DETECTION OF PLASMA MEMBRANE PHOSHATIDYLSERINE TRANSLOCATION IN RAM SPERMATOZOA AFTER IN VITRO TREATING WITH DEXAMETAZONE

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ABSTRACT

In vitro induced apoptosis by dexamethazone treating of ram spermatozoa provoked the phospatidylserine exposure which is mainly localized on sperm tail region of plasma membrane and is correlated negatively with the sperm motility and survival rate.

KEY WORDS: spermatozoa, plasma membrane, phosphatidylserine, Annexin V

РЕЗЮМЕ

Ин витро индуцирана апоптоза чрез третиране с дексаметазон на сперматозоиди от коч провокира експресия на фосфатидилсерин, която се локализира главно в областта на опашката на плазмената мембрана и корелира негативно с подвижността и преживяемостта на сперматозоидите.

ПОДРОБНО РЕЗЮМЕ

При нарушение на фосфолипидната асиметрия на плазмената мембрана (ПМ) фосфолипида фосфатидилсерин (ФС) се премества от вътрешния на външния монослой. Това е един от най- ранните белези на клетките, при които е стартиран процесът на клетъчна смърт, но също така е от голямо значение при оплождането на бозайниците. В настоящите изследвания е приложен Анексин V тест за определяне нарушенията в ПМ след ин витро третиране на сперматозоиди от коч с дексаметазон. Първоначалната експресия на молекули ФС на външната повърност на ПМ е много ниска и варира от 2.30%±0.89 за свежите сперматозоиди - контрола до 2.74%±0.65 за третираните с дексаметазон – опитна група. След 6 часовото съхранение тези проценти нарастват на 23.44±1.12 за контролата и 39.83±3.33 за опитната група ($p \leq 0.5$). Експресията на ФС на клетъчната повърхност, изследвана на 6-я час от съхранението корелира негативно с по-ниската подвижност и преживяемост на сперматозоидите rate ($r = -0.37 \pm 0.7$ – за контролана и $r = -0.58 \pm 0.09$ – за експерименталната група). Беше установена специфична локализация на ФС в областта на тялото на сперамтозоидите. При някои сперматозоидит беше засегната цялана ПМ. В заключение, ин витро индуцираната апоптоза чрез третиране на сперматозоиди с дексаметазон провокира експресия на ФС, която е локализирана главно в областта на тялото на сперматозоидите. Нарастването на промени във фосфолипидната асиметрия на ПМ корелира с намаляването на спермалната подвижност и преживяемост и тези факти вероятно са една от причините за слабата оплодително способност при разплодни кочове.

INTRODUCTION

The study of programmed cell death, or apoptosis has emerged as a topic of intense research activity at last years. The big variety of biological processes attended this phenomena are not fully studied, especially in sperm cells. It is known that apoptosis is a form of cell death in which an individual cell undergoes an internally controlled or "programmed" transition from intact metabolically active state into a number of shrunken remnants. This process is attended with a loss of plasma membrane integrity. The detectable characteristics of apoptosis are DNA fragmentation, the loss of mitochondrial integrity, changes in cell size and granularity, changes in plasma membrane permeability and cell surface modification (externalization of PS) and formation of apoptotic bodies [3, 6, 12]. Necrosis and apoptosis are two forms of cell death. Necrosis results from injury affected large number of cells, causing sell swelling and membrane rupture. In spermatozoa these processes are not clearly understand especially after ejaculation. It's known that in adult males, germ cell death during normal spermatogenesis. Some times during spermatogenesis spontaneous apoptosis may observe. The failure to remove apoptotic germ cells results in fall into ejaculates a number of defective (apoptotic) spermatozoa [15].

