# ANALYSIS OF PLASMA MEMBRANE INEGRITY BY USING OF FLUORESCEIN LABELED ANNEXIN V AND ITS RELATIONSHIP TO MOTILITY AND VIABILITY OF RAM SPERM AFTER CRYOPRESERVATION

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## ABSTRACT

The present study was conducted to detect sperm apoptosis in fresh, capacitated and frozen ram semen and to determine its relationship with other seminal parameters as motility and viability. The cryopreservation process induces the plasma membrane disruptions, attended with increasing of phoshpatidylserine molecules expression on the extracellular surface, wherefore some of the live spermatozoa loss their biological potential to fertilize the egg.

**KEY WORD: spermatozoa, cryopreservation, phosphatidylserine, Annexin V.**

## РЕЗЮМЕ

Настоящите изследвания имат за цел да се направин анализ на процеса апоптозата при свежи, капацитирани и криоконсервирани сперматозоиди от коч и да се установи взаимовръзката с промените в други сперматологични параметри, като подвижност и преживяемост. Криоконсервацията индуцира нарушения в плазмената мембрана, изразени с нарастване на експерсията на молекули фосфатидилсерин на външната и повърхност, при което някои живи сперматозоиди загубват биологичния си потенциал за оплождане на яйцеклетката.

## ПОДРОБНО РЕЗЮМЕ

Нарушенията в на интегритета на плазмената мембрана при сперматозоидите играе важна роля в процеса на опложданe и това е особено важно след стресови процедури като криоконсервацията. В настоящите изследвания са изследвани трансмембранното движение и нарушенията в подредеността на фосфатидилсерина от плазмената мембрана при свежи, еквилибрирани и криоконсервирани сперматозоиди, чрез прилагане на Анексин V теста. Резултатите показват, че при свежа и еквилибрирана сперма се запазва функционалността на плазмената мембрана в областа на акрозомата, постакрозомата и тялото на сперматозоидите. Има единични сперматозоиди, показващи по-бледа флуоресценция. Двата метода на криоконсервация (С трис-среда – протокол 1 и трис – ПВП среда – протокол 2) водят до значително понижаване в мотилитета на сперматозоидите, както и на процента живи сперматозоиди (An-/6CFDA+), докато нараства процента на апоптичните клетки (An+/6CFDA+), в сравнение със свежите и еквилибрирани сперматозоиди. Като разликите са статитически достоверни ($p<0.05$). Процента на тотално живите сперматозоиди - An-/6CFDA+ намалява от 84.25% за свежата сперма до 18.34% (Протокол 1) и 36. 65% (Протокол 2), като в същото време процента мъртви сперматозоиди нараства до 55.27% (Протокол1) и 29.18% (Протокол 2). От изследваните два протокола за криоконсервация, трис средата с ПВП съхранява в по-висока степен жизнеспособността на сперматозоидите. От изследванията се установява, че не всички живи сперматозоиди имат потенциал да оплодят яйцеклетката, защото при някои от тях ендогенният фосфатидилсерин е представен на външната повърхност на плазмената мембрана (Ann Cy3.18+/6 CF+). Този факт може да даде обяснение за ниската оплодителна споксобност на сперматозоидите от коч след криоконсервация.


## INTRODUCTION

The sperm plasma membrane (PM) is a key structures [16], affected the destructive processes during cryopreservation. The PM molecular components are influenced by different ways to the temperature lowering. The lipids are comparatively sensitive to the low temperature influence, while the proteins are more cryoresistant. I this way the glycolipids and the glycoproteins take an esculent place. The influence of the low temperatures upon the cells membrane structures is connected with the increasing of the phospholipids and cholesterol quantity [1]. This is a result of biochemical transformation of the phospholipids. During the temperature falling the degradation of phospholipids to the mono and dyglicerides, which predispose for constitution of the complex defect as triglyceride – protein is occurred. This complex disintegrates during thawing at a temperature about $50^0$C. It is considered that the forming of the mono and triglyceride is a result of lipid peroxidation. Their presence in PM may lead to changes in the plasma membrane plastic properties, permeability and the cell viability [17, 18]. Furthermore, the lipid phase transitions and phase deviation during cryopresrvation determine one unstable membrane, which predisposes to different structural defects and increased permeability [6].

