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TGF-β activity in cow milk and fermented milk products: An in vitro bioassay with oral fibroblasts

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ABSTRACT

Objective: Milk is a rich source of transforming growth factor (TGF)-β which supports intestinal mucosal homeostasis of infants. Milk may also have beneficial effects on the integrity of the oral cavity, its being part of the gastrointestinal tract. However, it is unclear if milk and fermented milk products provoke a TGF-β response in oral cells.

Material and Methods: Human gingival fibroblasts were exposed to pasteurized cow milk, yoghurt, sour milk, buttermilk and whey, followed by a reverse transcriptase polymerase chain reaction of the TGF-β target genes interleukin11 (IL11), proteoglycan4 (PRG4), and NADPH oxidase 4 (NOX4). Immunoassays were performed for IL11 and TGF-β in cell culture supernatant and milk products, respectively. Signaling was investigated with the TGF-β receptor type I kinase inhibitor SB431542.

Results: We report here that pasteurized cow milk and the aqueous fractions of yoghurt, sour milk, buttermilk and whey enhanced the expression of IL11, NOX4 and PRG4 in gingival fibroblasts. Moreover, IL11 protein levels in the respective supernatant were significantly increased. Cow milk, yoghurt, sour milk and buttermilk contain approximately 1–2 ng TGF-β1, whereas active TGF-β1 is hardly detectable in whey. SB431542 reduced the response of gingival fibroblasts to pasteurized cow milk and fermented milk products based on IL11 release into the supernatant.

Conclusions: These results demonstrate that gingival fibroblasts respond to pasteurized cow milk and to fermented milk products with an increased expression of TGF-β target genes.

1. Introduction

Milk produced by the mammary gland is a hallmark of mammalian evolution, providing infants and small children with essential nutritional components for their early developmental growth. Besides the nutritional aspects, milk is a rich source of growth factors including transforming growth factor (TGF)-β (Okada et al., 1991). Cow's milk, like human milk (Namachivayam et al., 2013), contains approximately ten times more TGF-β2 than TGF-β1 and can vary during different stages of lactation (Chockalingam, Paape, & Bannerman, 2005; Purup, Vestergaard, & Sejrsen, 2007). Oral administration of pasteurized cow's milk can reduce intestinal tissue damage and mortality in dextran sodium sulfate colitis and lipopolysaccharide-induced endotoxemia models involving TGF-β receptors I kinase activity (Ozawa et al., 2009). Thus, what is known for human milk, that TGF-β protects infants against necrotizing enterocolitis (Namachivayam et al., 2013; Sisk, Lovelady, Dillard, Gruber, & O'Shea, 2007), is also true for cow's milk (Ozawa et al., 2009). Moreover, the topical application of milk has beneficial effects for example, on umbilical cord separation in the newborn (Abbaszadeh, Hajizadeh, & Jahangiri, 2016; Aghamohammadi, Zafari, & Moslemi, 2012; Golshan & Hossein, 2013; Vural & Kisa, 2006), the healing of diaper dermatitis (Farahani, Ghobadzadeh, & Yousefi, 2013), on ulcerated haemangioma (Laws, Porter, Taibjee, & Clayton, 2012), and on atopic eczema (Kasrae, Amiri Farahani, & Yousefi, 2015).

Activation of latent TGF-β by the gastric acid is required to enable milk to protect infants against necrotizing enterocolitis (Nakamura et al., 2009). Pasteurization, exogenous neuramidase and chondroitase treatment cause activation of latent TGF-β in milk (Namachivayam et al., 2013, 2015). Active TGF-β exerts its pleiotropic function by binding and thereby dimerizing the two TGF-β receptors. The activated TGF-β receptor I kinase controls the phosphorylation and nuclear

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translocation of the transcription factor Smad2/3 that finally regulates the expression to the respective target genes including plasminogen activator inhibitor-1 (PAI-1) (Lund et al., 1987), CTGF (Igarashi, Okochi, Bradham, & Grotendorst, 1993) and MMP-13 (Uria, Jimenez, Balbin, Freije, & Lopez-Otin, 1998). In mice, Smad2 phosphorylation was observed in the intestine (Ozawa et al., 2009). Even though milk contains other growth factors and cytokines such as IGF-1 (Baxter, Zaltsman, & Turtle, 1984) and IL-10 (Garofalo et al., 1995), there is strong evidence that TGF-ß-regulated genes are responsible for the protective effects of milk in colitis (Ozawa et al., 2009). Now, the question arises: do fermented milk products deliver TGF-β activity and can this possibly be translated into beneficial effects for oral wound healing?

