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# RABBIT TRANSGENIC BROTHERS WITH DIFFERENT REPRODUCTIVE TRAITS

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## ABSTRACT

The aim of this study was to analyze reproductive traits of rabbit transgenic brothers with respect to their ejaculate characteristics, fertilizing capacity, occurrence of pathological spermatozoa and histological structure of the testis. Transgenic rabbit males (marked as no. 36-1 and no. 36-2) were produced by mating transgenic female founder carrying the mWAP-hFVIII gene construct with non-transgenic male. All reproductive characteristics of two transgenic brothers were compared with other transgenic and non-transgenic rabbit males of the same lines. Transgenic male no. 36-1 showed worse results in all analyzed reproductive parameters (volume of ejaculate and sperm concentration) in comparison with transgenic male no. 36-2 or the other transgenic and non-transgenic males. Also, significant differences ($p<0.001$) between both transgenic males were found in the occurrence of pathological spermatozoa and histological structure of the testis. None of the females bred with transgenic male no. 36-1 became pregnant, while transgenic male no. 36-2 and other transgenic males transmitted transgenic allele on their offspring at 46% frequency. In conclusion, a great difference among rabbit transgenic brothers was observed. It is assumed, that this phenomenon is probably not due to deleterious consequence of transgenesis, but may be a result of individual variability in reproductive state among rabbits. Therefore, to establish new transgenic herds, a detailed individual evaluation and selection of transgenic animals is required.

**Key words:** transgenic rabbit, reproductive trait, pathological sperm

## INTRODUCTION

Reproductive capabilities of transgenic rabbit founders affect the creation of stable lines of transgenic offspring, which can be used to produce different biologically active recombinant proteins, enzymes or to improve meat and milk quality and quantity or enhance resistance of such transgenic animals against disease. There are many factors influencing quality and quantity of rabbit semen, such as breed, sex, age, season, nutrition and collection rhythms (Castellini, 1993; Bhatt et al., 2002; Nizza et al., 2003). Thus, in order to provide large volumes of high quality semen, it is important to define a sexual regime for males, quality of rabbit spermatozoa, used for breeding or artificial insemination or heterospermic insemination with respect to the type of morphological abnormalities (Brun et al., 2002; Chrenek et al., 2005a). In previous studies, morphology of rabbit non-transgenic and transgenic spermatozoa has been characterized using light and electron microscope analysis (Kuzminsky et al., 1996; Chrenek et al., 2007), however no effect of transgenesis on reproductive traits (with respect to pathological and histological findings) of rabbit transgenic males within three generations was found (Chrenek et al., 2006; 2007). In this study, we describe reproductive traits of two transgenic rabbit males bearing the same genetic and epigenetic background. Our present goal was to determine possible reasons for the infertility found in one

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of two transgenic rabbit brothers based on analyses of ejaculate characteristics, fertilizing capacity, occurrence of pathological spermatozoa and testicular histology.

## MATERIALS AND METHODS

### Animals

Transgenic founders carrying the mWAP-hFVIII gene were produced as described by Chrenek et al. (2005b) and mated to produce F1 generation. In our experiments we tested two transgenic brother males (marked as no. 36-1 and no. 36-2) about 7 months old. The rabbits were housed in individual cages under a constant photoperiod of 14 h of day light. The 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. Both transgenic males were crossed with non-transgenic females of the same breed to test fertility rate and transgene transmission.

### Semen collection and analysis

Libido (reaction time) was estimated as the time elapsed between the introduction of the female into the male's cage and ejaculation. Semen was collected using artificial vagina, once a week (10 ejaculates per male were analyzed). Each sample was evaluated with respect to its volume, concentration and motility of fresh ejaculate (evaluated visually as the percentage of straight moving spermatozoa cells).

