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Brief Report

Reactive oxygen species and cell morphology of MC3T3E1 pre-osteoblast cell line exposed to methylgyoxal by laser scanning confocal microscopy

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AGEs precursor; MC3T3E1; Methylglyoxal; Pre-osteoblast

Abstract

Advanced glycation end products (AGEs) are known to increase in osteoporosis. Methylglyoxal is AGEs precursor that is toxic to osteoblasts. Methylglyoxal toxicity in osteoblasts involves oxidative stress. The involvement of reactive oxygen species (ROS) in changes of MC3T3E1 pre-osteoblast cell line morphology caused by methylglyoxal exposure is still unknown. This study was aimed to investigate a possible effect of methylglyoxal on ROS level and morphology of MC3T3E1 cell line. Pre-osteoblast MC3T3E1 cell line, obtained from American Type Culture Cell (ATCC), was exposed to methylglyoxal at several concentrations. Then ROS level and cell morphology were evaluated by laser scanning confocal microscopy using H_2DCFDA. The optimal dose and exposition time of MC3T3E1 pre-osteoblast cell subclones to methylglyoxal is estimated to be 5 µM for 6 h which showed fusiform, polygonal cells, a homogenous cytoplasm and a clear hole as nucleus in the center of the cell. In conclusion, methylglyoxal exposure of MC3T3E1 pre-osteoblast cell line increased ROS level at 5 µM for 6 h of exposure. Beginning with this dose, the cell presents morphologic markers of apoptosis.

INTRODUCTION

Methylgyoxal is an electrophilic reactive substance which attacks functional groups of macromolecules to change their biological activity. Protein modification by methylglyoxal induces loss of enzymatic activity, change of conformational structure, and development of fluorescence [1]. Methylglyoxal modifies lysine and arginine residues leading to changes in their characteristic structure [2]. At cellular level, methylglyoxal induces the arrest of cell growth, dysfunction, apoptosis, necrosis, mechanisms that almost involve protein modification [3]. In addition, methylglyoxal can irreversible bind into protein amino acids and nucleic acids resulting in methylglyoxal-derived advanced glycation end products (MAGEs) [4].

Oxidative stress due to reactive oxygen species (ROS) is a main factor for decreasing physiological functions such as ion transport, electrical activity and cellular signal transduction [5]. Previous *in vitro* studies showed that AGEs interaction with their receptors (RAGEs) induce oxidative stress and activation of the transcription factor NF-κB. Besides that, AGEs induces antioxidant depletion [6]. The effect of methylglyoxal on mature osteblasts is mediated by apoptosis through biochemical pathways, including c-Jun N-terminal kinase (JNK) activity, change of mitochondria membrane, release of cytochrom C, increased Bax/Bcl-2 ratio, and the activation of caspase 3 and 9. A

previous *in vivo* study showed that administration of methylglyoxal at concentrations of 100-200 µg via drinking water increases loss of bone mineral density of rats [7].

As far we know, there are no investigations on the effects of methylgyoxal exposure on ROS of MC3T3E1 pre-osteoblast cell line. To understand the involvement of methylgyoxal in osteoporosis, this study was aimed to evaluate the effect of methylgyoxal exposure on possible morphological changes and ROS of MC3T3E1 pre-osteoblast cell line by laser scanning confocal microscopy.

MATERIAL AND METHODS

Cell line

MC3T3E1 pre-osteoblast cell line subclone 4 was obtained from *American Type Culture Cell Collection* (ATCC). MC3T3E1 pre-osteoblast cell line was exposed to methylglyoxal at concentrations 2.5, 5, 10 and 20 µM for 1, 3, 6 and 12 h of exposition time, according to previous studies [7, 8]. The control group was set as MC3T3E1 pre-osteoblast cell line subclone 4 without methyglyoxal exposure.

Cell culture

MC3T3E1 pre-osteoblast cell line subclone 4 was grown in 75 cm^2 flasks in atmosphere humidification at 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented by 100 U/ml penicilin, 100 µg/ml streptomycin and 10% (v:v) fetal bovine serum (FBS). DMEM and FBS were obtained from Gibco® (Invitrogen). Subculture was done using 0.1% tripsin 1 mM EDTA in phosphate buffer saline free of Ca^{2+} and Mg^{2+}. In 70-80% confluent cultures, cell was harvesting into 6 or 24 well. These culture cell methods were done in Central Laboratory of Life Science, University of Brawijaya, Malang, Indonesia.

Reactive oxygen species analysis

ROS analysis was done using confocal laser scanning microscope [9]. For this reason, 25 µM 2,7-dichlorodi-hydrofluorescein diacetate (H_2DCFDA) was added to MC3T3E1 pre-osteoblast cell line subclone 4. After that, cells were washed with PBS and fixated by 10% buffer formalin for 10 min. Mounting was done on slide glass by ProLong® Antifade (Invitrogen). The evaluation of cell morphology was done using Zeiss LSM 510 laser scanning confocal microscopy. The green flourescence in MC3T3E1 pre-osteoblast cell line subclone 4, expressed as arbitrary units, was estimated as ROS level.

RESULTS

Reactive oxygen species level

The highest level of ROS was achieved at 5 µM for 6 h of exposure of the MC3T3E1 pre-osteoblast cell line subclone 4 to methylglyoxal (Fig.1). However, the levels of ROS is not significantly different between groups ($P > 0.05$).

