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*Original paper*

# Effect of Transgenesis on the Morphology and Function of the Rabbit Mammary Gland

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## Abstract

Transgenesis is a biotechnological process of transferring one or more genes from the DNA of a donor organism into a recipient DNA, in order to introduce genes into the genome of the recipient and exert their effects in the offspring, and to transfer that new characteristics to their progeny. This research was carried out in order to study the changes in the structure and function of the mammary gland of rabbit, in order to compare control of transgenic and non-transgenic animals. A transgenic rabbit group was created by microinjection of embryos with the WAP gene structure – hFVIII that determines synthesis of human factor VIII in the mammary gland of females. Quantitative microscopic structure and the relative volume of the glandular epithelium were examined in mammary gland tissues. There was no significant differences in the apoptotic cells percentage between transgenic and non-transgenic mammary gland tissues at third lactation stage, but the percentage of apoptotic cells at the involution stage was significantly higher in non-transgenic compared with transgenic mammary gland tissues. Analysis showed significant differences in the presence of mitochondria and vacuoles in the tissue of the mammary gland of transgenic and non-transgenic females, while the other parameters showed no significant differences. Genome manipulation and microinjection of WAP gene structure – hFVIII in transgenic rabbit embryos did not significantly affect mammary gland tissues morphology of lactating rabbits, and does not have significant expression in other organs.

**Keywords** Mammary gland, transgenesis, rabbits.

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# Introduction

The aim of the research is to investigate the morphology and function of the mammary gland of transgenic female rabbits, and then to compare it with a control group of standard genotype.

The study was based on an assumption that there are differences in the composition, ultrastructure and apoptotic status of the mammary gland of transgenic females and standard genotype. Compared with other mammalian species (pig, goat, sheep, and cow) that are currently being studied as bioreactors, rabbits offer many advantages: high fertility, easy generation of transgenic founders and offspring, insensitivity to prion diseases, relatively high milk production, and no transmission of severe diseases to humans (WANG et al, 2013). Milk production, reaches its maximum two weeks after birth (TVRDÁ et al 2014, CHRASTINOVA et al, 1997), stays on that level for 10-15 days, and falls considerably during the fourth week (CARA, 2003; CARA et al, 2004). In the eighth week after birth, young offspring can consume 90% of plant food and 10% of lactic diet. The technology for using the mammary gland as a bioreactor has been developed to the point that pharmaceuticals derived from the milk of several transgenic farm animal species, currently in the advanced stages of clinical tests (DOVE 2000; FAN 1999; BOSZE, 2003; CHRENEK and MAKAREVICH, 2008).

# Materials and Methods

### Biological material

Medium-sized transgenic animals including rabbits are used as bioreactors for production of human proteins (SUCKOW et al, 2012.).The experiment was carried out on a commercial consume hybrids created by crossing New Zealand white and Californian rabbits. Biological material was obtained from transgenic rabbits microinjected during the embryo phase with gene structure WAP-hFVIII that determines synthesis of human factor VIII (coagulation factor VIII blood HF) in the mammary gland of female rabbits. Gene construct WAP – whey acid protein originaly thought to be present only in rodents (VILOTTE et al, 2013.) hFVIII - human factor VIII was obtained from the laboratory al. Henry Lubon's, the American Red Cross. WAP – line hFVIII founders were created according to the recommendations of Chrenek et al. 2004. We used the transgenic founders and transgenic rabbits lines of F1 generation. These were obtained by growing both lines of transgenic founder lines (SM single microinjection and DM – double microinjection) with no transgenic rabbits of the same New Zealand line. The experiment involved the F2 generation of transgenic New Zealand white rabbits after crossing transgenic founder lines (genomic WAP-hFVIII) with non-transgenic rabbits of the same breed (CHRENEK et al, 2003). Detection of transgene integration in the offspring of the F1 generation was performed using PCR. DNA was extracted from the tissue of the ear (VASICEK et al, 2003) or from the hair (BAUEROVA et al, 1999) of newborn rabbits (F1 generation). The animals were kept in standard cages in facilities with controlled microclimate conditions,

Animals were selected from identical litters and after testing the integration of the genes were divided into two groups: positive integration and without integration of transgenes. Animals with positive integration were not split by gender, evaluated within a statistical group and compared with their non-transgenic relatives from identical litters. This study was carried out in compliance with Directive 86/609/EEC on the protection of animals used for scientific purposes.

