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Milk Exosomes Prevent Intestinal Inflammation in a Genetic Mouse Model of Ulcerative Colitis: A Pilot Experiment

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Keywords
Colitis · Genetic mouse model for colitis · Inflammation · Milk exosomes · Therapy

Abstract
Background: Milk is rich in nutrients and anabolic mediators rendering it essential for postnatal growth and metabolic programming. However, in adults, excessive consumption of milk is controversial as civilization disorders such as diabetes or prostate cancer may be promoted. A cytoprotective effect of milk could be utilized in inflammatory conditions, that is, chronic colitis. ***Objective:*** To evaluate the effect of bovine milk exosomes on intestinal inflammation in a genetic mouse model of ulcerative colitis. ***Methods:*** Intestinal-specific kindlin 2 knockout (KO) mice were exposed for 4 days to tamoxifen for induction of an ulcerative colitis phenotype. At the same time 4 other kindlin 2 KO mice were exposed to 33 µg/g cow milk derived exosomes in PBS by oral gavage. Both groups were compared to untreated wild-type controls. ***Results:*** Milk exosomes prevented the appearance of a severe ulcerative phenotype. The macroscopic colitis score dropped from a mean of 3.33 in untreated mice to 0.75 index points ($p < 0.01$) in exosome-treated mice, which included significant improvement of the subscores of stool improvement and colon weight and length. Treated mice featured a noninflamed appearance of the intestinal mucosa. ***Key Message:*** Milk exosomes have cytoprotective/anti-inflammatory activity in a genetic mouse model of ulcerative colitis. The mechanisms behind this need to be elucidated. This pilot study needs verification before a therapeutic strategy is developed.

Introduction

Milk of all mammalian species contains bioactive extracellular vesicles (EVs) [1, 2]. Among them, a special class of nanovesicles are exosomes (30–100 nm), which are derived via the endosomal route from mammary gland epithelial cells and secreted into the alveolar lumen [3]. They play a key role in epigenetic regulation for metabolic and immunological programming [4–10]. Bioactive milk exosomes contain and deliver microRNAs, mRNAs, and long noncoding RNAs, transforming

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growth factor-β, and a variety of unique proteins and lipids which are protected by the exosome lipid bilayer membrane [11–14]. Milk exosomes are, thus, extremely resistant against the harsh degradative conditions in the gastrointestinal tract [15–17] but can be taken up by intestinal epithelial cells [5, 18]. A substantial fraction of them reaches the systemic circulation of the milk consumer, as shown in rodents and humans [19–21]. Whether nonabsorbed exosomes also act from the luminal side is not elucidated. Recent evidence indicates that milk exosomes play a critical role for intestinal maturation and function in humans and rodent models [22–28]. It has been demonstrated in murine models that human, bovine, and porcine milk exosomes support intestinal cell growth [22–24], attenuate LPS-induced apoptosis [25], prevent intestinal endothelial cell damage [26, 27], enhance goblet cell numbers and mucin production [28], modify bacterial growth, and promote intestinal microbiota [29, 30]. Accumulating evidence indicates that milk exosomes exert barrier-protective and anti-inflammatory effects in rodent models of necrotizing enterocolitis (NEC) and NEC in human infants [25–32]. NEC exhibits severe tight-junction defects as shown in rodent models and human neonates [33–35]. As defective tight junctions are also reported in humans with ulcerative colitis (UC) [36–38], we became interested in the potential therapeutic usefulness of milk exosomes for the treatment of UC. For this purpose, we investigated the effects of oral exposure of bovine milk exosomes in an established genetic mouse model of UC [39]. This UC model, which operates by disruption of the tight-junction barrier between intestinal mucosal cells, employs a tamoxifen-inducible intestinal-specific deletion of intestinal expression of kindlin 2, resulting in a severe UC phenotype [39, 40].

Kindlin 2 as adapter protein for integrin β1 serves at the lateral side of mucosal cells for the establishment of tight junctions. As a consequence of kindlin 2 deletion, tight junctions are broken, the intercellular space is enlarged, cells become cuboidal, and crypt diameters are distended [39]. Functionally, phosphatidylcholine (PC) secretion to apical mucus was shown to be disturbed and invasion of microbiota to be facilitated. These are all also typical features in human ulcerative colitis [39]. Eventually, severe mucosal inflammation occurs. The difference of this mouse model in comparison to the human disease is (1) its rapid and severe phenotype and (2) that not only a hemorrhagic colitis but also an ileitis manifests. In previous studies, therapeutic strategies with topical PC supplementation as well as a luminally acting phospholipase inhibitor were shown to be effective [39, 40].