A temperature diapason between $-4^0$C and $-20^0$C, where the lipid phases transitions from liquid - crystalloid stages to gel stage are occurred take a special place in many investigations [1, 6, 3]. This process is accompanied with lipids phase distribution and takes a special place in the membrane defects forming. It is considered that lipases have initial capability for hydrolysis of lipids to gel condition [3]. In as a result of this the phospholipase activity increased suddenly in conditions of lipid phase transition and these is the reason for the forming of the membrane defects. In this way in erythrocytes it was established regular difference in the phosphlipids loss [5, 8, 12]. The phosphatidylcholine is the most sensitive to the influence of the low temperature, followed from the cholesterol and cardiolipine, while the phosphatidylserine (PS) doesn't change the values almost during cryopresrvation. As it is seen the lipids phase transition attended with their aggregation with proteins, lead to the forming of PM structural defects, which in the hiperosmolarity conditions of the environment, determined the changes of the cells form, together with the macrovasiculation of their membranes [5].

The aim of the present study is to investigate the plasma membrane PS behavior from ram spermatozoa, during cryopresrvation, by using of Annexin V, as marker for PS expression on the extracellular surface of the PM and the connection with other spermatozoa properties.

## MATERIALS AND METHODS

Semen samples were provided from 6 rams and used for experimental analysis. Only ejaculates with good motility >80% and high % of normal morphology >80% were used in the study. The semen samples were frizzed according to two cryoprotective protocols. Following the 1-th protocol, one pert of each semen samples was diluted 1:1 with a cryoprotective medium (glucose 2.01, lactose 8.0, tris 0.280 and 20%egg yolk). The second part was diluted with medium, without egg yolk, but containing

polivinilpirolidon 0.8 (PVP) – Protocol 2. After 210-min equilibration at $4^0$C, the semen samples were diluted with the second parts of the mediums, containing 9% glycerol and were frizzed according to Nagaze - Niwa technology. After thawing at $39^0$C the semen samples were investigated for sperm motility, survival rate and functional integrity of PM. For this purpose the fresh equilibrated and cryopreserved over two protocols sperm cells were suspend to concentration $1x10^6$ cell per ml. With pen marker were drown 2 circles of approximately 0.5 cm diameter on special polypro poly-L-lysine coated slides (Sigma, St. Louis, MO). 25 µl of the each tested cells suspension was placed in each circle. Then the slides were placed at a room temperature allowing the spermatozoa to be absorbed to the plates. After the incubation and washing with binding buffer the double label staining solution, containing 6CFDA and Annexin CY3.18. was used for coloring the sperm samples. The excess liquid was removed by carefully touching to the slide of the circle [13]. The results were observed by light and fluorescence microscope, by using correct filters and light source depending on the label.

## RESULTS AND DISCUSSION

The four groups of semen samples (fresh, equilibrated and cryopreserved over protocol 1 and 2) were characterized for sperm motility, survival rate at $39^0$C and functional integrity of PM (Figire 1). Depending on the various cryostorage protocols used the spermatozoa motility and survival rate decreased significantly compared to fresh and equilibrated semen. The two methods of cryostorage led to a significant decrease of sperm motility, as well as of percentage of live (An-/6CFDA+) spermatozoa, whereas the percentage of apoptotic (An+/6CFDA-) cells increased, compared to fresh and equilibrated semen.

The differences between the percentages of apoptotic spermatozoa in fresh semen and after thawing were statistically significant ($p<0.05$ Figure 1). The persentage of total vital spermatozoa An-/6CFDA+ decreased from 84.25% for fresh semen to 18.34% (Prot 1) and 36. 65% (Prot 2), at the same time the percent of necrotic spermatozoa increased to 55.27% (Prot 1) and 29.18% (Prot 2). Surprisingly the significantly differences between the percent of apoptotic spermatozoa according to the protocol 1 and Protocol 2 of cryopreservation (medium with PVP, $p<0.05$) were registered. The increased necrotic and apoptotic spermatozoa and the decreased number of live sperm cells indicate that almost half of the spermatozoa classified as live, by 6CFDA+ reaction, are effected by deterioration on their plasma membranes, after cryopreservation. During the period of equilibration the sperm motility and the functional integrity of PM protected values near to those of spermatozoa of fresh semen.

The stained with Annexin Cy3.18/6CFDA ram spermatozoa showed a variety of labeling patterns (Figure 2, 3). Those spermatozoa that remained motile were uniformly labeled over the whole head and tail (Figure 2 a) correspond to live – pattern classification. Other patterns observed include uniform staining on the head with strong fluorescence over mid piece and stronger fluorescence over the acrosome and strong fluorescence over whole region of mid piece, or interrupted on some places midpiece. When observed the same fields of the slides under the red fluorescence may be seen that some of the live spermatozoa (Figure 2 b), reacted also with Ann3.18 day, where such cells classified as apoptotic. The spermatozoa that had been cryopreserved showed much reduced green fluorescence in all regional domains (Figure 3 A), in comparison to spermatozoa from fresh semen (Figure 3 a). Moreover, the red fluorescence on the same fields is more intensive (Figure 3 b).

