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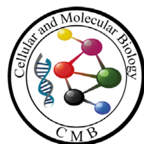

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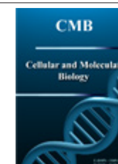

Original Article

Comparing the effect of using 2-Mercaptoethanol in the cell culture medium of different cell passages of human mesenchymal stem cells

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Abstract

Preparing a suitable cell culture medium that supports the biological needs of the growing cells is crucial to enhancing the success rate of any in vitro and in vivo experiments and minimizing undesirable interferences. Mesenchymal stem cells (MSCs) which are powerful regenerative stem cells require being grown in proper culture media to preserve their stemness and therapeutic properties. MSCs are usually grown in Dulbecco's Modified Eagle low glucose Medium (DMEM low glucose) which contains 5.6 mmol/L of glucose and is supplemented with Fetal Bovine Serum (FBS), antibiotics, and 2-Mercaptoethanol. The addition of 2-Mercaptoethanol to the cell culture medium was proposed long ago and has continued to be used until now. Despite the positive effects of adding 2-Mercaptoethanol in the cell culture medium, its use is still controversial and needs continuous updates to limit its interference with experimental treatments. Herein, we found that 2-Mercaptoethanol is beneficial to enhancing the proliferation and survival of MSCs at higher passage numbers while its effect is negligible for earlier passages. This concise study provides updates regarding the suitable time to add 2-Mercaptoethanol which can minimize its intermeddling with the experimental design and treatments.

Keywords: Mesenchymal stem cells, Cell passage, Culture medium, 2-Mercaptoethanol, Protocol, survival, Proliferation

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells that are extensively studied for their possible regenerative abilities [1]. These cells are well known for being highly accessible and can be isolated from different sources, such as bone marrow and adipose tissues[1, 2]. MSCs are plastic-adherent cells and have morphological similarities with fibroblasts [3]. MSCs secrete a cocktail of paracrine and growth factors that can activate the repairing mechanism in the damaged tissues [4]. These cells can also home at site of transplantation and adopt the internal machinery of the host tissues and start differentiating to restore the homeostasis of the damaged cells[3, 5]. The preservation of biological and molecular characteristics of MSCs is important to achieve a successful post-transplantation therapeutic outcome [6, 7]. Compliance with good cell culturing practices is an essential step towards achieving a successful MSCs transplantation. MSCs are usually cultured and expanded in Dulbecco's Modified Eagle low glucose Medium (DMEM low glucose) which contains 5.6 mmol/L of glucose and is supplemented with Fetal Bovine Serum (FBS), antibiotics, and 2-Mercaptoethanol [1, 8, 9]. This specialized cell culture medium was found to be efficient in supporting and maintaining the proliferation and survival of seeded MSCs.

2-Mercaptoethanol is a xenobiotic organosulfhydryl that has a powerful antioxidant ability to control the level of oxygen free radicals and prevent their toxic level [10–12]. The addition of 2-Mercaptoethanol to the cell culture medium was proposed 40 years ago to enhance the proliferation and survival of immune cells which is important to potentiate their immunological characteristics [11, 12]. Nowadays, 2-Mercaptoethanol is routinely used in the cell culture media of many cell types whether they are adherent or suspended cells[8, 10, 13]. Despite its regular use as an important constituent in the cell culture medium, its actual benefits are still controversial and need to be updated. In this concise and brief study, we addressed the effect of adding 2-mercaptoethanol in MSCs cell culture medium and its impact on different cell passages in order to update the current cell culture practices that can benefit both novice and experienced researchers.

2. Materials and Methods

2.1. Human mesenchymal stem cells

The Human Adipose tissue-derived MSCs (hAD-

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MSCs) used in this study were commercially purchased from Lonza (Cat# PT5006, Lot# 21TL138912) and were characterized for the presence of MSCs cell surface markers by the company.

