

Monovalent Ions and Stress-Induced Senescence in Human Mesenchymal Endometrial Stem Cells

Alla Shatrova

Institute of Cytology of the Russian Academy of Sciences

Natalja Pugovkina

Institute of Cytology of the Russian Academy of Sciences

Alisa Domnina

Institute of Cytology of the Russian Academy of Sciences

Nikolaj Nikolsky

Institute of Cytology of the Russian Academy of Sciences

Irina Marakhova (✉ imarakhova@mail.ru)

Institute of Cytology of the Russian Academy of Sciences

Research Article

Keywords: human mesenchymal endometrium stem cells, oxidative stress

Posted Date: December 8th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1087662/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

Monovalent ions are involved in growth, proliferation, differentiation of cells as well as in their death. This work concerns the ion homeostasis during senescence induction in human mesenchymal endometrium stem cells (hMESC): hMESCs subjected to oxidative stress (pulse H₂O₂ treatment) enter the premature senescence accompanied by persistent DNA damage, irreversible cell cycle arrest, cell hypertrophy, lipofuscin accumulation, enhanced β -galactosidase activity. Using flame photometry to estimate K⁺, Na⁺ content and Rb⁺ (K⁺) fluxes we found that during the senescence development in stress-induced hMESCs, Na⁺/K⁺ pump-mediated K⁺ fluxes are enhanced due to the increased Na⁺ content in senescent cells, while ouabain-resistant K⁺ fluxes remain unchanged. Senescence progression is accompanied by a peculiar decrease in the K⁺ content in cells from 800-900 μ mol/g to 500-600 μ mol/g. Since cardiac glycosides are offered as selective agents for eliminating senescent cells, we investigated the effect of ouabain on ion homeostasis and viability of hMESCs and found that in both proliferating and senescent hMESCs, ouabain (1 nM-1 μ M, 24-48 h) inhibited pump-mediated K⁺ transport (ID₅₀ 5x10⁻⁸ M), decreased cell K⁺/Na⁺ ratio to 0,1-0,2, however did not induce apoptosis. Comparison of the effect of ouabain on hMESCs with the literature data on the selective cytotoxic effect of cardiac glycosides on senescent or cancer cells suggests the ion pump blockade and intracellular K⁺ depletion should be synergized with target apoptotic signal to induce the cell death.

Introduction

Monovalent ions are involved in the control of cell growth, proliferation, and death. Unlike Ca²⁺, which is an important signaling player in the cell, the role of monovalent ions such as K⁺, Na⁺ and Cl⁻, in these cellular processes is not well understood. It is commonly suggested that ion transporters and ion channels are involved in the intracellular signaling network, and K⁺, Na⁺, and Cl⁻ are important for setting the membrane potential and the intracellular pH and Ca²⁺ concentrations during cell cycle progression. For example, the concentration of Na⁺ in cells can affect cell cycle progression by pH_i changes: it has been shown that Na⁺/H⁺ exchanger activity regulates G₂/M progression by increasing pH_i which, in turn, affects cyclin B1 expression and cdk2 activity¹⁻³. It is assumed that cellular Cl⁻ is involved in the hyperpolarization of cell membrane during G₁/S transition⁴.

In addition to signaling role, the transmembrane movement of monovalent ions may be important in context of cell volume control. Cell division depends on cell volume increase, and monovalent ions (such as K⁺, Na⁺, Cl⁻) play important role in the regulation of cell volume⁵⁻⁹. Cell volume is considered as an important component in the regulation of cell cycle progression. In studies of transformed cells of different origin and human mesenchymal endometrial stem cells we revealed significant changes in cell K⁺ content and K⁺ influxes which were related to cell accumulation in G₁ phase of cell cycle and proliferation slowing¹⁰⁻¹². Analysis of K⁺ content changes in cell cultures with different proliferative status and in human blood lymphocytes, stimulated to growth, suggested that K⁺ as the main

intracellular ion might be involved in regulation of cellular water content during cell transit from quiescence to proliferation^{13,14}. On the whole, being elements of cellular “housekeeping”, main monovalent permeable ions are able to modulate intracellular signaling and provide a specific intracellular ion context for development of peculiar cellular response.

In recent years, ion channels and transporters, Na⁺, K⁺-ATPase in particular, are suggested as anti-aging targets. It has been revealed that cardiac glycosides selectively kill senescent cells via apoptosis^{15,16}. It is proposed that cardiac glycosides damage harmful cells by blocking Na⁺, K⁺-ATPase, however the mechanism underlying their selective killing effect has not been established.

Cellular senescence is defined as an irreversible cell cycle arrest that can be triggered in cells in response to various intrinsic and extrinsic stimuli, as well as developmental signals¹⁷⁻²⁰. Senescence plays physiological role during normal cell development, it underlies stem cell aging and is also proposed as a tumor suppression mechanism²¹⁻²⁴. Senescence markers such as DNA damage, increased expression of the cell cycle inhibitors (p16, p21, p53) as well as distinct phenotypic alterations, including chromatin remodeling, metabolic reprogramming, characteristic messaging secretome can be used to identify senescent cells, but these markers are neither specific nor universal. Despite profound metabolic changes and impaired protein synthesis, alterations in mitochondria and lysosomes physiology, senescent cells remain metabolically active for a long time. Whether monovalent ions participate in senescence development as well as in maintaining the viability of senescent cells has not been investigated. To date, there are few studies on changes in ion content during senescence. Higher concentration of intracellular Ca²⁺ is observed in senescent cells compared to proliferating cells, and chelation of Ca²⁺ with BAPTA partly prevents senescence²⁵⁻²⁷. There are data on the activation of the Na⁺/H⁺ exchanger during stress-induced proliferation arrest³. The membrane potential of both cells and mitochondria appears to be lower in senescent cells than in cyclic cells¹⁶. Using fluorescent probes, an increased content of K⁺ and Na⁺ was detected in the senescent human lung fibroblast IMR90¹⁵. Direct analysis of K⁺ and Na⁺ in senescent cells has not yet been performed.

