DIETARY XYLITOL IN THE PREVENTION OF EXPERIMENTAL OSTEOPOROSIS

Beneficial effects on bone resorption, structure and biomechanics

PAULI MATTILA

Institute of Dentistry

OULU 1999

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Abstract

Dietary xylitol supplementation increases bone calcium and phosphorus concentrations in healthy rats, as well as protects against the decrease of bone minerals and bone density during experimental osteoporosis. This suggests that dietary xylitol might have a favorable effect on the prevention of osteoporosis. However, before any conclusions can be drawn about the usefulness of a compound, studies including structural evaluation and biomechanical testing of bones must first be performed.

Thus, the aim of the present study was to clarify whether dietary xylitol affects bone resorption, bone structure, and bone biomechanics in healthy rats, and whether dietary xylitol offers some preventive effects against the increased bone resorption, decreased bone trabeculation, and weakened bone biomechanical properties during experimental osteoporosis.

Dietary xylitol reduced bone resorption in 3-mo old healthy male rats, and protected significantly against the increase of bone resorption in 3-mo old ovariectomized rats, as measured by the urinary excretion of ^{3}H following [^{3}H]tetracycline-prelabeling. In addition, increased trabecular bone volume of proximal tibia in 4-mo old healthy male rats was detected after a 1-mo xylitol feeding period, and significant protection against the decrease of trabecular bone volume in 6-mo old ovariectomized rats was observed after a 3-mo xylitol feeding period. Furthermore, dietary xylitol increased the strength properties of long bones in 6-mo old healthy male rats after a 3-mo feeding period, without affecting the bone elastic properties as tested by three-point bending of tibia, torsion of femur, and loading of femoral neck. Accordingly, dietary xylitol protected significantly against the weakening of bone biomechanical properties in 6-mo old ovariectomized rats after a 3-mo feeding period.

In conclusion, the above results strongly support the hypothesis that oral administration of xylitol protects effectively against the progression of experimental osteoporosis. Dietary xylitol was effective both in increasing bone mass in healthy rats, and in preventing bone loss in ovariectomized rats, suggesting a favorable effect of xylitol on both main targets in the prevention of osteoporosis. As dietary xylitol was effective also in protecting against the experimental osteoporosis-caused changes in bone structure and weakening of bone biomechanical properties, oral xylitol administration seems to provide interesting possibilities when searching for new physiological choices for the prevention of osteoporosis.

Keywords: bone metabolism, ovariectomy, polyols, rat

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Abbreviations

ANOVA	analysis of variance
Bq	bequerel
BV/TV	bone volume/total volume
Ci	curie
EDTA	ethylene diamine tetraacetic acid
HPLC	high-performance liquid chomatography
IU	international unit (1IU of vitamin D_3 is 0.025µg)
M	molar
mo	month
N	newton
NAD	nicotinamide adenine dinucleotide (oxidized)
NADH	nicotinamide adenine dinucleotide (reduced)
NADP	nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
PLSD	protected least significant difference
S-D	Sprague-Dawley (rats)
SERM	selective estrogen receptor modulator
wk	week
w/w	weight/weight
1,25-$(OH)_2D_3$	1,25-dihydroxycholecalciferol

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Mattila P, Svanberg M & Knuuttila M (1995) Diminished bone resorption in rats after oral xylitol administration: A dose-response study. Calcif Tissue Int 56: 232-235.

II Mattila PT, Svanberg MJ, Mäkinen KK & Knuuttila MLE (1996) Dietary xylitol, sorbitol and D-mannitol but not erythritol retard bone resorption in rats. J Nutr 126: 1865-1870.

III Svanberg M, Mattila P & Knuuttila M (1997) Dietary xylitol retards the ovariectomy-induced increase of bone turnover in rats. Calcif Tissue Int 60: 462-466.

IV Mattila P, Knuuttila M, Kovanen V & Svanberg M (1999) Improved bone biomechanical properties in rats after oral xylitol administration. Calcif Tissue Int 64: (in press).

V Mattila PT, Svanberg MJ, Pökkä P & Knuuttila MLE (1998) Dietary xylitol protects against weakening of bone biomechanical properties in ovariectomized rats. J Nutr 128: 1811-1814.

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1. Introduction

Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture (Consensus development conference 1993). Typical characteristics of an osteoporotic stage are increased bone resorption in proportion to bone formation, reduced bone mineral density and decreased trabecular bone volume. Osteoporosis can be considered a major public health problem, causing over 1,3 million fractures and financial costs exceeding 10 billion dollars annually in the United States alone (Consensus development conference 1991). Osteoporosis is predicted to be an even greater problem in the future, due to the aging of the world population. Furthermore, the number of osteoporotic fractures, e.g. in Finland, is increasing more rapidly than can be accounted for by demographic changes alone (Kannus *et al*. 1995).

Since there are no effective, safe methods for restoring high quality bone to the osteoporotic skeleton, the prevention of osteoporosis is of utmost importance. Strategies for the prevention of osteoporosis consist of optimization of peak bone mass in early adulthood, and prevention of bone loss at menopause and with aging. Genetic, nutritional and life-style factors influence peak bone mass, and may be used in focusing the preventive efforts. An adequate calcium intake, good general nutrition and sufficient physical activity have been emphasized. From the current therapy modalities for the prevention of osteoporotic bone loss, most widely used are the treatments with estrogen (+progesterone), bisphosphonates and calcitonin.

On the grounds of recent studies, orally administred xylitol has some interesting properties that may be of use in the prevention of osteoporosis. Dietary xylitol supplementation in rats has been shown to increase calcium and phosphorus levels of bone (Knuuttila *et al*. 1989), to promote the restoration of bone calcium content during rehabilitation following dietary calcium deficiency (Hämäläinen *et al*. 1990), and to protect against the ovariectomy induced loss of bone mineral content during experimental osteoporosis (Svanberg & Knuuttila 1994).

However, before any conclusions can be drawn about the usefulness of a compound, studies including structural evaluation and biomechanical testing of the bones must first be performed. Thus, the present study was performed in order to clarify the effects of dietary xylitol supplementation on bone resorption, on bone trabeculation, and on bone biomechanical properties in healthy rats, and during experimental osteoporosis.

2. Review of the literature

2.1. Xylitol

Xylitol is a five-carbon polyalcohol, pentitol (Fig. 1), which is widely distributed in nature. Most fruits, berries and plants contain xylitol, the richest natural sources being plums, strawberries, raspberries, cauliflower and endives (Washüttl *et al.* 1973). Xylitol is also an intermediate of mammalian carbohydrate metabolism. In human body, 5-15 grams of xylitol is formed daily (Hollman *et al*. 1964).

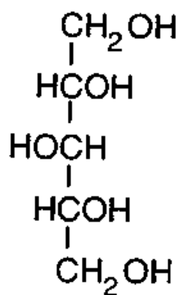


Fig. 1. Structural formula of xylitol.

Endogenous xylitol is produced in the liver from L-xylulose by an NADP-linked dehydrogenase, as a metabolite of the glucuronate-xylulose pathway (Touster *et al*. 1956). The function of this cycle is obscure, but production of glucuronic acid for synthetic processes and detoxification reactions has been assumed (Touster 1974, Sochor *et al*. 1979).

Ingested xylitol is absorbed by passive or facilitated diffusion from the intestine (Bässler 1969, Lang 1971). The absorption rate is quite slow, which means that high oral doses may induce transient osmotic diarrhea. Unadapted persons can consume 30-60

grams oral xylitol per day without side effects (Mäkinen & Scheinin 1975). A unique feature of xylitol is the adaptive enhancement of intestinal absorption by continuous xylitol administration (Lang 1971). Proposed adaption mechanisms involve induction of polyol dehydrogenase activity in the liver (Bässler 1969), and selection of intestinal microflora (Krishnan *et al.* 1980). After adaptation up to 400 grams of xylitol have been taken daily without side effects (Mäkinen & Scheinin 1975).

Xylitol is apparently excreted by simple glomerular filtration (Wyngaarden *et al.* 1957). Although there is no reabsorptive mechanism for xylitol (Lang 1969), very little is excreted in the urine, probably due to the fast diffusion from the blood to the tissues (Demetrakopoulos & Amos 1978). The net xylitol utilization in humans is over 90 % after moderate xylitol administration (Lang 1969).

Most of the exogenous xylitol is metabolized in the liver (Jakob *et al.* 1971, Wang & vanEys 1981), although other tissues like kidney, testes, adipose tissue, adrenal cortex, muscles and erythrocytes are also able to metabolize it (Lang 1969, Wang & Meng 1971). Xylitol is oxidized mainly to D-xylulose by a non-specific NAD-linked polyol dehydrogenase (Smith 1962, for review see Froesch & Jakob 1974), which then enters the pentose phosphate shunt via D-xylulose-5-phosphatase. Another possible pathway of xylitol metabolism is oxidation to L-xylulose by a specific NADP-linked polyol dehydrogenase. In both these reactions a reduced redox state is produced (The ratios NADH/NAD and NADPH/NADP increased), which has been regarded as a primary metabolic effect of xylitol (Froesch & Jakob 1974). The final metabolic products of xylitol in the liver are glucose and glycogen. (Froesch & Jakob 1974).

Xylitol is used as a sugar substitute because of its anticariogenic properties (for review see Mäkinen 1994). Xylitol can also be used in the diet of diabetic subjects, because it is slowly absorbed, its initial metabolic steps are independent of insulin, and it does not cause rapid changes in blood glucose concentration (Lang 1971, Förster 1974). Furthermore, xylitol is used as a source of energy in intravenous nutrition, because tissues can use xylitol under postoperative and posttraumatic conditions, when considerable insulin resistance prevents the effective utilization of glucose (Georgieff *et al.* 1985). Recently, xylitol-containing chewing gum has been shown to reduce the occurence of acute otitis media in day-care children (Uhari *et al.* 1996).

2.2. Xylitol in mineral metabolism

Xylitol increases calcium absorption from the gut in rats (Hämäläinen *et al.* 1985). This effect is independent of vitamin D action, and it is suggested to proceed via passive diffusion (Hämäläinen *et al.* 1985). The exact mechanism behind this is not known, but increased osmotic pressure in the intestine (Pansu *et al.* 1976), the removal of an energy barrier to calcium movement (Hauschildt *et al.* 1981), and complex formation between xylitol and calcium (Angyal 1974) have been suggested. Complexed multivalent metal cations may remain soluble at the nearly neutral pH of the gut lumen, leading to a prolonged absorption process (Hämäläinen & Mäkinen 1989a). In addition, the chelators protect the cation from forming complexes with other ligands that may inhibit absorption (Schubert 1981). To be utilized, the metal cation must, however, also be assimilated from

its complex. Thus, the chelators, which form complexes with cations with intermediate stabilities, seem to be the most suitable promoters of the absorption. This is also the case concerning comlexes with calcium and xylitol (Hämäläinen & Mäkinen 1989b). An additional explanation for the increased intestinal absorption of calcium may be better availability of calcium in the large intestine associated with decreased pH. This seems to be the case with lactitol (Ammann *et al.* 1988). However, no studies concerning xylitol in this aspect are available.

Dietary xylitol supplementation (20%) increases calcium and phosphorus levels of the bone in rats (Knuuttila *et al.* 1989). The xylitol-caused enhancement of calcium absorption is probably one of the main mechanisms behind this, but reduced intracellular redox state and changes in 1,25-dihydroxyvitamin D_3 concentration are also suggested to be involved (Svanberg & Knuuttila 1993). Bone and serum citrate concentrations elevate during xylitol administration (Knuuttila *et al.* 1989). This can be due to the xylitol metabolism-caused accumulation of NADH, enabling NADH to be used merely as an energy source in the respiratory chain. As a consequence, the rate of the citric acid cycle may retard, and lead to an accumulation of citric acid. Other suggested mechanisms of the elevated citrate levels include increased citrate synthesis, calcium-dependent inhibition of citrate decarboxylation, and increased rate of lipolysis (Knuuttila *et al.* 1989). Citrate can be considered a regulator of bone mineral metabolism, affecting mineralization, maturation and crystallization of bone inorganic fraction (Brecevic & Füredi-Milhofer 1979, Burnell *et al.* 1980, Johnsson *et al.* 1991).

