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EVALUATION OF FERTILIZING CAPACITY OF RABBIT SPERM ON THE BASIS OF ANNEXIN V-LABELLED MEMBRANE CHANGES

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ABSTRACT

The aim of this study was (1) to analyze fresh intact or differently influenced rabbit sperm samples, using Annexin V–binding method for the detection of membrane phosphatidylserine translocation, and (2) to estimate obtained results in relation to the conceptional rate of rabbit females fertilized with semen from different groups. Following sample groups were designed: 1. freshly collected semen; 2. semen after 90 min incubation in sperm diluent; 3. semen incubated in cryoprotective medium, without freezing; 4. semen frozen in floating chamber with liquid nitrogen; 5. Semen frozen using classical procedure in manual regime. Higher rate of annexin V-positive (AnV+) cells was observed in both cryopreserved groups (4, 5) when compared with groups 1, 2 and 3 ($p < 0.05$). The highest conceptional rate of rabbit females was obtained, when semen doses from groups 3, 2 or 1 were used for the insemination. Oppositely, conceptional rate was minimal when sperm was frozen in floating chamber (group 4; 16.67%), whilst using the sperm frozen in manual regime (group 5) no one of 13 inseminated females was parturited. A negative linear dependence between the rate of An-V+ cells and female conceptional rate was found. Therefore, Annexin V-labelling would be considered as a technique of choice to predict fertilizing capacity of rabbit sperm.

Key words: rabbit, sperm, annexin V, conceptional rate

INTRODUCTION

A main purpose of any sperm analysis is an exact, fast and objective prediction of fertilizing capacity of the semen. The success of estimation technique used is consisted in the existence of significant correlation between sperm parameter tested and the ability of the sperm to fertilize. One of these methods is a technique of binding of the membrane phospholipid – phosphatidylserine (PS) by fluorescence-labelled annexin V. This method enables the detection of early phase of apoptosis prior to loss of cell membrane integrity. At initial stages of apoptosis, PS migrates from the inner cell membrane to their outer part; this translocation is a primary feature of cells affected by apoptosis. Annexin V, by binding to PS, reveals the cells with early membrane alterations and the cells dead due to apoptosis (Muratori et al., 2004).

In rabbit sperm after the induction of acrosome reaction with progesterone the annexin V- binding sites were found almost only in the acrosomal region, but with number of binding sites in the equatorial area (Avalos-Rodriguez et al., 2004). On the contrary, after acrosome reaction induction with calcium-ionophore A23187, PS translocation was also observed in the post-acrosomal region. Plasma membrane destabilization during capacitation and acrosome reaction may be important for

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sperm-oocyte fusion. Annexin V method was successfully used for the detection of PS translocation in frozen-thawed sperm of bulls (Anzar et al., 2002) and boars (Peňa et al., 2003). Januskauskas et al. (2003) reported that a certain proportion of bull spermatozoa after thawing exhibits PS on their surface, e.g. they have altered membrane functions, and the incidence of such cells is inversely correlated to sperm viability and positively correlated to abnormal chromatin condensation.

The aim of our study was to analyze samples of either fresh or cryopreserved rabbit semen for annexin V- associated membrane changes. These results were estimated in relation to conceptional rates of females following insemination with respective sperm samples.

MATERIALS AND METHODS

Semen collected from rabbit males of M91 and P91 strains, cross-bred of New Zealand White and Californian breeds, was divided into following groups:

1. Fresh semen immediately after collection
2. Semen incubated in semen diluent (1:1) at room temperature for 90 min to reach a capacitation.
3. Semen incubated in a cryoprotective medium (0.5M dimethylsulfoxide + 0.014 M sucrose) at the 1:1 dilution.
4. Semen in 500 µl straws, placed into floating chamber containing liquid nitrogen.
5. Semen mixed with a cryoprotective medium and frozen using manual procedure: 90 min – at 5°C, 20 min – at -6°C, 20 min – at -18°C, plunging into liquid nitrogen.

