

Original Research Article

Putamen Ovi enhances biomineral formation of osteoblasts *in vitro*

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A B S T R A C T

After the age of 30, quantity and quality of bone decline, resulting in an increase in the risk of bone fracture. The bone loss can be slowed down or even prevented by hormone replacement, medications like alendronate or calcitonin that suppress osteoclast activity. This study investigated the effect of these medications and of putamen ovi (PO) on the osteoblastic function. PO, a specially processed matrix of the chicken eggshell, was compared with alendronate, calcitonin, estradiol, and calcium carbonate. Proliferation, expression of bone matrix proteins, and biomimetic formation of primary osteoblast stimulated by the different medications were characterized. Scanning electron microscopy and electron diffraction pattern validated apatite biomimetic formation. Cell proliferation and the concentration of bound calcium were found to be not significantly different with estradiol, alendronate, and calcitonin. Proliferation of PO-stimulated cells was significantly higher than in the control-culture. After one week a marked increase in proliferation was seen with the addition of PO. The application of PO alone and the combination of estradiol together with CaCO_3 led to a significant increase of osteoblastic function (osteoblast-proliferation and increased biomimeticization). Future *in vivo* studies should be conducted to evaluate the clinical impact of PO on patients with osteoporosis and osteopenia.

Keywords

Eggshell,
calcium
carbonate,
osteoblast,
cell culture,
osteoporosis,
mineralization

Introduction

The dynamic and complex bone metabolism is characterized by adaptive bone formation and resorption that is subject to many hormonal and nervous regulatory mechanisms (Spelsberg et al. 1999). Because of hormonal changes, calcium metabolism of seniors alters with an advanced age, especially in postmenopausal women, with a reduction in calcium absorption (Riggs et al. 1998).

At menopause, the balance between osteoclastic bone resorption and osteoblastic bone formation shifts and women undergo a rapid phase of bone loss. To prevent the progression of bone mass loss and osteoporosis, different strategies for treatment are still in discussion.

Currently, antiresorptive drugs, such as

bisphosphonates and calcitonin are frequently used for the treatment of osteoporosis.

The bisphosphonate alendronate binds selectively to bone mineral and is taken up by the osteoclasts during bone resorption. Inside the cell it inhibits farnesyldiphosphonate synthase that leads to osteoclast apoptosis and deactivation. Consequently, bone resorption is suppressed and bone mass is increased (Reszka et al. 2003). Conversely, bisphosphonates can occasionally cause osteonecrosis of the jaw especially in patients with prolonged treatment.

Calcitonin is also an inhibitor of osteoclast activity, inhibits bone resorption and can significantly decrease fractures of postmenopausal women with established osteoporosis (Chesnut et al. 2000). Another commonly prescribed medication used for the treatment of osteoporosis is hormones such as estrogens. Estrogen therapy inhibits bone loss by prolongation of the osteocytes and osteoblasts, with inhibition of osteoclast function and formation (Aloia et al. 1991, Lindsay et al. 1999). However, estrogen therapy is now being reevaluated in the light of an increased risk of cardiovascular outcomes or breast cancer (Rossouw et al. 2002). Thus estrogen therapy cannot be recommended for osteoporosis treatment unless other medications cannot be used (cauley et al. 2003). Since none of the described medications provides optimal features for long-term use, because of the growing incidence and prevalence of osteoporosis and osteopenia, and the fact that the number of fractures related to osteoporosis is increasing worldwide, there has been active interest in the development of novel anti-osteoporosis drugs.

One interesting substance is putamen ovi (PO), which has been traditionally used for some decades in Germany. Anecdotally, numerous treating physicians have observed the positive effect of putamen ovi on bone mineral density in patients with osteoporosis and osteopenia Ruepp et al. 1995). Putamen ovi is a specially purified and processed matrix from the mamillary and palisade layer of the chicken eggshell.

We hypothesize that due to its natural composition (especially proteins, calcium, trace elements), putamen ovi may have a positive effect on bone metabolism. In this context it is remarkable, that avian eggshells and their derivates were tested as bone graft material in numerous studies (Durmus et al. 2007, Prabaharan et al. 2005, Jin-Woo et al. 2007, Dupoirieux et al. 1999, Dupoirieux et al. 2001, Dupoirieux et al. 1995).

