Sperm DNA Fragmentation in Rams Vaccinated with Miloxan

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Abstract: Sperm DNA fragmentation was analysed in 113 semen samples obtained from different rams over a period of one year. Semen samples were collected from: unvaccinated rams between January and June (Control group 1; CG1); vaccinated rams at least 70 days after vaccination between October and December (Control group 2; CG2); vaccinated rams 20 days after vaccination (Vaccinated group 1; VG1); and vaccinated rams 40 days after vaccination (Vaccinated group 2; VG2). Results show Miloxan, the vaccine of interest in this study, increased the percentage of sperm cells with fragmented DNA by 10-fold on average (from 6.5±7.9 to 63.4±24.2). However, the negative impact of vaccination on sperm DNA fragmentation appeared to reversible; near normal levels of sperm DNA fragmentation had been restored 40 days after vaccination (21.7±10.6). On the basis of these data, the use of semen samples from vaccinated animals should be avoided until at least one month after vaccination.

INTRODUCTION

Sperm DNA Fragmentation (SDF) has been the subject of numerous studies because loss of fertility has been shown to be highly correlated with a high frequency of nuclei containing damaged DNA in human [1] and in other mammals [2, 3]. However, based on human studies, the relationship between a high frequency of sperm containing fragmented DNA and the capacity of the male to generate fertilization is controversial [4]. The origin of DNA fragmentation is mostly unknown, although apoptosis, oxidative stress, or persistence of DNA breaks produced during the chromatin protamination process in spermiogenesis, could be direct causes. It is also known that some external effectors such as exposure to environmental pollutants, smoking, or genetic characteristics such as a massive reduction of telomeric DNA sequences, may trigger sperm DNA fragmentation [5, 6]. The assessment of SDF in organisms used for reproductive purposes is therefore of interest, especially when the individuals concerned have encountered a stress episode. This is because the assessment of classical parameters of sperm quality does not fully correlate with the sperm DNA quality [7, 8]. In particular, SDF should be periodically assessed along the reproductive life of those animals used repeatedly to retrieve semen samples for assisted reproduction, in order to avoid using damaged sperm for fertilization.

Infectious agents may interfere with genetic programs controlling the organism's normal development with a clear detrimental effect on individual fitness. In terms of reproduction, it is known that infectious agents can affect embryonic survival, ovulation rates and fertilization [9,10]. From a similar viewpoint, immunological infertility in males has been associated with presence of auto- or iso-antibodies and their effect on sperm proteins. These antibodies can prevent the accessibility of spermatozoal proteins at various stages during the fertilization process and therefore induce infertility. A number of sperm-specific proteins are therefore being considered as candidates for the development of vaccines for reducing fertility [11,12].

Although vaccines are composed of weakened, dead germs or toxins, when injected to living animals they may produce side-effects, generally through the activation of the immune system. Vaccination may affect the various standard seminal parameters such as sperm motility, concentration and production rate of abnormal forms [13]. The aim of the present investigation was to analyse if subcutaneous vaccination of rams with Miloxan (*Clostridium perfringens* type C, D and *C. oedematiens* type B) had any effect on the percentage of sperm cells with fragmented DNA.

MATERIAL AND METHODS

Semen Samples, Collection and Preparation

One hundred and thirteen semen samples of different individuals from four main different races of ram (Castellana, Assaf, Lacaune and Churra) collected along one year were included in the analysis. All animals belong to the same Genetic Centre (Ovigen, Zamora, Spain). The animals were vaccinated with Miloxan (*Clostridium perfringens* type C, D and *Clostridium oedematiens* type B; Merial, Lyon, France). Two ml were subcutaneously administered to each animal. Sperm samples included in the analysis were collected at different periods throughout the year and classified into four groups: Control Group 1 (CG1): sperm samples obtained from January to June from unvaccinated animals. Vaccina-

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tion Group 1 (VG1): sperm samples obtained in July 20 days post-vaccination. Vaccination Group 2 (VG2): sperm samples obtained in August 40 days post-vaccination. Control Group 2 (CG2): sperm samples obtained in October to December at least 70 days post-vaccination. Unfortunately, during the experiment, it was only possible to assign semen samples to particular animals in 35% of cases. Samples within each pre-established groups were therefore considered to be independent clusters of individuals.

