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BERBERINE ENCAPSULATED NANOPARTICLES STIMULATE OSTEOBLAST DIFFERENTIATION *IN VITRO*

Nguyen Thi Hong Minh[1,2,✉], Tran Tuan Anh[2], Le Thi Thuy Duong[3], Nguyen Viet Linh[3], Nguyen Thi Mai Phuong[1, 3]

[1]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology*
[2]*University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology*
[3]*Institute of Biotechnology, Vietnam Academy of Science and Technology*

✉To whom correspondence should be addressed. E-mail: nguyen-thi-hong.minh@usth.edu.vn

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SUMMARY

Berberine has been known as a traditional component for treatment of intestinal-related diseases in Asian countries. Additionally, it possesses a variety of pharmacological properties, which were studied for treating tumor, diabetes, cardiovascular disease, hyperlipidemia, inflammation, bacterial and viral infections, cerebral ischemia trauma, and mental disease. Moreover, berberine has been known as an anti-osteoporotic agent by controlling both osteoclast (bone resorption cells) and osteoblast (bone-forming cells) functions. Beside the beneficial effects of berberine, it has some drawbacks that hindered its applications and resulted in low bioavailability. One of the most drawback characteristics of berberine is that it has poor watery solubility. To overcome these limits, nanotechnology has been used as the primary approach to deliver berberine in different nano-formulations. In this study, a novel berberine nanoparticle (nanoberberine, NBB) with good water dispersion was synthesized to enhance its bioavailability. The result showed that NBB was successfully developed in spherical shape and approximately 559 nm of mean size. Besides, *in vitro* release study revealed that berberine content release from NBB was 3 to 4 times higher than that from free berberine. Moreover, no cytotoxicity was observed for both NBB and berberine on osteoblast MC3T3-E1 cells at the tested concentrations. Additionally, alkaline phosphatase (ALP) activity, a marker for osteoblast differentiation process, was significantly higher in NBB compared to free berberine at the same test concentrations. This result indicated that NBB could be a potential biological agent for inducing bone formation. Overall, our data indicated that NBB could improve bioavailability, especially osteogenesis activity *in vitro* compared to free berberine.

Keywords: *Berberine, nanoparticles, osteoblast differentiation, osteogenesis, osteoporosis*

INTRODUCTION

Bone related problems due to numerous reasons, including accidents, nutritional issues, hormonal imbalance, and ageing are increasing recently (Sanyasi *et al.*, 2016). Bone disorders are among one of the most popular diseases and a public health concerned not only in Vietnam but also in all over the world. Normally, bone tissue in human body is renewable and maintains bone homeostasis by interactions between osteoblasts, bone-forming cells, and osteoclasts, bone-resorption cells. If the activity of osteoclasts is more than osteoblasts, it could lead to an imbalance between bone formation and bone resorption, resulting osteoporosis. The strategies for osteoporosis treatment are focused on reducing osteoclast activity, and/or increasing

osteoblast differentiation. Osteoblasts, cells that originate from mesenchymal stem cells, are responsible for bone formation. They are controlled by various growth factors, cytokines, and hormones (Kassem *et al.*, 1991). Osteoblast differentiation is a necessary process for bone strength and remodeling, which can be divided in three subsequent stages, including proliferation stage, maturation stage, and mineralization stage (Zamurovic *et al.*, 2004). Osteogenesis agents, which could activate osteoblast differentiation, have been used to recover bone loss and improve the microstructure of a cancellous bone (Kim *et al.*, 2014). Therefore, the research trend using osteoblast differentiation-induced factors in order to gain bone loss density and bone homeostasis is a promising strategy for osteoporosis treatment.

