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RESEARCH ARTICLE

The Viability and Cytotoxic Effect of High Mobility Group Box Protein 1 on MC3T3-E1 Pre-osteoblast Cells: an *In Vitro Study*

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Abstract

Osteoblast proliferation and migration are significantly involved in bone healing, regeneration and embryonic skeletal development. Recently, reports suggest anabolic properties of High Mobility Group Box Protein 1 (HMGB1) within the subsequent phase of tissue regeneration as well as HMGB1 has been shown to be highly expressed during bone fracture and regulates osteochondral ossification. In this study, the effect of various concentrations of Recombinant Human HMGB-1 (rhHMGB-1) (50, 100,150 and 200 µg/L) on the viability and proliferation of Pre-osteoblast cells MC3T3-E1 were examined over 24, 48 and 72 hours intervals in comparison with control, untreated cells. A cell count kit (CCk-8) was used to assess osteoblast viability and proliferation, results were quantified using a microplate reader and the viability %(survival rate) was calculated. All experiments were done in triplicate and outcomes were statistically analyzed. A direct positive impact of HMGB-1 on the viability and proliferation of osteoblast cells was in a dose dependent manner. Lower concentration of HMGB-1 (50 and 100 µg/L) showed a non-significance difference in the viability and proliferation of osteoblast cells over time, however, a higher concentration of HMGB-1 (150 and 200 µg/L) showed a significant increase in the proliferation and survival rate by 1.7 fold compared to control group in the 2nd and 3rd day of treatment. No significant difference was found between the 150 and 200 µg/L concentrations which suggested the appropriate dose will be 150 µg/L. Moreover, further *in vivo* and *in vitro* investigations will be required.

Key words: High Mobility Group Box Protein-1(HMGB-1), osteoblast, Viability, Proliferation, MC3T3-E1 pre-osteoblast cells

Introduction

The proliferation and migration of osteoblasts are a critical process for both skeletal development and bone fracture healing [1]. Several *In vitro* studies showed that many growth factors and extracellular cytokines such as bone morphogenic proteins (BMPs), insulin like growth factors and wingless and ant (Wnt) ligand are involved in the proliferation process of the bone cells and therefore involved in bone regeneration and healing process [2-4].

High Mobility Group Box Protein - 1(HMGB-1) which is also known as amphoterin or HMG-1 is a 30KDa abundant and non-histone nuclear protein that is expressed in almost all eukaryotic cells [5-7]. HMGB-1 could be passively secreted from necrotic or damaged cells [5] or actively secreted by activated monocytes or macrophages [8].

However, previous reports suggested that HMGB-1 as an endogenous molecule, is a lethal mediator of sepsis [9], extracellular HMGB-1 has been reported to act as an active cytokine that contributes to inflammation [10-12], ischemic reperfusion of skeletal muscle

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tissue [13], liver fibrosis [14] and tumor growth and metastasis [15, 16].

Previous reports suggested the anabolic effect of HMGB-1 within various phases of tissue regeneration and Degryse [17] found that HMGB-1 can promote proliferation and migration of myofibroblasts, skeletal myoblasts and mesoangioblasts [17-19]. In addition, a data is collected from cardiac infarction mouse model [19] and others from diabetic mouse wound model demonstrated the direct effect on pluripotent stem and progenitor cells streaming in the defect surroundings promoting the regeneration efficiency and that HMGB-1 promotes wound closure, granulation tissue formation and angiogenesis, eventually enhancing wound healing [20, 21].

In the fracture microenvironment, the expression of almost all HMGB-1 receptors including receptors for advanced glycation end products (RAGE) and Toll like receptors 2 and 4 (TLR2 and TLR4) are significantly upregulated [22]. When HMGB1 binds to RAGE, the mitogen-activated protein kinase (MAPK) signaling pathway is activated [23]. It has been shown that the p38 MAPK signaling pathway plays an important role in the osteoblastic differentiation of mesenchymal stem cells (MSCs) [24].

