

FLUORESCENT ANALYSIS OF TRANSGENIC AND NON-TRANSGENIC RABBIT SPERMATOZOA

P. CHRENEK^{1,2*}, A.V. MAKAREVICH¹, M. SIMON³

¹Animal Production Research Centre Nitra, Slovak Republic; ²Slovak University of Agriculture, Nitra, Slovak Republic; ³Institute of Animal Biochemistry and Genetics, Slovak Academy Science, Ivanka pri Dunaji, Slovak Republic

ABSTRACT

The aim of our study was to compare viability of sperm cells from transgenic (mWAP-hFVIII gene) or non-transgenic (normal) rabbit males using fluorescent analyses (SYBR-14/PI and annexin V/DAPI tests). These results were estimated in relation to conceptional rates of females following insemination with respective sperm samples.

In both, transgenic and non-transgenic groups, the highest proportion of annexin V positivity was recorded in head-tail joint (84.66 vs. 68.84%) of spermatozoa. The percentage of the sperm stained with SYBR-14/PI or with annexin V/DAPI was correlated with liveborn rate in transgenic ($R^2=0.6118$ and $R^2=0.2187$, resp.) or non-transgenic ($R^2=0.671$ and $R^2=0.3579$, resp.) rabbits.

These data indicate that the viability of rabbit transgenic and non-transgenic spermatozoa could be reliably quantified using viability (SYBR-14/PI) and apoptosis (annexin V/DAPI) tests.

Key words: transgenic rabbit; spermatozoa; SYBR-14; annexin V

INTRODUCTION

Effect of transgenesis on reproductive traits of rabbit males (occurrence of malformed spermatozoa, histological structure of the testis) was previously reported (Chrenek et al., 2006; 2007a; 2007b). Viability of spermatozoa may be evaluated on the basis of membrane integrity. The principle of the test is a differential staining of live and dead cells with fluorescent dyes SYBR-14 and propidium iodide (PI) (Garner and Johnson, 1995). SYBR-14 labels only viable cells, whereas PI labels only the cells with damaged membranes. Combination of these two dyes can provide a differentiation between live or dead cells.

More detailed analysis of mammalian spermatozoa for plasma membrane destabilization using

Annexin V was reported in fresh bull spermatozoa (Januskauskas et al., 2003) and in cryopreserved rabbit spermatozoa (Makarevich et al., 2008). This assay is based on the observation that after initiation of apoptosis, phosphatidylserine (PS) an obligatory element of the inner part of the cell cytoplasmic membrane migrates from the inner site of the membrane on the cell surface (Vermes et al., 1995). Fluorescently labelled annexin V can bind externalized PS and label membranes of apoptotic cells. This method enables the detection of early phase of apoptosis prior to loss of cell membrane integrity.

The aim of our study was to compare viability of transgenic and non-transgenic rabbit spermatozoa using test of viability (SYBR-14/PI) and apoptosis (annexin V/DAPI) and to evaluate these parameters in relation to conceptional rate of females following insemination.

*Correspondence: E-mail: chrenekp@yahoo.com
Peter Chrenek, Animal Production Research Centre Nitra, Hlohovecká 2,
95141 Lužianky near Nitra, Slovak Republic
Tel.: +421 37 6546 285 Fax: +421 37 6546 189

Received: July 16, 2010
Accepted: August 3, 2010

MATERIALS AND METHODS

Animals

Transgenic founders with the WAP-hFVIII gene were produced as described by Chrenek et al. (2005). Randomly selected transgenic (n=10) and non-transgenic (n=10) males of the same breed (New Zealand White) and age were used. The males were housed in individual cages, under a constant photoperiod of 14 h of light day. Temperature and humidity in the building were recorded continuously by means of a thermograph positioned at the same level as the cages. The rabbits were fed *ad libitum* with a commercial diet and water was provided *ad libitum* with nipple drinkers.

Semen collection and analysis

Semen was collected using an artificial vagina, twice per week (75 transgenic and 75 non-transgenic ejaculates were analyzed). We evaluated each sample of fresh ejaculate for concentration and motility by visual inspection as a percentage of straight moving spermatozoa).