One characteristic feature of the plasma membrane of

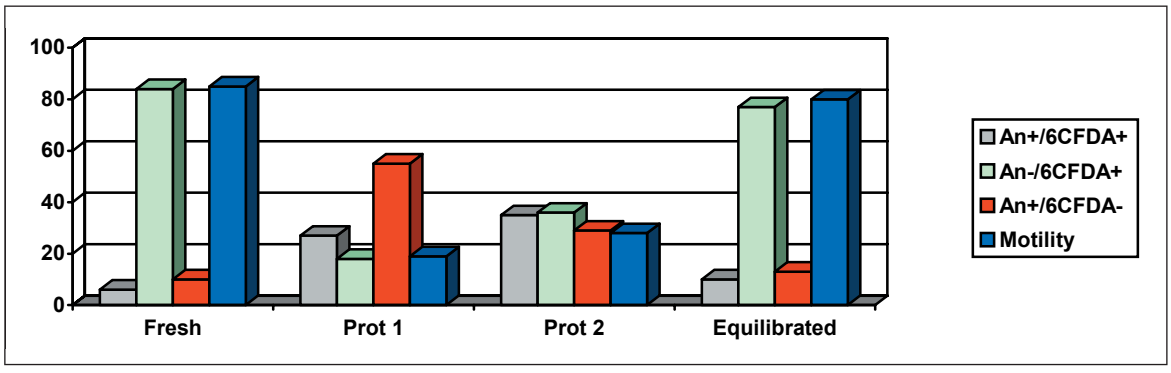


Figure 1. Motility and functional integrity of the plasma membrane in ram spermatozoa before and after cryopreservation.

mammalian sperm cells is the asymmetric transbilayer distribution of lipids that has suggested playing an important role in the fertilization process of spermatozoa [10, 11]. Here, we have investigated the transbilayer movement of PS in the membrane of fresh, equilibrated and cryopreserved ram spermatozoa according to the two cryoprotective protocols to analyze a possible disturbance of the recently characterized transbilayer asymmetry and movement of phosphatidylserine. In order to differentiate population of spermatozoa in the ejaculates, we characterize the expression of PS over the extracellular surface, as marker of early apoptotic cells [9, 13]. Using this assay in fresh and equilibrated semen about 80% of cells was defined as intact cells, while only 6-12% was apoptotic. Following cryopreservation over two protocols, however the relative amount of apoptotic and necrotic cells significantly decreased compared to fresh semen. Moreover, the half part of live cells seems to be apoptotic and this occasionally explaining the low fertility potential of the thawed ram spermatozoa.

After cooling of the biological membrane, a reordering of membrane components is likely [6]. The cryopreservation

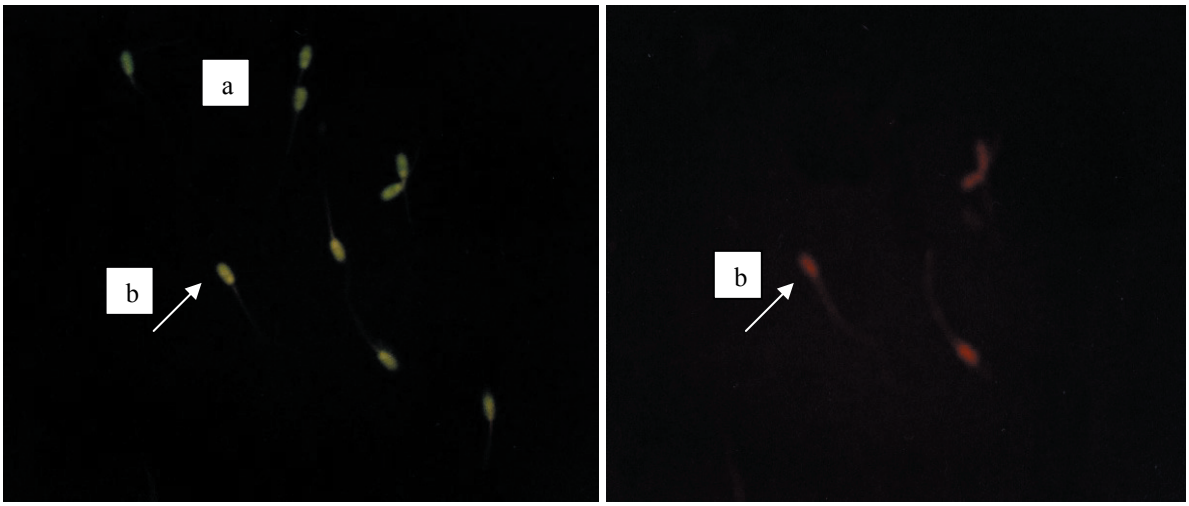


Figure 2. Fresh ram spermatozoa stained by Annexin V test (Ann Cy3.18 - red fluorescence and 6CF - green fluorescence) Different fluorescence patterns observed – a. it is seen strong green fluorescence on head and tail regions typical for live spermatozoa; b – number of spermatozoa, which are stained with the 6CF, were stained at the same time by Ann Cy3.18. These cells are labeled as apoptic.

