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Viability and apoptosis in spermatozoa of transgenic rabbits

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Summary

The aim of our study was to compare the viability of sperm cells from transgenic (*mWAP-hFVIII* gene) or non-transgenic (normal) rabbit males as assessed by viability (SYBR-14/PI) and apoptosis (annexin V) tests. These results were evaluated using female conception rates following insemination with the respective sperm samples. No significant differences were found in concentration and motility between transgenic and non-transgenic spermatozoa. Spermatozoa from both transgenic (63.05 ± 20.05%) or non-transgenic (65.75 ± 22.15%) males, stained with SYBR-14 (green), were found to be morphologically normal. In both groups, the highest proportion of annexin V-positive sperm staining was found in the post-acrosomal part of the sperm head (8.66 and 27.53%). The percentage of sperm that stained with SYBR-14/PI or with annexin V/DAPI was correlated with liveborn in transgenic rabbits ($R^2 = 0.6118$ and $R^2 = 0.2187$, respectively) or non-transgenic rabbits ($R^2 = 0.671$ and $R^2 = 0.3579$, respectively). These data indicate that there was no difference in the viability of rabbit transgenic and non-transgenic spermatozoa when determined by both fluorescence assays.

Keywords: Annexin V, Rabbit, Spermatozoa, SYBR-14, Transgenic

Introduction

Efficiency of transgenesis depends on several factors including integration and expression rate, health status or physical conditions, as well as quality and viability of transgenic gametes. The effect of transgenesis on reproductive traits of rabbit males (occurrence of pathological spermatozoa, histological structure of the testis) has been previously reported (Chrenek *et al.*, 2006, 2007a,b). Viability analysis of spermatozoa can be evaluated based on membrane integrity. The principle of the test is the differential staining of live and dead cells with two fluorescent dyes, SYBR-14 and propidium iodide (PI) (Garner & Johnson, 1995). SYBR-14 labels only viable cells, whereas PI labels only cells that have damaged membranes. The combination of these two dyes can provide a differentiation between live or dead cells.

A more detailed analysis of mammalian spermatozoa for plasma membrane destabilization using annexin V has been reported for fresh bull spermatozoa (Januskauskas *et al.*, 2003) and for cryopreserved rabbit spermatozoa (Makarevich *et al.*, 2008a). 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 to 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 the early phase of apoptosis prior to loss of cell membrane integrity.

The aim of our study was to compare viability in transgenic and non-transgenic rabbit spermatozoa using tests for viability (SYBR–14/PI) and apoptosis (annexin V). Obtained results were evaluated in relation to female conception rates following insemination with the respective spermatozoa samples.

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Material 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 New Zealand White breed and age were used. The males were housed in individual cages, under a constant photoperiod of 14 h of daylight. Temperature and humidity in the building were recorded continuously by 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 analysed). We evaluated each sample for concentration and motility of fresh ejaculate (evaluated visually as the percentage of straight moving spermatozoa).

Spermatozoa viability test (SYBR-14/PI)

Alive spermatozoa were assessed based on membrane integrity by staining with a Live/Dead cell kit (SYBR-14, Molecular Probes Inc.) as described by Garner & Johnson (1995). Semen samples were diluted 1:10 in HEPES-buffered saline (HBS) (10 mM HEPES, 150 mM NaCl, 1% bovine serum albumin (BSA), pH 7.4). A working solution of SYBR-14 was prepared by 50-fold dilution of a SYBR-14 stock solution in HBS. A total of 5 µl of the SYBR-14 working solution was added to 1 ml of diluted spermatozoa sample (final concentration 100 nM) and this sample was incubated at 37°C for 5–10 min. Then, 5 µl of PI (final concentration 12 µM) was added to spermatozoa sample, which was then incubated for a further 5–10 min. Afterwards, a 4 µl aliquot of the sample was placed between a microslide and coverslip and viewed under a Leica fluorescence microscope (at ×20 or ×40 magnification, with the excitation filter set at 450–490 nm and 510–580 nm). Minimally 200–400 spermatozoa cells were analysed per group.