In this study, we investigated changes in monovalent ion homeostasis during the development of premature senescence. Using flame photometry to assess K⁺ and Na⁺ content as well as Rb⁺ (K⁺) fluxes, we found peculiar changes in K⁺ content in cells during oxidative stress-induced senescence of human endometrial mesenchymal stem cells (hMESC). The significance of ouabain-induced disorders of ion homeostasis (in particular, cellular K⁺) for the death of senescent hMESC is also discussed.

Results

H₂O₂ - stressed hMESC enter premature senescence. It has recently been shown that hMESC subjected to sublethal oxidative stress enter irreversible cell cycle arrest and exhibited a senescent phenotype including persistent DNA damage foci, cell cycle arrest mediated by the p53/p21/Rb pathway, cell hypertrophy, enhanced β-galactosidase staining³². In the present study, the growth of hMESC, that

were treated with the hydrogen peroxide (200 μM H_2O_2 for 1 h) and then returned to H_2O_2 -free serum-containing DMEM, stopped by the 3d day and displayed typical senescence markers (Fig. 1A). Under these conditions, the cells enlarged as evidenced by increased forward scattering (Fig. 1B). Also, the enhanced autofluorescence of stressed cells indicated accumulation of lipofuscin, which is considered as a marker of cellular senescence^{33,34} (Fig. 1C). As can be seen in Fig. 1D, stress-induced arrested hMESC displayed

the decreased mitochondrial membrane potential as evaluated by increased TMRM fluorescence signal³¹. Finally, the SA- β -galactosidase activity, a common marker of senescence, was increased in arrested hMESC (Fig. 1E, F). After H_2O_2 treatment hMESC retain high viability. As evaluated by FACS analysis, at the 5th day the percentage of viable cells in stressed population was 91 ± 7 ($n=3$) instead of 96 ± 5 ($n=3$) in control proliferating cells. Taking into account all these data we considered hMESC, which received a short H_2O_2 pulse, as a model for studying the ion homeostasis during premature senescence progression.

Cell K^+ and Na^+ content during senescence progression of stressed hMESC. As recently shown and confirmed in this study, short oxidative stress (1 h treatment with 200 μM H_2O_2) leads to a decrease in K^+ content and an increase in the Na^+ content in proliferating hMESC¹² (Fig. 2A). As a result of reciprocal changes in the content of K^+ and Na^+ in H_2O_2 -treated cells, the intracellular K^+/Na^+ ratio decreased from 7-8 to 3-3.5 which indicates disordered ionic gradients during oxidative stress. After replacing the medium with a fresh medium that did not contain H_2O_2 , the ionic gradients were restored within a day; however, the K^+ content in stressed cells was lower than in control proliferating cells (Fig. 2A).

We then assessed the cellular monovalent ions during culturing stressed hMESC. In our experiments, during the first 2 days, the stressed cultures continued to grow, though at a slower rate than the control cultures, and then stopped growing (Fig. 1A). Under these conditions, the K^+ content in both proliferating and stressed cells decreases, which is associated with an increase in cell density and a decrease in the proliferative activity of the culture¹². By the 8th day, the K^+ content decreases to a constant level, but in stressed cells it is lower (652 ± 41 $\mu\text{mol/g}$) than in control cyclic cells (795 ± 39 $\mu\text{mol/g}$) (Fig. 2A). Thus, stress-induced cessation of cell proliferation is accompanied by a decrease in the K^+ content calculated for the protein content in the cell.

To assess the content of cations in a cell, in our studies the measured amount of cations was normalized to the mass of cellular protein in the same sample. In cell biology, such evaluation of intracellular ions is widely used. Indeed, there are significant difficulties in assessing intracellular ion concentrations (ion content per cell water content) because of the difficulties in measuring the volume and water in adherent cells. The most adequate method for assessing the water content of cells in suspension cultures - measuring the buoyant cell density - is not applicable for monolayer cell cultures. It should be noted here that there are attempts to assess the ionic and other physiological parameters of cells in monolayer cultures after their treatment with a trypsin-containing medium. Our experience has shown, that cells

shortly treated with trypsin (0.05%) have an increased Na^+ content (while maintaining a high K^+ content) and a K^+/Na^+ ratio close 1 (Table 1). It also turned out that a low K^+/Na^+ ratio persists if the cells are washed from trypsin in a fresh medium and then kept in suspension for up to 1-2 hours (longer observations were not carried out). It is noteworthy that only after attachment to the adhesive surface the low Na^+ content and the high K^+/Na^+ ratio are restored in these cells (Table 1). Based on these data, we believe that the flame emission method used to measure the intracellular ion content is the most appropriate for studying the ionic homeostasis of monolayer cultures. The method allows one to determine both the content of basic cations in cells and the ion influxes, using analogous cations (for example, Rb^+ to assess the influx of K^+). It is also important that normalization of the amount of ions (in our case, K^+) to the amount of protein in each sample allows us to obtain data that contribute to understanding the mechanism of K^+ participation in cell growth and proliferation.

Senescent hMESC_s remain viable in culture for a long time. We asked if the late stressed cells retain a high K^+/Na^+ ratio. As can be seen in Fig. 2A, in long-term cultures (up to 22 days), senescent hMESC_s had a K^+ content ($562 \pm 39 \mu\text{mol/g}$), comparable to that in early senescent cells. After oxidative stress, the content of intracellular Na^+ decreases and does not change for 8 days, however during senescence progression, the content of Na^+ in cells increases from $120 \pm 10 \mu\text{mol/g}$ to $160 \pm 19 \mu\text{mol/g}$ (Fig. 2C). Taken together, these data indicate that, during long-term culture, senescent hMESC_s maintain the high K^+/Na^+ ratio typical for functionally active animal cells. A peculiar feature of senescent hMESC_s in comparison with proliferating cells is a lower K^+ content per cellular protein.