It was therefore our objective in this study, to evaluate the effectiveness of putamen ovi on osteoblasts *ex vivo*, tested in comparison to estrogen, alendronate, calcitonin and calcium carbonate.

Materials and Methods

Cell culture

Osteoblast-like cells were derived from periosteum of calf metacarpus. The periosteum was cut into 3-6 mm pieces and transferred into culture dishes. Osteogenic layer was placed face downwards. Osteoprogenitor cells migrate from these tissue explants. Explants were cultured for 3 weeks in High Growth Enhancement Medium (ICN Biomedicals GmbH, Eschwege, Germany) supplemented with 10% fetal calf serum, 250 µg/ml amphotericin B, 10,000 IU/ml

penicillin, 10,000 µg/ml streptomycin, 200 mM L-glutamine (Biochrom KG seromed^(R), Berlin, Germany), 10 mM β-glycerophosphate, and 25 µg/ml ascorbic acid (Sigma-Aldrich, Deisenhofen, Germany) at 37°C and 5% CO₂ in humidified air. Medium was replaced once a week. For these experiments cells of the first passage were used. Primary culture cells were harvested by incubation with collagenase (Biochrom KG seromed^(R)) and tyrode solution, collected and pelleted by centrifugation. Resuspended cells were seeded on the bottom of culture dishes at a density of 10.000 cells per cm² for the proliferation study and at 60.000 cells per cm² for the other experiments. Cell culture conditions were similar to those used for the periosteum outgrowth culture, the investigated substances were put into the media.

The substances investigated were putamen ovi (Aar Pharma, Remscheid, Germany), calcium carbonate (Fresenius Medical Care GmbH, Bad Homburg, Germany), alendronate monosodium Fosamax[®] (MSD Sharp & Dohme GmbH, Germany), estradiol (Jenapharm[®] GmbH, Jena, Germany) and calcitonin (Ratiopharm GmbH, Ulm, Germany), each of which were added each in a concentration of 0.1 mg active substance/ml fluid. As 0.1-mg/ml concentration of alendronate was found to be cytotoxic, this substance was added in a concentration of 0.001 mg/ml media. Analyses of cell proliferation were performed after one week, of cell differentiation (immunohistochemistry) after one and two weeks and of biomineral formation after four weeks culture time.

Proliferation

For the proliferation experiments the cells were seeded at a concentration of

approximately 10.000 cells/cm². Cultures were examined regularly by light microscopy. After one week culture time the cells were harvested as described and counted (CASY[®] I computer counter system, Model TT, Schärfe System GmbH, Germany).

Histology and Immunohistochemistry

Osteoblast differentiation was characterized by determination of the synthesis of bone matrix proteins. For immunohistochemistry, medium was decanted and the specimens were washed three times with phosphate-buffered saline (PBS). Extracellular matrix proteins were detected by immunohistochemical staining with specific antibodies.

Anti-collagen type I, polyclonal was obtained from BioTrend Chemikalien GmbH, Germany and the monoclonal antibodies anti-osteocalcin from clone OC4-30 and anti-osteonectin from clone OSN4-2 were obtained from Takara, Shiga, Japan. For immunohistochemical staining, the DAKO EnVisionTM+-system was applied. The stained cell cultures were controlled with a light microscope. Quantitative evaluation was performed on a scanned and digitized image. Using an image processing program (Easy Win 32, Herolab, Germany) analysis of the staining intensity was performed.

Calcium determination

Calcium quantification was based on the interaction of calcium cations with Arsenazo III dye (Sigma-Aldrich). Under acidic conditions this chromogen reacts with calcium to give a colored complex with an absorption peak at 600 nm; the calcium concentration is directly proportional to the intensity of the color

read by the spectrometer. The calcium content of the sample was determined using a calibration curve obtained from calcium standard solutions produced by dilution of a 10 mg/dl calcium/phosphate standard solution (Sigma-Aldrich, Cat. no. 360-11) in deionized water. According to the manufacturer's information, Arsenazo III absorption is linear up to a calcium concentration of 18 mg/dl.

