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# CSN1S2 protein of goat milk inhibits the decrease of viability and increases the proliferation of MC3T3E1 pre-osteoblast cell in methyl glyoxal exposure

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## PEER REVIEW

## Peer reviewer

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## Comments

In this manuscript, the authors report beneficial effect of CSN1S2 on preosteoblast exposed to MG. The manuscript is well written, adequately illustrated and opens the ground for further evaluation of this topic.

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## ABSTRACT

**Objective:** To investigate whether the CSN1S2 protein of goat milk is able to inhibit the toxicity of methyl glyoxal (MG) towards MC3T3E1 pre-osteoblast cells.

**Methods:** At confluency, pre-osteoblast cells were divided into five groups which included control (untreated), pre-osteoblast cells exposed to 5 μmol/L MG, pre-osteoblast cells exposed to MG in the presence of CSN1S2 protein at doses of 0.025, 0.050, and 0.100 mg/L, respectively. Analysis of reactive oxygen species was done with 2,7-dichlorodihydrofluorescein diacetate fluorochrome. The proliferation and viability of MC3T3E1 cells were measured by trypan blue staining. Malondialdehyde analysis was done colorimetrically.

**Results:** Cell's viabilities were significantly lower in MG+0.050 mg/L CSN1S2 protein of goat milk compared to MG group ( $P<0.05$ ). MG+0.100 mg/L CSN1S2 protein of goat milk significantly increased the cells viability compared to MG group ( $P<0.05$ ). The levels of proliferation were significantly higher in MG+0.100 mg/L CSN1S2 protein of goat milk compared to control group and all treatment groups, respectively ( $P<0.05$ ).

**Conclusions:** High dose of CSN1S2 protein of goat milk (0.100 mg/L) in high MG environment inhibits the decrease of viability due to the increases of the proliferation of MC3T3E1 pre-osteoblast cell.

## KEYWORDS

Bone formation cells, Carbonyl compounds, Oxidative stress, Mitogenic

## 1. Introduction

Bone is a dynamic tissue that constantly undergoes remodeling, through the precise and localized coupling of resorption (removal of the aged material) with replacement by newly formed bone[1]. Patients with type I diabetes frequently shows a decrease in bone formation, with a reduction in the number and activity of osteoblasts[2,3]. Diabetes enhances the apoptosis of osteoblasts, mainly by activating caspase-8, -9

and -3 expression and activity[4]. Osteoblast proliferation and differentiation are tightly regulated by various hormones, cytokines, and multiple transcription factors[5-7].

Methyl glyoxal (MG) is a metabolite, which is a substance produced during participating in chemical processes that occur in living organisms and produced in the human body during glucose metabolism. MG is presented in many foods and drinks, including coffee, and is produced during glycolysis and sugar fermentation[8]. MG was more

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reactive than glucose and attacked the cluster of amino groups of intracellular and extracellular proteins to produce advanced glycation end products (AGEs)[9]. Soluble and matrix-associated AGEs can modulate osteoblastic growth and differentiation[10,11]. Long-term exposure to AGEs-modified proteins significantly inhibited the proliferation, differentiation and mineralization of osteoblastic cultures[12]. As far as we know, there is no study to explore the effect of long-term exposure to MG on bone formation cells.

Recently, the nutrigenomic studies were directed to find out the bioactive peptides for disease treatment. The casein alpha S2 (CSN1S2) protein which is isolated from Ethawah goat's milk has many functions, such as antimicrobial and antioxidative peptides. The CSN1S2 of goat milk has higher antioxidant amino acids (histidine, methionine, cysteine, tyrosine, phenylalanine) than cow's milk[13,14]. However, the function of CSN1S2 protein of goat milk to increase the viability, proliferation and differentiation in the long-term MG exposure remains unknown. This study focuses on elucidating the biological function of CSN1S2 protein of goat milk to inhibit the toxicity of MG towards MC3T3E1 pre-osteoblast cells.

