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Cytotoxic effect of cigarette smoke condensate on mice and rat mesenchymal stem cells and HeLa cells

Aftab AHMAD, Abdul Rauf SHAKOORI

Aim: To study the effect of cigarette smoke condensate (CSC) on HeLa cells and mesenchymal stem cells (MSCs) of mice (mMSC) and rats (rMSC). Cigarette smoking results in the deaths of millions of people annually. The constituents of cigarette smoke produce oxidants in the cells that result in inflammation, carcinogenesis, and apoptosis.

Materials and methods: CSC was collected on filter paper and dissolved in dimethyl sulfoxide, and 10-100 µg/mL was added to HeLa cells, mMSCs, and rMSCs in a 96-well plate. The cytotoxic effect of CSC was measured after 48 h using a neutral red uptake assay. Images of the cells were taken after growing in the neutral red medium.

Results: There was no significant change in cell morphology after exposing the cells to CSC for 48 h. The growth of the HeLa cells, mMSCs, and rMSCs was reduced by 14%, 38%, and 36%, respectively.

Conclusion: The present study demonstrates the growth inhibitory effect of CSC on stem cells compared to mature HeLa cells. The study also proves that CSC does not promote proliferation.

Key words: Cigarette smoke condensate, HeLa cells, mesenchymal stem cells, proliferation, cytotoxic effect

Introduction

The habit of tobacco smoking is very addictive and can become a life-long addiction if a person starts at an early age. The damaging effects of tobacco smoking are dose-dependent and if damages are not very severe, they could be reversed by smoking cessation (1). The estimated number of smokers is 1.3 billion, which results in more than 6 million deaths worldwide (2). Tobacco smoking can result in several diseases including oral and lung cancers and heart and lung diseases, and it can also diminish the regenerative function of different cells in the body (3). Smoking is injurious to health because cigarette smoke contains more than 4000 different chemicals and many of them are very toxic. More than 60 compounds of cigarette smoke are known human carcinogens. There are 2 phases of cigarette smoke, gaseous and particulate. The gaseous phase

is composed of several poisonous gases (carbon monoxide, ammonia, and hydrogen cyanide). The particulate phase mainly consists of nicotine and tar (4-6).

Exposure of cells to cigarette smoke condensate (CSC) results in the generation of oxidants within the cells, resulting in the imbalance of oxidants and antioxidants, which leads to oxidative stress in cells. Inflammation, apoptosis, and carcinogenesis are the outcomes of oxidative stress in the cells. Apoptosis could be triggered in cells by different means; it can be a spontaneous process or it can be due to an exposure to radiation, heat, and steroids. Apoptosis is usually characterized by DNA fragmentation, reduction in cell size, condensation of chromatin, and the formation of apoptotic bodies in cells. Exposure to CSC normally results in oxidative stress in the cells (7,8). The oxidative stress results in

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many inflammatory lung diseases, including chronic obstructive pulmonary disease (COPD). In cigarette smokers, this oxidative stress could be directly due to cigarette smoke or by the oxidants released by inflammatory cells that are recruited to the place where injury is caused by cigarette smoke (9).

Different systems have been used to study the effect of CSC. The easy and convenient way is to use cell lines as experiments, which can be performed under controlled conditions. Bronchial epithelial cells have usually been used to study the effect of whole CSC or individual components of CSC (10,11). Stem cells are rapidly dividing cells in the body, and therefore can be a good system to study the effects of environmental toxins. Studies have shown that exposure to cigarette smoke results in reduced proliferation of cells, reduced attachment, and apoptosis of stem cells (12). In this report, we show the effect of CSC on HeLa cells and mesenchymal stem cells (MSCs) from mice (mMSC) and rats (rMSC). We investigated and compared the effects of CSC on stem cells and mature cells.

Materials and methods

Isolation of mMSCs and rMSCs

The tibias and femurs of 16-week-old Swiss Webster female mice were excised aseptically and cleaned of adhering soft tissues. The epiphysis was cut and the bone marrow was flushed out using 5 mL of Dulbecco's modified Eagle's medium (DMEM) (incomplete medium). Single cell suspension was obtained by passing the flushed-out cells from a 22-gauge needle several times and the total cells were counted with a hemocytometer. Added to the cell suspension was 10 mL of complete medium (DMEM containing glutamine, 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin), and the suspension was transferred to a 75-cm² flask (Nunc). The flask was incubated at 37 °C with 5% CO₂ in a humidified environment for 24 h. The medium was removed and the cells were washed with phosphate-buffered saline (PBS). Fresh medium was added and the flask was incubated again.

A 7-week-old female Wistar rat was selected for the isolation of rMSCs. The rMSCs were essentially isolated by the same procedure as described above,

except for flushing the cells through a 16-gauge needle several times. Fetal bovine serum (FBS, 1 mL) was added to 10 mL of bone marrow, which was flushed out and spun at 450 × g for 5 min at room temperature. The supernatant was discarded, whereas the pellet was resuspended in complete medium as above.

HeLa cells

HeLa cells were grown in complete medium (DMEM, 2 mM glutamine, 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin) until they reached 80% confluency. The cells were trypsinized with trypsin-EDTA (PAA, Austria) and counted with a hemocytometer.