Test for apoptosis (annexin V/DAPI)

For the annexin V analysis, semen samples were centrifuged at 2000 rpm for 6 min and resuspended in an equal volume of annexin V-binding buffer (supplied with the kit). A semen suspension (5 µl) was mixed with 100 µl of the working solution of annexin V-Fluos (Annexin-V-Fluos staining kit, Roche Diagnostics) and incubated at 37°C for 15–25 min. Afterwards, aliquots of the semen suspension (5 µl) were placed between a microslide and coverslip into 5 µl of the Vectashield anti-fade medium that contained the DAPI fluorescent dye. Staining with annexin V and DAPI was checked under a Leica fluorescence microscope (Leica Microsystem) using 488 nm and 420 nm wavelength filters respectively. Spermatozoa with annexin V-positive membranes 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 commercial diluent (Minitub,) to a minimum concentration of 14×10^6/ml and used for the insemination of hormonally (PMSG and HCG, Werfaser and Werfachor) stimulated New Zealand White rabbit females. Conception rate was estimated by pregnancy diagnosis at 15 days after insemination.

Statistical analysis

Standard *t*-test was used to compare the spermatozoa concentration, motility, viability and apoptosis in different groups. The averaged values for transgenic and non-transgenic groups were compared between each group. Data obtained on conception rate was processed statistically using one-way ANOVA and analysed using the Group probability comparison test. Differences between related traits were analysed by linear regression analysis.

Results

Table 1 shows basic characteristics of rabbit transgenic and non-transgenic spermatozoa. The concentration of spermatozoa in the transgenic group was insignificantly lower (505.25 ± 45.05) than in the non-transgenic one (595.30 ± 38.15).

No significant difference was found either in motility (Table 1) or in the percentage of pathological spermatozoa (Table 2) between transgenic (20.75 ± 5.42) and non-transgenic (18.95 ± 5.25) groups. The most frequent abnormality in transgenic males was cytoplasmic drop retention (4.50%) and a knob twisted tail (4.35%). In case of non-transgenic males the more frequent sperm abnormality was a knob twisted tail (5.58%) and a separated tail (5.10%) respectively.

No differences in the percentage of viable (SYBR-14/PI) or apoptotic (annexin V) sperm were observed between groups. Annexin V-positive staining was observed in the following sperm compartments: acrosomal part of the sperm head; postacrosomal part

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Table 1 Basic transgenic and non-transgenic rabbit spermatozoa characteristics

Group	Concentration $n \pm$ SD ($\times 10^6$/ml)	Motility $n \pm$ SD (%)	Viability (SYBR-14/PI) no. $\pm$ SD (%)	Apoptosis (annexin V) no. $\pm$ SD (%)
Transgenic	505.25 ± 45.05	63.85 ± 22.42	63.05 ± 20.05	5.23 ± 1.82
Non-transgenic	595.30 ± 38.15	73.45 ± 25.05	65.75 ± 22.15	6.41 ± 1.03

Table 2 Occurrence of pathological spermatozoa in transgenic and non-transgenic rabbit males (%)

	Separated tail	Knob twisted tail	Torso tail	Rounded tail	Broken tail	RCD	Small head	Large head	Acrosomal changes	OF	Total
Transgenic rabbit males	3.55	4.35	1.78	3.15	0.80	4.50	0.23	0.25	1.18	0.96	20.75
Non-transgenic rabbit males	5.10	5.58	1.10	1.05	0.95	1.55	0.55	0.24	1.52	1.31	18.95

OF, other pathological spermatozoa; RCD, retention of cytoplasmic drop.

Table 3 Presence and localization of annexin V positivity in rabbit spermatozoa compartments

		Annexin V distribution through the sperm cell		
Group of spermatozoa	No. annexin V-positive sperm	Acrosomal part *n* (%)	Post acrosomal part *n* (%)	Tail *n* (%)
Transgenic	300	6 (2.00)	26 (8.66)[a]	14 (4.66)
Non-transgenic	276	4 (5.30)	76 (27.53)[b]	6 (2.17)

a vs. *b* significant differences at $p < 0.05$.

