CELL DEATH DIFFERENTIATION IN BLACK HEADED RAMS SPERMATOZOA, USING FLUORESCENT LABELED ANNEXIN V

ОПРЕДЕЛЯНЕ А ЕКСПРЕСИЯТА НА ФОСФАТИДИЛСЕРИН ПРИ СПЕРМАТОЗОИДИ В РАННА АПОПТОЗА ЧРЕЗ ФЛУОРЕСЦЕНТНО БИЛЯЗАН АНЕКСИН V

M. Ivanova-Kicheva, A. Kukov, D. Daskalova, E. Solenkova, I. Nikolov, L. Todorov

Institute of Biology and Immunology of Reproduction “Acad. Kiril Bratanov” – Bulgarian Academy of Sciences, Sofia Bulgaria

Manuscript received: February 15, 2008; Reviewed: April 20, 2008; Accepted for publication: April 20, 2008

ABSTRACT

Double staining kit of Annexin V Cy3.18/6-CFDA was used to investigate the changes in phospholipide asymmetry after treating sperm cells with dexamethasone. The % of spermatozoa with registered translocation of PS in treated with dexamethazone groups at the 10-th min and in control no treated varied from 2.74%±0.65 to 2.30%±0.89. After the 5 hour of incubation these % increased to 39.83±3.33 for the treated group and 23.44±1.12 for the control. It was concluded that Annexin V binding assay is more sensitive in the detection of deterioration in membrane function than other conventional methods such as motility analysis and supravital techniques.

Key words: spermatozoa, plasma membrane, and phosphatidylserine

РЕЗЮМЕ

Приложен е кит за двойно оцветяване с Анексин V Cy3.18 и 6-CFDA, даващ възможност за диференциране на апоптични от мъртви клетки.

Процентът сперматозоиди с регистрирана експресия на ФС при свежи нетретирани сперматозоиди – контролна проба и експериментални, третирани с дексаметазон на 10-тата минута, варира от 2.30%±0.89 до 2.74%±0.65. След съхранение, на 5-я час този процент нараства до 23.44±1.12 за контролата и до 39.83±3.33 за опитната група. С настоящето изследване се доказва, че Анексин V теста е по-чувствителен за определяне промените в ПМ, в сравнение с конвенционалните методи на изследване за подвижност и преживяемост на сперматозоидите.

Ключови думи: сперматозоид, плазмена мембрана и фосфатидилсерин

РАЗШИРЕНО РЕЗЮМЕ

При нарушаване на фосфолипидната асиметрия на плазмената мембрана (ПМ), настъпва транслокация на фосфолипида фосфатидилсерин (ФС) от вътрешния на външния монослой. Това е един от най-ранните белези при клетки, в които е стартиран процеса на клетъчна смърт. Използването на Анексин V – плацентарен антикоагулантен протеин, позволява този процес да се наблюдава, тъй като самият протеин има изразен афинитет на свързване в присъствие на Ca2+ йони към негативно заредения фосфолипид ФС. Белязането на Анексин V с флуоресцентни флуорохроми позволява да се наблюдават най-ранните процеси съпровождащи мембранната дисфункция. Прилагането в настоящето изследване на кит за двойно оцветяване с Анексин V Cy3.18 и 6-CFDA, дава възможност за диференциране на апоптични от мъртви клетки. Процесът апоптоза е индуциран чрез in vitro третиране на сперматозоиди с дексаметазон. Процентът сперматозоиди с регистрирана експресия на ФС при свежи, нетретирани сперматозоиди – контролна проба и експериментални, третирани с дексаметазон на 10-тата минута, варира от 2.30%±0.89 до 2.74%±0.65. След съхранение, на 6-я час този процент нараства до 23.44±1.12 за контролата и до 39.83±3.33 за опитната група. Нарастването на броя на апоптичните сперматозоиди води до промени в молекулната организация на ПМ. С настоящето изследване се доказва, че Анексин V теста е по-чувствителен за определяне промените в ПМ, в сравнение с конвенционалните методи на изследване за подвижност и преживяемост на сперматозоидите.