Spermatozoa viability test (SYBR-14/PI)

Alive spermatozoa were identified basing on membrane integrity using staining with Live/Dead cell kit (SYBR-14, Molecular Probes Inc., Eugene, OR, USA) as described by Garner and Johnson (1995). Semen samples were diluted 1:10 in a HEPES-buffered saline (HBS) (10 mM HEPES, 150 mM NaCl, 1% BSA, pH 7.4). Working solution of SYBR-14 was prepared by 50-dilution of SYBR-14 stock in HBS. 5 µl of SYBR-14 working solution was added to 1 ml of diluted spermatozoa sample (final concentration 100 nM) and incubated at 37 °C for 5-10 min. Thereafter, 5 µl of propidium iodide (final concentration 12 µM) was added to spermatozoa sample and incubated for further 5-10 min.

Test for apoptosis (annexin V/DAPI)

For the annexin V analysis semen samples were centrifuged at 2000 rpm for 6 min and resuspended in equal volume of annexin V binding buffer (supplied with the kit). Semen suspension (5µl) was mixed with 100 µl of working solution of annexin V-Fluos (Annexin-V-Fluos staining kit, Roche Diagnostics, Germany) and incubated at 37 °C for 15-25 min. Afterwards aliquots of the semen suspension (5 µl) were placed between microslide and coverslip into 5µl of the Vectashield anti-fade medium containing DAPI fluorescent dye. Staining with annexin V and DAPI was checked under a Leica fluorescent microscope (Leica Microsystem, Germany) using 488 nm or 420nm wave-length filters, respectively. The spermatozoa with the annexin V-positive membrane exhibited green fluorescence, whilst total spermatozoa count was identified by blue signal due to DAPI staining.

Insemination

Transgenic or non-transgenic spermatozoa were diluted in a commercial diluent (Minitub, Tiefenbach, Germany) up to the concentration minimum $14 \times 10^6/\text{ml}$ and used for the insemination of hormonally (PMSG and HCG, Werfaser and Werfacher, Austria, Chrenek et al., 2007a) stimulated New Zealand White rabbit females. Conceptional rate was estimated by pregnancy diagnosis at 15 days after insemination.

Statistical analysis

Obtained data on conceptional rate were statistically processed using one-way ANOVA and analyzed using Group probability Comparison test. Differences between related traits were analyzed by linear regression analysis with graphical representation.

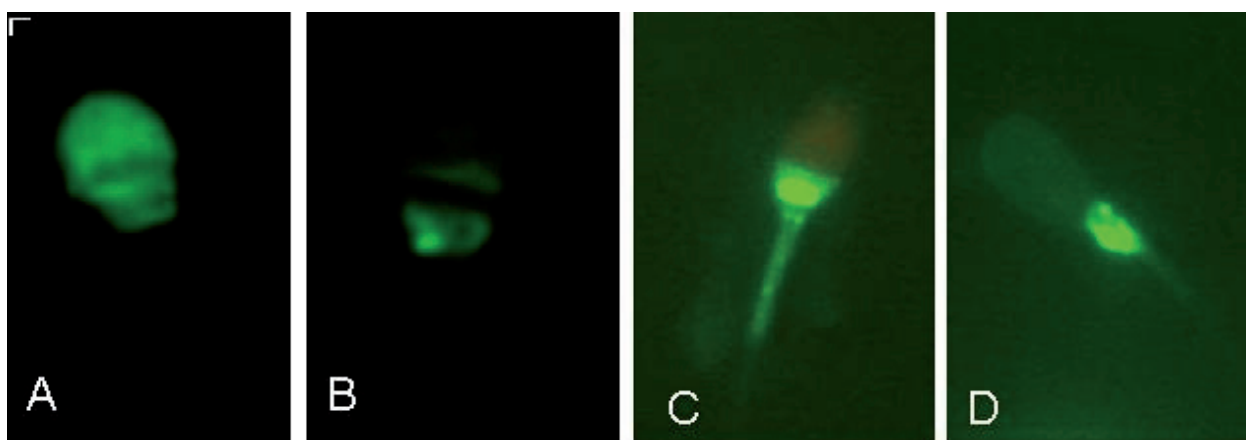


Fig. 1A-D: Annexin V positive staining in different rabbit sperm compartments: acrosomal part of the sperm head (A), post-acrosomal part (B, C), and a proximal cytoplasmic droplet (D)