The integrity of sperm plasma membrane has an important role in realizing of the fundamental spermatozoa purpose – egg fertilization. The assessment of membrane integrity is based on examination of the sperm morphology and motility and hypo-osmotic swelling tests. (9) Staining with combinations of fluorescent dyes is useful for evaluating viability and functionality of the spermatozoa. In the past, Rodamine 123 was used to assess mitochondrial membrane potential and ethidium bromide was used to determine membrane DNA integrity [1]. Later propidium iodide (PI) was combined with other stains as carboxyfluorescein diacetate to evaluate sperm functions. These methods enabled discrimination between live and dead or moribund spermatozoa but did not detect early phases of disturbed membrane functions [5]. During the early stages of membrane dysfunction the phoshpolipid asymmetry is disturbed such as intracellular phospholipide PS is translocated to the extracellular surface of the plasma membrane (13). This PS expression is one of the earliest features of cells undergoing apoptosis [15]. The registration of early stages of apoptosis is exceptionally important for defining of different spermatozoa populations in the ejaculates [16]. One of the typical sign of the cell apoptosis is the translocation of phosphatidylserine (PS), from the inner side of the plasma membrane (PM) to the outer leaflet, by which PS become exposed at the external surface of the cells [10, 11]. The phospholipids scrambling is observed as a result of different cellular phenomena, including cell adhesion and exocytose; during the capacitating process in spermatozoa (4), however it is often considered to be an early indicator of cell apoptosis [10]. While the Weil et al [14] were unable to find any evidence for the involvement of caspases (key apoptotic catalysts) in mouse sperm dead, a number of other recent studies have used the detection of PS exposure in sperm samples as evidence of apoptotic changes.

The aim of the present study is to describe different spermatozoa populations in the ejaculates from fresh sperm and from ejaculates with in vitro induced apoptosis by comparative investigations of PS translocation in sperm PM and the connection of changes in sperm motility and viability.

MATERIALS AND METODS

Semen samples from 10 mature rams were used for investigations. The classical semen parameters, including sperm concentration, motility and morphology were examined after the sperm receiving. The ejaculates were used for experiments after the sperm plasma removing by double washing with PBS. Each ejaculate was divided in to two parts and was diluted in Tris-citric acid diluent - pH 6.8, osmotic pressure – 320 mOsm/kg. To induce apoptosis the part one was incubated with 0.1% dexamethazone solution (Intervet, Netherland) – seria1, the other one was served as a control – sera 2. The sperm survival rate was monitored to the 6-th hour during the semen incubation at 39^0C. The PS expression on the external PM surface was investigated at 10-th min and at the 300-th min for the control and experimental groups. For this purpose the 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). 25 μl of the cells suspension (sera 1 and 2) was placed in each circle. Then the slides were placed at 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 (Sigma) for coloring the sperm samples was used. The excess liquid was removed by carefully touching to the slide of the circle. The results were observed by light and fluorescence microscope, by using correct filters and light source depending on the label.

RESULTS AND DISCUSSION

The investigated semen samples showed concentration of $245\text{-}378x10^7$ sp/ml and motile spermatozoa about 87.8 ± 3.23%. The results from sperm survival are presented on Figure 1. It's obvious that the initial values of sperm motility and survival rate are near in the two groups. The sperm incubation at 39^0C leads to decreasing the % spermatozoa motility and viability, but no significant were registered. However, the spermatozoa from sera 1 – experimental group, showed the lower values of these parameters, compared to spermatozoa from sera 2 – control group.

Depending on the control and experimental group the used of Annexin V/6CFDA test registered the following population of cell in the ejaculates: 6CF+/Ann- - live cells, 6CF+/Ann+ - apoptic cells and 6CF-/Ann+ - dead cells. The 6CFDA – 6-carboxyfluoresceine diacetate was used as supavital stain to distinguish the live and dead sperm cells. When this non-fluorescent compound enters