2.2. Human mesenchymal stem cell culturing procedure

Frozen hAD-MSCs at Passage 5 (P5) and Passage 10 (P10) were thawed, counted and cultured using Dulbecco's Modified Eagle's Medium Low Glucose (DMEM-Low Glucose, Euroclone) which contained 5.6 mmol/L glucose and was supplemented with 10% fetal bovine serum (FBS, Gibco), 0.1 mg/ml streptomycin and 100 units/ml penicillin G in standard cell culture incubators (5% CO_2/95% air; 37 °C). Each passage was cultured and expanded in the presence or absence of 2-Mercaptoethanol (Thermo Fisher, Cat# 31350010, 50mM) in the cell culture medium. Cells Medium was changed every other day, and cells were allowed to grow for 5 days. The final concentration of 2-Mercaptoethanol in the cell culture medium was 50µm [14, 15] and was added each time the medium was changed for the cells. The cells that were grown in cell culture medium without (w/o) 2-Mercaptoethanol were considered the control group.

2.3. Cytotoxicity assays

To measure the level of cytotoxicity in human MSCs after being seeded in 96-well plates at $5x10^4$ /well and cultured in cell culture media with or without 2-Mercaptoethanol for 5 days, we measured the lactate dehydrogenase (LDH) which was released from the damaged MSCs (LDH Assay Kit, Abcam, Cat# ab102526) and the absorbance values were obtained using Cytation 5 (BioTek, USA). The viability percentages of human MSCs were also assessed using 0.4 % Trypan blue, and the percentages of viable cells were obtained using Corning® CytoSmart Cell Counter.

2.4. Proliferation assay

Cell proliferation of expanding human MSCs was measured using the commercial kit (WST-1 Assay Kit, Abcam, Cat# ab65473). Briefly, human MSCs at different passages were seeded in 96-well plates (5×10^4 cells/well) and cultured in cell culture media with or without 2-Mercaptoethanol for 5 days, followed by the addition of 10 µl of WST1 solution to each well. After incubation for 4 hours, the absorbance values which are correlated with the number of proliferating cells were detected at 450 nm using Cytation 5 (BioTek, USA).

2.5. Reactive Oxygen Species detection assay

The level of Reactive Oxygen Species (ROS) was analyzed using ROS Detection Assay Kit (Abcam, Cat # ab287839). Briefly, human MSCs at different passages were seeded in 96-well plate (2×10^4 cells/well) and cultured in cell culture media with or without 2-Mercaptoethanol for 5 days. Media was aspirated and cells were washed in 100 µl of ROS Assay Buffer I/ROS Assay Buffer, followed by the addition of 100 µl of 1X ROS Label I/ROS Label diluted in ROS Assay Buffer I/ROS Assay cells/ml into each well. The working ROS label solution was incubated for 45 min at 37°C in the dark. After the incubation time was over, the ROS label working solution was removed and 100 µl of phosphate-buffered Saline (PBS) was added to each well. Fluorescent intensity and fluorescent images were detected at Ex/Em= 495/529 nm using Cytation 5 (BioTek, USA).

2.6. Statistical analysis

Data were reported as mean ± SD. Comparison of data between multiple groups was performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test, and analysis between two groups was made using Student's *t*-test (two-tailed). Statistical significance is determined as $P < 0.05$. Each figure represents one of at least three independent quantifiable experiments.

3. Results

3.1. 2-Mercaptoethanol differential effect on the proliferation of hAD-MSCs

MSCs are highly proliferating cells with a doubling time of around 36 hours depending on the source of these cells [16]. Maintaining proper MSCs proliferation is an essential step in preserving their stemness characteristics. Preparing a nourishing cell culture medium is crucial to supporting the proliferation of MSCs. Herein, we found that the proliferation of hAD-MSCs at P5 wasn't significantly different in the presence or absence of 2-Mercaptoethanol in the cell culture medium (Figure 1a), whereas the proliferation of cells at P10 was significantly higher when 2-Mercaptoethanol was added to the cell culture medium (Figure 1a).

3.2. 2-Mercaptoethanol differential effect on the survival of hAD-MSCs

The concept behind adding 2-Mercaptoethanol is to control the level of toxic free radicals that may impact the survival of growing cells [10]. MSCs can also secrete many antioxidant factors that can prevent the rise in the level

