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BERRIES AS A NATURAL SOURCE OF BONE SUPPORT - THE INVOLVEMENT OF ANTHOCYANINS IN THE MOLECULAR MECHANISMS OF THE HEALING AND REGENERATION PROCESSES

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Bone tissue has remarkable self-regenerating ability which, in case of injury, enables return to a completely functional, pre-injury state. However, aging, disease, obesity, traumas, multiple fractures, infections and tumor removal cause large bone defects that cannot be healed spontaneously. In order to achieve successful bone healing and regeneration, a plenty of approaches, including the application of autografts, allografts and bone tissue engineering (BTE), have been developed. One of the approaches is based on the findings that bone loss in humans and many animals during aging is partially caused by accumulation of reactive oxygen species (ROS). Due to the spectrum of biological activities, including antioxidative, essential polyphenolic components - anthocyanins (ACNs), are a part of a significant research area regarding means and methods for bone healing and regeneration. Berries are especially rich in ACNs. Based on *in vitro* and *in vivo* studies regarding molecular mechanisms involved in bone healing and regeneration supported with berries' ACNs and on observational research in human populations, it has been found that berries' ACNs enhance osteoblastogenesis, suppress osteoclastogenesis and have osteoimmunological activity. Therefore, berries' ACNs should be considered as naturally widespread therapeutics for bone support. Nevertheless, before implementation of berries as a natural source of bone support, there are some issues left to resolve: clarification of molecular mechanisms of ACNs action in bone metabolism, identification of effective doses of particular ACNs for bone regeneration therapies and performing clinical studies for determination of therapeutic efficacy of different types and concentrations of ACNs.

Keywords: bone healing, bone regeneration, berries, anthocyanins, molecular mechanisms

BOBIČASTO VOĆE KAO PRIRODNI IZVOR POTPORE KOSTIJU – UČEŠĆE ANTOCIJANA U MOLEKULARNIM MEHANIZMIMA PROCESA ZACELJENJA I REGENERACIJE

Koštano tkivo ima izuzetan potencijal samoregeneracije koji, u slučaju povreda, omogućava povratak u kompletno funkcionalno stanje koje je postojalo pre povrede. Međutim, starenje, bolesti, gojaznost, traume, višestruki prelomi, infekcije i uklanjanje tumora uzrokuju velike koštane defekte koji se ne mogu spontano zalečiti. U cilju postizanja uspešnog zaceljenja i regeneracije kostiju, razvijeno je mnoštvo pristupa, uključujući primenu autografta, alografta i tkivnog inženjerstva kosti. Jedan od pristupa se zasniva na saznanjima da je gubitak kostiju kod ljudi i mnogih životinja tokom starenja delimično uzrokovan akumulacijom reaktivnih vrsta kiseonika. Zbog spektra bioloških aktivnosti, uključujući antioksidativnu, esencijalna polifenolna jedinjenja - antocijani, deo su značajne oblasti istraživanja vezane za sredstva i metode za zaceljenje i regeneraciju kostiju. Bobičasto voće je posebno bogato antocijanima. Na osnovu *in vivo* i *in vitro* proučavanja molekularnih mehanizama uključenih u zaceljenje i regeneraciju kostiju potpomognutih antocijanima iz bobičastog voća i opservacionih istraživanja u humanim populacijama, utvrđeno je da antocijani bobičastog voća pospešuju osteoblastogenezu, suzbijaju osteoklastogenezu i imaju osteoimunološku aktivnost. Prema tome, antocijane iz bobičastog voća treba smatrati potencijalnim, široko rasprostranjenim terapijskim sredstvom za davanje potpore kostima. Ipak, pre primene bobičastog voća kao prirodnog izvora potpore kostima, ostala su još neka pitanja koja treba rešiti: pojašnjenje molekularnih mehanizama delovanja antocijana u metabolizmu kostiju, identifikacija efikasnih doza konkretnih antocijana za terapije regeneracije kostiju i izvođenje kliničkih studija za utvrđivanje terapijske efikasnosti različitih tipova i koncentracija antocijana.