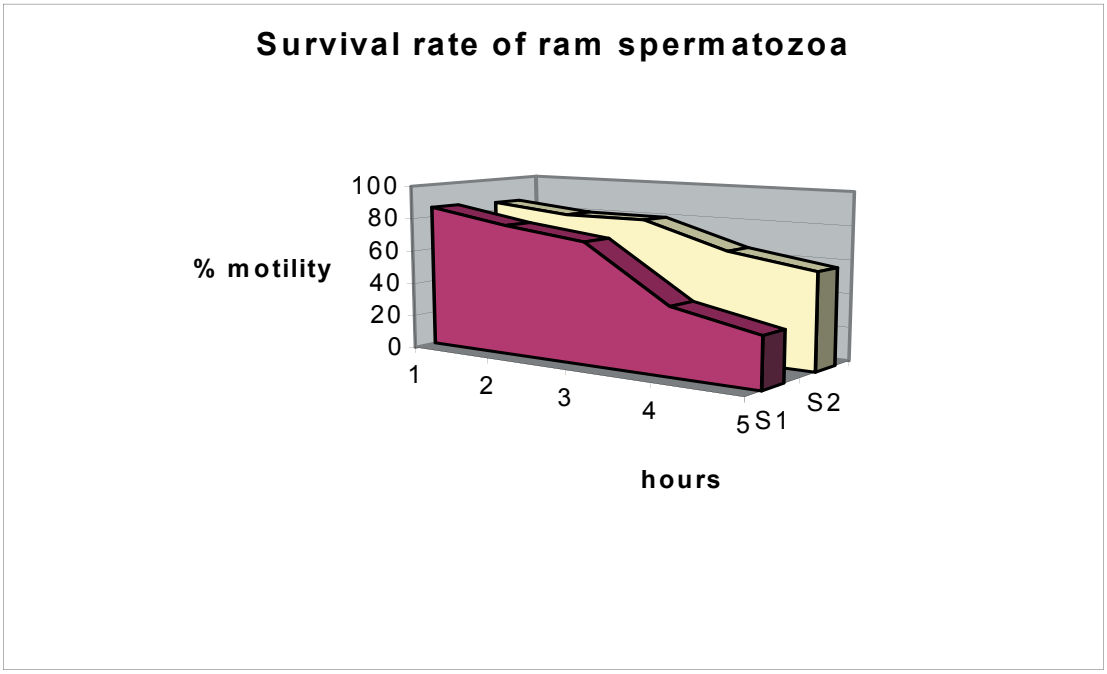

Figure 1. Investigation of ram sperm motility and survival rate of control group – sera 1 and experimental group – sera 2.

living cells, esterase present hydrolyzed it, producing the fluorescent compound 6-carboxyflourescein (6CF). This appears as a green fluorescence. Furthermore, the PS expression on the external PM surface is a sign for starting of the cell's apoptosis process [2]. The cells with PS expression may visualize by labeled with fluorescence conjugate Cy3.18 Annexin V. Under the fluorescence microscope the Ann Cy3.18 bind to the translocated PS macromolecule and observed as a red fluorescence. Live cells are labeled only with 6-CF (green), while dead are labeled only with Ann Cy3.18 (red). Cells in the early stage of apoptosis, however are labeled with both Ann Cy3.18 (red) and 6-CF (green) - Figure 2. Nevertheless, strong correlation between the low motility of sperm cells with in vitro induced apoptosis and specific localization of PS in the region of tail was observed. Some authors report that the surface region of the capacitated spermatozoon in which bicarbonate-induced PS exposure can be seen is the apical head region of PM. This is the region in which the PM and outer acrosomal membrane fuse at multiple foci during the zona pellucida-induced acrozome reaction [4]. In our experiments the presence of dexamethazone induces PS expression over the tail region of spermatozoa PM. Furthermore after the 6-th hour of preservation in some spermatozoa the whole PM was affected. These changes associated with the lower motility and viability of spermatozoa. It is not clearly understand the cellular mechanism and signal way of dexamethazone influence after in vitro treating of spermatozoa.

The sperm protection at 39^{0}C for 6 hours induced PS translocation in a high degree (Table 1). Although the initial sperm motility is protected, the Annexin V binding test gives possibility to differentiate some spermatozoa with started process of cell looses of PM asymmetry.