Berberine, a natural isoquinoline alkaloid present in several plants, is an essential phytochemical, with wide range of biological activities. Pharmacological and clinical uses of berberine have been extensive studied, which indicated for treating various kind of diseases, including tumor, diabetes, cardiovascular disease, hyperlipidemia, inflammation, bacterial and viral infections, cerebral ischemia trauma, mental disease (Imenshahid, Hosseinzadeh, 2016). In addition, berberine has also been studied its ability as an anti-osteoporotic agent by controlling osteoclast functions and to facilitate osteoblast differentiation (Li *et al.*, 2013; Han *et al.*, 2017; Han, Kim., 2019). Berberine inhibits osteoclast activity through amelioration of RANKL-mediated osteoclast formation and survival, resulting in reducing bone loss (Hu *et al.*, 2008). In osteoblastic cells, berberine enhances bone formation process by inducing osteoblast differentiation through activation of Runx2 by p38 MAPK, resulting in increasing bone density (Lee *et al.*, 2008). Thus, berberine could be a potential agent for treatment of osteoporosis.

Despite the advantages of berberine, it has poor intestinal absorption and oral bioavailability, which have been reported less than 1% in rat (Chen *et al.*, 2011). One of the most important drawbacks of berberine is that it has poor aqueous solubility. Moreover, in osteoporosis treatment, berberine did not show an outstanding result in clinical trials compared to existing osteoporosis agents (Nam *et al.*, 2020). Therefore, clinical use of berberine is limited. Considering all the foregoing hints, it is necessary to use nanotechnology, which has been considered as main strategy to form berberine encapsulated nanoparticles with better water dispersion and higher bioavailability. There have been many different types of formulation with specific nanocarriers to synthesize berberine in nanoparticles form, including polymeric based, magnetic mesoporous silica based, lipid based, dendrimer based, graphene based, silver and gold nanoparticles (Mirhadi *et al.*, 2018). In this study, berberine encapsulated nanoparticles were synthesized using lipid based nanocarriers. Lipid-based nanoparticles, which include emulsions, vesicular systems, as well as lipid particulate systems, endure the gain of being the smallest destructive in favour of *in vivo* applications (Puri *et al.*, 2009). Nanoemulsions, colloidal dispersion systems that compose of two immiscible liquids combined alongside with emulsifying agents (surfactants and co-surfactants) to form a solitary phase, are usefull in creating liquid formulations of weakly water-soluble drugs (Gurpreet, Singh, 2018). Oil-in-water nanoemulsions can additionally be utilized to stabilize drugs to facilitate hydrolytic and oxidative degradation (Tamilvanan, 2004). Nanoemulsions provide a number of benefits over other dosage forms, which includes improved rate of absorption, deminished variability in absorption, protection from oxidation and hydrolysis, aqueous dosage form for water insoluble drugs, enhanced bioavailability for many drugs, delivery systems to enhance efficacy whilst limit total dose and side effects (Gurpreet, Singh, 2018; Mirhadi *et al.*, 2018). Thus, considering the advantanges of nanoemulsion method as well as the simplicity in performing experiment, we have been synthesized NBB in nanoemulsion form for further evaluation. Morphology and particle size of NBB was also verified. *In vitro* release assay

of NBB was performed to compare its release rate to berberine free form. Finally, biological activity of NBB on osteoblast differentiation was preliminarily determined by investigating its effects on cell viability and alkaline phosphatase (ALP) activity, an important marker for bone generation.

MATERIALS AND METHODS

Materials

The normal cell line MC3T3-E1 (pre-osteoblast cells) was obtained from American Type of Culture Collection/ATCC (Manassas, USA). Minimum Essential Medium α (MEM α) was obtained from Gibco BRL (Life Technologies, USA). MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) reagent, berberine chloride, trypsin–EDTA, penicillin/streptomycin, p-Nitrophenyl Phosphate (pNPP) substrate, ascorbic acid, dexamethasone, β-glyceraol phosphate, and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, USA). Other chemicals and reagents were of analytical grade.

Synthesis of NBB

Berberine chloride was dissolved in ethanol using a magnetic stirrer at 400-500 rpm in parallel with heating at 40-50˚C until the solution was homogenous. In the next step, the carrier mixture of polyethylene glycol (PEG) 400 and ethylene glycol (EG) was prepared using a sonicator in 2 h at room temperature. Finally, mixture of berberine/PEG 400/EG/soybean oil/H_2O at a suitable ratio was formed at room temperature. The free-particles that were not encapsulated or not distributed well in water were removed by incubating overnight and centrifuging at 5000 *g* for 10 min.

