Transient lactose-reduced milk can be achieved through *in vivo* transfection of β-galactosidase gene in rat mammary gland

**Accepted 19th July, 2013**

**ABSTRACT**

Decreasing the lactose content in dairy products is a preferred strategy to cope with lactose intolerance. This is usually obtained by adding a lactase enzyme to food products intended for human consumption. Production of transgenic mice expressing lactase in the mammary gland to produce lactose-reduced milk *in vivo* has been successful and, therefore scaling up to livestock seems feasible. However, more data on the long term effects of the modified milk composition, particularly on progeny are needed. The aim of the study was to perform an *in vivo* transfection in rat mammary gland of a β-galactosidase gene. Expression of the enzyme would allow pups to suckle breast milk containing a lower lactose concentration and thus to study the metabolic effects of modifying milk composition in both mothers and offspring. Data show the feasibility of transfecting the mammary gland *in vivo* by the infusion of plasmid DNA encoding a recombinant β-galactosidase gene. Transient activity of the recombinant enzyme was achieved in milk during lactation, allowing 66% of the control levels by day 6 of lactation. However, induction of β-galactosidase in milk was associated with increased maternal body fat content and, more importantly, offspring showed a predisposition to develop obesity in adult life. This data outlines the importance to design long-term studies in order to assess the impact of early nutritional input.

**Key words**: lactose, milk, β-galactosidase, obesity

**INTRODUCTION**

Lactose is synthesized in the mammary gland by the lactose synthase complex and constitutes the main sugar present in milk (Jensen, 1995; Ninonuevo et al., 2006; Urashima et al., 2012). Many adults throughout the world show the symptoms associated with malabsorption of lactose and are lactose-intolerant (Hammer and Hammer, 2012; NDA, 2004; Tunick, 2009). For this reason, lactose is on the list of food allergens that have to be included in the labelling of food products under European legislation (NDA, 2004). Strategies to counteract lactose intolerance without reducing calcium intake, include recommendations of dairy products treated to decrease lactose content. However, a recent consensus report recognizes that although lactase-treated products may be tolerated better than non-treated products in intolerant individuals, more research is needed (NIH, 2010).

Early attempts to produce lactose-reduced milk have been developed. For example milk containing a lactase enzyme extracted from yeast and fungi was commercialized in 1979 (Tunick, 2009) and recently, a food constituent containing a lactase enzyme has been approved by the European Food Safety Authority (NDA, 2009). Several experimental methods are able to produce lactose-reduced milk, including *in vitro* procedures (Suarez et al., 1995; Tamm, 1994; Vilotte, 2002), as well as a transgenic mice expressing a lactose-hydrolyzing enzyme in the mammary gland (Jost et al., 1999b). Lactose-reduced milk can also be produced *in vivo* by targeting a reduction in the activity of
the lactose synthase complex. However, this approach brings about an overall reduction in the sugar content and results in highly viscous milk (L'Huillier et al., 1996; Palmer et al., 2006; Stinnakre et al., 1994).

The main aim of the present study was to perform an in vivo transfection in rat mammary gland mediated by the infusion of a plasmid encoding a recombinant β-galactosidase (β-Gal) gene. Expression of the heterologous protein would allow pups to suckle breast milk containing lower lactose concentration and thus, to study the metabolic effects of modifying milk composition in both mothers and offspring.

MATERIALS AND METHODS

Preparation of plasmid DNA

The commercially available β-Gal expression plasmid pTarget™ (5670 bp) (Promega, Madison, WI) was used. The pTarget™ Vector contains a modified version of the coding sequence of α-peptide of β-Gal and carries the human cytomegalovirus immediate-early enhancer/promoter region to promote constitutive expression in mammalian cells. Transformed JM109 (Promega, Madison, WI) bacterial cells were selected and grown. Plasmid was isolated and purified with the Plasmid Maxi Kit (Quiagen, Madrid, Spain). In brief, alkaline bacterial lysate was cleared by centrifugation and the supernatant loaded onto the anion exchange tip where plasmid DNA selectively binds under appropriate low-salt and pH conditions, allowing removal of impurities by a medium-salt wash, and then ultrapure plasmid DNA was eluted in high-salt buffer. DNA was resuspended in water and concentration determined using a NanoDrop® Spectrophotometer ND-1000 (NadroDrop Technologies, Wilmington, DE, USA).