Na^+ , K^+ -ATPase-mediated Rb^+ (K^+) transport is increased during senescence progression in stressed hMESC_s. Short-term Rb^+ uptake was used to assess changes in K^+ transport during senescence. In proliferating hMESC_s, ouabain-inhibitable Rb^+ influx, mediated by the Na^+ , K^+ -ATPase pump, accounts for more than half of the total Rb^+ flux. In the first days after stress, the ouabain-inhibitable Rb^+ flux decreases, as well as in proliferating cultures, the influx decreases (Fig. 2B). A decrease in pump-mediated K^+ transport in growing cell culture is associated with density-dependent inhibition of cell proliferation¹². In stress-induced hMESC_s, a decrease in ouabain-inhibitable Rb^+ influx also reflects the transition to cell cycle arrest and the cessation of cell proliferation.

We then investigated the transport activity of the Na^+ , K^+ -ATPase pump in hMESC_s during senescence development and compared ouabain-inhibitable Rb^+ influxes in proliferating and early stressed hMESC_s with that in late stressed hMESC_s. As can be seen in Fig. 2B, in late stressed cells, the ouabain-inhibitable Rb^+ uptake was increased accounting $65 \pm 4 \mu\text{mol/g}$, 30 min ($n=4$) instead of $40 \pm 4 \mu\text{mol/g}$, 30 min ($n=6$) in early stressed cells. These data indicate elevated pump-mediated K^+ transport in established senescent hMESC_s. To determine whether the observed increase in pump activity is proportional to changes in intracellular Na^+ concentration, we compared pumping rate coefficients calculated as the ratio of ouabain-inhibitable Rb^+ uptake to intracellular Na^+ content during senescence development³⁵⁻³⁷. It turned out that the rate coefficients do not differ for early and late stressed cells. Thus, the increased

ouabain-inhibitable K^+ transport in senescent cells is not associated with a change in the intrinsic properties of Na^+/K^+ -ATPase pump, but is a consequence of flux-concentration relations in existing ion pumps and is probably associated with an increase in cellular Na^+ in late senescent hMESC.

During senescence development, passive transport of $Rb^+(K^+)$, resistant to ouabain, decreases slightly and then remains unchanged (Fig. 2D).

Comparison of ion changes during the growth of hMESC culture with those in senescent cells shows that stress-induced cell cycle arrest and senescence progression are accompanied by a decrease in K^+ content per gram of cellular protein mass. The senescence progression is also associated with elevated cell Na^+ content and increased pump-mediated K^+ influxes.

Cell K^+ and Na^+ content and Rb^+ fluxes in ouabain-treated cycling and senescent hMESC.

Having data on ion homeostasis of hMESC, both proliferating and senescent, we analyzed the cytotoxic effect of ouabain on these cells. Increasing evidence suggests that cardiac glycosides are capable of inducing apoptosis and selectively killing senescent, but not cycling cells^{15,16}. We asked whether ouabain-induced changes in cellular K^+ and Na^+ content could contribute to apoptotic cell death in senescence, and examined the relations between changes in ionic homeostasis in the presence of ouabain and ouabain's ability to kill senescent hMESC. So far, direct measurements of intracellular K^+ and Na^+ content have not been performed with ouabain-treated senescent cells compared with cycling cells.

First, we confirmed that ouabain at high concentrations stops cell proliferation. Starting from concentration 10^{-7} M by 24 h ouabain inhibits the growth of hMESC cultures causing S-G₂/M delay in cell cycle (Fig. 3A, B). As expected, after incubating with ouabain for 24 h, there occur reciprocal changes in cell K^+ and Na^+ contents (Fig. 3C, D). With increasing the ouabain concentration from 10^{-9} to 10^{-7} M the cell K^+ decreased from 879 ± 30 $\mu\text{mol/g}$ (n=4) to 110 ± 12 $\mu\text{mol/g}$ (n=3) in control (young) cells and from 674 ± 48 $\mu\text{mol/g}$ (n=3) to 96 ± 17 $\mu\text{mol/g}$ (n=3) in senescent cells with the most significant decrease between 5×10^{-9} to 10^{-8} M concentrations in both cell populations. Simultaneously with a decrease in cellular K^+ content, the Na^+ content in

ouabain-treated cells increased reaching the highest level with 10^{-7} M ouabain (Fig. 3B). Ouabain induced dose-dependent decrease in ouabain-inhibitable Rb^+ influx with the same IC₅₀ (5×10^{-7} M) for both young and senescent hMESC (Fig. 3E, F). Within the wide range of ouabain concentrations, ouabain-resistant Rb^+ influx did not change (Fig. 3G). Thus, ouabain already at a concentration of 10^{-7} M completely stops ion pumping thus leading to disruption of cell K^+/Na^+ gradients.

Next, we tested how the long-term ouabain affects the viability of young and senescent hMESC. As evaluated by FASC analysis, 10^{-6} M ouabain led to an increase in number of PI-stained proliferating

(young) cells (8,66% PI+ and 14,43% PI+ after 1 and 2 days) whereas in the population of ouabain-treated senescent hMESC the number of PI-stained cells remained as low as in control (5,02% PI+ and 6,07% PI+) (Fig. 3H). Annexin V test carried out after 10^{-6} M ouabain treatment did not show apoptosis induction in proliferating (young) and senescent cells (Fig. 3I). Taken together, the above data indicate that in both proliferating and senescent hMESC, ouabain causes the same disturbances in monovalent ion homeostasis and does not induce apoptosis.