### Genetic analysis

Detection of the gene responsible for producing the aforementioned proteins was performed on subsequent generations after transfer of microinjected embryos in surrogate mothers. Identification of the transgene into the genome of the rabbit was carried out by PCR method from a sample of DNA obtained by notching the rabbit ear. The presence of human factor VIII has been identified in the milk by Western blott method.

### Sampling of the mammary gland tissue

From each mammary gland at abdomenal region of females (non-transgenic and transgenic), at 35$^{th}$ day of lactation, 3 samples were obtained. After collection, specimens were fixed in 10% formol and then dehydrated in the graded ethanol series (70, 80, 90 and 100%), then neutralized in benzene inserted (fixed) in paraffin. Blocks of samples are sectioned on a microtome into sections with the size of 7-12 micrometers that were subsequently stained with hematoxylin and eosin (MASSANYI et al, 2000). On the basis of morphological criteria (UHRIN, 2002) the quantitative value of the mammary gland structure were evaluated for each section separately. Tissue of the mammary gland was studied in terms of its quantitative microscopic structure, the relative volume (%) glandular epithelium, and the lumen is formed by interstitium fibrocolagenous and connective tissue.

### Analysis of cell death

Samples of transgenic and non-transgenic thick tissue sections (3-4 μm) of mammary gland during lactation and involution stages were processed for terminal deoxy-nucleotide transferase dUTP nick end labelled (TUNEL) using a MEBSTAIN Cell death kit Direct (Immunotech, Marseille, France). Samples were deparaffinized using xylene, rehydrated in ethanol series (100%, 90%, and 80%) and treated with proteinase K for permeabilization. Labeling of samples followed using terminal deoxynucleotidyl transferasereagent, counterstained with 4',6-diamidino-2-phenylindole (DAPI) stain and mounted into Vectashild anti-fade reagent (Vector Laboratories, Burlinghame, UK). Cell death was analysed under a Leica fluorescent microscope (Leica, Wetzlar, Germany) calibrated on a 10:1 or 20:1 objective and 10:1 eyepiece. Cell death rate was calculated as percentage of TUNEL-positive cells in total number of cells stained by DAPI.

### Electronic microscopy

Mammary gland tissue samples were fixed in 2.5% glutaraldehyde in 0.15M cacodylate buffer (pH 7.1-7.3) for 1 hour and washed in cacodylate buffer. This was followed by one-hour post-fixation in 2% osmium tetroxide, and cacodylate buffer, followed by washing in distilled water, dehydratation in acetone, samples were fixed in Durkupan ACM (Fluka, Buchs, SG, Switzerland). Blocks of tissue samples were cut on LKB-Nova ultramikrotome (LKB, Bromma, Switzerland) in section thickness of 1-2 μm and stained with toludin blue.

These sections (silver) were contrast stained with uranyl acetate and lead citrate and examined in JEM 100 CX II (Jeol, Tokyo, Japan) 80kV electron microscope, at magnification ratio of 18 000:1. Each sample of mammary gland produced at least 10 microphotographies.

### Morphometry

Photos of the mammary gland tissue obtained by electronic microscope were scanned by HP scanjet 3770 (Paolo Alto, CA, USA) and transfered in to digital format. All cell structures (mitochondria, Goldgy apparatus, endoplasmic reticulum, vacuoles, fat and protein micelles) necessary for this study were contrast painted and area digitally measured by Auto-Cad software (Autodesk, San Rafael, CA, USA). All parameters were statistically analyzed.

### Statistical analysis of data

After measuring and systematization, obtained data was statistically analyzed. Arithmetic calculations of individual data sets were performed, and values were compared using t-test (HADŽIVUKOVIĆ, 1991).

