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PRODUCTION OF RECOMBINANT HUMAN FACTOR VIII IN THE MILK OF TRANSGENIC RABBITS

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ABSTRACT

The stability of milk production, quality and content of recombinant human factor VIII (rhFVIII) in transgenic rabbits was determined. Transgenic rabbits carrying the mouse whey acid protein promoter (mWAP) and human factor VIII gene (hFVIII) were born after mating of transgenic and non-transgenic rabbits. PCR analysis of samples from F4 generation showed that the transgene was transmitted. Milk production, obtained at 10th, 15th, 20th and 30th day of the first lactation by weight-suckle-weight method, showed no significant difference between transgenic and non-transgenic does. No significant difference between transgenic and non-transgenic rabbit milk composition was also found. ELISA test confirmed rhFVIII at the level of 3 – 12 μg/ml. Milk sample from non-transgenic rabbit was hFVIII negative. In conclusion, the stabilty of hFVIII transgene transmission and production of rhFVIII was confirmed in F4 generation of transgenic rabbits, with lower efficiency than in F1, F2 and F3 generations, but without reduction in milk production.

Key words: rhFVIII, transgenic, rabbit, milk production,

INTRODUCTION

The mammary gland of farm animals is probably the most promising target tissue because it produces large amounts of protein in a temperature-regulated fluid that may be collected daily in a non-invasive fashion. Transgenic animals used as bioreactors to produce human recombinant proteins represent a new horizon in animal husbandry, but are often followed by low viability of newborn animals. Repercussions of that can also be irregular secretion and alteration of milk composition. Milk is usually the sole source of nourishment of young mammals, therefore offspring growth and development depends on milk. Rabbit milk yield may be affected by breed of doe (Lukafahr et al., 1983), nutrition (Chrastinova et al., 1997), number of kids suckling and their age of weaning and pregnancy during lactation (Lukafahr et al., 1983). Intensive recombinant human protein production by mammary gland of transgenic rabbit necessitates the knowledge of the lactation curve and quality (composition) of milk as a possible effect of transgenesis on milk yield (Chrenek et al., 2007a). Lactating rabbit female can produce 170-220g of milk per day and yield up to 10kg of milk per year under semiautomatic hygienic milking conditions. Some other criteria which influences selection of the species for transgenesis are reproductive

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capabilities focused on reproductive interval and number of offspring per litter (production of new generations with reasonable efficiency) with a stable transgene transmission in offspring's genome.

This study demonstrates stability of content of recombinant factor VIII in the milk of transgenic rabbits derived from F4 generation and compares milk production between transgenic and non-transgenic lactating females on the first lactation.

MATERIALS AND METHODS

Biological material

Transgenic rabbits derived from F4 generation were obtained following the mating of transgenic (F3 generation) and non-transgenic rabbits. Transgenic founders were produced by microinjection of mWAP-hFVIII gene construct into pronucleus of fertilized eggs (Chrenek et al., 2005). PCR technique was used to detect mWAP-hFVIII gene construct integration in the transgenic rabbit from F4 generation, as previously reported (Chrenek et al., 2007b). The animals of about 4-5 months age weighing 3.5-4 kg of were used in this experiment. All females gave rise to 7-9 pups per litter. They were housed in individual steel cages, with adjacent boxes facilitating the separation of litter during entire experiment, in controlled environment (constant temperature and light-dark regime). Food and water were offered *ad libitum.*

Milk production

Rabbit pups' milk consumption and litter growth were used to assess lactation performance. Measurements were realized in transgenic (n=3 females) and non-transgenic (n=3 females) animals during first and second lactation. Body weight of pups was determined on day 1, 2, 5, 10, 20, 30 *post partum*. It is known that rabbit pups are nursed only for about 3 min once every 24h. Milk consumption on 1st lactation was assessed on day 10, 20 and 30 *post partum* using the weight-suckle-weight method. Just before suckling, body weight of pups separated from their mother (24 h prior to suckling) was recorded and immediately after suckling the body weight of pups was again recorded. The difference between body weights reflected daily milk consumption (yield).

Rabbit milk analysis

Milk samples were collected (per 10ml) from each lactating transgenic and non-transgenic females on day 21 using "home made air vacuum pump" from several parts (nipples) of mammary gland. In order to stimulate milk letdown, an intramuscular injection of 5 IU of oxytocin (Veyx Pharma, Germany) was given, 10 min before milk collection.

Rabbit milk composition (content of fat, protein, lactose, dry matter as solids non fat, SNF) was investigated by infrared absorption instrument (Instrument Milko-Scan FT 120 Foss Electric Denmark, according to the manufacturer´s manual). Determination of minerals in ash was done after annealing (STN 57 0532) by atomic absorption spectrometer (UNICAM 939 Solar STN 57 0532).

