

The Effect of Milk and Fermented Milk on High Blood Pressure and Bone Mineralization

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I. High blood pressure

1. Epidemiological studies

Epidemiological studies suggest that consumption of milk and milk product is inversely related to the risk for hypertension. The association between milk consumption and blood pressure was reported in the analysis of first National Health and Nutrition Examination Survey (NHANES I) (1). In this cross-sectional study with over 10000 persons, low consumption of milk products was associated with a high incidence of hypertension. The prevalence of hypertension has also been reported to be twice as high in middle-aged men in Puerto Rico who drank no milk as in middle-aged men in Puerto Rico who drank at least one litre of milk a day (2). The consumption of milk has also been shown to be lower in hypertensive than normotensive persons in American and Italian population studies (3, 4). The milk product consumption has also been shown to relate to the risk of stroke. During 22-years of follow-up of 3000 men the consumption of milk was related to the lower rate of thromboembolic stroke (5). In this study and in a prospective Nurses' Health Study with 85000 American women (6) the inverse association between calcium intake and stroke was stronger for dairy calcium than for non-dairy calcium. It is therefore possible that some components of milk other than calcium e.g. other electrolytes proteins or peptides had been important in relation to the incidence of stroke. However beneficial relationship between consumption of milk and incidences of hypertension and stroke has not been seen in all studies (7, 8).

2. Intervention studies

Intervention studies have investigated the effects of milk and milk products on blood pressure. One of the most remarkable recent studies is the DASH (Dietary Approaches to Stop Hypertension) trial with almost 500 normotensive or mildly hypertensive subjects, which shows that a diet rich in fruits, vegetables and low-fat dairy products (the so-called combination diet) was shown to reduce blood pressure significantly. Mean systolic blood pressure and mean diastolic blood pressure decreased by 5.5 mmHg and 3.0 mmHg, respectively, in total study cohort. Among the hypertensive subjects the reductions were greater. Mean systolic blood pressure decreased by 11.4 mmHg and mean diastolic blood pressure by 5.5 mmHg. Blood pressure lowered more on the combination diet than on the diet rich only in fruits and vegetables (9, 10). In a follow-up study (DASH II), in which sodium intake was restricted to a maximum of 1.5 g/day, mean systolic blood pressure fell further in the normotensive subjects, by 7.1 mmHg (11).

Although it has been shown also in some other intervention studies that consumption of milk products reduces blood pressure (12-15), the relationship has not been demonstrated in all studies (16).

3. Protein and blood pressure

Milk contains many constituents including electrolytes, proteins and peptides, which could affect blood pressure beneficially. Milk contains about 3.5% of protein, which is consist of caseins (80%) and whey proteins (20%). Caseins have been classified as α -, β - and κ -caseins. Whey contains β -lactoglobulin, α -

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lactalbumin and several minor proteins with different biological activities like enzymes, mineral binding properties, immunoglobulins (17).

Some epidemiological studies suggest that protein intake is inversely related to the risk for hypertension (18-20). In two cross-sectional studies, the Honolulu Heart Program study and the Intersalt study have shown inverse relation between protein intake and blood pressure (18, 19). Association between protein intake and the risk of stroke was also seen in Nurses' Health Study (6). However, in some epidemiological studies, no inverse relationship between high protein intake and blood pressure has been seen (21).

Also in intervention studies the effects of dietary protein on blood pressure has been investigated (22, 23). The results of some of these studies show that a high dietary protein intake was associated with a low incidence of hypertension. However, in some intervention studies no significant effects of level of protein intake on blood pressure were observed (for review, see 24).

The antihypertensive mechanism of protein is still unknown. One possibility is the degradation of protein into peptides, which have antihypertensive effects. In this review we concentrate to discuss about the blood pressure lowering effect of milk derived peptides.

Peptides and blood pressure

1. Peptide production and absorption

Biologically active peptide fragments are formed when milk proteins are broken down by digestive enzymes, or by the proteinases formed by lactobacilli during fermentation of milk (24, 25). Lactic acid bacteria are suitable for milk fermentation because they have a proteolytic activity and capacity to produce peptides, e.g. a starter containing *L. helveticus* strain has reported to produce bioactive peptides (26, 27). Milk peptide activities include binding to opioid receptors, inhibition of angiotensin-converting enzyme (ACE), and modification of antithrombotic and immune responses (24, 25). Phosphopeptides, formed from casein, may enhance the absorption of minerals,

especially calcium, from the digestive tract into the circulation (24).