INTRODUCTION

The investigation of apoptosis in sperm cells in connection to determine different cell's types is very actually at last years. There are a big variety of biological processes attended this phenomena which are not fully understood. 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 that retain their membrane bound integrity. The detectable characteristics of apoptosis are DNA fragmentation, 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].

The biological role of sperm plasma membrane is an important in the realizing of the spermatozoa – egg fertilization process. 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. Rhodamine 123 is used to assess mitochondrial membrane potential and ethidium bromide is used to determine membrane integrity [1]. Later propidium iodide (PI) was combined with other stains such as carboxyfluorescein diacetate to evaluate sperm functions. These methods enabled discrimination between live and dead spermatozoa but did not detect early phases of disturbed membrane functions [5]. During the early stages of membrane dysfunction the phoshpolipid asymmetry is disturbed and the intracellular phospholipide PS is translocated to the extracellular surface of the plasma membrane [13]. This PS translocation is one of the earliest features of cells undergoing apoptosis. The registration of early stages of apoptosis is exceptionally important for defining of different sperm cell's types in the ejaculates. One of the typical signs of the cell apoptosis is the translocation of PS from the inner leaflet of the PM to the outer leaflet, by which PS become exposed at the external surface of the cells [4, 10, 11].

By using of fluorescent-labeled Annexin V, which binds specifically PS in the presence of Ca^{2+} ions, early stages of apoptosis may be determined [8]. The double staining by using of Annexin V – Cy3.18 together with fluorescence stain for living cells 6-Carboxyfluorescein diacetate (6-CFDA) give possibility to divide the following cell groups: live cells, necrotic cells and cells in early phases of apotosis.

The aim of the present study is todetermine different types of spermatozoa with in vitro induced apoptosis after dexamethazone treatment using of double staining Annexin V Cy3.18/6CFDA kit, with a view to make a differentiation between apoptosis and necrosis.

MATERIALS AND METODS

Semen samples from 10 mature rams (breed - Black headed) 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 sample was divided into two parts. To induce apoptosis the part one was incubated with 0.1% dexamethasone solution (Sigma D 2915) diluted in PBS – treated samples, the other one served as control – nontreated, only with PBS. The sperm survival rate was monitored until the 5-th hour during the semen incubation at 37^0C. The PS expression on the external PM surface was

Table 1. Analysis of ram sperm plasma membrane phospholipide asymmetry, by Annexin V Cy3.18/6 CFDA kit (n 9)

Group	6CF+/Ann- Live	6CF+/Ann+ Apoptotic	6CF-/Ann+ Necrotic
	nontreated		
10th min	90.17±5.75	2.30±0.89	6.53±2.13
300 min	52.40±7.80	23.44±1.12[a]	24.16±4.62
	treated		
10th min	92.58±6.39	2.74±0.65	4.68±1.98
300 min	37.80±6.75	39.83±3.33[b]	22.40±3.94

Mean ± SEM
Difference between a-b, $p<0.05$

investigated at 10-th min and 300-th min after treating with dexamethasone. For this purpose the sperm cells were suspend to concentration $1x10^6$ cells per ml. With pen marker were drown 2 circles of approximately 1 cm of diameter on poly-L-lysine precoated slides. 25 µl from each sample (treated and non-treated) was placed in each circle. Then the slides were placed at room temperature allowing the spermatozoa to be absorbed to the plates. After incubation and washing with binding buffer a double staining solution, containing 6CFDA and Annexin V Cy3.18 was used for labeling the sperm cells. The excess liquid was removed by carefully touching with filter paper to the end of the circle [1]. The results were analyzed at a magnification of x 400 by fluorescence microscopy with an ultraviolet filter (Laborlux Leika). At least 100 sperm cells were counted per slide. By fluorescence microscopy, 6-CFDA is observed as green fluorescence and Ann VCy3 as red. Three types of fluorescence are observed: a) live, normal cells that stain only with 6-CFDA (green); b) live cells with translocation of membrane phosphatidylserine that stain with both 6-CFDA (green) and Ann VCy3 (red); and c) dead cells that stain only with Ann VCy3 (red).

