Theriogenology 97 (2017) 148–153

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Detection of macrophages in rabbit semen and their relationship with semen quality

Lenka Kuželová [a, *], Jaromír Vašíček [a, b], Ján Rafay [d], Peter Chrenek [b, c]

[a] AgroBioTech Research Centre, Slovak University of Agriculture, Tr. A. Hlinku 2, 949 76, Nitra, Slovak Republic
[b] Institute of Farm Animal Genetics and Reproduction, Research Institute for Animal Production in Nitra, National Agricultural and Food Centre, Lužianky, Slovak Republic
[c] Department of Biochemistry and Biotechnology, Faculty of Biotechnology and Food Science, Slovak University of Agriculture, Tr. A. Hlinku 2, 949 01, Nitra, Slovak Republic
[d] University of Ss. Cyril and Methodius, Nám. J. Herdu 2, 917 01, Trnava, Slovak Republic

ARTICLE INFO

Article history:
Received 17 October 2016
Received in revised form
19 April 2017
Accepted 22 April 2017
Available online 26 April 2017

Keywords:
Macrophages
Rabbit
Spermatozoa
Flow cytometry
Fertility

ABSTRACT

We aimed at the evaluating the occurrence of macrophages in rabbit semen and finding possible relationship between macrophage concentration and spermatozoa quality. The concentration of macrophages in semen samples from broiler rabbit males of lines M91 and P91 (n = 30) without overt evidence of genital tract infections was determined using monocyte/macrophage lineage antigen CD14 and flow cytometry. Then the rabbits were assigned into three groups according to the macrophage concentration in semen (MΦ1 group with less than 1×10^6 macrophages/mL, MΦ2 group with $1.5–3.5 \times 10^6$ macrophages/mL and MΦ3 group with more than 8×10^6 macrophages/mL). Spermatozoa viability parameters such as occurrence of apoptotic (Yo-Pro-1) and dead/necrotic (propidium iodide) spermatozoa and plasma membrane integrity (PNA-Fluos) were evaluated using flow cytometry. Sperm motility parameters were determined by CASA (Computer Assisted Semen Analysis). Ultrastructural detection of macrophages was performed using transmission electron microscopy. Spermatozoa fertility potential was examined after intravaginal artificial insemination of rabbit doses. Significantly higher proportions of the apoptotic and necrotic spermatozoa and spermatozoa with lower plasma membrane integrity were revealed in the MΦ3 group compared to MΦ1 and MΦ2 groups. The percentage value of total motility and progressive movement was significantly highest in the MΦ1 group, whilst lowest in the MΦ3 group. The conception rate and the kindling rate were significantly decreased in the group with the highest macrophage concentration (MΦ3). Based on our results we can conclude that the abundance of seminal macrophages in the rabbit semen may be closely associated with poor spermatozoa quality.

1. Introduction

Macrophages (MΦ) originate from bone marrow stem cells by differentiating into monoblasts, pro-monocytes and monocytes. Monocytes circulate in the bloodstream for a short time (1–3 days), after which they die or emigrate into organs and tissues where differentiation into MΦ takes place [1,2]. Macrophages are considered as the first line of immunological defence and account for 20%–30% of seminal leukocytes in man [3,4]. Semen MΦ has been reported to occur widely in various groups of vertebrates, e.g. mammals [5,6], teleosts [7] and reptiles [8]. In mammals, spermiophagy takes place in various portions of the male reproductive tract, such as the seminiferous tubules [9], rete testis, efferent duct [9,10], ampulla of the vas deferens [5], seminal vesicle [11] and ejaculatory duct [6]. Macrophages are known to perform essential roles in a number of physiological processes including lipid metabolism, wound healing, scavenging, and host defence against microorganisms and neoplasms, but the relationship between macrophages and the fertilizing potential of semen remains highly controversial. The macrophages are capable of engulfing numerous spermatozoa and such phagocytosis could represent a process for removal of ageing spermatozoa [12]. Engulfment of dying cells prevents injury due to a burst of toxic products and enables apoptosis to operate without pro-inflammatory responses [13].

* Corresponding author.
E-mail addresses: lenka.kuzelova@uniag.sk (L. Kuželová), vasicek@is.uniag.sk (J. Vašíček), jan.rafay@ucm.sk (J. Rafay), peter.chrenek@uniag.sk (P. Chrenek).

http://dx.doi.org/10.1016/j.theriogenology.2017.04.032
0093-691X/

However, semen MΦ also have been associated with male infertility. An ultrastructural analysis showed that MΦ involved in spermatozoa phagocytosis are associated with altered spermatozoa features in non-leukocytospermic semen from sub-fertile couples [14]. We aimed at characterizing seminal MΦ cells basing on expression of CD14 antigen, which has been previously described as a useful marker to detect monocytes and MΦ diagnostically [15–17]. The objective of this study was to examine the relationship between the presence of MΦ and spermatozoa quality in rabbits asymptomatic for a genital tract infections.

2. Materials and methods

2.1. Animals and semen collection

Sexually mature broiler rabbit males (n = 30) and females (n = 123) both of lines M91 and P91 without overt evidence of genital tract infections were used in experiments. Animals were housed in individual cages, fed with a commercial diet (KV; TEKRO Nitra, s.r.o., Slovakia) and watered *ad libitum*. The photoperiod used was a ratio of 14 h light:10 h dark. The temperature and humidity in the area were 17–20 °C and 60–65%, respectively.

The experiments have been carried out in accordance with The Code of Ethics of the EU Directive 2010/63/EU for animal experiments [18].