Experimental animals

Animals were housed under controlled temperature (22°C) and a 12 h light-dark cycle (light on from 0800 to 2000), and they had unlimited access to water and standard chow diet (Panlab, Barcelona, Spain) containing: 23.5% proteins, 4.3% fat, 3.7% fibre, 5.8% minerals, 51% carbohydrates and 12% water. Female virgin Wistar rats (200-225 g) were mated with male rats (Charles River Laboratories, Barcelona, Spain). All experimental procedures were performed according to both national and institutional guidelines for animal care and use. The animal protocol followed in this study was reviewed and approved by the Committee of our University (approval 13/02/2006). Day of conception (day 0 of pregnancy) was determined by examination of vaginal smears for the presence of sperm, and from then on female rats were single-caged. Pregnancy evolved without disturbances and three days before the planned parturition, pregnant rats were divided into two groups, control and β-Gal (6 animals/group) according to the subsequent treatment.

Mammary gland in vivo transfection

Plasmid DNA was freshly diluted in PBS to attain a concentration of 1 μg/μl and was then complexed to a DEAE-dextran solution in a ratio 1.39 (Sigma, Saint Louis, Missouri). Transfection mixture was warmed to 37°C and then injected subcutaneously in the mammary tissue (50 μl in each mammary gland) of the β-Gal group. In vivo transfection was carried out four days before the expected date of parturition under anaesthesia. Control rats followed the same manipulation, but the injection mixture did not contain DNA. Parturition took place between 2-4 days after the injection. The number of pups was adjusted to ten within 24-48 h after parturition.

Follow up of the animals

Lactation

Milk and serum samples of dams were collected on day 6, 12 and 18 of lactation (birth was defined as day 0). For milk collection, nursing rats were separated from their pups for 6 h to guarantee that mammary glands were full of milk. Then, dams were anaesthetized and milk was obtained by manual milking of the mammary glands. For plasma collection, a blood sample was taken out from the end of the tail in heparinised tubes and centrifuged at 2500 rpm for 10 min at 4°C to obtain the plasma. All samples were stored at −20°C. Tail blood samples were also collected on day 12 from one randomly selected male and one female from each dam and processed as indicated above.

Food intake and maternal and offspring body weights were periodically recorded. In addition, body composition of dams (day 10 of lactation) and of a selection of pups was determined (days 12 and 24 of life) by EchoMRI-700™ (Echo Medical Systems, LLC, TX, USA). This equipment is based in nuclear magnetic resonance (NMR) and allows body composition analysis creating contrast between soft tissues by taking advantage of the differences in relaxation times of the hydrogen proton spins in different environments. The fat content measured is the mass of all fat molecules in the body expressed as equivalent weight of canola oil. Lean is a muscle tissue mass equivalent of all the body parts containing water, excluding fat, bone minerals, and such substances which do not contribute to the NMR signal, such as hair, claws, etc (http://www.echomri.com/specs/echo700_specs.aspx). The ratio fat/body weight was calculated by division of both values for each animal.
On day 21 of lactation, the dams plus one male and one female, randomly selected from each dam, were sacrificed and tissue samples collected and weighed. The remaining animals were grouped by sex and maternal treatment and kept in standard housing conditions.

**Post weaning**

Body weight and food intake of animals were followed until 5.5 months of age. Body composition was assessed on days 40 and 125 of age by EchoMRI-700™ (Echo Medical Systems, LLC, TX, USA). In addition, six animals from each group were randomly selected to carry out a test of tolerance to glucose at 125 days of age. A load of 1.5 ml of glucose (1.5 g/kg body weight) was orally given to the animals during the first hour of the beginning of the light cycle after overnight fasting (Lee et al., 2006). Blood glucose concentration was determined from tail blood samples at time 0, 30, 60, 120 and 180 min post injection using an Accu Check Sensor (Roche Diagnostics, Barcelona, Spain).

