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Short-term effects of 7-ketocholesterol on human adipose tissue mesenchymal stem cells *in vitro*

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ABSTRACT

Oxysterols comprise a very heterogeneous group derived from cholesterol through enzymatic and non-enzymatic oxidation. Among them, 7-ketocholesterol (7-KC) is one of the most important. It has potent effects in cell death processes, including cytoxicity and apoptosis induction. Mesenchymal stem cells (MSCs) are multipotent cells characterized by self-renewal and cellular differentiation capabilities. Very little is known about the effects of oxysterols in MSCs. Here, we describe the short-term cytotoxic effect of 7-ketocholesterol on MSCs derived from human adipose tissue. MSCs were isolated from adipose tissue obtained from two young, healthy women. After 24 h incubation with 7-KC, mitochondrial hyperpolarization was observed, followed by a slight increase in the level of apoptosis and changes in actin organization. Finally, the IC_{50} of 7-KC was higher in these cells than has been observed or described in other normal or cancer cell lines.

1. Introduction

Maintenance of the cellular redox balance is crucial for cell survival. Lipids are very sensitive to oxidative modifications [1]. Lipid oxidation is characterized by complex product patterns, including lipid peroxides, aldehydes, and many others. Moreover, molecules with regulatory functions are generated by the oxidation of lipids and fatty acids [2–6]. Oxidized phospholipids are recognized as important mediators of cellular signaling [7]. Oxidative stress, antioxidant efficiency, and lipid oxidizability are known to change in different pathophysiological conditions [8].

Cholesterol is an important structural element of cell membranes and an essential substrate for the biosynthesis of several molecules, such as bile acids and steroid hormones [9]. It is transported in plasma mainly by low-density lipoprotein (LDL). The cholesterol molecule is easily oxidized and may be transformed into numerous oxidation products known as oxysterols. They can be considered a way to route the cholesterol molecule for catabolism.

Oxysterols comprise a highly heterogeneous group derived from cholesterol through enzymatic and non-enzymatic oxidation [10]. Among them, 7-ketocholesterol (7-KC) is one of the most important, and is found in relatively large abundance in oxidized low-density lipoprotein (oxLDL) [11,12].

Oxysterols exhibit several biological activities. They play essential roles in a number of physiological processes, such as cholesterol homeostasis regulation, platelet aggregation, and protein prenylation [13]. They participate in the control of lipid metabolism and regulation of the immune system, and have been associated with several other pathophysiological processes [14].

Oxysterols have potent effects on cell death processes, including apoptosis induction [15,16]; reactive oxygen species (ROS) are reportedly involved in this effect [15]. In fact, oxysterols have been shown to exhibit cytotoxicity in a number of cell lines, including smooth muscle cells, fibroblasts, and vascular endothelial cells [17].

Mesenchymal stem cells (MSCs) are multipotent cells characterized by self-renewal and cellular differentiation abilities [18]. *In vitro*, according to the Society for Cellular Therapy, MSCs are described as cells that are capable of adhering to plastic with fibroblast-like morphology and differentiating into mesenchymal lineages (e.g., chondrogenic, osteogenic, and adipogenic lineages)

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under appropriate culture conditions. They are also characterized by the expression of CD105, CD73, and CD90, as well as by the lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II. The property of self-renewal is essential for the expansion of the stem cell pool during fetal development, as well for maintenance of the stem cell pool throughout the organism's lifespan [19].

Several oxysterols exert important effects on cell death, proliferation, and differentiation: 7-ketocholesterol is known to induces monocyte differentiation [11]; and 22(R)-hydroxycholesterol and 25-hydroxycholesterol are potent inhibitors of cell growth in thymocytes, lymphocytes, and keratinocytes, as well as inducers of keratinocyte differentiation [20]. However, very little is known about the effects of oxysterols in MSCs. There are few descriptions, and most are related to their osteogenic effect. In fact, some types of oxysterols can behave as anti-adipogenic signals by diverting pluripotent MSCs away from adipogenic and toward osteogenic differentiation [14].