The important role of HMGB-1 in bone restoration is more obvious as it promotes migration and differentiation of mesenchymal stem cells along the osteoblastic pathway [25]. The same conclusion was found by Wolf *et al.* [26], they found that periodontal ligament stem cell proliferation, migration and osteogenic differentiation were increased by HMGB-1 treatment [26].

In a previous study, HMGB-1 found to be able to promote osteoblast migration, the results showed that the migration rate of osteoblast increases 2.3 fold after HMGB-1 treatment [27]. In another study, it showed that the hypoxia environment developed during fracture stimulated the release of HMGB-1 and upregulate the expression of its receptors which suggested the critical role of HMGB-1 during fracture healing process [28].

This *in-vitro* study aimed to measure the viability/cytotoxic effect of various concentrations of HMGB-1 on osteoblast like cells MC3T3-E1 and to determine the optimal concentration of HMGB-1 for further *in vitro* and *in vivo* experimentation.

Materials and Methods

Cells and Reagents

Pre-osteoblast MC3T3-E1 cell line was purchased from RIKEN BioResource Research Center (BRC) (305-0074 -Tsukuba, Ibaraki, Japan), (cells resource number, RCB1126). The medium used was Dulbecco's modified eagle media (DMEM) with 10% Fetal Bovine Serum (FBS) and Antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Trypsin 0.05% + 0.53 EDTA (Mediatech Inc., A Corning, USA) were used for cell collection and sub-culturing. RhHMGB-1 protein was commercially purchased as a 50 µg lyophilized powder (R&D system, USA). Graded concentrations of RhHMGB-1 (50, 100, 150 and 200) µg/L were obtained through diluting HMGB1 powder with phosphate buffer saline (PBS).

Cells handling and culture procedures

Cell handling and culturing were performed following previous study [27] and recommendations provided by RIKEN BRC cell bank. All procedures were performed under strict sterile condition in a sterile laminar flow cabinet. Cells allowed to thaw at room temperature, then after multiple pipetting cells were transferred to a 15ml centrifugation tube containing 5 ml of growth medium (DMEM + 10% FBS + Antibiotic). The tube was then centrifuged at 1000rpm for 5 minutes, the supernatant was discarded and cells pellet was re-suspended in another 5 ml of growth medium. The previous step was repeated and the cells pellet was dissolved in 2 ml of growth medium by several pipetting. The cell suspension was dispensed in a 100mm culture dish containing 8 ml of growth medium. So, the total volume of the culture medium became 10ml. The culture dish was then incubated in a humidified atmosphere of 5% CO_2 and 95% relative humidity at 37^{o}C till it reached 70-80% confluence.

Cell viability/cytotoxicity assay

Cell viability and cytotoxicity assay was performed using cell count kits CCK-8 provided by (Dojin chemical laboratory – DOJINDO-, Japan) following instructions provided with the Kits [29]. When the cell confluence reached 70-80%, cells were collected by trypsinization by adding 1-2 ml of trypsin/EDTA solution and incubated for 5 minutes in humidified atmosphere at 37ºC and 5% CO_2. The collected cells were suspended in a 15ml centrifugation tube containing 2 ml of growth medium. 100µl of cell suspension was dispensed with a concentration of 5 x 10^3 cells/well in a sterile 96 well plate. The plate was incubated for 24 hours in a humidified atmosphere at 37ºC and 5% CO_2 to allow cell attachment. The cells were seeded with 10 µl of various concentration of RhHMGB-1 (50, 100, 150 and 200µg /L) and then the plate was incubated in the humidified atmosphere at 37ºC and 5% CO_2 for 24, 48 and 72 hours. Wells contain medium, cells and 10 µl PBS instead of HMGB-1 were made as control wells. Blank wells, that's contain the CCK-8 solution without cells were also made. At each time interval, 10µl of cell count kits solution was then added to each well of the plate, and the plate was incubated for 1-4 hours to allow development of the color from formazan dye generated by hydrogenases in cells.

