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Effect of metformin on cell growth and differentiation in cultured odontoblasts

조선대학교 대학원

치의학과

오 창 영

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배양 상아질모세포 성장과 분화에 미치는
metformin의 효과

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이 논문을 치의학 석사학위신청 논문으로 제출함.

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TABLE OF CONTENTS

LIST OF FIGURES

ABSTRACT in KOREAN

배양 상아질모세포 성장과 분화에 미치는 metformin의 효과

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상아질은 치아의 중요한 구성 성분으로 치관과 치근의 대부분을 구성하며, 상아질모세포는 유기기질과 당단백 및 dentin sialophosphoprotein을 합성하고 분비한다. 상아질의 기질이 침착됨에 따라 상아질모세포가 세포돌기를 원심 쪽으로 내어 상아세관에 묻히게 되고, 결과적으로 상아질모세포는 치수 가장자리 상아질의 내면에 위치하여 상아질을 유지하게 된다. 그러나 상아질모세포의 분화기전과 상아질의 형성과정을 조절하는 인자 그리고 그와 관련된 분자생물학적 기전은 아직까지 명확히 알려져 있지 않다. French lilac(*Galega officinalis*)에서 유래한 metformin(1,1-dimethylbiguanide hydrochloride)은 type 2 당뇨병 치료제 이며, 최근 들어 다낭성 난소증후군 및 비알코올성 지방간 질환에도 효과가 있고 특정 암세포의 성장을 억제할 수 있다고 보고되었

다. 그러나 상아질모세포의 분화기전과 관련된 metformin에 관한 자료는 매우 부족하다. 따라서 본 연구에서는 상아질모세포로서 MDPC-23 세포주의 분화과정에서 metformin에 의한 상아질모세포 분화촉진 효과를 밝히고자 하였다.

상아질모세포의 분화과정에서 metformin에 의한 상아질모세포의 특성변화를 분석하기 위해, 생쥐 치유두세포 유래의 상아질세포 전구세포주인 MDPC-23 세포주에서 분화유도와 metformin을 처리한 후, Alizarine red-S 염색, 세포형태 관찰 및 qPCR 분석을 시행하여 다음과 같은 결과를 얻었다.

1. MDPC-23 세포주의 분화유도에서 대조군에 비해 metformin 처리군에서 석회화 결절현상이 뚜렷이 증가하였다.
2. MDPC-23 세포주의 분화유도 과정에서 metformin은 ALP와 Col I mRNA의 발현을 증가시키면서 가속화시켰다.
3. MDPC-23 세포주의 분화유도 과정에서 metformin은 DSPP와 DMP-1 mRNA의 발현을 증가시키면서 가속화시켰다.
4. Metformin은 MDPC-23 세포주의 증식에는 영향을 주지 않았다.

본 연구의 결과로 상아질모세포 MDPC-23 세포주 분화과정에서 metformin이 상아질모세포의 분화를 촉진시킬 수 있음을 알 수 있었다. 또한 본 실험의 결과로, 상아질 수복과 재생에 관한 하나의 연구방향을 제시할 수 있을 것으로 사료된다.

중심어: metformin, 상아질모세포, 세포분화, 광화

I. INTRODUCTION

Tooth formation is regulated by sequential and reciprocal epithelial-mesenchymal interactions [1]. Dental epithelial cells from the dental organ differentiate into ameloblasts, while ectomesenchymal cells from the dental papilla differentiate into odontoblasts [1,2]. Dentin, which forms the bulk of the tooth, is a mineralized tissue composed of odontoblasts [3,4]. Odontoblasts are differentiated from ectomesenchymal cells and are involved in the secretion of the organic matrix during odontoblast differentiation [3-5]. This matrix contains a mixture of collagenous and non-collagenous proteins, which subsequently mineralize to form dentin, the main hard tissue of a tooth [3-5]. Dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP-1), which are synthesized and secreted by odontoblasts, are regarded as odontoblast differentiation markers [1,4,5].

Many researchers suggest that signaling molecules in the bone morphogenetic protein, fibroblast growth factor and wingless families as well as transcription factors such as Runx2 are involved in the odontoblast differentiation [5-7]. Indeed, the exquisite balance between conserved signaling pathways and transcription factors is important for all aspects of odontoblast differentiation [5-7]. However, the exact molecular mechanisms underlying odontoblast differentiation are not well understood.

Metformin (1,1-dimethylbiguanide hydrochloride), derived from French lilac (*Galega officinalis*), is an anti-diabetic drug that belongs to the biguanide class, and a first-line drug prescribed for patients with type 2 diabetes [8-10]. It exerts

its anti-diabetic effect by reducing hepatic glucose production [11,12]. Moreover, it has been known to used in the treatment of polycystic ovarian syndrome [13] and non-alcoholic fatty liver disease [14]. In addition, metformin has been reported to have anti-cancer effects in variety of cancers including colon cancer, ovarian cancer, lung cancer, breast cancer and prostate cancer and so on [15-20]. Nevertheless, at present, the role of metformin in regulating odontoblast differentiation remains unclear.