RESULTS

No differences between transgenic (63.05 %) and non-transgenic (65.75 %) groups in the percentage of viable (SYBR14-positive) sperm were observed. Similarly, a percentage of apoptotic (annexin V-positive) sperm in the transgenic group (5.23 %) did not differ from those in the non-transgenic group (6.41%). Annexin V positive staining was observed in the following sperm compartments: acrosomal part of the sperm head, post-acrosomal part of the head and a proximal cytoplasmic droplet (Fig. 1A-D). Sperm cells, labelled in such a pattern, were considered as annexin V positive ones (apoptotic). Sperm cells, which

did not show any staining of the head, were considered as intact ones. Significant differences ($p < 0.05$) between transgenic and non-transgenic spermatozoa were found in the post-acrosomal part.

Based on linear regression analysis, we found a positive correlations between sperm viability and rate of liveborn ($R^2=0.6118$; Fig. 2a), and between sperm apoptosis and liveborn rate ($R^2=0.2187$; Fig. 3a) in transgenic rabbit. The similar correlations between liveborn and sperm viability ($R^2=0.671$) as well as between liveborn and apoptosis ($R^2=0.3579$) were observed in case of non-transgenic rabbit sperm (Fig. 2b and 3b).

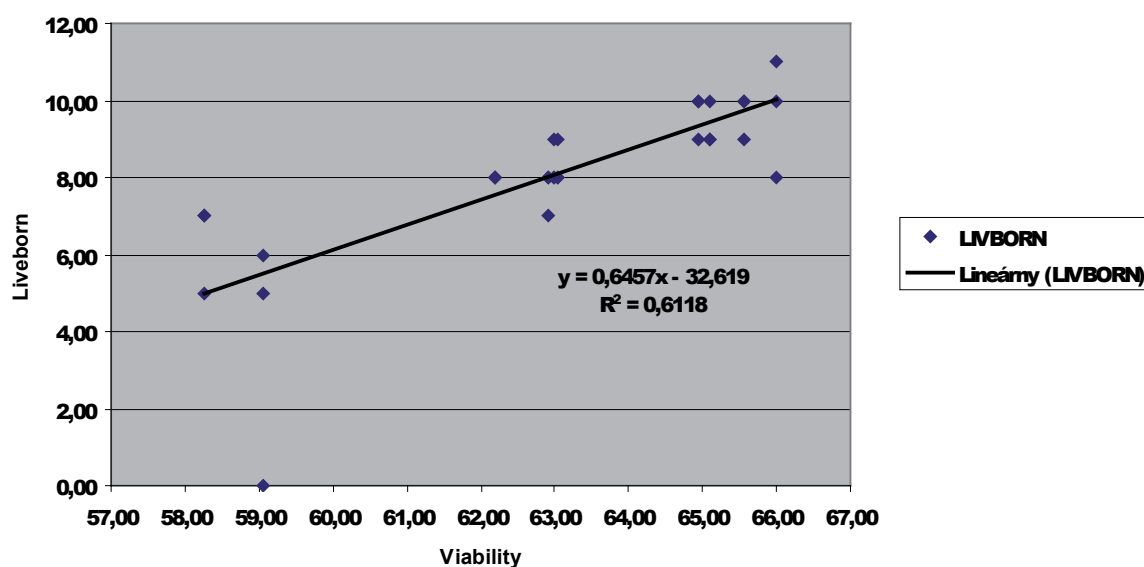


Fig. 2a: Linear regression of liveborn rate on viability in transgenic rabbits

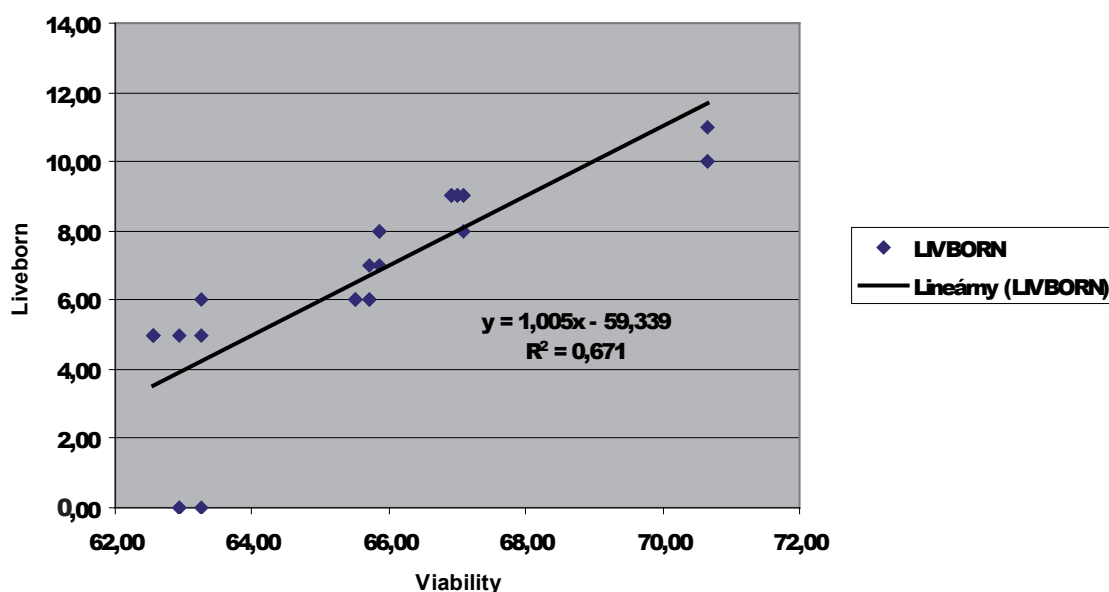


Fig. 2b: Linear regression of liveborn rate on viability in non-transgenic rabbits