All procedures were performed in triplicate and the mean values were obtained. The absorbance values were measured using a microplate reader (Multiskan FC microplate reader, Thermo fisher, FINLAND) at 450 nm. The cell viability was calculated using the following equation: Cell viability (%) = (Absorbance sample (As) - Absorbance blank (Ab) / Absorbance control (Ac) - Absorbance blank (Ab)) x 100.

Statistical analysis

One-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (Tukey's HSD) test as post hoc test was used. Analysis was done using Statistical Package for Social Sciences version 22.0 (SPSS, IBM Corp., Armonk, NY, USA). Results were expressed as mean ± standard error (SE). P value < 0.05 was used to indicate statistical significance**.** The statistical analysis was based on the intention-to-treat population.

Results

Results from the findings of viability/cytotoxic assay showed a direct effect of various concentration of HMGB-1 on the viability of osteoblast cells. Table 1 showed Comparison of the average absorbance among different HMGB 1 concentrations during a period of three days. HMGB-1 showed to increase the viability and proliferation of MC3T3-E1 cells in a dose dependent manner reaching the plateau at 200µg/L. Treatment with a lower dose of HMGB-1 (50µg/L) showed a non- significance difference in the proliferation and viability of the osteoblast cells. At day one, there were non-significant differences of the average absorbance among the different concentrations of HMGB 1 ($P < 0.05$). However, in the third day, the concentrations of 150 and 200 µg/L showed a significantly higher absorbance value that is indicating a higher effect on viability and proliferation of osteoblast cells than the lower concentrations. However no significant difference between these concentrations (150 and 200µg/L) was observed (Figure 1).

The survival rate of the cells were calculated through the equation of (Survival rate (%) = A s- A b/ A c - A b x 100). As the survival rate was estimated depending on the absorbance value, results from Table 2 showed non-significant differences of the average viability % among the different concentrations of HMGB 1 ($P < 0.05$) at day one. However, a significantly higher viability % was observed in the second and third day at concentrations 150 and 200 µg/L compared to (100 µg/L) and lower ones, with no significance difference between the concentrations of 150 and 200 µg/L (Figure 2).

Table 1: Comparison of the average absorbance value from microplate reader results among different High Mobility Group Box Protein 1 (HMGB 1) concentrations within time points of 24, 48 and 72 hours post-treatment

HMGB-1 concentration	24 hours	48 hours	72 hours
Control	0.57±0.03	1.86±0.41^{b}	2.0±0.33^{b}
50 µg/L	0.52±0.04	1.67±0.04^{b}	1.85±0.02^{c}
100 µg/L	0.58±0.02	2.98±0.22^{a}	2.58±0.02^{b}
150 µg/L	0.54±0.01	2.92±0.05^{a}	2.73±0.07^{a}
200 µg/L	0.58±0.01	3.33±0.08^{a}	3.06±0.04^{a}

Means± standard error, carrying different superscripts, within the same column are significantly different at (P < 0.05).

Table 2: Comparative viability% (survival rate) among different High Mobility Group Box Protein 1 (HMGB 1) concentrations within time points of 24, 48 and 72 hours post-treatment.

HMGB-1 concentrations	24 hours	48 hours	72 hours
Control	100±2.14	100±1.02^{c}	100±1.45^{c}
50 µg/L	89.02±3.18	89.62±6.2^{c}	92.52±2.34^{c}
100 µg/L	100.55±4.5	159.73±2.4^{b}	128.62±5.6^{b}
150 µg/L	92.63±6.1	156.82±4.6^{a}	136.32±2.4^{a}
200 µg/L	102.13±5.5	178.96±6.2^{a}	152.69±4.1^{a}

Means± standard error, carrying different superscripts, within the same column are significantly different at (P < 0.05).

