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The Effect of Mammary Gland-Specific Transgene Expression on Rabbit Reproductive Gland Structure

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Transgenic rabbits are excellent animal models for human diseases and suitable bioreactors for the production of recombinant proteins on an experimental and commercial scale. The aim of this study was to compare the structure of the mWAP-hFVIII transgenic and non–transgenic rabbit ovarian and testicular tissue. Ovarian and testicular tissue samples were taken from transgenic and non-transgenic New Zealand White rabbits, examined by optical microscopy and analyzed morphometrically. An increase of the relative volume of primary follicles and a decrease of the relative volume of antral follicles was detected in the transgenic ovarian structure ($P<0.05$), but other developmental follicular stages and follicular diameters were not affected ($P>0.05$). In the testes a significant decrease ($P<0.05$) of the epithelial height was detected in the transgenic testicular structure, but the relative volume of all basic structures (germinal epithelium, interstitium and lumen) was unaltered ($P>0.05$). Generally, this study demonstrates a weak negative effect of mWAP-hFVIII transgenesis on rabbit gonadal structure.

Key words: Histology, mWAP-hfVIII, ovaries, testes, rabbit, transgenesis.

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Transgenesis is a relatively new technology based on the fundamental principle that genes can be transferred between completely unrelated organisms to produce new varieties of organisms with new characters which otherwise would not be possible to find in nature. The aim of such technology in animals is to improve the quality of animal products or to increase resistance to diseases. At the same time transgenic animals are seen as possible "bioreactors" in which recombinant products such as biologically active human proteins and oligosaccharides could be conveniently produced (VENKATESH 2008; PRIETO 2012). The general approach is to target the expression of the desired protein to the mammary gland using regulatory elements derived from a milk protein gene and then collect and purify the product from milk (CLARK 1998; HIRIPI *et al*. 2003).

Transgenic rabbits are considered excellent animal models for human diseases as well as suitable bioreactors for the production of pharmaceutical proteins (HOUDEBINE 1995). An excellent example is the transgenic rabbit whose mammary gland produces human factor VIII (hFVIII) used for haemophilia type A treatment (FALLAUX *et al*. 1995).

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Most studies regarding "molecular farming" primarily examine the quantity and/or quality of the produced recombinant protein, not the effects of transgenesis on the physiological state of the recipients (SIROTKIN *et al.* 2008). Nevertheless, recent publications show that the presence of mWAP-hFVIII (the human clotting factor VIII transgene under the murine whey acidic protein promoter) in rabbits may increase the incidence of pathological changes in the animal (PARKANYI *et al.* 2004; MARTINIAKOVA *et al.* 2005; JURCIK *et al.* 2007). The effect of this transgene on rabbit ovarian and testicular tissue has not been previously reported.

Successful mWAP-hFVIII gene construct integration should cause expression of the recombinant protein in the mammary gland only. However, transgenic rabbits should have the gene integrated into each cell with a nucleus, gonads included. Since effective reproduction, high pregnancy rates and stability of transgene transmission to the offspring are important factors limiting the efficiency of transgenic animal production, the objective of this work was to evaluate possible effects of mWAP-hFVIII integration on the ovarian and testicular structure.

Material and Methods

All the experiments related to the study had been previously approved by the State Veterinary and Food Administration of the Slovak Republic (Permission no. SK P 28004 for the research facility and no. Ro 6115/04-220 for manipulations with the animals) as well as by the Biosafety Department of the Ministry of Environment of the Slovak Republic (Permission for GMO research no. 1330/247/2004-5.2.-13-PPZ20).

Ten transgenic rabbits (5 males, 5 females) (CHRENEK *et al.* 2005a) were selected for this study. The animals were obtained after breeding a mWAP-hFVIII transgenic founder rabbit (No. 10, line SM) with non-transgenic New Zealand White rabbits. DNA was isolated from ear tissue of the offspring and PCR was used to detect the integration of the mWAP-hFVIII gene construct (CHRENEK *et al.* 2005a; MARTINIAKOVA *et al.* 2005). Littermate non-transgenic rabbits (5 males, 5 females) were used as controls. As the study was designed to evaluate the histological development of the gonadal structures, we chose young and sexually immature animals (1.5-2 months old) with 1.5-2.0 kg of weight.

