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Short communication

IMPACT OF THE PRESENCE OF MACROPHAGES IN RABBIT EJACULATES ON FEMALE FERTILITY

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ABSTRACT

The present study was designed to investigate the occurrence of macrophages in rabbit sperm and their impact on female fertility parameters *in vivo*. Activated macrophages, engaging in sperm phagocytosis (spermiophages), might represent a marker of innate immunosystem activation. Semen samples were collected from rabbit males using an artificial vagina, and spermiophages were identified with fluorescent dye Alexa Fluor 488-AcLDL (Acetylated Low Density Lipoprotein Molecular Probes, USA). For artificial insemination the sperm samples were divided into two groups basing on number of spermiophages: the group R1 - ejaculates with the occurrence of spermiophages less than 20 % of all cells and the group R2 - ejaculates with the occurrence of spermiophages at about 20 - 40 %. The increased occurrence of spermiophages, observed in the R2 group, was associated with decreased female fertility parameters (conceptional rate, liveborn pups). The differences in conceptional rates (73.08 vs. 68.18 %) as well as the differences in average number of liveborn pups per doe between groups of R1 and R2 does were not statistically significant. These preliminary results may indicate the negative impact of higher spermiophage presence in semen on female fertility.

Key words: rabbit; spermatozoa; spermiophages; conceptional rate

INTRODUCTION

Phagocytosis, the process by which cells engulf foreign particles, occurs in eukaryotes ranging from unicellular organisms, which use it for nutrition, to mammals, where it plays a key role in innate immunity (Gagnon *et al*., 2002). Leukocytes are present throughout the male reproductive tract, are found in most ejaculates, and are thought to play an important role in immune surveillance and phagocytic clearance of abnormal sperm (Gallegos-Avila *et al*., 2010). Polymorphonuclear (PMN) granulocytes account for 50 % to 60 % of all white blood cells (WBC) in semen, macrophages (MF) account for 20 % to 30 % and T-lymphocytes for 5 % (Smith *et al*. 1989; Wolff, 1995). Macrophages belong to the mononuclear phagocyte system and they represent a mechanism to remove senescent, malformed or degenerative sperm. They appear to be involved in immunologic surveillance, immunoregulation, and tissue remodelling. Early morphological studies described the presence of macrophages in the interstitium and occasionally in the *lamina propria* of seminiferous tubules of human testis (Hermo *et al*., 1978; Pollanen *et al*., 1987; Frungieri *et al*., 2002). Phagocytosis of sperm by epithelial cells has been described previously in *ductal tubules testis* of various animal species (Dym, 1974; Hoffer and Hamilton, 1974; Hoffer *et al*., 1975; Holstein, 1978; Riva *et al*., 1981; Goyal, 1982). Spermiophagy by macrophages has been described in the male extragonadal ductal system (Holstein, 1978); in prostate and in boar

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ejaculate (Hrudka and Post, 1983). In cases of male infertility there has not been found a correlation with the percentage of abnormal spermatozoa in the semen or the motility of the spermatozoa and the number of spermiophage cells, in cases of necrozoospermia they were not dominant. Samples of semen voided after a prolonged period of abstinence did not show an increase in the spermiophage cells (Phadke, 1961). There is lack of information about occurrence of spermiophages in rabbit ejaculate and effect of spermiophages on female reproduction.

The aim of the study was to identity spermiophages in rabbit ejaculates using specific staining and to evaluate the impact of spermiophagy on fertility of rabbit does.

MATERIAL AND METHODS

Animals

Sperm samples were obtained from sexually mature (4 – 5 months old) and clinically healthy rabbit males of M91 and P91 lines from the breeding farm of Animal Production Research Centre Nitra. The males were housed in individual cages, under a constant photoperiod of 14h of daylight. Temperature and humidity in the building were recorded continuously by means of a thermograph positioned at the same level as the cages (average relative humidity and temperature during the year were maintained at 60±5 % and 17±3°C). The rabbits were fed *ad libitum* with a commercial diet (KV; TEKRO Nitra, s.r.o.) and water was provided *ad libitum* with nipple drinkers.

