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REGULAR ARTICLE

# EFFECT OF DIFFERENT DILUENTS ON ROOSTER SPERMATOZOA APOPTOSIS

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## ABSTRACT

The aim of this study was to observe the apoptosis of rooster spermatozoa and to compare the effect of commercial insemination diluent and saline solution on rooster spermatozoa apoptosis. Semen samples were collected once a week from roosters lines Lohmann Light (n=30). The one heterospermic sample was diluted with saline solution at a ratio of 1:100 and the second was diluted with commercial insemination diluent (Avian diluents; IMV Technologies, France) in the same ratio 1:100 at room temperature. The percentage of apoptotic spermatozoa was estimated by fluorescent staining (Yo-Pro-1) 0.5, 1 and 2 hours after semen collection and analyzed by fluorescent microscopy. There were no significant differences in sperms' membranes quality after half an hour and one hour between diluted semen in both heterospermic samples. Significantly higher rate ($P<0.05$) of Yo-Pro-1 – positive cells was observed in sample diluted in commercial diluents (22.03% vs. 35.91% of apoptotic spermatozoa) after two hours. We concluded that rooster semen could be diluted by saline solution which is cheaper and more accessible for practical use.

**Keywords:** rooster, spermatozoa, apoptosis, diluents

## INTRODUCTION

Fertility is one of the most important economic traits in poultry production, together with egg hatchability. Male fertility potential may be defined as the capability to produce and to ejaculate spermatozoa that are able to fertilize eggs, which includes accomplishment of all steps of the fertilization process: sperm moving across the female reproductive tract and reaching the sperm storage tubule, binding and penetration into the perivitelline layer, and fertilization (**Celeghini *et al.*, 2005**). In birds, as in mammals, the testes are responsible for the production of spermatozoa and the secretion of androgen. Spermatocytogenesis consists of the mitotic divisions involving proliferation and maintenance of spermatogonia (**Senger, 1999**). Spermatogonia then undergo meiosis to form primary spermatocytes and then secondary spermatocytes, which differentiate into spermatids. This clonal expansion is excessive, requiring that a mechanism exist to match the number of germ cells with the supportive capacity of Sertoli cells. Sertoli cells and germ cells, the only cell types within the seminiferous epithelium, are in close contact. Sertoli cells, spanning the thickness of the seminiferous epithelium, orchestrate spermatogenesis by providing structural and nutritional support to germ cells (**Lee *et al.*, 1997**). Overproliferation of early germ cells is tempered by selective apoptosis of their progeny (**Bartke, 1995; Allan *et al.*, 1992; Billig *et al.*, 1995**).

Apoptosis is physiologically programmed cell death that affects single cells without any related inflammation in the surrounding tissue (**Wyllie *et al.*, 1980**). It is a complex phenomenon that is characterized by distinct ultra-structural and biochemical changes. The changes in the plasma membrane permeability and mitochondrial membrane potential constitute an essential stage of apoptosis (**Baccetti *et al.*, 1996; Blanc-Layrac *et al.*, 2000**). The most significant changes related to apoptosis are the externalization of the phosphatidylserine (PS), DNA fragmentation, caspase activation, loss of mitochondrial membrane potential, and increase in sperm membrane permeability (**Bratton *et al.*, 1997; Glander and Schaller, 1999; Martin *et al.*, 2004**). During the early phases of disturbed membrane function, asymmetry of the membrane phospholipids occurs, before the integrity of the plasma membrane is progressively damaged (**Martin *et al.*, 1995**). When the cell membrane is disturbed, the phospholipid PS is translocated from the inner to the outer leaflet of the plasma membrane (**Desagher and Martinou, 2000**). Although there is growing knowledge on the apoptosis of the testes in mammals (**Wyllie, 1987; Coucouvanis *et al.*, 1993; Wang *et al.*, 1998**), very little information is currently available pertaining to the apoptosis of the testes in avian species.