**Determinations**

Lactose and galactose levels in milk were measured by a colorimetric method (Galactose and Lactose Assay Kit (Biovision, Deltaclon K617-100)). Glucose in milk was determined with a commercial kit (D-glucose UV-method from Roche). Leptin plasma concentrations were assessed by ELISA using the commercial kit Mouse Leptin Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA).

**Statistical analysis**

All data are expressed as the mean ± SEM. Student's test was used to assess statistical differences between control and β-Gal animals. Multiple comparisons were assessed by repeated measures ANOVA. After 4 months of age, the number of offspring being followed was reduced. Therefore, to consider the maximum number of animals under the statistical analysis of the body weight evolution, ANOVA analysis was performed in two timing sets (from day 0 to 109 and from day 123 to 165) and is indicated in the corresponding legend. The analyses were performed with SPSS for Windows (SPSS, Chicago, IL). Threshold of significance was defined at P<0.05 (*).

**RESULTS**

**Dams**

Transfection with β-Gal gene in mammary gland of pregnant rats caused a transient decrease in the milk concentration of lactose (66% of control values at day 6, p<0.05) and galactose (59% of control values at day 6, p<0.05). Both sugar concentrations showed control values from day 12 of lactation onwards (Figure 1). In contrast, milk glucose concentration was not altered during the whole period (Figure 1) and the same was seen concerning the levels of total proteins in milk (data not shown).

Body weight did not reach statistical significant differences between control and β-Gal dams during lactation (data not shown). However, analysis of body composition revealed a higher body fat content in β-Gal dams at day 10 of lactation, in absolute terms (29% higher than controls, p<0.05) and also in relationship with the body weight (Figure 2A). In accordance with increased body fat content, plasma leptin levels were also higher in β-Gal dams during lactation at day 6 (by 24%) and, specifically at day 12 (by 54%, p<0.05), whereas at day 18, recovery of control levels was seen (Figure 2B).

At the end of the lactation, blood glucose levels were not affected by the treatment (Table 1). In addition, no significant differences in body weight were observed between groups and this was in correspondence with the weight of the main organs such as liver, mammary gland and stomach. However, a higher retroperitoneal fat mass (1.9 times higher, p<0.005) was still found in β-Gal dams with respect to the controls (Table 1) at the end of lactation, in accordance with the higher body fat content previously seen and indicating a specific effect on adipose accumulation (as also seen in the sum of the collected fat depots).

**Offspring**

Transfection with β-Gal gene in mammary gland of pregnant rats was not associated with changes in plasma leptin or glucose in offspring at day 12 of lactation (data not shown), plasma glucose was also unaltered at day 20 of age (Table 2). Body and tissues weights at the end of lactation showed no major differences between groups with the exception of heavier BAT in males (p<0.05) and slightly heavier liver in females (p<0.05) with a more developed epididymal WAT in females from β-Gal dams (1.6 times, p<0.05) (Table 2).

Follow up of offspring after weaning, showed that animals from β-Gal treated dams had higher body weight than the respective controls (Figure 3), a condition that was not accompanied by increased food intake (Figure 3). Furthermore, a clear impact on body fat was observed. Body composition during the perinatal period (measured at days 12 and 24) did not show differences between groups. However, from day 40 and, particularly at adult age (4 months), increased body fat content was observed in animals from β-Gal dams [25% higher in females (p<0.05) and 15% in males] (Figure 3). No disturbances in the
Lactose, galactose and glucose levels were determined in milk at days 6, 12 and 18 of lactation of control and β-Gal transfected dams. Four days before parturition animals were in vivo transfected in mammary gland with a gene construct able to express β-Gal gene. Then, animals were housed and treated under standard conditions. Data are expressed as the mean ± SEM of 6 animals per group. * = p<0.05, from the respective control group (Student’s t test).
homeostasis of glucose were observed after a glucose tolerance test was performed in adult (Figure 4).