Scanning electron microscopy

For scanning electron microscopy (SEM), chemically unfixed cell culture specimens were cryofixed in liquid nitrogen cooled propane, freeze-dried at -80°C and slowly warmed up to room temperature. Then the freeze-dried specimens were covered with a 3-5 nm chrome layer (GATAN evaporator). A field effect scanning electron microscope (LEO 1530) was used for morphological assessment. Acceleration voltages of 1-2 kV were applied.

Statistical analysis

Values given in this study are in means, \pm standard deviation of the mean. Data in the various experiments were compared using the t-test for independent samples. The data processing programs used were "Excel 2000" and "SPSS 10.0 for Windows".

Results and Discussion

Increase or decrease in cell count with time is shown in figure 1. This allows for identification, mainly of clear differences between the various cell populations with respect to stimulatory effects and the increase/decrease in cell density induced by the added substances. Proliferation in the putamen ovi stimulated cell culture

was significantly higher than in the control culture. After one week of treatment a marked increase in cell proliferation was seen with the addition of PO (+30%; $p < 0.05$).

The cell count of cultures stimulated with calcium carbonate (CaCO_3) and estradiol are clearly exceeded. However, the increase was not significant (+40%; $p < 0.1$). In comparison with the untreated control, almost no increase/decrease in cell count was seen with calcitonin. In contrast, there was a significant decrease in cell proliferation with alendronate (-59%; $p < 0.05$).

Semi quantitative immunohistochemical demonstration of the extracellular matrix (ECM) proteins was carried out for each of the cell populations and each treatment period. After application of the active substances putamen ovi, calcium carbonate, alendronate, estradiol, and calcitonin for one or two weeks, respectively, the cells were stained immunohistochemically. Collagen type I, osteonectin, and osteocalcin remained almost constant with respect to the untreated control, the expression values measured for alendronate stimulated cultures were slightly but not significantly below those of the control ($p < 0.05$). The tested substances seem to have no additional stimulatory effects on the synthesis of ECM proteins.

To test the effect of the different additives (putamen ovi, calcium carbonate, alendronate, estradiol, and calcitonin) with regard to biominerization formation, osteoblasts were cultured for 4 weeks with the growing medium plus ascorbic acid and β -glycerophosphate as external supply of phosphate. In all cultures biominerization was found. Results of

the spectrometric calcium measurements are given in figure 3, which shows the mean value of the bound calcium in the cell sample for each of the treatments carried out. The untreated control acts as reference. In all the treated cell populations the mean total concentration of bound calcium is similar or exceeded to that of the untreated control.

While the calcium concentration with calcitonin fell just below the reference value, the mean calcium concentration of the other samples was increased with respect to the control. The application of putamen ovi (121%; $p<0.05$) and the combination of CaCO_3 and estradiol led to a significant increase in the calcium concentration in the cell culture (113%, $p<0.05$).

Observation of mineralization phenomena in cell cultures by SEM (figure 4) revealed areas of globular mineral deposition (mineral-spherules) that were interspersed among the osteoblasts. Small mineral nodules with an approximate diameter of 200 nm were found as early indicators of newly formed biomimetic in direct contact with the cell surface. In successive stages of mineralization, mineral-spherules with a diameter of up to 700 nm appeared to surround the cells. These matured spherules were partly covered by cell membranes or were found to occur with a clear distance from the cell margins. Mineral formation was seen in colocalization with the bone-like extracellular matrix. Electron diffraction analysis (figure 4, insert) revealed apatite mineral formation at early stages that could be well distinguished from an artificial calcium phosphate accumulation or precipitation (exemplified by a supersaturated calcium-phosphate solution).

After peak bone mineral density (bone mass) is achieved in the 30s, bone architecture is conserved by a constant remodeling process. This process of bone remodeling is the key phenomenon in bone biology. Understanding this remodeling process and its regulation will clarify not only control of osteoblast and osteoclast function, but also the physiology of age-related bone mass loss and osteoporosis. Thus, for the discussion of bone loss treatment both the osteoblast and the osteoclast function are important. When using supplements and replacement therapy, knowledge of the local effects of the substances on bone metabolism and target structures – in this case, the osteoblasts – is a prerequisite for the interpretation of study results. Earlier publications have been concerned primarily with clinical experience or interaction with osteoclasts in the treatment of bone mass loss or osteoporosis, but for the most part, interactions with osteoblasts at a cellular level have not been sufficiently identified. The experimental design of the work presented here allowed the reactions of osteoblast-like cells to the different related substances used in mineral supplementation and hormone replacement therapy for osteoporosis to be investigated. It could be confirmed that not only the application of putamen ovi or calcium carbonate, but also the addition of estradiol, alendronate, and calcitonin enable biomimetic formation in the osteoblast cultures. The evidence of cell-regulated biomimetic formation is an important characteristic of osteoblasts (Wiesmann et al. 2005, Wiesmann et al. 2004). Thus, the spectrometric determination of calcium is a meaningful feature of bone mineral formation, which may be regarded as a semi quantitative end-product control of cell culture

