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COMPARISON OF SEMEN CHARACTERISTICS AND HISTOLOGICAL STRUCTURE OF THE TESTIS FROM TRANSGENIC AND NON-TRANSGENIC RABBITS

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Abstract: The aim of this study was to compare semen characteristics including sperm quantity, quality, and abnormalities, as well as histological structure of the testis of three-year old transgenic (human clotting factor, hFVIII, gene) and non-transgenic rabbits. For the experiment, 10 transgenic rabbits of F2 and F3 generations and 10 randomly selected non-transgenic males of the same breed and age were used as controls. All males were housed in individual cages, under a the same environmental conditions: photoperiod (14L:10D), temperature (18-20°C), and humidity (65-70%). Semen samples, collected once a week for 20 wk from each control and transgenic male, were analyzed by computer assited semen analysis within a few minutes following natural ejaculation into an artificial vagina. Concentration of spermatozoa was higher in the transgenic than in the non-transgenic group (P<0.001; 316.6±148.8 and 126.7±64.4×10^6/mL, respectively). Significant differences (P<0.1) between transgenic and non-transgenic males were observed also in spermatozoa motility (63.08 *vs.* 32.60%). Significantly higher (P<0.05) relative volume (8.08±2.89%) and diameter of testicular lumen (36.89±23.11 µm) were found in the transgenic animals compared to control animals (16.69±4.70%, 53.89±25.42 µm). Our results show that spermatozoa parameters and histological structure of the testis can be used for the characterization of male reproductive traits of older transgenic rabbits.

Key words: transgenic rabbit, spermatozoa quality, testicular histology.

INTRODUCTION

Reproductive capabilities of transgenic male rabbits affect the creation of stable lines of transgenic offspring. These lines can be used to produce different biologically active recombinant proteins, including enzymes, improve meat and milk quality and quantity, or enhance the resistance of such transgenic animals to disease. As well as transgene integration of microinjected gene construct, the second greatest factor limiting the efficiency of the production of transgenic animals is a low stability of transgene transmission to their offspring.

There are many factors influencing the quality and quantity of rabbit semen, such as breed (Amin *et al*., 1987), individual (Castellini, 1996), age (Gogol *et al*., 2002), season (Safaa *et al.,* 2008), photoperiod (Theau-Clement *et al*., 1995), nutrition (Nizza *et al.,* 2000) and collection rhythms (Nizza *et al*., 2003; Castellini *et al.,* 2006). The influence of transgenesis on spermatozoa quality and quantity of young rabbit males (F1, F2 and F3 generations) has been already documented (Chrenek *et al*., 2007a; 2007b).

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Therefore, it is important to define a sexual regime with respect to age for the transgenic males to be able to provide large volumes of high quality semen.

The type of morphological abnormalities is sometimes considered, among other factors, during the evaluation of rabbit spermatozoa quality for breeding or for artificial insemination (Brun *et al*., 2002). Morphology of rabbit transgenic spermatozoa has already been characterized using a light and electron microscope analysis (Chrenek *et al*., 2005a).

The aim of this study was to compare semen characteristics involving sperm quantity, quality, and abnormalities, as well as the histological structure of the testis of three-year old transgenic and non-transgenic rabbits.

MATERIAL AND METHODS

Transgenic males with the WAP-hFVIII gene were produced as described by Chrenek *et al*. (2005b). For the experiment we used 10 three-year old transgenic rabbits from the F2 and F3 generations. They were obtained by breeding the transgenic founders with non-transgenic rabbits of the same New Zealand White strain. Ten randomly selected non-transgenic males of the same breed and age were used as controls. All males were housed in individual cages, under a constant photoperiod of 14 h of light per day. Temperature (18-20°C) and humidity (65-70%) 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.

From each control and transgenic male the semen was collected once a week for 20 wk, from February to June. Thus, 200 transgenic and 200 non-transgenic ejaculates were analyzed.

Computer–assisted semen analysis (CASA)

Semen samples were analyzed within a few minutes following natural ejaculation into the artificial vagina. Well-mixed semen was transferred by a pipette to a Makler counting chamber with a depth 10 μm. The sample was placed on a heating plate at 37°C set under an Olympus CX 51 phase-contrast equipped microscope (Olympus Europe, Germany) at 240× magnification and images were transferred to the computer through the video camera. Records were analyzed using SpermVision® software (MiniTube, Germany) set at 10 s tracing time. An average 1000 spermatozoa per sample were analyzed for the concentration, motility (motile: > 20 μm s^{-1}; local motility: 5–20 μm s^{-1}; circular motility: %), distance (distance average path in μm; DIS), curvilinear velocity (VCL), straight-line velocity (VSL) and average path velocity (VAP) in μm s^{-1} and motion parameters of linearity (LIN = VSL:VCL) (Roychoudhury and Massanyi, 2008).