Discussion

In present study, we investigated the ion homeostasis during stress-induced cell cycle arrest and premature senescence development in hMESC. Using flame photometry to estimate cellular K^+ and Na^+ content and transmembrane Rb^+ (K^+) fluxes we found that senescent hMESC maintain high K^+/Na^+ ratio typical for functionally active animal cells. The senescence progression is accompanied by elevated Na^+ content in cells and increased pump-mediated K^+ influxes. Stress-induced cell cycle arrest does not affect passive, ouabain-resistant K^+ fluxes across plasma membrane. A peculiar feature of senescent in comparison with proliferating hMESC is lower K^+ content per cell protein mass.

Premature senescence caused by various stresses in cells is associated with the cessation of cell proliferation. The lower ratio of the K^+ content to protein mass in senescent cells is in good agreement with our previous finding that a decrease in this index correlates with a decrease in cell proliferation^{12,13,38,39}. Recently, when studying the activation of human T lymphocytes, we also found that the transition of cells from quiescence to proliferation is necessarily accompanied by an increase in the content of both K^+ and water per g of protein so that during the growth of lymphocytes and an increase in their volume, the intracellular concentration of K^+ remains constant¹⁴. These data allow us to suggest that K^+ may be involved in maintaining cell growth and proliferation as an intracellular ion, which participates in the regulation of cell volume by adjusting the water balance of cell.

In the present study, by analogy with our previous experimental data when reliable measurements of K^+ , water and volume were carried out simultaneously on proliferating and resting cells cultured in suspension^{14,40-42}, relying on a theoretical analysis of ion and water balance in animal cells^{5,9,43-46}, and also taking into account that K^+ is the main cation compensating intracellular anions we assume that the lower ratio of cell K^+ content to cell protein mass can be interpreted as lower water content in senescent hMESC. To find out if this is really so, it is necessary to take reliable measurements of the volume of senescent cells. Various experimental approaches to measuring cell volume have been developed; however, this is still not an easy task to measure the volume of adherent cells in growing monolayer cultures⁴⁷. Nevertheless, we can discuss our assumptions on the significance of the ratio of K^+ content to protein in cells, taking into account the recent theoretical studies of cell volume regulation by Model and Petrucci⁴⁵. Using the fundamental basic equations of the osmotic balance of the cell and the electroneutrality of the system, the authors investigated the relationship between cellular organic matter,

cell volume and inorganic ions, which made it possible to estimate how the water content in the cell changes with the change in the content of small intracellular cations. Applying this approach it can be concluded that the higher the intracellular content of K^+ and Na^+ , the higher the water content in the cell. As found in the present study stress-induced senescence is accompanied by a decrease in the sum of K^+ and Na^+ , mainly due to a decrease in the K^+ content in cells, which indicates a lower water content in non-proliferating senescent cells.

There are few experimental data on changes in cell hydration state associated with a change in the proliferative status of cells. Based on some evidence of higher embryonic and cancer cell hydration, increased cell hydration has been proposed as an important factor in cell malignant growth and carcinogenesis^{48,49}. The relationship between hydration and cell proliferation may reflect the effect of macromolecular crowding on cellular metabolism: macromolecular crowding reduces metabolic processes and cytoplasm fluidity⁵⁰⁻⁵⁷. It has also been hypothesized that macromolecular crowding plays a role in signaling volume perturbations⁵⁸⁻⁶². Finally, measurements of water content in aging erythrocytes have suggested that cell water loss and macromolecular crowding may be a common mechanism of cellular senescence⁶³.

To further investigate the role of monovalent ions in the maintenance of senescence, we decided to test the participation of the Na^+/K^+ pump in the survival of senescent hMESC. To access further the involvement of monovalent ions in maintaining senescence we wanted to test the role of Na^+/K^+ pump in survival of senescent hMESC. This problem is discussed in view of proposed anti-aging effects of cardiac glycosides: cardiac glycosides have been reported to selectively kill senescent cells via apoptosis and can be used as senolytics in the treatment of aging-related diseases^{15,16}. In our experiments, in proliferating and senescent hMESC, ouabain inhibits pump-mediated K^+ transport in a dose-dependent manner, which leads to profound changes in ionic homeostasis, such that Na^+ substitutes K^+ inside the cell and after a 24 h incubation with 0.1 μ M ouabain, in cells of both types, intracellular K^+ becomes only 90-100 instead of 800-900 μ mol/g protein in untreated cells. Despite such serious disturbances in ion homeostasis, as assessed by PI/Annexin staining, long-term ouabain (1 μ M, up to 48 h) did not induce death and apoptosis in senescent hMESC.

Earlier it was reported about high apoptosis resistance of human mesenchymal stem cells^{32,64-66}. However, the high resistance of senescent hMESC to ouabain is not consistent with proposed selective cytotoxic (senolytic) activity of cardiac glycosides in relation to senescence^{15,16}. Note that there is a lot of data in the literature that cardiac glycosides also kill high proliferating cancer cells, but not cells that normally cycle⁶⁷⁻⁷². The question arises as to what may underlie the selective toxic effect of cardiac glycosides on senescent or cancer cells, and whether disturbances in cell ion homeostasis (in particular, K^+ content) can be involved in the anti-aging or anticancer effects of these drugs.

The Na^+/K^+ -ATPase pump, a cellular target of cardiac glycosides, is a major player in glycoside cytotoxic effects. Depletion of Na^+/K^+ -ATPase by RNA interference inhibited glycoside-induced apoptosis in cells⁷².