Materials and Methods

Exosome isolation from commercial cow milk was performed as described [41]. In brief, 10-mL milk was diluted with an equal volume of phosphate-buffered saline (PBS) and centrifuged at 4°C for 30 min at 2,000 *g*. The supernatant was centrifuged for 45 min at 12,000 *g*. The supernatant was then centrifuged for 2 h at 110,000 *g*. After that, the pellets were resuspended in PBS and pooled. After filtration through a 0.22-μm filter, the suspension was again centrifuged for 70 min at 110,000 *g* and the resuspended pellet once more centrifuged for 70 min at 110,000 *g*. The final pellet was resuspended in 200-μL PBS. The collected samples of 5 preparations yielded a protein concentration of 1.1 ± 0.3 mg protein/mL.

Characterization of exosomes employed EXOCET as an exosome quantification kit (Exocet 96A-1-SBI; System Biosciences, Heidelberg, Germany). It revealed a 79.8 ± 6.9% recovery of exosomes in the purified pellet compared to the original milk source. Within the enriched pellet suspension, Western blotting [42] revealed a dose-dependent signal of the enriched pellet suspension using antibodies to CD9 (PA5-85955), ADAM10 (PA5-28161) (both from Thermo Fisher Scientific) at a dilution of 1:500. Anti-β-actin (AC15, Sigma) at a dilution of 1:100,000 served as loading control.

Characterization of the UC Phenotype in Genetic Mouse Models

Animal studies followed the "ARRIVE" guidelines and were approved by the Heidelberg ethics committee (Ref-# 35-9185.81/6123/10 and 6284/11) (Ref-# S-211/2010). Tamoxifen-inducible, villin-Cre-dependent, kindlin 2 intestine-specific knockout mice were a gift of R. Faessler (Max-Planck Institute for Biochemistry, Munich) and propagated after embryonic transfer in the animal facilities of the University of Heidelberg [39, 40]. Mutant kindlin $2^{(-/-)}$ mice received LasVendi Rod 18 complete diet (Soest, Germany) ad libitum and were kept in conventional caging with AB-BEDD LT-E-001 (Vienna, Austria) bedding at 22°C, with a 12-h/12-h light/dark cycle. Mutant mice (12-week-old mice with a comparable body weight of 30 ± 2 g) were intraperitoneally (i.p.) injected at 9:00 a.m. with 0.2 mg tamoxifen daily for up to 4 days before being sacrificed 1 day later. Ileal mucosal cells were isolated [43] and characterized by Western blotting using antibodies to mouse kindlin 2, to ensure complete depletion in the mutant mice [39, 40]. As control, 12-week old wild-type mice were used. All mice were male of a C57BL/6 background obtained from Charles River.

Evaluation of Disease Activity

Quantitative evaluation of the UC phenotype was performed in resected gut segments and included the macroscopic colitis score with determination of the subscores total colon weight (including stool content), length, and stool appearance [39, 40].

Treatment of Mutant Mice with Milk Exosomes

The same disease activity parameters were assessed when the mutant mice were treated with the exosome suspension daily in 200 μL PBS (1 mg protein/mL) or just 200 μL PBS (control) by oral gavage during the 4 days after start of tamoxifen treatment [39, 40] to assess improvement of the UC phenotype. The application by oral gavage – using a 6.5-cm 18G × 2″ flexible elastic tube (9918; Cadence Science, Cranston, RI, USA) – ensures that the full dose of exosomes is provided to the small intestine. The dosage of 33 μg/g body weight corresponds to the amount of exosomes in