Milk peptides that are not degraded in proteolysis can theoretically be absorbed intact. It has been suggested that dipeptides and tripeptides (Ile-Pro-Pro and Val-Pro-Pro) are absorbed in the intestine (28, 29). Val-Pro-Pro has been reported to transport via the Caco-2 cell monolayer via paracellular diffusion (29). Also the absorption of longer peptides has been studied. The ACE inhibitory peptide, lactokinin, Ala-Leu-Pro-Met-His-Ile-Arg has been found to be transported intact through the Caco-2 monolayer (30).

2. Animal studies

Ile-Pro-Pro and Val-Pro-Pro have been shown to reduce blood pressure in spontaneously hypertensive rats (SHR) after a single oral administration (27). They also prevent the development of hypertension in SHR after long-term, twelve and thirteen weeks, oral feeding (31, 32). At the end of the twelve-week treatment period systolic blood pressure was 17 mmHg lower in the group receiving *L. helveticus* LBK-16H fermented milk containing Ile-Pro-Pro and Val-Pro-Pro than in the control group receiving water ($p < 0.001$) and 12 mmHg lower in the group receiving the tripeptides in water than in the control group (31). In a thirteen-week study, *L. helveticus* LBK-16H fermented milk-containing Ile-Pro-Pro and Val-Pro-Pro tripeptides attenuated the development of hypertension more effectively than water ($p < 0.001$) or the *L. helveticus* and *S. cerevisiae* fermented milk containing half as much the same peptides than *L. helveticus* LBK-16H fermented milk ($p < 0.001$) (32). It has been shown that α -Lactorphin (Tyr-Gly-Leu-Phe) lowers blood pressure dose-dependently in SHR and in normotensive Wistar-Kyoto (WKY) rats. The blood pressure was measured with continuous radiotelemetric monitoring and the maximal reductions in systolic and diastolic blood pressure were 23 ± 4 and 17 ± 4 mmHg, respectively (33). Two *L. helveticus* strains, *L. helveticus* CHCC637 and *L. helveticus* CHCC641 in fermented milk have an ACE inhibition effect. This fermented milk reduced blood pressure more than the control group in SHR (34).

3. Clinical studies

Lactobacillus helveticus fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptide has also been shown to decrease systolic and diastolic blood pressure in hypertensive subjects (35-38). In a placebo-controlled study on hypertensive subjects, *L. helveticus* and *S. cerevisiae* fermented milk reduced systolic and diastolic blood pressure ($p < 0.05$) during the eight-week intervention more than placebo-fermented milk (35). In an eight-week placebo-controlled study on 17 hypertensive subjects systolic and diastolic blood pressures were lowered more in the group receiving *L. helveticus* fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides than in the control group receiving normal fermented milk fermented with *Lactococcus sp.* mixed culture ($p = 0.05$ and $p < 0.05$) (37) and also in the long-term clinical study (21 weeks) systolic and diastolic blood pressure were decreased more in *L. helveticus* fermented milk group ($n = 22$) than in the control group receiving fermented milk ($n = 17$) (SBP 6.7 ± 3.0 , $p = 0.030$ and DBP 3.6 ± 1.9 , $p = 0.059$) (36). Tablets, containing Ile-Pro-Pro and Val-Pro-Pro tripeptides, have been shown to decrease blood pressure in mild or moderately hypertensive subjects (39). Milk that has been fermented using *Lb. casei* and *Lc. lactis* and that contains γ -aminobutyric acid (GABA) reduced blood pressure during a 12-week treatment period. Systolic blood pressure lowered more in fermented milk group than in the control group ($p < 0.05$), but diastolic blood pressure of the fermented milk group did not differ from the control group (40). In another eight-week-long study systolic blood pressure was significantly lower in the group receiving yoghurt fermented with two strains of *Streptococcus thermophilus* and two strains of *Lactobacillus acidophilus* and in the group receiving yoghurt fermented with one strain of *Enterococcus faecium* and two strains of *Streptococcus thermophilus* compared to the group receiving yoghurt fermented with two strains of *Streptococcus thermophilus* and one strain of *Lactobacillus rhamnosus* (41).