In this study, therefore, the effect of metformin on regulating odontoblast differentiation was investigated in MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells.

II. MATERIALS AND METHODS

1. Materials

Metformin, Alizarine red S and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). All other reagents were of analytical grade. In preliminary studies to investigate the effect of metformin on the differentiation of MDPC-23 odontoblastic cells, the 100 μM metformin started to show the difference from the control group (metformin non-treated control group). In the subsequent experiments of this study, therefore, MDPC-23 odontoblastic cell differentiation was examined using the concentration of 100 μM metformin.

2. Cell cultures

MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells were kindly provided by Dr. J. E. Nör (University of Michigan, Ann Arbor, MI, USA). The MDPC-23 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and antibiotics (Invitrogen, Carlsbad, CA, USA) in a 5% CO_2 atmosphere at 37℃ [21,22]. To induce cell differentiation and mineralization, confluent MDPC-23 cells were treated with 50 ㎍/ml ascorbic acid and 10 mM β-glycerophosphate for up to 7 days [4,23].

3. Alizarine red S staining

The cells were fixed with 70% ethanol for 20 min and stained with 1% Alizarine red S (Sigma - Aldrich Corp., St. Louis, MO, USA) in 0.1% NH_4OH at pH 4.2 - 4.4. The mineralization assays were performed by staining MDPC-23 cells with Alizarin red S solution. The cells were evaluated at 0, 4 and 7 days. To quantify the intensity of mineralization, we measured density of stained nodules by colorimetric spectrophotometry. The stained cells were collected by centrifugation at 13,000 rpm for 10 min at 4°C. Cell lysate was solubilized with 0.5 ml of 5% SDS in 0.5 N HCl for 30 min at room temperature. Solubilized stain (0.1 ml) was transferred to wells of a 96-well plate, and absorbance was measured at 405 nm.

4. Quantitative PCR (qPCR) analysis

To perform qPCR, the total RNA was isolated using TRIzol reagent (Gibco BRL, Grand Island, NY, USA) according to the manufacturer's instructions. Reverse transcription was carried out with 1 ㎍ of total RNA using the TermoScript RT-PCR system (Invitrogen, Carlsbad, CA, USA). The levels of alkaline phosphatase (ALP) (ALP-F, 5'-CTCTCCGAGATGGTGGAGGT-3'; ALP-R, 5'-GTC TTCTCCACCGTGGGTCT-3'), type I collagen (Col I) (Col I-F, 5'-TAAGTTGCC AAGAACGTGCC-3'; Col I-R, 5'-AATTGAAAGCCAGGAGGCAT-3'), dentin sialophosphoprotein (DSPP) (DSPP-F, 5'-ATAGCACCAACCATGAGGCT-3'; DSPP-R, 5'-CTTTTGTTGCCTTTGTTGGG-3'), dentin matrix protein-1 (DMP-1)

(DMP-1-F, 5'-CGGCTGGTGGTCTCTCTAAG-3'; DMP-1-R, 5'-ATCTTCCTGGG ACTGGGTCT-3'), bone sialoprotein (BSP) (BSP-F, 5'-AAGAAAATGGAGACGGC GAT-3'; BSP-R, 5'-CACCTGCTTCAGTGACGCTT-3') and GAPDH (GAPDH-F, 5'-TGCATCCTGCACCACCAACT-3'; GAPDH-R, 5'-CGCCTGCTTCACCACCTTC -3') inductions were measured by qPCR, and visualized by DNA agarose gel electrophoresis. The differences in expression were presented as a histogram after densitometry using a VersaDoc™ imaging system (Bio-Rad, Hercules, CA, USA).

5. Cell proliferation assay (MTT assay)

The MDPC-23 cells were seeded at a density of 4 X 10^4 cells/well in 24-well plates and allowed to attach to the well overnight. After incubation, the cultured cells were treated with metformin. The cells were incubated with metformin at various defined concentrations (1, 3, 10, 30, 100 and 300 μM) for 1 and 2 days at 37℃. After incubation under the defined conditions, the cells were incubated for another 4 h in 20 ㎕ of 5 mg/ml MTT solution. To dissolve the formazan transformed from MTT, the cells were resuspended in 150 ㎕ dimethyl sulfoxide and the optical density of the solution was determined using a spectrometer at an incident wavelength of 495 nm [24]. The experiments were repeated four times, independently.

6. Statistical analysis

All experiments were performed at least in triplicate. The results were presented

as the mean ± SEM. Statistical significance was analyzed using a Student's t-test for two groups and one-way analysis of variance for multi-group comparisons. All statistical analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). A p value <0.05 was considered statistically significant.