### Assessment of pathological spermatozoa

A drop of ejaculate was diluted with a drop of physiological saline and then placed on a slide tilted at a 45° angle. The samples were dried at 38°C, fixed in Hancock´s solution and rinsed with distilled water and stained as described by Hancock (1957) and viewed under a light microscope at a magnification of 500x. A minimum of 500 spermatozoa randomly collected form each male was evaluated (Massanyi et al., 2000). Following changes in spermatozoa morphology were considered as pathological: separated tail (ST), knob twisted tail (KT), torso tail (TT), rounded tail (RT), retention of cytoplasmic drop (RCD), broken tail (BT) and other forms (OF) of pathological spermatozoa (teratogenic changes, club bag tumor, tail ball, etc.).

### Real-time PCR

Total RNA from the mammary gland of transgenic female (as a positive control) and from the testis of transgenic male no. 36-1 was extracted using TRIzol Reagent (Invitrogen, Austria) according to manufacturers´ protocol. Isolated RNA was subsequently treated with DNase I (Fermentas MBI, Lithuania) to remove the genomic DNA contamination. About 900 ng of treated total RNA was reversely transcribed using the Gene Amp RNA PCR kit (Perkin Elmer, USA) and the presence/absence of hFVIII mRNA in different tissues of transgenic and control rabbits was analyzed using sense: 5´ – TGC CTG ACC CGC TAT TAC TC – 3´ and anstisense: 5´ – TGA GGT ACC AGC TTC GGT TC – 3´ primers. For quantification, following sense: 5´- CTT TGC TGA CCT GCT GGA TT - 3´ and antisepse: 5´- GCT TGA CCA AGG AAA GCA AG - 3´ primers located in different exons of rabbit HPRT gene, thus avoiding co-amplification of genomic DNA under amplification protocol, were used. Real-time PCR was performed using LightCycler (Roche Molecular Biochemical, USA). The obtained PCR products were subsequently analyzed by agarose gel electrophoresis.

### Testicular and epididymal histology

Qualitative study of testicular and epididymal structure was conducted on adult transgenic as well as non-transgenic rabbit males (no. 36-1, no. 36-2). Testes and epididymis were fixed in 10% formalin, dehydrated in a graded series of ethanol, saturated in benzene, benzene-paraffin and embedded in paraffin wax (Massanyi et al., 2000). Samples were sectioned on a microtome and serial 10μm thick sections were stained with haematoxylin and eosin.

### Statistical analysis

Statistical analysis was performed for the following traits: LIB (libido), VOL (volume), CON (concentration), MOT (motility), and pathological abnormalities of spermatozoa. Linear model and one-way analysis of variance was used to analyse the data. Least square mean estimates with standard errors of the estimates were produced. Differences among least square means estimates were tested using Scheffe-s multiple range test. The statistical package SAS (SAS, 2001) was used for the analysis.

## RESULTS

### Ejaculate characteristics

Basic ejaculate characteristics of two transgenic and one non-transgenic rabbit males with statistical differences between both transgenic brother males are presented in Table 1. Intervals between introduction of the female into the male's cage and ejaculation (libido) were statistically different ($p<0.001$) between both (no. 36-1 vs. no. 36-2) transgenic males. The same result was noted in case of the ejaculate volume (200±28.00 vs. 900±36.00 μl) and the sperm concentration (4.00±2.00 vs. 855±25.00 $x10^6$/ml) among both transgenic males. Significant differences ($p<0.01$) were observed also in the sperm motility.

**Table 1: Basic ejaculate characteristics of transgenic and non-transgenic rabbit males**

| Male no. | Libido (s) | Volume (µl) | Sperm concentration ($x10^6$/ml) | Motility (%) | Author |
|---|---|---|---|---|---|
| Transg. (n=10) | 7.90±1.00 | 615.00±38.00 | 685.20±24.00 | 65.00±1.00 | Chrenek et al., 2007 |
| Non-transg. (n=5) | 6.20±1.00 | 710.33±31.00 | 455.50±26.00 | 54.30±1.00 | Chrenek et al., 2007 |
| 36-1 | 50±1.00a | 200±28.00a | 4±1.80a | 0a | Present study |
| 36-2 | 11±1.00b | 900±36.00b | 855±25b | 80±2.00b | |