Pasteurized cow's milk is frequently subjected to a fermentation process ending up with various milk products including yoghurt, sour milk, and buttermilk. Lactose fermenting bacteria produce lactic acid (Bouteille, Gaudet, Lecanu, & This, 2013). The lowering of the pH causes the denaturation of casein, which gives the characteristic rheological properties of fermented milk products (Pepe et al., 2013). Furthermore, lowering the pH of milk by gastric acid can support the conversion from the inactive latent form into active TGF-β (Nakamura et al., 2009). Heating milk to 80 °C activates latent TGF-β (Namachivayam et al., 2013). Pasteurization of cow's milk usually occurs when heated at around 72 °C for 15 s (Ranieri, Huck, Sonnen, Barbano, & Boor, 2009) and for the preparation of yogurt, milk is heated up to more than 80–95 °C for 10 to 30 min, respectively (Bonczar, Walczycka, & Duda, 2016; Jablonski & Jackson, 2008). Yoghurt, sour milk, and buttermilk may thus provoke a differential TGF-β response in oral fibroblasts.

For the production of cheese, casein, usually in pasteurized milk, may be cleaved by rennet extract, although today enzymes from plants, fungi and microbial sources are used (Hsieh & Pan, 2012). What remains from milk upon coagulation are the whey proteins, which are basically serum proteins. Whey protein concentrate contains TGF-β activity (Hering et al., 2011) and attenuates inflammation-induced intestinal injury by improving mucosal barrier function (Xiao et al., 2016). Whey-containing toothpastes have been introduced (Elsadek et al., 2009). Until now, there is no data about the role of whey in changing the expression of TGF-β target genes in a bioassay with oral fibroblasts.

There is indirect support for a beneficial effect of TGF-β in fermented milk, for example as yogurt inhibits acute gastric lesions and ulcers in rats (Uchida & Kurakazu, 2004; Uchida, Shimizu, & Kurakazu, 2010), and can suppress squamous hyperplastic change and inflammation associated with C. albicans infection in the forestomach in rats (Terayama, Matsuura, Uchida, Narama, & Ozaki, 2016). Yoghurt also reduced the disruption of the small intestinal barrier in methotrexate-treated rats (Southcott, Tooley, Howarth, Davidson, & Butler, 2008) and in inflammation-disrupted cell barriers in vitro (Putt, Pei, White, & Bolling, 2017). Yoghurt can prevent local inflammation in the intestine of mice and consequently, colon carcinogenesis (Del Carmen, de Moreno de LeBlanc, & LeBlanc, 2016). Whey protein concentrates decrease inflammation-induced intestinal injury (Xiao et al., 2016). Overall, existing knowledge suggests that fermented milk products protect against inflammatory lesions, possibly by their TGF-β activity. The question arises as to whether fermented products can target oral fibroblasts?

As a first step towards answering this question we refer to our bioassay, where gingival fibroblasts respond with an increased expression of TGF-β target genes interleukin 11 (IL11), proteoglycan4 (PRG4), NADPH oxidase 4 (NOX4) (Sawada et al., 2015), and also by the established genes PAI-1, CTGF and MMP-13. The promoter of the IL11 (Zhang et al., 2015), NOX4 (Bai, Hock, Logsdon, Zhou, & Thannickal, 2014) and PRG4 (Chavez, Coricor, Perez, Seo, & Serra, 2017) genes has Smad binding elements. This bioassay with oral fibroblasts offers an alternative to the reported assays with NRK47 F cells and RAW 264.7 macrophages, confirming TGF-β activity in human milk (Namachivayam et al., 2015; Okada et al., 1991). Studying the role of cow's milk and its respective fermentation products on the bioavailability of TGF-β for fibroblasts of the oral mucosa may be of clinical relevance in growing infants and adults in maintaining oral health. Moreover, cow's milk and its respective fermentation products might reduce mucositis in patients, including those suffering from radiation therapy (Maria, Eliopoulos, & Muanza, 2017), chemotherapy (Lalla, Saunders, & Peterson, 2014) and chronic inflammatory disorders such as Crohn's disease (Harty et al., 2005). This in vitro research is inspired by the vision to ease the burden of oral mucositis by local application of TGF-β derived from cow's milk and fermented milk products.