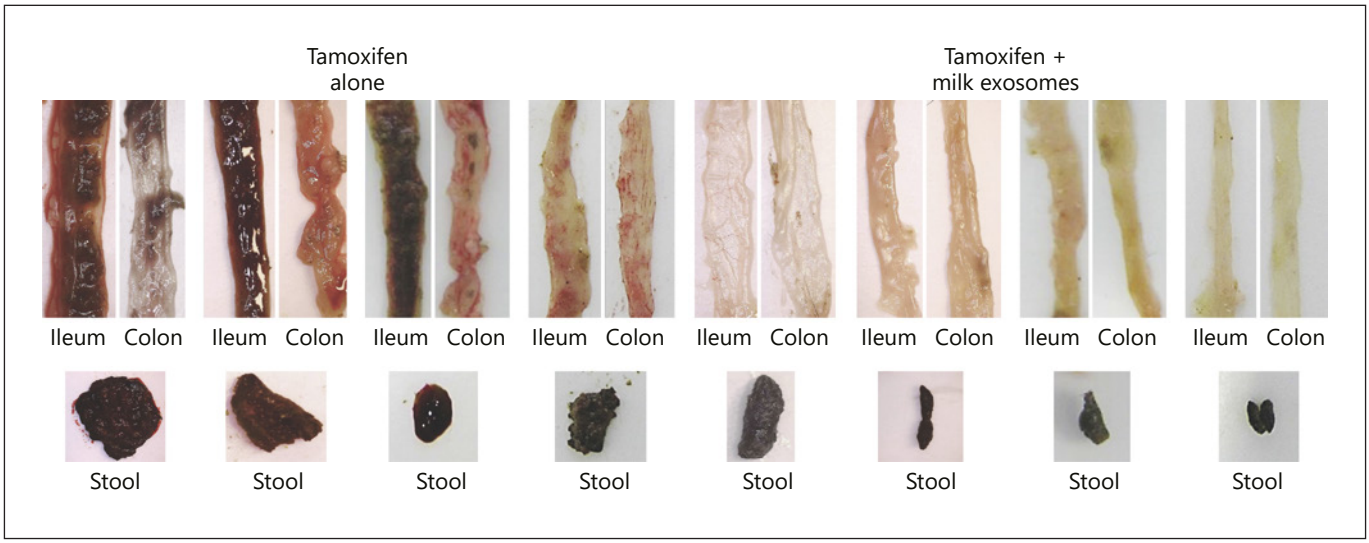

Fig. 1. Macroscopic appearance of opened ileal and colonic segments and stool samples. Depicted are specimens of 4 animals each of tamoxifen-induced intestinal-specific kindlin $2^{(-/-)}$ mice in the absence (left panels) and presence (right panels) of simultaneous oral administration of bovine milk exosomes for 4 days.

7.1 mL milk. This excessive amount overpowers the absorptive capacity and, thus, due to functional malabsorption a substantial fraction enters the colonic lumen.

Statistical Analyses

Statistical analysis was performed using Prism 4.0 software (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were evaluated using the Mann-Whitney U test. Multiple groups were compared by 1-way ANOVA with Dunnett's post hoc test. Data are presented as means ± SD, and $p < 0.05$ was considered statistically significant.

Results

The intestinal mucosa-specific kindlin $2^{(-/-)}$ mice showed – as expected – a severe inflammatory phenotype as it was observed in 2 preceding studies [39, 40]. This was compared to kindlin $2^{(-/-)}$ which were treated with tamoxifen and simultaneously with milk exosomes. Here exosomes prevented the inflammation (Fig. 1, upper panel). This is also seen at stool consistency (Fig. 1, lower panel). Untreated and exosome-treated kindlin $2^{(-/-)}$ mice were compared to wild-type mice in regard to the macroscopic appearance of stool as well as colon weight and length (Fig. 2). The total macroscopic colitis score of these 3 parameters showed a mean drop from 3.33 to 0.75 index points ($p < 0.01$).

The data of Figures 1 and 2 indicate that milk exosomes protect against intestinal inflammation.

Discussion

Previous epidemiological studies examining milk and dairy products and the development of Crohn's disease or UC are sparse and showed conflicting results. Some investigations report that a higher intake of milk is associated with a moderately increased risk of inflammatory bowel disease [44, 45], whereas others find an inverse association or none [46–50]. In comparison to individuals that do not consume milk, the European Prospective Investigation into Cancer and Nutrition cohort including 401,326 participants reported that individuals consuming milk had significantly reduced odds of Crohn's disease (OR 0.30, 95% CI: 0.13–0.65) and nonsignificantly reduced odds of UC (OR 0.85, 95% CI: 0.49–1.47) [51]. Notably, pasteurized fresh milk in contrast to fermented milk products such as yoghurt contains bioactive milk exosomes [52].