Here we described the short-term cytotoxic effect of 7-ketocholesterol on MSCs derived from human adipose tissue.

2. Materials and methods

2.1. Human adipose tissue mesenchymal stem cell isolation and characterization

Adipose tissue was obtained from two young, healthy women (20 and 22 year old) that underwent abdominal plastic surgery for esthetic reasons. The Ethical Committee of the Institution approved the protocol for this study, and patients provided written informed consent. With more than 90% of the volume, adipocytes have been described to represent the major cell type in lipoaspirate material [21]. From each patient, 30 ml of fatty material was collected in a sterile flask. The tissue was dissociated with 30 mg of collagenase type IA diluted in 30 ml of Dulbecco's Modified Eagle Medium (DMEM) for 45 min, and then centrifuged to isolate the cells. Medium consisting of 12 ml DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotics/antifungal (100 μg/mL streptomycin, 100 UI/mL penicillin, 0.25 μg/mL amphotericin B) was added to the cell pellet. After transference to 75 cm^2 culture flasks, cells were incubated at 37 °C in 5% CO_2 atmosphere [22]. After reaching confluence, cells were detached with a solution containing 0.05% trypsin in 0.02% EDTA and seeded at a density of 5×10^3 cells/cm^3. Cells were used for experiments at the 4th passage.

Total RNA was extracted from undifferentiated adipose tissue mesenchymal stem cells (hAMSCs) using Trizol (Invitrogen, Caltag Laboratories, Carlsbad, CA) according to the manufacturer's instructions. Oct-4 and Nanog gene expression were determined using RT-PCR, as described [23].

Cell surface markers were measured using flow cytometry in a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ). After trypsinization and washing with PBS, approximately 5×10^5 cells were stained for 15 min in the dark with primary monoclonal antibodies against CD34, CD49d, CD73, and CD90, conjugated with fluorescein isothiocyanate (FITC) or phycoerythrine (PE), all from Invitrogen. The number of acquired events was 10,000 per acquisition using the BD CellQuest Pro software. Finally, hAMSCs were also characterized by their osteogenic and chondrogenic differentiation capability *in vitro*, as described [23].

2.2. Stem cell treatments

The purity of 7-KC (Sigma–Aldrich, St. Louis, MO) was determined to be ~98% by GS/MS. For all experiments, a 7-KC stock solution was prepared at a concentration of 1000 μM in absolute ethanol. The concentrations used in the experiments were in the range of those described to induce cell death on several cell lines [24]. hAMSCs from each donor were plated at a density of 5×10^3 - cells/cm^3 in 96-well Black Flat Bottom Polystyrene Microplates (Corning, MA) and incubated as described above. After 24 h, the medium was replaced with fresh DMEM without FBS for 24 h. Several concentrations of 7-KC (0–100 μM, 200 μl final volume) were added, followed by incubation for another 24 h. At the end of this experimental period, several parameters were determined in each of the two samples, as described hereafter.

2.3. Cell viability assay

Cell viability was determined using MTT (3-[4,5 dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) [25]. Briefly, 10 μl MTT reagent (Sigma–Aldrich) were added to each well to a final concentration of 5 mg/ml, incubated for 4 h at 37 °C and centrifuged at 2000 rpm for 10 min. The medium was discarded and 100 μl of dimethyl sulfoxide (Sigma–Aldrich) were added to each well. The experiment was performed using six replicates for each oxysterol concentration and was repeated three times. Cholesterol at the same concentrations was also used as control. The amount of formazan was determined by measuring the absorbance at 570 nm refereed to 630 nm using an Elx800™ Absorbance Microplate Reader (Biotek, Winooski, VT). For IC_{50} calculations, survival data were evaluated by variable slope curve-fitting with GraphPad Prism (GradPad Software, CA).