2. Material and methods

2.1. Cow's milk, fermented milk products, and cells

Three different batches of pasteurized cow's milk (Billa Bergbauern Heumilch; Spar Halbfett Milch; Hofer Milfina Halbfett Milch), yoghurt (Ja Natürlich Naturjoghurt; Clever Joghurt; Nöm Naturjoghurt), sour milk (Hofer Milfina Sauermilch; Nöm Sauermilch; Schärdinger Sauermilch), buttermilk (Ja Natürlich Buttermilch Natur; Clever Buttermilch; Spar Buttermilch) and whey (Lattella Naturmolke; Clever Fruchtmolke; Spar free from Molke) were centrifuged at 20,000 G for 10 min at 4 °C. The aqueous fractions were stored at −20 °C until testing. We consider the aqueous fractions to be the milk and fermented products thereof after centrifugation. Samples were subjected to not more than two freeze-thaw cycles. Gingival fibroblasts were incubated with the aqueous fraction of cow's milk (0.04–10%) and fermented products (1%) prior to analysis of TGF-β signaling in oral fibroblasts, similar to previous studies with enamel matrix derivative (Stahli, Bosshardt, Sculean, & Gruber, 2014) and bone conditioned medium (Peng et al., 2015). Serum-free DMEM was used to dilute the aqueous fraction. Stimulation of oral fibroblasts was performed under serum-free conditions. Active TGF-β1 in the aqueous fraction of cow's milk and fermented milk products was measured by Quantikine ELISA kit (R &D Systems, Minneapolis, MN). An immunoassay from Genorise Scientific (Berwyn, PA) failed to provide reliable TGF-β2 data. ELISA data were not normalized to an internal compound.

2.2. Primary gingival fibroblasts

Human gingival fibroblasts were prepared from explant cultures of three independent donors after approval of the Ethical Committee of the Medical University of Vienna (EK Nr. 631/2007). Cells were cultured in a humidified atmosphere at 37 °C in growth medium consisting of DMEM, 10% fetal calf serum and 1% antibiotics (Invitrogen Corporation, Carlsbad, CA, USA). Cells were plated in growth medium at 30,000 cells/cm^2 into culture dishes. The following day, cells were exposed to 1% processed cow's milk and fermented milk products, if not otherwise indicated, or recombinant human TGF-β1 and TGF-β2 (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel) at 5 ng/ml in growth medium for 24 h, before gene expression analysis was performed. SB431542, a TGF-β receptor I kinase inhibitor, was used at 10 μM (Calbiochem, Merck Millipore). Supernatant was harvested, centrifuged and stored frozen until subjected to immunoassay.

2.3. Viability assay

For viability experiments, gingival fibroblasts were incubated with the aqueous fraction of cow's milk at the indicated concentrations. After 24 h, a MTT (3-[4,5-dimethythiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) solution at a final concentration of 0.5 mg/ml was added to each well of a microtiter plate (CytoOne, Starlab International, Hamburg) for 2 h at 37 °C. Medium was removed and formazan crystals were solubilized with dimethyl sulfoxide. Optical density was

normalized to unstimulated control values.

2.4. qRT-PCR analysis and immunoassay

Total RNA was isolated with the ExtractMe total RNA kit (Blirt S.A., Gdańsk, Poland). Reverse transcription was performed with SensiFAST™cDNA (Bioline, London, UK). Polymerase chain reaction was done with the SensiFAST™ SYBR ROX Kit (Bioline) on a 7500 Real-Time PCR System (both Applied Biosystems, Life Technologies Corporation, Carlsbad, CA). Primer sequences are hIL11_F GGACAGGGAAGGGTTA AAGG, hIL11_R GCTCAGCACGACCAGGAC; hPRG4_F CAGTTGCAGGT GGCATCTC, hPRG4_R TCGTGATTCAGCAAGTTTCATC; hNOX4a_F TCTTGGCTTACCTCCGAGGA, hNOX4a_R CTCCTGGTTCTCCTGCTTGG; hPAI-1_F AAGGCACCTCTGAGAACTTCA, hPAI-1_R CCCAGGACTAGGC AGGTG ; hCTGF_F CTCCTGCAGGCTAGAGAAGC, hCTGF_R GATGCAC TTTTTGCCCTTCTT; hMMP-13_F CCAGTCTCCGAGGAGAAACA, hMMP-13_R AAAAACAGCTCCGCATCAAC; hGAPDH_F AAGCCACATCGCTCA GACAC, hGAPDH_R GCCCAATACGACCAAATCC. The mRNA levels were calculated by normalizing to the housekeeping gene GAPDH using the ΔΔCt method after exponential expression transformation. For the immunoassay, the human IL11 Quantikine ELISA kit was used (R&D Systems, Minneapolis, MN). ELISA data was not normalized to an internal compound.