Several studies correlate the expression levels of the α -1 Na^+ , K^+ -ATPase to the susceptibility of cells towards cardiac glycosides. Indeed, the expression level of Na^+ , K^+ -ATPase is dependent on cell proliferative status: quiescence is characterized by a lower expression of α 1- and β 1- Na^+ , K^+ -ATPase subunits, while the transition of cells to proliferation is accompanied by increasing their transcription and the synthesis of new α 1- Na^+ , K^+ -ATPases^{29,73,74}. As a result, highly proliferating cancer cells are characterized by a higher Na^+ , K^+ -ATPase expression and may be more sensitive to glycosides than quiescent, differentiated or normally cycling cells. As for senescent cells, there are no studies indicating an increased expression of Na^+ , K^+ -ATPase during senescence: the expression level of α 1- Na^+ , K^+ -ATPase did not differ between cycling and arrested senescent lung cancer cells A549 cells¹⁶. In the present study, no differences were found in the relationship between K^+ influx and ouabain dose, or in the rate coefficient for the Na^+ , K^+ -ATPase pump in proliferating and senescent hMESC. Thus, it is unlikely that the Na^+ , K^+ -ATPase pump in senescent cells is more sensitive to cardiac glycosides. In addition, the glycoside concentrations which are toxic to senescent cells but do not to normal cycling cells fall into a narrow window¹⁶. These features of drugs indicate a rather low toxic specificity of glycosides in relation to senescent cells.

The Na^+ , K^+ -ATPase pump is the primary ion transport system responsible for the maintaining the Na^+ and K^+ concentrations and the resting membrane potential in the cell⁷⁵⁻⁷⁹. Animal cell stability and survival depend on the operation of the Na^+ / K^+ pump through the pump-leak mechanism⁵. Impermeant anionic molecules in cells establish an unstable osmotic condition (the Donnan effect), which is counteracted by the operation of Na^+ / K^+ pump, thus creating an asymmetric distribution of Na^+ and K^+ and preventing excessive water influx. In most animal cells, the Na^+ / K^+ pump blockade leads to cell swollen, membrane disruption and necrotic death. To understand the mechanism of selective toxic action of glycosides on dangerous cancer or senescent cells, it is important to know what type of death they cause.

Accumulating evidence indicate that in senescent and cancer cells, cardiac glycosides induce apoptosis or "mixed" cell death with signs of apoptosis and necrosis or autophagy, causing mitochondria dysfunction^{15,69-73,80-84}. In this context, K^+ transport plays a central role in mitochondrial physiology. Under normal physiological conditions, the high electric potential difference generated by proton pump across the inner mitochondrial membrane is used to make ATP and determines also the inwardly directed K^+ flux into matrix; changes in matrix volume due to this K^+ influx and concomitant water entry are compensated by activity of the electroneutral K^+ / H^+ antiporter (so called K^+ cycle)⁸⁵. Disruption of K^+ cycle inevitably leads to disturbances in mitochondria function and contribute to intrinsic apoptosis induction. It is important, that the activities of K^+ / H^+ antiporter and K^+ channels are regulated by the apoptotic Bcl-2 proteins: pro-apoptotic Bax can interacts with K^+ channels in the inner mitochondrial membrane, thus blocking K^+ influx⁸⁶⁻⁹⁰.

The question is what might be the role of cellular K^+ depletion due to ion pump inhibition in mitochondria dysfunction. Induction of apoptosis by valinomycin alone suggests that disruption of K^+ homeostasis may be sufficient to induce apoptosis in cancer cells⁸¹. Excessive K^+ efflux and intracellular K^+ loss are key early step in apoptosis induction^{7,91-96}. It is noteworthy that this early loss of K^+ occurs simultaneously with the loss of water by cells and does not lead to a decrease in the concentration of K^+ in cells⁴⁰. At later apoptosis, however, before the release of cytochrome c, a significant decrease in the content of K^+ and Cl^- assayed by X-ray microanalysis or cryo-correlative microscopy was found in the cytoplasm and mitochondria⁹⁷⁻⁹⁹. Late stages of apoptosis are also associated with cell shrinkage, a decrease in water content in cells and a simultaneous decrease in the cell K^+ concentration¹⁰⁰. Altogether, these data suggest that in cells with the Na^+/K^+ pump turned off, a sever decrease of cytoplasmic K^+ could promote intrinsic apoptosis by disruption of the mitochondria ion and volume homeostasis.

Apoptosis as a programmed cell death is controlled by a well-orchestrated genetic program so that the cell fate is dependent on the balance between pro- and anti-apoptotic members of Bcl-2 family proteins, and the ability of cardiac glycosides to induce apoptosis depends on whether the pro-apoptotic proteins are expressed in cells treated with glycoside. It has been revealed that in cancer cells, cardiac glycosides induce apoptosis by down-regulating the anti-apoptotic proteins Bcl-XL and Bcl-2 as well as Mcl-1 and increasing the pro-apoptotic proteins Bid and Bax^{68-70,83,84,101-104}. In senescent human lung fibroblast IMR90 cells, cytotoxic ouabain increased the pro-apoptotic NOXA protein¹⁵. Based on these studies, we can conclude that if a cell is prone to apoptosis (i.e. pro-apoptotic proteins are in proper functional position in cell) cardiac glycosides can contribute to the apoptosis.

Cardiac glycosides do induce apoptotic death in human embryonic stem cells (hESCs) but not in hESC-derived mesenchymal stem cells and human bone marrow mesenchymal stem cells (hBMMSCs)⁶⁶. As reported recently and also shown in our study, cardiac glycosides do not induce apoptosis in both hMESC and their senescent partners¹⁰⁵. Obviously, the high resistance of hMESC to cardiac glycosides is provided by a highly expressed anti-apoptotic program in these cells^{32,64,65,106,107}. Taken together, the above data suggest that the depletion of cellular K^+ is necessary event in cardiac glycoside-induced apoptosis; however, a separate apoptotic signal is required to kill a cell, which acts together with low cellular K^+ .