Mechanisms of the antihypertensive effects of milk peptides

1. Angiotensin-converting enzyme inhibition

One mechanism by which milk-derived peptides can reduce blood pressure is inhibition of ACE (26, 27, 32, 33, 42-45). This is the mechanism that has been studied most in relation to the antihypertensive effects of milk peptides. ACE is an enzyme that plays a crucial role in the function of the renin-angiotensin system (RAS). The RAS is an important regulator of blood pressure and fluid and electrolyte balance (46). In the RAS angiotensin I is converted to angiotensin II by ACE. Angiotensin II is a strong vasoconstrictor that induces release of aldosterone and therefore increases sodium concentration and furthers the blood pressure. ACE inhibitors have two effects on the renin-angiotensin system. They reduce production of angiotensin II and inhibit the degradation of the vasodilator bradykinin by Kininase II, the same enzyme as ACE.

Several antihypertensive peptides that inhibit ACE have been isolated from milk products and the ACE inhibition activity of these peptides has been determined. The IC_{50} values are the concentrations at which ACE activity *in vitro* is inhibited by 50%. The relationship between ACE inhibitory peptides and the chemical structure has not been confirmed, but it has been suggested that peptides, with hydrophobic amino acids at the C terminal position could be the most likely ACE inhibitors (47).

Peptides derived from casein by *L. helveticus* proteases have been shown to have ACE inhibitory activities (48). ACE inhibitory activity of casein-derived tripeptides Ile-Pro-Pro and Val-Pro-Pro has been shown *in vitro* (26). *L. helveticus* fermented milk-containing Ile-Pro-Pro and Val-Pro-Pro raised plasma renin activity in SHR during long-term treatment (32). Also ACE activity in aorta of SHR was reduced after single oral administration and after long-term treatment with *L. helveticus* and *S. cerevisiae* fermented milk containing Ile-Pro-Pro and Val-Pro-Pro tripeptides (28, 49). Antihypertensive peptides with insignificant ACE-inhibitory activity have also been

isolated from milk products (33, 43, 45). Therefore milk-derived peptides could also affect blood pressure by mechanisms other than ACE inhibition.

2. Other possible mechanisms

Several milk peptides have opioid-like activities. Typical opioid peptides originate from three precursor proteins: pro-opiomelanocortin (endorphins), pre-enkephalin (enkephalins) and prodynorphin (dynorphins). All have the same N-terminal amino acid sequence, Tyr-Gly-Gly-Phe (25). Opioids bind to opioid receptors and have morphine-like effects. The opioid system contains several different endogenous opioid peptides and receptors. Opioids are present in the central nervous system and in peripheral tissues, where they are involved e.g. in the regulation of circulation (50). Opioids also affect blood pressure (51). Opioid-like activity has been discovered in many peptide fragments from casein and the first characterized opioid milk peptide agonist was derived from β -casein (β -casomorphin). The peptides with opioid-like activity derived from α -casein are called α -exorphins and from γ -casein derived are called casoxins. In addition opioid peptides can be derived from the whey proteins α -lactalbumin and β -lactoglobulins. α -Lactorphin, found from α -lactalbumin, has been shown to lower blood pressure in SHR. Because the antihypertensive effect of α -lactorphin was completely prevented by an opioid receptor antagonist naloxone, it has been proposed that the antihypertensive effect is mediated via opioid receptors (33).

Some peptides, like caseinophosphopeptides, have also been shown to increase the solubility of calcium and enhance the absorption of calcium (52, 53) and some milk peptides have antithrombotic effects by e.g. inhibiting the aggregation of ADP-activated platelets (54). This might also have some role in the beneficial cardiovascular effects of milk-derived peptides.

References

1. Sipola M. Effects of milk products and milk protein-derived peptides on blood pressure and arterial function in rats. Doctoral Thesis supervised by Docent Riitta Korpela, Ph.D, Institute of Biomedicine, Pharmacology, University of Helsinki, Finland.
2. The list of other references can be obtained from Dr. R. Korpela.