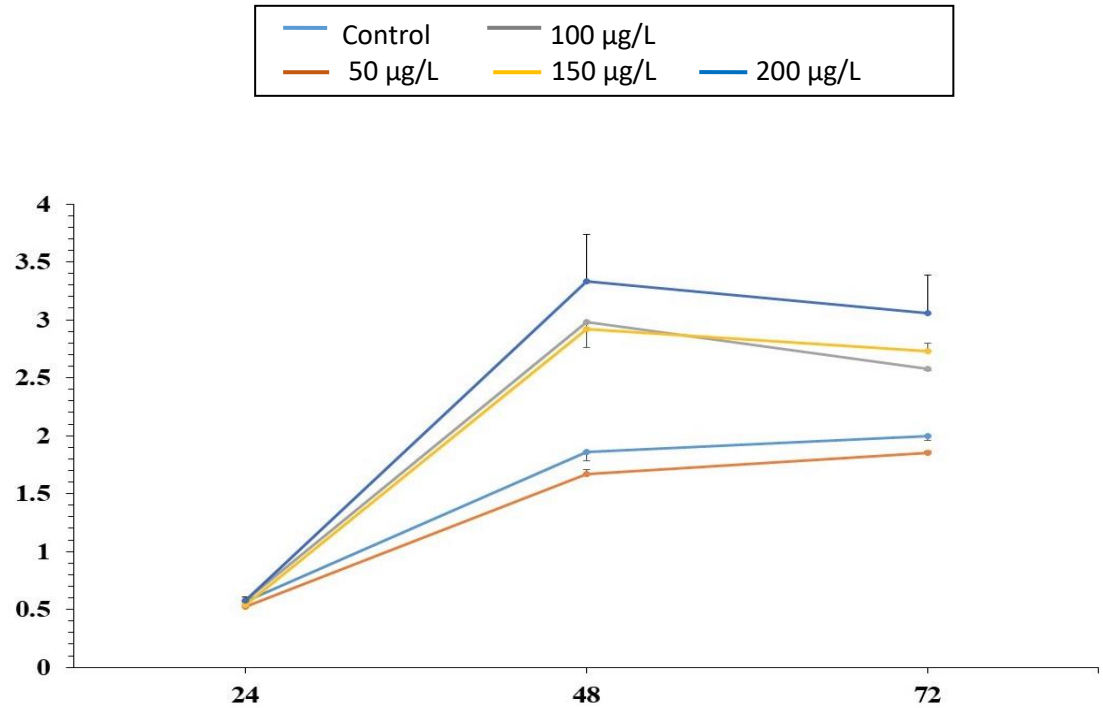

Figure 1: Showing the average absorbance value from microplate reader using concentrations of (50, 100, 150 and 200) µg/l of High Mobility Group Box Protein 1 (HMGB-1) and from control wells at time interval of 24, 48 and 72 hours post-treatment.