Ⅲ. RESULTS

1. Metformin accelerates mineralization in MDPC-23 odontoblastic cells.

To examine the effect of metformin on odontoblastic cell mineralization, the mineralization assays were performed by staining MDPC-23 cells with Alizarine red S solution. MDPC-23 cells were cultured for 7 days in differentiation media treated with 100 μM metformin and the mineralized nodules were evaluated by Alizarin red S staining. In control MDPC-23 cells and metformin treated MDPC-23 cells, mineralized nodules appeared after 4 days of culture (Fig. 2). The metformin-treated MDPC-23 cells showed mineralized nodules after 4 and 7 days, and the mineralized nodules significantly increased compared to control MDPC-23 cells (Fig. 2).

2. Metformin changes the expressions of differentiation marker genes in MDPC-23 odontoblastic cells.

To study the potential role of metformin in MDPC-23 cell differentiation, the qPCR analyses were performed using ALP, Col I, DSPP, DMP-1, BSP and GAPDH primers in MDPC-23 cells treated with metformin. In control MDPC-23 cells, the ALP expression appeared after 7 days of culture (Fig. 3). In contrast, to control MDPC-23 cells, the ALP expression appeared after 4 days of culture in metformin-treated MDPC-23 cells, and significantly increased compared to control

MDPC-23 cells (Fig. 3). Furthermore, in control MDPC-23 cells, the Col I expression appeared after 7 days of culture (Fig. 4). In contrast to control MDPC-23 cells, the Col I expression appeared after 4 days of culture in metformin treated MDPC-23 cells, and significantly increased compared to control MDPC-23 cells (Fig. 4). In addition, the qPCR results showed that metformin enhanced the higher expression of DSPP, along with an increase in their activities compared to control cells (Fig. 5). And also, the qPCR results showed that the expression of DMP-1 was gradually up-regulated in MDPC-23 cells treated with metformin (Fig. 6). On the other hand, the expression of BSP, one of osteogenic differentiation marker gene, was not observed in control MDPC-23 cells and metformin-treated MDPC-23 cells (Fig. 7)

3. Metformin does not alter the cell proliferation in MDPC-23 odontoblastic cells.

To investigate the effect of metformin in MDPC-23 cell proliferation, the MTT assays were performed in MDPC-23 cells treated with metformin. As shown in Fig. 8, metformin did not alter the cell proliferation in the MDPC-23 cells. Taken together, these results were suggesting that metformin was promoting the odontogenic specific differentiation without alteration the cell proliferation in MDPC-23 odontoblastic cells.

Ⅳ. DISCUSSION

Metformin, derived from French lilac, is an oral hypoglycemic drug that is widely used in the world [8-10]. Moreover, it has been studied in biological events, including anti-cancer activities [15-20], polycystic ovarian syndrome [13] and non-alcoholic fatty liver disease [14]. However, the physiological role of metformin in the regulation of odontoblast differentiation is not entirely clear. In this study, therefore, the effect of metformin on regulating odontoblast differentiation was examined in MDPC-23 odontoblastic cells. This study reports here that metformin promoted the MDPC-23 odontoblastic cell differentiation.

The MDPC-23 cells are immortalized undifferentiated dental papilla cells that are capable of differentiating into odontoblasts that express DSPP (a dentin-specific gene) and forming mineralized nodules [1]. To determine the effect of metformin on the stage of odontoblast differentiation *in vitro*, MDPC-23 cells were cultured in differentiation medium for up to 7 days with metformin, and the formation of mineralized nodules was evaluated by Alizarin red S staining. Our results revealed the presence of mineralized nodules from 4 days in control MDPC-23 cells and metformin treated MDPC-23 cells, and the mineralized nodules significantly increased compared to control MDPC-23 cells in the metformin-treated MDPC-23 cells (Fig. 2).

In addition, the expression levels of ALP and Col I mRNAs, well known markers of odontoblastic differentiation [1,4], gradually increased from days 4 to 7, and up-regulated compared to control MDPC-23 cells in the metformin-treated

MDPC-23 cells (Fig. 3 and 4). Furthermore, to determine whether metformin induces the odontogenic specific differentiation in MDPC-23 cells, it was measured the expressional levels of DSPP and DMP-1, which are well known representative markers to identify the odontogenic differentiation [1,4,6,25]. Our qPCR results showed that the expressions of DSPP and DMP-1 were gradually up-regulated in MDPC-23 cells treated with metformin (Fig. 5 and 6). By contrast, the expression of BSP, one of osteogenic differentiation marker genes [1,4,26], was not observed in control MDPC-23 cells and metformin-treated MDPC-23 cells (Fig. 7). Taken together, these data suggest that metformin may positively accelerate the differentiation of MDPC-23 odontoblastic cells.