Ključne reči: zaceljenje kostiju, regeneracija kostiju, bobičasto voće, antocijani, molekularni mehanizmi

Introduction

Bone-forming osteoblasts (OBs) and bone-resorbing osteoclasts (OCs) control normal bone metabolism (1). Remarkable bone tissue self-regenerating ability is maintained throughout adulthood to a certain degree which, in case of injury, enables return to a completely functional, pre-injury state (2). However, high degradation rate of bone tissue and, at the same time, decreased production of new bone, leads to bone loss (1). Bone mass declines and bone microarchitecture weakens with aging so that, in advanced age, bone resorption rate surpasses bone formation rate (3). Besides aging, other systematic factors, such as disease or obesity, also have the influence on bone healing and regeneration (4). Moreover, traumas, multiple fractures, infections and tumor removal cause large defects that cannot be healed spontaneously (5).

Bone healing and bone regeneration approaches

In order to achieve successful bone healing and regeneration, a plenty of approaches have been developed. The gold standard in the treatment of posttraumatic conditions – fractures, delayed unions and nonunions, is the application of autografts – the grafts constructed out of patient's own bone (6). Autografts' structure is similar to the original bone, so the bone growth and regeneration are enabled (7), but taking autologous bone is usually associated with health risks to the patient and the source of the tissue is limited (8). Processed bone allografts are good alternative because of higher availability and the lack of donor site complications in the recipient (6). However, the limitation of allografts is the induction of immune reactions which is followed by graft rejection, possibility of infection and the chance of disease transmission (9). In the field of bone tissue engineering (BTE), great efforts have been made in order to avoid complications caused by bone grafts. The idea of BTE is to mimic the structure of a natural bone and construct an implant based on biological triad – biomaterial as a scaffold, regulatory molecules and cells (10). In the light of that, implants based on combinations of different biomaterials, various regulatory molecules and different types of cells were constructed. The promising results in BTE include application of calcium phosphate-based biomaterials as a scaffold due to their similarity with the natural bone structure (11, 12, 13). Also, the inclusion of natural sources of growth factors, such as platelet-rich plasma (PRP) (14) and blood (15) into the implants' structure deserves a special attention in BTE. Biomaterials and regulatory molecules can be combined with macrophages (13, 16) or stem cells, including adipose tissue-derived mesenchymal stem cells (ADSCs). ADSCs can be applied either freshly isolated (17), *in vitro* expanded prior to implantation (18) or *in vitro* differentiated into various types of cells, including OBs and endothelial cells, prior to implantation (11, 12, 18).

Another approach for bone healing and regeneration is based on findings that bone loss in humans and many animals during aging is partially caused by accumulation of reactive oxygen species (ROS) (19). Cells normally prevent the excessive presence of ROS thanks to their antioxidant defense system that includes vitamins, enzymes and other substances (20). Excessive ROS accumulation leads to DNA damage, lipid peroxidation and oxidation of amino acids with consequent changes in the cells' structures and functions (21). In bone tissue, oxidative stress leads to OBs and osteocytes apoptosis (22) and stimulate osteoclastogenesis (19). During osteoclastogenesis, transcription factor nuclear factor-kappa B (NF-κB) signaling pathway plays a crucial role (1). The existence of such an association between oxidative stress and bone mass loss during aging points out to the need of improving an individual's antioxidant defense. Standard pharmacological agents that improve bone mass and reduce fractures may show side effects and/or poor efficacy in bone healing and regeneration (23).

Folk medicine has its various approaches to bone healing, and some of them are a source of valuable facts for creating more effective modern bone treatments. For example, water extracts of flavonoids-rich plants, such as *Labisia pumila* (Blume) Fern.-Vill. (Myrsinaceae) and *Piper sarmentosum* Roxb. (Piperaceae), are used in folk medicine by Malay women since these plants maintain the estrogen level at the post-menopausal stage and consequently increase bone formation and reduce bone resorption (24). Root barks and stem barks rich in flavonoids are traditionally used for bone fracture healing by the indigenous people of Eastern Ghat (25). Several flavonoid-rich plants, including *Pholidota articulata* Lindl. and *Coelogyne cristata* Lindl. (Orchidaceae), are the part of folk tradition in India for the treatment of bone-related disorders and fractured bones (26).