36-1 and 36-2 - transgenic males (per 10 ejaculates), Tr. – transgenic, N-Tr. – non-transgenic a - vs, b - difference is significant at $p<0.001$

**Pathological spermatozoa abnormalities**

The percentage of pathological spermatozoa abnormalities in transgenic and non-transgenic rabbit males is presented in Table 2. Statistical differences in the percentage of total spermatozoa abnormalities were found between transgenic males no. 36-1 vs. no. 36-2 (36.50 vs. 15.61%). The most frequent abnormalities in both the transgenic males (Figure 1) were separated tail (7.10 vs. 6.47%) and knob twisted tail (7.30 vs. 2.60% resp.).

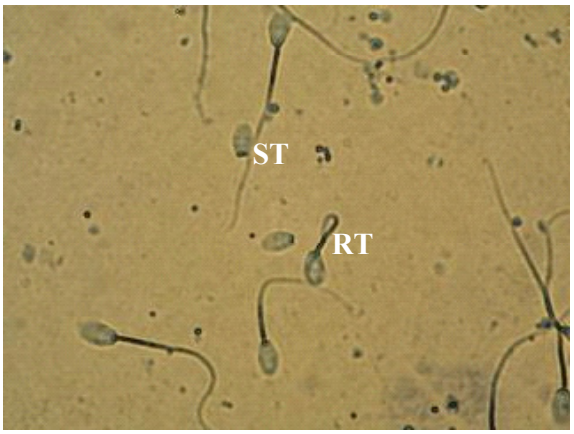


**Fig. 1: Transgenic rabbits spermatozoa. ST- separated tail and RT – rounded tail**

**Fertilizing capacity of spermatozoa**

Significant differences (Table 3) in fertilizing capacity and transgene transmission between transgenic males were found. The ability of spermatozoa of transgenic male no. 36-2 to transmit transgene into the offspring was 45% (25/55). None of the females mated with transgenic male no. 36-1 became pregnant.

**RT-PCR**

As expected, hFVIII mRNA expression was confirmed in the mammary gland of the transgenic female used as positive control, while no rhFVIII mRNA expression in the analysed testes from both transgenic rabbit males was found (data not shown).

**Table 2: Analyses of spermatozoa in transgenic and non-transgenic rabbit males (%)**

| Males | ST | KT | TT | RT | BT | RCD | OF | Total | Author |
|---|---|---|---|---|---|---|---|---|---|
| Transg. (n=10) | 3.87 | 4.33 | 1.68 | 3.19 | 0.54 | 5.00 | 0.63 | 20.25 | Chrenek et al., 2007 |
| Non-transg. (n=5) | 6.12 | 4.58 | 1.08 | 1.08 | 0.93 | 1.85 | 1.05 | 18.80 | Chrenek et al., 2007 |
| 36-1 | 7.10 | 7.30 | 5.60 | 4.50 | 2.50 | 6.00 | 2.50 | 36.50A | Present study |
| 36-2 | 6.47 | 2.60 | 1.07 | 1.27 | 0.47 | 2.13 | 1.00 | 15.61B | |

36-1 and 36-2 - transgenic males, Tr. - transgenic, N-Tr. – non-transgenic a - vs, b - difference is significant at $p<0.001$; ST-separated tail, KT-knob twisted tail, TT-torso tail, RT-rounded tail, BT-broken tail, RCD–retention of cytoplasmic drop, OF–other pathological spermatozoa

**Table 3: Fertilizating capacity and transgene transmission ability of transgenic rabbit males**

| Male/trait | No of mated Females, N | No of newborn offspring, N | No of transgenic offspring, n/N (%) | Author |
|---|---|---|---|---|
| Transg. (n=10) | 20 | 136 | 63/136 (46) | Chrenek et al., 2007 |
| Non-transg. (n=5) | 14 | 115 | 0/115 (0) | Chrenek et al., 2007 |
| 36-1 | 7 | 0 | 0A | Present study |
| 36-2 | 7 | 55 | 25/55 (45)B | |

