Original paper

EFFECT OF ROOSTER MACROPHAGES ON THE MOTILITY CHARACTERISTICS OF SPERMATOZOA

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Abstract

The aim of our study was to detect macrophages in rooster semen and subsequently to asses their effect on the spermatozoa motility parameters. Semen samples were collected from Ross PM3 breeder males (n = 24) by the dorso-abdominal massage into prepared sterile tube. Macrophages were identified using fluorescent dye Alexa Fluor-AcLDL and evaluated under a Leica fluorescent microscope (Leica Microsystem, Germany). Roosters (n=8) with the occurrence of macrophages above 20% per samples were classified to the group R1 and roosters (n=16) with the occurrence of macrophages 0-10% were classified to the group R2. The mean content of macrophages was 23.43±1.82 vs. 7.59±1.39 (group 1 vs. group 2, $P<0.001$) during experiment. The concentration (10^9 per ml), percentage of motile spermatozoa (motility > 5 μm/s) and percentage of progressive motile spermatozoa (motility > 20 μm/s) of two heterospermic samples were measured using computer-assisted sperm analysis (CASA system, Sperm Vision™). We observed significantly ($P<0.05$) lower progressive motile of rooster spermatozoa in the group R1 in comparison to group R2 (25.04±5.98 vs. 46.38±3.28). Based on the observed preliminary results, we hypothesized that the higher presence of macrophages in semen may have a negative effect on the quality of rooster spermatozoa in terms of their motility. Further experiments are required in order to prove our hypothesis.

Key words: *CASA, macrophages, motility, rooster, spermatozoa*

Introduction

Monocytes-macrophages, cells belonging to the mononuclear phagocytic system, are considered as the first line of immunological defense. Being mobile scavenger cells, macrophages participate in innate immunity by serving as phagocytic cells. These cells arise in the bone marrow and subsequently enter the blood circulation as blood monocytes. Upon migration to various tissues, monocytes mature and differentiate into tissue macrophages (Qureshia et al., 2000). Activated macrophages, engaging in sperm phagocytosis (spermiophages), might represent a marker of innate immune system activation (Pelliccione et al., 2008). Macrophages are known to perform essential roles in a number of physiological processes including lipid metabolism, wound healing, scavenging, and host defense against microorganisms and neoplasms but no clearly defined role has been assigned to macrophages normally present in the reproductive tract. Spermiophagy has been reported to occur widely in various groups of vertebrates, e.g., teleosts (Porawski

et al., 2004), amphibians (Sever, 1992), reptiles (Akbarsha et al., 2007), mammals (Murakami et al., 1985; Goyal, 1982; Abou-Elmagd and Wrobel, 1990). In mammals, spermiophagy takes place in various portions of the male reproductive tract, such as the seminiferous tubules (Holstein, 1978), *rete testis*, efferent duct (Holstein, 1978; Goyal, 1982), ampulla of the *vas deferens* (Murakami et al., 1985), seminal vesicle (Murakami et al., 1978), and ejaculatory duct (Abou-Elmagd and Wrobel, 1990). In avian species, spermiophages have been identified in the semen of guinea fowl (Hess et al., 1986) and in the *rete testis* of normal cockerels (Aire and Malmqvist, 1979). Aire (2000) reported active spermiophagy by the non-ciliated cells in the epithelial lining of the epididymal efferent ducts of the normal chicken.

The macrophages are capable of engulfing numerous spermatozoa (Hughes et al., 1981), and such phagocytosis could represent a process for removal of ageing spermatozoa (Tomlinson et al., 1992). However, current evidence indicates that it can also be pathological (Bronson, 1999; Turek and Lipshultz, 1994).

The aim of the study was to detect macrophages in rooster semen using fluorescent staining and to asses their effect on the spermatozoa motility and concentration using CASA method.