The objective of our study was to evaluate the effect of diluents on the apoptosis of rooster spermatozoa.

## MATERIAL AND METHODS

### Semen collection and dilution

Semen samples were collected from clinically health and sexually matures roosters (n=30) lines Lohmann Light, aged 61-67 weeks. The roosters were housed in individual cages, under a constant photoperiod of 14 h of light day and were fed a commercial standard diet with water given *ad libitum*.

Semen was routinely collected from each rooster once a week by dorso-abdominal massage into prepared sterile tube. The heterospermic pool was transported to the laboratory for analysis. Pooled semen was then divided into two groups. The one heterospermic sample was diluted with saline solution (Sodium chloride 0.9 %; B. Braun, Gemany) at a ratio of 1:100 and the others was diluted with commercial insemination diluents (Avian diluents; IMV Technologies, France) in the same at a ratio 1:100 at room temperature.

### Test for apoptosis (Yo-PRO-1/PI/DAPI)

Apoptotic cells were detected using specific nuclear fluorochrome Yo-Pro-1 in combination with PI half an hour after ejaculate collection. Following washing in saline-FBS solution, sperm samples were incubated 20 min at room temperature in staining solution containing 1 µl Yo-Pro-1 and 10 µl of PI in 189 µl PBS-PVP (solutions PBS supplemented with polyvinylpyrrolidone, 4 mg/ml). After incubation, the sperm samples were washed in a saline-FBS solution, centrifuged and 4µl of sperm suspension were placed onto microslide, mixed with 4µl of Vectashield with DAPI and immediately evaluated under a Leica fluorescent microscope. This procedure was then repeated after 1 and 2 hours after collection semen. Using Yo-Pro-1/PI/DAPI, three populations of sperm cells were detected: the apoptotic sperm cells (green or yellow stained), the dead (necrotic) spermatozoa (colored red) and all spermatozoa (colored dark-blue).

### Statistical analysis

Obtained data were statistically analyzed using $\chi^2$ – test. The level of significance was set at *** (P<0.001); ** (P<0.01); * (P<0.05).

## RESULTS AND DISCUSSION

In the present study, we assessed the early apoptotic changes (Figure 1) in the membrane of rooster spermatozoa based on the increased membrane permeability. Apoptosis detection using Yo-Pro-1 staining was previously tested on somatic cells (**Idziorek *et al.*, 1995**) and later on boar (**Peňa *et al.*, 2005; Trzcinska *et al.*, 2008**) and bull (**Martin *et al.*, 2004)** spermatozoa and showed to be inexpensive, quick and easy to perform. Propidium iodide (PI), a membrane impermeate dye (red fluorescence) was used to identify dead cells. The staining pattern resulting from the simultaneous use of these dyes in combination with DAPI, a cell permeate DNA dye (blue fluorescence), makes it possible to distinguish normal, early apoptotic and dead (DAPI/Yo-Pro-1/PI) cells by fluorescence microscopy.