II. Bone mineralization

Milk protein and bone

1. Correlations with milk consumption and bone

Milk is a good source of several nutrients, such as protein, calcium, phosphate, magnesium, potassium and zinc that are considered important for bone health. In epidemiological studies milk consumption in childhood and during adolescence has been related to higher bone mineral density in adulthood (Sandler et al. 1865, Hirota et al. 1992, Stracke et al. 1983, Murphy et al. 1994, Soroko et al. 1994, Teegarden et al. 1995, New et al. 1997, Kalkwarf et al. 2003). This effect has been particularly established in white women (Optowsky and Bilezikian 2003). In cross-sectional studies milk intake has been shown to correlate to bone mineral density in children (Black et al. 2002), in adult men (Egami et al. 2003) and in women (Lacey et al. 1991, Tylavsky et al. 1992, Davis et al. 1996, Hawker et al. 2002).

Intervention studies show that milk and milk products increase bone mineral density in adolescents, both in girls (Chan et al. 1995, Cadogan et al. 1997, Merrilees et al. 2000) and in boys (Renner et al. 1998). In pre- and postmenopausal women, 2-4-year interventions with milk and milk products prevented the bone loss that occurred in the control group, who had no dietary intervention, although there was no increase in bone mineral density (Baran et al. 1990, Chee et al. 2003). The control groups of these intervention studies did not have adequate calcium intake, thus the result of the interventions may be the result of a higher calcium intake by the groups receiving milk products. In calcium-controlled studies, calcium supplementation and ingestion of dairy products increased bone mineral density in adolescents (Matkovic et al. 2004) and

reduced bone loss in postmenopausal women (Prince et al. 1995). Dairy products have also been shown to increase not only bone mineral density but also bone growth (Matkovic et al. 2004).

The effect of milk and milk products on fracture risk has been evaluated in children and in women. It has recently been found that children with low milk intake are at higher risk of prepubertal bone fractures (Gouding et al. 2004). Higher milk consumption by women during the past 12-14 years has been shown to prevent fractures in Japanese population (Fujiwara et al. 2003). In North America retrospectively obtained information about childhood milk intake was associated with lower fracture risk in females over the age of 50 (Kalkwarf et al. 2003), but milk consumed during adulthood did not relate to fracture risk (Feskanich et al. 1997, 2003).

These results show that the consumption of milk and milk products correlates with higher bone mineral density during growth and prevents age-related bone loss. This may be the result of a higher calcium intake, in view of the fact that in western countries milk contributes 80% of dietary calcium (Fleming and Heimbach 1994). However, there are other components in milk that may contribute to the effect on bone.

2. Protein intake and bone

Protein intake is important for bone health. Both inadequate and excess intakes have been postulated as having detrimental effects on bone (for reviews see Dawson-Hughes 2003, Ginty 2003). The effect of protein on bone is dependent on the intake of calcium (Meyer et al. 1997). Increase in protein intake causes increased calcium excretion through acid production, but this effect is not significant when calcium intake is adequate (Heaney 1998, Dawson-Hughes and Harris 2002, Dawson-Hughes et al. 2004). In addition to the effect on calcium, protein affects bone through insulin-like growth factor I (IGF-1) (for review see Bonjour et al. 1997), which regulates bone growth and bone mineral density (Yakar et al. 2002). Protein supplementation increases serum IGF levels (Schürch et al. 1998) and restriction of protein decreases IGF levels

(Bourrin et al. 2000). The intake of milk has been associated with higher IGF-1 levels, possibly due to the effects of protein (Cadogan et al. 1997, Heaney et al. 1999).

Protein intake has been associated with higher bone mineral density in adults (Promislow et al. 2002), in postmenopausal women (Kerstetter et al. 2000) and in the elderly (Hannan et al. 2000, for review see Bell and Whiting 2002). The effect of protein on fracture risk is controversial (negative: Feskanich et al. 1996, Sellmeyer et al. 2001, positive: Munger et al. 1999).