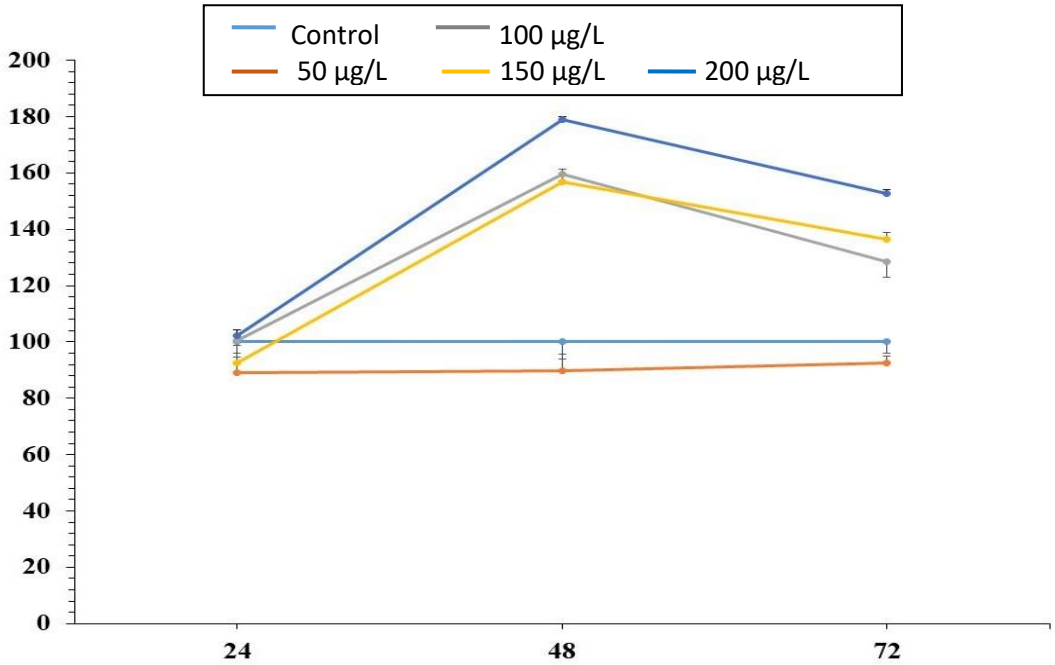

Figure 2: Showing viability% (survival rate) of MC3T3-E1 cells after 24, 48 and 72 hours of treatment with different concentrations of High Mobility Group Box protein 1 (HMGB-1) (50, 100, 150 and 200) µg/l compared with control, untreated cells

Discussion

The present study aimed to determine the effect of various concentrations of HMGB-1 on the viability and proliferation of osteoblast cells as well as to determine the optimum concentration of HMGB-1 to be used for further in-vitro and in-vivo investigations.

Previous reports had been recognized the Extracellular HMGB1 as a multifunctional proinflammatory cytokine [30-32]. It participates in the processes of embryonic development [33], vasculogenesis and angiogenesis [17, 18, 34, 35], hematopoietic stem cell mobilization [36-38]. HMGB-1 has been also known to be involved in the inflammatory process, migration and activation of monocytes, macrophages and dendritic cells [5, 9, 39-42], tumorogenesis [31, 35, 43] and ischemic reperfusion of skeletal muscle [44].

Osteoblast proliferation and migration are a critical process during embryo skeletal development and fracture healing process [1]. Previous reports showed that osteoblast and osteoclast cells expressed receptors for HMGB1 which support the hypothesis that higher local concentration of HMGB1 which reported to be induced by the hypoxic microenvironment developed during fracture might induce osteoblast migration and there by regulate bone repair and skeletal development [28, 45].

In the present study, various concentrations of HMGB-1 had been tested for their effect on viability and proliferation of MC3T3-E1 osteoblast like cell line. The concentrations used were (50,100, 150 and 200 µg/L), the reason of using these graded concentrations that the serum level of HMGB-1 in patients subjected to gram negative bacterial endotoxin had been reported to be approximately 30-150 µg/L [9].

HMGB-1, also known as alarmin, had been reported as an endogenous molecule released passively by dead and necrotic cells that alert the innate immune system to tissue damage and subsequently the need for repair [46]. Meanwhile, several reports concluded that HMGB-1 was a bone active cytokine which involved in fracture repair as well as bone resorption signals [45]. Our finding showed that HMGB_1 had a direct effect on viability and proliferation of osteoblast like cells (MC3T3-E1). It had been shown that HMGB-

1 increase the viability and proliferation activity of MC3T3-E1cells in a dose dependent manner.