The semen samples were collected from rabbit males once a week with a pre-heated artificial vagina during two months (from April to May 2015). Males were excluded if semen was transparent or contaminated by urine or blood. The semen samples were transported to the laboratory in a water bath at 37 °C.

2.2. Experimental design

Firstly, all semen samples (n = 30) were evaluated individually for the presence of MΦ as indicated below, then the samples were combined to create a pools. Semen samples were routinely collected from selected rabbits once a week during two months (from April to May 2015) into the prepared sterile tubes and mixed in order to create heterospermic pool of the respective groups. The part of heterospermic pools was used immediately for intravaginal artificial insemination (AI) of rabbit doses. The second part were transported to the laboratory, where semen suspensions were divided and evaluated by flow cytometry and CASA-system. Obtained seminal parameters were compared between groups.

The rabbits were classified into three groups according to the seminal macrophage concentration.

Group MΦ1: Low MΦ number ($<1 \times 10^6$ MΦ/mL) (n = 16)
Group MΦ2: Intermediate MΦ number ($1.5–3.5 \times 10^6$ MΦ/mL) (n = 9)
Group MΦ3: High MΦ number ($>8 \times 10^6$ MΦ/mL) (n = 3)

Two rabbits with macrophages number 6×10^6 MΦ/mL and 4.5×10^6 MΦ/mL were discarded from the experiment, because of rabbits with these concentrations of seminal macrophages were not suited into respective groups.

2.3. Flow cytometry analysis

The heterospermic pools were washed and centrifuged (600× *g* for 7 min) in a saline solution (sodium chloride 0.9%; B. Braun Medical Ltd. Bratislava, Slovak Republic) enriched with 1% of fetal bovine serum (saline-FBS, Sigma-Aldrich, Germany) and divided into three aliquots for subsequent flow cytometric assessment of MΦ concentration and percentage of apoptotic, necrotic spermatozoa and spermatozoa with plasma membrane damage, as described below.

Macrophages were determinated by phycoerythrin (PE)-conjugated CD14 monoclonal antibody (Monoclonal Mouse Anti-Human CD14, Clone TÜK4 DAKO, Germany). Cell suspensions (1×10^6 cells/mL) were incubated for 30 min at room temperature (RT) with CD14 antibody at a final concentration of 10 μg/mL. As shown in Fig. 1(A, B, C), the plots represent the fluorescence of CD14 antibody versus side scatter of the three spermatozoa samples (MΦ1, MΦ2, MΦ3 group).

The concentration of MΦ/mL was calculated by applying the following formula [19]:

$$\frac{\%\,\text{CD14 positive cells} \times \text{no. of spermatozoa/ml}}{100}$$

The detection of apoptotic spermatozoa was performed using the specific nuclear fluorochrome Yo-Pro-1 (Molecular Probes, Lucerne, Switzerland). The assay was performed according to the method described by Mahfouz et al. [20] and Kuzelova et al. [21]. Briefly, the cell density was adjusted to 1×10^6 cells/mL in saline-FBS. One microliter of the Yo-Pro-1 solution (100 μmol/L) and 4 μL of the PI solution (50 μg/mL, Molecular Probes, Eugene, Oregon, USA) were added to 1 mL of the cell suspension. The samples were mixed and incubated in the dark at room temperature for 15 min. Three different populations can be identified by using this assay: viable spermatozoa - negative for both PI and Yo-Pro-1, apoptotic spermatozoa are positive for Yo-pro-1 but negative for PI, and dead spermatozoa are positive for both PI and Yo-Pro-1 or positive for PI and negative for Yo-Pro-1 (Fig. 1D).

The evaluation of plasma membrane damage was accomplished using a fluorescein-labelled lectin (Alexa Fluor) from peanut agglutinin (*Arachis hypogea*; PNA, Molecular Probes, Lucerne, Switzerland). 1 μL of PNA working solution (1 μg/mL) was added to 250 μL of diluted semen samples (1×10^6 spermatozoa/mL) and incubated for 15 min at the room temperature in the dark [22]. PNA binding is limited to the acrosomal cap of the spermatozoa and confined to the outer acrosomal membrane [23]. The samples were not fixed, allowing PNA Alexa Fluor green labelling only in the spermatozoa with damaged plasma membranes and exposed acrosome, whilst the spermatozoa with intact plasma membranes remained unstained (Fig. 1E).

After incubation samples were washed in saline-FBS and centrifuged at 600× *g* for 6 min, the supernatant was discarded and stained semen samples were analysed by a Becton Dickinson flow cytometer FACSCalibur (BD Biosciences, USA) equipped with a 488-nm argon laser as a light source. Acquisitions were done using the Cell Quest Pro (Becton Dickinson, Germany). At least 50 000 events (cells) were analysed in each sample. The spermatozoa population was gated using forward and side scatter to exclude debris and aggregates. CD14/PI emitting red fluorescence and Yo-Pro-1/PNA emitting green fluorescence were recorded in the FL-2 (650-nm filter) and FL-1 (525-nm filter) channels, respectively.

2.4. Computer assisted sperm analysis

The samples were diluted at the ratio of 1:100 (v:v) in a saline-FBS. The concentration and motility characteristics of diluted rabbit spermatozoa with different macrophage content were analysed using the Computer Assisted Semen Analysis (CASA) system. A subsample of this solution (3 μL) was placed on a Standard Count Analysis Chamber Leja (depth of 20 μm) (MiniTüb, Tiefenbach, Germany) and evaluated using the CASA software under a Zeiss Axio Scope A1 microscope (Sperm Vision™; MiniTüb, Tiefenbach, Germany). A frame rate of 60 Hz was used for analysis. For each