2.5. Immunofluorescence

Gingival fibroblasts exposed to pasteurized cow milk for 24 h were incubated with anti-SMAD3 antibody (D7G7 XP® Rabbit mAb, Cell Signalling Danvers, MA) for over night at 4° C. Following blocking by 1% BSA and permeabilisation with 0.1% Triton X, an Alexa Fluor® 488-conjugated secondary antibody (Cell Signalling) was added for 1 h at room temperature. Images were captured under a fluorescent microscope (Axio Imager M2, Carl Zeiss AG, Oberkochen, Germany).

2.6. Statistical analysis

The Kruskal–Wallis test comparing the mean rank of each treatment group with the mean rank of the untreated control group was used for data presented in Figs. 1A and 2A and B. The Mann–Whitney U test was used to compare the data presented in Fig. 1B. The p-values are indicated in the respective figures. Descriptive statistics were used to present all other data.

3. Results

3.1. Pasteurized cow's milk stimulates TGF-β target gene expression in gingival fibroblasts

Gingival fibroblasts were exposed to various concentrations of the aqueous fraction of pasteurized cow's milk and a viability assay was performed. At 1%, pasteurized cow's milk had no significant impact on cell viability (Supplement Table 1). Next, the expression of TGF-β target genes was determined. Based on a whole genome gene array, IL11, NOX4 and PRG4 were among the most strongly up-regulated genes induced by 1% pasteurized human milk (unpublished observations). Recombinant human TGF-β1 and TGF-β2 caused an expected increase of IL11, NOX4 and PRG4 expression on gingival fibroblasts (Supplement Table 2). With 1% cow's milk, transcript levels of IL11, NOX4 and PRG4 were increased in gingival fibroblasts with GAPDH used for normalization (Fig. 1A, Table 1). Moreover, IL11 protein levels in the respective supernatant were significantly increased (Fig. 1B). One percent cow's milk also notably increased the expression of typical TGF-β target genes CTGF (12.4 ± 8.7-fold), PAI-1 (9.1 ± 3.3-fold), and MMP-13 (12.0 ± 9.6-fold) in three independent experiments (data not shown). Time-response experiments showed a maximum expression of TGF-β target genes between 3 and 6 h with a moderate decrease at 24 h (Table 2A). Dose-response experiments revealed that even 0.04% of pooled cow's milk caused a strong activation of TGF-β target genes (Table 2B). The strong increase in the expression of TGF-β target genes suggests that gingival fibroblasts are highly susceptible to pasteurized cow's milk.

3.2. Fermented milk products stimulate TGF-β target gene expression in gingival fibroblasts

We next investigated whether fermentation affects the TGF-β activity of cow's milk. As shown in Fig. 2A, the aqueous fractions of yoghurt, sour milk, buttermilk and whey increased the expression of IL11, NOX4 and PRG4 in gingival fibroblasts. The variations between the different experiments and batches from the three dairies are indicated in Supplement Table 3. Again, increased IL11 protein levels were detected in the respective supernatants (Fig. 2B). These results demonstrate that fermentation maintains the activity necessary to drive the expression of TGF-β target genes in gingival fibroblasts.