We observed an anti-inflammatory effect of milk exosomes in our murine model of UC. As demonstrated, macroscopic colitis scores decreased significantly after the exposure of milk exosomes including stool consistency.

A shortcoming of this pilot study is the small number of animals and the lack of opportunity to support the obvious macroscopic findings with histology and quantitative biochemical measurements, for example, TNFα, IL-6, IL-10, IL-12p40, and mucin 2. Therefore, the anti-in-

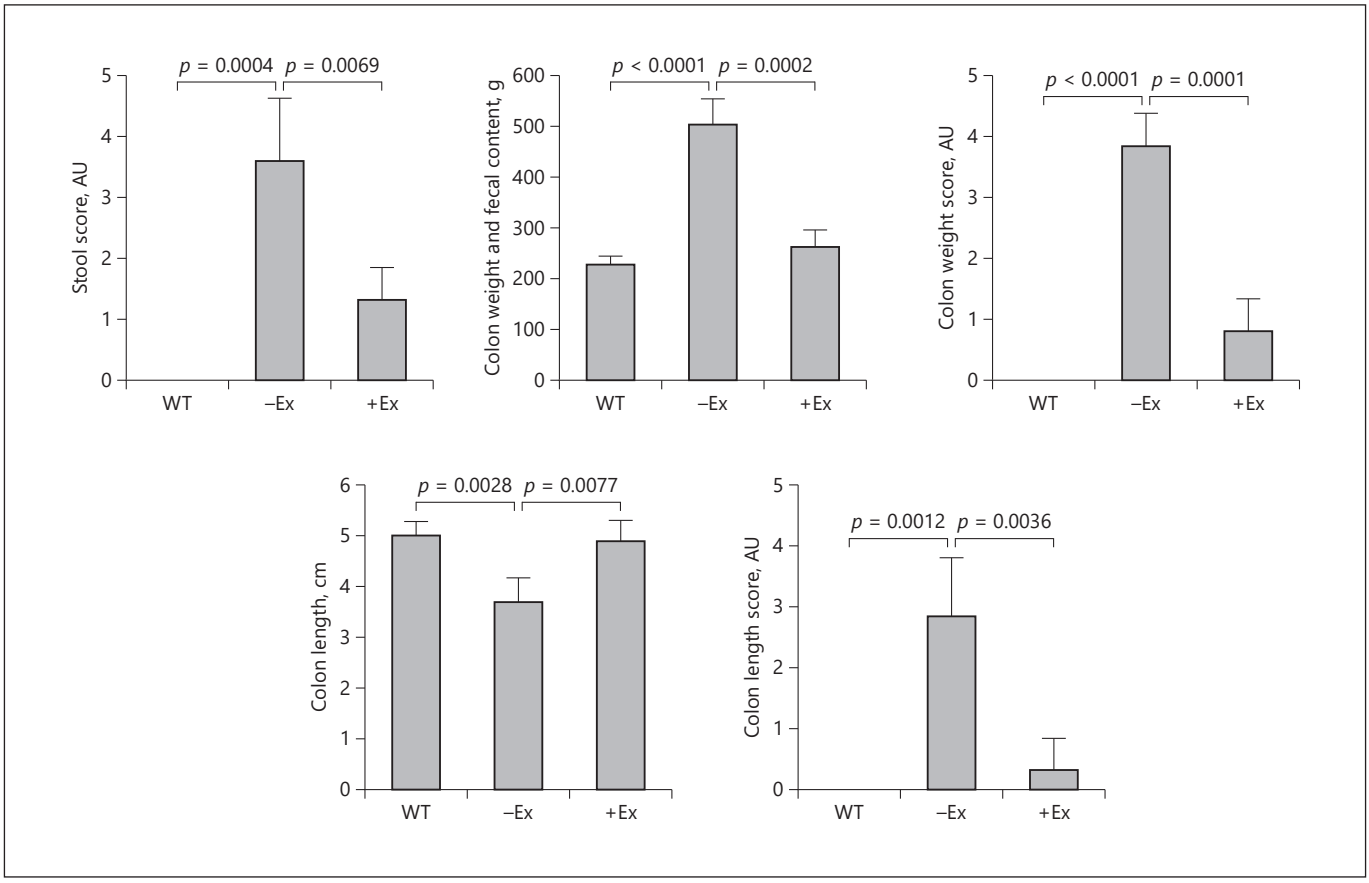

Fig. 2. Stool score, colon weight with fecal content, colon weight score, colon length, and colon length score. Mean and standard deviations for each parameter are given for wild-type animals (WT) and kindlin $2^{(-/-)}$ mice with (+Ex) or without (–Ex) milk exosome treatment ($n = 4$ in each group). Scores for stool (0 = normal, 1 = loosely shaped, 2 = amorphous, 3 = diarrhea, +1 = for blood), colon weight (0 = <10%, 1 = <10–50%, 2 = >50–100%, 3 = >100–150%, 4 = >150% weight gain compared to the wild-types' mean), and colon length (0 = <5%, 1 = 5–14%, 2 = 15–24%, 3 = 25–35%, 4 = >35% shortening compared to the wild-types' mean) were arbitrary defined. The *p* values for comparing 2 groups (wild-type vs. kindlin $2^{(-/-)}$ or kindlin $2^{(-/-)}$ without treatment versus kindlin $2^{(-/-)}$ with treatment) were calculated with unpaired *t* test.

flammatory mechanism of milk exosomes is still elusive. However, 3 major modes of action are conceivable:

Low mucus PC content is due to disturbed tight-junction barrier [37–40] and causes insufficient translocation of PC from systemic lipoproteins to the luminal side of mucosal cells. The reduced surface hydrophobicity allows attack of microbiota to induce mucosal inflammation [53, 54]. This may be counterbalanced by the transfer of milk exosome-derived PC [55]. This conception is supported by clinical improvement of colitis by topical application of PC in UC patients [56–58] and in genetic UC models [39]. Furthermore, inhibition of the ectophospholipase activity of the microbiota by ursodeoxycholate-lysophosphatidylethanolamide (UDCA-LPE) prevented the colitis in this murine UC model [40][59].

Another mode of action may concern inadequate production of mucin 2 (MUC2) as MUC2-deficient mice exhibit clinical and cellular features of active UC [60]. Remarkably, improvement of goblet cell activity and MUC2 production has been reported in an experimental model of NEC by application of bovine milk exosomes [28]. Mucus does not only form a nonspecific physical barrier, but constrains the immunogenicity of gut antigens by delivering tolerogenic signals [61].