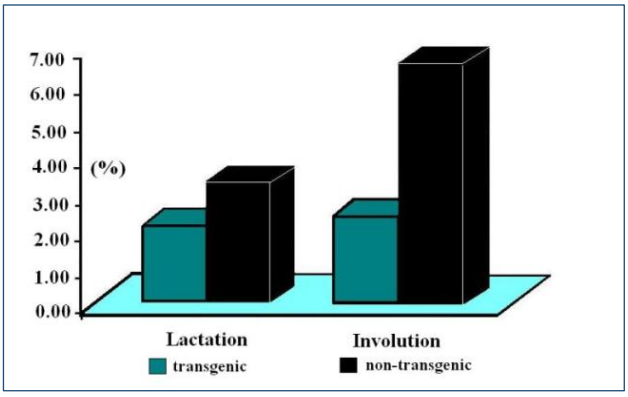
## Results and Discussion

### Features of the mammary gland of transgenic rabbits

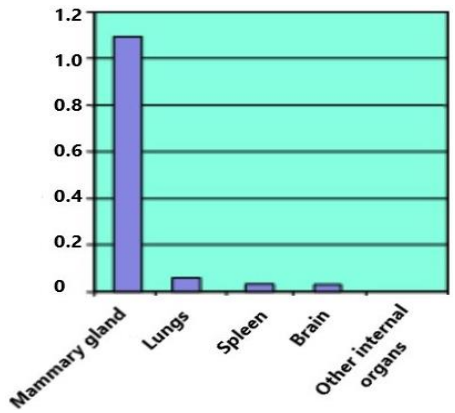
Mammary gland is a rare body structure, which is characterized by repeated cycles of structural development, functional differentiation and regression. Transgenic animals that show recombinant protein secretion in the mammary gland were genetic "re-coded" so that the changes in milk composition was inevitable. These animals produce milk with different protein profiles. The amount of secretion was not impaired to transgenesis (CARA, 2003), as well as most cell organelles (except vacuoles and mitochondria), and concluded that the ultrastructure of secretory epithelium of the mammary gland remains stable after the change of the genome. Cell death at the lactation or involution stage was found in epithelial cells of mammary gland tissues from transgenic and non-transgenic females. No significant differences in the of apoptotic cells percentage between transgenic and non-transgenic mammary gland tissues at third lactation stage were found (2.1% versus 3.3%, Graph 1). On the other hand, the percentage of apoptotic cells at the involution stage was significantly higher ($P<0.01$) in non-transgenic compared with transgenic mammary gland tissues (6.5% versus 2.4%). This result is in accordance with our earlier reports, that the mWAP-hFVIII gene construct does not bring any negative effect on transgenic rabbit milk quantity and quality (CHRENEK et al, 2007 a, b). Process of the mammary gland involution may be accompanied by an increased rate of cell death in this tissue. Probably increased rate of apoptotic cells in nontransgenic tissue, reflects existing difference in the dynamics of involution process compared with transgenic tissues.

Large, cristae-rich mitochondria with dense matrix were seen in secretory epithelium, unlike the elongated mitochondria of developing adipocytes. Table 1 presents the results related to differences in the secretory epithelium of the mammary gland of transgenic and non-transgenic females.

Statistical analysis showed significant differences ($P<0.01$) in the presence of mitochondria and vacuoles in the tissue of the mammary gland of transgenic and non-transgenic females, significant differences ($P<0.05$) in the presence of endoplasmatic reticulum and protein globules, while the other observed parameters showed no statistically significant differences. Table 2 shows the quantitative differences in the microscopic morphology of the tissues of the mammary gland of transgenic and non-transgenic rabbits (relative volume (%) glandular epithelium, lumen and interstitium formed fibrocolagenous and connective tissue).




**Graphic 1.** Cell death rate (terminal deoxynucleotide transferase dUTP nick end labeling index) in transgenic and non-transgenic rabbit mammary glands on D35 of third lactation (lactation stage) and 6 weeks after the end of lactation (Involution stage).




**Graphic 2.** Expression of mWAP - rhFVIII in different tissues of transgenic females

**Table 1.** Ultrastructure of the mammary gland tissues of transgenic and non-transgenic rabbits (%)

| Morphology (%) | Non-transgenic animals $\bar{x}_1$ | Transgenic animals $\bar{x}_2$ | t-test |
|---|---|---|---|
| mitochondria | 0.50 | 0.76 | 2.409443 ** |
| E.R. | 0.33 | 0.45 | 1.626307 * |
| Golgi apparatus | 0.05 | 0.03 | 0.530482 ns |
| fat droplets | 0.27 | 0.27 | 0.069643 ns |
| protein globules | 0.18 | 0.11 | 1.334961 * |
| vacuoles | 4.60 | 3.03 | 2.787644 ** |

Ns - no statistically significant difference; * - statistically significant difference P<0.05;
** - statistically significant difference P<0.01