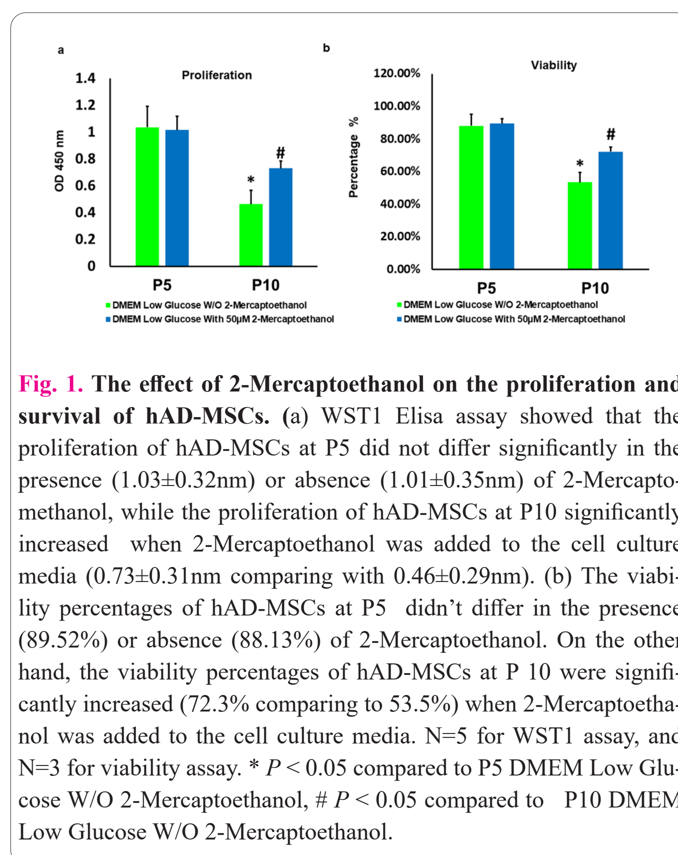

Fig. 1. The effect of 2-Mercaptoethanol on the proliferation and survival of hAD-MSCs. (a) WST1 Elisa assay showed that the proliferation of hAD-MSCs at P5 did not differ significantly in the presence (1.03±0.32nm) or absence (1.01±0.35nm) of 2-Mercaptomethanol, while the proliferation of hAD-MSCs at P10 significantly increased when 2-Mercaptoethanol was added to the cell culture media (0.73±0.31nm comparing with 0.46±0.29nm). (b) The viability percentages of hAD-MSCs at P5 didn't differ in the presence (89.52%) or absence (88.13%) of 2-Mercaptoethanol. On the other hand, the viability percentages of hAD-MSCs at P 10 were significantly increased (72.3% comparing to 53.5%) when 2-Mercaptoethanol was added to the cell culture media. N=5 for WST1 assay, and N=3 for viability assay. * $P < 0.05$ compared to P5 DMEM Low Glucose W/O 2-Mercaptoethanol, # $P < 0.05$ compared to P10 DMEM Low Glucose W/O 2-Mercaptoethanol.

of reactive oxygen species (ROS) [17, 18]. The passaging of MSCs can change some of their well-known therapeutic characteristics[19, 20]. We found in this study that the survival of hAD-MSCs at P5 was similar in the presence or absence of 2-Mercaptoethanol in the cell culture media (Figure 2b). The viability percentage was around 85% whether 2-Mercaptoethanol is added or not (Figure 2b). Conversely, the survival of hAD-MSCs at P10 was higher when 2-Mercaptoethanol was added to the cell culture media (Figure 2b). The viability percentage was around 56% in the absence of 2-mercaptoethanol, and it increased significantly to almost 73% when 2-mercaptoethanol was there in the cell culture media (Figure 2b). Cells that get damaged and go through a cell death pathway will start releasing Lactate dehydrogenase (LDH). We measured the LDH activity using LDH Elisa kit. The cytotoxicity and LDH activity values were almost comparable in the present or absent of 2-Mercaptoethanol in the cell culture media where hAD-MSCs at P5 were grown (Figure 2). Nevertheless, the cytotoxicity and LDH level values were remarkably higher when 2-Mercaptoethanol was omitted from the cell culture media where hAD-MSCs at P10 were cultured (Figure 2).

To figure out the mechanism of reduced cytotoxicity and enhanced survival after adding 2-Mercaptoethanol in the cell culture media of hAD-MSCs at P10, we measured the level of ROS in these cells. The level of ROS was remarkably higher in MSCs at P10 compared to MSCs at P5 (Figure 3).