Morphology of MC3T3E1 pre-osteoblast cell line subclone 4

Morphology of normal MC3T3E1 pre-osteoblast cell line subclone 4 was regular fusiform, polygonal; the surface of cytoplasm was homogenous and clear hole as nucleus was found in the center of the cells. At highest level of ROS, which achieved at 5 µM for 6 h, the cell showed swelling, oval shape, and cytoplasm condensation as morphology markers for apoptosis (Fig.2).

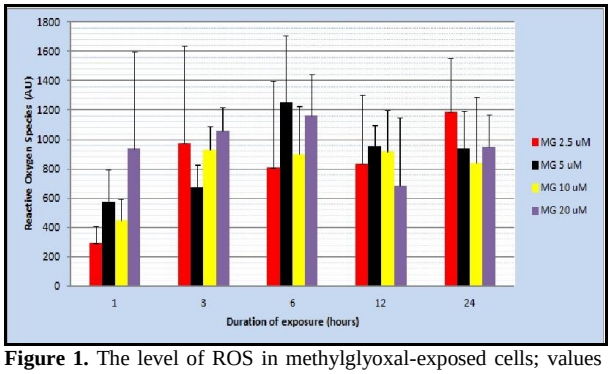

Figure 1. The level of ROS in methylglyoxal-exposed cells; values are presented as mean ± SD; MG: methylgyoxal; AU: arbitray units. The optimal dose of methylglyoxal for highest production ROS in MC3T3E1 pre-osteoblast cell line subclone 4 is 5 µM for 6 h of exposure.

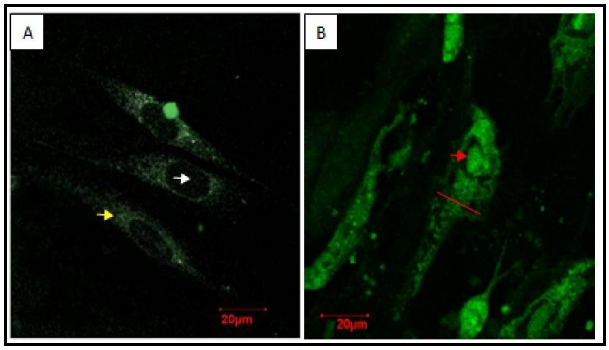

Figure 2. Confocal micrograph of MC3T3E1 pre-osteoblast cell line subclone 4 not exposed **(A)** and exposed to methylglyoxal at optimal dose **(B)**. Morphology of normal MC3T3E1 pre-osteoblast cell line subclone 4 was regular fusiform, polygonal; the surface of cytoplasm was homogenous (yellow arrow) and clear hole as nucleus was found in the center of cell (white arrow). At this optimal dose, the morphology of MC3T3E1 pre-osteoblast cell line subclone 4 showed swelling, oval shape (red line), and cytoplasm condensation as apoptosis marker (red arrow). [Magnification x400; bar is 20 µm]

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DISCUSSION

The main finding of this study is that methlyglyoxal was able to increase ROS in MC3T3E1 pre-osteoblast cell line although not reachead statistically difference. In addition, methylgyoxal also changed the morphology of MC3T3E1 pre-osteoblast cell line at the optimal dose. Increasing of ROS was probably due to activation of NADPH oxidase [6]. This activation resulted in superoxide radical catalyzed by its endogenous antioxidant superoxide dismutase forming hydrogen peroxide or another ROS. Besides, a previous study showed that the reaction between methlyglyoxal and arginine increases methylglyoxal radical and superoxide radical [10]. The optimal dose of methlyglyoxal exposure was found to be 5 μM for 6 h of exposure. This finding indicates that ROS will achieve intracellular maximum levels at this dose. The levels of ROS was the result of the depletion of glutathione and the acivity of a glyoxalase system for conversion and detoxification of methlylglyoxal in cytoplasm [10-12]. Methylglyoxal was toxic to the cell due to induction of ROS for apoptosis signaling [13]. Former studies showed that methylglyoxal induced cytotoxicity at longer times of exposure (4-24 hours) [14, 15], whereas it was not toxic in short time of exposure to myoblast L6 cell line [13], hepatocytes [16], and human endothelial cells [17].

The normal morphology of MC3T3E1 pre-osteoblast cell line is fusiform, polygonal and regular shaped. The increase of ROS exposure induced swelling and apoptosis. A recent study reported that at 400 μmol/l of H_2O_2 exposure no marked morphological change was observed under a light microscope; cells started swelling with 800-1000 μmol/l; and at 1000 μmol/l, cells underwent apoptosis [18]. In the present study, the morphology of MC3T3E1 pre-osteoblast cell line at highest intracellularly ROS level from methlyglyoxal exposure resulted in swelling, oval shape, cytoplasm condensation, and the margin between cytoplasm and nucleus became unclear as morphologic markers of apoptosis.

In conclusion, methylglyoxal expsoure at MC3T3E1 pre-osteoblast cell line increased ROS level at 5 μM for 6 h of exposure. At this dose, the cells start showing evidence of morphologic apoptosis markers.

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CONFLICT OF INTEREST

None to declare

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