Analysis of spermiophages

The ejaculates of sperm were washed in a saline solution. Fetal calf serum (FCS) was added and the samples were centrifuged at 1000 rpm for 3 min to separate it from the rest of seminal fluid. Pellets were resuspended in 2 $\mu g.ml^{-1}$ of Alexa-AcLDL in a saline solution, added with FCS and incubated in incubator for 2 – 4 hours. The samples were subsequently centrifuged at 1000 rpm for 3 min and resuspended in a cold saline solution. The suspension was afterwards placed onto a microscope slide, mixed with an equal volume of Vectashield antifade medium (Vector Laboratories, Burlingame, CA) containing DAPI fluorochrome. The drop was covered with a coverslip. Samples were evaluated under a Leica fluorescent microscope (Leica Microsystem, Germany).

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 spermiophages (macrophages) that possess "scavenger" receptors specific for modified LDL. The superior fluorescence output by Alexa Fluor AcLDL provides easier identification of spermiophages and endothelial cells in mixed cell population.

Insemination

Sexually mature and clinically healthy rabbits does (n=48) that were included in the rearing programme were used for artificial insemination. Spermatozoa were diluted in a commercial diluent (MiniTüb, Tiefenbach, Germany) up to the concentration minimum of $14x10^6$/ml and used for the insemination of hormonally stimulated rabbit females. Females of rabbits were inseminated with fresh doses of filtered heterospermic semen (0.5ml I.D. per female), divided into two groups R1 (n=2) and R2 (n=2). PMSG at 25 I.U. (Sergon, Bioveta, Czech Republic) was administrated to each doe 48 hours before artificial insemination (A.I.). Immediately following AI, synthetic GnRH (2.5 μg; Supergestran, Ferring-Pharmaceuticals, Czech Republic) was intramuscularly injected into each doe. The ratio of kindled does to the number of inseminated does (kindling rate) and also the average number of liveborn kits per 1 inseminated doe were recorded. Conceptional rate was determined basing on pregnancy diagnostics on 17^{th} day following insemination.

Obtained data were statistically processed by χ^2 –test using Excel software.

RESULTS AND DISCUSSION

According to spermiophage occurrence in sperm ejaculates the samples were divided into two groups: the ejaculates with the occurrence of spermiophages (SP) less than 20 % of all cells - the R1 group, and the ejaculates with the occurrence of spermiophages in the range of 20-40 % - the R2 group (Fig. 1).

Results of insemination trials showed that higher occurrence of spermiophages led to decrease in female fertility parameters: conceptional rate and liveborn pup rate compared to the group with less count of spermiophages (R1). However, the differences in conceptional rates and in average number of liveborn kits per doe between R1 and R2 groups were not statistically significant. Values of fertility parameters within the groups R1 and R2 are shown in Table 1.

Tomlinson *et al.* (1992) suggested the possibility that macrophages may play a positive role in the control of semen quality. Specifically, the authors postulated that phagocytes might shape the quality of the human ejaculate by phagocytosing morphologically abnormal spermatozoa. Tomlinson *et al.* identified three types of seminal phagocytic cells: small PMN leukocytes, monocytes of similar size, and much larger macrophages capable of engulfing multiple sperm heads. These authors suggested