## 2. Materials and methods

### 2.1. Cell culture

Mouse pre-osteoblast cells MC3T3E1 sub clone 4 were purchased from American Type Culture Cell Collection. MC3T3E1 mouse calvaria derived cells were grown in alpha-modified eagle medium (Sigma Life Sciences, USA), 2 mmol/L L-glutamine (Gibco, USA), 1 mmol/L sodium pyruvate, 10% fetal bovine serum (Gibco, USA), and 10% penicillin-streptomycin (Sigma Life Sciences, USA), and were incubated in 5% CO<sub>2</sub> at 37 °C[10]. The cells were sub-cultured with trypsin, incubating for 5 min and resuspended at a density of 21×10<sup>5</sup> cells/mL. When the confluency of the cells reached 70%–80%, the experiment could be started.

### 2.2. Isolation of CSN1S2 protein of goat milk

The isolation of CSN1S2 protein from goat milk 36 kDa were done using sodium dodecyl sulphate-polyacrilamide gel electrophoresis discontinuous system according to the previous study[15].

### 2.3. Amino acid analysis

The measures of amino acid were performed according to the previous study with modification[16]. The samples were hydrolyzed by 6 mol/L HCl, at 110 °C during 24 h in vacuum condition. Then the tube was cooled, and the mixed solutions were filtered by Spartan-HPLC 13-mm syringe filter. Amino acid samples were diluted with aquadest 1:20 v/v and

then continued to the derivatization procedure by adding O-phthaldehyde and 9-fluoronylmethyl chloro-formate to the samples. The solutions were placed in the cuvet after derivatization, each sample was detected with excitation wave at 335 nm and emission wave at 450 nm during 30 min.

### 2.4. MG treatment

The MG exposure was performed according to the previous studies with modification in duration of treatment[17]. The pre-osteoblast cell at sub passage of eleven was exposed to 5 µmol/L MG (Sigma Life Science, Germany) for 72 h. The CSN1S2 protein of goat milk at doses of 0.025, 0.050, and 0.100 mg/L was added at the cell's culture start at 6-h incubation, respectively[18].

### 2.5. Analysis of cell proliferation and viability

The proliferation and viability of MC3T3E1 cells were measured by trypan blue staining and counted by hemocytometer[19].

### 2.6. Analysis of reactive oxygen species (ROS) and cell differentiation

The cells were stained with 2,7-dichlorodihydrofluorescein diacetate 25 µmol/L (Sigma Life Science, Israel) and incubated for 1 h. Cells were observed under confocal laser scanning microscope (Olympus, Japan) with Olympus Fluoview software version 1.7a (Olympus, Japan)[17]. The numbers of cell differentiation were then counted.

### 2.7. Malondialdehyde analysis

The medium of cells were added with 15% trichloroacetic acid (Sigma Life Sciences, USA) and 0.67% thiobarbituric acid (Sigma Life Sciences, USA). Then the solutions were vortexed and boiled with waterbath at 95 °C. The solutions were cooled, and the absorbance was read at 532 nm[20].

### 2.8. Statistical analysis

The statistical test were analyzed using SPSS 16.0 by One-way ANOVA test, with  $P < 0.05$  that indicates significant differences between groups, and then followed up by a *post hoc* test if the result was significant.

## 3. Results

### 3.1. Amino acid component

Table 1 shows the amino acid composition in CSN1S2 protein of goat milk, they are L-aspartic acid, L-serine, L-glutamic acid, glycine, L-histidine, L-arginine,

L–threonine, L–alanine, L–proline, L–cystine, L–tyrosine, L–valine, L–methionine, L–lysine, L–isoleucine, L–leucine, L–phenylalanine. The highest amino acid content is L–glutamic acid (34 483.83 mg/L).

**Table 1**

The composition of amino acid in CSN1S2 protein of goat milk.

Amino acid	Level (mg/L)
L–aspartic acid	11 760.00
L–serine	7 906.60
L–glutamic acid	34 483.86
Glycine	2 478.75
L–histidine	4 111.40
L–arginine	4 475.64
L–threonine	6 066.06
L–alanine	4 655.53
L–proline	16 088.44
L–cystine	Not detected
L–tyrosine	5 896.56
L–valine	10 638.59
L–methionine	4 121.66
L–lysine	Not detected
L–isoleucine	7 376.41
L–leucine	13 704.61
L–phenylalanine	7 524.58

### 3.2. Effect of CSN1S2 or MG on ROS and oxidative stress

The exposure of MG to pre–osteoblast cells affected the ROS (Figure 1) and malondialdehyde level. The level of ROS and

malondialdehyde were not significantly different between groups ( $P>0.05$ ).