Next, to examine the effect of metformin on MDPC-23 cell proliferation, the MTT assays were performed in MDPC-23 cells treated with metformin. In the present study, while metformin accelerated mineralization in MDPC-23 odontoblastic cells, metformin did not alter the cell proliferation in the MDPC-23 cells (Fig. 8).

In conclusion, these results suggested that the metformin in MDPC-23 mouse odontoblastic cells derived from mouse dental papilla cells facilitate the odontoblast differentiation and mineralization without alteration the cell proliferation. However, the mechanisms of odontoblast differentiation induced by metformin are not yet completely understood. Further studies will reveal the precise cellular and molecular mechanisms of odontoblast differentiation induced by metformin. Overall, metformin might be considered a critical candidate as an odontoblastic cell differentiation molecular target for the development of therapeutic agents in the dental medicine.

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Ⅵ. FIGURE LEGENDS

Fig. 1. Chemical structure of metformin.

Fig. 2. Effect of metformin on the mineralization in MDPC-23 odontoblastic cells. Confluent cultures of MDPC-23 cells were maintained in complete medium with the addition of differentiation cocktail (50 ㎍/ml ascorbic acid and 10 mM β-glycerophosphate). MDPC-23 odontoblastic cell mineralization was altered by metformin. (A) Mineralized nodule formation in MDPC-23 cells. MDPC-23 cells were treated with 100 μM metformin for 7 days, and the mineralization was evaluated by Alizarine red S staining (0; 0 day after treatment with differentiation media, 4; 4 days after treatment with differentiation media, 7; 7 days after treatment with differentiation media, Control; treatment with differentiation media only, Metformin; treatment with differentiation media and 100 μM metformin). (B) Quantification of mineralization was accessed by colorimetric spectrophotometry. Each data point represents the mean ± SEM of four experiments. $^{**}p<0.01$ vs. control (the control cells were measured in the absence of metformin treatment).

Fig. 3. Effect of metformin on alkaline phosphatase (ALP) mRNA expression. Total RNA was isolated using TRIzol reagent and reverse transcription was carried out with 1 ㎍ of total RNA using the TermoScript RT-PCR system. The PCR products were electrophoresed on a 1.5% agarose gel and

visualized with ethidium bromide. (A) Agarose gel electrophoresis was performed on the RT-PCR products from MDPC-23 cells. (B) The quantitative data for (A) were analyzed by using Imagegauge 3.12 software after GAPDH normalization. The percentage of ALP mRNA expression was calculated as a ratio of GAPDH band. Each data point represents the mean ± SEM of four experiments. $^{*}p<0.05$ vs. control (the control cells were measured in the absence of metformin treatment).

Fig. 4. Effect of metformin on type Ⅰ collagen (Col Ⅰ) mRNA expression. (A) Agarose gel electrophoresis was performed on the RT-PCR products from MDPC-23 cells. (B) The percentage of Col Ⅰ mRNA expression was calculated as a ratio of GAPDH band. Each data point represents the mean ± SEM of four experiments. $^{*}p<0.05$ vs. control (the control cells were measured in the absence of metformin treatment).

Fig. 5. Effect of metformin on dentin sialophosphoprotein (DSPP) mRNA expression. (A) Agarose gel electrophoresis was performed on the RT-PCR products from MDPC-23 cells. (B) The percentage of DSPP mRNA expression was calculated as a ratio of GAPDH band. Each data point represents the mean ± SEM of four experiments. $^{*}p<0.05$ vs. control (the control cells were measured in the absence of metformin treatment).

Fig. 6. Effect of metformin on dentin matrix protein-1 (DMP-1) mRNA expression. (A) Agarose gel electrophoresis was performed on the

RT-PCR products from MDPC-23 cells. (B) The percentage of DMP-1 mRNA expression was calculated as a ratio of GAPDH band. Each data point represents the mean ± SEM of four experiments. $^{**}p<0.01$ vs. control (the control cells were measured in the absence of metformin treatment).

Fig. 7. Effect of metformin on bone sialoprotein (BSP) mRNA expression. (A) Agarose gel electrophoresis was performed on the RT-PCR products from MDPC-23 cells. (B) The percentage of BSP mRNA expression was calculated as a ratio of GAPDH band. Each data point represents the mean ± SEM of four experiments.

Fig. 8. Measurement of MDPC cell proliferation by metformin. The cells were treated with metformin at various defined concentrations (1, 3, 10, 30, 100 and 300 μM) for 1 and 2 days at 37°C. The cell proliferation was determined by MTT assays after metformin treatment. The experiments were repeated four times, independently.