All semen samples were collected with the use of an artificial vagina. Immediately after collection, the semen was diluted to a final concentration of 43×10^{8} spermatozoa/ml with a commercial egg yolk-based extender (Triladylt; Minitube Canada, Woodstock, Canada) and frozen using 0.2 ml straws. Only sperm samples considered of sufficiently high quality for standard parameters, including volume, concentration, motility, acrosomes and positive endosmosis, are currently frozen at the Ovigen Center. All animals were aged 2 to 4 years, healthy, and controlled for feeding, housing, and photoperiod conditions. These stocks are currently being maintained for breeding purposes.
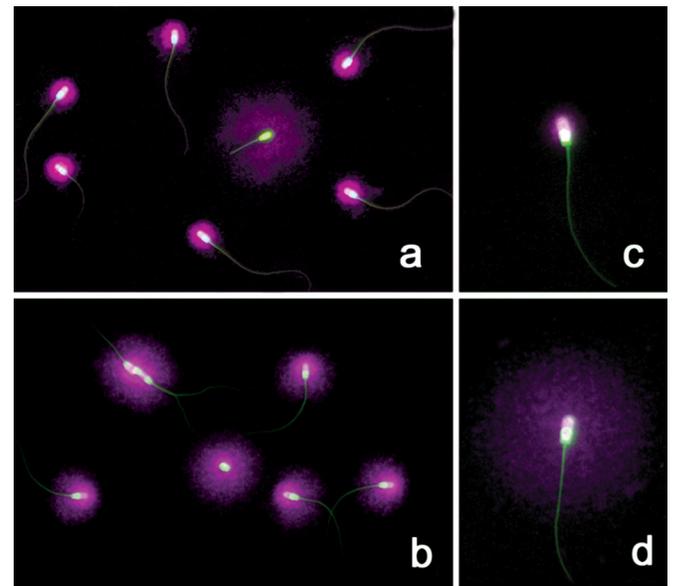
Sperm DNA Fragmentation Analysis

To determine the percentage of sperm cells with fragmented DNA, i.e. DNA Fragmentation Index (SDFi), the Sperm-Halomax® kit (Halotech-DNA SL, Madrid, Spain) was used. This methodology is a variant on the SCD (Sperm Chromatin Dispersion) test and has been used to assess sperm DNA fragmentation in mouse [6], pig [14, 15] and bull [16]. For each experiment, frozen straws were thawed and different aliquots were prepared to a final concentration of $10\text{-}15 \times 10^{6}$ spermatozoa/ml. Twenty five microliters of semen diluted to $10\text{-}15 \times 10^{6}$ spermatozoa per millilitre were added to a vial containing low melting point agarose and mixed. Pre-treated slides were placed onto a metallic plate cooled to 4ºC. A drop of the agarose-sperm mix was then spread onto the treated face of the slide and covered with a glass coverslip for 5 minutes at 4ºC. The coverslip was smoothly removed, and the slide was horizontally placed in 10 ml of the lysing solution provided in the kit, to remove membranes and proteins. Finally, slides were washed for 5 min, dehydrated in sequential 70, 90 and 100% ethanol baths. The sperm were then stained for fluorescence microscopy with DAPI in Vectashield Mounting Medium H-1000 (Vector, USA) followed by 2, 7 - dibrom - 4 - hydroxyl - mercuryfluorescein di-sodium salt (DMF; Sigma, USA). The dual emission fluorochrome combination used in the present experiment, allowed simultaneous visualization of DNA (blue) and proteins (green) respectively using a dual band pass fluorescence filter block or, alternatively, a single emission could also be observed using a single band pass fluorescence filter block.