36-1 and 36-2 - transgenic males, Tr.- transgenic males, N-Tr. – non-transgenic males, a - vs, b - difference is significant at $p<0.001$

**Testicular and epididymal histology**

Significant difference in testicular histology between transgenic males was found (Figure 2A and 2B). Testis of transgenic male no. 36-2 was formed with active germinal epithelium typical for developmental stages of spermatogenesis. In the lumen, sperms were occurred and also interstitium was normally formed. In the epididymis the lumen was filled with sperms, demonstrating normal course of spermatogenesis in the testis. In transgenic male no. 36-1 significant alterations were found: germinal epithelium was destroyed containing very few cells and many empty spaces. Lumen of the seminiferous tubules was empty. Only in some seminiferous tubules a few signs of cell development were detected. No sperms in the epididymal lumen were found (Figure 3A and 3B).

## DISCUSSION

The present study compares reproductive traits: semen characteristics, occurrence of abnormality of spermatozoa and histological structure of the testis from two rabbit transgenic brothers. Generally, seminal characteristics are affected by several factors (breed, feeding, health status, rearing condition, season and sperm collection frequency) and there is a wide variety in semen traits (Alvarino, 2000). Moreover, semen evaluation is a very difficult procedure and differences in laboratory methodologies can introduce substantial variations in the evaluation of sperm parameters such as motility, concentration and morphology (WHO, 1999).

Significantly different results were found when we compared seminal characteristics, fertilization and transgene transmission ability and abnormality between two transgenic rabbit brothers. These data were supported by histological findings of their testes. Reproductive traits of transgenic male no. 36-2, particularly the volume of ejaculate, sperm concentration, motility, were also higher, whilst pathological abnormality frequency were lower, than we reported previously on 10 analyzed transgenic

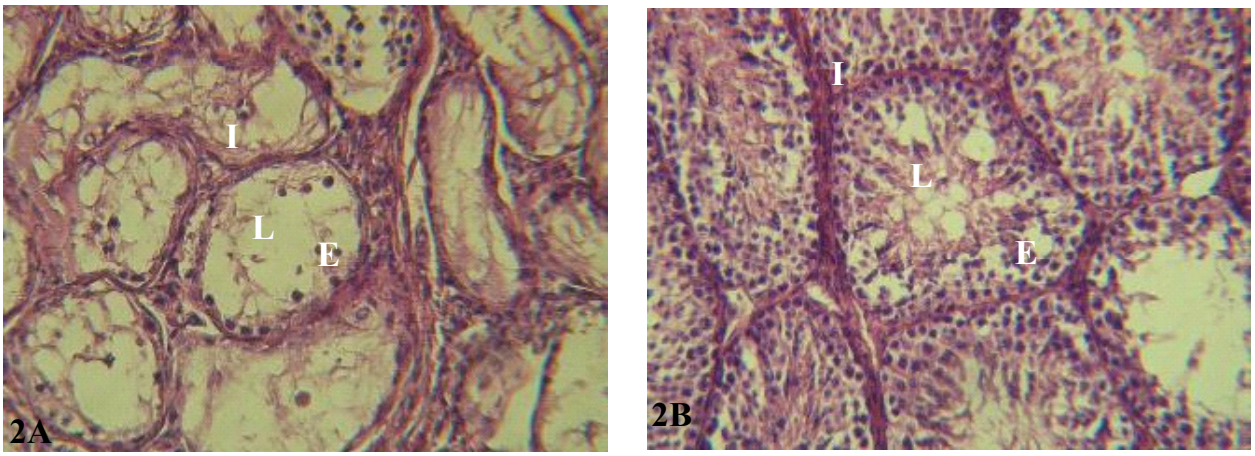


**Fig. 2: Testicular histology. Fig. 2A – transgenic male no.36-1 and Fig. 2B – transgenic male 36-2. Lumen (L), germinal epithelium (E) and interstitium (I); structural alteration of germinal epithelium [320X]**