In the recent decades, there has been a noticeable trend in the application of a suitable diet for the additional treatment of certain bone conditions and diseases. Nutritional therapies that are natural are expected to be safer therapeutic options for bone loss and restoration of normal bone metabolism. A plethora of preclinical and clinical researches indicate that fruits and vegetables-rich diets could help bone fracture healing (27). Due to the spectrum of observed biological activities, flavonoid substances, especially anthocyanins (ACNs) as bioactive components of natural origin, are a part of a significant research area regarding means and methods for bone healing and regeneration.

Anthocyanins: classifications, biological activities and health effects

With around 8000 polyphenols among which nearly 500 are bioactive, plants represent abundant source of antioxidants (28). Berries are especially rich in polyphenols. Due to the health benefits associated with polyphenols, the intake of berries and possibility of their application in medicine have gained much interest within scientific circles (29). Phenolic compounds have a spectrum of biological activities including anticancer, antidiabetic, anti-inflammatory, anti-platelet, and represent one of the most powerful natural antioxidants (30).

Polyphenols are categorized into four groups: phenolic acids, flavonoids, stilbenes, and lignans (31). It has been found that flavonoids are able to promote bone formation and stimulate osteogenic differentiation of mesenchymal stem cells (MSCs) (32). The idea for the application of flavonoids for bone healing and regeneration in clinical medicine comes from the experience of using flavonoids-rich compounds in folk medicine worldwide (33).

Flavonoids can be classified into flavones, flavonols, isoflavones, flavanones, flavanes, chalcone, isoflavanes and ACNs (34). ACNs are essential polyphenolic water-soluble plant pigments which consist of an anthocyanidin (aglycone) bound to sugar fraction (35). According to the position and number of hydroxyl and methoxy groups, more than 635 ACNs have been identified (1). Cyanidins are the most abundant group of ACNs found in food, afterwards delphinidins, pelargonidins, peonidins, malvidins, and petunidins (36). These plant pigments are responsible for the wide range of colors (from dark red to blue) visible to the human eye. ACNs are indicators of the ripeness as well as the quality of the fruit (37, 38). The berry skin contains the highest amount of ACNs, although they are also present in the pulp (39, 40). Their production is affected by various environmental conditions, such as light, temperature, presence of minerals, climate and many other factors.

In vitro and *in vivo* studies indicate that ACNs have anti-inflammatory and antioxidative properties (41) that prevent or delay the onset of chronic diseases involving oxidative stress and inflammation (29, 42). ACNs stop pro-inflammatory mediators by blocking their production or activity, which is the foundation of their anti-inflammatory potential (43). Some types of ACNs possess ROS scavenging properties thus preventing DNA damage (44).

Among berries, blackcurrant (*Ribes nigrum* L.) stands out as a rich source of ACNs (45), with 250 mg of ACNs per 100 g of fresh fruit (46) and ACNs' concentrations that are up to fourfold higher in comparison with other similar fruits (47). Blackcurrant contains delphinidin-3-*O*-rutinoside (del-3-rut), cyanidin-3-*O*-rutinoside (cya-3-rut), delphinidin-3-*O*-glucoside (del-3-glc) and cyanidin-3-*O*-glucoside (cya-3-glc), contributing to approximately 98% of total ACNs (48). Delphinidins contribute to approximately 74% of total ACNs in blackcurrant and are of particular importance for the prevention of bone resorption (49) due to the higher free-radical scavenging capacity in comparison with all other major ACNs (50).

A review of the literature revealed other berries valuable as a beneficial source of ACNs. Blueberries and grapes are abundant in delphinidin, cyanidin, petunidin and malvidin glycosides, making up over 90% of the total ACNs content (51, 52). Malvidins account for 16% of the total blueberry ACNs (48), whereas strawberries' attractive color and health benefits are derived from pelargonidin-3-*O*-glucoside as the major ACN of this popular red berry (38, 53). Cranberries' dominant ACNs are peonidin glycosides (54), whilst cyanidin glycosides are most represented in red currants, blackberries and raspberries (48, 55, 56). The role of these ACNs in bone healing and regeneration will be thoroughly elucidated in this paper.