The rabbits were housed in specialized halls used for rabbit breeding, kept in individual steel cages and in a controlled environment (constant temperature and light-dark regime). Food and water were supplied *ad libitum*.

The animals were paralyzed using electric stunning and sacrificed by cutting the carotid artery. Subsequently, 70 ovarian tissue samples were obtained; 7 from each transgenic and non-transgenic female rabbit. Similarly, 70 testicular tissue samples were collected; 7 from every transgenic and non-transgenic male rabbit. The samples were fixed in 10% formol, dehydrated in a grade series of ethanol (70%, 80%, 90% and 100%), saturated with benzene and embedded into paraffin. Paraffin blocks were sectioned on a microtome into 7-12 μm thick sections which were stained with haematoxylin and eosin. Photographs were taken using a NU2 optical microscope (VEB Carl Zeiss, Jena, Germany) and Olympus C-5060 digital camera (Olympus, Tokyo, Japan). Fine quantification was performed using the Quick PHOTO MICRO system (Olympus, Tokyo, Japan) (MASSANYI *et al.* 2000a; MASSANYI *et al.* 2000b).

Quantitative microscopic structures, the relative volume (%) of primary follicles, follicles with less than 2 layers of granulosa cells, follicles without antrum formation, antral follicles and interstitium, as well as the diameter (μm) of developmental ovarian structures in relation to oocytes and follicles were investigated in ovarian tissue samples.

The relative volume (%) of germinal epithelium, lumen and interstitium were studied in testicular tissue samples. The seminiferous tubule and lumen diameters (μm), as well as epithelial height (μm) of the germinal epithelium were counted as well.

Statistical analysis of the obtained data was carried out using the SAS statistical program (SAS Institute Inc., Cary, USA). Basic statistical parameters (mean, standard deviation, coefficient of variation) were calculated at first. Subsequently, a paired *t*-test was used to compare the results between the transgenic and non-transgenic group. Average values of the experimental and control group were tested against each other. Differences among the groups at $P<0.05$ were considered as significant.

Results

Testes

The testes were completely enclosed by the *tunica albuginea*, which was thickened posteriorly to form the *mediastinum* of the testis. Fibrous septa formed by the *mediastinum* divided the testicular body into lobules, with each lobule containing seminiferous tubules. Each seminiferous tubule

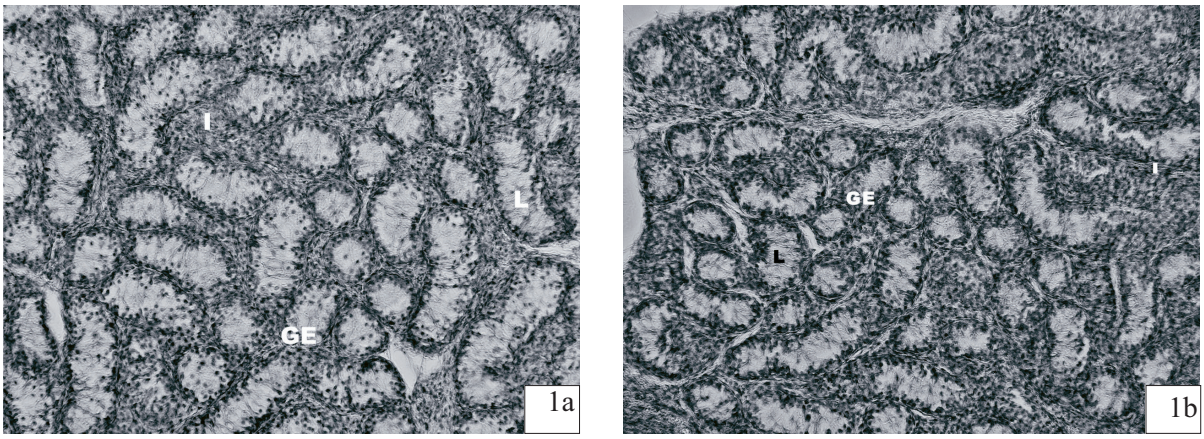

Fig. 1. Microscopic structure of the rabbit testes composed of germinal epithelium (GE), interstitium (I) and lumen (L). The structures were not significantly altered in non-transgenic animals (1a) in comparison to transgenic (1b) ones. Light microscopy. Original magnification ×100.