Ⅶ. FIGURES

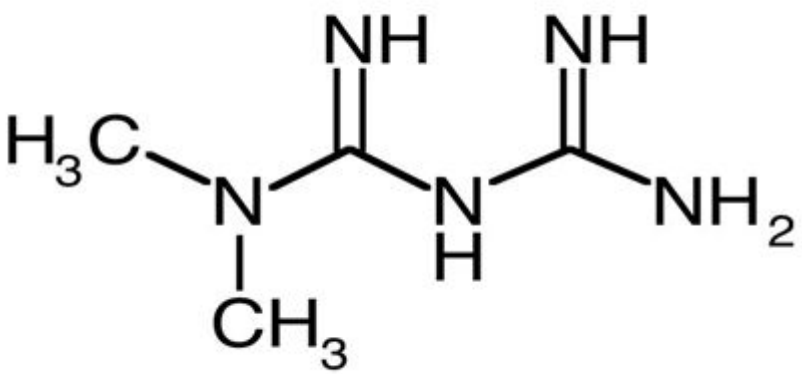

Fig. 1. Chemical structure of metformin.

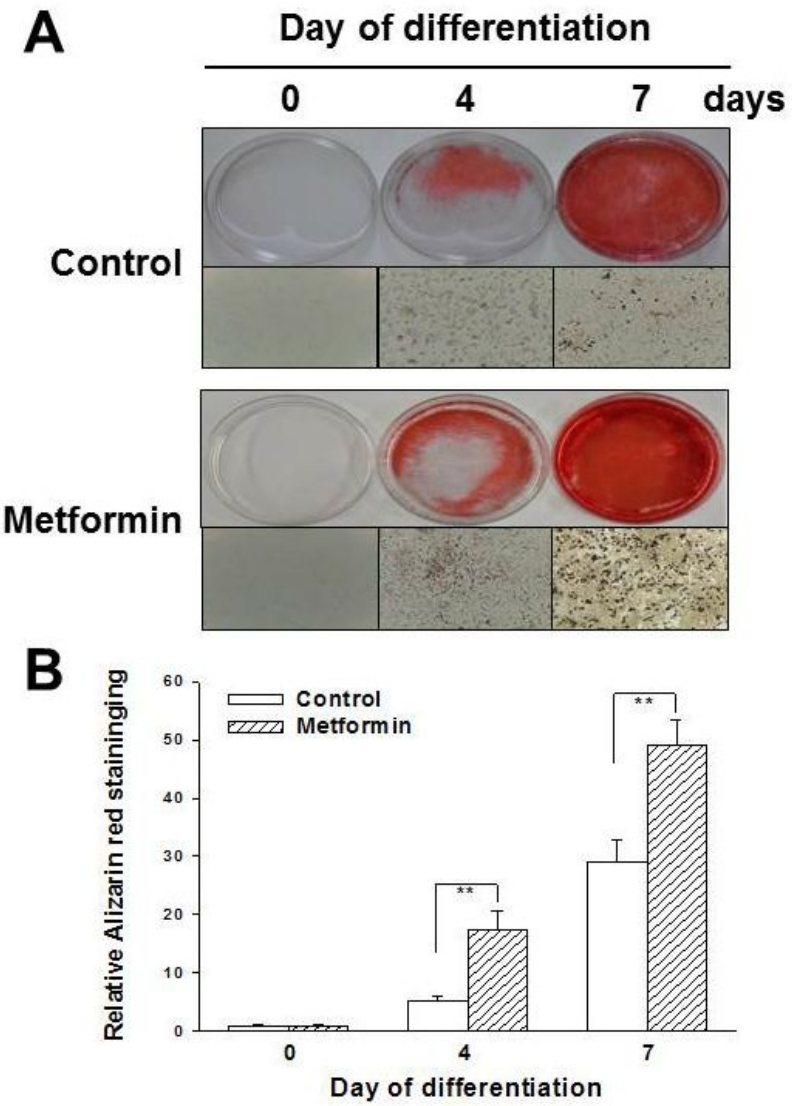

Fig. 2. Effect of metformin on the mineralization in MDPC-23 odontoblastic cells.