**Table 2.** Analysis of differences in the microscopic morphology of the mammary gland of transgenic and non-transgenic rabbits (%)

| Tissue (%) | Non-transgenic animals $\bar{y}_1$ | Transgenic animals $\bar{y}_2$ | t-test |
|---|---|---|---|
| Epithelium | 26.51 | 20.22 | 0.680366 ns |
| Lumen | 64.13 | 73.14 | 0.600676 ns |
| Collagen fibers | 2.30 | 1.86 | 0.473460 ns |
| Connective tissue | 7.06 | 4.78 | 0.789639 ns |

ns - no statistically significant difference

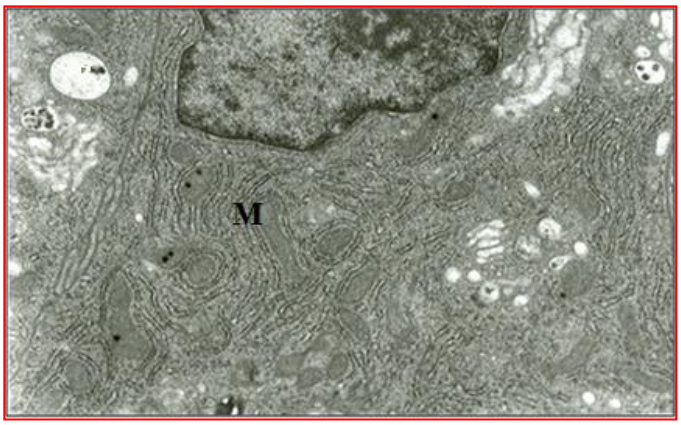



**Figure 1.** The mitochondria (M) in the tissue of the mammary gland of non-transgenic females as Durcupan ACM thin (silver) section. Magnification: x 18 000.

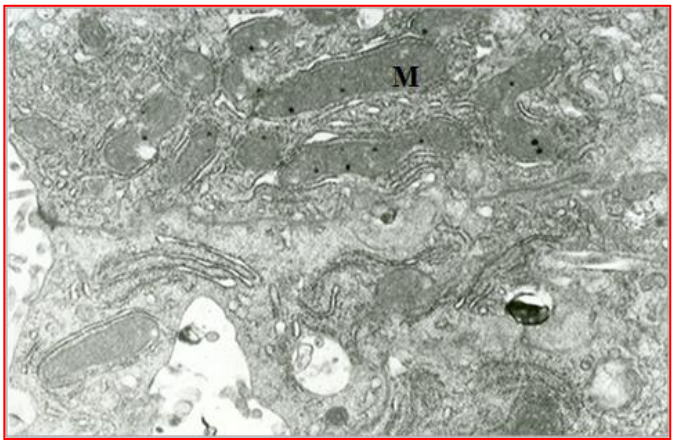



**Figure 2.** The mitochondria (M) in the tissue of the mammary gland of transgenic females as Durcupan ACM thin (silver) section. Magnification: x 18 000.

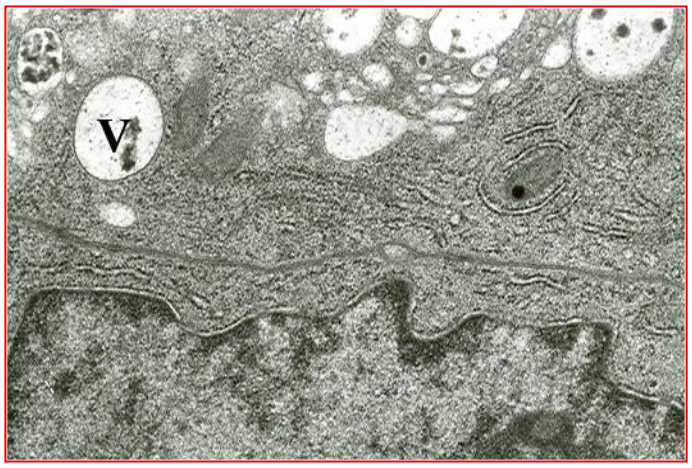



**Figure 3.** Vacuoles (V) in the mammary gland tissue of non-transgenic females as Durcupan ACM thin (silver) section. Magnification: x 18 000.

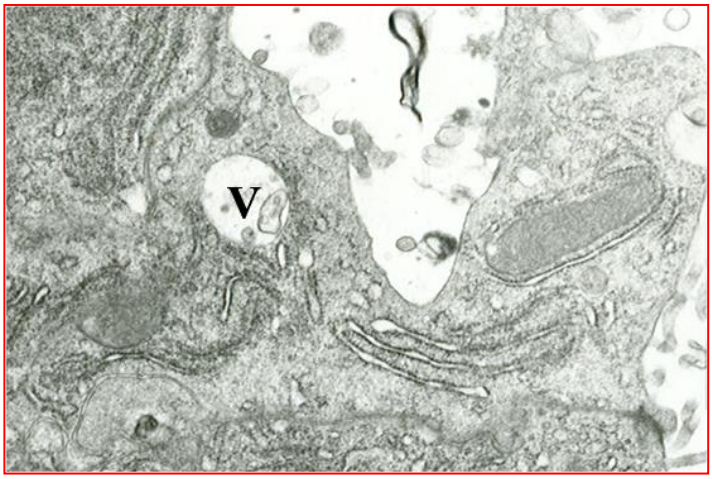



**Figure 4.** Vacuoles (V) in the tissues of the mammary gland of transgenic females as Durcupan ACM thin (silver) section. Magnification: x 18 000.