Fluorescence microscopy was used for automatic sperm counting using the Sperm Class Analyzer DNA module (Microptic SL, Barcelona, Spain). The SCA software discriminated between spermatozoa that showed a small halo of spreading of chromatin loops (sperm containing unfragmented DNA) and large or medium halo of diffused chromatin fragments (sperm containing fragmented DNA). This software was coupled to a Leica DMLA (Leica SA, Germany) motorized fluorescence microscope, controlled with

Leica based software, for automatic scanning and image digitalization of each slide. Magnification lenses 20x were used for facility of cell counting. A minimum of 800 spermatozoa per sample were counted. The SDFi (percentage of sperm cells with fragmented DNA) was then calculated. Statistical analysis was performed using Minitab version 14 for Windows.

RESULTS AND DISCUSSION

Sperm samples processed for DNA fragmentation visualization showed basically two different spermatozoa morphologies. Those nuclei that remained compact or displayed very small halos of chromatin spreading corresponded to spermatozoa harbouring unfragmented DNA (Fig. **1a**,**c**). By contrast, those sperm nuclei which exhibited medium or large peripheral halos of diffusion of chromatin spots (Fig. **1b**,**d**), contained fragmented DNA [16].

Fig. (1). Visualization of the sperm DNA fragmentation in ram under fluorescence-microscopy. (**a**) Sperm sample showing a low SDFi. (**b**) Sperm sample of an individual after 20 days of vaccination where most of the sperm cells exhibit a large dispersion of the chromatin. (**c**) Selected nuclei from a sperm cell containing unfragmented DNA. (**d**) Selected nuclei from a sperm cell containing fragmented DNA. In all cases, blues colour corresponds to DNA, whereas protein fluoresces in green and is mainly labelling the flagelum and sperm core after the SCD test.

The observed range of SDFi in the CG1 (January to June), was from individuals presenting nearly 0 % of sperm DNA fragmentation, to others exhibiting about 41% % of SDFi,. The mean value of SDFi or this period was 6.4+7.9. The animals studied between October and December (CG2) showed a similar distribution of SDFi values (5.7+4.5) and significant differences between both periods of analysis were not obtained based on arc-sine transformed data (one-way ANOVA: F = 0.16; p = 0.7 for 1 and 68 df). The condition of SDF 20 and 40 days post vaccination was very different (Fig. **2**). SDFi post vaccination (VG1) significantly increased with a mean value of 63.4+24.2 (one-way ANOVA, compared to CG1 and CG2 combined: F = 484.9; p<0.0001 for 1 and 101 df).
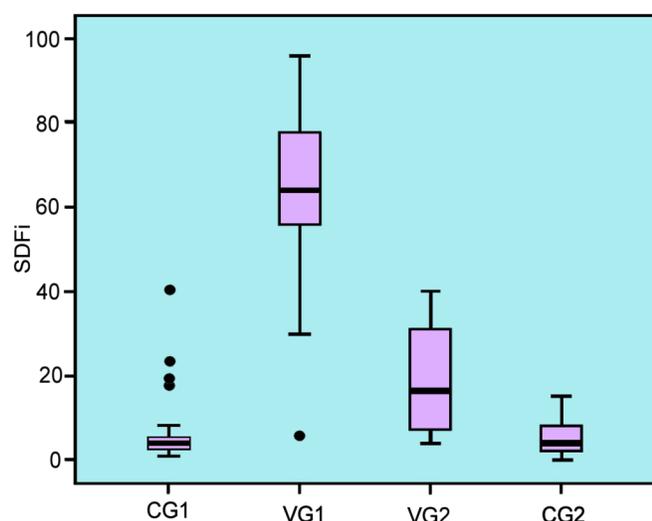
Fig. (2). Descriptive statistics (box-and-whisker plots) of sperm DNA fragmentation index in the four established groups (CG: Control Group; VG: Vaccinated group). See text for details.

