Animal Reproduction Science 144 (2014) 54–58

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Flow cytometric evaluation of sperm apoptosis in semen of silver foxes in the breeding period

K. Kostro[a,*], L. Krakowski[b], U. Lisiecka[a], A. Jakubczak[c], A. Żmuda[a], P. Wojtaszczyk[a], A. Wąchocka[b]

[a] Department of Epizootiology and Clinic of Infectious Diseases, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Głęboka 30, 20-612 Lublin, Poland
[b] Department of Andrology and Biotechnology of Reproduction, Chair and Department of Animal Reproduction, Faculty of Veterinary Medicine, University of Life Sciences in Lublin, Głęboka 30, 20-612 Lublin, Poland
[c] Department of Biological Bases of Animal Production, Faculty of Biology and Animal Breeding, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland

ARTICLE INFO

Article history:
Received 6 September 2012
Received in revised form
27 September 2013
Accepted 16 October 2013
Available online 26 October 2013

Keywords:
Silver fox
Semen
Reproduction
Apoptosis
Necrosis
Flow cytometry

ABSTRACT

The objective of the study was to evaluate cytometrically the percentage of apoptotic and necrotic spermatozoa in fresh semen of silver foxes in the breeding season. In males F3 and F4 with high percentages of early apoptotic (A+Pi−), late apoptotic (A+Pi+) and necrotic (A−Pi+) spermatozoa as well as 56–65% of living spermatozoa (A−Pi−) with progressive motility, the semen was characterised by reduced fertility. In males F1 and F2 with spermatozoa showing the motility and viability of 89–90% and high percentages of living cells that do not bind Annexin V and propidium iodide, the semen was assessed as valuable and useful for artificial insemination. Amongst 16 females of group I and II inseminated with semen from F1 and F2 males, 15 (93.75%) had multi-cub litters – on average 6.1 and 4.8, respectively. In contrast, amongst 16 females of group III and IV inseminated with semen from F3 and F4 males, only 10 (62.5%) had litters with few cubs (on average 2.6 in group III and 2.1 in group IV). Our findings explicitly indicate that semen of farm male foxes should be evaluated before the breeding season, as one of the causes of reproduction failures is likely to be a high percentage of apoptotic and necrotic spermatozoa. Thanks to flow cytometry, fresh ejaculates can be speedily evaluated and their usefulness for artificial insemination determined.

1. Introduction

Reproduction disorders of farm-raised foxes depend on many factors, including genetic conditions, female age, impaired hormonal metabolism, dietary errors (Gliński and Kostro, 2002), mineral–vitamin deficiencies (Cybulski et al., 2009b), heavy metal intoxications (Cybulski et al., 2009a), poor zoo-sanitary conditions, bacterial or viral infections (Kostro et al., 1997; Wawrzkiewicz et al., 1997), and parasite invasions (Gliński and Kostro, 2002). Moreover, improper organisation of reproduction and resultant low rates of effective mating are also relevant (Farstad, 1998; Gliński and Kostro, 2002). Artificial insemination commonly used for reproduction of farm-raised foxes, and the effectiveness is primarily dependent upon proper detection of oestrus and ovulation in females and evaluation of male fertility, i.e. sperm quality. Semen of good quality should be characterised by a high percentage of living spermatozoa with progressive motility and the lowest percentage of morphologically changed sperm cells (Gliński and Kostro, 2002; Janicki et al., 2006a,b). The standard examinations of ejaculates are often simplified and limited to evaluation of

* Corresponding author. Tel.: +48 81 4456201; fax: +48 081 445 62 01.
E-mail address: kkostro@wp.pl (K. Kostro).

0378-4320/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.anireprosci.2013.10.006

viability and concentration of spermatozoa. According to many authors, the general evaluation of sperm only indirectly discloses its functional ability to fertilise the egg cell. At present, however, it is considered a minimum requirement to make decisions on the usefulness of ejaculates for artificial insemination (Farstad, 2000; Gliński and Kostro, 2002).

Much attention has been paid to sperm apoptosis and necrosis. The processes are associated with maturation of spermatozoa and occur in all stages of development, i.e. in spermatogonia, spermatocytes and spermatids (Anzar et al., 2002). The significant sperm changes include nuclear chromatin integration and DNA fragmentation. Many recent studies have demonstrated the clinical importance of evaluation of DNA integrity and apoptosis in ejaculated and cryopreserved spermatozoa (Boue et al., 2000; Gillan et al., 2005). Any DNA damage may cause morphological changes in spermatozoa leading to reduced survival or embryonic genetic defects, which results in cell death and resorption during early pregnancy (Oosterhuis and Vermes, 2004; Rodriguez-Marinez et al., 2009). This aspect of male fertility is underestimated in reproduction of farm-raised foxes.