In summary, oxidative stress-induced senescence in hMESC is associated with specific changes in cell ion homeostasis, which primarily concerns intracellular K^+ . In senescent cells, pump-mediated K^+ transport is enhanced due to the increased Na^+ content, while passive ouabain-resistant K^+ influxes remain unchanged. In the course of senescence development, cellular K^+ content is decreases and in established senescent cells, the ratio of K^+ content to cellular protein becomes lower that of in cycling cells which may indicate a decrease in the hydration of senescent cells. Evaluating the cytotoxic selectivity of cardiac glycosides toward senescence, we conclude that K^+ being a key ion in mitochondria physiology is involved in intrinsic apoptotic events in cells treated with cardiac glycosides. However, to kill

senescent cells, the ion pump blockade and intracellular K^+ depletion should be synergized with the target apoptotic signal. Given the pleiotropic effects of cardiac glycosides, their use as selective drugs for eliminating the dangerous cells warrants further study: cardiac glycosides as inhibitors of the ion pump that ensures the stability and survival of all animal cells are able to kill not only harmful tumorous or senescent cells but also functionally important differentiated cells.

Methods

Cells and experiment design. The procedures involving human cells were performed in accordance with the standards of the Declaration of Helsinki (1989) and approved by the Institute of Cytology Ethics Committee. Written informed consent was obtained from all patients who provided tissue.

All the experiments have been performed on human mesenchymal endometrial stem cells (hMESC) established from human menstrual blood²⁸. As shown in our lab, the cells are multipotent, capable for self-renewal, express CD13, CD29, CD44, CD73, CD90, CD105 and are negative for the hematopoietic markers CD34 and CD45 (Zemelko et al., 2011). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM)/F12 (Gibco) supplemented with 10% fetal calf serum (HyClone), 1% penicillin-streptomycin (Gibco BRL, MD, USA) and 1% glutamax (Gibco BRL, MD, USA) and subcultured at 1:3–1:4 ratio twice a week.

To induce premature senescence, the subconfluent cultures of hMESC were treated with 200 μ M H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) for 1 h, then washed twice with serum-free medium to remove H_2O_2 , and re-cultured in fresh complete culture medium (Burova et al., 2013). Cells were analyzed either immediately after H_2O_2 shock or at selected time points after prolonged cultivation, depending on the aim of the study. To assess the effect of ouabain on the viability of hMESC, young proliferating cells (passage 10) and cells whose senescence was induced by peroxide after an additional 5 days of cultivation were used.

Analysis of cell K^+ and Na^+ content and K^+ influx. Measurements of ions were performed essentially as described previously^{13, 29}. To estimate K^+ influx Rb^+ was used as the physiological analog of K^+ . $RbCl$ (final concentration 5 mM) was introduced into the culture medium for 20 min. To evaluate the Na^+ , K^+ -ATPase K^+ influx, prior to $RbCl$, 10^{-4} M ouabain (Sigma-Aldrich, USA) was added to culture medium. Then, cells were rapidly washed 5 times with ice-cold isotonic $MgCl_2$ and cations were extracted with 1 ml of 1% trichloroacetic acid (TCA). TCA extracts were analyzed for Rb , K and Na by emission flame photometry on a Perkin-Elmer AA 306 spectrophotometer. TCA precipitates were dissolved in 0.1 N $NaOH$ and analyzed for protein by Lowry procedure. Ouabain-sensitive Rb^+ uptake was calculated as the differences between the mean values measured in samples incubated with and without ouabain. The intracellular ion content was expressed as amount of ions per amount of protein in each sample analyzed.

FACS analysis of cell viability, cell proliferation, mitochondrial health and apoptosis. For FACS analysis, adherent cells were rinsed twice with PBS and harvested by trypsinization. Detached cells were pelleted

by centrifugation and suspended in PBS. Samples were analyzed with CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) or CytoFLEX S flow cytometer (Beckman Coulter, Brea, CA USA).

To determine cell viability propidium iodide (PI) staining was used. PI is excluded by viable cells but can penetrate cell membranes of dead cells and intercalates into double-stranded nucleic acids. 50 µg/mL of PI was added to each sample just before analysis. At least 3000 events were usually collected as the main cell population. Triplicate counts were obtained for each procedure. Representative PI versus FSC dot plots allowed us to distinguish between PI-negative “live” cells and PI-positive “dead” cells.

For cell cycle analysis, each cell sample was suspended in 300 µl PBS/serum-free medium containing 200 µg/mL of saponin (Fluka, NY, USA), 250 µg/mL of RNase A (Sigma-Aldrich, MO, USA) and 50 µg/mL of PI, incubated from 30 to 60 min at a room temperature (in dark) and subjected to FACS analysis. Data were analyzed using CytExpert software (versions 1.2 and 2.0, Brea, California, USA). Dot plots (FSC versus PI) were generated to assess the distribution of cell cycle phases. For this, the cells were gated in accordance with the DNA content. At least 15,000 cells are collected for research. The experiments were repeated three times.

For detection of lipofuscin accumulation, the samples were analyzed for autofluorescence (AF, 488 nm laser). To evaluate the increase in the cell size which accompanies the senescence, forward scatter signal (FS) was monitored. Tetramethylrhodamine (TMRM; Invitrogen, Carlsbad, CA, USA) dye was used as mitochondrial membrane potential indicator (MMP)^{30,31}. Healthy cells have functioning mitochondria and the bright fluorescence signal, respectively. Briefly, to prepare a 1×staining solution (100 nM) TMRM stock solution (100 µM) was diluted in 1000 times with growth medium, which added to the cells.

Apoptosis was assayed using Annexin V/Alexa Fluor™ 647 conjugate in accordance with the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). Treated and untreated cells were harvested by trypsinization, washed with PBS, pelleted by centrifugation and adjusted to a concentration 1×10^6 cells/mL in 1×Annexin binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4). 1×10^5 cells (100 µL of cell suspension) were stained with 5 µL of Annexin V conjugate and 2 µL of DAPI (final concentration 2 µg/mL) for 15 min in the dark at room temperature. Then, 400 µL of 1×buffer was added to each sample, gently vortexed and analyzed by flow cytometry as soon as possible.