### 3.3. Effect of CSN1S2 or MG on cell viability, proliferation, and differentiation

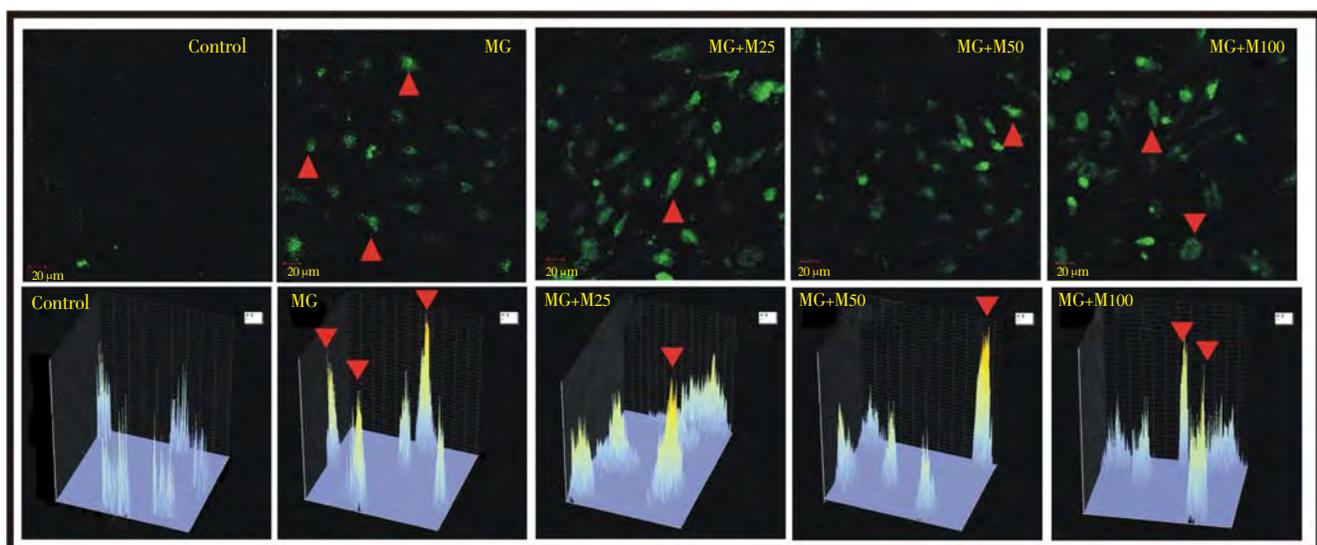
Table 2 summarizes the level of cell viability, proliferation, and differentiation in each group. Out of the 0.025, 0.050, and 0.100 mg/L doses of CSN1S2 protein of goat milk, only the two highest doses significantly prevented MG–induced increase in viability level ( $P<0.05$ ). The proliferation levels were significantly greater in the MG+M100 group compared to all groups ( $P<0.05$ ). The levels of differentiation were not significantly different between groups ( $P>0.05$ ).

**Table 2**

The level of cells viability, proliferation, and differentiation in each group. cells/mL.

Group	Viability	Proliferation	Differentiation
Control	83.33±28.86	116.66±28.86	32.66±26.76
MG	166.66±28.86	116.66±76.37	20.83±15.45
MG+M25	100.00±50.00	133.33±28.86	24.73±23.81
MG+M50	133.33±28.86 <sup>b</sup>	133.33±28.86	19.16±18.76
MG+M100	150.00±50.00 <sup>b</sup>	316.66±104.08 <sup>abcd</sup>	25.00±18.02

Values are presented as mean±SD; M25: 0.025 mg/L CSN1S2 protein of goat milk; M50: 0.050 mg/L CSN1S2 protein of goat milk; M100: 0.100 mg/L CSN1S2 protein of goat milk; <sup>a</sup>:  $P<0.05$  in comparison with control group; <sup>b</sup>:  $P<0.05$  in comparison with MG group; <sup>c</sup>:  $P<0.05$  in comparison with MG+M25 group; <sup>d</sup>:  $P<0.05$  in comparison with MG+M50 group.