Adding 2-Mercaptoethanol in the cell culture media reduced the level of ROS of MSCs at P10 and enhanced their survival, with no significant effect observed in MSCs at P5 (Figure 4). These results indicate that the positive effects of adding 2-Mercaptoethanol in the cell culture media of MSCs vary depending on cell passage number and is needed more with increasing cell passage number.

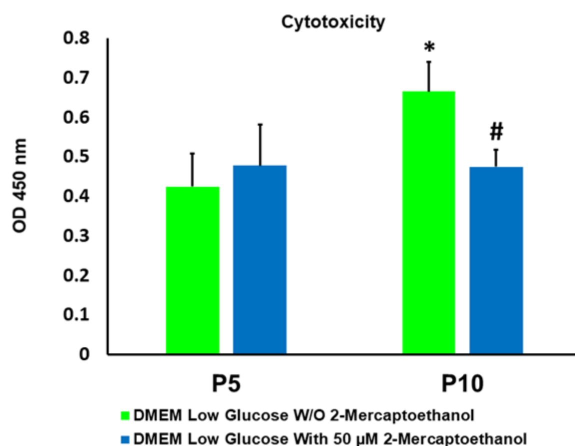

Fig. 2. The cytotoxicity level in the presence or absence of 2-Mercaptoethanol. LDH cytotoxicity assay revealed that the LDH release from hAD-MSCs at P5 and the level of cytotoxicity were similar whether 2-Mercaptoethanol was omitted (0.42nm±0.081) or added (0.47nm±0.089) to the cell culture media, whereas, the LDH release from hAD-MSCs at P10 and the level of cytotoxicity were higher in the absence (0.66nm±0.087) of 2-mercaptoethanol in the cell culture media comparing to the cells in culture media with 2-mercaptoethanol (0.47nm±0.079). N=5, * $P < 0.05$ compared to P5 DMEM Low Glucose W/O 2-Mercaptoethanol. # $P < 0.05$ compared to P10 DMEM Low Glucose W/O 2-Mercaptoethanol.

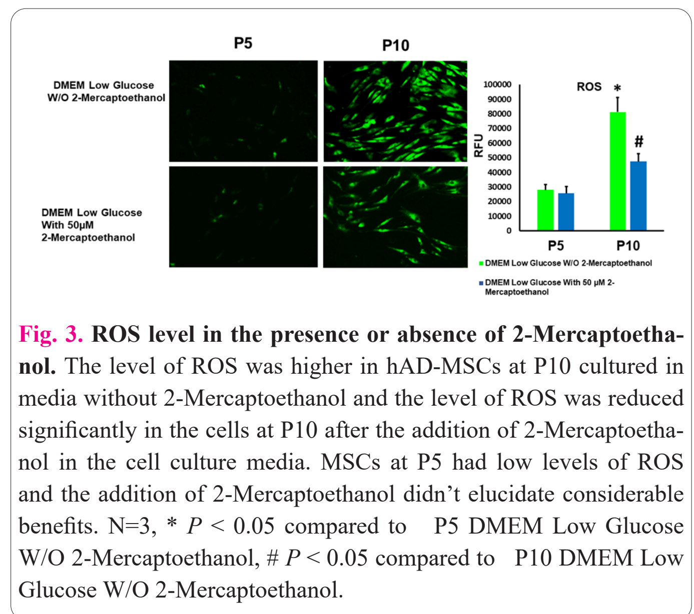

Fig. 3. ROS level in the presence or absence of 2-Mercaptoethanol. The level of ROS was higher in hAD-MSCs at P10 cultured in media without 2-Mercaptoethanol and the level of ROS was reduced significantly in the cells at P10 after the addition of 2-Mercaptoethanol in the cell culture media. MSCs at P5 had low levels of ROS and the addition of 2-Mercaptoethanol didn't elucidate considerable benefits. N=3, * $P < 0.05$ compared to P5 DMEM Low Glucose W/O 2-Mercaptoethanol, # $P < 0.05$ compared to P10 DMEM Low Glucose W/O 2-Mercaptoethanol.