**DISCUSSION**

Some intent to decrease lactose concentration in milk has been proposed as a suitable way to eliminate the problems related to lactose intolerance (Jost et al., 1998; Jost et al., 1999b; NDA, 2009; Sinha et al., 2007). Nowadays, one potential strategy to overcome lactose intolerance, without excluding milk and dairy products from diet, consists in the consumption of low-lactose milk, preincubated in presence of exogenous lactase. This strategy avoids the nutritional disadvantages associated with reduced calcium and vitamins intake when milk products are excluded from diet. Low-lactose milk produced by exogenous enzyme is considered a valid and effective tool in the therapeutic management of lactose intolerance (Montalto et al., 2006; Shaukat et al., 2010; Usai-Satta et al., 2012). Although lactose may be eliminated *in vitro* by post-harvest processes, direct production of milk reduced in lactose can be more efficacious (Onwulata et al., 1989). In this context, our interest was to assess the long term nutritional effects in rats of suckling milk synthesised in the mammary gland potentially with lower lactose levels by encoding a recombinant galactosidase enzyme.

Nowadays, mammary gland is described as the best bioreactor for the production of foreign proteins (Wang et al., 2013). To develop the low-lactose milk we used a method based on the *in vivo* transfection in rat mammary gland of a DNA/DEAE-dextran solution. This constitutes a good method for transient transfections with promoter/reporter plasmids and in addition, is suitable for overexpression of recombinant protein in mammalian cells (Selden, 2001). Furthermore, this technique has been...
Table 1. Body and tissue weights of female rats and blood glucose levels at the end of lactation (day 20)\textsuperscript{a,b,c}

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>(\beta\text{-Gal})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>315(\pm)5</td>
<td>320(\pm)8</td>
</tr>
<tr>
<td>Mammary gland (g)</td>
<td>15.4(\pm)1.6</td>
<td>15.4(\pm)1.5</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>15.5(\pm)0.6</td>
<td>15.4(\pm)0.4</td>
</tr>
<tr>
<td>Stomach (g)</td>
<td>1.84(\pm)0.1</td>
<td>2.03(\pm)0.09</td>
</tr>
</tbody>
</table>

**Adipose tissue**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>(\beta\text{-Gal})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epididymal (g)</td>
<td>3.00(\pm)0.1</td>
<td>4.40(\pm)0.6</td>
</tr>
<tr>
<td>Mesenteric (g)</td>
<td>1.54(\pm)0.2</td>
<td>1.64(\pm)0.2</td>
</tr>
<tr>
<td>Retroperitoneal (g)</td>
<td>1.72(\pm)0.3</td>
<td>3.26(\pm)0.5*</td>
</tr>
<tr>
<td>Total white adipose (g)</td>
<td>6.72(\pm)0.3</td>
<td>10.1(\pm)1.0*</td>
</tr>
<tr>
<td>Brown adipose tissue (g)</td>
<td>0.27(\pm)0.05</td>
<td>0.26(\pm)0.04</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.83(\pm)0.61</td>
<td>5.72(\pm)0.28</td>
</tr>
</tbody>
</table>

\(\text{a}\) Four days before planned parturition, animals were in vivo transfected in mammary gland with a gene construct able to express \(\beta\text{-Gal}\) gene. Then, animals were housed and treated under standard conditions.

\(\text{b}\) Blood and tissue samples were collected at sacrifice on day 20 of lactation.

\(\text{c}\) Data are expressed as the mean \(\pm\) SEM of 6 animal per group. * = \(p<0.05\), from the respective control group (Student’s \(t\) test).