Analysis of abnormal spermatozoa

For analysis of abnormal spermatozoa, histological preparations were fixed with Hancock's solution and stained with Giemsa. All slides were analyzed at 500× magnification. For each rabbit male at least 1000 spermatozoa were evaluated and the percentage of abnormal spermatozoa was determined according to criteria of abnormal spermatozoa routinely used at breeding institutes (Massanyi *et al*., 2004; Chrenek *et al*., 2005a). These abnormal changes were classified as follows: knob-twisted flagellum, separated flagellum, flagellum torso, broken flagellum, retention of cytoplasmic drop and flagellum ball. Acrosomal changes, large heads, small heads, classified as abnormalities of the sperm head and other abnormal forms of spermatozoa (teratoid spermatozoa, a spiral twisted flagellum, deformation of the mitochondrial part and others) were identified (Massanyi *et al*., 2000).

Histological analysis

Testes were collected at the end of the experiment after killing the animals (by electroshock and subsequent bleeding) and fixed in 10% formalin solution. After fixation, the samples were dehydrated in a graded series of ethanol (70, 80, 90 and 100%), saturated in benzene, benzene-paraffin and embedded into paraffin. Blocks of samples were then sectioned on a microtome into 10 μm thick sections stained with hematoxylin-eosin. After staining, the sections were mounted on solacryl and digital microphotographs were evaluated using an Olympus BX51 microscope (Japan) and morphometrical image analyzer software Micro Image version 4.0 (Massanyi *et al*., 2000). For each pair of testes, the relative volume of germinal epithelium, interstitium and lumen, as well as the diameter of seminiferous tubules, lumen and the height of germinal epithelium were determined.

Fertilizing capacity of spermatozoa and transgene transmission

Transgenic and non-transgenic males were mated with non-transgenic females from the same breed to test the fertility rate and transgene transmission. Fertilized rabbit eggs were recovered from mated females at 20 h post coital (p.c.) and the flushed eggs were cultured *in vitro* in k-DMEM medium + 10% FCS (both from Gibco BRL) up to blastocyst stage (96 h p.c.). The WAP-hFVIII gene integration into the embryos was assessed using the PCR method (Chrenek *et al*., 2007b).

Statistical methods

Overall, data on transgenic and non-transgenic samples were analyzed using basic statistical parameters (mean, SD, SEM). General linear model (SAS 1989) and one-way ANOVA with Scheffe's test were performed for each variable.

RESULTS

Evaluation of rabbit spermatozoa

Concentration of spermatozoa in the transgenic group (316.6±148.8×10^6/mL) was higher ($P<0.001$) compared to the control group (126.7±64.4×10^6/mL). Significant differences ($P<0.01$) were also observed in the motility of spermatozoa between the transgenic (63.08±23.88%) and non-transgenic groups (32.60±17.78%). No differences were found in local and circular motility among transgenic (35.92±24.09% and 1.00±0.95%, respectively) and non-transgenic groups (65.00±16.80% and 2.40±0.66%, respectively).

Detailed analysis of spermatozoa motion showed that each parameter was better in transgenic than in non-transgenic rabbits. The linearity of transgenic rabbit spermatozoa was higher ($P<0.01$) than that of the non-transgenic animals (33.78±0.48 *vs*. 14.27±0.63%, respectively). Distance covered by the evaluated spermatozoa in the analyzed time was similar in both transgenic and control animals (56.48±0.66 *vs*. 54.65±1.52 μm, respectively). Significantly higher values ($P<0.01$) for curvilinear velocity (49.45±0.55 and 29.92±0.86 μm s^{-1}, respectively), straight line velocity (20.08±0.47 and 5.47±0.44 μm s^{-1}, respectively) and average path velocity (29.22±0.51 and 10.86±0.52 μm s^{-1}, respectively) were found in the transgenic spermatozoa compared to the non-transgenic spermatozoa.

The percentage of abnormal spermatozoa of transgenic and non-transgenic rabbit males is shown in Table 1. No statistical differences in the percentage of total spermatozoa abnormality were found between the transgenic and non-transgenic male groups (13.47±2.48 *vs*. 24.07±6.33%, respectively).

Fertilizing capacity of spermatozoa and transgene transmission

Table 1: Occurrence of abnormal rabbit spermatozoa (%).

	Transgenic animals		Non-transgenic animals	
Abnormality	Mean	S.D.	Mean	S.D.
Total number of abnormalities	13.47	2.48	24.07	6.33
Tail abnormalities				
Separated tail	3.30	2.10	4.95	2.76
Tail torsion	0.31	0.28	0.90	0.28
Knob-twisted tail	5.37	3.97	11.67	7.15
Broken tail	0.86	0.78	1.50	1.13
Tail bail	2.43	1.71	4.20	2.55
Retention of cytoplasmic drop				
	0.44	0.39	0.10	0.01
Abnormalities of spermatozoa head				
	0.77	0.38	0.75	0.50

The ability of spermatozoa to fertilize the eggs ranged between 93 and 100% for transgenic males, and between 94 and 100% for non-transgenic males. Stability of transgene transmission via transgenic sperm, confirmed by the PCR method, was in the range of 43 to 47% in all transgenic males.

Histological analysis (qualitative and quantitative micromophological analysis)

Subjective evaluations of histological preparations revealed a normal structure of germinal epithelium containing all developmental stages of spermatogenesis (data not shown). The shape of the tubules was oval, and without alterations. The interstitium contained collagen fibers and fibrocytes as well as Leydig cells in both groups.