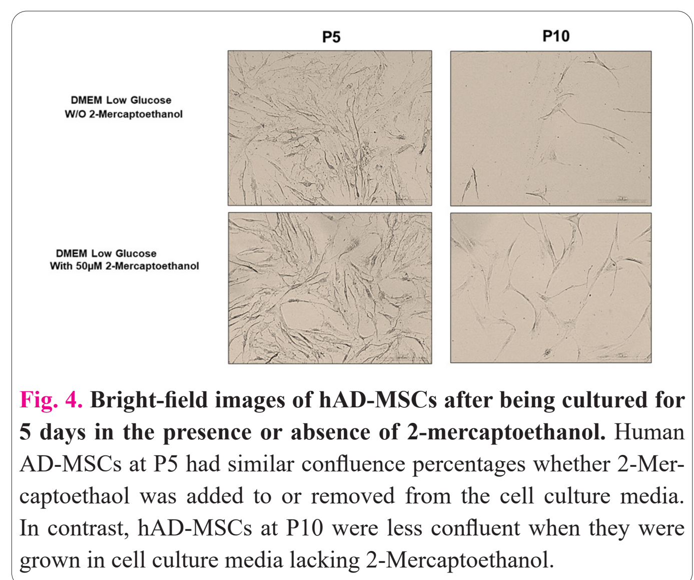

Fig. 4. Bright-field images of hAD-MSCs after being cultured for 5 days in the presence or absence of 2-mercaptoethanol. Human AD-MSCs at P5 had similar confluence percentages whether 2-Mercaptoethaol was added to or removed from the cell culture media. In contrast, hAD-MSCs at P10 were less confluent when they were grown in cell culture media lacking 2-Mercaptoethanol.

Furthermore, these findings update the frequently used protocol to prepare cell culture medium and delineate the suitable time to add certain supplementing constituents to the cell culture medium to improve the cells biological characteristics.

4. Discussion

Choosing the suitable cell culture medium that contains the required and necessary supplements while minimizing additives that can affect the biological and molecular characteristics of the growing cells is an essential step to increase the success rate of any in vitro and in vivo studies. MSCs, which are ideal stem cell types for regenerative purposes, have inherited characteristics that should be maintained and preserved in the pre and post-transplantation phases[3, 21]. MSCs are grown and expanded in specific culture media that support their stemness characteristics[1, 2]. MSCs are usually cultured in DMEM low glucose medium that contains FBS, antibiotics cocktail, and 2-Mercaptoethanol. This culture medium formula can ensure supporting the proliferation and proper survival of MSCs. 2-Mercaptoethanol, which is a strong reducing agent, can help in controlling the level of free radicals in the cell culture [22]. Introducing 2-Mercaptoethanol as a beneficial additive in the cell culture medium was based on

its positive impact on increasing the proliferation of lymphocytes[11, 12]. The exact mechanisms of how 2-Mercaptoethanol increases the survival and proliferation rate of cells have not been clarified yet, and it is proposed to be logically related to its ability to expunge toxic products [23, 24]. Despite its routine use in the cell culture medium of immune and non-immune cells, there is a lot of controversy surrounding its use as it is highly toxic for some cells and can affect the molecular features of the growing cells which can impact the outcomes of experimental treatments [25, 26]. The addition of 2-Mercaptoethanol in the cell culture medium was established 40 years ago, and it has not been updated by recent studies. In this study, we revealed that 2-Mercaptoethanol is not required for MSCs early passages, and the possible explanation is the ability of these cells at early passages to secrete enough antioxidants and growth factors to support their survival and proliferation [14, 16, 18]. As the passage number increases, the addition of 2-Mercaptoethanol can produce more positive outcomes and speed up the growth of MSCs by reducing the level of ROS that has been accumulated as MSCs passage increases. This can be explained by the reduced ability of these cells to support their growth and proliferation with increased passage numbers. Compromising the unnecessary additives in the cell culture media can help in reducing the chances of changing their biological characteristics and minimize their possible interference with the outcomes of certain experimental treatments.

Conflict of interest
The author declares that there is no conflict of interest.

Consent for publications
The author read and approved the final manuscript for publication.

Ethical approval
The ethical approval was not needed in this study. The human cell lines used for this study were commercially purchased.

Informed Consent
The authors declare that no patients were used in this study.

Availability of data and material
The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author contributions
E.A.E.R conceptualized, carried out experiments, analyzed the data, and wrote the manuscript. R. R.K drafted the manuscript. All authors read and approved the final version of manuscript.

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