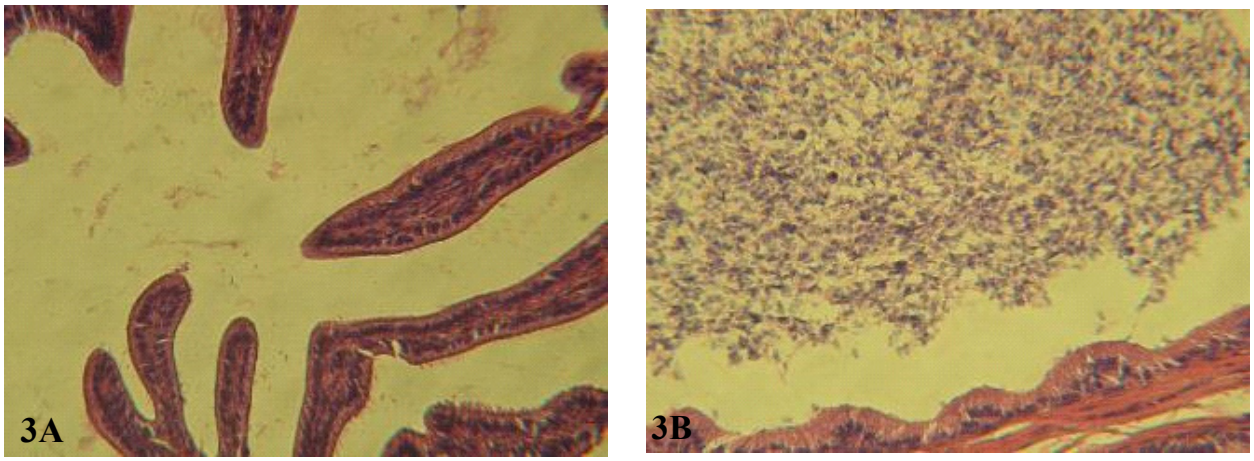


**Fig. 3: Epididymal histology. Fig. 3A – transgenic male no.36-1 and Fig. 3B – transgenic male 36-2. In transgenic male no. 36-1 sperms in lumen are absent. [320X].**

rabbit males, derived from three generations (Chrenek et al., 2006). We detected no negative effect of transgenesis on reproductive traits of transgenic rabbit males. In this work we have found no expression of hFVIII mRNA gene in the testis of transgenic male no. 36-1 by RT-PCR. These results confirmed our previous observation (Chrenek et al., 2005c), that hFVIII mRNA was detected only in mammary gland samples. Based on our results we may eliminate any effect of non-specific expression on reproductive traits of transgenic male no. 36-1. The transgenic male no. 36-1, despite no any health problems (respiratory distress, any malformation) or body weight deviations, was infertilite. Significantly worse semen characteristic of the male no. 36-1 may be due to unsufficient spermatogenesis, which has been proved by histological analysis of the testis. Therefore, infertility of transgenic male no. 36-1 may not be a consequence of transgene effect.

Although rabbits are not a conventional dairy livestock, it is agreed that the short generation time, multiple offsprings per litter, stable paternal transmission of the transgene and milk yield offer advantages over our conventional dairy livestock for the establishment of a line producing a therapeutic recombinant protein in sufficient concentration and biological activity. High differences observed between transgenic brothers bearing the same genetic and epigenetic background may suggest that this phenomenon is probably not due to a deleterious effect of transgenesis, but because of individual variability in reproductive healths state of rabbits. Therefore, to establish new transgenic herds an individual evaluation and selection of transgenic animals is required.

## *ACKNOWLEDGEMENT*

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