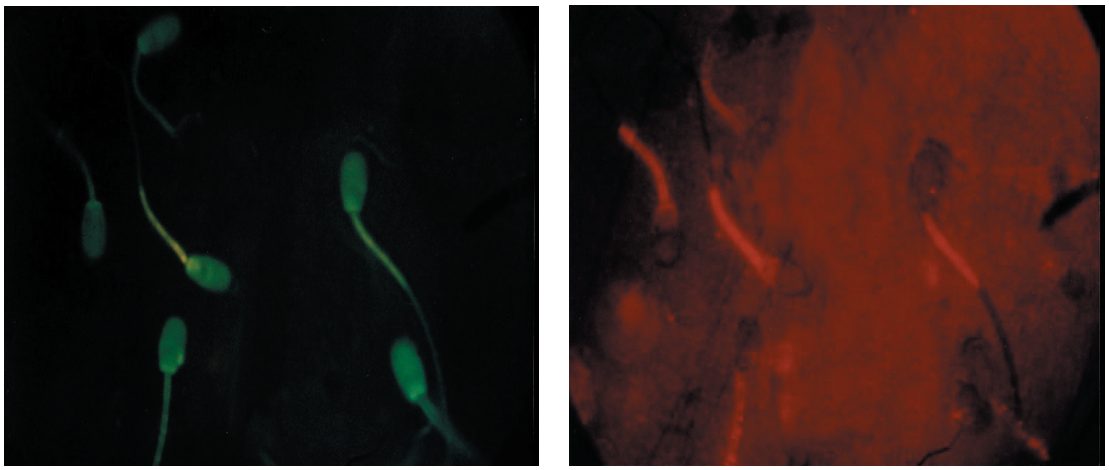

Figure 3. Comparison of green – 6CF (A) and red – Ann Cy3.18 (B) fluorescence in frozen - thawed semen samples. Note that the more of the motile live spermatozoa (green fluorescence - a) were stained by Ann Cy3.18 (strong red fluorescence - b) also.

usually caused sublethal cryodamage to spermatozoa, decreasing post-thaw cell viability [2]. More precise information is needed on the exact proportion of only slightly disturbed spermatozoa and how this ratio might be affected by cryopreservation [7]. Despite different methodological improvements for cryopreservation of ram spermatozoa, the frozen semen has significantly lower fertility than fresh. The present results demonstrate that even when ram spermatozoa survive freezing apparently undamaged, lipid diffusion of their PM is significantly compromised. Such changes, albeit subtle, would have an important iterative effects on the recovery of fertilizing capacity as they would affect phenomena such as spatially depended signaling pathways required for acrosomal exocytosis, development of membrane fusigenicity and antigen migration [14]. It is not clear in present whether the cryoprotectants as egg yolk are having a direct effect or whether low temperature, or both cause the changes. Whatever the reasons, they are not irreversible in spermatozoa cryopreserved in medium with PVP and probably restored the lipid infusibility over the whole membrane to near normal level. PVP has found a range of use as non-penetrating cryoprotectant, for preserving the activity of purified enzymes and for preserving cells sticking none specifically to plastic ware [15]. Its effect in the present context is probably indirect, e.g. such as displacing loosely-bound proteins (most likely seminal plasma proteins) from the surface membrane thereby altering its properties [7].

## CONCLUSION

In conclusion the cryopreservation process induces the PM disruptions, attended with increasing of PS expression on the extracellular surface. The Annexin V – binding assay is more sensitive method and gives additional information for alteration or dysfunctional PM, than other supravital staining techniques. Furthermore, immotile spermatozoa were occasionally stained in the annexin- binding assay, suggesting that not all of the immotile spermatozoa represent irreversibly dead cell [4]. On the other hand not all of the live spermatozoa have potential to fertilize the egg, because in some of them the endogenous PS is given to be expressed over the exoplasmic PM leaflet of impaired cells (Ann Cy3.18+/6 CF+). Additionally the Annexin V – binding assay gives possibility to differentiate all cells subpopulations in the ejaculates. It will be interest in future studies to investigate the effects of other macromolecules on lipid diffusion in low temperature stressed sperm membranes.

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