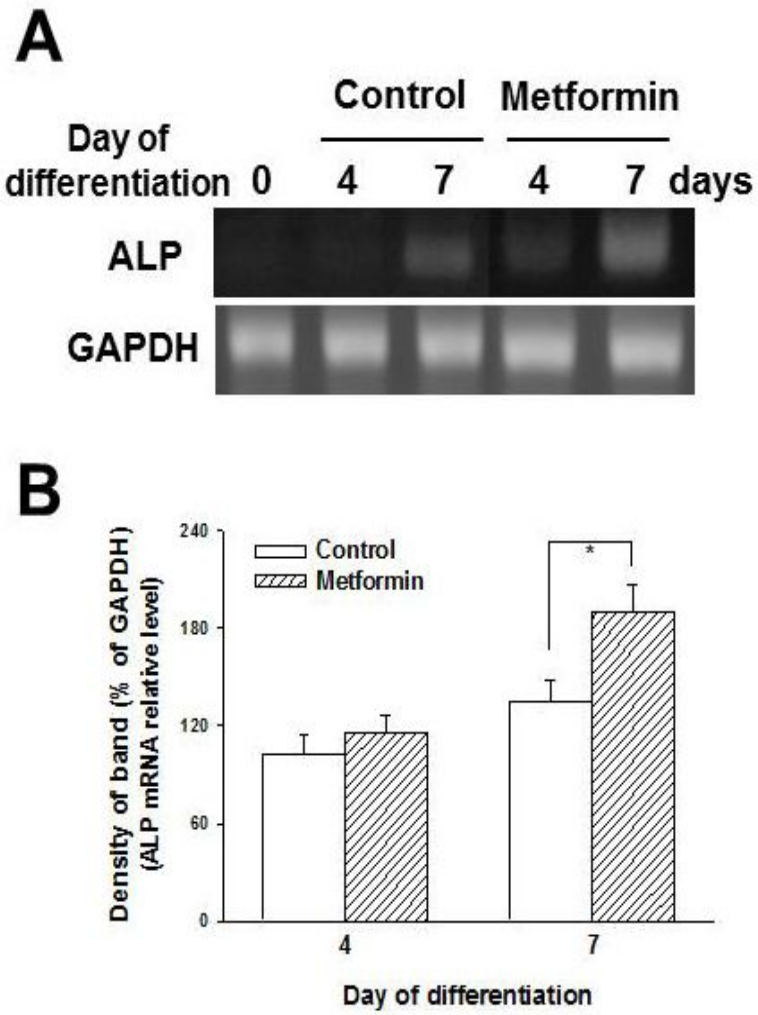

Fig. 3. Effect of metformin on alkaline phosphatase (ALP) mRNA expression.

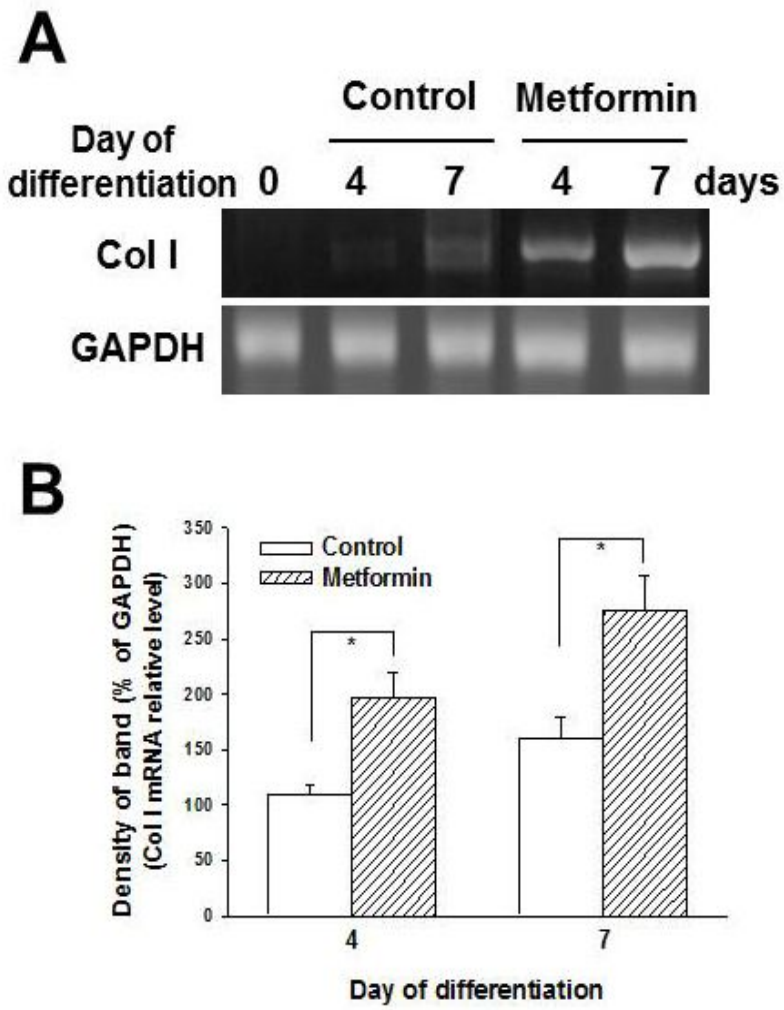

Fig. 4. Effect of metformin on type Ⅰ collagen (Col Ⅰ) mRNA expression.