Sperm samples of all groups were diluted (1:6) in a commercial diluent (DMRS (Minitub, Germany) up to concentration of 38.86 x 10^6 /0.5ml. Thereafter, sperm aliquots were picked up for the Annexin V analysis and the rest of semen was used for the artificial insemination of rabbit does. Females were treated with 25 I.U. PMSG (Sergon, Bioveta, Czech Republic) 48 h prior to insemination. After insemination they were injected with 2.5 µg of synthetic GnRH (Supergestran, Ferring-Léčivá, Czech Republic).

For the Annexin V analysis semen samples were centrifuged at 5000 rpm for 6 min and resuspended in equal volume of HEPES-buffered saline. Semen suspension (5µl) was mixed with 100 µl working solution of annexin V-Fluos (Annexin-V-Fluos staining kit, Roche Diagnostics, Germany) and incubated for 15 min at 37°C. Afterwards aliquot of the semen suspension was placed between microslide and coverslip into 5µl of the anti-fade medium Vectashield containing DAPI fluorescent dye. Staining with annexin V was checked under the Leica fluorescent microscope (Leica Microsystem, Germany) using 488 nm wave-length filter. Sperm with disordered membrane exhibited green fluorescence, whilst live sperm was unstained.

Obtained data were statistically processed using one-way ANOVA and analyzed using Group Probability Comparison test (u-test).

RESULTS AND DISCUSSION

The presence of fluorescent staining by annexin V was observed in following sperm compartments: acrosomal part of sperm head, post-acrosomal segment, sperm membrane along the entire head, an equatorial segment (Fig. 1A-D). Sperm cells, labelled in such a pattern, were considered as annexin V-positive (AnV+) ones (apoptotic). Sperm cells, which did not show a staining of the head, were considered as intact ones (viable, Fig. 1E).

Most of AnV+ sperm was found in groups 4 and 5, i.e. following freezing by both methods (Fig. 2). In fresh (group 1), capacitated (group 2) or incubated in cryoprotective medium (group 3) semen, the rate of AnV+ cells was significantly lower ($p< 0.05$) than in frozen-thawed semen. The sperm, frozen using manual procedure, did not differ significantly from that frozen in floating chamber. In capacitated or cryoprotective-incubated groups the rate of AnV-positive sperm was lower compared to fresh semen, what may be explained by the fact that fresh semen was not mixed with a diluent, as other groups did.

Fertilizing ability of sperm was proved by the results of female parturition following insemination (Table 1.). A highest conceptional rate was recorded when either cryoprotective- incubated or capacitated or fresh semen was used for insemination. Oppositely, using floating chamber-frozen semen, a conceptional rate was minimal; when the semen was frozen using manual procedure no one of 13 inseminated females was parturited.

A range frame scatter plot, which we used for the characterization of interrelations between the rate of apoptotic cells (AnV+) and conceptional rate of females, demonstrates a negative linear dependence between these parameters (Fig. 3). Our results show that the analysis of sperm membrane integrity, on the basis of membrane localization of PS by means of annexin V binding, enables a prediction of fertilizing potential of rabbit sperm.

The method of annexin V labelling has been previously used for the evaluation of vitality of frozen-thawed semen of bulls (Anzar et al., 2002, Martin et al., 2004) and boars (Peňa et al., 2003). These authors indicate that the presence of apoptotic sperm in the semen may be a one of reasons for poor fertility in bulls (Anzar et al., 2002), and the process of cryopreservation triggers a mechanism of apoptosis in bovine sperm. It is assumed that liquid nitrogen vapour and also sperm dilution or equilibration steps are potential initiators of the apoptosis-