Dietary xylitol addition (5%) in the rat diet during $CaCO_3$ rehabilitation promotes restoration of bone calcium content following dietary calcium deficiency as compared with $CaCO_3$ supplementation alone (Hämäläinen *et al.* 1990, Svanberg *et al.* 1993). This effect is most pronounced in the newly formed bone, indicating no major evidence of recalcification (Svanberg & Knuuttila 1993). The calcium deficiency-caused elevation in the serum 1,25-$(OH)_2D_3$ concentration fell as much as 65% below the control level after the xylitol supplementation period (Svanberg *et al.* 1993). The decrease in the 1,25-$(OH)_2D_3$ concentration may slow down the remodeling process by retarding the generation of osteoclasts (Bar-Shavit *et al.* 1983), and by retarding the maturation of organic matrix (Mechanic *et al.* 1975). The reduction in the 1,25-$(OH)_2D_3$ concentration may also be associated with the increased bone calcium content (Svanberg & Knuuttila 1994). The serum citrate level, as in the former study with normocalcemic diets, remained elevated in the xylitol-fed rats.

The supplementation of rat diet with xylitol (5%) has also been shown to protect against ovariectomy-induced decreases of bone calcium and phosphorus concentrations, of bone density, and of bone ash weight during experimental osteoporosis (Svanberg & Knuuttila 1994). While diminished absorption of calcium from the gut is suggested to be one of the major mechanisms in the pathogenesis of ovariectomy-induced osteoporosis (Nordin & Morris 1989), the enhanced calcium absorption associated with dietary xylitol administration could partly explain the preventive effect of xylitol on osteoporosis. The retarded remodeling rate associated with the decreased 1,25-$(OH)_2D_3$ concentration and the altered mineralization status associated with the increased citrate levels may also be involved.

2.3. Other sugar alcohols

There are several naturally occuring sugar alcohols, including erythritol, D-arabitol, ribitol, xylitol, sorbitol, D-mannitol, galactitol and rhamnitol (for review see Mäkinen 1994). All the sugar alcohols are acyclic polyols, containing three or more hydroxyl groups. Within the scope of the present study were, besides xylitol, a four-carbon sugar alcohol (tetritol) erythritol, and the six-carbon sugar alcohols (hexitols) sorbitol and D-mannitol (Fig. 2). Polyols occur naturally in many plants. Sorbitol is found in numerous berries and higher plants (Lohmar 1962). D-Mannitol is also widely distributed, being most frequently present in plant exudates (Lohmar 1962). Erythritol exists in fruits (Shindou *et al.* 1989) and mushrooms (Yoshida *et al.* 1986), but the amounts are extremely low. Like xylitol, sorbitol (Winegrad *et al.* 1972) and D-mannitol (Laker & Gunn 1979) are also endogenous metabolites in mammals.

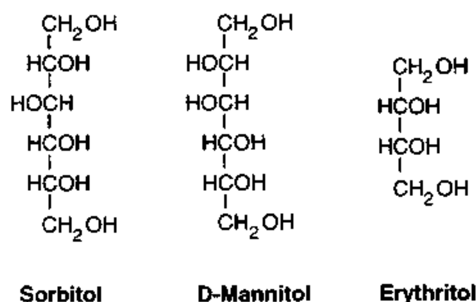


Fig. 2. Structural formulas of sorbitol, D-mannitol and erythritol.

Exogenous polyols are absorbed slowly, and metabolized to the corresponding 2-keto-sugars by the action of nonspecific NAD-dependent polyol dehydrogenase mainly in the liver (McCorkindale & Edson 1954). Unlike xylitol, sorbitol and D-mannitol, erythritol is not a precursor of liver glycogen (Mäkinen 1994). Xylitol and sorbitol are metabolized completely after moderate administration, while D-mannitol is poorly utilized due to its low affinity for L-iditol dehydrogenase, causing an increased D-mannitol concentration in the urine (Dills 1989). Exogenous erythritol is very poorly metabolized, being excreted almost completely in urine without degradation (Noda *et al.* 1994).

Polyols are used as dietary sugar substitutes. They are also used as ingredients in the diets of diabetic subjects, and in infusion therapy solutions. Comparing to xylitol and sucrose, which are approximately of equal sweetness (Moskowitz 1971), erythritol is 75-80% (Kawanabe *et al.* 1992), D-mannitol 45-57% (Moskowitz 1974) and sorbitol 35-60% (Wright 1974) as sweet at equal weight. Like xylitol, sorbitol has an energy content similar to that of sucrose. D-Mannitol, when consumed as part of a mixed diet, has a reduced energy value (Dills 1989). Erythritol is a very low-energy sweetener, the available energy value being under 10% of that of sucrose (Noda & Oku 1992).

Other polyols share some properties with xylitol, also regarding their association with calcium metabolism. Sorbitol and D-mannitol increase calcium absorption and urinary calcium excretion in rats (Vaughan & Filer 1960, Hämäläinen & Mäkinen 1986, Knuuttila *et al.* 1989). Dietary sorbitol also increases the concentration of bone calcium, although less than xylitol (Knuuttila *et al.* 1989).

2.4. Osteoporosis

Osteoporosis is a systemic skeletal disease characterized by low bone mass, microarchitectural deterioration of bone tissue leading to enhanced bone fragility, and a consequent increase in fracture risk (Consensus development conference 1991, Consensus development conference 1993). Furthermore, aspects concerning impaired bone quality (Kann *et al.* 1994, Turner *et al.* 1995) have also been emphasized (for review see Sherman *et al.* 1993, Marcus 1996). Osteoporosis is a major cause of morbidity and medical expense worldwide. Moreover, negative disease outcomes, like pain, depression, loss of self-esteem and loss of independence, must not be ignored (Gold & Drezner 1995). Osteoporosis affects 75 million people in the USA, Europe and Japan combined, including 1/3 of the postmenopausal women and a majority of the elderly (Consensus development conference 1991). Furthermore, osteoporosis is predicted to pose an even greater problem in the future, with aging of the world population (Consensus development conference 1991). In Finland, for example, the whole population incidence rate of hip fractures almost tripled between 1970 and 1991 (Kannus *et al.* 1996). Furthermore, interestingly, the age-standardized incidence rates of hip fractures almost doubled at the same time (Parkkari *et al.* 1994). The immediate costs of hip fractures in Finland in 1991 were 47,336 FIM (about 9500 USD) per patient (Kannus *et al.* 1996), and of all the surgical beds in Finland, hip fracture patients are predicted to take up 11-13% by the year 2000 (Lüthje 1991).

Osteoporosis can be considered a consequence of multiple genetic, physical, hormonal and nutritional factors (Marcus 1996). Typical symptoms of an osteoporotic stage are increased bone resorption in proportion to bone formation, reduced bone mineral density, decreased trabecular bone volume, and as a consequence, impaired mechanical properties of bone resulting in an increased risk of bone fractures. Osteoporosis affects both sexes along with aging. However, in women, estrogen deficiency following the loss of ovarian function in menopause or after surgical ovariectomy, results in the most profound alterations in the skeletal metabolism. The main determinants in the pathogenesis of osteoporosis are achieved peak bone mass and subsequent rate of bone loss (for review see Väänänen 1991). Accordingly, the strategies for osteoporosis prevention consist primarily of optimization of the peak bone mass in the early adulthood, and prevention of the bone loss at menopause and with aging (Sambrook 1995).

2.4.1. Peak bone mass

Peak bone mass is the highest level of bone mass each individual has attained as a result of normal growth (Burckhardt & Michel 1989). In humans, it is achieved during the first two to three decades of life (Bonjour *et al.* 1991). The peak bone mass has been shown to be regulated mostly by genetic factors, but nutritional, behavioral, environmental and mechanical loading factors are also of importance (for review see Chesnut 1991). Twin studies have suggested that genetic factors account for up to 80% of the bone mineral density variance in young adults (for review see Ralston 1997). However, the relative contribution of these factors to the peak bone mass in comparison to other determinants is largely unknown (Chesnut 1991). A number of possible genes has been suggested for being responsible for the low bone density, including genes influencing the metabolism of osteocalcin (Kelly *et al.* 1991) and type 1 collagen (Tokita *et al.* 1994), as well as polymorphism associated with the vitamin D receptor gene (Eisman 1995), and with the estrogen receptor gene (Kobayashi *et al.* 1996).

Of the nutritional factors involved, a sufficient calcium intake is of greatest importance. Adequate calcium nutrition is essential for the development and maintenance of a normal skeleton (Consensus development conference 1993). Furthermore, calcium supplementation to levels above habitual intake has been shown to increase peak bone mass in children and adolescents (Consensus development conference 1993). However, calcium deficiency is assumed to exist in the majority of adolescent females, most likely throughout the world (Chesnut 1991). The recommended dietary allowance for females between the ages 11 and 18 in the USA is 1200 mg daily (Commitee on Dietary Allowances, Food and Nutritional Board, National Research Council 1989). However, many recent clinical trials (Johnston *et al.* 1992, Lloyd *et al.* 1992) have shown that this recommended allowance is not high enough to sustain maximal skeletal accumulation (for review see Heaney & Matkovic 1995). In recent consensus conferences, daily allowances of calcium between 1400 to 1500 mg/d have been recommended (NIH Consensus Conference 1994, Heaney 1998). In addition to an adequate calcium intake, good general nutrition, including adequate intakes of e.g. vitamin D, vitamin B_6, vitamin B_{12} and vitamin K, is important for attaining the optimal peak bone mass. (Consensus development conference 1993).

Sufficient practise of physical exercise has been associated with increased bone density values among adolescents (Block *et al.* 1986, Recker *et al.* 1992). Furthermore, bone density values of the dominant arm or leg in different groups of adolescent athletes have been found to be increased (Nilsson & Westlin 1971, for review see Heaney & Matkovic 1995). On the other hand, excessive exercise, which leads to reduced body weight and impaired ovarian function in young women, may actually reduce bone mass (Drinkwater *et al.* 1984). Normal skeletal growth requires a normal endocrine status, including pituitary, adrenal, thyroid and gonadal functions (for review see Lam *et al.* 1988, Heaney & Matkovic 1995). Nowadays, a particular problem in many western countries is an acquired gonadal hormone deficiency found in many young women associated with eating disorders (Davies *et al.* 1990). Some life-style factors, like smoking (Välimäki *et al.* 1994) and alcohol abuse (Klein 1997) may also decrease peak bone mass (for review see Riggs & Melton 1986, Consensus development conference 1991). Systemic diseases, like

hyperthyroidism and primary hyperparathyroidism, as well as medication, e.g. excessive exposure to glucocorticoids, can also be reasons for an inadequate peak bone mass (Consensus development conference 1991).

2.4.2. Bone loss

Adult skeleton is constantly being remodeled. Normal homeostasis is a balance between bone resorption and bone formation, which is controlled mainly by hormonal and mechanical factors (for review see Rodan 1996). The purpose of remodeling is thought to be the enabling of functions such as supporting the calcium homeostasis and hematopoesis, and maintaining the load-bearing capacity of bone by preventing and repairing microscopic structural damage (Parfitt 1996). Bone remodeling is carried out by temporary anatomic structures known as basic multicellular units (Frost 1986). Remodeling (for review see Väänänen 1993) is initiated by activation of osteoclastic stem cells to differentiate into mature multinucleated osteoclasts. A resorption phase includes tight attachment of the activated osteoclasts to bone (Lakkakorpi *et al.* 1989), followed by actual bone absorption during which solubilization of hydroxyapatite crystals is done by acidification of resorption lacuna (Väänänen *et al.* 1990), followed by degradation of organic bone matrix by lysosymal hydrolases and collagenases (Blair *et al.* 1986, for review see Vaes 1988). During a reversal phase, mononuclear cells prepare the resorption lacunae for bone formation (Eriksen *et al.* 1990). Bone formation starts with the activation of preosteoblasts to differentiate into osteoblasts, which secrete bone-matrix proteins to form the organic matrix, which is later mineralized. At each remodeling site, bone resorption is coupled with bone formation, locally released growth factors and cytokines acting as mediators of this process (for review see Canalis *et al.* 1988, Mundy 1995). The decrease of bone mass is a consequence of an imbalance between the amount of mineral and matrix removed, and subsequently incorporated into each resorption cavity (for review see Kanis *et al.* 1990).

After reaching its peak, bone mass begins a gradual decline in both men and women as a natural part of the aging process (Christiansen 1992). In men and premenopausal women, bone loss is relatively slow, and primarily related to the gradual thinning of trabecular plates and cortical bone, caused by the age-related decline in the amount of matrix synthesized by the osteoblasts (Aaron *et al.* 1987, Mellish *et al.* 1989). Age-related osteoporosis has been defined as type II osteoporosis (Riggs & Melton 1983). It is characterized, in addition to the impaired bone formation, by secondary hyperparathyroidism caused by an age-related decrease in calcium absorption, and leading to increased bone turnover (Delmas *et al.* 1983, for review see Kassem *et al.* 1996). As bone formation at the cellular level is defective, increased bone turnover results in increased bone loss (Kassem *et al.* 1996). The impaired calcium absorption is probably caused by an age-related intestinal resistance to 1,25-dihydroxyvitamin D_3 (Eastell *et al.* 1991), and by a decrease in intestinal vitamin D receptor concentration with aging (Ebeling *et al.* 1992). Furthermore, aging is associated with a decline in the levels of growth hormone (Zadic *et al.* 1985, Rudman & Rao 1991) and insulin-like growth factor-1 (Quesada *et al.* 1992), which may also explain some of the variability in calcium homeostasis and bone turnover

(for review see Blumsohn & Eastell 1995). Other factors suggested to affect bone turnover during aging include nutritional deficiencies and a low level of physical activity (for review see Blumsohn & Eastell 1995).