synthesis (Wiesmann et al. 2001). A significant increase of bound calcium (represents apatite formation) was found only for putamen ovi and for calcium carbonate alone or in combination with estradiol. A significant variation due to an addition of alendronate, estradiol or calcitonin was not obvious. However, especially for estradiol it was shown that physical activity i.e. mechanical stress is necessary for neo-bone formation (Prince et al. 1991). The highest biomimetic increase shows putamen ovi, which acts also as a significant stimulator for osteoblast proliferation. In contrast, alendronate was in the first tested concentration toxic for the osteoblasts and for the tested concentration of 0,001mg/ml showed a suppression of the proliferation. Further, no significant variation from control was found for the tested medications concerning the extracellular matrix protein formation.

With respect to calcium supplements, however, a distinction must be made whether the women treated were more than five years postmenopausal or not. Many authors have demonstrated rapid bone loss with a significant reduction in the absolute bone mineral density during the first five years after menopause, despite giving extra calcium (Gambacciani et al. 1995, Dawson-Hughes et al. 1990). In women who had menopause more than five years earlier, calcium supplements not only led to a significant increase in calcium levels in 24-hour urine and serum, but also to increased bone mineral density when compared with controls (Riggs et al. 1998, Dawson-Hughes et al. 1990). This is supported by the results of the present ex vivo study for osteoblasts, which found evidence at the cellular level of a strong

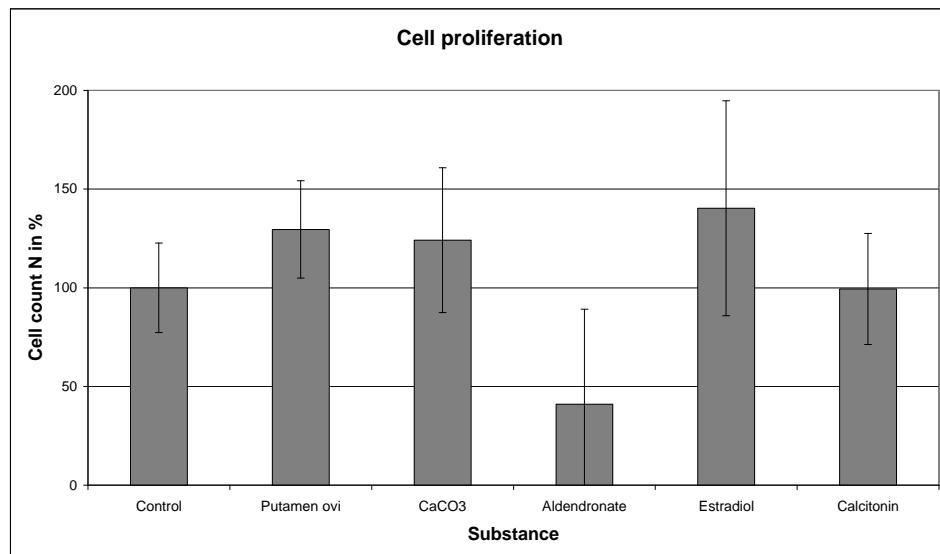
increase in minerals when osteoblasts were treated with either putamen ovi or with calcium carbonate.