Statistics

Differences between the transgenic and non-transgenic groups were determined by ANOVA followed by Duncan´s multiple range test. Differences between groups at $p<0.05$ were considered as significant.

Detection of hPC gene expression

ELISA

Secretion of rhFVIII into the transgenic milk was determined quantitatively using an ELISA-kit (Asserachrom FVIII:Ag, Diagnostica Stago, France) as per the manufacturer´s manual. Milk samples were diluted in the range of 1:10 - 1:20.

RESULTS

Transgenic animal production

Integration of mWAP-hFVIII gene in ear samples of transgenic rabbits from F4 generation was detected by PCR (data not shown). The transgenic rabbits were apparently normal and crossing with non-transgenic male yielded litter of normal size without any disturbance during lactation.

Rabbit milk production

Transgenic females showed gradual increase of milk production until day 20th (182 vs. 315 g) afterwards it was decreased next 10 days upon the end of experiment (Table 1). No significant differences in milk production were obtained between transgenic and non-transgenic females at the first lactation. Milk production of transgenic females was comparable with those of non-transgenic does.

Table 1: Milk production of transgenic and non-transgenic does at 1st lactation (g)

Female	10th	15th	20th	30th
Transgenic (n=3)	182±0.2	228±0.4	315±0.1	168±0.4
non-transgenic (n=3)	198±0.4	235±0.2	284±0.2	157±0.4

Rabbit milk composition

To analyze milk composition of transgenic and non-transgenic rabbit females, the content of fat, protein,

Table 2: Milk composition of transgenic and non-transgenic rabbits on the day 20 at 1st lactation

Females		Fat g/100g	Protein g/100g	Lactose g/100g	SNF g/100g
Transgenic	(n=3)	11.58±0.25	9.85±0.25	1.95±0.03	12.68±0.24
Non-transgenic	(n=3)	11.68±0.22	9.68±0.15	2.06±0.03	12.09±0.23

lactose and SNF was determined (Table 2). No significant differences for analyzed traits between transgenic and non-transgenic rabbits from milk samples were found.

Detection of hFVIII gene expression

The concentrations of rFVIII produced in the mammary gland of transgenic females from F4 generation, determined by ELISA, ranged between 3-12 µg/ml depending on lactation day (table 3). No signal was detected in milk of non-transgenic females.

Table 3: Concentration of rhFVIII in milk of transgenic rabbit females (µg/ml) at several days of lactation

Female		15th	20th	30th
Transgenic	(n=3)	3	12	9
Non-transgenic	(n=3)	0	0	0

DISCUSSION

Stable transgene integration in transgenic rabbits using different gene constructs (hPC, hFVIII) in different generations was also detected in the same laboratory (Chrenek et al., 2002, 2005, 2006). Zinovieva et al. (1998) obtained six generations of transgenic rabbits with stable integration and production of biologically active IGF-1 without any negative effect on their physiological or reproductive performance. Phenotypic and genotypic stability of hPC and hFVIII gene expression has been reported in several multiple lines of transgenic animals including mouse and pigs (Chen et al., 2002; Chrenek et al., 2007b). Our results correspond to data obtained from earlier studies.

Milk production of transgenic females was comparable with those of non-transgenic does. Schranner (1993) reported similar results (day 10 - 0.18 kg, day 20 - 0.21 kg, day 30 - 0.17 kg), and this observation is in agreement with previously published reports by other authors (Chrastinova et al., 1997; Dragin et al., 2004; Chrenek et al., 2006). Generally, rabbit milk yield shows gradual increase until 20th day of lactation, afterwards it decreases by next 10 days (Lukafahr et al., 1983; Chrastinova et al., 1997). This observation is in agreement with our recent data on transgenic and non-transgenic rabbit milk yield in both of the investigated lactations (Chrenek et al., 2007a). The peaks of milk production were at 315g and 284g for transgenic and non-transgenic females respectively on first lactation. These results are also consistent with previous reports, where 250 - 300g yields for New Zealand White (NZW) non-transgenic rabbits (Sanchez et al., 1985) or 225 - 250g yields for New Zealand White crossbred with Californian rabbits (Partridge et al., 1986) were obtained. Our present observation is in accordance with our previously published report (Dragin et al.2004), where we compared milk yield of NZW transgenic rabbit females over-expressing mammary gland specific hPC (human protein C) with non-transgenic females.