Surface morphology of NBB

Morphology of NBB was assessed by observing under high resolution field emission scanning electron microscope (FE-SEM, Hitachi-S4800, Japan). The size of NBB was deduced from the FE-SEM picture by using ImageJ software.

In vitro drug release study

In vitro release study was performed in different media, including PBS pH 5.4, PBS pH 7.4, and distilled water following the membrane dialysis method in order to examine the release of berberine from NBB compared with berberine free form (Lam *et al.*, 2015). The samples were packed separately into dialysis bags, which were soaked in double-distilled water before use. The berberine content will be diffused into dissolution media by using 3500 Da dialysis bag (Biotopped, USA), whereas other content (carriers) are retained in the bag. The levels of berberine release content at desired time intervals were measured by UV/VIS spectrophotometer at 345 nm.

Cell culture and cell viability assay

Cells were cultured in α-MEM containing 10% FBS and antibiotics at 37 ˚C in a 5% CO_2 humidified incubator. To induce osteogenic differentiation in MC3T3-E1 cells, culture media were changed to ODM (alpha MEM supplemented with 50 µg/mL ascorbic acid, 10^{-8} M dexamethasone, and 10 mM β-glycerolphosphate). MC3T3-E1 cells were seeded at concentration of 1×10^4 cells/well in a 96-well plate. They were incubated after seeding and treating with different concentrations of samples. After incubation time, the cells were harvested for each experiment. The untreated cells and the carrier without berberine loaded were used as controls.

Cell viability was determined by using MTT assay. In brief, MC3T3-E1 cells were seeded and then treated with berberine and nanoberberine at different concentrations. After 48 h incubation and treatment, medium was removed and the cells were incubated with a solution of 0.5 mg/mL MTT for 4 h at 37 ˚C and 5% CO_2. The formazan crystals were then solubilized in dimethyl sulfoxide (DMSO), and the optical density was measured by monitoring the signal at 570 nm using a microplate reader (PowerWave XS model; BioTek Instruments, Inc., Winooski, USA). Relative cell viability treated with berberine or nanoberberine was calculated

compared to the non-treated group. Results were presented as mean ± SD, and each value is the average of triplicate cultures.

ALP activity

To evaluate ALP activity, MC3T3-E1 cells were seeded into 96-well plates in ODM medium supplemented with 10% FBS. The culture medium was then replaced by a new ODM medium supplemented with/without the agents to be tested and incubation continued for 7 days. Cells were then rinsed with phosphate-buffered saline (PBS) and adding 100 µL pNPP following the instruction of the manufacture's protocol. Finally, the ALP activity in the cells was measured at 405 nm using a spectrophotometer. ALP activity is calculated following the below equation:

ALP activity (%) = (A-Ao) / Ao x 100%

Where: A is the relative absorbance with sample, and Ao is the relative absorption without sample.

Statistical analysis

All data are presented as means ± standard deviation. Statistical analyses among the groups were determined, and statistical significances were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Characterization of NBB

In this study, particle size and morphology of nanoparticles were analyzed by FE-SEM. The result showed that the nanoparticles were uniform and homogenous with spherical shape (Figure 1A). However, the size of particles has been in wide range variation (Figure 1B). We observed most of the synthesized particles were at 200 - 600 nm in size, but there was also a small population of particles at around 800 - 1000 nm. The mean size was found to be 559 nm using ImageJ software, therefore, NBB was successfully synthesized in nanoscale.

The use of nano-formulations to encapsulate berberine has been previously reported. Pund and others (2014) has developed other formulations to delivery berberine in liquid and solid form by self-nanoemulsifying drug delivery system. This synthesized beberine formulation has globule size of 17 - 45 nm with the improved anti-inflammatory and anti-angiogenic activities (Pund et al., 2014). Another study published in 2014 by Wang and others showed that berberine has been delivered in a solid lipid-based system with mean size of 81.44 nm. This formulation has improved antitumor activity compared to free berberine (Wang et al., 2014). Besides, berberine has also been delivered by bovine serum albumin with an average nanoparticle size of approximately 395 nm for liver fibrosis therapy (Lam *et al.*, 2015). Moreover, berberine has been delivered by conjugation and encapsulation approach to form G4 PAMAM dendrimers (Gupta *et al.*, 2017). The obtained G4 PAMAM-berberine formulations have approximately 100-200 nm in size. This formulation was found to have anticancer activity against MCF-7 and NDA-MB- 468 breast cancer cells (Gupta *et al.*, 2017). Overall, there are different formulations to delivery berberine in nanoparticles with variety of size range in nanoscale for better bioactivities compared to free berberine. However, all NBB products synthesized using the reported formulations did not show osteogenic activity in our study. Therefore, although the obtained NBB in our study had bigger particle size, it showed an induction of ALP activity in osteoblasts."