Anthocyanins in bone healing and regeneration: mechanisms of action

Discovering the possibilities for bone healing and regeneration supported with berries' ACNs opens the door for exploring the potential use of ACNs in therapeutic interventions for people that suffer from bone degeneration related with inflammation, menopause or aging (57). Potential benefits of ACNs are especially important for those populations that are more and more prone to osteoporosis (57) - the most common bone disease characterized by low bone mineral density (BMD) and bone matrix fragility that predisposes patients to increased risk of fractures (28, 58). Osteoporosis' incidence is influenced by various factors including aging, insufficient estrogen levels, increased oxidative stress, chronic inflammation, and genetics (58).

Human (59) and animal (60) studies have shown that a strong positive correlation exists between excessive ROS and bone loss. ROS directly contribute to bone degradation by osteoclast (OC)-generated superoxide or can cause an increase in OCs' differentiation and function (60). At the same time, excessive ROS inhibit osteogenic differentiation through extracellular signal-regulated kinases (ERK) and ERK-dependent NF-κB signaling pathways (22). Also, an important factor in the oxidant-antioxidant balance is the capacity of OBs to produce antioxidants in response to ROS (61).

It has been reported that ACNs are able to enhance osteoblastogenesis, suppress osteoclastogenesis and also have osteoimmunological activity (28). The main modes of ACNs' action on osteogenesis are achieved by involving in the molecular mechanisms of bone morphogenetic protein (BMP-2), WNT-β catenin and fibroblast growth factor (FGF). Del-3-rut and cya-3-glc activate FGF pathway thus accomplishing the influence on OBs' differentiation. Most of ACNs that can promote osteogenesis also up-regulate expression of genes for transcription factors Sox9, Runx2, and Osterix (Osx) and genes for type 1 collagen (Col1), osteopontin (OPN), osteocalcin (OCN), and alkaline phosphatase (ALP) (1). The effects of different ACNs on osteoclastogenesis are mainly achieved by involvement in the molecular mechanisms of some pathways such as: c-Fos, NF-κB, JNK, Ca^{2+} and ROS (1). In addition, three subfamilies of mitogen-activated protein kinases (MAPKs) are important in RANK signal-mediated OC generation (62), while nuclear factor of activated T-cells 1 (NFATc1) represents major pathway that regulate osteoclastogenesis.

Bone healing and regeneration supported with ACNs and berries compounds – *in vitro* studies

The involvement of ACNs and berries compounds in bone healing and regeneration was examined by using various *in vitro* models. One type of the applied *in vitro* models mimics the conditions in an organism that are the consequence of estrogen deficiency and/or microdamage. In one of such models, oxidative stress was induced in human OB-like cell line SaOS-2 by an intracellular depletion of glutathione (GSH), in the period before the beginning of osteogenic differentiation and during the early mineralization process (63). In GSH-depleted SaOS-2 cells, blueberry juice (BJ) rich in ACNs prevented inhibition of osteogenic differentiation and mineralization process caused by oxidative stress. BJ also modulated signals which up-regulate the expression and activity of osteogenic factors. Likewise, increased expression of sirtuin type 1 deacetylase, an enzyme that regulates osteogenic differentiation of tendon and MSCs and represents a positive Runx2 regulator (64), is probably related to the osteogenic action of BJ (63).

Inhibitory effects of delphinidin, cyanidin and peonidin on osteoclastogenesis were examined and compared – delphinidin suppressed *in vitro* OC formation, while cyanidin and peonidin did not showed such strong impact on osteoclastogenesis (58). On the other hand, Ostos Mendoza and associates (65) revealed that, peonidin-3-*O*-glucoside applied in low concentrations improved OBs' viability and reduced apoptosis in serum-starved human OBs. This treatment favored the cell growth and OBs differentiation as well as alteration in the expression of proinflammatory interleukins and downregulation of RANKL-expression, which suggests the possible use of peonidin as therapeutic in bone diseases.

In vitro osteogenic effects of delphinidin glycoside-enriched maqui berry extract (MBE) were also examined (66). Up-regulated bone-related gene expression for proteins such as BMP-2, OSX, and OCN indicated that MBE stimulated osteogenic differentiation of MC3T3-E1 cells. The research conducted on RAW264.7 cell line showed that petunidin (>5 μg/ml) significantly suppressed OCs' differentiation and down-regulated expression of genes for c-Fos, NFATc1, matrix metalloproteinase 9 and cathepsin K (67).