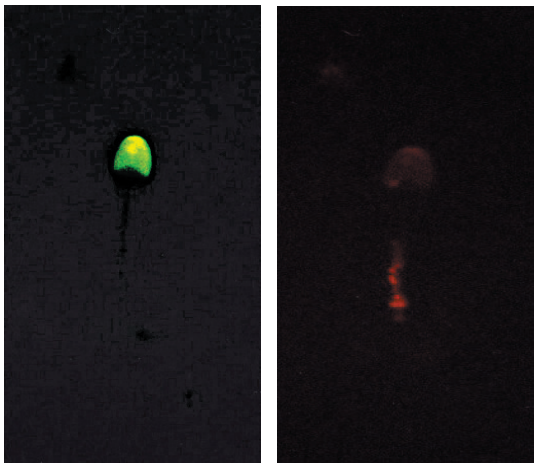

Figure 2. Live ram spermatozoa with PS eternalization - 6-CF (green) and Ann Cy3.18 (red) fluorescence

PS is translocated and exposed on the outer leaflet of PM preliminary in the tail region. When compared the results between fresh and treated with dexamethasone spermatozoa, there were registered insignificant differences in the percent of the apoptotic spermatozoa at the 10-th minute (2.30%±0.89 to 2.74%±0.65). However, after the 6-th preservation at 39^{0}C the percent of apoptotic spermatozoa significantly increased (23.44±1.12 for the controls and 39.83±3.33 for the experimental group), compared to fresh semen - $p \leq 0.5$ (Table 1). Special interest represents the group of spermatozoa with positive 6CF+/Ann+ reaction. It's obviously that to the 6-th hour of preservation the degree of changes in aminophoshpolipid asymmetry increased notability. These changes lead to a collapse of plasma membrane asymmetry suggesting the PS exposure of whole plasma membrane (Figure 3 a, b). In the same time a negative moderate correlation between sperm survival and PS expression in live cells was registered ($r = -0.37 \pm 0.7$) in the control group, while in the experimental group this negative correlation was significant ($r = -0.58 \pm 0.09$).

It is supposed that the sperm PM is disturbed and the first signs of membrane dysfunction are started, subsequently led to cell's death. Although the high primary values of live spermatozoa with protected motility in the two groups – control and experimental were registered, after the 6-th hour preservation at 39^{0}C, occurred significant differences in these parameters. It is make an impression that the increasing of cells with PS scrambling is at the expense of decreased motile spermatozoa. In the same time the percent of dead cells in the control and experimental groups are with nearly values. Moreover, in ejaculates with near values of the sperm motility and viability, there are a high variety of different cell populations [7]. The results implicate that a considerable number of spermatozoa might exist with altered and possibly dysfunctional plasma membrane besides moribund or dead cells. Such membrane disturbance accompanied with PS translocation on whole the external PM surface means for starting of the cell dysfunction [2, 12].

CONCLUSION

Functional assay of plasma membrane integrity can potentially characterize the quality of spermatozoa. Several tests have been report for evaluation of PM as supravital techniques [1, 5], or the hypo-osmotic test [9]. These methods can discriminate viable from death spermatozoa or damaged, but not monitor early phases of membrane dysfunction or initial phases of apoptosis, like Annexin V – binding assay.

In conclusion, the present Annexin V test may use

as prognostic assay for fertilizing potential of the spermatozoa. The Annexin V- binding assay seems to provide additional information about sperm PM deterioration besides conventional motility analysis and supravital staining. Furthermore, not all of motile spermatozoa were occasionally stained with Ann Cy3.18, but were stained with 6-CF suggesting that the process of cell death may be visualized during different preserving conditions. In other hand the in vitro induced apoptosis by dexamethazone treating of spermatozoa provoke PS exposure which is mainly localized on sperm tail region. The increasing of the changes in aminophospholipide asymmetry of PM correlated with the decreasing of sperm motility and survival rate. This event seems to be important for prognostic the semen quality to reduce the risk of using poor-fertility rams in artificial insemination programs.

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REFERENCES

1. Evenson D. P., Darzynkiewicz Z. and Melamed M. R. Simultaneous measurement by flow cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. (1982) J. Histochem. Cytochem. 30, 279-280.

2. Fadok VA, Voelker DR, Campell PA, Bratton DL, Henson PM. Exposure of phosphatilserine on the surface of appoptic lymphocytes triggers specific recognition and removal by microphages. (1992)J Imunol 148: 2207-11.