Table 4 Parameters of rabbit female fertility following insemination with transgenic or non-transgenic semen

Group of spermatozoa	No. mated females	No. parturited does/ conceptional rate, *n* (%)	Liveborn *n*	Deadborn *n*	Average no. liveborn pups/litter
Transgenic	30	25 (83)	207	6 (2.9)	8.3
Non- transgenic	30	26 (87)	218	7 (3.2)	8.4

of the head; and a proximal cytoplasmic droplet (data not shown). Sperm cells, labelled in such a pattern, were considered as annexin V positive (apoptotic). Sperm cells that did not show head staining were considered to be intact.

Significant differences ($p < 0.05$) between transgenic and non-transgenic spermatozoa were found in the postacrosomal part of the head and in the tail region (Table 3).

No significant difference was found in the conception rates (83 vs. 87%) between the transgenic and non-transgenic spermatozoa used for inseminations (Table 4).

Based on linear regression analysis, we found a positive correlations between liveborn and sperm viability ($R^2 = 0.6118$), and between liveborn and sperm apoptosis ($R^2 = 0.2187$) in transgenic rabbits. Similar correlations between liveborn and sperm viability ($R^2 = 0.671$), as well as between liveborn and apoptosis ($R^2 = 0.3579$), were observed for non-transgenic rabbit sperm.

Discussion

The sperm plasma membrane may be important for evaluating the biological quality of spermatozoa, and the introduction of fluorescence staining techniques opens up 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). This stain enters through pores in the nuclear membrane that are located in the diverticulum, or membrane folds, of the posterior region of the spermatozoa head

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(Garner & Johnson, 1995). The motility of rabbit transgenic and non-transgenic spermatozoa varied from 64 to 73%, whereas viability/membrane integrity assessed with SYBR-14 was similar (63 to 66%).

A significant correlation has previously been found with the semen of bulls, turkeys or boars stained with SYBR-14 and with motility (Garner & Johnson, 1995, Garner *et al.*, 1996). Conversely, Hong *et al.* (1988) compared human sperm motility with viability and found that many vital spermatozoa were immotile. Plasma membrane integrity in culture 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 & Vickers, 1990). In this study, a similar percentage number of sperm from transgenic or non-transgenic male rabbits stained for motility and SYBR-14 viability. Vetter *et al.* (1998) reported that membrane integrity is a necessary, although insufficient, criterion for predicting motility.

Changes in sperm quality that depend on membrane integrity may be also be analysed by annexin V. Although there is a finding that the annexin V test in sperm cells may indicate capacitation-like, rather than apoptotic, membrane changes (Gadella & Harrison, 2002), in numerous reports annexin V binding has been considered as an indicator of apoptotic changes in sperm. In particular, the annexin V labelling method has been used previously to evaluate the viability of frozen–thawed semen from bulls (Martin *et al.*, 2004) or rabbits (Makarevich *et al.*, 2008a). These authors indicate that the presence of apoptotic spermatozoa in the semen may be one of the reasons for poor male fertility. Live annexin V-positive human sperm cells were mainly represented by damaged spermatozoa, as revealed by a negative correlation between PS exposure and normal morphology and the motility of the sperm (Muratori *et al.*, 2004). Peňa *et al.* (2003) analysed 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 does PI. Moreover, the annexin V-labelling technique is more sensitive when compared with the current SYBR-14/ PI method, and it represents a new approach for membrane status determination in sperm. In our study around 5 to 6% of rabbit transgenic and non-transgenic spermatozoa were annexin V positive, which corresponded to obtained conception rates (83 vs. 87%) of spermatozoa from both of the groups 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), was correlated with liveborn transgenic or non-transgenic rabbits. Therefore, annexin V labelling and SYBR-14/PI may be used as markers of rabbit sperm viability.

In conclusion, obtained data indicate that there was no difference in the viability of rabbit transgenic and non-transgenic spermatozoa, as determined by both fluorescence assays. This study demonstrates that the viability of rabbit transgenic and non-transgenic spermatozoa can be evaluated reliably using the SYBR-14/PI and annexin V tests.

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