Estrogen, alendronate, calcitonin, and an intake of calcium are well known to be useful in treatment or prevention of age-related bone loss or osteoporosis (Anderson et al. 2004, Black et al. 1996, Yang et al. 1998, Meschia et al. 1993). However, estrogen, alendronate, and calcitonin inhibit bone resorption by interfering with osteoclast function or formation, or leading to apoptosis (Delaney 2006). This suppression of osteoclast activity conserves bone mass but the bone turnover is also elongated and for example micro-fracture healing or bone adaptation to mechanical stress is retarded. Thus, the question arises, is a bone with minor bone remodeling still as functional (physiologically responsive) and healthy as normal bone?

In a review article about bisphosphonates, Morris and Einhorn reported that there is a common misconception, that the mechanism of action of the bisphosphonates is specific to the osteoclasts. The authors outlined the need to further investigate the effect of bisphosphonates on osteogenic and other cells (Morris et al. 2005).

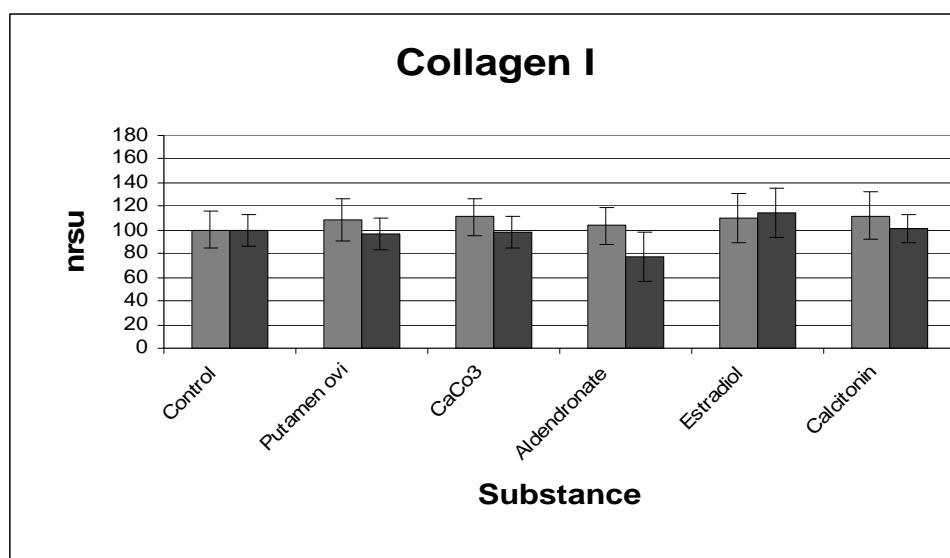
We have shown in this study, that the bisphosphonate alendronate in a 0.1-mg/ml concentration was absolutely cytotoxic to osteoblasts, and that a 0.001-mg/ml concentration caused a significant decrease of almost 60% in the osteoblast cell proliferation.

Figure.1 Number of cells in cultures treated continuously for one week with putamen ovi, calcium carbonate, alendronate, estradiol, and calcitonin. The untreated control acts as a reference. The cell count is given as a percentage of the control value. Statistically significant differences between cell count in the treated populations and the control are indicated by symbols