***In vitro* profile of NBB**

The pH varieties flanked by diverse human body tissue and cellular environments can be a restricting factor in the profile of drug release. Thus, the release profile of NBB was examined in different media, including water, PBS pH 7.4, and PBS pH 5.4 for up to 100 h duration of time. Berberine content release from NBB and berberine was measured by UV-vis spectrophotometer at 345 nm wavelength, and presented in Figure 2. The results showed that berberine released from NBB was ca. 3 times higher than that from free berberine in PBS media and ca. 4 times higher in distilled water. Berberine released from NBB has initially

burst in the first 2 h, reaching to 80% and no more release was observed after that time point. In contrast, berberine released in water from unloaded form was almost not recorded in the first 2 h, and release rate was very slowly, reaching to only approximately 20% after 50 h. In PBS, berberine release content from NBB and free berberine was quite the same pattern in the first 4 h, but increase distance between them after that. The highest release content in PBS media was about 50% for NBB and 20% for free berberine. The obtained data revealed that nanoparticles form could enhance bioavailability of berberine compared to the original one. Besides, the release content of berberine from nanoparticles in both water and PBS media was strongly higher compared to free berberine. These obtained data could be resulted from smaller size (nano size) and better water distribution of berberine loaded in nanoparticles than that of berberine free form.

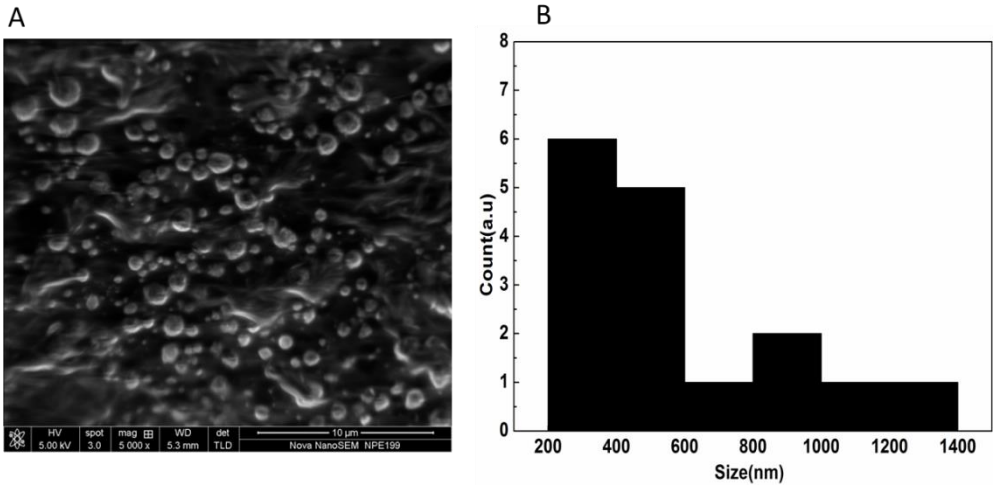

Figure 1. FE-SEM image of nanoberberine. (A) FE-SEM image under magnification of 50k. (B) Particle size of NBB deduced from FE-SEM picture using ImageJ software.

In vitro cytotoxicity of NBB on MC3T3-E1 cells

To test whether NBB/berberine has toxic to the normal osteoblastic cells MC3T3-E1, MTT assay was performed. The result in Figure 3 indicated that both NBB and berberine did not show any cytotoxicity on the test cell line at concentration of 0.1; 0.25; 0.5; and 1.0 µg/mL. Moreover, at low concentration (0.1 – 1 µg/mL), both test agents could even enhance cell proliferation up to 20% compared to non-treated cells. Thus, these concentrations were suitable for the next experiment on osteogenesis activity of NBB/berberine on MC3T3-E1 cells.