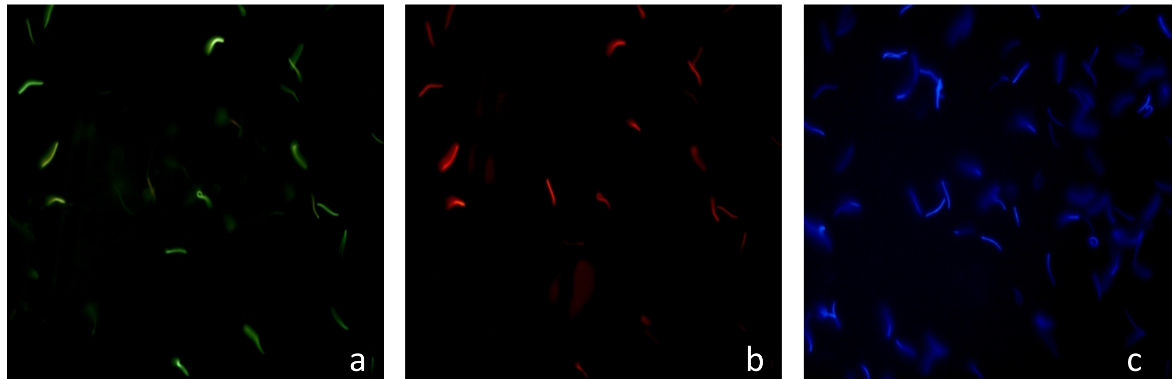
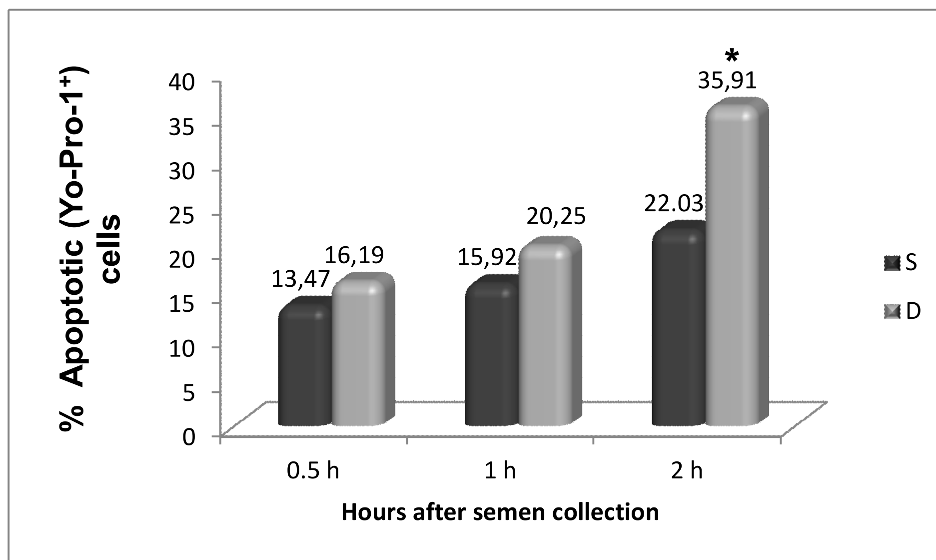



**Figure 1** Rooster spermatozoa stained with a) Yo-Pro-1, b) PI, c) DAPI dye

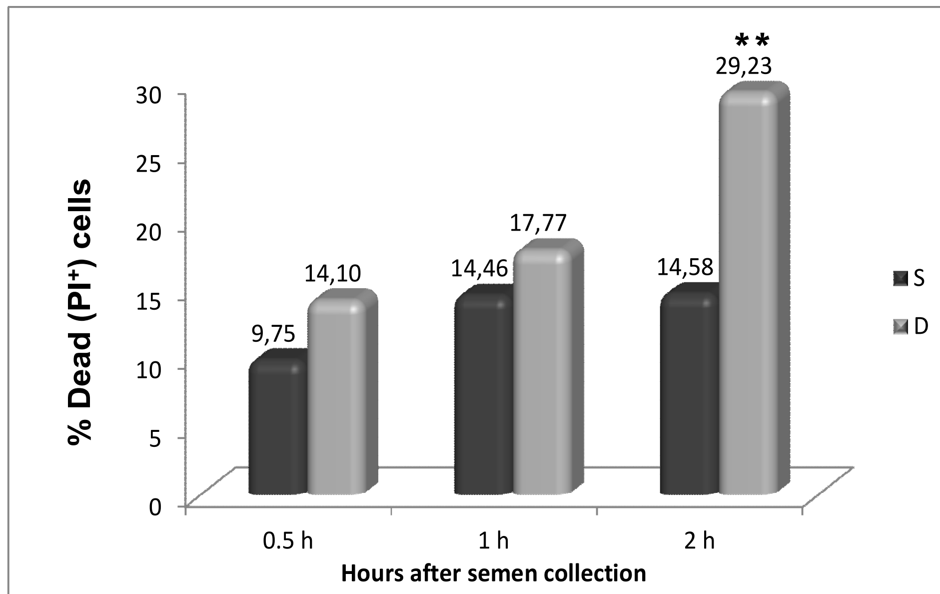
Mammalian male germ cells undergo apoptosis at 37 °C internal body temperature; birds, however, are unique among homeothermic animals in developing spermatogenesis at the elevated avian internal body temperature of 40-41°C (**Bakst**, **2007**). **Wei *et al.* (2011)** described the apoptotic features of testicular cells in ostrich chick testis from day 1 to day 90. Apoptosis of testicular cells took place at any developmental period from 1-day-old to 90-day-old ostrich age, but more apoptosis testicular cells were observed at day 45. That is to say, apoptosis varied depending on the developmental stage.