2.4. Detection of apoptosis

The Annexin V: FITC Apoptosis Detection Kit II (BD Biosciences) was used to determine the percentages of apoptotic cells, as described by the manufacturer. Cells were incubated with 0.5 μl of FITC Annexin V and 0.5 μl PI and incubated for 15 min at room temperature in the dark. The nuclei were counterstained with 0.1 μg/mL Hoechst 33342 (Molecular Probes, NY) for 10 min. The presence of apoptosis was analyzed within 1 h using an ImageXpress Micro high content screening system (Molecular Devices, Sunnyvale, CA). Nine sites per well and three wells per treatment were acquired. Apoptosis (%) was determined by the MetaXpress Cell Health software application.

2.5. Detection of caspase-3/7 activity

Caspase-3/7 activity was measured using the NucView 488 caspase-3 Assay kit for live cells (Biotium, Hayward, CA). The nuclei were counterstained with 0.1 μg/mL Hoechst 33342. Fluorogenic substrates were determined using ImageXpress. Nine sites per well and three wells per treatment were acquired. Caspase-3/7 activity was determined using the cell scoring MetaXpress software.

2.6. Measurement of transmembrane mitochondrial potential

Mitoscreen (BD Biosciences) was used to evaluate the transmembrane mitochondrial potential ($\Delta\psi$). JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide) is a lipophilic fluorochrome that is used to evaluate the status of $\Delta\psi$. JC-1 can exist in two different states, aggregates (at higher dye concentrations) and monomers (at low concentrations), each with different emission spectra. Uptake of JC-1 monomers into mitochondria is driven by the $\Delta\psi$. The $\Delta\psi$ of normal, healthy mitochondria is polarized, and JC-1 is rapidly taken up by such mitochondria. This uptake increases the concentration of JC-1, leading to the formation of JC-1 aggregates within the mitochondria. JC-1

aggregates exhibit a red spectral shift, which results in higher levels of red fluorescence.

The cells were incubated with JC-1 working solution for 15 min at 37 °C in an atmosphere of 5% CO_2. ImageXpress was used to determine the presence of JC-1 aggregates in hATSCs after treatments. Nine sites per well and three wells per treatment were acquired. The presence of JC-1 aggregates was determined using the transfluor MetaXpress software. $\Delta\psi$ was expressed as a percentage of JC-1 aggregates per cell.

2.7. Cell cycle evaluation with Hoechst 33342

Cells were fixed in a solution of 4% paraformaldehyde (Sigma–Aldrich) for 15 min. After washing twice with DPBS, the cells were incubated with Hoechst 33342 (0.1 μg/mL) for 15 min. ImageXpress was used to determine the cell cycle. Nine sites per well and three wells per treatment were acquired. Cell cycle MetaXpress software was used to analyze the different phases of the cell cycle.

2.8. Changes in F-actin organization

Changes in actin organization were investigated using Alexa Fluor 488 phalloidin (Molecular Probes). Cells were fixed in a solution of 4% paraformaldehyde for 15 min. After rinsing twice with DPBS, the cells were permeabilized with a 0.1% Triton X-100 solution (Sigma–Aldrich) at 4 °C for 10 min, followed by incubation with 3 U/mL phalloidin in DPBS for 30 min. After washing with DPBS, cell nuclei were stained with 300 nM DAPI. The plates were then washed twice with DPBS and analyzed using ImageXpress. Nine sites per well and three wells per treatment were acquired.

2.9. Statistical analysis

Results from a representative of at least three independent experiments of each sample are shown as mean ± SD. Means were compared with the Mann–Whitney *U* test using GraphPad Prism (GradPad Software, CA). P-values $\leqslant 0.05$ were considered significant.

3. Results

We used several methods to characterize hAMSCs. First, hAMSCs were isolated from the two samples by their adherence to plastic. Second, hAMSCs expressed the transcription factors Oct-4 and Nanog, as determined by RT-PCR (data not shown). Both markers confirm the undifferentiated state. Third, hAMSCs were cultivated in specific osteogenic and chondrogenic differentiation media to evaluate their plasticity and multipotency. Differentiation was confirmed after 21 days (data not shown). Finally, hAMSCs expressed CD90 and CD73 (mesenchymal markers) and were negative for CD49d and CD34 (which are absent in mesenchymal cells) (data not shown). Altogether, the cells isolated from adipose tissue exhibited the characteristics of MSCs.