In women, bone loss progresses much more rapidly after the menopause. This is related to estrogen deficiency-caused increase in bone turnover, bone resorption exceeding bone formation (for review see Heaney *et al.* 1978, Christiansen 1992). The loss of ovarian function leads to enhanced development of both osteoclast and osteoblast progenitors in bone marrow (for review see Manolagas & Jilka 1995), to increased activation frequency of new basic multicellular units (Eriksen *et al.* 1990), as well as to an imbalance between bone resorption and formation at each remodeling unit, with the former exceeding the latter (Eriksen *et al.* 1990, Jilka *et al.* 1992). Because of its large surface area and relatively thin network structure, cancellous bone is especially sensitive to this kind of disturbance (for review see Parfitt 1988). As a consequence, there is complete removal of some trabecular plates, and significant disruption of the trabecular lattice, the trabeculae becoming more widely separated and trabecular connectivity reduced (Dempster 1995). Consistent with biomechanical principles, this results in reduction of bone strength that is disproportionately greater than the reduction in bone mass (Melton & Riggs 1988). Estrogen regulates bone remodeling by modulating the production of cytokines and growth factors from bone marrow and bone cells (for review see Pacifici 1996a). The bone sparing effect of estrogen seems to be due to its ability to block osteoclastogenesis and the activation of mature osteoclasts and to promote apoptotic osteoclast death (Pacifici 1996a). On the other hand, estrogen deficiency-caused increase in the levels of interleukin-1 and tumor necrosis factor are proposed as causative factors underlying the accelerated bone loss (Pacifici 1996a). Postmenopausal osteoporosis has been defined as type I osteoporosis (Riggs & Melton 1983). It is characterized by decreased parathyroid hormone secretion, decreased 1,25 hydroxyvitamin D_3 production, and, as a consequence, decreased calcium absorption (for review see Kassem *et al.* 1996). Postmenopausal osteoporosis is probably not a result of the menopause only, but also of additional factors that are present in some of these women, and that exacerbate and prolong the rapid phase of the bone loss induced by the estrogen deficiency (Khosla *et al.* 1995, Kassem *et al.* 1996). Such factors may include defects of osteoblasts, impairing their ability to increase bone formation in order to compensate for the increased bone resorption (Cohen-Solal *et al.* 1991), and genetic predisposiotion for a pattern of cytokine secretion (Pacifici *et al.* 1991, Jilka *et al.* 1992, Kassem *et al.* 1996).

2.4.3. Prevention and treatment of bone loss

Sufficient intake of dietary calcium (1500 mg/day), along with sufficient supply of vitamin D to optimize calcium absorption, are among the most important factors in the prevention of osteoporosis at the population level (for review see Johnston 1996, Masi & Bilezikian 1997). Malabsorption of calcium is a common disorder in osteoporotic subjects, and thus treatment with calcium supplements alone is often ineffective (Horowitz *et al.* 1987). Malabsorption can be corrected to some extent by the addition of 1,25 dihydroxyvitamin D_3 to the calcium regimen (Need *et al.* 1985). On the other hand, reduced

calcium absorption efficiency has also been linked with a heritable polymorphism at the vitamin D receptor genotype, being conductive especially during low calcium intake levels (Dawson-Hughes *et al.* 1995). Other helpful modes of preventive action at the population level include maintaining an adequate rate of weight-bearing physical activity (Dalsky *et al.* 1988, Stevenson *et al.* 1989, for review see Stevenson *et al.* 1990), avoiding smoking and abuse of alcohol (Riggs & Khosla 1995), as well as paying attention to such efforts that minimize the likelihood of an individual to fall, e.g. concerning balance, eyesight and environmental factors (Masi & Bilezikian 1997).

Pharmacological intervention to decrease postmenopausal and age-related bone loss should be undertaken in persons who, because of low bone density, are deemed to be at increased risk for osteoporosis (Riggs & Khosla 1995). Hormone replacement therapy has been the most widely used form of therapy for the prevention of postmenopausal osteoporosis since the early 1990s (Eriksen *et al.* 1996), with current consensus recommendations indicating that this is the treatment of choice (Rozenbaum & Birkhäuser 1996). Estrogen intervention in postmenopausal women reduces accelerated bone remodeling and subsequent bone loss (for review see Lindsay 1995, Christiansen 1996). This is mainly related to the ability of estrogen to retard bone resorption (Riggs & Melton 1986, Pacifici 1996b), although stimulation of bone formation is likely to play a contributory role (Chow *et al.* 1992, Bain *et al.* 1993). The effects of estrogen therapy continue for as long as estrogens are given, but discontinuation of the therapy results in a bone loss rate comparable to that in the early postmenopausal years (Lindsay *et al.* 1978). On the other hand, increased age is not a contraindication to this therapy (Quigley *et al.* 1987, for review see Lindsay 1995). However, in older women, side effects, such as breast tenderness and recurrence of menstrual bleeding may be poorly tolerated (Prestwood *et al.* 1995), and in fact, no more than one third of women accept long-term hormone replacement therapy even in those countries where it is most widely used (Consensus development conference 1993). Estrogen therapy accelerates intestinal absorption of calcium either directly (Heaney *et al.* 1978), or indirectly by increasing the supply of 1,25-dihydroxyvitamin D_3 (Cosman *et al.* 1990). Progestins are usually given along with estrogen for endometrial protection in nonhysterectomized women, because of the estrogen-related increased risk of endometrial hyperplasia and cancer (Whitehead *et al.* 1981, for review see Agarwal & Judd 1995). Long term use of estrogens is also associated with an increased risk of developing breast cancer in postmenopausal women (for review see Bergkvist & Persson 1996). The risk is assumed to increase by 30% after 10 years' use, and by 50% after 20 years' use (Brinton *et al.* 1986). On the other hand, estrogen therapy has been associated with protective effects against cardiovascular diseases (for review see Bergkvist & Persson 1996). This effect stems mainly from favorable alterations in plasma lipid concentrations, and from direct effects on arteries (Limacher 1998).

In order to utilize the beneficial effects of estrogen on bone metabolism, but to avoid the undesirable side effects, selective estrogen receptor modulators (SERMs) have been the focus of many recent studies. The most promising of these preparates so far is raloxifene. It seems to have estrogen agonist effects on bone and cholesterol metabolism, and estrogen antagonist effects on uterine and mammary tissue (Delmas *et al.* 1997). The antagonist activity is probably mediated via classical pharmacological competition for estrogen receptor binding, and the agonist activity appears to involve novel post-receptor

pathways and non-classical estrogen response elements which are activated by SERMs (Bryant & Dere 1998). However, these findings are only preliminary, and large clinical trials are currently in progress.

Intranasal administration of salmon calcitonin has also been shown to inhibit osteoclastic bone resorption. It is used to some extent by postmenopausal women unable or unwilling to tolerate long-term hormone replacement therapy. A problem in its use, in addition to its expensiveness, is an occurance of resistance, associated with formation of neutralizing antibodies (Muff *et al.* 1991), downregulation at receptor sites (Gruber *et al.* 1984), or counterregulatory mechanisms (Singer *et al.* 1980). However, it has only slight side effects, like transient facial flushing, nausea, rare vomiting and diarrhea, which should not necessiate drug discontinuation. Thus, calcitonin is considered perhaps the safest of all currently available pharmacological therapies for osteoporosis (for review see Chesnut 1995). Calcium supplements are often given along with calcitonin to prevent induction of secondary hyperparathyroidism (Riggs & Khosla 1995).

Bisphosphonates are used increasingly to treat bone diseases characterized by increased bone resorption. They attenuate bone turnover by suppressing the activity of osteoclasts. The most common adverse events include abdominal pain, nausea, dyspepsia, constipation and diarrhea. More alarming have been the reports of some patients developing esophageal ulceration, although as a result of unrecommended administration of the second-generation bisphosphonates (for review see Jeal *et al.* 1997). Long-term high-dose treatment with a first-generation bisphosphonate, etidronate, has been associated with defects in mineralization (Canfield *et al.* 1977). A hypothetical concern regarding the long-term use of agents such as bisphosphonates that suppress bone remodelation, is a decreased ability of bone to respond to microfractures, which might then lead to progression of macrofractures (Chesnut 1995). However, no increase in fracture rates has been detected following bisphosphonate therapy so far (Harris *et al.* 1993).

Fluoride has the potential to increase skeletal mass. However, it has been shown to affect the crystalline structure of bone, and thus to increase its fragility (Hedlund & Gallagher 1989, for review see Riggs & Khosla 1995). This has been explained by toxic actions of fluoride ion on skeletal mineralization, impairment of the normal processes of bone resorption, and fluoride-induced decrease in strength per unit of bone (for review see Kleerekoper 1996). Other problems concerning the use of fluoride include gastrointestinal effects and a peripherial bone pain (Consensus development conference 1993). The problems in the use of fluoride stems also partly from its narrow therapeutical window, and with strict dosing, also favorable effects on fracture rates have been achieved (Reginster *et al.* 1998). Insulin-like growth factor and transforming growth factor are also substances that increase bone formation, but their possible usefulness in the therapy of osteoporosis is still under clinical investigation (Boonen *et al.* 1997).

As seen from the above, the drugs that are used for the prevention of osteoporosis are obviously effective against the progression of the disease. On the other hand, their use is not unproblematic, because of the many side effects they have expressed. Thus, developing more physiological preparations for the prevention of osteoporosis is still a challenging task.

2.5. Experimental osteoporosis

The Food and Drug Administration of the United States has established guidelines for using animals in preclinical testing of agents intended for the prevention or treatment of human postmenopausal osteoporosis (Guidelines for preclinical and clinical evaluation of agents used in the prevention or treatment of postmenopausal osteoporosis 1994). It recommends the use of ovariectomized rats and larger animals, because of the relationship of human osteoporosis to estrogen depletion. Studies must include histologic evaluation of the bones. Furthermore, in addition to determinations of bone density and biochemical markers of bone turnover, the use of biomechanical testing is suggested to evaluate the propensity to develop fragility fractures.

The ovariectomized rat is considered an appropriate model for studying human menopausal osteoporosis because of many similarities in their pathophysiological mechanisms (Kalu 1991, Wronski & Yen 1991, Frost & Jee 1992). In both species, bone loss is most rapid after the onset of estrogen deficiency, and it is characterized by an increased bone turnover, resorption exceeding formation (Gallagher *et al.* 1972, Delmas *et al.* 1983, Wronski *et al.* 1988a, for review see Kalu 1991). Furthermore, in both species, bone loss is greater in the trabecular than in the cortical bone (Yamazaki & Yamaguchi 1989), intestinal absorption of calcium is decreased (Arjmandi *et al.* 1994), and the skeletal responses to current prevention and treatment modalities are similar (Wronski *et al.* 1989b). These regimens include exercise (Donahue *et al.* 1988, Tuukkanen *et al.* 1991, Peng *et al.* 1994a, for review see Tuukkanen 1993), estrogen replacement therapy (Aitken *et al.* 1972, Wronski *et al.* 1988b, Kalu *et al.* 1991a), as well as treatment with bisphosphanates (Seedor *et al.* 1991, Wronski *et al.* 1991a) and calcitonin (Hayashi *et al.* 1989, Mazzuoli *et al.* 1990).

Two models of experiments are widely used with the ovariectomized rats. The aged rat model uses animals 6-24 months of age, and the mature rat model uses animals about 3 months of age (for review see Kalu 1991). Although the aged rat model has many of the characteristics to look for in an animal model of postmenopausal osteoporosis, mature rats are used more often, because they, in comparison to the aged rats, are cheaper, easily available, and the effects of ovariectomy on their skeleton are manifested within a reasonable time. Furthermore, the characteristics of bone loss are mostly similar to those of the aged rat model (Kalu 1991).

In ovariectomized rats, the proportion of cancellous bone decreases significantly, the trabecular bone volume in the secondary spongiosa of the long bones being about 10-15 %, as compared with 20-30% of the sham-operated controls (Wronski *et al.* 1993, Tsurukami *et al.* 1994, Yamaura *et al.* 1994). However, this decrease is greatly inhibited by therapeutic interventions with estrogen (Kalu *et al.* 1991b, Evans *et al.* 1994), calcitonin (Wronski *et al.* 1991b) and bisphosphonates (Wronski *et al.* 1993, Lepola *et al.* 1995). Partial prevention is also achieved by moderate exercise (Tuukkanen 1993).