3.3. TGF-β receptor I kinase inhibitor SB431542 in gingival fibroblasts

As indicated in Fig. 3, cow's milk, yoghurt, sour milk and buttermilk contain approximately 1–2 ng TGF-β1, whereas TGF-β1 was hardly detectable in whey. No normalization for milk protein was performed

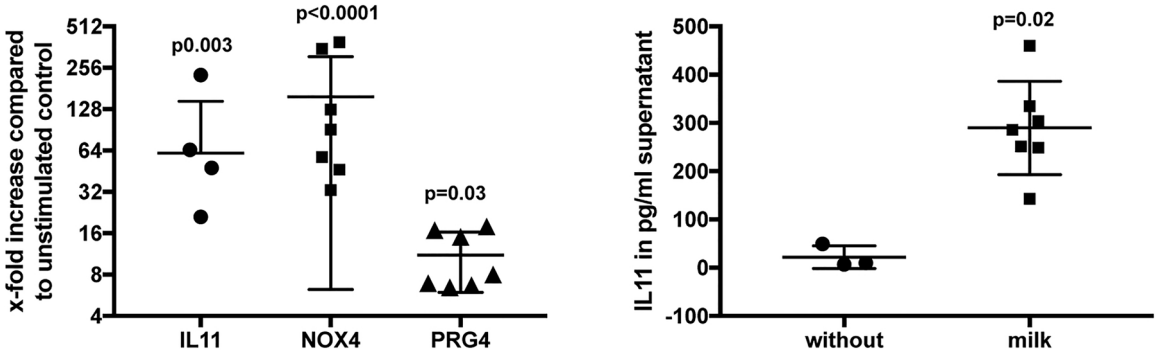

Fig. 1. *Pasteurized cow's milk stimulates TGF-β target gene expression in gingival fibroblasts.*
Gingival fibroblasts were exposed for 24 h to the aqueous fraction of 1% pasteurized cow's milk from three different dairies. (A) Transcript levels of IL11, NOX4 and PRG4 were measured by RT-PCR. (B) IL11 protein level in the respective supernatant was determined by immunoassay. Graphs represent the mean and standard deviation of three independent experiments done with cells from three different donors. Kruskal–Wallis test (A) and Mann–Whitney U test (B) were used to compare the mean rank of the treatment group with the mean rank of the control group.

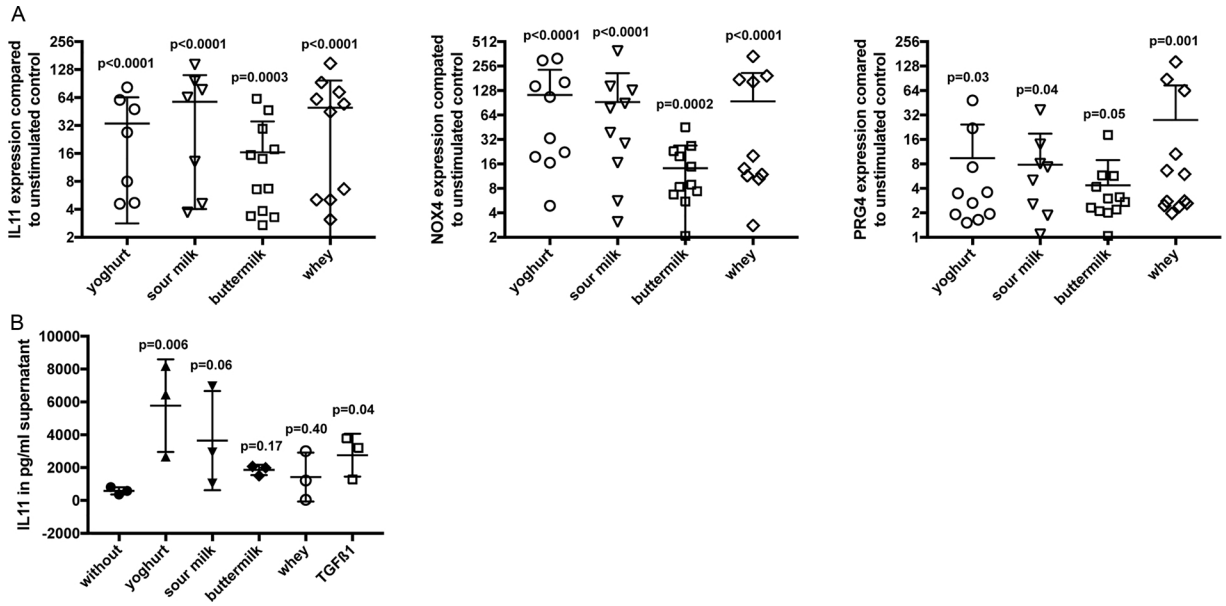

Fig. 2. *Fermented milk products stimulate TGF-β target gene expression in gingival fibroblasts.*
Gingival fibroblasts were exposed for 24 h to the aqueous fraction of 1% fermented milk products from three different dairies. (A) Transcript levels of IL11, NOX4 and PRG4 were measured by RT-PCR. (B) IL11 protein levels in the respective supernatant were determined by immunoassay. Graphs represent the mean and standard deviation of three independent experiments done with cells from three different donors. Kruskal–Wallis test was used to compare the mean rank of the treatment group with the mean rank of the control group.