Forty days post vaccination (VG2) the SDFi decreased with respect to the VG1 score (one-way ANOVA: F = 37.6; p<0.0001 for 1 and 41 df), but was still significantly increased, with a 3.6 fold increase in mean compared to the two outside controls (one-way ANOVA: F = 36.8; p<0.0001 for 1 and 78 df). The mean SDFi value (21.7+10.6) for VG2 was also still very high compared with the standard values.

Two main conclusions may be obtained from this work 1) vaccination can temporarily increase sperm DNA fragmentation in ram and 2) sperm DNA fragmentation levels can be restored to normal levels after being elevated following an acute stress event, such as vaccination. Practically, the sudden large increase in the percentage of sperm cells with fragmented DNA after vaccination is another reason to avoid using sperm samples retrieved from animals for artificial reproduction until at least, one month or forty days following vaccination.

It is known that germ line cells are significantly affected by infectious agents as well as by vaccination episodes against those same agents [10]. In boar [17] and in ram [18], the percentage of forward movement and normal spermatozoal morphology and motility were significantly reduced in vaccinated animals. In the male germ line, only spermiogenesis is affected. Meiosis is also likely to be affected by the collateral effects of vaccination, as suggested by the increased occurrence of univalents involving autosomes, as well as sex chromosomes, in a group of mice which were antirabic vaccinated, in comparison with control stocks [19]. In this situation, vaccines could be considered as clastogenic effectors. In the case analyzed here, the time of recovery to a normal rate of sperm DNA fragmentation seems to be related to duration of spemiogenesis, more than to possible mutations or alterations occurring during meiosis. Duration of spermiogenesis varies in different species but is quite constant within each species. Thus, in ram the spermiogenesis encompasses around 18 days, while in bull and stallion it is about 21 days [20, 21]. The timing here for sperm to reach the maximum levels of sperm DNA fragmentation, coupled with the quick recovery of sperm quality, seems to indicate that spermiogenesis rather than spermatogenesis was af-

fected by vaccination. If meiotic cells or gonial cells were affected the time to recover normal cells would be longer than observed here. In the case analysed here from 20 to 30 days was the delay before SDF started to decrease. This suggests that histone/protamine replacement and assembly during sperm maturation were affected. Interestingly, when the effect of foot-and-mouth disease vaccination on the various seminal attributes of buffalo bulls was studied, it was observed that vaccination also produces adverse effects on the standard seminal parameters, up to one month after vaccination [13]. This duration for semen quality recovery is quite similar to that reported here.

The effect of vaccinations on semen parameters is not fully understood. Probably the type of vaccine and genetic background of the animal is determinant of the immune reaction of the individual. Inoculation of live *Brucella ovis* into the epididymus of rams generated intense epididymal lesions. In addition, an increased numbers of neutrophils, macrophages and lymphocytes were detected, being indicative of induced inflammatory response. Unfortunately, such effects on spermatozoa were not determined in this experiment [22]. However, it is known that leucocytospermia produces oxidative stress that may result in DNA damage [23, 24]. Additionally, vaccination may trigger elevated temperature, either locally through the inflammation process, or generally, inducing fever in some cases. In fact, concentration, motility and morphology may be adversely affected by fever, especially during the period of spermiogenesis [25]. Also sperm chromatin structure seems to be affected by an increase of temperature, causing extensive DNA strand breaks, altering protein synthesis [26] and decreasing the protamine disulfide bonding [27]. Interestingly, in bulls, scrotal mild-thermal stress caused sperm morphological abnormalities after 11 days after insulation [28]. They increased during the following days, decreasing near 40 days after scrotal insulation.

The effects of vaccination on sperm quality and particularly on sperm DNA integrity probably consists of many factors and effectors, such as the genetic background, and the capacity to respond to oxidative stress or temperature variations, that finally determines a response for each individual. Substantial interactions between these factors could be an explanation for the high levels of variance in response for sperm DNA fragmentation values observed for animals post-vaccination.

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