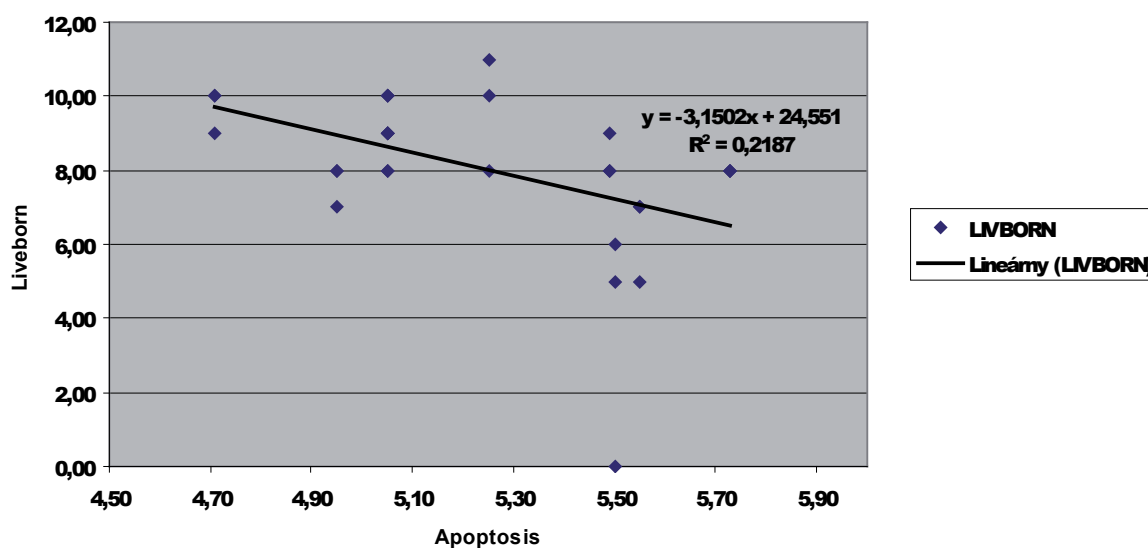


Fig. 3a: Linear regression of liveborn rate on apoptosis in transgenic rabbit

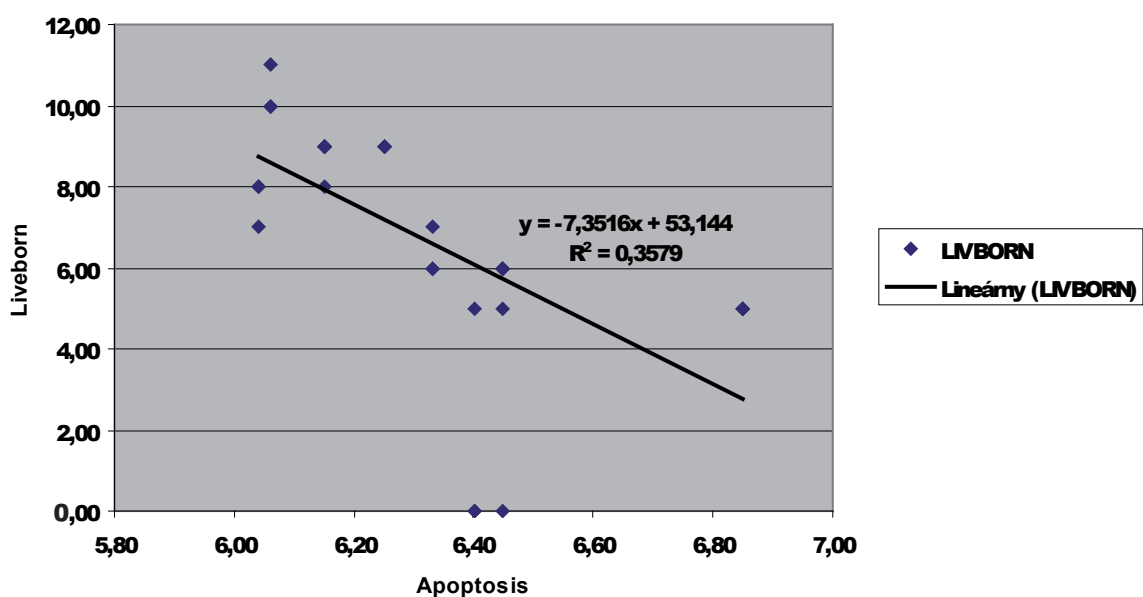


Fig. 3b: Linear regression of liveborn rate on apoptosis in non-transgenic rabbits

DISCUSSION

Generally, the sperm plasma membrane may be important for evaluating the biological quality of spermatozoa, and the introduction of fluorescent staining techniques opens new possibilities for this evaluation. The change in SYBR-14 staining in relation to PI is evident because when spermatozoa die, they lose their ability to resist the influx of the membrane penetrating PI stain (Bialkowska et al., 2004, Chrenek et al., 2010).

It enters through pores in the nuclear membrane that are located in the diverticulum, or membrane folds, of the posterior region of the spermatozoa head (Garner and Johnson, 1995). Plasma membrane integrity in a medium with a normal K^+ and Na^+ ratio needs a supply of intracellular ATP. Therefore, membrane damage measured by fluorescent probes may indicate prior metabolic failure as well as a concomitant loss of vital intracellular metabolites (Harrison and Vickers, 1990). Changes in sperm quality depend on membrane integrity