Table 1
Pasteurized cow's milk from three dairies.

Pasteurized cow milk	IL11	NOX4	PRG4
Billa Heumilch	34.4 ± 13.3	109.1 ± 18.2	12.4 ± 5.5
Spar Halbfett Milch	3.6 ± 0.3	45.1 ± 12.1	10.9 ± 4.2
Hofer Halbfett Milch	145.8 ± 81.2	264.1 ± 154.8	10.4 ± 4.6

Gingival fibroblasts were exposed to 1% aqueous fractions of three different batches of pasteurized cow's milk for 24 h before gene expression analysis was performed. Numbers indicate the means and standard deviation of expression changes compared to unstimulated controls of two independent experiments with cells from pooled donors.

Table 2
Time-response and dose-response experiments.

Time-response	IL11	NOX4	PRG4
1h	1.1 ± 0.4	1.4 ± 0.2	0.7 ± 0.2
3h	1.9 ± 0.2	27.5 ± 1.0	6.2 ± 3.8
6h	1.6 ± 0.6	30.4 ± 3.5	4.9 ± 3.5
24h	1.4 ± 1.3	17.9 ± 2.0	5.5 ± 1.2
Dose-response	**IL11**	**NOX4**	**PRG4**
10%	4.8 ± 4.3	5.9 ± 3.4	6.5 ± 6.0
5%	4.2 ± 3.4	5.2 ± 0.1	3.0 ± 0.9
1%	7.5 ± 5.5	17.1 ± 11.6	9.0 ± 5.0
0.20%	3.7 ± 2.7	3.0 ± 0.1	2.8 ± 1.8
0.04%	4.8 ± 1.6	2.8 ± 0.7	3.0 ± 2.8

Gingival fibroblasts were exposed for (A) the indicated time points to 1% aqueous fractions and (B) to various concentrations of pooled pasteurized cow's milk before gene expression analysis was performed. Numbers indicate the mean and standard deviation of expression changes compared to unstimulated controls of two experiments.

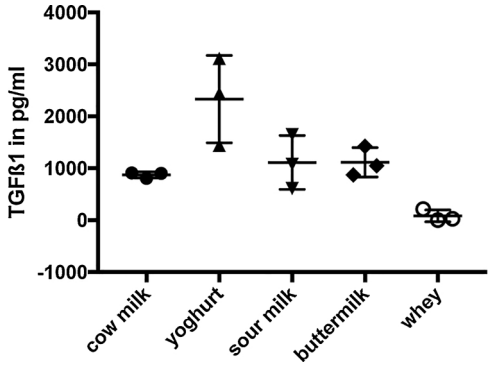

Fig. 3. *Active TGF-β1 in the aqueous fraction of cow's milk and fermented products.*
Active TGF-β1 in cow's milk and fermented milk products was determined by immunoassay. Data represent the mean and standard deviation of preparations of three dairies. TGF-β1 is indicated in pg/ml.

Table 3
TGF-β receptor I kinase inhibitor SB431542 in gingival fibroblasts.

	W/O	CM	Y	SM	BM	W	TGF-β1
Experiment 1	581	1140	6454	2938	2077	1219	3208
(+) SB431542	144	137	264	204	315	188	141
Experiment 2	810	1685	8195	6959	1999	3005	3785
(+) SB431542	676	146	961	852	307	281	812

Gingival fibroblasts were exposed to 1% aqueous fractions of pooled pasteurized cow's milk and fermented milk products for 24 h. IL11 in the supernatant was measured by immunoassay. Abbreviations are w/o (without control), (CM) cow's milk, Y (yoghurt), SM (sour milk), BM (buttermilk), W (whey), TGF-β1 (5 ng/ml). Data from two independent experiments are shown and given in pg IL11/ml culture medium.