3. Whey protein and bone

3% of milk is protein, of which 80% is casein and 20% whey protein. Whey proteins, such as alpha lactalbumin and beta lactoglobulin, bind calcium, but this has also no effect on calcium balance and retention *in vivo* (Takada et al. 1997a, for review see Gueguen and Pointillart 2000). In fact, whey protein has been shown to increase the bone-breaking energy of rats compared to casein, by increasing the total amino acid content of the femur (Takada et al. 1997a). In growing rats, a whey protein supplementation did not make any difference to femoral density, bone mineral content or the biomarkers of bone metabolism after a 7-week intervention (Kelly et al. 2003). However, in the same study the whey protein-supplemented casein diet increased bone formation after 14 days in a model of ectopic bone formation (Kelly et al. 2003), implying that the intervention was too short to demonstrate the effect on bone. These results suggest that whey protein does not increase mineral bioavailability but supports bone by increasing bone protein, especially bone collagen content.

Whey protein reduces the formation of osteoclasts and their activity (Takada et al. 1997b). Osteoclast formation measured by multi-nuclei cell formation decreased dose-dependently with the highest concentration of 1 mg/ml whey. Whey protein has also been shown to activate osteoblasts (Takada et al. 1996).

The effects of whey protein on bone could be derived from the basic part of the protein (milk basic protein, MBP). Isolated MBP decreases bone deterioration in

ovariectomised rats (Kato et al. 2000). In human studies short-term ingestion of MBP increases radial and calcaneus BMD and decreases bone resorption markers (Aoe et al. 2001, Toba et al. 2001, Yamamura et al. 2002). The active component of MBP has been found to be kininogen, belonging to high-mobility-group-like proteins (Yamamura et al. 1999, 2000). One of the mechanisms by which this active compound affects bone is through cystatin C, which inhibits osteoclast activity by decreasing the secretion of collagen-digesting cathepsin (Matsuoka et al. 2002).

To summarise the effect of milk on bone, milk consumption has a positive correlation with bone mineral density. In addition to its calcium content, milk contains proteins, which are beneficial to bone health in the presence of adequate calcium.

Milk-derived bioactive peptides on calcium and bone metabolism

In the gastrointestinal tract, food protein is digested into smaller peptides, some of which have physiological effects (for review see Korhonen and Pihlanto 2003). These bioactive peptides can be formed by enzymatic hydrolysis with digestive enzymes, but also by fermentation with starter cultures (for review see Korhonen and Pihlanto 2003). In fermentation, the formation of peptides depends on the bacteria used (Matar et al. 1996). The active peptides are usually small, consisting of 3-20 amino acid residues. Bioactive peptides have been found in many different foods, such as milk, eggs, beans, fish and corn (for review see Kitts and Weiler 2003), but milk protein is the most important source of bioactive peptides (for review see Korhonen and Pihlanto 2003).

The bioavailability of peptides most often requires that they should not be digested in the gastrointestinal tract. The absorption of small peptides is well known (for review see Fricker and Drewe 1996). Peptides can be absorbed through the gastrointestinal wall by different mechanisms, such as passive diffusion through the enterocytes, paracellularly, through cytosol or through a carrier (for review see Fricker and Drewe 1996). Some peptides, such as caseinphosphopeptides,

express their activity in the gastrointestinal tract without being absorbed.

Bioactive peptides have been shown to have various physiological effects both *in vitro* and *in vivo* (for review see Pihlanto-Leppälä 1999, Korhonen and Pihlanto 2003, Maisel and FitzGerald 2003). Opioid peptides from casein or whey proteins possess an affinity to opiate receptors as well as opiate-like effects. They act either as agonists or antagonists in the opioid receptors. Opioid peptides influence the nervous system, gastrointestinal transit time, nutrient intake and the secretion of insulin and glucagons. Antithrombotic peptides derived from casein have been shown to suppress platelet aggregation. Immunomodulating peptides stimulate the proliferation of human lymphocytes and the phagocytic activities of macrophages. Antimicrobial effects have been shown with small whey proteins binding iron, an essential nutrient of micro-organisms (for review see Clare et al. 2003). Other casein-derived antimicrobial peptides have shown anticarcinogenic effects (for review see Aimutis 2004). The antioxidative and hypocholesterolemic effects of bioactive peptides have only recently been discovered (for review see Korhonen and Pihlanto 2003). One bioactive peptide may have several physiological effects, e.g. casein-derived beta-casomorphin 7 has both angiotensin converting enzyme (ACE)-inhibitory effects and opioid-like effects (for review see Meisel and Bockelmann 1999). The only milk-derived bioactive peptides that have been studied on calcium metabolism are caseinphosphopeptides (CPP) (for review see Scholz-Ahrens and Schrenzenmeier 2000).