Bone resorption has been shown to increase about 40% within a week following ovariectomy, as monitored by measuring the urinary excretion of [^{3}H] of previously [^{3}H]-tetracycline prelabeled rats (Cecchini *et al.* 1997). The increase of bone resorption is also significantly inhibited by treatment with estrogen (Turner *et al.* 1993, Goulding *et al.* 1996, Cecchini *et al.* 1997), calcitonin (Mühlbauer & Fleisch 1995), and bisphosphonates (Mühlbauer & Fleisch 1995, Frolik *et al.* 1996).

After ovariectomy, decreased bone biomechanical properties have been detected in rats. Peng *et al.* (1994b), using the mature rat model, found a decrease of maximum load in the bending of tibial shaft (8,7%) and in the loading of femoral neck (15,8%) in ovariectomized rats. The values for stiffness did not differ among the groups. Accordingly, using the same model, Sœgaard *et al.* (1994) revealed a significant decrease in the maximum load of femoral neck in the ovariectomized rats. No differences in the values of deformation at maximum load or of energy absorption capacity were found. In the study of Lepola *et al.* (1995) ovariectomy reduced the maximum load values in compression of the femoral neck by 12%. No significant effects by ovariectomy were observed concerning deformation or rigidity. Bagi *et al.* (1997) found reduced values for strength and stiffness of femoral necks obtained from ovariectomized rats as compared with those from the sham-operated controls. Lauritzen *et al.* (1993), using 6-month-old rats, showed a significant reduction of femoral midshaft ultimate load and stiffness, as well as of femoral neck stiffness in ovariectomized rats relative to non-ovariectomized controls. Furthermore, ovariectomy induced a 16,6% loss of the maximum torque capacity of tibia in the study of Peng *et al.* (1994a).

On the other hand, there are also findings indicating no significant ovariectomy-induced changes in the biomechanical properties of long bones (Toolan *et al.* 1992, Bagi *et al.* 1993). Sato *et al.* (1997) found no differences in the strength of the femoral neck between baseline, sham-operated and ovariectomized rats when 9-month-old rats were used, and suggested limited utility of this measurement in aged rats. In fact, even findings showing improved biomechanical properties after ovariectomy have been published. Aerssens *et al.* (1993), using the mature rat model, found a significant increase in torsional strength, in deformation energy to fracture, and in angular deformation of the torsional tested femurs. In the study of Lepola *et al.* (1995) the maximum load in three-point bending of femur was elevated by 7% after ovariectomy compared with sham-operated rats. Also the values for rigidity and deformation were increased. Accordingly, using 4-month-old rats, Bagi *et al.* (1995) found increased ultimate torque values in the ovariectomized rats relative to the shams.

Altered bone cross-sectional geometry, usually seen as widening of the medullary cavity of long bones (Turner *et al.* 1989), affects the cross-sectional moment of inertia, and may thus affect the bone biomechanical properties (Hayes & Gerhart 1985). The contradictory findings regarding bone biomechanical properties may, in addition to variations in the age and strain of the rats, and in the time after operation, be a consequence of these geometrical properties. For example, in all above studies showing improved biomechanical properties, the moment of inertia was increased, obviously due to geometrical adaptation of the bone to altered conditions (Aerssens *et al.* 1993, Bagi *et al.* 1995, Lepola *et al.* 1995). The ovariectomy-induced decline in bone mechanical strength seems to be more evident in the femoral neck than in the long bones of the rats. This may be explained by the fact that mechanical strength of the femoral neck is considerably determined by trabecular bone (Martens *et al.* 1983), which is more susceptible to estrogen deficiency than cortical bone (Turner *et al.* 1987). However, the cancellous bone of the femoral neck in rats may be less important for strength properties than in humans, because the femoral neck of rats contains much more cortical bone than that of humans (Bagi *et al.* 1997).

Ovariectomy-induced reduction of bone biomechanical strength has been shown to be suppressed by treatment with estrogen (Shen *et al*. 1995) and bisphosphonates (Katsumata *et al*. 1995, Kaastad *et al*. 1997).

3. Aims of the study

Dietary xylitol supplementation has been shown to increase calcium and phosphorus levels of the bone in rats, as well as to promote restoration of bone calcium content following dietary calcium deficiency. Furthermore, it has been shown to protect against ovariectomy-induced decreases of bone calcium and phosphorus concentrations, of bone density, and of bone ash weight during experimental osteoporosis. These findings support the hypothesis that dietary xylitol might have a favorable effect on the prevention of osteoporosis.

However, before any conclusions can be drawn about the usefulness of a compound assumed for the prevention of osteoporosis, studies including structural evaluation and biomechanical testing of the bones must first be performed.

Thus, to evaluate further the possibility of preventing progression of osteoporosis by means of dietary xylitol supplementation, the aims of the present study were:

1. To evaluate the effects of dietary xylitol supplementation on bone resorption in healthy rats, including determinations of time- and dose-response.
2. To compare the effects of different dietary polyol supplementations on bone resorption.
3. To evaluate whether dietary xylitol supplementation offers a preventive effect against ovariectomy-induced increase of bone resorption during experimental osteoporosis.
4. To evaluate the effects of dietary xylitol supplementation on bone structure in healthy rats.
5. To evaluate whether dietary xylitol supplementation offers a preventive effect against ovariectomy-induced changes of bone structure during experimental osteoporosis.
6. To evaluate the effects of dietary xylitol supplementation on bone biomechanical properties in healthy rats.
7. To evaluate whether dietary xylitol supplementation offers a preventive effect against ovariectomy-induced weakening of bone biomechanical properties during experimental osteoporosis.

4. Materials and methods

4.1. Animals

Outbred Wistar (Bkl) rats were used in studies I, IV and V, and outbred Sprague-Dawley (Mol) rats in studies II and III, according to availability from the supplier. Male rats were used in studies I, II and IV, and female rats in studies III and V, which included the use of ovariectomy. The rats were provided by the Laboratory Animal Center, University of Oulu, Finland. The main background information about the animals, and about the experimental design in different studies is presented in table 1.

Table 1. Main background data of the animals and of the experimental design.

	Study I	Study II	Study III	Study IV	Study V
Rats					
strain	Wistar	S-D*	S-D	Wistar	Wistar
sex	male	male	female	male	female
baseline age	9 wk	9 wk	10wk	12wk	12wk
final age	13 wk	13 wk	22wk	24wk	24wk
basal diet	R3**	RM1***	RM1	R3	RM1
Design					
xylitol supplementation	5, 10, 20%	1M****	10%	5, 10, 20%	10%
ovariectomy			x		x
[^{3}H]-tetracycline labeling	x	x	x		
trabecular bone volume			x	x	x
bone biomechanical tests				x	x

*S-D is Sprague-Dawley rat

**R3 by Lactamin (Labfor, Stockholm, Sweden)

***RM1 by Special Diet Services (Witham, Essex, UK)

****also 1M sorbitol, 1M D-mannitol and 1M erythritol were included in the study design

The animals were housed in cages (Makrolon III; Tecniplast, Buguggiate, Italy), two or three to a cage, on a bed of European aspen shavings in a temperature- and light-controlled room (21-23°C, 12-hour light-dark cycle). They were weighed weekly, and their food intake was measured. At the end of the experimental period the rats were killed using carbon dioxide, followed by decapitation.

The study protocols were approved by the Ethical Committee on Animal Experiments of the University of Oulu.

4.2. Experimental design

4.2.1. Dietary regimen

4.2.1.1. Basal diet

The animals were fed a basal powder diet, Lactamin R3 (Labfor, Stockholm, Sweden) in the studies I and IV, and RM1 (Special Diet Services, Witham, Essex, UK) in the studies II, III and V, according to the diet in use at the Laboratory Animal Center of the University of Oulu.

The Lactamin R3 diet consists of 28% barley meal, 20% wheat meal, 20% wheat germs, 10% wheat middlings, 7% soya meal, 7% fish meal, 3% fodder yeast, 3% minerals, 1% vitamins and trace elements, and 1% fat. The diet contains 1,1% calcium, 0,8% phosphorus, and 1500 IU/kg Vitamin D_3.

The RM1 diet consists of 88,5% cereal products (wheat, barley and wheatfeed), 6% vegetable proteins, 2,5% animal proteins (whey powder), 0,5% soybean oil, and 2,5% vitamins, minerals and amino acids. The diet contains 0,71% calcium, 0,5% phosphorus, and 600 IU/kg vitamin D_3.

The rats in all studies had free access to tap water ad libitum.

4.2.1.2. Polyol supplementation

To study the effects of orally administered xylitol, the diet of rats in studies I, III, IV and V was supplemented with xylitol (Xyrofin Co., Kotka, Finland). Xylitol concentrations in different groups were 5, 10 and 20% by weight (w/w). This corresponds to 50 g, 100 g and 200 g xylitol per 1 kg of the diet.

To compare the effects of different polyols, rats in study II were given a diet supplemented with either xylitol, D-glucitol (generally called sorbitol) (Serestar, Kreseld, Germany), D-mannitol (Sigma Chemical Co., St.Louis, MO) or meso-erythritol (generally called erythritol) (Fluka Chemie AG, Buchs, Switzerland). The polyol concentration in the diet was 1 mol/kg, corresponding to 152 g xylitol, 182 g sorbitol or D-mannitol and 122 g erythritol per 1 kg of the diet.

4.2.2. Ovariectomy

To study the effects of dietary xylitol during experimental osteoporosis (studies III and V), three-month old female rats were bilaterally ovariectomized by the dorsal approach (Waynforth 1980). A single longitudinal skin incision was made on the dorsal midline at the level of the kidneys. The ovaries were exposed, and after ligating removed together with their surrounding fat, oviduct and a small portion of the uterus. Animals of the control group underwent sham operations, during which the ovary was exposed but left intact. The surgery was done under anesthesia, using a 1:1:2 mixture of Hypnorm (Janssen Pharmaceutica, Beerse, Belgium), Dormicum (F. Hoffmann-La Roche AG, Basel, Switzerland), and water, 0.2-0.4 ml/100 g body weight, intraperitoneally. At the end of the experimental period, the success of ovariectomy was confirmed by verifying the absence of any ovarian tissue, and noting the marked atrophy of uterine horns.

4.3. Analyses

4.3.1. Bone resorption rate

Four-week old rats were injected subcutaneously with 1 ml of a solution containing 5µCi/ml (185 GBq/L) of [7-^{3}H(N)]-tetracycline (NEN Life Science Products, Boston, MA) dissolved in distilled water. The injections were repeated weekly for 5 weeks. One week after the last [^{3}H]tetracycline injection, the rats were housed in individual metabolic cages for a 24-hour baseline urine collection. After that, urine was collected twice a week for 31 days, and in study III, additionally once a month for two months. The urine collection period was always 24 hours, so as to avoid possible confusion caused by the diurnal rhythm (Mühlbauer and Fleisch 1990).

The volume of urine excreted was measured, and the amount of ^{3}H radioactivity present in a 1-ml aliquot was determined with a 1215 Rackbeta II scintillation counter (Wallac Co., Turku, Finland), using Hydrofluor (National Diagnostics, Manville, NJ) as the liquid scintillation counting solution. The total excretion of ^{3}H was calculated as an indicator of the amount of resorbed bone mineral as described by Klein and Jackman (1976).

After the urine collection period, the rats were killed, and their tibiae and scapulae were prepared for the determination of the ^{3}H content of bone. The total ^{3}H radioactivity per whole bone was determined using a modification of the method described by Klein and Jackman (1976). The tibial epiphyses and the bone marrow were carefully removed. After drying at 60 °C for 24 hours, the bones were weighed and pulverized with a micromill Mixer Type III 695 (Retsch, Haan, Germany). Thereafter, 25 mg of the pulverized bone was suspended in a sealed tube in a 3:1 mixture of concentrated HCl (pro analyse, Riedel-de Haen, Seelze, Germany) and concentrated HNO_3 (pro analyse, Merck, Darmstadt, Germany). The ^{3}H radioactivity of the resulting suspension was measured as described above. The total amount of ^{3}H left in the tibiae and the scapulae was calculated.

4.3.2. Analyses of bone inorganic fraction

Whole bone wet weight (with the marrow) of tibia was determined with a weighing machine (Mettler A30; Mettler Instrumente, Zürich, Switzerland), followed by a pycnometric measurement of bone density. Humerus was used for the other analyses. After epiphyses and bone marrow of the humerus had been carefully removed, and samples dried at 60°C for 24 h, the bone sample was pulverized with a micromill Mixer Type III 695 (Retsch, Haan, Germany). Bone calcium was determined by atomic absorption spectrophotometry (Perkin Elmer model 2380, Perkin Elmer Corporation, Norwalk, CT), using samples dissolved in a solution of HCl and HNO_3 (2:1 conc.). Bone phosphorus was analyzed as described by Fiske & Subbarow (1925). Bone ash weight was determined by ashing 25 mg of the pulverized bone at 900°C for 24 h.