Alkaline phosphatase (ALP) activity of NBB/berberine on MC3T3-E1 cells

Bone cell (osteoblast) differentiation can be identified in three stages, including cell proliferation; matrix maturation; and matrix mineralization (Olivares-Navarrete *et al.*, 2012). During cell proliferation and matrix maturation stage, ALP activity is highly expressed. Thus, ALP activity could be used as a marker to determine whether the sample treatment could induce osteoblast cell differentiation or not. The results in Figure 4 indicated that both NBB and berberine treatments could induce ALP activity in MC3T3-E1 cells in a dose-dependent manner. However, enhanced ALP activity by NBB treatment was higher than that induced by free berberine. NBB could induce ALP activity approximately 30% at concentration of 1 µg/mL, whereas free berberine induced the enzyme activity only 20% at the same concentration. The obtained results indicated that berberine loaded

nanoparticles enhanced osteogenic activity of osteoblast cells compared to the unloaded form. This result was also consistent with previous studies on improvement of pharmacological effects by berberine-loaded nanoparticles (Mirhadi *et al.*, 2018). For example, berberine in nanoemulsion form was found to enhance anti-inflammatory in rats and anti-angiogenic activity in chick embryo (chick chorioallantoic membrane assay) (Pund *et al.*, 2014); solid lipid nanoparticle encapsulation of berberine improved its anti-tumor activity by inhibiting cell proliferation on MCF-7, HepG 2, and A549 cancer cells (Wang *et al.*, 2014). In our study, we indicated that nanoberberine could enhance osteogenic activity in MC3T3-E1 cells by increasing ALP activity more pronounced compared to free berberine

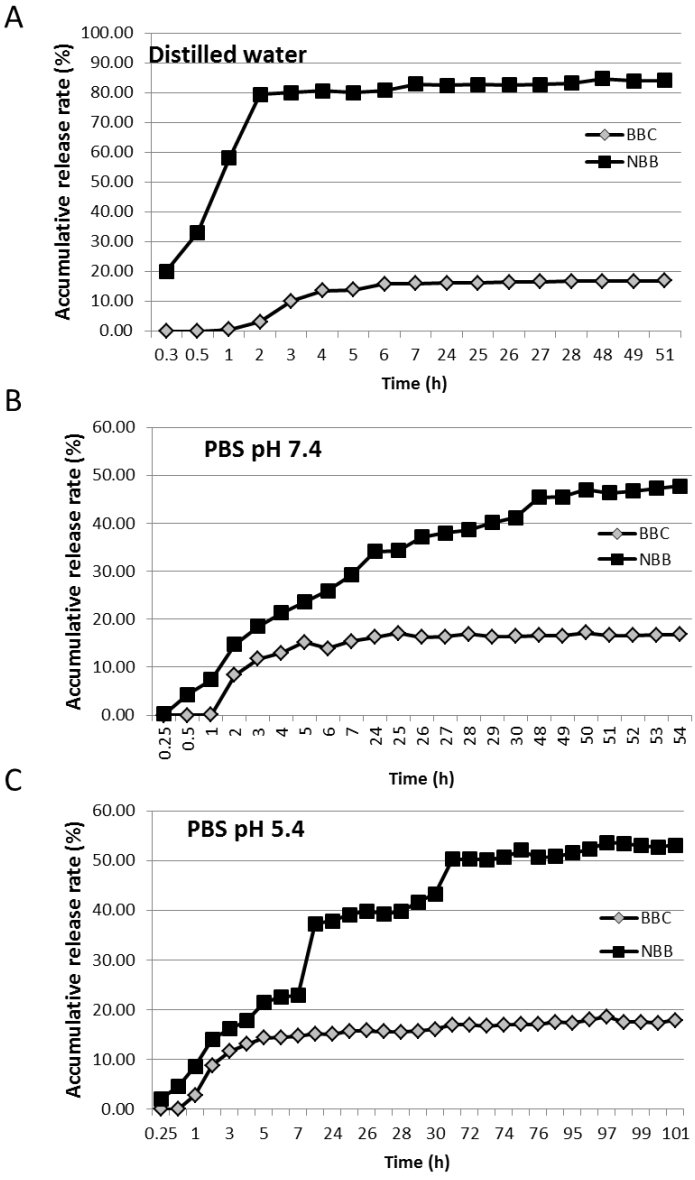

Figure 2. *In vitro* release profile of nanoberberine (NBB) and berberine (BBC) in different media.