Rabbit milk composition varies depending on various factors, such as breed, nutrition, lactation stage or number of pups (Chrastinova et al., 1997). To investigate the difference in milk composition between transgenic and non-transgenic rabbit females derived from F4 generation, basic analysis of milk was performed under same conditions. Analysis of transgenic rabbit milk samples have showed that all the transgenic females tested in this work produced lower or higher concentration of rhFVIII ranged between 3 to 12µg/ml depending on transgenic females with confirmed biological activity and no significant differences were found in the content of milk fat, protein and lactose. This concentration was lower compared to our previous results (Chrenek et al., 2005, 2007b). The higher variability and lower rhFVIII concentration in the F4 generation may be explained by different copies of integrated gene, the site of transgene insertion or its genomic environment, which could influence its expression (Salvo-Garrido et al., 2004).

In conclusion, present study confirms the stability of rhFVIII transgene transmission and proves that rhFVIII can be steadily secreted over multiple generations without any interference on milk production and quality.

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REFERENCES

CHEN, C. M. – WANG, C. H. – WU, S. C. – LIN, C. C. – LIN, S. H. – CHENG, W. T. K. 2002. Temporal and spatial expression of biologically active human factor VIII in the milk of transgenic mice driven by mammary-specific bovine alfa-lactalbumin regulation sequences. In: *Transgenic Res*., vol. 11, 2002, p. 257-268.

CHRASTINOVÁ, L. – SOMMER, A. – RAFAY, J. – SVETLANSKÁ, M. 1997. Avotan exploitation in rabbit nutrition. II. Nutrient digestibility and lactation performance of does rabbit. In: *J. Farm Anim. Sci*., vol. 30, 1997, p. 80-86.

CHRENEK, P. – VAŠÍČEK, D. – MAKAREVICH, A. – UHRÍN, P. – PETROVIČOVÁ, I. – ČUBOŇ, H. – BINDER, B.R. – BULLA, J. 2002. Integration and expression of the WAP-hPC gene in three generations of transgenic rabbits. In: *Czech J. Anim Sci*., vol. 47(2), 2002, p. 45-49.

CHRENEK, P. – VAŠÍČEK, D. – MAKAREVICH, A. V. – JURČÍK, R. – SÜVEGOVÁ, K. – BAUER, M. - PARKÁNYI, V. – RAFAY, J. – BÁTOROVÁ, A. – PALEYANDA, R. K. 2005. Increased transgene integration efficiency upon microinjection of DNA into both pronuclei of rabbit embryos. In: *Transgenic Res*., vol. 14, 2005, p. 417-428.

CHRENEK, P. – DRAGIN, S. – MAKAREVICH, A. 2006. Rreproductive characteristics of Transgenic rabbit males with human protein C gene. *In: Slovak J. Anim. Sci*., (accepted November 2006), in press.

CHRENEK, P. – CHRASTINOVA, L. – KIRCHNEROVA, K. – MAKAREVICH, A.V. – FOLTYS, V. 2007a. The Yield and Composition of Milk from Transgenic Rabbits. *Asian-Australasin J Anim. Sci.*, vol. 20(4), 2007a, p. 482-485.

CHRENEK, P. – RYBAN, L. – VETR, H. – MAKAREVICH, A.V. - UHRÍN P. – PALEYANDA, R. K. – BINDER, B. R. 2007b. Expression of recombinant human factor VIII in milk of several generations of transgenic rabbits. In: *Transgenic Res*., (accepted January 2007b), in press.

DRAGIN, S. – BOZIC, A. – CHRENEK, P. 2004. Effect of transgenesis on F2 and F3 rabbit offspring generations. 5th. scientific conference of PhD. students. Constantine the Philosopher University : Nitra, Slovakia, 2004, p. 28-32.

LUKEFAHR, S. – HOHENBOKEN, W. D. – CHEEKE, P. R. – PATTON, N. M. 1983. Doe reproduction and preweaning litter performance of straightbred and crossbred rabbits. In: *J. Anim. Sci.,* vol. 57, 1983, p. 1090-1096.

PARTRIDGE, G. G. 1986. Meeting the protein and energy requirements of the commercial rabbit for growth and reproduction. Proc. 4th World congress of Animal Feeding. Madrid, Spain, p. 271-277.

SALVO-GARRIDO, H. – TRAVELLA, S. – BILHAM, L. J. – HARWOOD, W. A. – SNAPE, J. W. 2004. The distribution of transgene insertion sites in barley determined by physical and genetic mapping. In: *Genetics*, vol. 167, 2004, p. 1371-1379.

SANCHEZ, W. – CHEEKE, P. R. – PATTON, N. M. 1985. Effect of dietary crude protein level on the reproductive performance and growth of New Zealand White rabbits. In: *J. Anim. Sci.*, vol. 60, 1985, p. 1029-1039.

SCHRANNER, S. 1993. Untersuchungen zum maschinellen Milchentzug beim Kaninchen als Grandlage zur Bestimmung von Laktationsleistungen und Milchinhaltsstoffen. Inaugural-Dissertation, Ludwig-Maximilians-Universität : München, 1993, p.120.

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