Materials and methods

Animals

Sexually mature (37 - 40 weeks old) Ross PM3 breeder males (n = 24) reared in a private breeding facility (Liaharenský podnik Nitra Ltd., Močenok Slovak Republic) were used in experiments. The roosters were housed in individual cages, under a constant photoperiod of 14 h of day light and were fed commercial standard diet with water given *ad libitum*.

Semen collection

The semen samples were collected from all roosters by dorso-abdominal massage into prepared sterile tubes. All the semen samples were at first analyzed for the presence of macrophages using fluorescent dye. The samples were divided into two groups on the basis of macrophage's content as follows: roosters (n=8) with the occurrence of macrophages above 20% per samples were classified to the group R1 and roosters (n=16) with the occurrence of macrophages 0-10% were classified to the group R2. Then heterospermic samples were routinely collected from these roosters twice a week into two prepared sterile tubes. The heterospermic pools were transported to the laboratory for fluorescent and CASA analysis.

Analysis of macrophages

Semen samples were washed in a saline solution (Sodium chloride 0.9 %, B. Braun Medical Ltd. Bratislava, Slovak Republic) and centrifuged at 300 x g for 3 min from the rest of seminal fluid. Pellets were resuspended in 2 $\mu g.ml^{-1}$ of Alexa-AcLDL (Acetylated Low Density Lipoprotein, Molecular Probes, USA) in saline solution and incubated in incubator for 2 hours. The samples were subsequently centrifuged at 300 x g for 3 min and resuspended in a cold saline solution. The suspension was afterwards placed on to a microscope slide, mixed with an equal volume of Vectashield antifade medium (Vector Laboratories, Burlingame, CA) containing DAPI fluorochrome. DAPI was used to identify the whole spermatozoa population. The drop was covered with a coverslip. Samples were evaluated under Leica fluorescent microscope (Leica Microsystem, Germany). The

percentage macrophages were determined for each heterospermic sample as the percentage of stained macrophages within the 400 spermatozoa counted.

CASA

The samples were diluted at the ratio of 1:100 in a saline solution. The concentration and motility characteristics of diluted rooster spermatozoa with different macrophage content were analyzed using CASA system (Sperm Vision™; MiniTüb, Tiefenbach, Germany). A subsample of this solution (4 μL) was placed on a Standard Count Analysis Chamber Leja 20 micron (MiniTüb, Tiefenbach, Germany) and evaluated using the CASA system under a Zeiss Axio Scope A1 microscope. In each sample the following parameters were evaluated: the concentration (10^9 per ml), percentage of motile spermatozoa (motility > 5 μm/s), percentage of progressive motile spermatozoa (motility > 20 μm/s), VCL (velocity curved line, μm/s), VSL (velocity straight line, μm/s), STR (straightness - VSL:VAP, velocity average path), LIN (linearity - VSL:VCL), BCF (beat cross frequency, Hz).

Statistical analysis

The experiment with heterospermic samples was replicated 4 times. Observed results were evaluated statistically using t-test by means of SigmaPlot software (Systat Software Inc., Germany) and expressed as the means ± SEM. P-values at $P<0.05$ were considered as statistically significant.

Results and discussion

The semen quality of the rooster is affected by different factors like age, breed, feed, environmental stressors, temperature and humidity (Zhang et al., 1999; Karaca et al., 2002; Shanmugam et al., 2012). The assessment of semen quality characteristics of poultry birds gives an excellent indicator of their reproductive potential and has been reported to be a major determinant of fertility and subsequent hatchability of eggs (Peters et al., 2004). Among the parameters evaluated in the seminal analyses, the white blood cell count is considered, as are sperm concentration, motility and morphology. It has been determined that neutrophils and macrophages constitute 95% of seminal leukocytes (Cavagana et al., 2012).