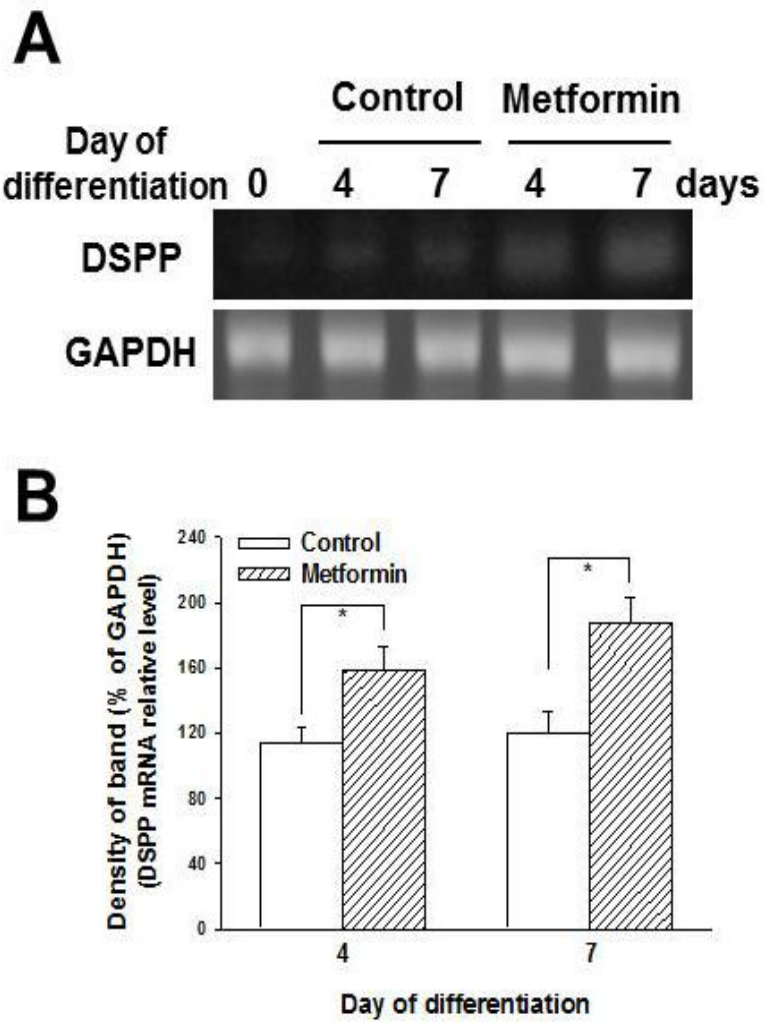

Fig. 5. Effect of metformin on dentin sialophosphoprotein (DSPP) mRNA expression.

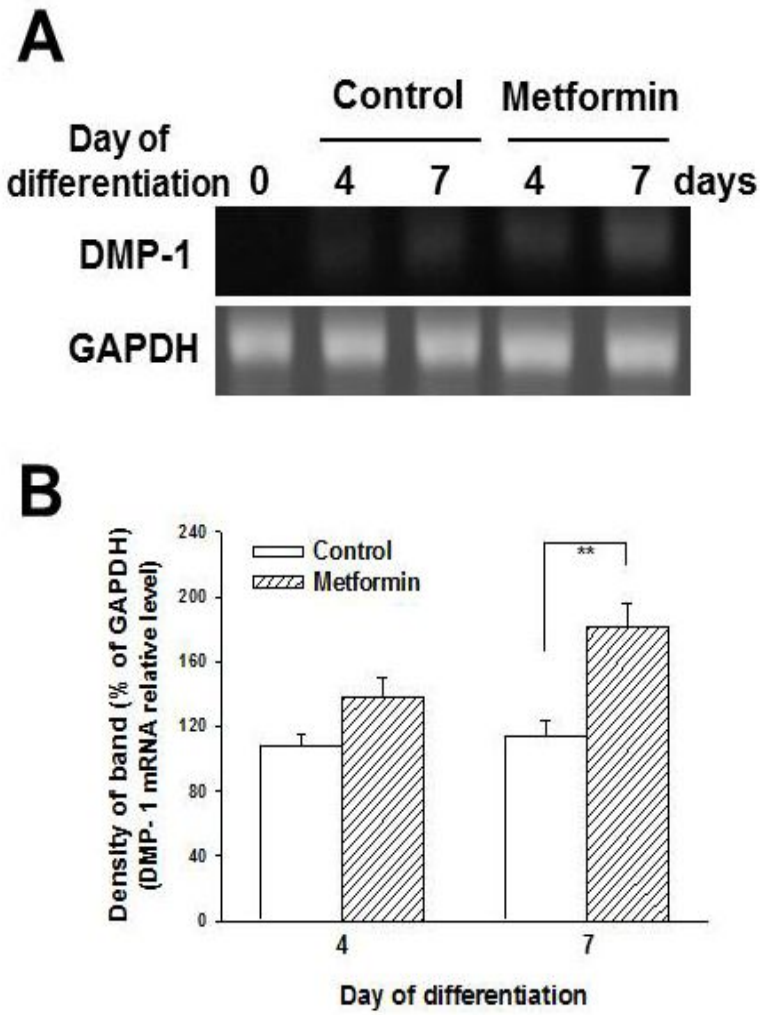

Fig. 6. Effect of metformin on dentin matrix protein-1 (DMP-1) mRNA expression.