Table 1

Relative volume (%) of testicular structures in transgenic (n=35) and non-transgenic (n=35) samples

Parameter	x	SD	CV	minimum	maximum
Non-transgenic rabbits					
Germinal epithelium	71.09	3.58	5.03	62.30	79.90
Interstitium	13.56	2.48	18.31	9.10	20.60
Lumen	15.35	2.21	14.42	10.80	19.20
Transgenic rabbits					
Germinal epithelium	69.06	4.09	5.93	62.50	76.50
Interstitium	15.45	2.57	16.61	11.40	20.30
Lumen	15.49	2.29	14.80	11.60	20.90

P>0.05; x – mean; SD – standard deviation; CV – coefficient of variation (%).

had a central lumen lined by the germinal epithelium, mixed with supporting cells – the Sertolli cells (Fig. 1). Quantitative morphometric analysis revealed some differences between transgenic and non-transgenic males (Tables 1, 2). The epithelial height was significantly (P<0.05) higher in non-transgenic animals compared to the transgenic ones (25.87±6.72 μm versus 25.55±4.04 μm, and 26.93±6.01 μm versus 23.24±4.14 μm, respectively). Other structures were not significantly altered in transgenic animals (P>0.05).

Ovaries

The surface of the ovaries was covered by a single layer of epithelial cells. A basal membrane separating the surface cells from the underlying ovarian tissue was divided into the inner *medulla* and outer *cortex*. The *cortex* was composed of follicles and interstitium (Fig. 2). Evaluation of the ovarian tissue revealed that in both cases, the interstitium was the dominant histological structure (80.51±8.03% in non-transgenic rabbits and 81.55±6.48% in transgenic rabbits). A significantly higher (P<0.05) value was obtained for the antral follicles in non-transgenic (8.56±8.57%) compared to transgenic (4.42±5.08%) animals. On the other hand, a significantly higher (P<0.05) value was detected for the transgenic primary follicles (4.56±2.78% versus 3.45±1.63%). Other differences were not statistically relevant (P>0.05). No significant differences between non-transgenic and transgenic females were observed in the diameter of the developmental follicular ovarian structures (Tables 3, 4). Our data in both groups demonstrate a fluent increase in the oocyte and follicular diameters related to the developmental stage. Generally, the oocyte and follicular diame-

Table 2

Diameter of seminiferous tubules, lumen diameter and epithelial height in the testes (μm) of transgenic (n=35) and non-transgenic (n=35) rabbit samples

Parameter	x	SD	CV	minimum	maximum
Non-transgenic rabbits					
Seminiferous tubules	101.57	15.96	15.71	77.30	181.40
Lumen	48.77	10.98	22.51	25.00	74.40
Germinal epithelium I	25.87	6.72	25.97	2.70	56.00
Germinal epithelium II	26.93	6.01	22.31	13.50	51.00
Transgenic rabbits					
Seminiferous tubules	90.96	13.93	15.32	69.80	129.70
Lumen	45.17	11.41	25.25	19.40	74.90
Germinal epithelium I	22.55*	4.04	17.94	13.50	36.80
Germinal epithelium II	23.24*	4.14	17.81	12.20	38.20

*P<0.05; x – mean; SD – standard deviation; CV – coefficient of variation (%).