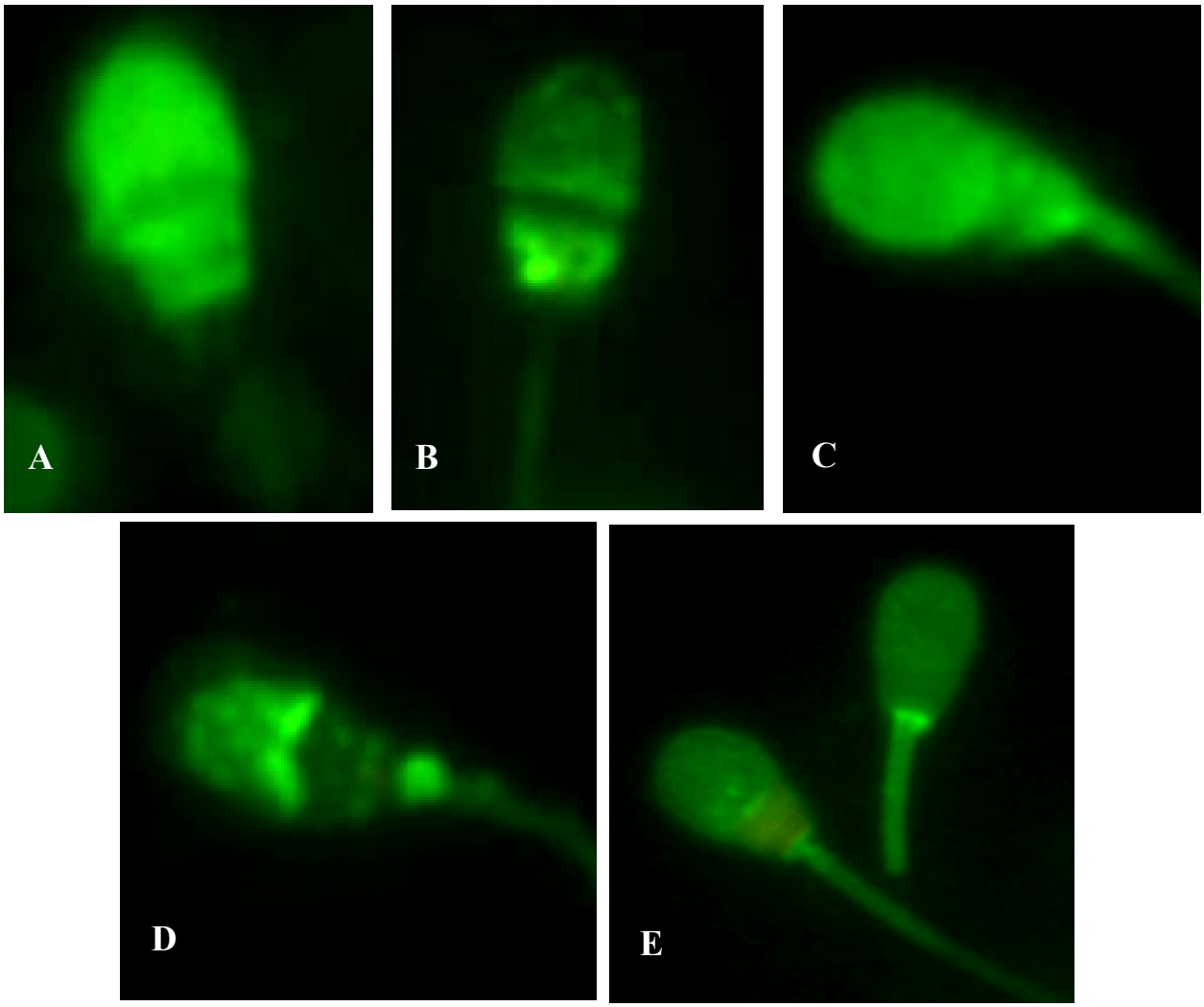
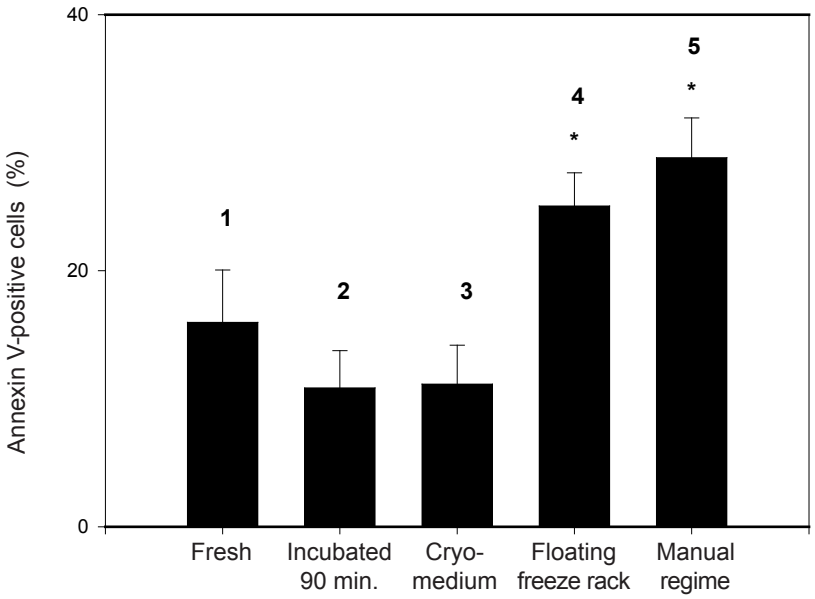