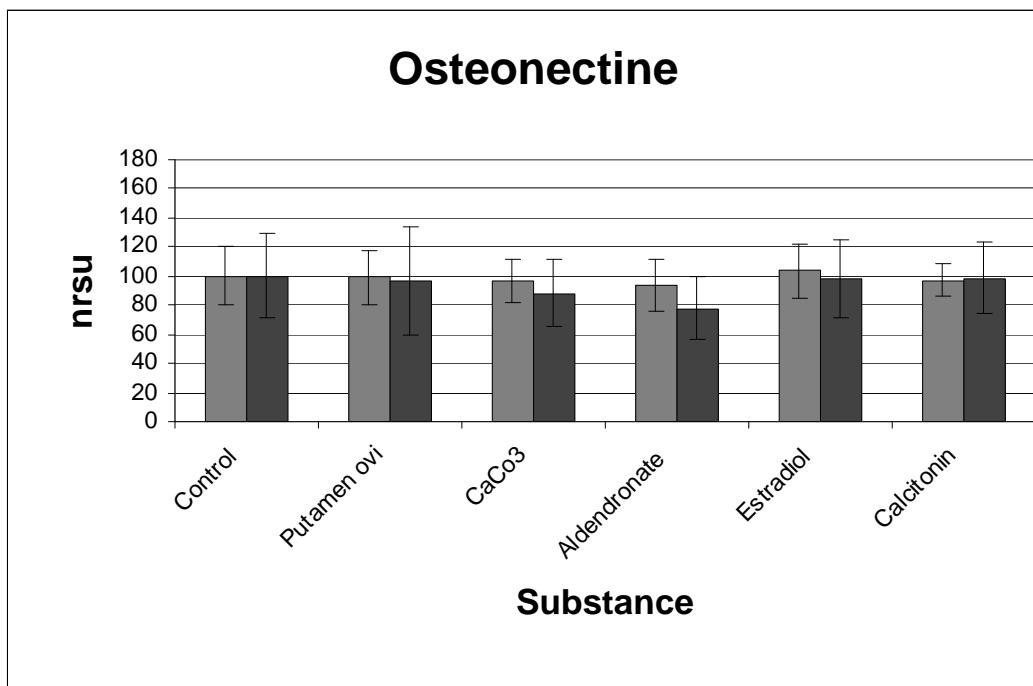


(* p<0.05).

Figure.2 (A, B, C): Immunohistochemical demonstration of extracellular matrix protein expression of (A) collagen type I, (B) osteonectine, and (C) osteocalcine (measured in normalized relative staining units [nrsu]). Osteoblast cultures were treated with different substances over periods of one and two weeks. Statistically significant differences in synthesizing ability between the stimulated cell cultures and untreated control are indicated by a symbol (* p<0.05)



(B) Osteonectine



(C) Osteocalcine

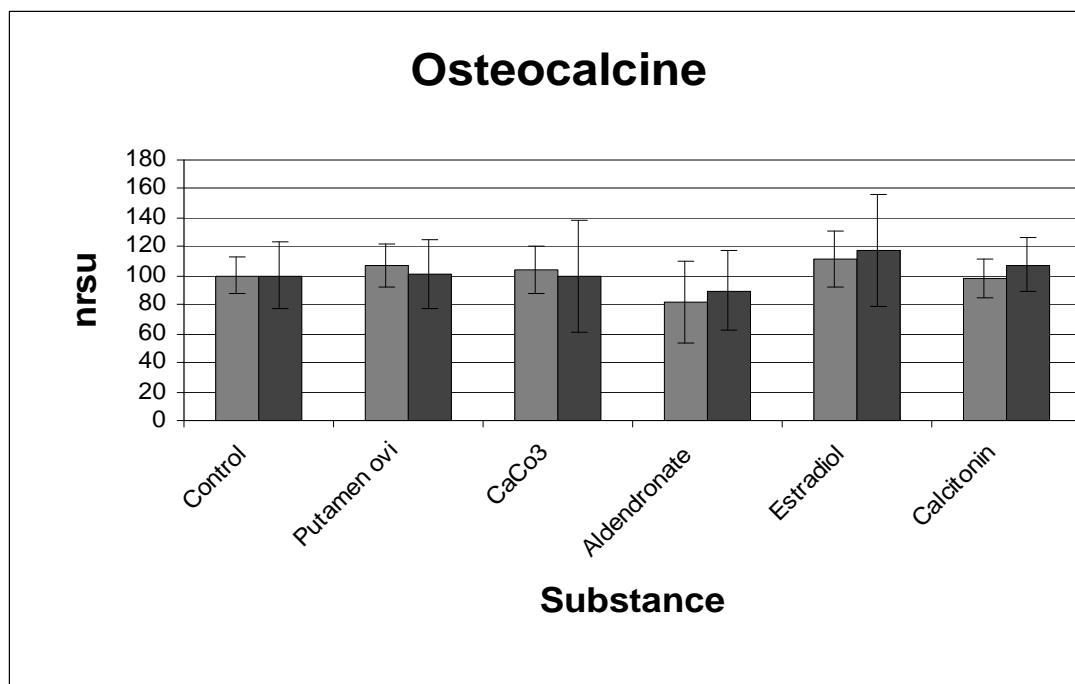


Figure.3 Mean concentrations of bound calcium in 4-weeks-old osteoblast cell culture as a function of treatment. Duration of treatment was 2 weeks, started after the first week. Statistically significant differences in calcium concentration between the stimulated cell cultures and the untreated control are indicated with a symbol (* p<0.05).