**Figure 1.** Micrograph illustrating the intensity of ROS in MC3T3E1 pre–osteoblast cells exposed to MG.

M25: 0.025 mg/L CSN1S2 protein of goat milk; M50: 0.050 mg/L CSN1S2 protein of goat milk; M100: 0.100 mg/L CSN1S2 protein of goat milk; Immunofluorescence of ROS was labeled with 2,7–dichlorodihydrofluorescein diacetate staining (green) (400×; bar is 20 μm).

## 4. Discussion

In the present study, we found that there was no significant difference of ROS and malondialdehyde levels in pre–osteoblast MC3T3E1 cells exposed to high doses of MG. Our finding showed that MG does not induce ROS

and subsequent oxidative damage in pre–osteoblast MC3T3E1 cells. One research reported that ROS may act as an important role in bone loss by generating bone microenvironment oxidation<sup>[21]</sup>. Meanwhile, other studies showed that MG has no effect on the respiration as one source of ROS in nonmalignant tissues and cells<sup>[22]</sup>. We

hypothesized that behavior of pre-osteoblast MC3T3E1 cells in long time MG exposure does not involve ROS and oxidative stress.

Recent study showed that methylglyoxal induced cytotoxicity at long time exposure (4–24 h of exposure), and the highest level of ROS was detected at 5  $\mu\text{mol/L}$  MG on the MC3T3E1 pre-osteoblast cells for 6 h exposure[23–25]. In short time of exposure among myoblast L6 cell line, hepatocyte, and human endothelial cells showed no cytotoxic effect[26–28]. Our finding showed that 72 h of 5  $\mu\text{mol/L}$  MG exposure tended to increase the viability of pre-osteoblast, although there was no significant difference. Administration of CSN1S2 protein of goat milk at two highest doses significantly decreased cell's viability compared to MG group ( $P < 0.05$ ). Besides, at the highest dose's treatment on CSN1S2 protein of goat milk significantly increased cells proliferations compared to untreated group and all treatment groups, respectively ( $P < 0.05$ ). Our finding revealed that MG alone or in combination with the highest dose of CSN1S2 protein of goat milk has mitogenic activity. Therefore, the treatment of the highest dose CSN1S2 protein of goat milk in the presence of MG may have potency for bone loss treatment. We hypothesized that several amino acids in CSN1S2 protein of goat milk may be acted as a mitogenic agent. Threonine, methionine, and arginine can modulate the growth of osteoblasts cultured *in vitro*[29–32]. Osteoblast-like, MG63, cells cultured on the hydroxyapatite–aspartic acid and hydroxyapatite–glutamic acid nanocrystals display good proliferation[33].

In conclusion, CSN1S2 protein of goat milk at dose 0.100 mg/L in high MG environment inhibits the decrease of viability due to increases the proliferation of MC3T3E1 pre-osteoblast cell.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgements

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### Comments

#### Background

The treatment with new substance especially functional food to inhibit toxic effect of methyl glyoxal is applicable to osteoporosis related diabetes mellitus.

#### Research frontiers

MG is one of pre-AGEs compounds and related with osteoporosis in diabetic patients. The application of biopeptide is novel and it can provide functional food.

#### Related reports

The previous report clearly stated the effect of MG on pre-osteoblast. The treatment with new substances to inhibit toxic effect of methyl glyoxal is reasonable.

#### Innovations & breakthroughs

The treatment with new substance especially functional food to inhibit toxic effect of methyl glyoxal is reasonable.

#### Applications

The treatment with new substance especially functional food to inhibit toxic effect of methyl glyoxal is applicable to osteoporosis related diabetes mellitus.

#### Peer review

In this manuscript, the authors report beneficial effect of CSN1S2 on preosteoblast exposed to MG. The manuscript is well written, adequately illustrated and opens the ground for further evaluation of this topic.

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