3. Fadok VA, de Cathelineau A, Daleke DL, Henson PM, Bratton DL. Loss of phosphatidylserine is required for phagocytosis of apoptic cells by macrophages and fibroblasts. (2001) J Biol Chem 276(2): 1071-77.

4. Gadella BM, RAP Harrison. Capacitation induces Cyclic Adenosine 3'. 5' – Monophosphate – Depend, but Apoptosis – unrelated, Exposure of Amonophospholipids at the Apical Head Plasma Membrane of Boar Sperm Cells. (2002) Biology of Reproduction, 67, 340-350..

5. Garner D. L., Pinkel D., Johnson L. A. and Pace M. M. Assessment of spermatozoal function by using

Table 1. Functional integrity of sperm plasma membrane, measured by Ann Cy3.18/6 CF fluorescence test (n 9)

Group	6CF+/Ann- Live	6CF+/Ann+ Apoptic	6CF-/Ann+ Dead
		10-th minute	
Control	90.17±5.75	2.30±0.89	6.53±2.13
Expreimental	92.58±6.39	2.74±0.65	4.68±1.98
		6-th hour	
Control	52.40±7.80	23.44±1.12*	24.16±4.62
Experimental	37.80±6.75	39.83±3.33	22.40±3.9

*Significant at $p \leq 0.05$ confidence level – between vertical rows.

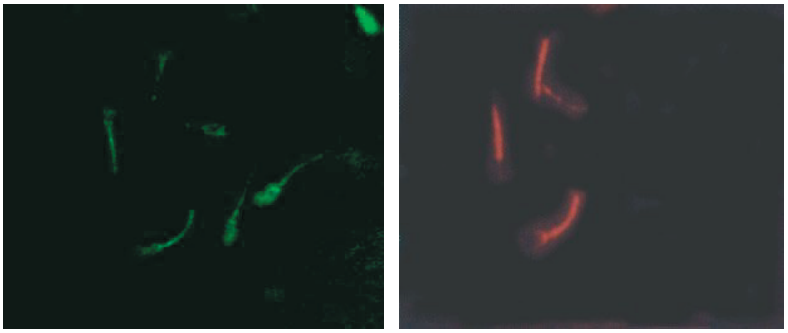

Figure 3. Localization of PS surface exposure over tail region of sperm plasma membrane

dual staining and flow cytometric analyses.1986) Biol. Reprod, 34, 127-138.

6. Hengartner OM. The biochemistry of apoptosis. (2000) Nature V 407, 10: 770-76.

7. Holt WV. Membrane heterogeneity in mammalian spermatozoon. (1984) Int Rev Cytol 87:159-94.

8. Huber R, Romisch J, Paques EP. The crystal and molecular structure of human annexin V anticoagulant protein that binds to calcium and membranes. (1990) The EMBO Journal 9: 3867-74.

9. Jeyendran R. S., Van der Ven, H. H., Perez-Palaez M. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. (1984) J. Reprod. Fertil, 70, 219-228.

10. Martin S. J., Reutelingsperger, C. P. M., McGahon A. J. et al. Earlier distribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of initialing stimulus: inhibition by over expression of Bcl-2 and Abl. (1995) J. Exper. Med, 182, 1545-1556.

11. Muller K, Pomorski T, Muller P, Zachowski A, Herrmann A. Protein-dependent translocation of aminophospholipids and asymmetric transbilayer distribution of phospholipids in the plasma membrane of ram sperm cells. (1994b) Biochemistry 33: 9968-74.

12. Op de Camp JAF. Lipid asymmetry in membranes. (1979) Annu Rev Biochem 48: 47-62.

13. Vermes I., Haanen C. and Reutelingsperger C. P. M. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescence labeled annexin, (1995) J Immunol Methods 180 39-52.

14. Weil M, Jacobson MD, Raff MC. Are caspases involved in the death of cell with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes. (1998) L Cell Sci, 111, 2707-2715.

15. Print CG, Loveland KL. Germ cell suicide: new insights into apoptosis during spermatogenesis. (2000) Bioassays 22, 423-430.

16. Barroso G, Morshedi M, Oehninger S, Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. (2000) Hum. Reprod. 15: 1338-1344.