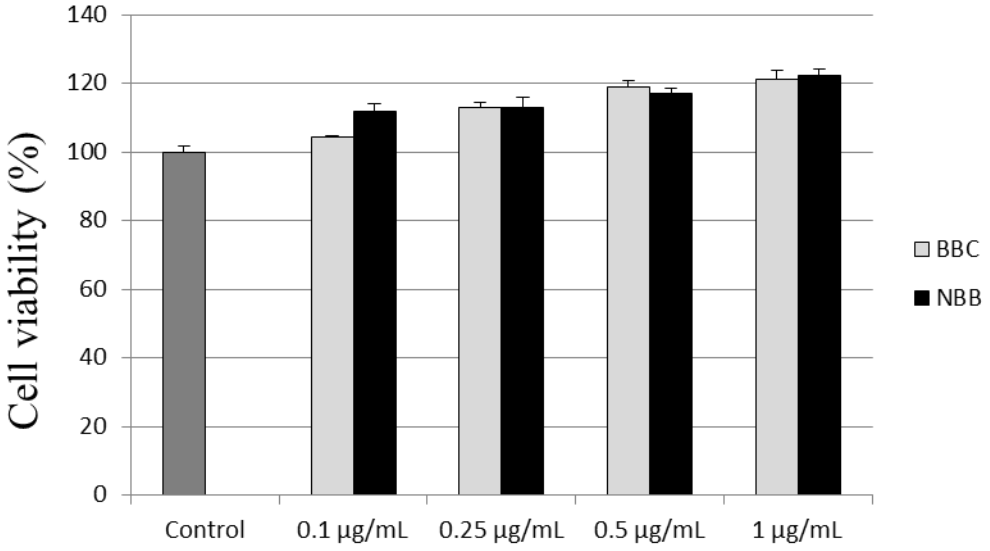

Figure 3. Cell cytotoxicity of nanoberberine (NBB) and berberine (BBC) on MC3T3-E1 cells. Data of relative cell viability was calculated as percentage compared to the non-treated group (Control).

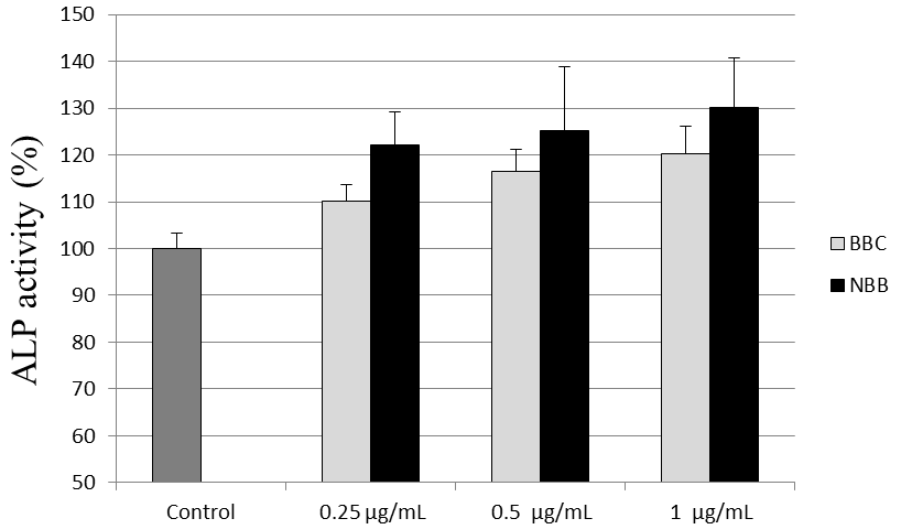

Figure 4. Alkaline phosphatase (ALP) activity of nanoberberine (NBB) and berberine (BBC) on MC3T3-E1 cells. MC3T3-E1 cells were treated with different concentration of NBB/BBC (0.25 – 1 µg/mL) for 48 h. Data of ALP activity were calculated as percentage compared to the non-treated group (Control). Each value is the average of triplicate cultures, and each bar indicates means ± S.D.