Preparation of CSC

CSC was collected using filter paper that was cut to size and placed in front of a cigarette filter, which was then smoked by a person. The filter paper was weighed before and after smoking and the increase in weight (5 mg) was taken as the weight of the CSC particles deposited on the filter paper. The filter paper was added into 0.25% dimethyl sulfoxide (DMSO) to dissolve the CSC particles. The CSC solution was incubated at 50 °C to dissolve it completely and was later filter sterilized using a 0.22-µm filter (Millipore).

Effect of CSC on HeLa, mMSCs, and rMSCs

The HeLa, mMSC, and rMSC cultures were grown to 80% confluency. The growth inhibitory effect of CSC was determined using a neutral red assay. Briefly, cells (1×10^4 cells/well for HeLa and 5×10^3 cells/well for mMSCs and rMSCs) were inoculated into a 96-well cell culture plate. Cells were grown in complete medium for 24 h and then the medium was replaced with complete medium (200 µL) containing 10-100 µg/mL CSC for 48 h. The medium was aspirated and the cells were incubated with neutral red medium (100 µL) for 3 h at 37 °C. The cells were washed with PBS and images were taken. Into each well, 200 µL of neutral red destaining solution (50% ethanol (96%), 49% dH₂O, and 1% glacial acetic acid) was added, and the plates were placed on a shaker at 100 rpm for 10 min at room temperature. The absorbance of the supernatant was taken at 492 nm and 630 nm using an ELISA reader (HumaReader Plus, HUMAN). All of the assays were performed in triplicate. Growth percentage and standard deviation (SD) of the cells was calculated from the optical density (OD) values.

Results and discussion

Isolation and growth of mMSCs and rMSCs

After growing the cells in standard culture conditions for 24 h, flushed-out bone marrow stem cells started adhering to the surface of the plates. All of the nonadherent cells were removed by changing the medium and washing the cells 2 times with PBS. After 6-8 days, the cells became confluent and were subcultured by treating them with trypsin-EDTA. The mMSCs and rMSCs had similar morphology. Both showed fibroblast-like cells.

Effect of CSC on HeLa cells

There was no significant change in the morphology of the HeLa cells (Figure 1). There was also no

reduction in the growth of the cells. Only a 14% decrease in proliferation was observed at a 100 $\mu\text{g}/\text{mL}$ concentration of CSC. The effect of CSC on HeLa cells is dose-dependent, as there is a gradual decrease in the proliferation of the cells with an increase in the concentration of CSC (Figure 2).

Effect of CSC on mMSCs

After growing the cells in the presence of CSC for 48 h, a neutral red uptake assay was performed. Change in the morphology of the cells was observed at a concentration of 80 $\mu\text{g}/\text{mL}$, as there was shrinkage in the size of the cells. There was also a reduction in the proliferation of the cells with an increase in the concentration of CSC, and a maximum reduction in the cell proliferation was observed at