Cholesterol did not change hAMSC viability (evaluated by MTT after 24 h of incubation), independently of the concentration (Fig. 1A). The same was observed with 7-KC at concentrations lower than 25 μM. Cell viability decreased at higher concentrations. The IC_{50} of 7-KC was 59.54 ± 2.09 μM.

Fig. 1B depicts the characteristics of hAMSC cycle phases after 7-KC treatment for 24 h. 7-KC did not produce cell cycle changes at any of the tested concentrations.

7-KC induced cell death by apoptosis, evaluated by Annexin V, only at 50 μM (Fig. 2A). Lower concentrations did not affect the externalization of phosphatidylserine, a hallmark of apoptosis.

To further examine the mechanism underlying 7-KC-induced cell death in hAMSCs, we investigated caspase-3/7 activity. We did not observe changes in caspase-3/7 activity in any of the tested conditions (Fig. 2B).

Mitochondrial changes promoted by 7-KC after 24 h were investigated by measuring $\Delta\psi$, using JC-1 as a probe. 7-KC led to a concentration-dependent increase in JC-1 aggregate formation (Fig. 2C). Therefore, by promoting $\Delta\psi$ increase, a hyperpolarized mitochondrial membrane state was obtained.

Changes in actin organization after 24 h treatment with 7-KC are shown in Fig. 3. Untreated hAMSCs were characterized by the homogeneous distribution of actin fibers and alignment in the same direction (Fig. 3A). After cells were treated with 17 μM and 25 μM 7-KC, actin fibers lost their unidirectional alignment, although no actin fiber disruption was observed (Fig. 3B and C). Treatment with 50 μM 7-KC led to a loss of intracellular contact, hAMSCs became less flattened, and in several cells actin fibers became difficult to detect (Fig. 3D).

4. Discussion

MSCs were isolated from two human adipose tissue samples. They exhibited all characteristics of MSCs: adherence to plastic, fibroblast-like morphology, expression of the undifferentiated genes Oct-4 and Nanog, membrane markers ($CD90^+$, $CD73^+$, $CD49d^-$, and $CD34^-$), and osteogenic and chondrogenic differentiation ability.

Oxidative stress is a key pro-apoptotic factor. It results in excessive accumulation of ROS, which directly damage cell membranes, protein, and DNA [26]. There are several reports on the effects of oxLDL in stem cells. In adult rat bone marrow multipotent progenitor cells, oxLDL treatment resulted in a time- and dose-dependent reduction of cell population through a combination of decreased cell proliferation and decreased apoptosis [27]. Therefore, oxLDL has modified the behavior of these cells by inhibiting self-renewal, one of the most important features of stem cells. In contrast, preincubation of rat bone marrow MSCs with high-density lipoprotein increases cell viability, reduces apoptotic indices, and decreases ROS [26].

The toxic components of oxLDL may include several components, including oxysterols [28]. The effect of oxLDL on its target cells is highly variable, and depends on cell type and oxLDL composition.

Different oxysterols may have cytotoxic and oxidative effects, or none whatsoever [29]. A number of oxysterols are biologically active, especially those oxidized at C7, such as 7-KC. Several lines of evidence indicate that oxysterols contribute to the regulation of numerous biological activities, including cell differentiation, proliferation, and death [9,24].

Oxysterols interfere with proliferation and cause the death of many cancer cell types, although they have little or no effect on senescent cells [30]. However, oxysterols are not highly toxic to normal, non-proliferating cells at doses that are cytostatic or toxic to malignant cell lines [31]. In fact, when their mitotic frequency is reduced, cancer cells become much less responsive to oxysterols [32], suggesting that oxysterol toxicity is somehow linked to proliferation.