because milk, yoghurt, sour milk and buttermilk have more protein than whey, the latter lacking casein. Moreover, the amount of protein is rather constant among cow milk and is not changed by fermentation. Cow milk and fermented milk products have around 3.5% total protein with 2.7% casein. Whey holds around 0.8% of the total milk protein (De Marchi et al., 2008). We further determined that the TGF-β receptor I kinase inhibitor SB431542 can neutralize the effects of pasteurized cow's milk and fermented milk products. Pharmacological inhibition of TGF-β receptor I kinase reduced the response of gingival fibroblasts to processed cow's milk and fermented milk products based on IL11

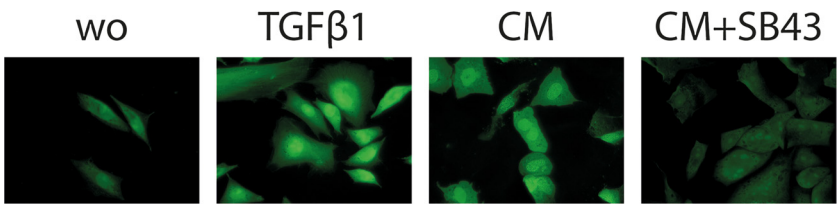

Fig. 4. *Nuclear translocation of Smad-3 in response pasteurized cow milk.*
Gingival fibroblasts were exposed for 24 h to the aqueous fraction of 1% pasteurized cow milk (CM) and recombinant TGF-β1. Note the nuclear translocation of Smad3 into the nucleus that was suppressed by TGF-β receptor I kinase inhibitor SB431542 (SB43).

release into the supernatant (Table 3). Moreover, pasteurized cow's milk caused the translocation of Smad3 into the nucleus of fibroblasts but not in the presence of the TGF-β receptor I kinase inhibitor SB431542 (Fig. 4).

4. Discussion

The study was inspired by the overall concept that milk and possibly fermented milk products are a rich source of TGF-β in supporting oral health (Okada et al., 1991). Cow's milk induces a TGF-β response in reporter cells (Ozawa et al., 2009), however whether cells of the oral cavity respond to TGF-β in milk has not been investigated so far. Moreover, it was unclear if fermentation of milk affects TGF-β activity. The main finding of this report is that gingival fibroblasts respond to cow's milk and fermented milk products with an increased expression of TGF-β target genes. These data confirm previous findings that pasteurized cow's milk (Ozawa et al., 2009) and human milk (Nakamura et al., 2009; Namachivayam et al., 2013, 2015) contain a TGF-β activity. However, no reports are available on TGF-β activity in fermented milk products such as yoghurt, sour milk, buttermilk and whey. Thus, the data presented here advance existing knowledge about cow's milk by the fact that TGF-β activity is maintained upon its heating and fermentation.

The data from the IL11 immunoassay suggest that the production of yoghurt might even increase the intrinsic TGF-β activity. To produce yogurt, milk is heated to about 80–95 °C (Bonczar et al., 2016; Jablonski & Jackson, 2008), a temperature where latent TGF-β is activated (Brown, Wakefield, Levinson, & Sporn, 1990). Moreover, like in sour milk, lactic acid fermentation lowers the pH which may in turn activate latent TGF-β (Brown et al., 1990; Lyons, Keski-Oja, & Moses, 1988). These observations led to the hypothesis that a combination of heating and lactic acid fermentation activates latent TGF-β in cow's milk. Further studies are necessary to determine to what extent, if at all, lactic acid fermentation affects TGF-β activity in milk products. Moreover, since commercial products were tested, we have no clear information about the pasteurization protocol, as the processing regime may affect TGF-β activity.

We report here that not only milk but also fermented milk products contain TGF-β1, and presumably also TGF-β2 (Chockalingam et al., 2005; Purup et al., 2007). Moreover, TGF-β receptor I kinase inhibitor SB431542 experiments show significant reduction of IL11 levels in whey, although little TGF-ß1 was found. SB431542 also blocked the translocation of Smad3 into the nucleus of fibroblasts in response to pasteurized cow's milk suggesting an activation of TGF-β receptor I kinase signaling. Even though we cannot show to what extent TGF-β1 and TGF-β2 contribute to the changes in gene expression, our data suggest that whey and the other milk products involve the TGF-β receptor I kinase in mediating their effects on IL11 production in oral fibroblasts.