may be analysed by annexin V also. The method of annexin V labelling has been previously used for the evaluation of viability of frozen-thawed semen of bulls (Martin et al., 2004) and rabbit (Makarevich et al., 2008). These authors indicate that the presence of apoptotic spermatozoa in the semen may be a one of reasons for poor male fertility. 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). Peña et al. (2003) analyzed membrane integrity using annexin V combined with PI staining and came to the conclusion that annexin V is able to detect changes in spermatozoa 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. In our study about 5-6 % of rabbit transgenic and non-transgenic spermatozoa were annexin V- positive, what corresponds to the obtained conceptional rate (83 vs. 87%), when spermatozoa of the both groups were used for inseminations.

Linear regression analysis of our data showed that the viability of sperm (SYBR-14/PI), as well as apoptotic index (annexin V/DAPI) were correlated with liveborn rate of transgenic or non-transgenic rabbits. Therefore, annexin V labelling and SYBR-14/PI may be used as markers of rabbit sperm viability.

CONCLUSION

According to the results of this study, it is concluded that the viability of rabbit transgenic and non-transgenic spermatozoa could be reliably quantified using viability (SYBR-14/PI) and apoptosis (annexin V/DAPI) tests. Positive correlations between liveborn rate and the sperm viability (SYBR-14/PI) or sperm apoptosis (annexin V/DAPI) have been found.