Very few works have reported the effects of oxysterols in MSCs. Most of these are related to the osteogenic effects of some types of oxysterols. Some oxysterols are osteoinductive molecules that can induce the lineage-specific differentiation of cells into osteoblasts [33]. Naturally occurring osteogenic oxysterols, such as 22(S)-hydroxycholesterol and 20(S)-hydroxycholesterol, have been described to trigger events leading to osteoblast differentiation and function *in vitro* and bone formation *in vivo* [34]. These specific

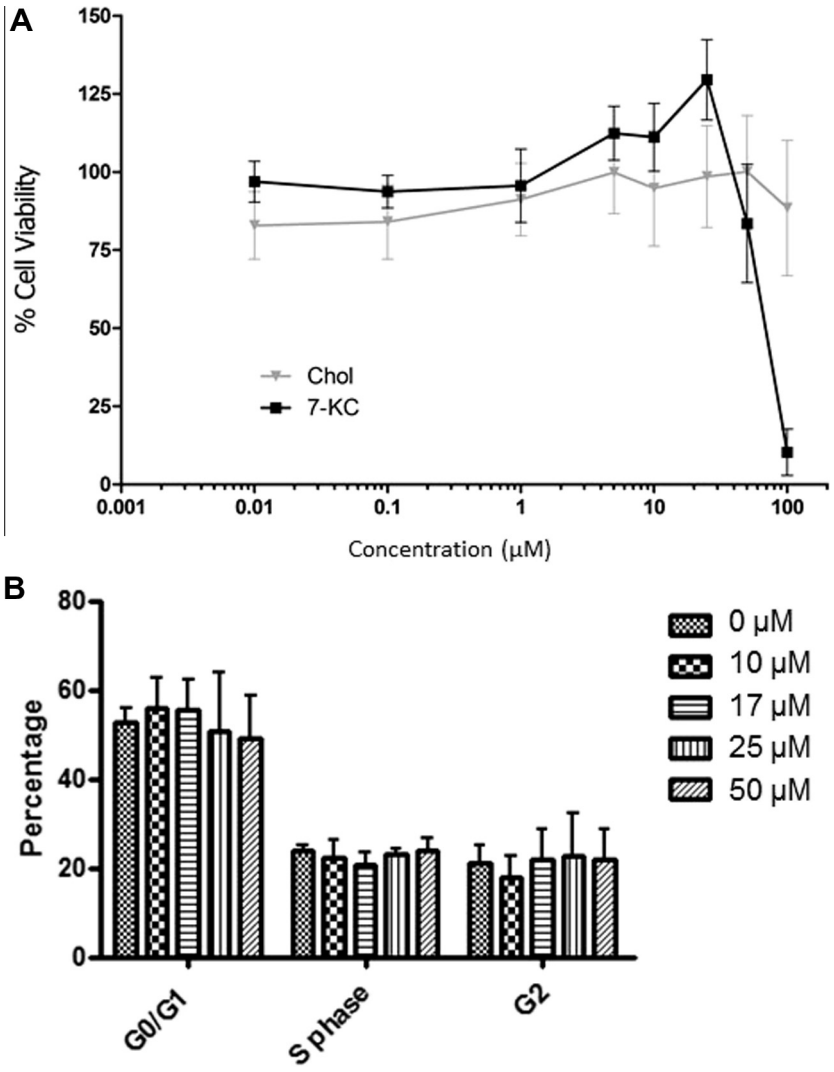

Fig. 1. Effects of 24-h 7-ketocholesterol (7-KC) incubation on cytotoxicity and cell cycle phases of human adipose mesenchymal stem cells. (A) Cytotoxicity evaluated by MTT. Chol, cholesterol. (B) Cell cycle phases were examined by Hoechst 33342 staining (B). Data are presented as mean ± SD.

oxysterols also have anti-adipogenic effects. However, cholestane-3β,5α,6β-triol promotes apoptosis in rat bone marrow MSCs and inhibits osteoblastic differentiation [19]. A combination of oxysterols can reportedly completely enhance osteoblastic differentiation of osteoprogenitor cells [17]. Taking all of these results into account, it is likely that oxysterols' effects on osteoblastic differentiation are specific, and vary with different oxysterols.

Among the several oxysterols that are biologically active, 7-KC is known to induce oxidative stress and cell death. It is present at enhanced levels in oxLDL and is involved in several pathophysiological processes.