Senescence-associated β-galactosidase assay. Cells expressing senescence-associated β-galactosidase (SA-β-gal) were identified using a β-galactosidase staining kit (Cell Signaling Technology, Beverly, MA, USA) according to the manufacturer's recommendations. The kit allows determining the activity of β-galactosidase at pH 6.0, which is well detected in senescent cells.

Statistical Analysis. All data are presented as the mean with standard error of the mean from at least three independent experiments. Statistical significance was assessed using either ANOVA-Tukey test in case of multiple comparisons or Student's t-test in case of pair comparisons.

Declarations

Acknowledgments

We are very grateful to Dr. Vinogradova T.A. for ion assay by flame photometry and Dr. Egorova L.I. (Institute of Cytology, RAS, St.-Petersburg) for excellent technical assistance. Some experiments with late senescent hMESC were carried out on cultures prepared by Dr. Borodkina A.P. and Dr. Derjabin P.I. (Institute of Cytology, RAS, St.-Petersburg). We thank Dr. Lyublinskaya O.G. (Institute of Cytology, RAS, St.-Petersburg) for analysis of β -galactosidase activity in hMESC.

Author contributions

I.M. conceived and designed the study and drafted the manuscript. A.S., N.P., A.D., I.M. performed the experiments and analyzed data. A.S. and I.M. wrote the main manuscript text and prepared figures. N.N. supervised the work. All authors approved the submitted version.

Competing interests

The authors declare no competing interests.

Funding

This work was supported by the Russian Science Foundation (Grant #19-14-00108). NP acknowledges support from the Russian Science Foundation (Grant # 21-74-20178).

Additional information

Correspondence and requests for materials should be addressed to I.M.

References

1. Putney, L. K. & Barber, D. L.. Na-H exchange-dependent increase in intracellular pH times G_2/M entry and transition. *J. Biol. Chem*, **278**, 44645–44649 (2003).
2. Darborg, B. V., Rentsch, M. L. & Rasmussen, M. Regulation of mitogen-activated protein kinase pathway by the plasma membrane Na/H exchanger. NHE1. *Arch. Biochem. Biophys*, **462**, 195–201 (2007).
3. Pedersen, S. F., Darborg, B. V., Rentsch, M. L. & Rasmussen, M. Regulation of mitogen-activated protein kinase pathways by the plasma membrane Na/H exchanger, NHE1. *Arch. Biochem. Biophys*, **462**, 195–201 (2007).
4. Klausen, T. K., Preisler, S., Pedersen, S. F. & Hoffmann, E. K. Monovalent ions control proliferation of Ehrlich Lettre ascites cells. *Am. J. Physiol. Cell. Physiol*, **299**, C714–C725 (2010).

5. Tosteson, D. C. & Hoffman, J. F. Regulation of cell volume by active cation transport in high and low potassium sheep red cells. *J. Gen. Physiol*, **44**, 169–194 (1960).
6. Lang, F. *et al.* Functional significance of cell volume regulatory mechanisms. *Physiol. Rev*, **78**, 247–306 (1998).
7. Lang, F. *et al.* Ion channels in cell proliferation and apoptotic cell death. *J. Membrane Biol*, **205**, 147–157 (2005).
8. Lang, F. *et al.* Cell volume regulatory ion channels in cell proliferation and cell death.. *Methods Enzymol*, **428**, 209–225 (2007).
9. Hoffman, T. K., Lambert, I. H. & Pedersen, S. F. Physiology of cell volume regulation in vertebrates. *Physiol Rev*, **89**, 193–277 (2009).
10. Troshin, A. S. *et al.* Culture density and ion transport through the plasma membrane in transformed cells. *Doklady Akademii nauk SSSR*, **282**, 709–711 (1985).
11. Marakhova, I. I., Vinogradova, T. A. & Yefimova, E. V. Early and delayed changes in potassium transport during the initiation of cell proliferation in CHO culture. *Gen. Physiol. Biophys*, **8**, 273–282 (1989).
12. Marakhova, I. *et al.* Proliferation-related changes in K⁺ content in human mesenchymal stem cells.. *Sci. Rep*, **9**, 346 (2019).
13. Vereninov, A. A. *et al.* Transport and distribution of monovalent cations in human peripheral blood lymphocytes activated by phytohemagglutinin. *Tsitologija*, **33**, 78–93 (1991).
14. Marakhova, I. *et al.* Intracellular K⁺ and water content in human blood lymphocytes during transition from quiescence to proliferation. *Sci. Rep*, **9**, 16253 (2019).
15. Triana-Martinez, F., Picallos-Rabina, P., Silva-Álvarez, D. & Llanos, S. P. F. Identification and characterization of cardiac glycosides as senolytic compounds. *Nature Communications*, **10**, 4731 (2019).
16. Guerrero, A. *et al.* Cardiac glycosides are broad-spectrum senolytics. *Nat. Metab*, **1**, 1074–1088 (2019).
17. Kuilman, T., Michaloglou, C., Mooi, W. J. & Peeper, D. S. The essence of senescence. *Genes Dev*, **24**, 2463–2479 (2010).
18. Fridlyanskaya, I., Alekseenko, L. & Nikolsky, N. Senescence as a general cellular response to stress: A minireview. *Exp Gerontol*, **72**, 124–128 (2015).
19. Hernandez-Segura, A., Nehme, J. & Demaria, M. Hallmarks of cellular senescence. *Trends Cell Biol*, **28**, 436–453 (2018).
20. Davan-Wetton, C. S. A., Pessolano, E., Perretti, M. & Montero-Melendez, T. Senescence under appraisal: hopes and challenges revisited. *Cell Mol. Life Sci*, **78**, 3333–3354 (2021).
21. Ermolaeva, M., Neri, F., Ori, A. & Rudolph, K. L. Cellular and epigenetic drivers of stem cell ageing. *Nat. Rev. Mol. Cell. Biol*, **19**, 594–610 (2018).