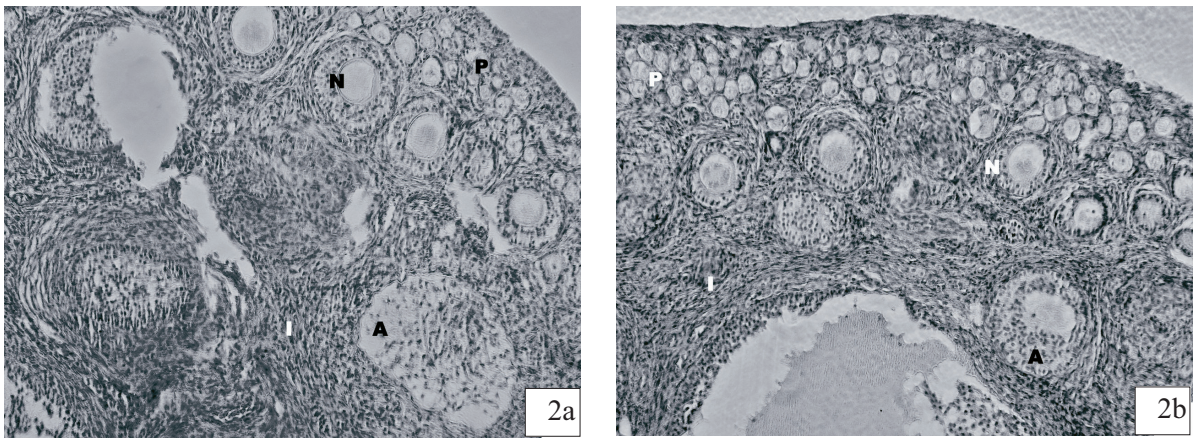

Fig. 2. Microscopic structure of the non-transgenic (2a) and transgenic (2b) rabbit ovaries containing primary follicles (P), follicles without antrum formation (N), antral follicles (A) and interstitium (I). Light microscopy. Original magnification ×100.

Table 3

Relative volume (%) of ovarian structures in transgenic (n=35) and non-transgenic (n=35) samples

Parameter	x	SD	CV	minimum	maximum
Non-transgenic rabbits					
Primary follicles	3.45	1.63	47.11	0.90	6.70
Follicles layers GC	2.51	1.91	76.14	0.00	7.00
Follicles without antrum	4.88	3.38	69.29	0.00	12.40
Antral follicles	8.65	8.77	101.39	0.00	31.80
Interstitium	80.51	8.03	9.98	58.00	93.50
Transgenic rabbits					
Primary follicles	4.56*	2.78	60.90	0.80	9.80
Follicles <2 layers GC	2.25	2.25	100.22	0.00	7.50
Follicles without antrum	7.22	2.91	40.23	1.40	13.30
Antral follicles	4.42*	5.08	114.77	0.00	17.20
Interstitium	81.55	6.48	7.95	68.30	92.10

*P<0.05; x – mean; SD – standard deviation; CV – coefficient of variation (%); GC – granulosa cells.

Table 4

Diameter (μm) of developmental stages of oocytes and follicles in transgenic (n=35) and non-transgenic (n=35) samples

Parameter	x	SD	CV	minimum	maximum
Non-transgenic rabbits					
Oocytes					
– in primary follicles	26.37	4.57	17.32	16.40	39.70
– in follicles without antrum formation	76.61	16.69	21.79	39.80	110.00
– in antral follicles	145.64	81.07	55.67	86.10	317.00
Follicles					
Primary follicles	36.57	5.72	15.65	24.90	51.20
Follicles without antrum formation	123.21	28.99	23.53	63.00	224.50
Antral follicles	402.27	128.38	31.91	256.20	650.30
Transgenic rabbits					
Oocytes					
– in primary follicles	26.27	5.03	19.17	16.40	39.90
– in follicles without antrum formation	65.03	17.56	27.01	23.60	96.80
– in antral follicles	97.80	24.00	24.62	73.50	121.50
Follicles					
Primary follicles	35.44	5.38	15.19	23.10	51.90
Follicles without antrum formation	112.40	28.86	25.68	58.80	161.80
Antral follicles	361.97	128.85	35.60	233.10	490.80

x – mean; SD – standard deviation; CV – coefficient of variation (%).

ters of transgenic rabbits was lower compared to non-transgenic rabbits, but no difference was statistically significant (P>0.05).

Discussion

The rabbit has become an appealing alternative to study a variety of biological functions and human diseases. Rabbits are closer to humans and larger than mice or rats, thus more appropriate for surgical experiments. Cloning in rabbits is now possible and its complete genome is about to be sequenced. Moreover, rabbits are already being used to produce specific pharmaceutical proteins at an industrial scale (BOSZE *et al.* 2003; BODROGI *et al.* 2006). However, most of the studies focused on the rabbit as a bioreactor only evaluate transgene integration and expression in the animal. The health state of the animals is mentioned only occasionally. Only a handful of studies inform about the impact of recombinant protein production on the overall fertility of the animal models (MASSOUD *et al.* 1996; MIKUS *et al.* 2004).

In our case, the lactating mammary gland of transgenic rabbits successfully expressed the human clotting factor VIII, a protein essential for blood clotting (NIEMANN *et al.* 1999; HIRIPI *et al.* 2003).