Anti-inflammatory effects of ACNs contained in blueberry, blackberry and blackcurrant were compared, and the relationship between their antioxidant capacity and anti-inflammatory effect in macrophages was determined (48). These berries achieved anti-inflammatory effects in macrophages, at least partially, due to inhibition of nuclear translocation of NF-κB independent of the nuclear factor E2-related factor 2 (NRF2)-mediated pathways.

Multiple effects of cya-3-glc on OCs are well-known, but mechanisms of its impact on OBs are not yet completely clarified. Therefore, the effects of cya-3-glc on proliferation and differentiation of the hip joint-derived OBs taken from osteoporotic patients and on mice OB cell line MC3T3-E1 were examined (68). The ability of OBs to mineralize after cya-3-glc treatment as well as the role of ERK signaling pathway in cya-3-glc regulation of OBs were also evaluated. ERK, a crucial member of MAPKs cascades, positively regulates OB differentiation and bone formation (69). Cya-3-glc enhanced OBs' proliferation rate and OBs' mineralization points, up-regulated OCN gene and protein expression, and increased the level of ERK phosphorylation (68), which proves that ERK pathway is involved in cya-3-glc regulation of osteogenic differentiation and indicates that OBs can be targets for prevention and treatment of osteoporosis.

Del-3-rut protects MC3T3-E1 from oxidative damage and promotes osteogenic differentiation of this cell line via PI3K/AKT pathway (1), which means that del-3-rut could be used as dietary supplement for the prevention of OBs' dysfunction in age-related osteoporosis (70). Also, malvidins are considered to be responsible for bone formation by inducing significantly higher calcium deposits in MSCs (71).

Bone healing and regeneration supported with ACNs and berries compounds – *in vivo* studies

Numerous *in vivo* studies reveal valuable relationship between ACNs and bone health. Bone-protective roles of phenolic and flavonoid ingredients derived from dried plum have been shown in rat osteoporosis models (72). The fact that dried plum and blueberry have several identical phenolic and flavonoid ingredients was used in order to perform a research on an ovariectomized (OVX)-rat model of postmenopausal osteoporosis (42). It was hypothesized that blueberry-derived phenolic compounds can prevent bone loss in ovarian hormone deficiency (42). OCN, Col1 and bone-specific ALP were chosen as markers of bone formation, and tartrate-resistant acid phosphatase (TRAP) as a bone resorption marker. Analyses at gene and protein expression level indicated that treatment with 5% blueberry (w/w) prevented bone loss by suppression of ovariectomy-caused bone turnover. OVX-rat model was also applied in order to examine the effects of rabbiteye blueberry on osteoporosis (73). Rabbiteye blueberries effectively inhibited bone resorption, bone loss, and reduction of bone strength of OVX-rats. In another study, blackcurrant extract supplementation reduced trabecular and cortical bone loss in an OVX-mice model (74), which was the same effect estimated upon supplementation of OVX-mice with delphinidin glycoside-enriched MBE (66). On the other hand, bilberry extract that has 15 various ACNs didn't show an impact on bone metabolism on OVX-rat model (19).

Besides OVX-model, which mimics postmenopausal estrogen loss but does not specifically mimic the effects of aging, an age-related model of bone loss can also be used in order to evaluate the influence of ACNs on bone loss prevention. In this model, the influence of blackcurrant extracts on the improvement of mice bone mass was evaluated (75). Young and old female C57BL/6J mice were fed with either a standard chow diet or a chow diet enriched with 1% (w/w) blackcurrant extract for four months. Since supplementation with blackcurrant extracts improved glutathione peroxidase and catalase activity and led to increase in trabecular bone volume, OB surface, and bone mineral content in young mice, it was concluded that consumption of blackcurrant early in life – when substantial amount of bone mass is still present, could prevent aging-associated bone loss.