The macrophages in rooster semen were detected by fluorescent dye Alexa-AcLDL in this work. If the LDL (Low-Density Lipoprotein Complexes) has been acetylated, the LDL complex no longer binds to the LDL receptor, but rather is taken up by macrophages that possess “scavenger” receptors specific for modified LDL. The superior fluorescence output by Alexa Fluor AcLDL provides easier identification of macrophages and in mixed cell population. The experimental roosters were divided into two groups on the basis of macrophages content, that was observed using fluorescent dyes Alexa-AcLDL (green stained) and DAPI (blue stained). Roosters (n=8) with the occurrence of macrophages above 20% per samples belong to the group R1 and roosters (n=16) with the occurrence of macrophages 0-10% belong to the group R2. The average content of macrophages was 23.43±1.82 vs. 7.59±1.39 (group 1 vs. group 2, $P<0.001$) during experiment.

Present work evaluates effect of rooster macrophages on selected sperm parameters. Tomlinson et al. (1992) suggested the possibility that macrophages may play a positive role in the control of semen. The authors postulated that phagocytes might shape the quality of the human ejaculate by phagocytising morphologically abnormal spermatozoa. In our study, there was no statistically significant difference in motility of spermatozoa between R1 and R2 group. On the other hand, the progressive motility was significantly

lower (P<0.05) in the group R1 (25.04±5.98) in comparison to the group R2 (46.38±3.28). There were no significant differences in other motility parameters (VCL, VSL, LIN, STR, BCF) between analyzed groups (Table 1).

Table 1. *CASA parameters of rooster heterospermic samples in the groups R1 and R2*

Semen sample	R1	R2
Concentration (10^9 per ml)	3.15±1.29	3.69±1.07
% of motile spermatozoa (motility μm/s)	43.99±3.88	55.51±8.43
% of progressive motile spermatozoa (motility 20 μm/s)	25.04±5.98*	46.38±3.28
VCL (μm/s)	87.06±6.73	86.09±6.11
VSL (μm/s)	31.15±1.23	30.29±2.27
LIN (VSL/VCL)	0.36±0.02	0.35±0.17
STR (VSL:VAP)	0.66±0.03	0.64±0.01
BCF (Hz)	26.84±1.53	28.63±1.61

Results are expressed as means ± SEM; * statistically significant at P<0.05

Pelliccione et al. (2008) reported a significant positive correlation between macrophage counts in men semen and sperm tail defects, acrosome damage and high sperm deformity index. The presence of spermiophages was associated with a lower total sperm count and sperm concentration, a lower forward motility and a high percentage of disrupted sperm compared to ejaculates without macrophages. Nevertheless, in our study no significantly negative effects of the macrophages on the concentration of rooster spermatozoa were observed (Table 1).

The polymorphonuclear macrophages are the main components of seminal leukocytes which can generate significantly higher (>100-fold) quantities of ROS, overwhelming the ROS-scavenging mechanisms in seminal plasma and resulting in oxidative stress and damage to spermatozoa (Sharma et al., 2001). Immune activation induced a steeper decline in sperm swimming velocity, thus highlighting the potential costs of an induced immune response on sperm competitive ability and fertilizing efficiency. Oxidative damage negatively correlated with sperm swimming velocity as reported for Great Tit (Losdat et al., 2011). Oxidative damage to spermatozoa can reduce fertility in domestic animals and humans (Tremellen, 2008; Aitken, 1999), and has been demonstrated recently in a wild bird species to affect sperm quality by a reduction of sperm motility and swimming velocity (Helfenstein et al., 2010). But on the contrary, the addition of macrophages to normal turkey semen had no effect on fertilizing capacity, hatchability, or embryonic mortality of unstored semen or semen stored for 6 h (Barnes et al., 1996). Seshadri et al. (2012) analyzed the effect of human leucocyte subpopulations on fertilization rates in an IVF cycle and they reported that the macrophages and the monocytes were significantly elevated in the good fertilizers group in comparison with the poor fertilisers.

Conclusion

These preliminary results may indicate the negative effect of higher macrophages presence in semen on the quality of rooster spermatozoa. Nevertheless, it is necessary to more precisely determine the effects of seminal macrophages on the quality parameters of rooster semen and fertilization.

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