The aim of the study was to determine the percentage of apoptotic spermatozoa in fresh semen of silver foxes in the breeding period using flow cytometry, and to assess sperm effectiveness for artificial insemination of females.

2. Material and methods

2.1. Animals

The study was carried out in a fox farm of eastern Poland with the herd consisting of 250 female and 20 male silver foxes at the turn of January and February 2011. In this herd, artificial insemination had been applied for 8 years. During the study period, it was found that despite macroscopic and microscopic evaluation of ejaculates collected from some male foxes, the females undergoing artificial insemination did not become pregnant or their litters were not numerous. The farm sanitary conditions were assessed as good. All foxes of the basic herd were kept in the same conditions and received identical food, according to the recommended standards for the breeding period; moreover, mineral–vitamin additives and continuous access to water were provided. In the second half of November, the basic herd foxes received antiparasitic agents and were vaccinated against distemper and infectious encephalitis.

The initial examinations comprised 32 females and 8 males, weighing 10–12 kg and aged 2 years; 4 males were selected after initial semen examinations. The experimental female groups included females, which in the previous breeding period had given birth and brought up multi-cub litters. The experimental males, which without any difficulties gave ejaculates, were from earlier multi-cub litters and their reproductive systems were anatomically normal. Before the oestrus cycle, females were divided into 4 experimental groups, 8 individuals each (group I, II, III, IV). Groups I and II contained females artificially inseminated with ejaculates from 2 males, F1 (group I) and F2 (group II), whose evaluation of sperm apoptosis with Annexin V demonstrated a high percentage of living spermatozoa (A−Pi−) and a low percentage of early apoptotic (A+Pi−), late apoptotic (A+Pi+) and necrotic (A−Pi+) spermatozoa. Groups III and IV included females artificially inseminated with ejaculates from another two males, F3 (group III) and F4 (group IV) with a high percentage of early apoptotic (A+Pi−) and late apoptotic (A+Pi+) spermatozoa as well as a low percentage of living spermatozoa (A−Pi−). After artificial insemination, females were under clinical observation throughout the pregnancy; special attention was paid to the number of mothers with cubs and the number of cubs in a litter.

2.2. Macro- and microscopic evaluation of semen

The semen was collected from eight male silver foxes by masturbation at room temperature (20–25 °C). The collected ejaculates were macro- and microscopically examined by assessing their volume as well as concentration, motility and morphology of sperm. The sperm concentration was assessed photometrically (SpermaCue® – GmbH, Tiefenbach, Germany) using a calibration cuvette for canine semen. The sperm motility and the percentage of living spermatozoa were analysed using a computer assisted sperm analyser (SCA) (MICROPTIC S.L., Barcelona, Spain).

The morphological tests were performed using the Diff Quik® kit (Sigma–Aldrich, Vienna, Austria) which is based on a modification of the Wright Giemsa stain and is commonly used in histological staining to rapidly stain and differentiate a variety of smears. The percentage of living/dead spermatozoa was determined by staining with eosin and nigrosin. After macro- and microscopic evaluation, the ejaculates were diluted with boar semen extender – the BTS extender (Minitüb Abfüll-und Laboretechnik GmbH, Tiefenbach, Germany) to preserve semen fertilising ability. BTS extender extends semen viability for up to 3 days. The final sperm concentration in the insemination dose was 120×10^6/ml.

2.3. Flow cytometric evaluation of semen

The integrity of sperm cell membrane in fresh undiluted semen was assessed using the Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen 556547). Semen samples were washed twice in the saline buffer – HEPES (0.15 M NaCl, 0.01 M Hepes/NaOH, pH 7.0) and centrifuged at $500 \times g$ at 25 °C for 10 min. The obtained spermatozoa were re-suspended in the Annexin V Binding Buffer (0.01 M Hepes/NaOH, pH 7.2, 0.15 M NaCl, 2.5 mM $CaCl_2$) at room temperature to a concentration of 2×10^6 spermatozoa/ml of solution. One hundred microliters of fresh diluted semen was used for the determinations, to which 5 μl of Annexin V-FITC was added followed by 5 μl of propidium iodide, 50 μg/ml. The samples were gently mixed and the entire reaction mixture incubated at room temperature in the dark for 15 min. After incubation, 500 μl of the Binding Buffer solution was added to each sample and determinations were carried out in a flow cytometer (Epics XL, Beckman-Coulter, FL, USA). The control samples were prepared in the same way yet without Annexin V-FITC and