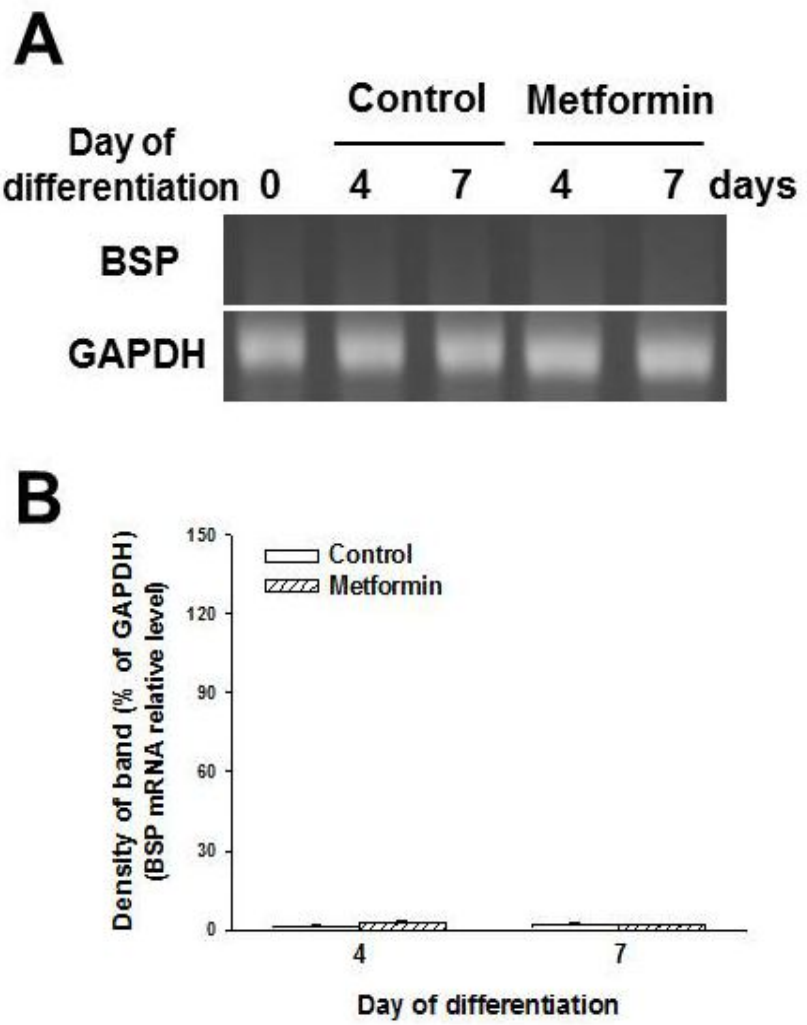

Fig. 7. Effect of metformin on bone sialoprotein (BSP) mRNA expression.

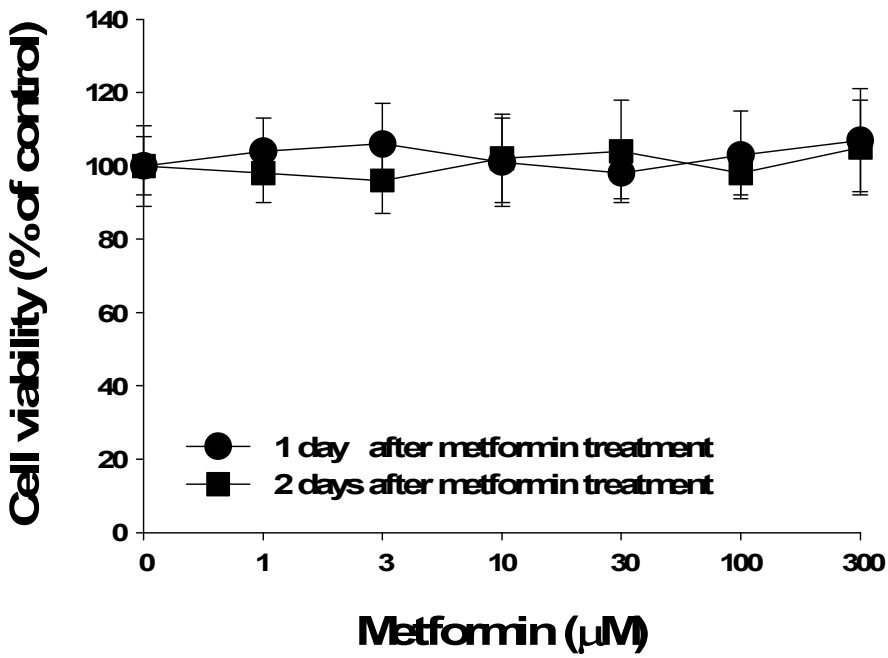

Fig. 8. Measurement of MDPC cell proliferation by metformin.