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 motility and survival rate are presented on Figure 1. It is obviously that the initial values of sperm motility and survival rate are near in the two groups. The incubation of samples at 37^0C lead to decreasing of the sperm motility and viability, but no significant difference was registered. However the spermatozoa from nontreated samples showed the lower values of these parameters, compared to the treated sperm cells.

Depending on the nontreated and treated groups the used of Annexin V/6CFDA test registered the following cell types in the ejaculates: $6CF^+$/Ann V^- - live cells, $6CF^+$/Ann^+ - apoptotic cells and $6CF^-$/Ann^+ - necrotic cells. 6-Carboxyfluoresceine diacetate was used as a supravital stain to distinguish the live from dead sperm cells. When this non-fluorescent compound enters living cells, esterases present hydrolize it, producing the fluorescent compound 6-carboxyflourescein (6CF). This appears as green fluorescence. Furthermore the PS expression on the external PM surface is a sign for starting of the cell's apoptosis [2]. The cells with PS translocation can be visualized by labeling with Annexin V Cy3.18. Annexin V Cy3.18 can bind to the translocated PS molecules and this may be observed as red fluorescence under the fluorescence microscope, Live cells are labeled only with 6-CF (green), while necrotic are labeled only with Ann V Cy3.18 (red). Cells in the early stage of apoptosis however are labeled with both Ann V Cy3.18 (red) and 6-CF (green) - Figure 2 – A, B, C.

Incubation of sperm samples at 37^0C induced PS translocation in a high degree (Table 1). Although the preliminary sperm motility is preserved, the Annexin V binding test gives possibility to differentiate some spermatozoa with started process of cell's destruction. When compared results between (treated and nontreated) at the 10th min with dexamethason spermatozoa, there were registered insignificant differences in the percent of the apoptotic spermatozoa (2.30%±0.89 to 2.74%±0.65). However, after incubation at 37^0C the percent of apoptotic spermatozoa significantly increased in treated samples (39.83±3.33 for the treated group and 23.44±1.12 for the controls), compared to control samples.

Special interest represents the group of spermatozoa with positive $6CF^+$/Ann V^+ reaction (Figure 2 A,). It is supposed that the sperm PM is disturbed and the first signs of membrane dysfunction are started, subsequently leading to cell's death. Although the high primary values of live spermatozoa with good rate of motility in the two groups – control and experimental, after the 5-th hour incubation at 37^0C, occurred significant differences in these parameters. It is make an impression that the

increasing of apoptotic cells is at the expense of decreased motile spermatozoa. In the same time the percent of necrotic cells in the control and experimental groups are with nearly values.

These results of the present study showed that the Annexin V binding test might differentiate with a high precision the processes carried out in spermatozoa after ejaculation. 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