4.3.3. Trabecular bone volume

The proximal tibiae were cut sagittally into two equal halves with a diamond saw, dehydrated with ethanol (40%), and embedded in methylmethacrylate as described by Baron *et al*. (1983). Undecalcified sections of 5µm were cut with a Polycut S heavy duty microtome (Reichert-Jung, Leica Instruments GmbH, Nussloch, Germany), and stained according to the von Kossa method (Dickson 1984). Sections were taken near the sagittal midline of the tibia at five levels, 50 µm apart, and one microscopic field per section was evaluated. Trabecular bone volume was measured in an area of 5 $mm^{2,}$ at 4x objective magnification, using a computer image analyzer (MCID, Model M1, Imaging Research Inc., Brock University, Ontario, Canada). The area situated within 1 mm from the upper surface of growth plates, as well as all trabeculae in contact with the cortices, were excluded from the measurements.

4.3.4. Analyses of bone organic fraction

Tibia was prepared for the determination of bone collagenous structures in study IV, and humerus in study III. The bones were decalcified at 4 °C with several changes of 0.5 mol/L EDTA, pH 7.2 for 5 weeks. After washing with 0.9 % NaCl, the bones were lyophilized, and pulverized with the micromill. The bone samples were weighed, freeze-dried, and hydrolyzed in 6 mol/L HCl at 110 °C for 24 hours in sealed tubes. A part of the hydrolyzate was subjected to partition chromatography for the analysis of pyridinium crosslinks according to the method of Black *et al*. (1988). Reversed phase high-performance liquid chromatography (HPLC) was carried out by the method of Eyre *et al.* (1984), with the modifications of Palokangas *et al*. (1992). Hydroxyproline was analyzed by HPLC, following derivatization by means of the *o*-phthalaldehyde/9-flurenylmethoxycarbonyl chloride system, originally described by Teerlink *et al*. (1989), and modified by Palokangas *et al*. (1992). The HPLC analyses were performed using the Merck Hitachi chromatograph, which included a pump, model 655 A-12 Liquid Chromatograph, the L-

5000 LC Controller, the AS-4000 Intelligent Auto Sampler, the F-1000 Fluorescence Spectrophotometer and the D-2000 Chromato Integrator. The Ultrashere ODS column (C18, 5 μ, 250 x 4.6 mm) from Beckman, and the sample loops of 50 μl and 100 μl, were used for the analysis of hydroxyproline and pyridinium crosslinks. The flow rate used was 1 ml/min.

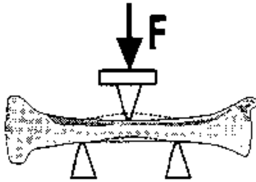
4.3.5. Bone biomechanical properties

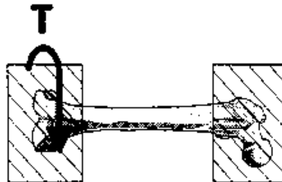
After preparation, left tibia and both femurs were wrapped in gauzes saturated with physiological saline and stored at -20°C until used. The storage at -20°C has been shown not to affect bone biomechanical properties (Peng *et al.* 1994b). Before testing, the bones were thawed at room temperature, and kept moist until the test was completed. To avoid confusion of earlier loadings, all mechanical tests were performed with different bones. The left femur was used for the torsion test, the right femur for the loading test of femoral neck, and the tibia for the three-point bending test.

The three-point bending test, and the loading test of femoral neck were performed using a material testing machine constructed by Timo Jämsä, PhD, at the Technical Services Department of the Medical Faculty, University of Oulu (Jämsä 1998). The testing machine is based on the lever arm principle, as described by Peng *et al.* (1994b). The interchangeable compression head, mounted on the pressing rod for different tests, transmits compressive force to the specimen moving at a constant speed of 0.155 mm/sec. The compressive force is measured by a sensor attached to the stationary part of the compression stage. A load-deformation curve was recorded during the tests by a plotter (Perkin-Elmer 165, Hitachi Ltd., Japan). Prior to testing, the machine was calibrated using a standard weight.

In the three-point bending test (Fig. 3), performed as described by Peng *et al.* (1994b), a supporter with two loading points, 13mm apart from each other, was used on the stage of the testing machine. Lateral surface of the tibia at tibiofibular junction was placed upon the first point, and proximal tibia upon the other. A press head compressed the middle of the tibial shaft until fracture occurred. The press head was rounded to avoid cutting into the bone when loaded.

Stress, strain and Young's modulus were derived from load-deformation curves obtained, by using equations described by Turner and Burr (1993). Stress, which is defined as force per unit area, was calculated as FLc/4I, where F is applied force (from the load-deformation curve), L is the distance between the loading points, c is the distance between the bone surface and the bone cross-sectional center, and I is the bone cross-sectional moment of inertia. Roundness of tibia cross-sections was within 0.92-0.94 and cross-sectional moment of inertia was determined by the equation of round specimens. Strain, which is defined as percentage change in length, or relative deformation, was calculated as $12cd/L^2$, where d is displacement (from the load-deformation curve). Young's modulus, which is defined as the slope of the stress-strain curve within the elastic region, and measures the intrinsic stiffness of the material, was calculated as $F/d \times L^3/48I$.





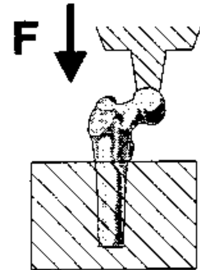


Fig. 3. Schematic presentation of the mechanical tests of the bones. F is the applied force, T is the applied torque, and the arrows represent the direction of the loading.

In the loading test of femoral neck (Fig. 3), performed as described by Tuukkanen *et al.* (1994), the head of the femur was loaded with a force parallel to the shaft of the femur until failure. A thick polymethyl methacrylate plate with several holes of different sizes, and grooves for the third trochanter of the femur, was used as a supporter for the bone. The femur was cut between the middle and lower third of the shaft, and the proximal part was inserted perpendicularly and tightly into a suitable hole until the lesser trochanter of the bone touched the surface of the plate. The concave compressing head used was 2.5 mm in diameter. Area of the femoral neck was measured at the point of fracture and the ratio load/area was calculated.

The torsion test of femur was performed using a method reported by Lepola *et al.* (1993), with a machine constructed by Timo Jämsä, Ph.D., at the Technical Services Department of the Medical Faculty, University of Oulu (Jämsä 1998). One of the heads of the machine rotates at an angular velocity of 6° per second, and the other head is stationary. The sensors for measuring the torsional load are attached to the stationary head. A load-deformation curve was recorded during the tests by a paper recorder (Goerz RE 511, Austria). Prior to testing, the machine was calibrated against a given torque of 0.5 Nm.

Each end of the femur was placed concentrically in a cavity of a nut (M8, Kanthal AB, Hallstahammar, Sweden) by a standardized method, and fixed with dental stone (Fujirock, G-C Dental Industrial Co., Tokyo, Japan). The femur with the nuts was inserted into two head sleeves of the torsion machine, and torsion was performed by twisting the bone inward.

Shear stress and shear modulus of elasticity were derived from load-deformation curves obtained by using equations described by Turner and Burr (1993). Shear stress was calculated as Tr/J, where T is applied torque (from the load-deformation curve), r is the radius of the bone cross-section, and J is the polar moment of inertia of the bone cross-section, which was determined by using the equation of round specimens. Shear modulus

of elasticity was calculated as $T/\theta \times L/K$, where T/θ is the slope of the load-deformation curve, L is the length of the unembedded portion of the bone, and K is the torsional constant, which is equal to the polar moment of inertia for circular cross-sections.

Length of the bones was measured with calipers. Cross-sectional views of the bones at the point of fracture were photographed under a microscope. The point of fracture in the three-point bending test was standardized by always placing the bone similarly in the testing machine. In the loading test of femoral neck and in the torsion test, however, the cross-section had to be measured using a standardized position of the opposite bone. This was done by cutting the opposite femur and femoral neck perpendicularly at their narrowest position. The cross-sectional areas and diameters of the bones were measured from the micrographs using Image Measure Computer Program (Microscience, Washington DC).

4.4. Statistical analyses

Information from a series of urinary ^{3}H measurements on each rat (in studies I, II and III) was summarized as the area under the curve as described by Altman (1990).

Statistical significances of the differences between the groups concerning all measured variables in studies I, II, IV and V were calculated using the analysis of variance, and further comparison was made using Fisher´s Protected Least Significant Difference (Armitage & Berry 1994).

In study III, statistical significances of the differences between the groups were calculated using unpaired t-test.

The statistical computer program used was StatView II for Macintosh (Abacus Concepts, Inc., Berkeley, CA).

5. Results

5.1. General findings

A slight tendency toward diminished weight gain was detected with increasing dietary xylitol concentration (studies I and IV). However, the differences in weight gains between xylitol-fed rats and controls were not particularly great, being significant ($p<0.05$) only when using the highest xylitol concentration (20%) in the study with the longest duration (3 months).

The weight gain of the rats that were fed the diet supplemented with 1M sorbitol ($p<0.01$) or 1M erythritol ($p<0.01$) for one month, was significantly smaller than that of the controls (study II).

Ovariectomized rats exhibited an increased weight gain as compared to the sham-operated controls ($p<0.01$) (studies III and V). Weight gain of the ovariectomized rats fed a diet supplemented with 10% xylitol was smaller than that of the ovariectomized rats without dietary xylitol supplementation. This difference was significant in study V ($p<0.05$), but not in study III.

The average food intake of the rats in different dietary xylitol supplementation groups did not differ from that of the controls. However, the food intake was greater in the 1M erythritol group as compared to the other groups ($p<0.05$), except for the 1M sorbitol group. The average food intake of the ovariectomized rats exceeded the food intake of both the sham-operated controls and the ovariectomized rats fed a diet with 10% xylitol supplementation ($p<0.05$).

Slight diarrhea was detected during the first week in the rats that had been fed the diet supplemented with 10% or 20% xylitol. Accordingly, slight diarrhea was detected during the first week in all 1M polyol supplementation groups. However, as distinct from the other groups, in the sorbitol group, the diarrhetic effect was maintained throughout the experimental period of one month.

Dietary administrations of 1M D-Mannitol ($p<0.01$) and 1M erythritol ($p<0.01$) caused diuresis in the rats. Dietary 1M xylitol and 1M sorbitol, however, did not affect the excretion of urine as compared to the rats fed the basal diet alone. Consequently, the mean water intake was greatest in the D-mannitol ($p<0.01$) and erythritol groups ($p<0.01$).

5.2. The effects of polyols on bone resorption

The urinary ^{3}H excretion of the [^{3}H]tetracycline-prelabeled rats, reflecting the rate of bone resorption, was significantly reduced in the 10 % and 20 % dietary xylitol supplementation groups as compared to the controls (study I). This was detected already in the first measurement, made two days after the onset of xylitol feeding, and the diminished level was maintained throughout the experimental period of one month (Fig. 4). However, no significant effect was seen in the 5% xylitol supplementation group. The retardation of bone resorption was about 25% in the 10% xylitol group ($p=0.0138$), and about 40% in the 20% xylitol group ($p<0.0001$). Accordingly, there was significantly more ^{3}H radioactivity left in the tibiae of the 10% ($p<0.05$) and 20% ($p<0.01$) xylitol supplementation groups as compared to the controls at the end of the experimental period.

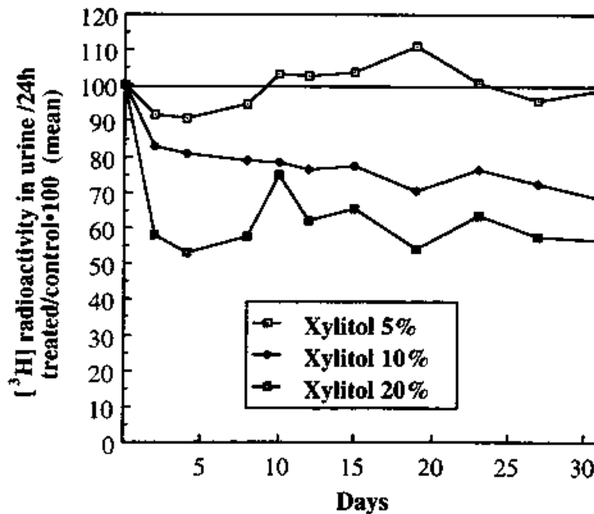


Fig. 4. Urinary ^{3}H excretion in different xylitol supplementation groups compared with the control group. All values are means, n=10 in each group. The mean value of the control group is set to 100 in each time point (Study I, Fig. 2.).