Fig. 1. Presence of Annexin V-positivity in sperm compartments. A: acrosomal part of sperm head; B: post-acrosomal segment; C: sperm membrane along the entire head; D: an equatorial segment of sperm cell; E: viable sperm cells with the unstained head

Table 1: Rabbit female fertility following insemination with differently treated semen

Experimental groups	No. mated	No.parturited does/ conceptional rate, %	Live-born, pieces	Dead-born, pieces/%	Average no. live-born pups/ litter
1. Fresh	72	32/44,44	227	13/5,42	8,41
2. Capacitated	25	14/56,00	118	8/6,35	9,08
3.Cryoprotective medium	26	17/65,38	138	4/2,82	8,63
4. Frozen – floating chamber	36	6/16,67	13	0	6,50
5. Frozen – manual procedure	13	0/0	0	0	0

** - differences versus fresh semen ($p < 0.01$; u-test)



* - differences versus fresh semen ($p < 0.05$; u-test)

Fig. 2. Occurrence of apoptotic sperm in differently treated semen

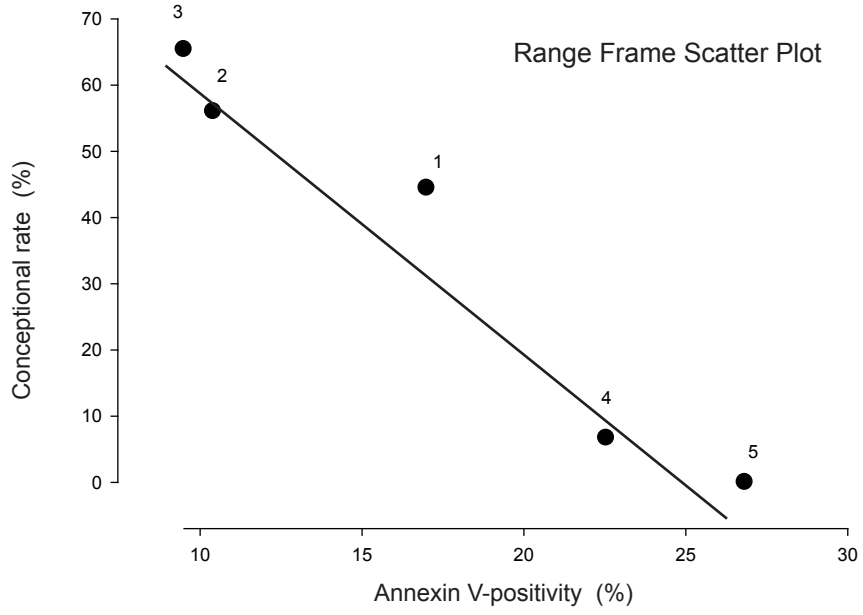


Fig. 3. Interrelationship between Annexin V index and conceptional rate of females fertilized with differently treated semen

like phenomenon (Martin et al., 2004). Live annexin V positive human sperm cells were mainly represented by damaged spermatozoa, as revealed by the occurrence of a negative correlation between PS exposure and normal morphology and motility of the sperm (Muratori et al., 2004). Peňa et al. (2003) analyzed membrane integrity using Annexin V combined with propidium iodide (PI) staining and came to the conclusion that annexin V is able to detect changes in sperm membrane earlier than PI does. Moreover, Annexin V- labelling technique is more sensitive when compared to currently used SYBR-14/ PI method, and it represents a new approach of membrane status determination in sperm.

CONCLUSION

Since a negative linear dependence between a percentage of annexin V- positive sperm and conceptional rate of rabbit does was determined, we may propose the Annexin V – labelling as a method of choice to predict fertilizing potential of rabbit sperm.

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