In the current study, a higher concentration of HMGB-1 of 150 and 200 µg/L showed a significance increase in the viability and proliferation of MC3T3-E1 cells by 1.5 to 1.7 fold than the untreated group at the 2nd and 3rd day of HMGB-1 treatment. In another study by Li et al., they found that the osteoblast migration was increased by 2.3 fold in HMGB-1 treated cells with concentration of 150 and 200 µg/L [27]. This effect of HMGB-1 on osteoblast cells could be attributed to the fact that extracellular HMGB-1 can induce complex signaling cascades by binding to its receptors including RAGE, TLR2 and TLR4 [47]. When HMGB1 binds to RAGE the Mitogen Activated Protein Kinase (MAPk) is activated [23] which play an important role in osteoblastic differentiation of MSC [24]. Moreover, activation of TLR2/4 – Nuclear factor-Kabba B signaling pathway (NF-κB) is known to facilitate cell proliferation [22]. As an exogenous, recombinant growth factor, it has a reduced biological activity. So that, a higher concentration is required to produce more effect till reaching plateau [48].

Conclusion

Our results demonstrated the direct effect of HMGB-1 on the proliferation and viability of osteoblast cells which suggested a significant functional role of HMGB1 in skeletal development and bone healing and regeneration. This effect has a dose dependent manner that increases by increasing the concentration of HMGB-1 used till reach plateau at 150µg/L.

The mechanism of action of the HMGB-1 on osteoblast cells and the signal pathways involved with HMGB-1 effect during bone restoration needs to be clarified more. Therefore further in vivo and in vitro studied are required.

Conflict of interest:

None of the authors have any conflict of interest to declare

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الملخص العربي

تأثير بروتين (HMGB-1) على حيوية و تكاثر الخلايا العظمية الأولية (MC3T3-E1) فى المختبر

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يؤثر نمو الخلايا العظمية الأولية و هجرتها على التئام العظم و نموه و كذلك على تطور الهيكل العظمى الجنينى . فى الآونة الأخيرة تشير الدراسات الحديثة على أن بروتين HMGB-1 له الكثير من الخصائص البنائية التى تساهم بشكل كبير فى مراحل مختلفة من نمو و تجديد أنسجة الجسم ، كما وجد أن بروتين HMGB-1 ينتشر بكثرة أثناء التئام العظم كما أنه له دور كبير فى تنظيم تعظم الجزء العظمى الغضروفى من العظم. فى هذه الدراسة، قمنا باختبار تأثير تركيزات مختلفة من HMGB-1 (50، 100، 150 و 200 ميكرو جرام /لتر) على نمو و تكاثر الخلايا العظمية الأولية (pre-osteoblast) على مدى 24، 48، 72 ساعة . و قد تم استخدام اداة لعد الخلايا مقدمة من مختبر دوجين الكيميائى لتقييم حيوية و تكاثر خلايا العظم الأولية بعد معالجتها ببروتين HMGB-1 و تقييم النتائج باستخدام قارئ الصفائح الميكرو و مقارنتها بالمجموعة الضابطة، و قد تم تكرار التجارب ثلاث مرات و تحليل النتائج إحصائيا. تشير النتائج الى التأثير المباشر ل HMGB-1 على نسبة الحيوية و عملية التكاثر للخلايا العظمية بشكل طردى يزيد بزيادة التركيز المستخدم. فقد وجد انه فى حالة التركيزات الصغيرة المتمثلة فى 50، 100 ميكروجرام/لتر لوحظ انخفاض فى نسبة الحيوية و التكاثر و لكنه يعتبر فرق غير جوهرى عند مقارنته بالمجموعة الضابطة و من ناحية اخرى فان التركيزات العالية من HMGB1 اظهرات زيادة إحصائية فى نسبة حيوية و تكاثر الخلايا العظمية الأولية حيث أشارت النتائج إلى زيادة نسبة الحيوية 1.7 مرة اكثر من المجموعة الضابطة فى التركيزات 150 و 200 ميكروجرام / لتر على حد سواء و ذلك فى اليوم الثانى و الثالث من تعرض الخلايا لبروتين HMGB-1 مع عدم وجود فرقا جوهريا بين التركيزين 150 و 200 ميكروجرام / لتر. و بالتالى يمكن استخدام التركيز 150ميكروجرام/لتر كتركيز مفضل لاجراء المزيد من الفحوصات داخل الجسم الحى و فى المختبر.