ACKNOWLEDGEMENT

This study was supported by the Slovak Research and Development Agency APVV under contracts No.VVCE-0064-07-05 "Biomembranes" and APVV-0514-07. The authors are grateful to the Slovakian branch of Leica (MIKRO spol. s.r.o. Bratislava, Slovak Republic) for their contribution to this study.

REFERENCES

- BIALKOWSKA, J. – DEMIANOWICZ, W. – GLOGOWSKI, J. 2004. Determining the viability of zebra mussel (*Dreissena polymorpha*) spermatozoa and changes in the integrity of its membrane using the fluorescence method. *Archives of Polish Fisheries*, vol. 12, 2004, p. 23-30.
- CHRENEK, P. – VASICEK, D. – MAKAREVICH, A. – JURCIK, R. – SUVEGOVA, K. – BAUER, M. – PARKANYI, V. – RAFAY, J. – BATOROVA, A. – PALEYANDA, R.K. 2005. Increased transgene integration efficiency upon microinjection of DNA into both pronuclei of rabbit embryos. *Transgenic Res.*, vol. 14, 2005, p. 417-428.
- CHRENEK, P. – DRAGIN, S. – MAKAREVICH, A. V. 2006. Reproductive characteristics of Transgenic rabbit males with human protein C gene. *Slovak J. Anim. Sci.*, vol. 39, 2006, p. 131-134.
- CHRENEK, P. – TRANDZIK, J. – MASSANYI, P. – MAKAREVICH, A. – LUKAC, N. – PESKOVICOVA, D. – PALEYANDA, R. K. 2007a. Effect of transgenesis on reproductive traits of rabbit males. *Anim. Reprod. Sci.*, vol. 99, 2007a, p. 127-134.
- CHRENEK, P. – MASSANYI, P. – MAKAREVICH, A. V. – LUKAC, N. – ZAHRADNIKOVA, M. – SCHNEIDGENOVA, M. – RYBAN, L. 2007b. Rabbit transgenic brothers with different reproductive traits. *Slovak J. Anim. Sci.*, vol. 40, 2007b, p. 113-117.
- CHRENEK, P. – MAKAREVICH, A. V. – SIMON, M. 2010. Viability and apoptosis in spermatozoa of transgenic rabbits. *Zygote*, accepted August 2010, in press.
- GARNER, D. L. – JOHNSON, L. 1995. Viability assessment of mammalian spermatozoa using SYBR-14 and propidium iodide. *Biol. Reprod.*, vol. 53, 1995, p. 276-284.
- HARRISON, R. A. – VICKERS, S. E. 1990. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *J. Repr. Fert.*, vol. 88, 1990, p. 343-352.
- JANUSKAUSKAS, A. – JOHANNISSON, A. – RODRIGUEZ-MARTINEZ, H. 2003. Sibley membrane changes in cryopreserved bull semen in relation with sperm viability chromatin structure, and field fertility. *Theriogenology*, vol. 60, 2003, p. 743-758.
- MAKAREVICH, A. V. – PARKANYI, V. – ONDRUŠKA, L., KUBOVIČOVÁ, E. – FĽAK, P. – SLEZÁKOVÁ, M. – PIVKO, J. – RAFAY, J. 2008. Evaluation of fertilizing capacity of rabbit sperm on the basis of annexin V – labelled membrane changes. *Slovak J. Anim. Sci.*, vol. 41, 2008, p. 1-5.
- MARTIN, G. – SABIDO, O. – DURAND, P. – LEVY, R. 2004. Cryopreservation induces an apoptosis-like mechanism in bull sperm. *Biol. Reprod.*, vol. 71, 2004, p. 28-37.
- MURATORI, M. – PORAZZI, I. – LUCONI, M. – MARCHIANI, S. – FORTI, G. – BALDI, E. 2004. Annexin V binding and merocyanine staining fail to detect human sperm capacitation. *J. Androl.*, vol. 25, 2004, p. 797-810.
- PENA, F. J. – JOHANNISSON, A. – WALLGREN, M. – RODRIGUEZ-MARTINEZ, H. 2003. Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. *Theriogenology*, vol. 60, 2003, p. 677-689.
- VERMES, I. – HAANEN, C. – STEFFENS-NAKKEN, H. – REUTELINGSPERGER, C.P.M. 1995. A novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Meth.*, vol. 184, 1995, p.39-51.