CONCLUSIONS

Berberine was successfully synthesized in nanoparticle form of ca.559 nm in size and spherical in shape using a new formulation. The nanoparticle did not show any cytotoxicity effect on the normal MC3T3-E1 cells at concentrations less than 1 µg/mL, and induce ALP activity more pronounced than free berberine, suggesting that it is has potential osteogenic activity. However, further studies will be required to fully evaluate osteogenic activity of NBB in both *in vivo* and *in vitro* experiments for practical applications.

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HẠT NANO BỌC BERBERINE CẢM ỨNG BIỆT HÓA TẾ BÀO TẠO XƯƠNG *IN VITRO*

Nguyen Thi Hong Minh[1, 2], Tran Tuan Anh[2], Le Thi Thuy Duong[3], Nguyen Viet Linh[3], Nguyen Thi Mai Phuong[1, 3]

[1]*Học viện Khoa học và Công nghệ, Viện Hàn lâm Khoa học và Công nghệ Việt Nam*
[2]*Trường Đại học Khoa học và Công nghệ Hà Nội, Viện Hàn lâm Khoa học và Công nghệ Việt Nam*
[3]*Viện Công nghệ sinh học, Viện Hàn lâm Khoa học và Công nghệ Việt Nam*

TÓM TẮT

Berberine đã được biết đến như một thành phần truyền thống để điều trị các bệnh liên quan đến đường ruột ở các nước châu Á. Bên cạnh đó, nó chứa nhiều tính chất dược lý được sử dụng để điều trị một số bệnh như ung thư, tiểu đường, tim mạch, tăng lipid máu, viêm nhiễm do vi khuẩn và virus, chấn thương do thiếu máu não và bệnh thần kinh. Hơn nữa, berberine còn được biết đến như một chất chống loãng xương bằng cách kiểm soát chức năng của cả tế bào hủy xương và tạo xương. Bên cạnh các tác dụng có lợi của berberine, nó chứa một số nhược điểm gây cản trở các ứng dụng và dẫn đến tính sinh khả dụng thấp. Một trong những điểm hạn chế của berberine là tan kém trong nước. Để khắc phục những giới hạn này, công nghệ nano đã được sử dụng như là phương pháp chính để vận chuyển berberine trong các công thức khác nhau ở dạng nano. Trong nghiên cứu này, hạt nano berberine mới (nanoberberine, NBB) có sự phân tán tốt trong nước đã được tổng hợp để làm gia tăng tính khả dụng sinh học của nó. Kết quả cho thấy NBB đã được tổng hợp thành công ở dạng hình cầu và kích thước trung bình khoảng 559 nm. Bên cạnh đó, nghiên cứu nhả hạt *in vitro* cho thấy hàm lượng berberine được giải phóng từ NBB cao gấp 3 đến 4 lần so với berberine dạng tự do. Hơn nữa, NBB và berberine đều không gây độc đối với các tế bào xương MC3T3-E1 ở các nồng độ thử nghiệm. Ngoài ra, hoạt tính phosphatase kiềm (ALP), một dấu hiệu phân tử của quá trình biệt hóa tế bào tạo xương, khi xử lý với NBB là cao hơn đáng kể so với berberine ở cùng nồng độ thử nghiệm. Kết quả này chỉ ra rằng NBB có thể là một tác nhân sinh học tiềm năng cho cảm ứng sự hình thành xương. NBB có thể cải thiện tính sinh khả dụng, đặc biệt là hoạt tính tái tạo xương *in vitro* so với berberine ở dạng tự do.

Từ khóa: *Berberine, hạt nano, biệt hóa tế bào tạo xương, tái tạo xương, loãng xương*