1. Caseinphosphopeptides (CPP)

CPP are a large group of peptides that have a phosphoserine residue in common. Phosphopeptides are formed either from casein by proteolytic enzymes during fermentation or in the gastrointestinal tract. CPP increase calcium absorption by forming a hydrophobic complex with calcium, thus preventing the formation of insoluble calcium phosphates (Meisel and Bockelmann 1999). *In vitro* studies have shown the effects of CPP on calcium absorption by inhibiting the precipitation of

calcium in the intestine (Sato et al. 1986).

In animal studies, the effect of CPP has produced inconsistent results. In most of the studies the CPP have increased calcium absorption (Mykkänen and Wasserman 1980, Lee et al. 1980, 1983, Kitts et al. 1992, Hirayama et al. 1992, Tsuchita et al. 1993, 2001, Bennett et al. 2000), but some studies failed to show any effect on calcium absorption (Brommage et al. 1991, Kopra et al. 1992). Only one study examined the effect of CPP on bone. In this study CPP was found to prevent bone loss in ovariectomised rats (Tsuchita et al. 1996).

An early as 1950 Mellander reported the first finding of the effect of CPP in humans in his study on rachitic children (Mellander 1950). Increased calcium absorption was independent of the effect of vitamin D, suggesting that CPP increase calcium absorption in the distal small intestine. In later human studies, the effect of CPP has been shown to be influenced by the calcium status of the subjects and by other nutrients affecting calcium absorption (Heaney et al. 1994, Hansen et al. 1997a, 1997b). A CPP preparation increased calcium absorption in women whose calcium absorption rate was low (Heaney et al. 1994). The effect of the addition of 1-2 g of CPP to different food products has produced conflicting results on calcium absorption. Calcium was shown to be more efficiently absorbed from rice meal compared to whole-grain whey (Hansen et al. 1997a); however, in later study CPP did not show any greater effect on calcium absorption from low or high phytate foods (1g) (Hansen et al. 1997b).

In conclusion, CPP increase calcium absorption in animal studies but the effect in humans is not conclusive.

2. *L. helveticus* fermented milk

During fermentation, bioactive peptides with physiological effects are formed. These peptides have been shown to enhance such processes as mineral absorption. Nevertheless, there are no prior studies on the effects of bioactive peptides and bone. The aim of our study was to clarify the effect of *Lactobacillus helveticus* (*L. helveticus*) fermented milk, phosphopeptides on the

bone metabolism of rats *in vivo*, on osteoblast and osteoclast precursor cells *in vitro* and on acute changes in calcium metabolism in man.

The following conclusions can be drawn from the series of studies:

1. *L. helveticus* fermented milk supports bone mineral density in growing rats and reduces bone loss in ovariectomised rats. The effect of *L. helveticus* fermented milk may be mediated through the isoleucyl-prolyl-proline (IPP) and valyl-prolyl-proline (VPP) peptides formed during fermentation.
2. The IPP and VPP peptides increase the bone formation of osteoblastic precursor cells. However, the IPP and VPP peptides given in water have no effect on bone metabolism *in vivo* in animal models.
3. *L. helveticus* fermented milk increases calcium absorption in postmenopausal women acutely. However, it does not increase calcium bioavailability, measured as the calcium content of the bones in rats.

To summarise, *L. helveticus* fermented milk increases calcium absorption and supports bone mineral density. This study suggests that *L. helveticus* fermented milk may have an additional value as part of a healthy diet in achieving high peak bone mass and in preventing bone loss during the aging process.

References

1. Narva M. Effects of *Lactobacillus helveticus* fermented milk and milk-derived bioactive peptides (CPP, IPP and VPP) on calcium and bone metabolism. Doctoral Thesis supervised by Docent Riitta Korpela, PhD, Institute of Biomedicine, Pharmacology, University of Helsinki, Finland.
2. The list of other references can be obtained from Dr. Riitta Korpela.