Besides being the direct bone remodeling mechanisms, high oxidative stress and chronic inflammation can also lead to obesity and, consequently, to bone loss. Low levels of vitamins C and E, carotenoids, superoxide dismutase, glutathione peroxidase, catalase and other plasma antioxidants and antioxidant enzymes in obesity can cause augmented bone resorption (76). Therefore, the effects of blueberry, blackberry and blackcurrant on bone health were examined on a diet-induced obesity mice model. High-fat (HF) diet-induced obese C57BL mice were fed a

HF diet, with or without berry supplementation, for 12 weeks. The results confirmed that there is a negative correlation between fat and bone mass, but that consumption of berries with different ACNs' composition can affect bone turnover via mechanisms that should be clarified in future (76).

It was discovered that pelargonidin-3-*O*-glucoside acts as an anti-inflammatory agent by suppressing the NF-κB pathway in an experimental model of osteoarthritis. In this way, the inflammation and cartilage damage can be reduced as well as the progression of osteoarthritis (77). Petunidin prevented bone mass loss in a RANKL-induced osteopenic mice model (67). Cyanidin-chloride (CC) and cya-3-glc, can regulate bone homeostasis, but the literature data regarding their specific role in osteoclastogenesis are controversial. According to Cheng and associates (78), CC inhibits osteoclastogenesis, hydroxyapatite resorption, and RANKL-induced signal pathways *in vitro* and protects against OVX-induced bone loss *in vivo*. Other data indicate that, in high dosage (> 10 μg/ml), cyanidins suppresses osteoclastogenesis and OCs fusion, but at low dosage (< 1 μg/ml) the effect is opposite (79). Moreover, cya-3-glc improved OBs proliferation and up-regulated OCN gene and protein expression, mainly via ERK1/2 pathway (68).

The application of flavonoids, including ACNs, in the field of BTE is becoming an increasingly attractive way to promote bone healing. Their role, in addition to protecting cells from oxidative stress, is also reflected in the promotion of proliferation and osteogenic differentiation of MSCs (80). Attempts to incorporate flavonoids into different types of biomaterials in order to promote bone defects' healing have proven to be more than successful. Their beneficial effect is reflected in the increase in osteogenic and angiogenic markers' expression, activation of Wnt signaling pathway and reduction of inflammatory factors' levels (34).

The effects of anthocyanins and other flavonoids on bone healing and regeneration in population studies

The information regarding the effects of different flavonoid subclasses on bone health in humans are limited. An observational research conducted in the group of 3160 female twins revealed that total flavonoid intake was positively correlated with BMD of the hip and spine (81). Also, a strong positive correlation between consummation of fruits and BMD and bone mineral content (BMC) in boys and girls (11-14 years), young women (20-34 years), and postmenopausal women (50-70 years) has been reported (82). In addition, positive association between high fruit intake and high BMD in men and women aged 25-64 years was found (83). Another study was conducted only in women — women that consumed high amounts of fruit in childhood had higher BMD of the femoral neck compared to the women that had medium or low intake of fruits during childhood (84).

Conclusion

Based on *in vitro* and *in vivo* studies regarding molecular mechanisms involved in bone healing and regeneration supported with berries' ACNs and on observational research in human populations, it is unequivocally clear that berries' ACNs could possibly be used for the prevention and/or treatment of the certain bone conditions and diseases. Berries' ACNs are part of nature, so they should be considered as naturally widespread therapeutics for bone support. Furthermore, research into the possibility of bone healing and regeneration using substances from natural sources, such as ACNs, may contribute to the development of new, less invasive therapeutic methods. However, some discrepancies are noticed regarding the influence of ACNs on bone healing and regeneration which can be explained by the different experimental models that were chosen, different classes of ACNs that were used, or different concentrations of the same type of applied ACNs. Despite that, a plenty of encouraging results speaks in favor of medical use of ACNs in bone healing and regeneration as safer and cheaper solution for human health in comparison with standard medical therapies. Nevertheless, before implementation of berries as a natural source of bone support, there are some issues left to resolve. First, further studies regarding clarification of molecular mechanisms of ACNs' action in bone metabolism are needed. Then, identification of effective doses of particular ACNs for bone regeneration therapies needs to be determined. Finally, clinical studies for determination of therapeutic efficacy of different types and concentrations of ACNs must be performed. Resolving these issues will make an additional contribution to the prevention and treatment of osteoporosis, which is a global public health problem primarily in the elderly population. Also, it will contribute to the quality of life of people with bone injuries, osteoporosis or other bone diseases.

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