Here we have described the early effects of 7-KC on MSCs derived from human adipose tissue. 7-KC led to decreased cell viability at concentrations higher than 17 μM, with no effect on the cell cycle. Although 7-KC promoted an increase in apoptosis, this mode of cell death was observed only at the concentration of 50 μM, without any change in caspase-3/7 activity.

The ability of 7-KC to induce apoptosis in several cell types is now well established. Moreover, the term oxiapoptophagy was suggested by Lizard to define the complex mode of cell death induced by 7-KC on U937 cells and murine oligodendrocytes, which includes oxidative stress, apoptosis, and probably autophagy [24]. In fact, it is unlikely that other forms of cell death are not present in our present experiment. Accumulating evidence suggests that autophagy plays a critical role in the homeostatic control of stem cell functions during aging, tissue regeneration, and cellular reprogramming. Autophagy may also participate in stem cell differentiation and proliferation [35].

In apoptosis induced by oxLDL, the early hyperpolarization of mitochondrial membrane represents a necessary event; caspase-3 activation is a later event. This was previously proposed in lymphoid cells and Caco-2 intestinal cells [36]. OxLDL has also been shown to induce mitochondrial-mediated apoptosis in tumor cells by provoking an increase in mitochondrial membrane potential followed by apoptosis-associated depolarization [37]. This effect has also been described in A7R5 rat smooth muscle cells; 7-KC induced a biphasic variation of the mitochondrial potential characterized by an early hyperpolarization followed a few hours later by a rapid depolarization [38].

Our data in adipose-derived MSCs support these findings. In the short term, we found a dose-related increase in JC-1 aggregation index promoted by 7-KC without alterations in caspase-3/7 activity. Altogether, these findings suggest that 7-KC, induces apoptosis in the short term by directly acting on mitochondria, in particular, to result in a peculiar alteration (i.e., hyperpolarization).

We have also observed that increased concentrations of 7-KC led to gradual changes in the morphology of hAMSCs, beginning with the loss of unidirectional alignment at lower concentrations,