We compared effect of commercial inseminations diluents (sample D) and saline solution (sample S) on rooster spermatozoa apoptosis. There was not find any significantly differences in sperms' membranes quality half an hour and one hour after semen collection between S and D sample according to fluorescence analysis. However, the percentage of spermatozoa in a sample diluted in commercial diluent significantly increased after two hours from semen collection (P<0.05). The results are shown in Figure 2. These sperms also demonstrated significantly higher percentage of dead cells in the time of two hours after ejaculate collection (Figure 3). The changes were statistically significant (P<0.01).




*P<0.05

**Figure 2** The proportion of apoptotic spermatozoa among semen diluted by saline solution (sample S) or commercial diluent (sample D) in different time intervals (0.5, 1 and 2 hours)




**P<0.01

**Figure 3** The proportion of dead spermatozoa among semen diluted by saline solution (sample S) or commercial diluent (sample D) in different time intervals (0.5, 1 and 2 hours)

According to our data, the presence of apoptotic spermatozoa was increased in both samples (S and D) proportionally with the period of their storage. The occurrence of apoptotic cells was significantly increased depending on time period in S sample (13.47% vs. 22.03%; P<0.05) and in D sample (16.19% vs. 35.91%; P<0.001). The occurrence of dead cells was significantly increased depending on time period only in D sample (14.10% vs. 29.23%; P<0.001).

Diluents are buffered salt solutions used to extend semen, maintain the viability of spermatozoa in vitro, and maximize the number of hens that can be inseminated. Semen extension is important since poultry semen is viscous and highly concentrated, containing 6 (roosters) to 12 (toms) billion spermatozoa/ml. Semen diluents are based on the biochemical composition of chicken and turkey semen (**Lake, 1995**). There are many diluents available for poultry semen, both published recipes and commercially available products. Several authors have compared the composition of various diluents and summarized fertility data across studies. What is evident from these reviews is that there is no standard diluents for poultry semen and that studies are so variable in experimental design including time of insemination, vaginal depth and frequency of AI, number of spermatozoa inseminated and duration of fertility analyzed that discerning the benefits of different diluents is difficult (**Howarth, 1983; Bakst, 1990; Bootswala and Miles, 1992**). There are basic characteristics common to nearly

all diluents: factors to maintain pH, osmolarity and provide an energy source for spermatozoa (**Christensen, 1995**). **Giesen and Sexton** (**1982**) observed a disappearance of turkey spermatozoa over an 18-h storage period suggesting that spermatozoa are swelling and bursting in vitro. These investigators hypothesized that hypertonic diluents could reverse the swelling and improve survival *in vitro* (**Donoghue and Wishart, 2000**).

## CONCLUSION

These preliminary results may indicate that saline solution is better solvent for rooster spermatozoa than our tested commercial diluent. Also saline solution is cheaper and more accessible for practical use. However, further research is needed to evaluate effect of diluents on the quality parameters and fertilizing capacity of rooster semen.

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