No statistical differences in relative volume of epithelium and interstitium and diameter of seminiferous epithelium between transgenic and non-transgenic animals were found (Table 2). Significant difference ($P<0.05$) in relative volume of the lumen and diameter of the lumen between transgenic (8.08 %, 36.89 μm) and non-transgenic animals (16.69 %, 53.89 μm), respectively was observed. Significant differences in height of seminal epithelium ($P<0.05$) were found between observed tissue samples.

DISCUSSION

It is generally known, that sexual maturation of rabbit males starts at 4-5 mo and declines after 24 mo, depending on breed and line of rabbits. The efficiency of male reproduction at 36 mo of age is expected to be even lower. In the case of transgenic rabbits, however, it is economically advantageous to use these males for reproduction (including insemination or semen freezing) as long as possible.

The most variable parameter of male rabbit reproductive traits is spermatozoa concentration, ranging from 10 to more than 1000 10^6/mL (Alvariño *et al*., 2000). Normally, an increase in semen collection frequency is associated with a decrease in spermatozoa concentration (Arroita *et al*., 2000). Our results of semen volume and spermatozoa motility parameters in transgenic and non-transgenic three-year old males correspond to previously published observations (Chrenek *et al*., 2005a) suggesting that the ageing has no negative effect at least up to three years.

Table 2: Testicular histology of rabbit testes.

	Transgenic animals		Non-transgenic animals	
	Mean	S.D.	Mean	S.D.
Relative volume of epithelium (%)	83.58	3.21	74.36	9.80
Relative volume of interstitium (%)	8.32	1.75	8.95	7.65
Relative volume of the lumen (%)	8.08	2.89	16.69***	4.70
Diameter of seminiferous tubules (μm)	102.70	44.95	102.40	44.57
Diameter of the lumen (μm)	36.89	23.11	53.89***	25.42
Height of germinal epithelium (μm)	36.58	13.03	29.23*	12.65

Significant differences among transgenic and non-transgenic group: *P<0.05; ***P<0.001.

Spermatozoa motility is the other important parameter of semen quality. Visual evaluation of the motility by an operator is rather subjective; therefore for objective evaluation of the motility the use of the CASA system is necessary. Motility parameters, determined by this method, in combination with spermatozoa morphology analysis can provide more accurate information about the fertilizing potential of rabbit spermatozoa (Lavara *et al*., 2005). In our study, significant differences were also observed in the motility of spermatozoa between the transgenic and non-transgenic groups. In fact, detailed analysis of spermatozoa motion in this study showed that each parameter was better in transgenic rabbit spermatozoa versus non-transgenic rabbit spermatozoa. In our previous experiments (Chrenek *et al*., 2007b), using transgenic rabbit males of various ages, we found a similar percentage of spermatozoa motility (65 *vs*. 63%). Similar motility in the non-transgenic spermatozoa was also reported by Nizza *et al*. (2003).

No statistical differences in the percentage of total spermatozoa abnormality were found between transgenic and non-transgenic male groups confirming our previous observations on this specific line (Chrenek *et al*., 2007b). The occurrence of pathological spermatozoa in transgenic males is in agreement with the findings obtained from different non-transgenic rabbit lines (Nizza *et al*., 2003).

The spermatozoa fertilizing capacity of transgenic males in this work showed no differences compared to non-transgenic males. These observations agree with our earlier reports (Chrenek *et al*., 2005b, 2007b), where transgenesis had no effect on the fertilizing capacity of transgenic spermatozoa. Similar results on the ability of transgenic founder males to produce offspring and to transmit integrated gene constructs via spermatozoa were also recently reported (Chrenek *et al*., 2005a). Stability of transgene transmission, proven in transgenic embryos or transgenic offspring, is one of the requirements for the definition of transgenic animals.

Differences in relative volume and diameter of the lumen of testes observed between transgenic and non-transgenic animals might be related to many factors: and it is noticeable that the activity of germinal epithelium is generally very sensitive. Observed differences, however, were quite stable: from higher relative volume of germinal epithelium, through higher spermatozoa motility parameters, to lower occurrence of abnormal forms of spermatozoa, which indicate no negative impact of transgenesis on rabbit male reproductive traits.

All our findings of statistically significant differences among transgenic and non-transgenic males are in the range of normal variability that is typical for the rabbit species. This was also confirmed in our previous and other reports (Chrenek *et al*., 2005b; 2007a; 2007b; Arroita *et al*., 2000; Nizza *et al*., 2003). A possible cause of these differences may be the low number of analyzed males. Moreover, the rabbit transgenic line in our study was derived from one founder, whilst the non-transgenic males where randomly chosen and originated from several ancestors.

CONCLUSION

Our results show that spermatozoa parameters and the histological structure of the testis can be potentially used for characterization of male reproductive traits of older transgenic rabbits. Better testicular and semen parameters found in three-year old transgenic rabbit males suggests that these animals are still able to produce stable transgenic lines without any reproductive alterations.

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