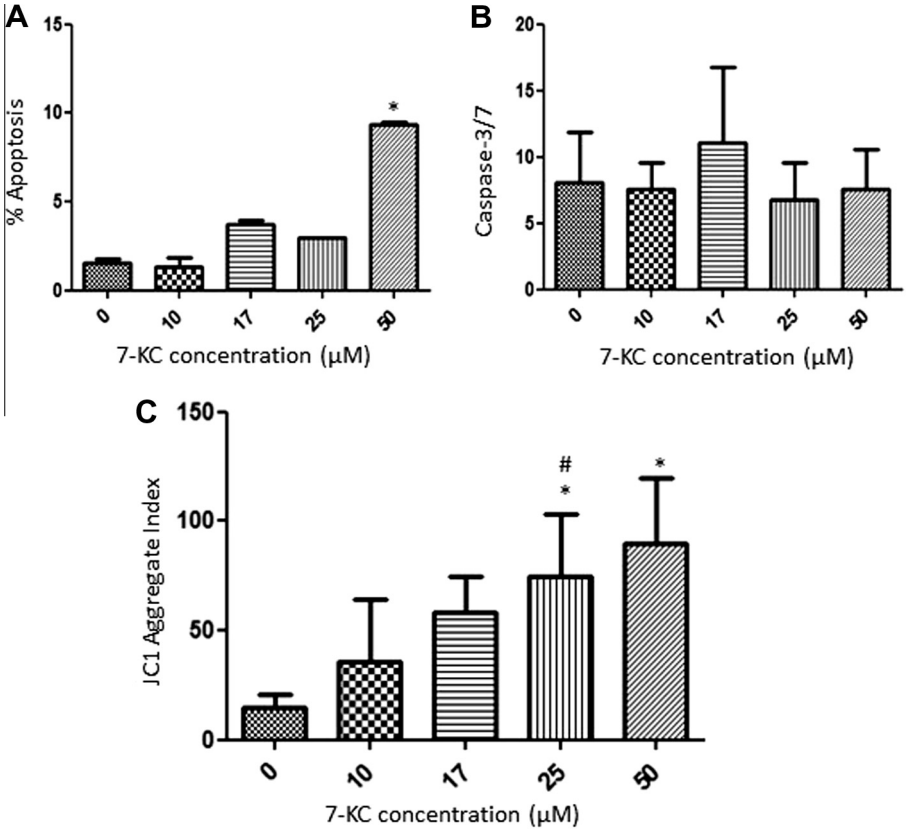

Fig. 2. Effects of 7-KC on apoptosis, caspase-3/7, and the mitochondrial transmembrane potential of human adipose tissue mesenchymal stem cells after 24 h culture. (A) Percentage of cells with apoptosis determined by the externalization of phosphatidylserine. (B) Caspase-3/7 activity. (C) Mitochondrial transmembrane potential as evaluated by JC1. Data are presented as mean ± SD from two independent experiments conducted in triplicate. $^{*}p < 0.001$; $^{\#}p < 0.05$.

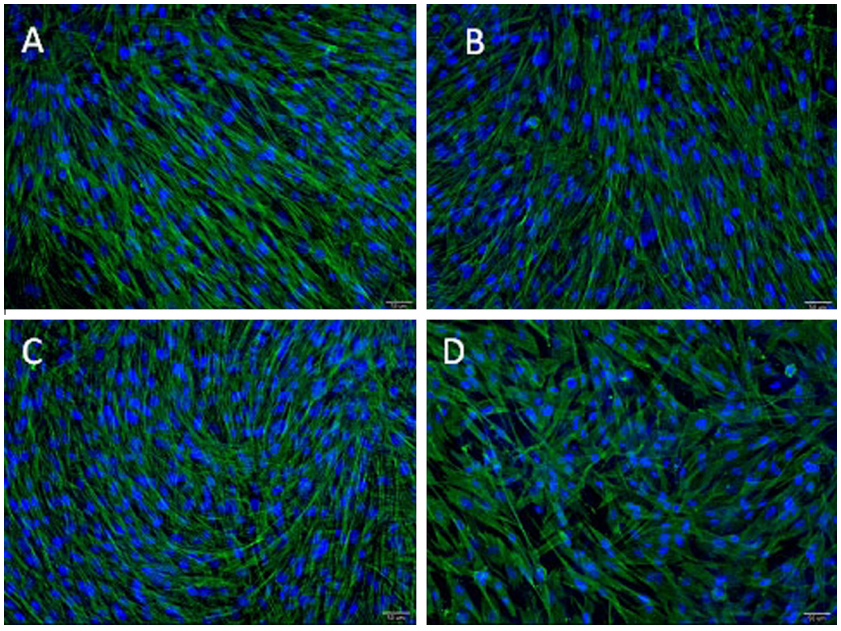

Fig. 3. Changes in actin organization after the treatment of human adipose tissue mesenchymal stem cells with 7-KC. Actin organization was investigated by staining with fluorescein–phalloidin. (A) Untreated cells. (B) Treatment with 17 μM 7-KC. (C) Treatment with 25 μM 7-KC. (D) Treatment with 50 μM 7-KC. Scale bar, 50 μM.

and including loss of actin organization and loss of intracellular contact, which were more pronounced at 50 μM. These changes were also described in rat smooth muscle cells [38]. However, we observed no changes in cell shape or cell adhesion.

The IC_{50} of 7-ketocholesterol in these cells was 59.54 ± 2.09 μM, which was higher than we had observed in human normal fibroblasts (47.66 ± 1.67 μM) or described in murine oligodendrocytes (10 μM), rat glioma (20 μM) or human neuroblastoma (20 μM)

[9]. It is tempting to postulate that hAMSCs are less susceptible to the death-promoting effect of 7-KC than other cell types.

Altogether, these results clearly suggest that 7-ketocholesterol promotes short-term mitochondrial hyperpolarization in human adipose tissue-derived MSCs, which might be a first step in the apoptotic process. Moreover, this was followed by a slight increase in the level of apoptosis and changes in actin organization. The pathophysiological importance of these findings remains to be investigated.

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