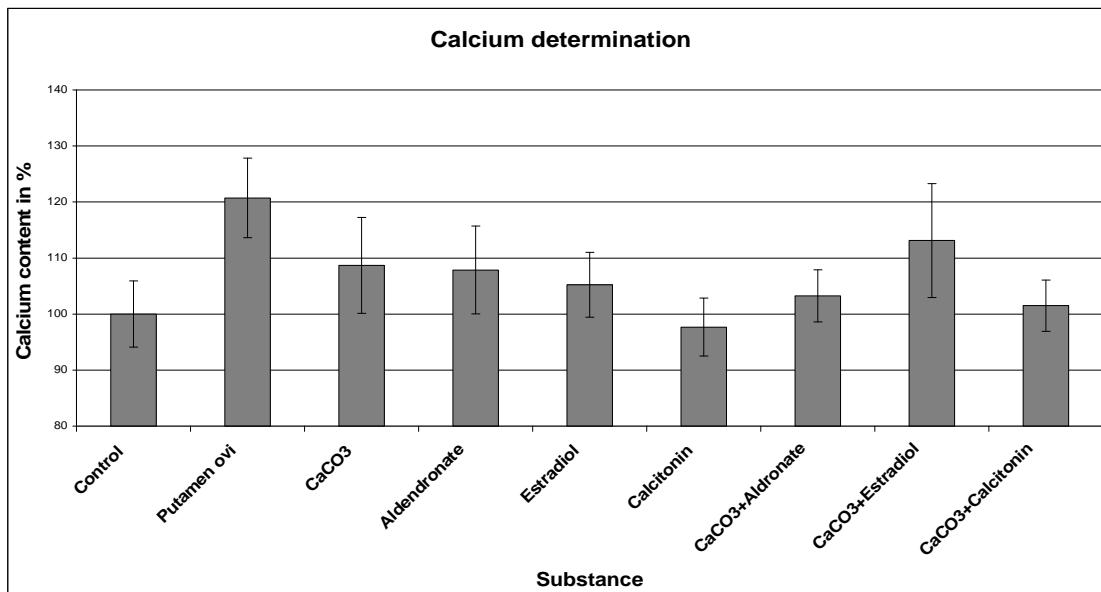
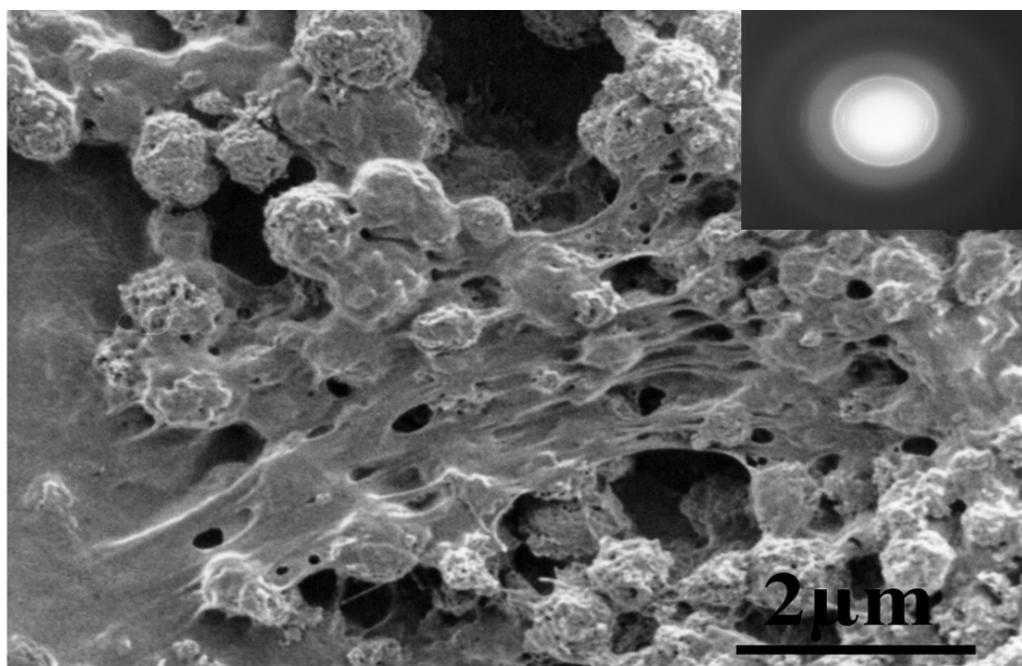


Figure.4 Scanning electron micrograph of globular bone-like biomimetic formation. Culture time of osteoblasts was 4 weeks, putamen ovi was added. The electron diffraction pattern shows a diffuse apatite ring scattering, which is characteristic for early bone mineral.