The mammary gland histology of transgenic rabbits producing recombinant human factor VIII in comparison with that of non-transgenic rabbit females with a several significant differences were reported (DRAGIN et al, 2006; DRAGIN 2007; CHRENEK et al, 2009). However, these results indicate no any harmful effect of the mWAP-hFVIII transgene expression on the state of mammary gland of transgenic rabbit females. The number and size of vacuoles are normally highly variable and in positive correlation with milk production. The size and number of vacuoles varies in a short period of time and should be positively correlated with the production of milk. The fact that transgenic and standard genomes in this trial have the same lactation averages, it can be assumed that the difference in size between the experimental groups of vacuoles are random. Differences in the size of mitochondria may affect a change in the balance of energy between the two experimental groups, but the difference in the number of mitochondria gives the assumption that these changes occurred in the period immediately preceding the biopsy. Different size of mitochondria may suggest an alteration in the energy balance between two experimental groups or a different mechanism. Further studies on mammary tissue over several stages of lactation and involution are required to determine whether this is a constant feature. These results are interesting in light of the recent reports that reversible transdifferentiation of secretory epithelial cells into adipocytes occurs in the mammary gland (GIORDANO et al, 2017; MORRONI et al, 2004).

Genome manipulation and microinjection of additional structure in transgenic rabbit embryos did not significantly affect mammary gland tissues morphology of lactating rabbits. Although there are few studies focused in this direction, some authors confirm these results. LAURENCE et al (2000) have carried out research on the gene expression of human hepatocarcinoma-intestin-pancreas / pancreatic associated protein (HIP/ PAP) in the mammary gland of transgenic mice during lactation. In order to confirm that the reduction in the secretion of HIP/ PAP consequence of the accumulation of the protein

in milk cells, the researchers compared the ultrastructure of milk epithelial HIP/ PAP transgenic mice during lactation with them than non transgenic mice. No significant morphological changes were observed in the milky epithelium of transgenic mice. Observed numerous bags filled Golgi and secretory vesicles when compared with normal mammary cells of mice. In terms of ultrastructure of milk epithelial cells are different observations and conclusions. The histological analysis of mammary gland HPC/ PACE mice did not show any difference in their morphology when compared to the control group. The tissue of transgenic animals showed slight mismatch, had less filled alveoli with larger epithelial cells of hPC as in transgenic mice is performed as PALEYANDA et al (1997). Other authors have performed similar studies with various types of transgenic animals. CHEN et al (2002) investigated the presence of bioactive hFVIII in the milk of transgenic mice, the tissue samples of freshly excised mammary glands by SASSON et al (1988) protocol. This experiment demonstrated that the transgenes were products accumulated in the lumen of the lactiferous tubules and thereby influence the volume of the cell. In addition to the tissue samples from the mammary gland, the team analyzed the tissue samples of heart, liver, lung, muscle, brain, pancreas and found no homologous transcripts hFVIII. Our research has shown that, in addition to the mammary gland, where it recorded the highest attendance, low rhFVIII presence in the spleen, lungs, and brain (Grafic 2.).

At the micro photos can be observed that the tissue of the mammary gland of transgenic females contain a larger number of mitochondria than mammary gland tissue of non-transgenic females during lactation (Figures 1 and 2).

Also, in the tissue of the mammary gland of transgenic females was noticed fewer vacuoles than in the tissues of non-transgenic animals (Figures 3 and 4).

## Conclusion

On the basis of these obtained results, following conclusions could be defined:

- There was no significant differences in the apoptotic cells percentage between transgenic and non-transgenic mammary gland tissues at third lactation stage, but the percentage of apoptotic cells at the involution stage was significantly higher in non-transgenic compared with transgenic mammary gland tissues;
- Organelles area measurement showed significant differences in the presence of mitochondria and vacuoles in the tissue of the mammary gland of transgenic and non-transgenic females, while the other parameters showed no significant differences;

Genome manipulation and microinjection of WAP gene structure - hFVIII in transgenic rabbit embryos did not significantly affect mammary gland tissues morphology of lactating rabbits, and does not have significant expression in other organs.

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