The structure of several mWAP-hfVIII rabbit organs has been studied before. Specific attention was dedicated to the mammary gland as the target organ for transgene expression. DRAGIN *et al.* (2006) showed no significant differences in a quantitative examination of the transgenic and non-transgenic mammary gland tissue structure using electron microscopy. Furthermore CHRENEK *et al.* (2009) examined transgenic milk characteristics, as well as selected histological, ultrastructural and apoptotic features of the transgenic mammary gland tissue during three lactations in the F4 generation. No negative effects of the transgene construct on transgenic rabbit milk performance and/or mammary gland structure were observed.

On the other hand, MARTINIAKOVA *et al.* (2005; 2008) examined the femoral bone structure of WAP-hFVIII transgenic rabbits. A new type of bone tissue was identified around the osteons in transgenic animals. Furthermore, a significantly higher rate of aneuploid cells from bone marrow was observed in the WAP-hFVIII rabbits, confirming a similar phenomenon detected in the peripheral blood lymphocytes of the same transgenic animals (PARKANYI *et al.* 2004). These results in-

dicate physiological changes possibly caused by genetic manipulations.

The main function of the ovaries and testicles is to contribute to the events leading to fertilization, but they also significantly affect a variety of other vital processes in the organism. At the same time it is necessary to realize that their proper function is dependent on a wide range of exogenous and endogenous factors. The presence and expression of a foreign gene in the organism may easily be one of them.

We found a significantly higher number of primary (immature) ovarian follicles in transgenic rabbits. On the other hand, a significantly higher number of antral follicles was found in non-transgenic rabbits. The antral follicle stage represents the early tertiary phase of folliculogenesis, and it may become a mature or preovulation follicle. Our results show that there was a presumably higher hormonal stimulation towards folliculogenesis and earlier development of mature follicles in the non-transgenic rabbits. Higher antral follicle count may also suggest a higher ovarian reserve and subsequently higher fertility. However, this difference was not supported by other results, since we found a relatively high count for the transgenic tertiary follicles without antrum formation, suggesting normal folliculogenesis. Also, the analysis did not reveal other significant differences therefore we may assume that the changes could be related to the current physiological and individual state of the animals. Additionally, WEBB *et al*. (2004) stated that variability of numbers of antral follicles growing during follicular waves is very high amongst animals, but remarkably highly repeatable within individuals. Thus, some females have relatively low or high numbers of antral follicles growing during follicular waves. Finally, all the transgenic rabbits obtained by the same breeding approach were overall healthy, fertile and after reaching sexual maturity, gave birth to healthy offspring with successful transmission of the transgene.

The evaluation of the testicular structure did not reveal significant changes between transgenic and non-transgenic animals, except for the height of the seminal epithelium. A higher germinal epithelium means higher epithelium activity and possibly elevated spermatogenesis. Equally as in females, we assume that there might be a higher hormonal stimulation in favor of sex cell production. According to the results, more spermatozoa were produced in non-transgenic rabbits. However, a higher concentration of spermatozoa does not mean a higher quality or a better fertilization rate. Based on no additional significant changes found, we assume that the differences between the epithelial heights were due to individual variability in the reproductive health states of the animals. This hypothesis is also proven by the fact that after reaching sexual maturity, all the transgenic males obtained from the mWAP-hFVIII transgenic founder rabbit were fertile. Moreover, CHRENEK *et al.* (2005b; 2007a;b) examined the effects of WAP-hFVII transgenesis on the reproductive traits and semen quality of rabbit males, likewise without finding any significant differences compared to non-transgenic rabbits.

In conclusion, this study demonstrates a weak negative effect of the mWAP-hFVIII transgenesis on the rabbit ovarian and testicular structure, which is revealed as relatively lower folliculogenesis and spermatogenesis. However, no important histological alterations were found, which together with previous studies, suggest that this effect did not influence the fertilization capacity of the rabbits. Since the transgene was successfully integrated, the recombinant product was produced and fertility was not altered by the presence of the transgene, we suggest that the mWAP-hFVIII transgenic rabbits may become an available alternative source of rhFVIII for pharmaceutical applications. As a result, this development constitutes another successful example of the application of transgenic animal technology.

Nevertheless, transgenesis is a relatively new biotechnology with many unpredictable consequences. Long-term effects of transgenesis have to be examined in the future and further observations will be required. Based on possible individual characteristics influencing the overall health state of the animals, we suggest particular evaluation and selection of transgenic animals when establishing new transgenic lines. It is also essential to establish the legislative safety of allowing practical applications of recombinant proteins before any of the transgenic technologies are implemented at the farm level.

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