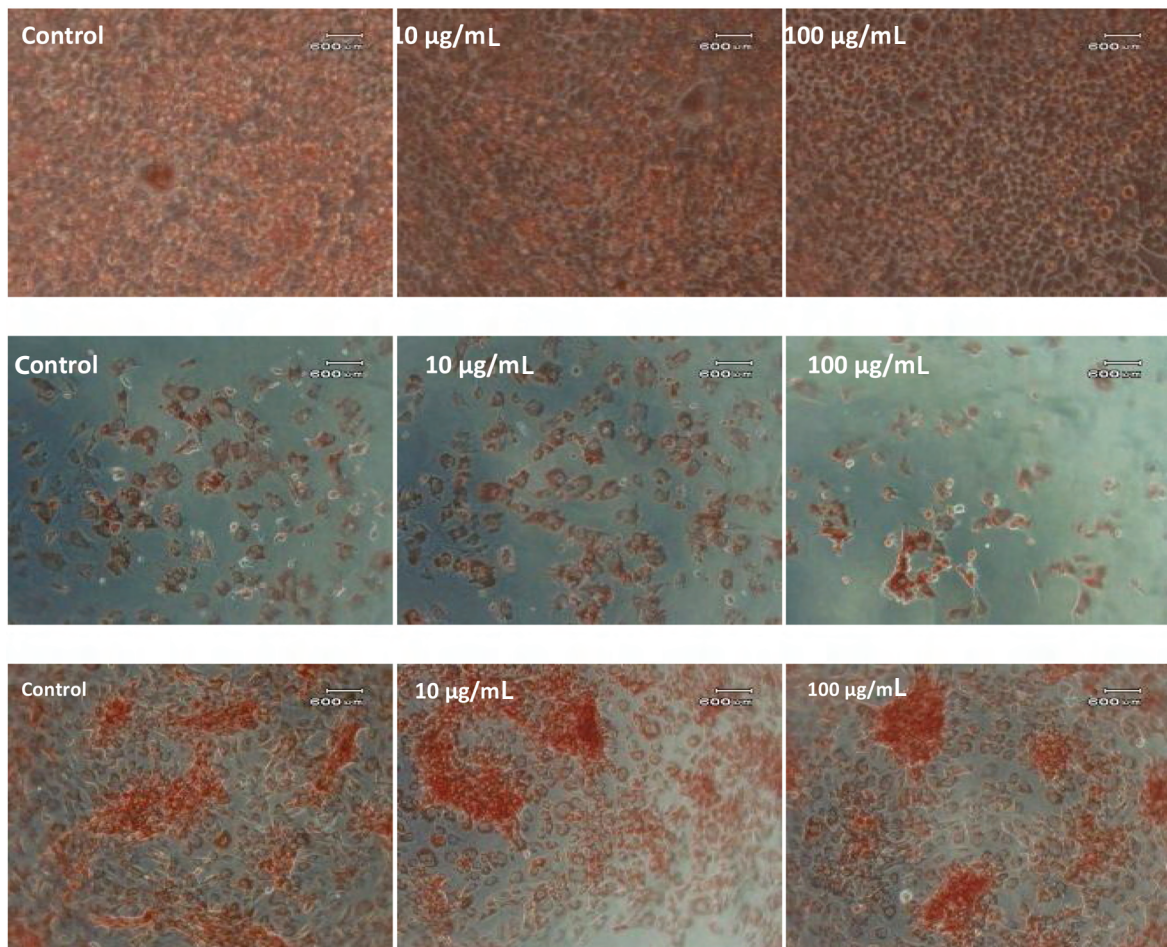


Figure 1. Effect of CSC on the growth of HeLa, mMSCs, and rMSCs. The cells were treated with CSC at concentrations of 10-100 $\mu\text{g}/\text{mL}$. Control cells and cells treated with 10 and 100 $\mu\text{g}/\text{mL}$ are shown. The top row is CSC-treated HeLa cells, the middle is mMSCs, and the lower is rMSCs.

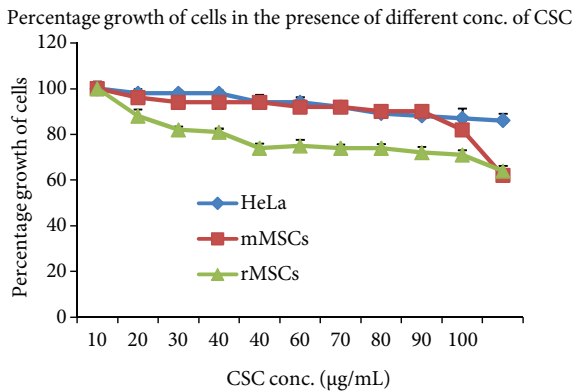


Figure 2. Effect of different concentrations of CSC on the growth of HeLa cells, mMSCs, and rMSCs. The error bars indicates the standard deviation.

a concentration of 100 µg/mL (Figure 1). The effect of CSC on mMSCs was not very significant until the CSC reached a concentration of 80 µg/mL; however, after this percentage, proliferation of the cells decreased. At a concentration of 100 µg/mL, there was a 38% reduction in the proliferation of mMSCs (Figure 2). Shrinkage in the size of the cell is one of the characteristics of apoptosis, so these cells may undergo apoptosis if exposed to CSC for a longer duration.

Effect of CSC on rMSCs

When rMSCs were exposed to CSC for 48 h, there was no significant effect on their morphology (Figure 1). All of the cells appeared normal, but there was a reduction in the proliferation of the cells with an increase in the concentration of CSC (Figure 2). In the case of rMSCs, there was a gradual reduction in the percentage of proliferating cells, and at a concentration of 100 µg/mL, there was a 36% reduction. The reduction percentage was comparable to that of the mMSCs (Figure 2). The observed reduction rates for mMSCs (38%) and rMSCs (36%)

at 100 µg/mL CSC were much higher than the 14% seen in the proliferation of HeLa cells.

Some studies have shown that short exposure to CSC in epithelial cells results in rapid proliferation of the cells compared to the control (13). However, in this study, there was no such effect on the HeLa cells, mMSCs, or rMSCs, and in all of these cases, the CSC inhibited the proliferation of the cells. The effect of CSC could be different on MSCs and osteoblasts. According to one study, lesser concentrations of CSC have a stimulatory effect on the growth of osteoblasts (14). In accordance with the present results, some other studies also proved that cigarette smoke reduces the proliferation of cells, and in addition, it also impairs the differentiation process (15). The response of CSC to different cells might be different. Researchers have used various concentrations of CSC, from 0 to 500 µg/mL, on different cells in order to check the effect of CSC on cells at the molecular level (16,17). Although in the current study higher concentrations of CSC were not used, future experiments will aim to use higher concentrations of CSC to attain IC_{50} values for HeLa cells and MSCs.

In the present study, 3 different cell lines, HeLa, mMSC, and rMSC, were exposed to CSC. According to the results, MSCs are more sensitive to CSC, and there is much more reduction in the growth of MSCs when exposed to higher concentrations of CSC as compared to HeLa cells. Further studies can be conducted by exposing cells to still higher concentrations of CSC and for different periods of time to attain more detailed information on the effects of CSC on animal cells, and in particular stem cells.

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