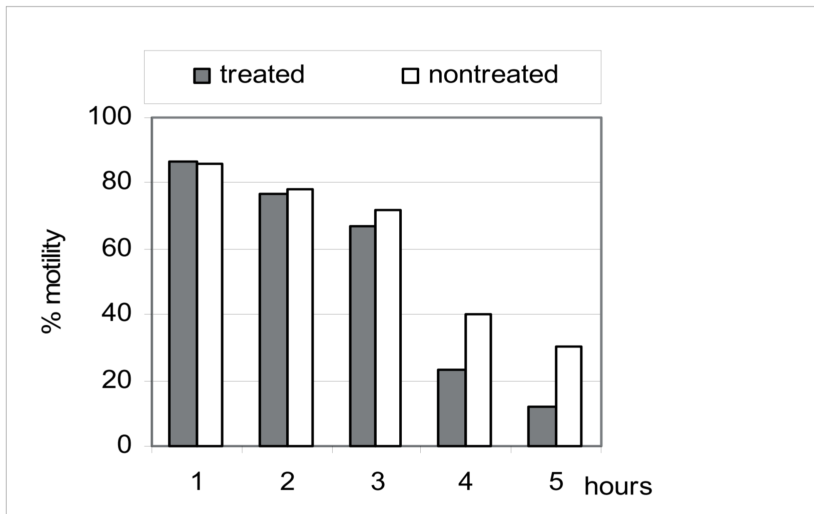

Figure 1. Sperm motility and survival rate of ram spermatozoa treated with dexamethasone (n=9)

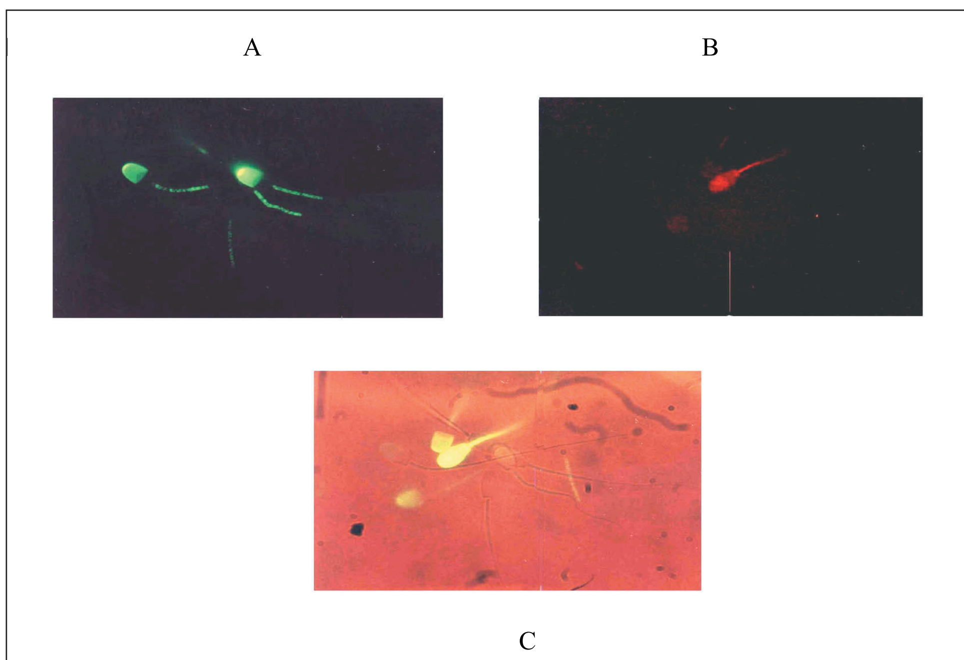

Figure 2. The different fluorescence patterns observed under fluorescence microscope of ram spermatozoa. The semen samples with induced apoptosis showed in live spermatozoa strong green fluorescence - A, while the death spermatozoa are with slight light. At the same time under the red fluorescence all spermatozoa had strong staining on the head, mid-piece and tail - B. Uniform staining over the head and tail region is typical for live spermatozoa. The red fluorescence over the all subdomens of the spermatozoa and the strong green staining only on the acrosome or equatorial segment - means that the plasma membrane disruption was started - C

a considerable number of spermatozoa might exist with altered and possibly dysfunctional plasma membrane besides the dead cells. Such membrane disturbance accompanied with PS translocation on the external PM surface means for starting of the cell destruction [2, 12].

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.

CONCLUSIONS

The present Annexin V – binding assay may use as prognostic for fertilizing potential of the spermatozoa. The Annexin V- binding assay seems to provide additional information about sperm 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.

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