When comparing the effects of different polyols (study II), dietary supplementation of 1M xylitol ($p<0.0001$), 1M sorbitol ($p<0.0001$), and to a less degree 1M D-mannitol ($p<0.05$) reduced the amount of excreted ^{3}H as compared to the basal diet alone (Fig. 5). However, the ^{3}H excretion was not affected by dietary erythritol. Preserved ^{3}H radioactivity in the tibiae and scapulae was significantly greater after the experiment only in the xylitol group ($p<0.05$) as compared to the controls.

Ovariectomy doubled the excretion of ^{3}H as compared to the sham-operated rats ($p<0.0001$) (study III). However, the ovariectomized rats that were fed a diet supplemented with 10% xylitol expressed a significantly reduced ^{3}H excretion rate as compared

to the ovariectomized rats without xylitol ($p<0.0001$) (Fig. 6). Accordingly, there was significantly more preserved ^3H radioactivity in the tibiae and scapulae of these xylitol-fed rats at the end of the experimental period ($p<0.0001$). In fact, no significant difference was detected between the xylitol-fed ovariectomized rats and the sham-operated controls.

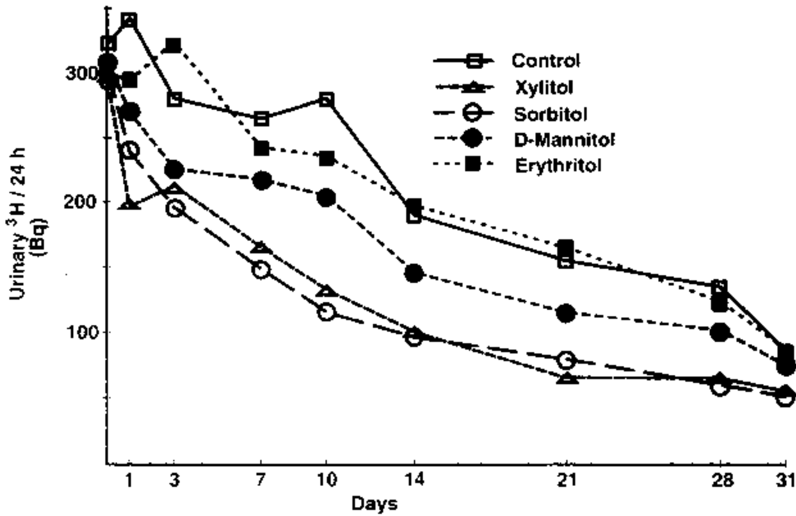


Fig. 5. Urinary ^3H excretion of the rats fed different polyols (1 mol/kg dry diet) for 1 mo. Values are means, n=10 in each group (Study II, Fig. 2.).

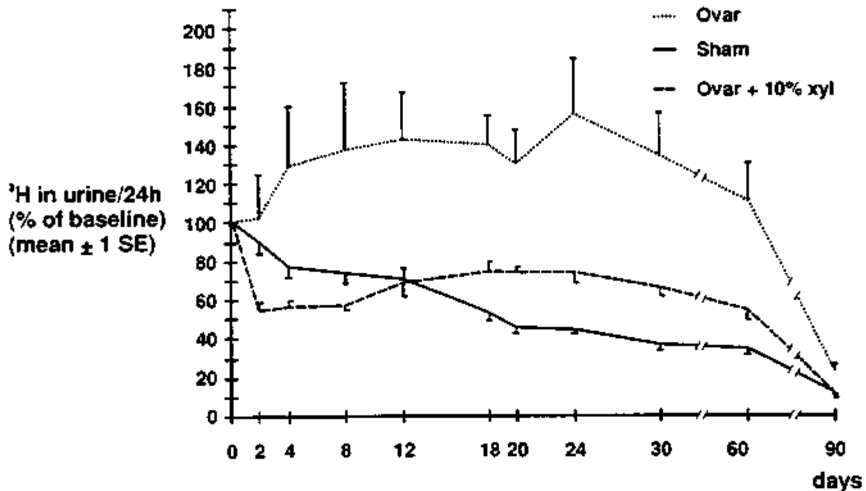


Fig. 6. Urinary ^3H excretion of rats with sham-operatation, ovariectomy or ovariectomy followed by 10% dietary xylitol supplementation. The values are presented in proportion to the baseline levels (%), n=10 in each group (Study III, Fig. 1.).

5.3. The effects of xylitol on bone composition and structure

5.3.1. The effects on bone inorganic fraction

The weight of the tibia in the rats that were fed xylitol supplemented diets was similar to that of the controls (study I). When comparing the rats fed diets of different polyol supplementations (study II), the weight of scapula was significantly smaller in the sorbitol administration group than in the control ($p<0.05$) and D-mannitol ($p<0.05$) groups. In experimental osteoporosis studies, tibial weights of the ovariectomized rats with and without dietary xylitol supplementation were similar, but both exceeded those of the sham-operated controls in study V ($p<0.05$).

Tibial density and humeral ash weight of the ovariectomized rats were significantly smaller than those of the sham-operated rats ($p<0.05$) (Study V). However, in the ovariectomized rats fed the diet supplemented with 10% xylitol, the decrease of tibial density was eliminated, and the decrease of humeral ash weight significantly reduced. Furthermore, dietary xylitol supplementation inhibited the decrease of humeral calcium and phosphorus concentrations observed after ovariectomy (Table 2).

Table 2. Tibial density and biochemical data of the inorganic fraction of humerus in ovariectomized rats (ovx), in ovariectomized rats fed a diet supplemented with 10% xylitol (ovx+xyl), and in sham-operated rats (sham)[*]

	Sham	Ovx	Ovx + Xyl
Tibia			
Density, g/cm^3	1.661 ± 0.031^b	1.637 ± 0.024^a	1.655 ± 0.020^b
Humerus			
Ash weight, g/100g	62.2 ± 1.0^b	58.8 ± 1.4^a	60.6 ± 1.4^b
Calcium, mg/g	204.9 ± 10.9^b	188.5 ± 15.4^a	203.2 ± 17.3^b
Phosphorus, mg/g	113.3 ± 5.0^b	105.9 ± 3.1	112.4 ± 5.8^b

[*]All values are expressed as mean±SD, n = 14. Group means in a row with different superscript letters differ significantly ($p<0.05$). Statistical test used was one-way ANOVA, further comparisons made by Fisher's PLSD.

5.3.2. The effects on bone collagen

No quantitative changes of bone collagenous structures were detected among the rats that were fed the basal diet or the diets with 5, 10 or 20% xylitol supplementation. The measured variables were the amount of collagen per dry weight of organic matrix, the concentrations of pyridinoline and deoxypyridinoline in collagen, and the ratio of these crosslinks in the humerus (study IV).

Ovariectomy, as compared to the sham-operated controls, caused a significant decrease in the amount of hydroxyproline in the organic fraction of tibia ($p<0.0001$) (Study III). The concentrations of pyridinoline ($p=0.0007$) and deoxypyridinoline ($p=0.0028$) were decreased in proportion. The 10% dietary xylitol supplementation normalized the above

mentioned decreases, so that no significant difference was seen between the xylitol-fed ovariectomized rats and the sham-operated controls. The ratio between the crosslink analogs remained unchanged in all groups.

5.3.3. The effects on bone trabeculation

The histomorphometrical data for the secondary spongiosa of the proximal tibia (Fig. 7) revealed that trabecular bone volume (BV/TV) was significantly increased after the 5% ($p=0.025$), 10% ($p=0.004$) and 20% ($p<0.001$) dietary xylitol supplementation as compared to the controls (study IV).

Fig. 7. Proximal tibia (representing average) of a control rat (A), and of a rat fed a diet supplemented with 5% (B), 10% (C), or 20% xylitol (D). Trabecular bone volumes (%, mean±SD) of different groups were 15.3±2,5, 18.4±4.1, 19.3±2.6 and 25.9±2.3, respectively (n=10 in each group) (Study IV, Fig. 2.).

In the experimental osteoporosis study, the trabecular bone volume of the ovariectomized rats was only about one half of that of the sham-operated controls ($p<0.0001$) (Studies III and V). However, in the ovariectomized rats fed the 10% xylitol-supplemented diet, the trabecular bone volume was significantly higher than that of the ovariectomized rats without xylitol ($p<0.0001$). In study III (Fig. 8), the trabecular bone volume (%, mean ± SD) was 27.5 ± 6.0 in controls, 13.5 ± 3.2 in ovariectomized rats, and 20.2 ± 1.9 in xylitol-fed ovariectomized rats.

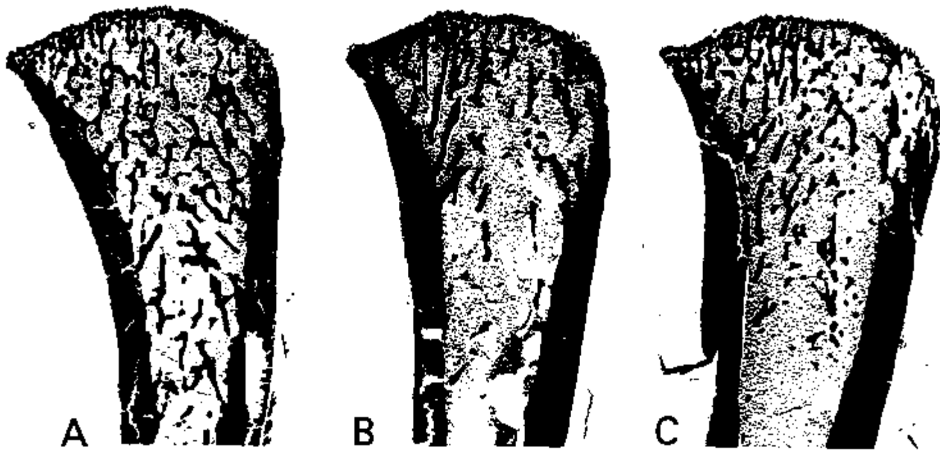


Fig. 8. Proximal tibia (representing average) of a sham-operated (A), of an ovariectomized (B), and of a 10% xylitol-fed ovariectomized rat (n=10 in each group) (Study III, Fig. 2.).

5.4. The effects on bone biomechanical properties

In the three-point bending test, there was a significant increase of the tibial stress in the 10% (p=0.022) and in the 20% (p=0.004) xylitol supplementation groups as compared to the control rats (study IV). A parallel, but not a significant effect was also seen in the 5% xylitol supplementation group (p=0.062). No significant differences in strain or Young's modulus were detected between the groups (Fig. 9).

In the torsion test, the femoral shear stress also increased along with the increasing xylitol content, 10% (p=0.031) and 20% (p<0.001) xylitol supplementation groups differing significantly from the controls (Fig. 9). A trend towards an increased shear modulus of elasticity was detected in all xylitol groups, although only the 20% supplementation group differed significantly from the controls (p=0.031).

The loading of the femoral neck showed a significant increase in the stress of the neck (load/area) in the 10% (p=0.031) and in the 20% (p<0.001) xylitol supplementation groups as compared to the controls (Fig. 9). A parallel, but not a significant effect was seen in the 5% xylitol supplementation group.

In study V, the three-point bending test showed a significant preventive effect of 10% dietary xylitol supplementation against the ovariectomy-caused decrease of tibial stress (p<0.01) (Fig. 10). No significant differences were found between any groups in the values of strain or Young's modulus.

Accordingly, 10% dietary xylitol supplementation prevented the ovariectomy-caused decrease of femoral shear stress in the torsion test (p<0.01) (Fig. 10). A trend towards an increased shear modulus of elasticity in the 10% dietary xylitol supplementation group was detected, but differences between the groups were not statistically significant.

Furthermore, dietary xylitol diminished the ovariectomy-caused decrease in the stress of the femoral neck by 40 % ($p<0.01$) (Fig. 10).