Table 2. Body and tissue weights and blood glucose levels of offspring of control and \(\beta\text{-Gal}\) rats at the end of lactation (day 20) \textsuperscript{a,b,c}

<table>
<thead>
<tr>
<th></th>
<th>Male Control</th>
<th>Male (\beta\text{-Gal})</th>
<th>Female Control</th>
<th>Female (\beta\text{-Gal})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>49.9 (\pm) 2.1</td>
<td>50.5 (\pm) 2.3</td>
<td>46.8 (\pm) 1.1</td>
<td>50.9 (\pm) 1.8</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>2.20 (\pm) 0.20</td>
<td>2.17 (\pm) 0.16</td>
<td>2.01 (\pm) 0.03</td>
<td>2.23 (\pm) 0.09*</td>
</tr>
<tr>
<td>Stomach (g)</td>
<td>0.37 (\pm) 0.01</td>
<td>0.35 (\pm) 0.02</td>
<td>0.37 (\pm) 0.02</td>
<td>0.39 (\pm) 0.03</td>
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</table>

**Adipose tissue**

<table>
<thead>
<tr>
<th></th>
<th>Male Control</th>
<th>Male (\beta\text{-Gal})</th>
<th>Female Control</th>
<th>Female (\beta\text{-Gal})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epididymal (g)</td>
<td>0.117 (\pm) 0.01</td>
<td>0.114 (\pm) 0.02</td>
<td>0.067 (\pm) 0.08</td>
<td>0.105 (\pm) 0.01*</td>
</tr>
<tr>
<td>Mesenteric (g)</td>
<td>0.262 (\pm) 0.03</td>
<td>0.259 (\pm) 0.02</td>
<td>0.223 (\pm) 0.03</td>
<td>0.297 (\pm) 0.03</td>
</tr>
<tr>
<td>Retroperitoneal (g)</td>
<td>0.091 (\pm) 0.01</td>
<td>0.084 (\pm) 0.01</td>
<td>0.071 (\pm) 0.06</td>
<td>0.085 (\pm) 0.01</td>
</tr>
<tr>
<td>Inguinal (g)</td>
<td>0.378 (\pm) 0.06</td>
<td>0.315 (\pm) 0.03</td>
<td>0.305 (\pm) 0.04</td>
<td>0.407 (\pm) 0.1</td>
</tr>
<tr>
<td>Total white adipose (g)</td>
<td>0.848 (\pm) 0.09</td>
<td>0.800 (\pm) 0.06</td>
<td>0.667 (\pm) 0.04</td>
<td>0.820 (\pm) 0.1</td>
</tr>
<tr>
<td>Brown adipose tissue (g)</td>
<td>0.204 (\pm) 0.02</td>
<td>0.264 (\pm) 0.01*</td>
<td>0.203 (\pm) 0.02</td>
<td>0.249 (\pm) 0.01</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>7.60 (\pm) 0.32</td>
<td>7.72 (\pm) 0.31</td>
<td>7.72 (\pm) 0.24</td>
<td>7.55 (\pm) 0.48</td>
</tr>
</tbody>
</table>

\(\text{a}\) \(\beta\text{-Gal}\) group has been formed with offspring animals from female rats transfected with \(\beta\text{-Gal}\) gene in mammary gland at the end of pregnancy. Control group received the same manipulation but the transfection did not contain plasmid.

\(\text{b}\) Blood and tissue samples were collected at sacrifice on day 20 of lactation.

\(\text{c}\) Data are expressed as the mean \(\pm\) SEM of 6 animals per group. * = \(p<0.05\), from the respective control group (Student’s \(t\) test).
Figure 3. Evolution of body weight, body composition and food intake in offspring of control and β-Gal dams. Male and female offspring are presented in left and right side panel, respectively. Evolution of body weight (A), body fat content (B) and food intake (C) are expressed as the mean ± SEM of the averaged 6 litters from each group along time. ANOVA significances are shown with the following capital letters: T (time) and G (group). Significance was set at p<0.05 and p value is indicated in the corresponding graph.

shown to work efficiently in guinea pigs to obtain the expression of the corresponding protein secreted in milk (Hens et al., 2000). The enzyme β-Gal was selected because it is used in multiple industrial and biotechnological applications, most of them concerning the removal of lactose from milk products (Husain, 2010; NDA, 2009).