The present study leads to new questions: (i) It is unclear if the consumption of milk or fermented milk products supports oral health, particularly in reducing mucositis following radiation therapy (Maria et al., 2017), chemotherapy (Lalla et al., 2014) and chronic inflammatory disorders such as Crohn's disease (Harty et al., 2005). (ii) In vivo, gingival fibroblasts are embedded in an extracellular matrix and it is not clear if the TGF-β activity can provoke a cellular response at the tissue level. (iii) Milk and fermented milk products contain multiple growth factors including IGF-1 (Baxter et al., 1984) and IL10 (Garofalo et al., 1995) that presumably cause a complex cell response that goes far beyond TGF-β signaling (Ballard & Morrow, 2013). (iv) Moreover, it remains to be proven if in whey, TGF-β2 which accounts for over 85% of the total TGF-β activity, caused the robust activation of the gene expression (Rogers, Goddard, Regester, Ballard, & Belford, 1996).

Limitations are that we have used the aqueous fraction of milk and milk products and what remains is the fat layer presumably containing growth factors. Analyzing this fraction has not been done. There is a large variability of gene expression changes between the independent experiments, and the responsiveness of the fibroblasts from different donors. The variation between the same products from different dairies remains challenging. Nevertheless, all independent experiments basically support the main conclusions, namely that isolated oral fibroblasts are susceptible to the TGF-β activity in pasteurized cow's milk and fermented milk products. Moreover, we have used gingival fibroblasts to detect the TGF-β target genes as oral keratinocytes are more readily exposed to milk. The oral epithelial cell line HSC2 does not respond to milk by changes of IL-11, NOX4 and PRG4 expression (unpublished). For future research, we need a gene array strategy to identify potential target genes for milk and milk products in oral epithelial cells.

It is relevant to understand if the beneficial effects of probiotic yogurt and the other milk products, for example, in reducing gastric lesions and ulcers (Uchida & Kurakazu, 2004; Uchida et al., 2010), in preventing local inflammation in the intestines (Del Carmen et al., 2016), in suppressing hyperplastic change and inflammation in the forestomach of the rat (Terayama et al., 2016), and in protecting intestinal barriers in vivo and in vitro (Putt et al., 2017; Southcott et al., 2008), involve a TGF-β activity. In vivo support for this concept comes from studies showing that the beneficial effects of cow's milk in preventing colitis in mice require TGF-β signaling and Smad2 phosphorylation in mouse intestine (Ozawa et al., 2009). It is reasonable to assume that fermented milk products exert a similar activation of Smad-signaling in the intestine. Nevertheless, the overall question of whether TGF-β activity in cow's milk and fermented products can provoke a response in the oral cavity, similar to the intestine, leaves room for future research.

What could be the possible role of IL11, NOX4 and PRG4 in the regulation of oral health? As already cited (Zimmermann et al., 2014), IL11 is a member of the IL6 family of cytokines and together with BMP-2, can accelerate bone regeneration (Suga et al., 2004). Recombinant IL11 was associated with decreased mucosal damage and accelerated healing following irradiation (Burnett, Biju, Lui, & Hauer-Jensen, 2013). PRG4 protects synovial joints from arthropathic changes (Rhee et al., 2005) and supports bone formation (Novince et al., 2012). PRG4 also has an anti-inflammatory role in synovial fibroblasts (Alquraini et al., 2017). NOX4 generates intracellular superoxide, which modulates not only osteoblast BMP-2 activity (Mandal et al., 2011) but also is involved in wound healing (Levigne, Modarressi, Krause, & Pittet-Cuenod, 2016). TGF-β1 requires NOX4 for angiogenesis (Peshavariya, Chan, Liu, Jiang, & Dusting, 2014) and fibrosis (Jiang, Liu, Dusting, & Chan, 2014). Overall, it remains open if the biological function of genes that are regulated by milk and fermented milk in oral fibroblasts could translate into a beneficial effect for tissue homeostasis and repair.

Taken together, our in vitro data suggest that pasteurized cow's milk, yoghurt, sour milk, buttermilk and whey activate TGF-β signaling in oral fibroblasts. These findings provide a scientific basis to further

investigate the possible role of cow's milk and fermented milk products on all aspects of oral tissue health.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.archoralbio.2018.07.005.

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