Effect of Cow Milk-Derived Extracellular Vesicles on Arthritis

Fons van de Loo
Rheumatoid arthritis (RA)

- Multifactorial, chronic inflammatory disease
- Affects 1-2% of world population
- Symptoms:
  - Pain
  - Immobility
- Disease characteristics:
  - Synovial inflammation
  - Cartilage destruction
  - Bone destruction

Strand et al. Nature Reviews Drug Discovery 6, 75–92 (January 2007)
RA is a complex multifactorial disease

- Genetics
  - HLA-DRB1
  - HLA-DR4
- DIET
  - Age
  - Sex F:M 3:1
- Environment
  - Odds ratio 2.0 men
  - Odds ratio 5.47
- Peridontitis and RA odds ratio 1.8-8.1

Role of food components, protective or deleterious?
Claims of foods that fight RA

Mediterranean diet, less meat.. more vegetables... and olive oil

Fish, omega-3 fatty acids

A meta-analysis published in Archives of Medical Research 43 (2012) 356-362 shows that Omega-3 PUFAs reduces NSAID consumption by RA patients. Most likely an effect on pain!
Milk is not just food

- Human breast milk contains anti-microbial and immune development compounds\(^1\), \(^2\)

- Extracellular vesicles, including exosomes, are present in human breast milk and bovine colostrum\(^3\), \(^4\)

**Semi skimmed cow milk 200 ml**

Extracellular  40 mg protein
Vesicles  5×10\(^9\) particles

\(^1\) Field, 2005  
\(^2\) Lönnerdal, 2003  
\(^3\) Admyre, *et al.*, 2007  
\(^4\) Hata, *et al.*, 2010
EVs are phospholipid bilayer vesicles

Membrane vesicles (100–2,000 nm)
- **Shape:** Irregular
- **Markers:** Integrins, selectins, CD40 ligand
- **Lipids:** Phosphatidylserine
- **Origin:** Plasma membrane

Apoptotic bodies (1–4 μm)
- **Shape:** Cup shaped
- **Markers:** Tetraspanins (CD63/CD9), Alix, TSG101, ESCRT
- **Lipids:** Cholesterol, sphingomyelin, ceramide, lipid rafts, phosphatidylserine
- **Origin:** Multivesicular endosomes

Exosomes (40–100 nm)

EVs in intercellular communication

Regulation of the Immune system

Secreting cell

Recipient cell

Exosomes

Antigen transfer

mRNA, microRNA transfer

Multi-vesicular endosomes

Cytosolic and membrane proteins

Fusion mediators

Regulation of the Immune system
Milk EVs contain miRNAs

Olivieri et al. Immunity & Ageing 2013 10:11
The hypothesis

Cow milk-derived EVs modulate the (mucosal) immune system and thereby affect disease in RA patients.
Isolation protocol milk EVs

1. Skimmed milk
   - 3,000g, 15 mins (3-4x)

2. Defatted milk
   - 12,000g, 60 min
   - 35,000g, 60 min
   - 70,000g, 60 min
   - 0.2μm filtration

3. Milk ‘Supernatant’
   - ExoQuick Overnight or 100,000g

Extracellular vesicles

Analysis:
- NanoSight NTA
- mRNA detection
- miRNA detection

Adapted protocol for exosome isolation from bovine milk ¹

¹ Yamada, et al., 2012
Characterization of cow milk EVs

Particle size

EM

NTA

Tetraspanin

Capture + NTA

CD63+ vesicle conc. (x10^6 ml⁻¹)

EVs (µg/ml)

Intensity (%) vs Diameter [nm]

Latex

Exosomes
RNA characterization of cow milk EVs

mRNA content EVs

miRNA content EVs

\( \Delta Ct \, (GAPDH) \)

\( \beta \) Casein \( \beta \) LG EF-1 \( \alpha \)

Mammary gland cells Somatic cells

Ct-value

Raw Milk Commercial Milk

miR-16 miR-21 miR-30a miR-92a miR-99a miR-223

NID
Protein profiling of cow milk EVs

1D-LC-MS/MS identified 64 proteins

1. Transmembrane proteins
   CD36, CD59, CD81

2. Cytosolic proteins
   Rab-1b, Rab-11a

3. Intracellular structural proteins
   actin, myosin-XIX

4. Precursor of milk proteins
   casein, α-lactalbumin, βLG, osteopontin
Stability of milk EVs

NanoSight Particle Tracking

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<th>Condition</th>
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<tr>
<td>Heat (boiling)</td>
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<td>30 mins</td>
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<tr>
<td>Freeze-thawing</td>
<td>Liquid NO₂</td>
<td>3 times</td>
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NFκB-Luciferase reporter assay

RAW-264.7 cell line containing a NFκB-luciferase construct
Cellular uptake of PKH-68 labelled EVs

RAW 264.7

37°C

4°C

10m

10m

1h

1h

3h

3h

24h

24h

Flow cytometry

Convocal microscopy

PKH-67

F4/80
EV uptake by murine intestinal explants

1. Isolation murine small intestine
2. Cleaning and stitching bottom
3. PKH-67 labeled EVs
4. 24h incubation at 37°C
5. Extensive rinsing
6. Imaging (CCD camera)
Cow milk-derived EVs:

- Diameter around 100 nm
- All have the characteristics of genuine exosomes
- Possess miRNAs and mRNAs (e.g. milk specific proteins)
- Remain intact under harsh conditions
- Are taken up by numerous different cell types

**BUT ARE THEY FUNCTIONAL?**
Milk EVs posses active TGF-β

(CAGA)12-Luciferase reporter in NIH-3T3 fibroblasts
T-cell immunity in RA patients

Treg Foxp3

CD4+ Th

Th17 RORγT

TGFβ

TGFβ

IL-6

IL-10

IL-2

CD25

Inflammation (autoimmunity)

Tolerance

1 Betteli, et al., 2006
2 Mangan, et al., 2006
Milk EVs induce Th17 differentiation

EVs also enhance IL-22 expression, but neither IL-21, TGFβ nor FoxP3
Milk EVs also induce Tregs in vitro

Differentiation into Th17 and Tregs induced by milk EVs is TGF-β mediated
Functional properties of milk EVS:

- Are able to activate a TGFβ-specific reporter bioassay
- Can mediate differentiation of naive T-cells into both Th17 or regulatory T-cells
- This T-cell differentiation effect by EVs is partially mediated by TGF-β

WHAT WILL BE THE EFFECT OF ORALLY APPLIED COW MILK EVs ON ARTHRITIS?
1st model: IL-1 receptor antagonist KO mice

Daily oral administration of EVs start at week 5 of age.

High dose = 1200 µg EVs / mouse
Low dose = 171 µg EVs / mouse
Results IL-1Ra arthritis model

A. Disease Incidence (%) as a function of weeks after treatment with PBS, Milk (Low), and Milk (High).

B. Macroscopic score (0-4) for Weeks after treatment with PBS, Milk (Low), and Milk (High).

C. Microscopic score (0-3) for Infiltrate, Exudate, Cartilage depletion, and Bone Marrow Cellularity with PBS, Milk (Low), and Milk (High).

D. Microscopic images showing PBS, Milk (low), and Milk (high) at 100 µm scale.
2\textsuperscript{nd} model: Collagen-induced arthritis

**Immunization i.d.**
bovine CII/CFA

**Booster i.p.**
CII/PBS

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Macroscopic score (paw 0-2)

**Administration of EVs via drinking water**

**High dose** = 115 µg EVs/ml ~ 400 µg EVs/mouse

**Low dose** = 38 µg EVs/ml ~ 140 µg EVs/mouse
Results CIA model

A

B

C

D

Histological score of only the affected mice
Effect of milk EVs on innate immunity

Isolated adherent murine splenocytes were incubated 24, 48, and 72 h with milk EVs (20, 200 μg/mL) and thereafter stimulated with LPS (10 ng/mL) for 6 h, and supernatants used in Luminex.
Effect of milk EVs on innate immunity

**IN VIVO**

**IL-1RA KO MICE**

![Graph showing mRNA expression levels for IL-6, KC, and MCP-1 with different treatments: PBS, Milk (low), Milk (High)].

**IN VIVO**

**CIA MICE**

![Graph showing serum levels of MCP-1 and IL-6 with different treatments: PBS, Milk (low), Milk (High)]
Milk EVs decrease the adaptive immunity in CIA mice

- **Total IgG absorbance (450nm)**
- **IgG1 absorbance (450nm)**
- **IgG2a absorbance (450nm)**

Dilution: 10, 100, 1000, 10000
Milk EVs decrease the adaptive immunity in CIA mice

Mixed Splenocytes mRNA relative expression ($2^{\Delta \Delta CT}$)

- **PBS**
- **Milk (low)**
- **Milk (High)**

- **T-bet**
- **GATA3**
- **FOXP3**
- **RORyT**

**Th1**
**Th2**
**Treg**
**Th17**
Therapeutic potential of milk EVs:

- Oral delivery (prophylactic) delays onset of disease in two models of rheumatoid arthritis
- Diminishes cartilage pathology and bone marrow inflammation
- Reduces the innate cytokine response to LPS (TLR4 signaling)
- Evidence for reduced Th1 (Tbet, IgG2a) and Th17 levels in mice
Can cow milk EVs be used to treat RA?

✓ Cow milk EVs are bioactive:  ✓ anti-inflammatory
✓ immunoregulatory

✓ Oral uptake of cow milk EVs ameliorate disease in 2 RA models.

New questions emerge:

Is complete milk different from their EVs?
Is raw milk different from processed?
Are milk EVs a heterogenous group?
What and where is the mode of action?
Can other diseases benefit from cow milk EVs?
Part II. Beneficial effect of milk on osteoporosis is debatable

Regular milk consumption throughout life, from adolescence to old age, is associated with higher BMC and BMD in old age (Eysteinsdottir et al. 2014)

Associated to calcium and protein consumption

Role of EVs?

Increase in the risk of hip fractures associated to the consumption of milk (Michaëllsson et al. 2014)

Associated to galactose consumption
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