Finally, microRNAs of milk exosomes may improve inflammation and intestinal barrier function. MicroRNA-148a and microRNA-21 are 2 major signature microRNAs of cow's milk [62], which target Rho-associated coiled-coil containing protein kinase 1 (ROCK1) [63, 64]. Notably, microRNA-21 increases the expression level of

occludin via ROCK1 suppression and, thus, stabilizes the intestinal barrier [65]. On the other hand, ROCK1 overexpression is associated with colorectal cancer (CRC) [66, 67]. Moreover, microRNA-148a and microRNA-21 inhibit DNA methyltransferase 1 (DNMT1) [68], an oncogene which is also overexpressed in CRC [69–71]. Indeed, incubation of CRC cells (Lim 1215) with human milk exosomes increased the cellular content of microRNA-148a [5], and addition of milk exosomes to normal intestinal cells (CRL 1831) significantly decreased DNMT1 expression [5, 24]. Three large meta-analyses came to the conclusion that milk consumption but not the consumption of fermented milk products, where exosomal microRNAs including microRNA-21 are depleted [52], has a protective effect against the development of CRC [72–74].

Although this pilot study proved a beneficial effect of milk exosomes in a model of UC, long-term oral application of milk exosomes may bare the risk of other diseases of civilization such as prostate cancer and type 2 diabetes mellitus [9, 75–77]. Nevertheless, oral exosome administration may be a promising new approach for the treatment of inflammatory bowel diseases as it has recently been confirmed by autologous exosome transfer in a murine colitis model [78].

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Statement of Ethics

Animal studies followed the "ARRIVE" guidelines and were approved by the Heidelberg ethics committee (Ref-# 35-9185.81/6123/10 and 6284/11) (Ref-# S-211/2010).

Disclosure Statement

The authors have no conflicts of interest to declare.

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Author Contributions

W.S. and B.C.M. created the hypothesis, W.S. performed the experiments, and B.C.M. contributed in the introduction and discussion with his knowledge about milk exosome biology. R.W. performed the statistics and generated Figure 2. W.S., R.W., and B.C.M. had access to the data of the study, contributed to writing of the manuscript, and read and approved the final manuscript.

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