda epididymidis from eight Swedish tomcats. In our study Halomax generaly pointed towards a higher presence of DFI, 20.88 % ± 2.87 DFI in cauda epididymis compared to Thuwanut *et al.* (2008). The flow cytometry result in this study closely resembles those of Sringam *et al.* (2011). The epi-fluorescence microscopy results in our study of the spermatozoa in cauda were 15.71 % ± 2.87 DFI in cauda comes close to Hingst *et al.* (1995) and Thuwanut *et al.* (2008) who also used the Acridine orange method.

The only previous cat study on Halomax® used TUNEL assay for comparison and found that the results were correlated (Vernocchi *et al.,* 2014). This correlation was also found in Pérez-Cerezales *et al.* (2011) mouse study. A study in human patients showed correlation between flow cytometry with Acridine orange and the TUNEL assay (Hamidi *et al.*, 2014). This may lead to the conclusion that flow cytometry with Acridine orange and Halomax® results could be correlated, but that is not the case in our study, nor in others (Garcías-Macía *et al.*, 2006; Pérez-Cerezales *et al.*, 2011).

A study in humans showed no correlation between Halomax® and Acridine orange using flow cytometry (Ribas-Maynou *et al.*, 2013). This is in line with our present work. No correlation have been found between Halomax® and SCSA in earlier animal studies (Garcías-Macía *et al.*, 2006; Pérez-Cerezales *et al.*, 2011, bull and mouse

sperm, respectively). However Garcías-Macía *et al.* (2006) correlated their results to fertility in bulls.

There have been theories of different methods targeting different kinds of DNA damage (Pérez-Cerezales *et al.*, 2011), and it would explain the fail to correlate the methods with each other. The idea of different methods measuring different changes in DNA could be further investigated. Because there has been correlation between DFI results from Halomax® and fertility in bulls (Garcías-Macía *et al.,* 2006; Karoui *et al.*, 2012) it would be interesting with future investigation of the correlation between Halomax® results and fertility or IVF success in cats.

Kapuscinski *et al.* (1983) realized the equilibrium needed between Acridine orange dye and the phosphate group in damaged DNA for a correct result was precise. Evenson and Wixon (2006) concluded that this could mean that the Acridine orange test is not suitable for staining on a glass slide because it is not entirely flat on a molecular level. The dye will pool in some regions and therefore skew the results. Villaverde *et al.* (2013) used Acridine orange and epi-fluorescence microscopy to assess DNA integrity in fresh and frozen thawed ejaculated cat semen. The result was approximately 97 % spermatozoa with good DNA integrity in the fresh sample, and 92-95 % in frozen samples.

Penfold *et al.* (2003) tried to define groups of DFI in relation to proportion of morphologically abnormal sperm, classifying tomcats with >60 % morphologically abnormal sperm as teratozoospermic and tomcats with <40 % abnormal sperm as normozoospermic. The results were 48.5 % ± 6.0 and 13.8 % ± 2.4 respectively, for thawed ejaculated sperm. The males counted as teratospermic had higher DFI than both regions in our study using Halomax®. Penfold *et al.* (2003) showed a lower fertilization rate with these ”teratospermic” males, as mentioned earlier.

Because there is no set DFI level for fertility in felids, more research is needed. We do not know if DFI 30.88 % ± 1.83 is too much, or if it could be sufficient for fertilization. Penfold *et al.* (2006) showed a reduced fertilization rate in a very small test group where mean DFI was 48.5 % ± 6.0. But they were not infertile, considering there was embryo development when tested in IVF. The testing was not carried out *in vivo* and not as a fullterm pregnancy. In humans the threshold has been tested to be around 27 % DFI. Over 27 % DFI predicts low fertility (Larson-Cook *et al.*, 2003). In bulls much lower threshold values has been reported, Karoui *et al.* (2012) concluded a threshold of 7-10 % for predicting low fertility in bull ejaculates. A high proportion of morphological abnormalities is related to higher DFI in cats, and even spermatozoa with normal appearance from these males has lower fertilization rate *in vitro*, individuals with high prevalence of these morphological abnormalities could still be fertile and produce kittens (Penfold *et al.*, 2003; Axnér and Linde Forsberg, 2007).

If SCSA should be used as a reference and "gold standard" for measuring DFI in cat spermatozoa, because of the good statistical material, with many counted sperm per sample, then Halomax® is not the best method. However, in other species, where Halomax® did not correlate with SCSA, Halomax® still predicts high fertility (Garcías-Macía *et al.*, 2006). So Halomax® might be correlated to fertility and success of ART in felids too, even if it is not correlated to the two other methods in our study. Garcías-Macía *et al.* (2006) also observed that SCSA and Halomax® could be used as complements to optimize the evaluation to get an accurate result on DNA and chromatin structure status. Halomax® is a subjective method, and the definition of chromatin patterns has proven to be not as easy as in the instructions.

The range of patients used for sampling is narrow in age and geography, so this might not be representative for the Swedish male cat population. The main goal was to test the Halomax® against the more commonly used DFI methods (Acridine orange in flow cytometry and epi-fluorescence microscopy), and the cat itself was its own control. However, we could not make indisputable assumptions about the population, concerning the significant difference between DNA fragmentation in corpus and cauda. The animals in this study are young.

When we decided which staining protocol to use we tried fluorescence staining a couple of times with the same sample. Every time it was difficult to distinguish good spermatozoa from fragmented spermatozoa. The benefit with fluorescence though, was that the background staining from coloured egg yolk from the extender was minimised compared with the light microscopy stain. A couple of weeks after the pre-study staining test the fluorescence microscope had to be calibrated because of trouble with distinguishing different colours when using Acridine orange. This may have affected the stain testing we had done.

Lastly, in the future, it would be interesting to compare Halomax® before and after freezing and thawing, to see if the results differ, and to correlate the results to success of IVF or pregnancy.

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