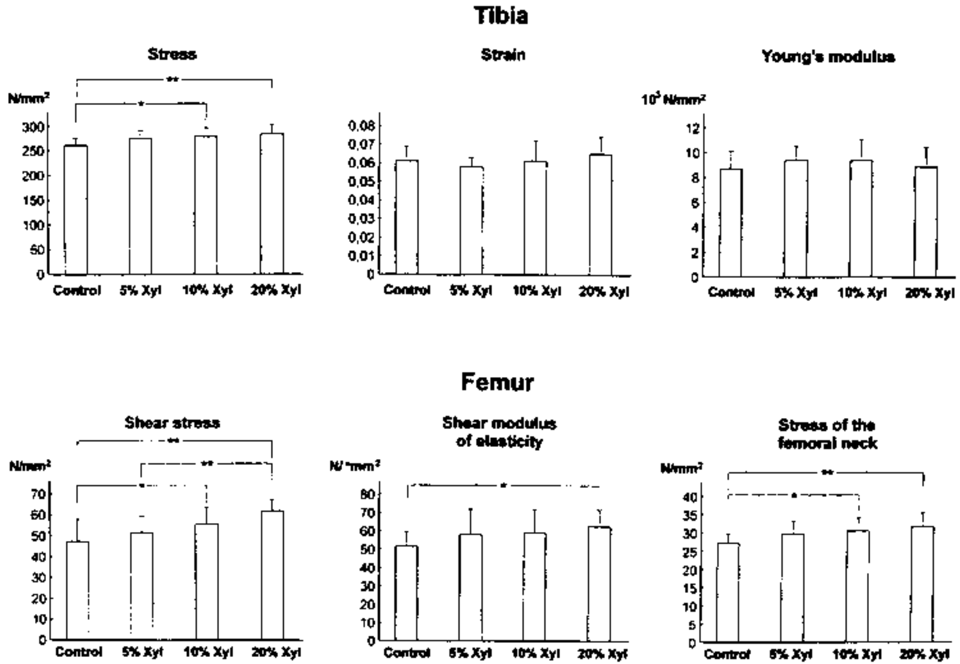


Fig. 9. Biomechanical results of three-point bending of tibia, of torsion test of femur, and of loading test of femoral neck in controls, and in rats fed a diet supplemented with 5, 10, or 20% xylitol (n=10 in each group) (Study IV, Fig. 1.).

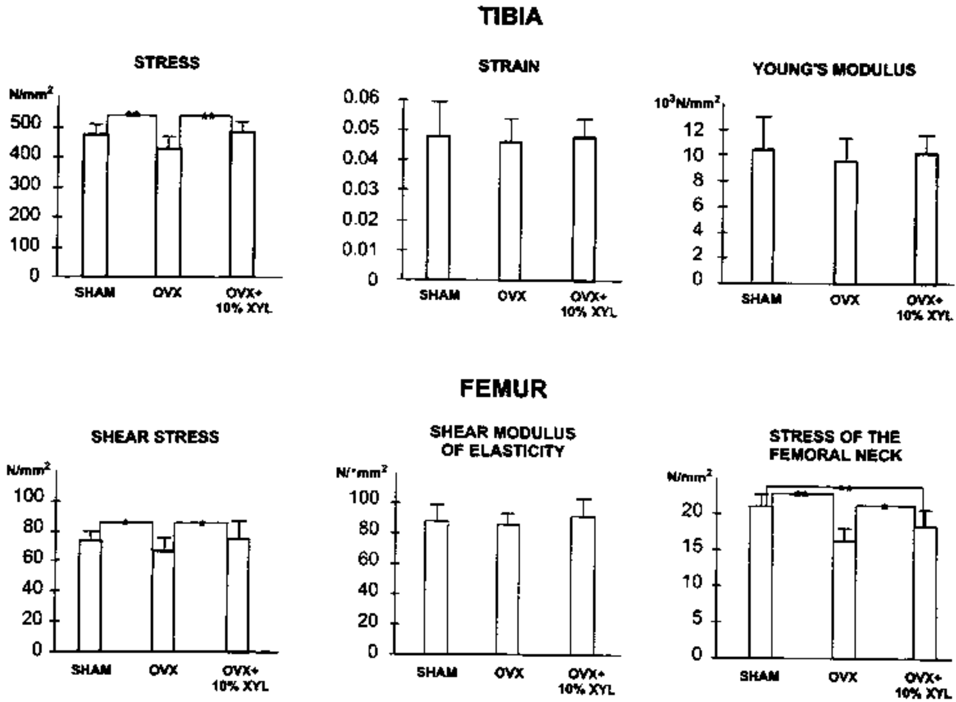


Fig. 10. Biomechanical results of three-point bending of tibia, of torsion test of femur, and of loading test of femoral neck in sham-operated rats, in ovariectomized rats, and in 10% xylitol-fed ovariectomized rats (n=14 in each group).

6. Discussion

6.1. Methodological aspects

6.1.1. Measuring bone resorption

The method used to monitor bone resorption is based on urinary excretion of ^{3}H radioactivity from [^{3}H]tetracycline-prelabeled rats. This method has been described by Klein and Jackman (1976), and further developed by Mühlbauer and Fleisch (1990). Tetracycline forms strong complexes with bone mineral once it incorporated into the bone (Ibsen & Urist 1964), and is removed only during resorption (Frost 1965). The use of multiple prelabeling of rapidly growing rats permits homogenous distribution of [^{3}H]tetracycline throughout the bones (Klein & Jackman 1976).

The validity of the method used is dependent on the stability of tritium in [^{3}H]tetracycline. Kelly and Buyske (1960) have demonstrated that [^{3}H]tetracycline does not undergo metabolic transformation in rats. Furthermore, Klein and Reilly (1976) have found that the molecular exchange with nonradioactive tetracycline in vitro does not induce any significant loss of tritium as water from the [^{3}H]tetracycline of bones of [^{3}H]tetracycline-prelabeled rats.

The efficiency of renal excretion of [^{3}H]tetracycline that is removed during bone resorption assures that the ^{3}H, unlike isotopes of calcium, will be only minimally reused at new sites of bone formation (Klein *et al.* 1985). Furthermore, the [^{3}H]tetracycline released from bone is unbound or poorly bound to apatite, and is thus not reincorporated into the newly formed bone (Klein & Wong 1986, Mühlbauer & Fleisch 1990).

The use of this method was preferred also because, unlike pyridinium crosslinks which are continuously generated in bone, the excretion of [^{3}H]tetracycline stems only from the bone formed during the labeling period, without a possible confusing effect of subsequent bone formation (Egger *et al.* 1994).

The possible confusing effect of tetracycline in the study protocol must not be ignored, because tetracyclines are known to affect bone metabolism in many ways. For example, minocycline given 10-20 mg/day orally has shown to protect against cancellous bone loss and to maintain a normal bone formation rate in ovariectomized aged rats (Williams *et al.*

1996) and in streptozotocin-diabetic rats (Bain *et al.* 1997). Doxycycline has shown to reduce extracellular matrix breakdown, including bone loss, in adult periodontitis during long-term clinical trials (for review see Ryan *et al.* 1996). Furthermore, chemically modified tetracyclines have been discovered to be potent inhibitors of severe classes of matrix metalloproteinases, preventing collagen breakdown and bone loss in a variety of animal models (for review see Ryan *et al.* 1996). In the [^{3}H]tetracycline studies, however, the risk for the tetracycline-caused confusion is minimized by using very low doses of tetracycline. Furthermore, in the present studies, all the groups were given identical tetracycline doses. The possible influence of tetracycline should thus be similar in all of the groups, and should not affect the comparisions made between the different groups.

In the present studies, the initial ^{3}H measurement of each rat served as a baseline value for their individualized patterns of ^{3}H excretion. This was done, instead of using mean values at each time point, to avoid possible confusing effects caused by the biological variations between the animals. The urine collection period was always 24 hours, to avoid possible confusion caused by the diurnal rhythm, and the possible confusing effect of different urine volumes between the animals was eliminated by calculating the amount of ^{3}H radioactivity in proportion to the whole daily volume of urine. Furthermore, the bone resorption values were confirmed by measuring the amount of ^{3}H radioactivity preserved in bones at the end of the experiment.

6.1.2. Measuring bone biomechanics

Of the biomechanical methods used in the present studies (IV and V), the three-point bending and torsional tests of long bones have been used as indicators of cortical bone strength (Saville 1969, Strömberg & Dalen 1976, Paavolainen 1978, Danielsen *et al.* 1992). The loading test of femoral neck measures also the properties of trabecular bone (Hou *et al.* 1991, Peng *et al.* 1994b), although the proportion of cortical bone in the femoral neck in rats is much higher than in humans (Bagi *et al.* 1997).

The results of the present studies, concerning bone strength properties of healthy and ovariectomized rats, were in accordance with previous studies performed either with the same testing machine (Lepola *et al.* 1993, Peng *et al.* 1994b), or like equipment (Strömberg & Dalen 1976, Paavolainen 1978), indicating valitidy and repeatability of the present measurements. The precision, accuracy and reproducibility of the testing machines used have been evaluated by Jämsä *et al.* (1996, 1998).

It should be noted that it is not possible to fully account for geometric effects in whole bone tests. However, unlike the values of strength and stiffness, the values of stress, strain, Young's modulus, shear stress and shear modulus of elasticity take into account bone geometric properties, and thus represent the intrinsic strength of bone materia. In the present study the accuracy of derivations was improved by measuring the actual cross-sectional areas of the bones by a computerized planimeter.

The bone biomechanical parameters are influenced by the histological structure of cortical and trabecular bone, as well as by the biochemical composition of bone mineral and organic structures. As a consequence, the biomechanical properties of bone can not be entirely understood without knowledge of the existing background variables. To get a

more reliable overall view, in the present studies, measurements were made also concerning the effects of dietary xylitol supplementation on bone weight, bone density, bone concentrations of calcium and phosphorus, trabecular bone volume, and on the amounts of bone collagen and its pyridinoline crosslinks.

6.2. General and side effects

Dietary administration of xylitol caused a temporary, slight diarrhea resulting from its slow absorption from the intestine (Mäkinen 1994). However, an intestinal adaptation process occurred during the first week, after which no diarrhea was observed. This adaptive phenomenon has been suggested to be caused by induction of increased activity of polyol dehydrogenase (Bässler 1969), or by selection of more suitable intestinal microflora (Krishnan *et al*. 1980).

The 5, 10 and 20% concentrations of xylitol used in the present studies correspond to a daily intake of approximately one, two and four grams of xylitol, respectively. These are about 3.5, 7 and 14% of the total daily caloric intake of the rats. These might suggest a daily intake of about 20, 40 and 80g of xylitol in middle-aged women, which amounts have been proven to be well tolerated (Mäkinen 1976). However, it should be observed that no direct decisions regarding human metabolism can be drawn from the present studies.

The presence of no significant differences in weight gain (except in study IV in the 20% xylitol supplementation group), in weights of the bones, in food and water intake or in excretion of urine between the control rats and the rats fed xylitol, indicates that there were no major xylitol-induced changes in the development of these animals. The slight reduction in the weight gain along with increasing xylitol concentration of the diet can be attributed to slighty lower availability of energy from ingested xylitol caused by the partial escape of this slowly absorbed polyol from the intestine to the colon (Krishnan *et al.* 1980), to slightly reduced food intake caused by the reduction in gastric emptying rate (Shafer *et al*. 1987), and to reduction in fat deposits ascribed to lowered lipid synthesis or increased lipolysis (Hämäläinen & Mäkinen 1983). Furthermore, while xylitol does not cause an insulin-stimulating action, the insulin-caused expedition of fat tissue accumulation is not strengthened (Dwivedi 1977).

In ovariectomized rats, the weight gain was significantly increased as compared to sham-operated controls. This is in accordance with previous studies, and has also been found in studies where pair fed animals were used (Wronski *et al*. 1989a, Yamazaki & Yamaguchi 1989). This ovarian hormone deficiency-caused obesity has been thought to provide a partial protection against osteopenia in the long bones of the ovariectomized rats (Wronski *et al*. 1987). This is probably related to the increased body weight, but may also be linked to the increased body fat, which may augment the conversion of adrenal androgens to estrogens (Avioli & Lindsay 1990). 10% dietary xylitol supplementation somewhat diminished the ovariectomy-induced increase of weight gain, suggesting that the xylitol-induced benefical effects on bone metabolism are not likely related to the changes in rat body weights.

Like xylitol, 1M D-mannitol and 1M erythritol also caused a temporary diarrhea. In the case of 1M sorbitol, however, no adequate adaptation process took place, resulting in continuous diarrhea. This was probably also reflected in reduced weight gain, and in associated decrease in bone weight of the rats given sorbitol. This phenomenon is in accordance with previous studies, and is suggested to be a consequence of unaltered turnover rate of sorbitol despite the increased polyol dehydrogenase activity (Bässler 1969). The lower weight gain and the greater food intake in the erythritol group probably resulted from the very low amount of energy available from this polyol (Noda & Oku 1992). D-Mannitol is known to be a strong diuretic agent and an effective osmoregulator in mammalian tissues (Dills 1989). The present results indicate that erythritol also is strongly diuretic in rats, and that enteral administration of erythritol substantially increases water intake. In earlier studies a diuretic effect has also been detected when using xylitol and sorbitol (Hämäläinen & Mäkinen 1986). However, in those studies, a higher dietary polyol concentration (20%) has been used. The above general metabolic differences between the studied polyols should not be ignored when comparing their effects on bone metabolism.