Considering our results, we suggest that instead of pharmacologically inhibiting osteoclasts, a support of osteoblast function and bone formation should rather be sought for the treatment of osteoporosis.

This study supports putamen ovi as a proper substance for osteoblast stimulation *ex vivo*. This ranged from greater proliferation of the cells to the promotion of mineralization. This is not a surprising result in view of the fact that Akins and Tuan showed in 1993 that the eggshell plays an important role in the chick embryogenesis. The authors demonstrated that the eggshell provides calcium, which penetrates through the chorioallantoic membrane to the embryo in order to stimulate skeletal development (Akins et al. 1993).

The mechanism of action of putamen ovi may not only be due to the calcium and/or trace minerals, but particularly due to the protein fraction of putamen ovi.

Megan et al reviewed the eggshell specific matrix proteins in 2009 (Megan et al. 2009). They outlined the beneficial properties and the two main functional roles of these proteins, the regulation of eggshell mineralization and the antimicrobial protection of the egg and its contents.

A substance like putamen ovi, with the mode of action of stimulating osteoblasts, could be an alternative medication for various bone disorders. For example, we anticipate that the anabolic features of PO may not only be useful for the treatment of osteoporosis and osteopenia, but probably also for Paget's disease of bone and Osteogenesis Imperfecta. Furthermore, PO may be effective for use as a stimulant in

bone grafting. Numerous authors published their positive *in vitro* and *in vivo* study results in the last years. They mainly remarked the beneficial properties of this inexpensive (Durmus et al. 2007, Dupoirieux et al. 1995), safe and easily available material (Durmus et al. 2007). Studies of Park et al. indicates the potential efficacy of surface-modified hen eggshell particles as an osteoconductive bone substitute for treating osseous defects during bone regenerative surgery in dental field. Moreover, other authors described eggshell-originated HAP as a potential ceramic, which could be useful as an inexpensive ceramic for biomedical applications (Prabaharan et al. 2005) and eggshell particles as resorbable biomaterial in extra skeletal and skeletal sites (Dupoirieux et al. 2001) with an excellent biocompatibility without any signs of toxicity in various experiments (Dupoirieux et al. 1995).

However, further clinical studies are necessary to confirm these hypotheses. The cell culture analyses carried out in this study confirm the results of other *in vivo* studies on postmenopausal women with respect to increased mineral content following the administration of hens' eggshell powder (Schaafsma et al. 2002). In a 12-month study, Schaafsma et al. compared daily dietary supplements of calcium carbonate and hens' eggshell powder in women who had undergone menopause more than five years previously. The administration of eggshell powder alone caused an increase in bone mineral density in the lumbar spine (Schaafsma et al. 2002). The positive effects of the eggshell powder were supported by the work of Ruepp et al.. They found a significant increase of bone mineral density of the lumbar vertebrae of postmenopausal women treated with the

hens' eggshell preparation for an average of 304 days (Yang et al. 1998, Morris et al. 2005). The superiority of the preparations from birds' eggshells compared with pure calcium carbonate described in the above-mentioned studies and in the present work is further confirmed by the experiments of Dawson-Hughes and co-workers. According to this group, treatment with pure calcium carbonate inhibits loss of bone mass only very slightly, unlike organic calcium compounds (Dawson-Hughes et al. 1990).

In conclusion, we have found that commonly used medications for osteoporosis, such as estradiol, alendronate, or calcitonin suppress osteoclast activity but do not support osteoblastic function *ex vivo*. Putamen ovi, however, significantly enhances osteoblast activity *ex vivo* and may therefore be advantageous for patients with osteoporosis, osteopenia and other disorders related to altered bone growth or bone healing. Further *in vivo* studies should be conducted to evaluate the true clinical impact of this very promising substance.

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