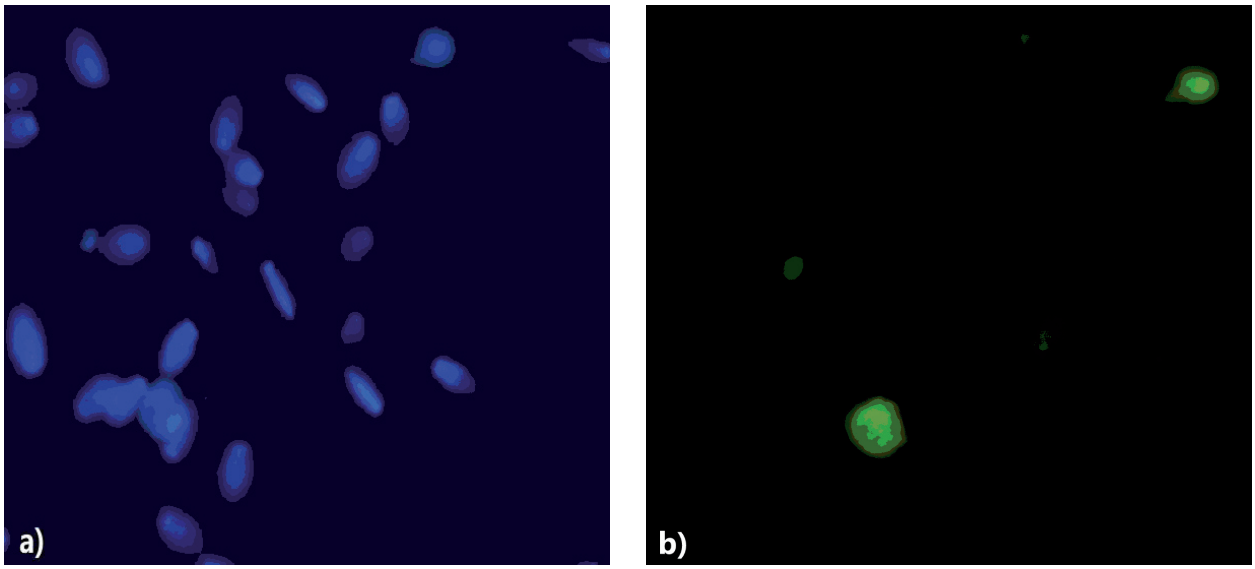

Fig. 1: a) Sperm population stained with nuclear fluorochrome DAPI
b) Colony of macrophages specifically stained with Alexa-AcLDL fluorescent dye

Table 1: Impact of spermiophage occurrence in male ejaculates on female fertility *in vivo*

Rabbit males	No. inseminated females	No. fertilized females	Conceptional rate %	Kindling rate %	Liveborn pups (n)/ average per doe (%)	Stillborn pups (n)/ average per doe (%)
Group R1 (n=2)	26	19	73.08	69.23	(182)/9.58	(9)/0.47
Group R2 (n=2)	22	15	68.18	68.18	(105)/7.00	(4)/0.27

that the sperm morphology is directly correlated with the size of the seminal leukocyte population (Gallegos-Avila *et al.*, 2010). Chrenek *et al.* (2010) evaluated sperm viability of rabbit males using fluorescent analysis (SYBR-14/PI and annexin V/DAPI tests). The present results show that female fertility parameters were decreased in group with higher occurrence of spermiophages, but these differences were not statistically significant. Once seminal leukocyte concentration rises above a threshold of $1x10^6$/ml (leucocytospermia), they have significant potential to damage sperm and cause infertility (Plante *et al*., 1994; Wolff, 1995; Ochsendorf, 1999; Sharma *et al*., 2001; Henkel *et al*., 2003). The presence of spermiophages was associated with lower total sperm count and sperm concentration, lower forward motility and high percentage of disrupted sperm compared to ejaculates without spermiophages (Pelliccione *et al*., 2008). These authors showed that spermiophages engulfing sperm are frequently observed in ejaculates from non-leukocytospermic men complaining for couple infertility.

CONCLUSION

Our data suggest that spermiophages might have a negative biological impact on female fertility regardless to their count in semen. Since these are preliminary and not statistically significant results, therefore further experiments are required in order to prove the impact of spermiophages on doe's reproductive traits.

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