6.3. Bone resorption

Dietary xylitol supplementation (10 and 20 %) diminished bone resorption in healthy rats, measured as urinary excretion of ^{3}H radioactivity of the [^{3}H]tetracycline-prelabeled rats. Furthermore, 10% dietary xylitol supressed significantly the ovariectomy-induced increase of bone resorption. Accordingly, significantly more ^{3}H radioactivity was preserved in the bones of the xylitol-fed rats as compared to the controls, and to the ovariectomized rats without xylitol at the end of the experimental period. This further confirms the retarded bone resorption, and also that the xylitol-induced effect can not be explained by any major disturbances in general metabolism or by failure in renal function of these animals. Dietary sorbitol, and to a lesser degree D-mannitol also retarded bone resorption in healthy rats, but the side effects associated with the use of these polyols were more harmful than those when using xylitol. Furthermore, no significant increase of presereved bone ^{3}H radioactivity at the end of the experiment was detected in rats fed sorbitol or D-mannitol as compared to the controls.

Dietary supplementation with 1M sorbitol led to reduced weight gain and decreased weight of the bones as compared to the control rats. This suggests a slower growth rate of the sorbitol-fed rats, accompanied by slower bone metabolism. This, most likely, also partly explains the diminished bone resorption values during dietary sorbitol supplementation.

However, the effects of other polyols suggest that the diminished bone resorption is not a xylitol-specific phenomenon, but more likely, a polyol-associated effect, which is greatly dependent on the metabolic differences between the polyols.

The detailed chemical mechanism of decreased bone resorption caused by dietary polyols is obscure, but an overload of calcium is most likely involved. A parallel, rapidly expressing decrease in the urinary excretion of ^{3}H was detected by Mühlbauer and Fleisch (1990) when dietary calcium supplementation was given to animals that were first fed a

low-calcium diet. Increased calcium absorption (Hämäläinen *et al.* 1985) and increased bone calcium content (Knuuttila *et al.* 1989) has been observed during xylitol administration. Accordingly, increased calcium absorption has been detected during dietary administrations of sorbitol and D-mannitol (Vaughan & Filer 1960, Hämäläinen & Mäkinen 1986, Knuuttila *et al.* 1989), and increased bone calcium content during dietary administration of sorbitol, although to a lesser degree than with xylitol (Knuuttila *et al.* 1989).

The enhanced calcium absorption may be related to complex formation between calcium and the polyols. It has been suggested that the complexed calcium remains soluble in the gut lumen for prolonged periods of time promoting its absorption (Hämäläinen & Mäkinen 1989a). The metal cation must, however, be able to assimilate from the complex to prevent it from being excreted in urine or in feces. Thus, complexes with intermediate stability are the most effective promotors of calcium absorption. Briggs *et al.* (1981) have shown that the relative complexation coefficients (measuring relative stabilities of complexes) of xylitol, sorbitol, D-mannitol and erythritol with calcium are 0.30, 0.40, 0.20 and 0.19, respectively. Accordingly, the polyols, xylitol and sorbitol, that form stronger complexes, were also more effective in retarding bone resorption.

Another interesting metabolic point of view is that xylitol and sorbitol, which are mostly metabolized, seem to retard bone resorption more effectively than the poorly utilized D-mannitol and the unmetabolized erythritol. The first step of the metabolism of absorbed polyols is their oxidation by L-iditol dehydrogenase to the corresponding 2-ketoses with concomitant production of NADH. Ingested xylitol and sorbitol will thus increase the cellular NADH/NAD ratio, while the low oxidation rate of D-mannitol should not elevate the cellular NADH level significantly. The high cellular NADH/NAD ratio may lead to many metabolic reactions and hormonal effects that may affect bone metabolism. One of the possible targets is the citric acid cycle (Jakob *et al.* 1971). Increased urinary and bone citrate levels have been detected in xylitol-fed rats (Hämäläinen & Mäkinen 1986, Knuuttila *et al.* 1989). However, the actual significance of the increased NADH achieved during xylitol feeding and its possible mechanism affecting bone metabolism are not known.

Postmenopausal osteoporosis and ovariectomy are accompanied by reduced intestinal absorption of calcium, probably contributing to the accompanying bone loss (Heaney *et al.* 1978, Ash & Goldin 1988, Gallagher 1990). This estrogen deficiency-induced effect has been linked to decreased serum levels of 1,25-dihydroxyvitamin D_3 (Gallagher *et al.* 1980), or to intestinal resistance to the action of 1,25-dihydroxyvitamin D_3 (Gennari *et al.* 1990). It has also been suggested, that estrogen may play a physiological role in the regulation of intestinal calcium absorption, and that its deficiency in postmenopausal osteoporosis, and following ovariectomy, may result directly in calcium malabsorption (Arjmandi *et al.* 1993). Furthermore, increased intestinal secretion of calcium has been suggested to be essential in the impaired calcium balance in these situations (O'Loughlin & Morris 1994).

Dietary xylitol, on the other hand, is known to increase calcium absorption independently of vitamin D action (Hämäläinen *et al.* 1985). Thus, xylitol is able to fulfill its bone resorption inhibiting action despite the state of estrogen deficiency.

6.4. Bone structure

Bone trabecular volume was significantly greater in all xylitol-fed groups than in the controls, the volume increasing along with increasing xylitol content. Furthermore, 10% dietary xylitol supplementation prevented significantly the accelerated trabecular bone loss in ovariectomized rats.

This bone conserving action of dietary xylitol in experimental osteoporosis is different from those provided by calcium supplements, which have not been able to show reduced loss of bone trabeculae (Recker *et al*. 1977, Riis *et al*. 1987, Breslau 1994). As estrogen deficiency is accompanied with malabsorption of calcium attributed to impaired vitamin D response, dietary administration of pure accessory calcium would probably not lead to increased bone calcium levels. However, as mentioned above, dietary xylitol increases calcium absorption independently of vitamin D action (Hämäläinen *et al*. 1985). Thus, dietary xylitol is probably able to provide sufficient calcium levels also during estrogen deficiency.

The xylitol-induced increase of bone trabeculation is, most likely, partly a consequence of diminished bone resorption, but it probably also reflects an anabolic effect of xylitol. This was suggested in a previous study (Svanberg & Knuuttila 1993), where the increase of cortical bone mineral content after dietary xylitol administration was greater in newly synthetized bone than in formerly formed bone when compared to the controls. The anabolic effect of intravenously and parenterally administered xylitol has been detected in many studies (Lang 1971, Wilkinson 1972, Förster 1974). A positive nitrogen balance, which is related to increased bone mineral content (Morris *et al*. 1992), has also been detected during intravenous and parenteral xylitol nutrition (Georgieff *et al*. 1985, Almdal *et al*. 1993).

Increased NADH/NAD ratio, occuring also during the metabolism of xylitol, has been linked with the initiation of bone mineralization (Shapiro *et al*. 1982). Furthermore, the reduced redox state is associated with increased collagen synthesis and decreased collagenase activity (Hernández-Munôz *et al*. 1994). Unfortunatelly, no dynamic bone histomorphometry or other determinations of bone formation were done in the present studies.

Dietary xylitol did not cause quantitative changes of hydroxyproline, pyridinoline or deoxypyridinoline in healthy rats. The volume of these collagenous elements was decreased in ovariectomized rats, but largely maintained if 10% xylitol was included in their diet. This suggests a dietary xylitol-associated normalizing effect on the rate of bone turnover in these rats. The relative volumes of collagenous elements remained unchanged in all groups. This is in accordance with previous findings (Robins & Duncan 1987), where no significant differences in crosslink concentrations, or their ratios were found between ovariectomized and sham-operated rats, when the crosslink concentrations were expressed as the number of residues per collagen molecule. Thus, no profound selective changes in the structure of bone collagen were suggested either during xylitol feeding, or following ovariectomy.

6.5. Bone biomechanical properties

Reduction of mechanical strength is the ultimate reason for traumatical bone fractures (Melton *et al.* 1986, Alho *et al.* 1988, Lotz & Hayes 1990). Prevention of this reduction is the final determinant of the usefulness of antifracture therapy, and should be evaluated with respect to each compound before clinical use.

The 10 and 20 % dietary xylitol supplementation increased the tibial stress, the femoral shear stress, and the stress of femoral neck significantly as compared to the control rats fed the same diet without xylitol. Accordingly, 10% dietary xylitol protected significantly against the ovariectomy-caused decline of these properties. None of the measured biomechanical variables was weakened after dietary xylitol administration. These indicate a beneficial effect of xylitol on the mechanical properties of both the cortical and trabecular part of the bone, and negate the possibility that dietary xylitol in spite of the increased bone mineral content could cause such qualitative changes in bone that might compromise the mechanical properties of bone. In this respect the present results with xylitol differ from the results of recent rat model studies with fluoride (Einhorn *et al.* 1992, Jiang *et al.* 1996), where the increased bone mass and geometric properties did not unambiquously lead to an increased bone strength.

Our results, concerning increased bone density, increased ash weight and increased bone mineral content in the xylitol-fed healthy rats, and concerning a dietary xylitol-induced protective effect against the decrease of these values in ovariectomized rats, partly explain the advanced biomechanical properties. These results are derived mainly from the cortical part of the bone, and are thus associated especially with the results of the three-point bending test. The increased trabecular bone volume along with increasing dietary xylitol concentration, and the 10% dietary xylitol-induced preventive effect against the loss of bone trabeculae in ovariectomized rats must be partial explanations for the biomechanical results as well. Above all, they are very important reasons for the improved biomechanical properties of the trabecular bone, seen especially in the loading test of the femoral neck. Furthermore, it is of interest that the cancellous component of the femoral neck in humans is probably even more important for the strength properties, because the femoral neck of humans consists of much more cancellous bone than that of the rats (Bagi *et al.* 1997).

No significant differences in strain or Young's modulus of the three-point bending test of the tibiae were detected between the groups. This indicates that the plastic-elastic properties of the cortical bone are not largely affected either by ovariectomy or by dietary xylitol, and neglects the possibility of decreased bone elasticity during dietary xylitol administration. Changes in the above parameters have previously been shown to depend on changes in bone collagenous structures (Burstein *et al.* 1975). Accordingly, no qualitative changes in bone collagen structures were detected in the present studies.

As seen from the results above, besides retarding bone resorption, and increasing bone trabeculation, the oral administration of xylitol also improves bone biomechanical properties, and protects against weakening of these properties during experimental osteoporosis. Thus, considering the results of these experimental studies, dietary xylitol seems to meet all critical requirements of a useful antifracture preparate for the use in the prevention of osteoporosis.

7. Summary and conclusions

To attain the aims of the study, the following results were gained:

1. Dietary xylitol supplementation reduced significantly the rate of bone resorption in healthy rats. The effect was achieved when using xylitol concentrations of 10 and 20%, but not when using a 5% concentration. The effect was detected already after two days, and it was maintained throughout the experimental period of one month.
2. Dietary supplementation of 1M xylitol, 1M sorbitol, and to a less degree 1M D-mannitol reduced the rate of bone resorption. However, bone resorption rate was not affected by 1M erythritol. As distinct from xylitol, sorbitol administration caused continuous diarrhea, and D-mannitol and erythritol caused diuresis.
3. Dietary xylitol supplementation (10%) protected significantly against the ovariectomy-induced increase of bone resorption during experimental osteoporosis.
4. Dietary xylitol supplementation (5, 10 and 20%) increased significantly the trabecular bone volume in healthy rats. A greater increase was attained with an increased xylitol concentration.
5. Dietary xylitol supplementation (10%) protected significantly against the ovariectomy-induced decreases of bone density, bone ash weight, bone calcium and phosphorus concentrations, and of the amounts of bone collagen and its crosslinks. Accordingly, the decrease of the trabecular bone volume was significantly prevented during experimental osteoporosis.
6. Dietary xylitol supplementation (10 and 20 %) increased the strength properties of long bones in healthy rats, without affecting the bone elastic properties.
7. Dietary xylitol supplementation (10%) protected significantly against the ovariectomy-induced weakening of the bone biomechanical properties during experimental osteoporosis.

In conclusion, the above results further strongly support the hypothesis that the oral administration of xylitol protects effectively against the progression of experimental osteoporosis. Although other sugar alcohols share some properties, when their accurate effects and side effects are taken into account, xylitol seems to be superior to the other polyols studied. Dietary xylitol supplementation was effective both in increasing bone mass of healthy rats, and in preventing bone loss of ovariectomized rats. This suggests a

favorable effect of xylitol on both main targets in the prevention of osteoporosis. As dietary xylitol was effective also in protecting against the experimental osteoporosis-caused changes in bone structure and weakening of bone biomechanical properties, oral xylitol administration seems to provide interesting possibilities in the search for new physiological choices for the prevention of osteoporosis.

8. References

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