Our data show the feasibility of transfecting the mammary gland in vivo by the infusion of plasmid DNA encoding a recombinant β-Gal gene. Transient activity of the recombinant enzyme was achieved in milk during lactation, allowing lactose levels of 66% the control values, by day 6 of lactation. Total absence of lactose was not desirable because, lactose, together with other sugars and diffusible ions, is responsible for the osmotic pressure of milk. Furthermore, lactose together with fat represent the critical elements providing milk energy (Rudolph et al., 2007). Therefore, the approach used was enough to produce a significant reduction in lactose, at least during the first third of lactation and at a similar extent to that found in transgenic animal models (Jost et al., 1999a).

In addition, hydrolysis of lactose by the heterologous enzyme would produce glucose and galactose in proportion...
Figure 4. Blood glucose levels after a glucose tolerance test in offspring of control and β-Gal dams. Data are expressed as the mean ± SEM of 6 animals per group. ANOVA significances were observed along T (time); no effect of group was found. Significance was set at p<0.05 and p value is indicated in the corresponding graph.

to the decrease in lactose content. However, partial reabsorption of those monosaccharides by the alveolar cells in mammary gland seemed to be produced after lactose hydrolysis, as seen in a mice transgenic model expressing intestinal lactase in milk (Jost et al., 1999a). In fact, galactose reabsorption was also present and a decrease (59% at day 6) in milk content was found, whereas concentration of glucose was not altered throughout lactation, which suggests the presence of mechanisms to keep glucose concentration unaltered.

Unexpectedly, the induction of β-Gal in milk was associated with a tendency to increase maternal body fat content (which was also reflected in higher plasma leptin levels). By the end of lactation, a few adipose depots still reflected the excess body fat in the transfected animals. Maybe the availability of more glucose and galactose, because of the hydrolysis of lactose, delayed the mobilization of maternal fat resources for milk synthesis, contributing to the higher fat content in β-Gal expressing animals.

Interestingly, the growth rate of offspring was not altered during lactation as seen in transgenic mice (Jost et al., 1999a). No changes in milk protein concentration suggest that the nutritional value of the milk could fulfil the amino acid requirements of growth for offspring. However, longer follow up until adulthood was associated with higher body weight in offspring from transfected animals. Body fat content of the progeny was not different between groups at mid lactation, but started to diverge after weaning and was completely apparent at 4.5 months of life. Because, no increased food intake was found; other mechanisms affecting the efficiency of nutrient use as metabolic substrates should be involved. Data suggest the implementation of early nutritional programming induced by suckling low-lactose milk during the lactation stage, which contributed to adult obesity. This is certainly a
relevant issue that shows the relevance to perform long term follow up studies after nutritional interventions at early ages. Although, it was beyond the scope of the current study, further research would be necessary to characterize the underlying mechanisms.

Conclusions

We have shown that in vivo transfection of a reporter gene in rat mammary gland allows a transient expression of a recombinant and functional protein in milk. Although mammary gland is recognised as the best bioreactor, most of the approaches used up to now are dedicated to the generation of transgenic animals (Wang et al., 2013). Therefore, the present study constitutes a novelty in the field and shows that DNA/DEAE dextran transfection may be a very useful approach for potential application in laboratory animals at lower cost and difficulty than the generation of transgenics (Hens et al., 2000; Houdebine, 2002). In addition, the in vivo transfection with β-Gal gene has fulfilled the aim of the study and milk containing lower lactose content than in control animals was transiently available to offspring during lactation.

However, further follow up of the progeny indicated that the achieved modification in milk composition was associated with development of obesity in adulthood, a fact that requires specific investigation for further characterization of the involved mechanisms.

ACKNOWLEDGEMENTS

This work was supported by the Spanish Government (grants AGL2009-11277 and AGL2012-33692) and Instituto de Salud Carlos III, Centro de Investigación Biomédica en Red Fisiopatología de la Obesidad y Nutrición, CIBERObn. A. Palou and F. Serra belong to the Nutrigenomics-group, awarded as “Group of Excellence” of CAIB and supported by “Direcció General d’Universitats, Recerca i Transferència del Coneixement” of Regional Government (CAIB) and FEDER funds (EU).

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