22. Lee, C. H. *et al.* Bufalin induces apoptosis of human osteosarcoma U-2 OS cells through endoplasmic reticulum stress, caspase- and mitochondria-dependent signaling pathways. *Molecules*, **22**, 437 (2017).
23. Rhinn, M., Ritschka, B. & Keyes, W. M. Cellular senescence in development, regeneration and disease. *Development*, **146**, dev151837 (2019).
24. Wang, B., Kohli, J. & Demaria, M. Senescent cells in cancer therapy: friends or foes? *Trends Cancer*, **6**, 838–857 (2020).
25. McCarthy, D. A., Clark, R. R., Bartling, T. R., Trebak, M. & Melendez, J. A. Redox control of the senescence regulator interleukin-1 α and the secretory phenotype. *J. Biol. Chem*, **288**, 32149–32159 (2013).
26. Yu, X. *et al.* Isradipine prevents rotenone-induced intracellular calcium rise that accelerates senescence in human neuroblastoma SH-SY5Y cells. *Neuroscience*, **246**, 243–253 (2013).
27. Borodkina, A. V. *et al.* Calcium alterations signal either to senescence or to autophagy induction in stem cells upon oxidative stress. *Aging*, **12**, 3400–3418 (2016).
28. Zemel'ko, V. I. *et al.* Multipotent mesenchymal stem cells of desquamated endometrium: isolation, characterization and use as feeder layer for maintenance of human embryonic stem cell lines. *Tsitologiya*, **53**, 919–929 (2011).
29. Marakhova, I. I., Vereninov, A. A., Toropova, F. V. & Vinogradova, T. A. Na,K-ATPase pump in activated human lymphocytes: on the mechanisms of rapid and long-term increase in K influxes during the initiation of phytohemagglutinin-induced proliferation. *Biochim. Biophys. Acta*, **1368**, 61–72 (1998).
30. Scaduto, R. C. & Grotyohann, L. W. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. *Biophys. J*, **76**, 469–477 (1999).
31. Creed, S. & McKenzie, M. Measurement of mitochondrial membrane potential with the fluorescent dye tetramethylrhodamine methyl ester (TMRM). *Methods Mol. Biol.* 1928, 69-76 (2019)
32. Burova, E., Borodkina, A., Shatrova, A. & Nikolsky, N. Sublethal oxidative stress induces the premature senescence of human mesenchymal stem cells derived from endometrium. *Oxid. Med. Cell. Longev.* 474931(2013)
33. Bertolo, A., Baur, M., Guerrero, J., Pötzel, T. & Stoyanov, J. Autofluorescence is a reliable in vitro marker of cellular senescence in human mesenchymal stromal cells. *Sci. Rep*, **9**, 2074 (2019).
34. Shatrova, A. N. *et al.* Outcomes of deferoxamine action on H₂O₂-induced growth inhibition and senescence progression of human endometrial stem cells. *Int. J. Mol.Sci*, **22**, 6035 (2021).
35. Jakobsson, E. Interactions of cell volume, membrane potential, and membrane transport parameters. *Am. J. Physiol. Cell Physiol*, **238**, C196–C206 (1980).
36. Lew, V. L., Bookchin, R. M. & Volume pH, and ion-content regulation in human red cells: analysis of transient behavior with an integrated model. *J Membr Biol*, **92**, 57–74 (1986).
37. Vereninov, I. A., Yurinskaya, V. E., Model, M. A., Lang, F. & Vereninov, A. A. Computation of pump-leak flux balance in animal cells. *Cell. Physiol. Biochem*, **34**, 1812–1823 (2014).

38. Troshin, A. S. *et al.* Culture density and ion transport through the plasma membrane in transformed cells. *Doklady Akademii nauk SSSR*, **282**, 709–711 (1985).
39. Marakhova, I. I., Vereninov, A. A., Toropova, F. V. & Vinogradova, T. A. Long-term enhancement of Na,K-ATPase pump during blasttransformation of human lymphocytes is controlled first by translational, then by transcriptional mechanisms. *FEBS Lett*, **368**, 110–112 (1995).
40. Yurinskaya, V. E. *et al.* Potassium and sodium balance in U937 cells during apoptosis with and without cell shrinkage. *Cell Physiol Biochem*, **16**, 155–162 (2005).
41. Yurinskaya, V. E. *et al.* Thymocyte K⁺, Na⁺ and water balance during dexamethasone- and etoposide-induced apoptosis. *Cell. Physiol. Biochem*, **16**, 15–22 (2005).
42. Yurinskaya, V. *et al.* A comparative study of U937 cell size changes during apoptosis initiation by flow cytometry, light scattering, water assay and electronic sizing. *Apoptosis*, **22**, 1287–1295 (2017).
43. Jentsch, T. J. VRACs and other ion channels and transporters in the regulation of cell volume and beyond. *Nat. Rev. Mol. Cell. Biol*, **17**, 293–307 (2016).
44. Vereninov, I. A., Yurinskaya, V. E., Model, M. A. & Vereninov, A. A. Unidirectional flux balance of monovalent ions in cells with Na/Na and Li/Na exchange: experimental and computational studies on lymphoid U937 cells. *PLoS One*, **11**, e0153284 (2016).
45. Model, M. A. & Petruccelli, C. Intracellular macromolecule in cell volume control and methods of their quantification. *Curr. Top. Membr*, **81**, 237–289 (2018).
46. Yurinskaya, V. E., Vereninov, I. A. & Vereninov, A. A. Balance of Na⁺, K⁺, and Cl⁻ unidirectional fluxes in normal and apoptotic U937 cells computed with all main types of cotransporters. *Front. Cell Dev Biol*, **8**, 591872 (2020).
47. Model, M. A. Methods for cell volume measurement. *Cytometry A*, **93**, 281–296 (2018).
48. McIntyre, G. I. Cell hydration as the primary factor in carcinogenesis: A unifying concept. *Med. Hypotheses*, **66**, 518–526 (2006).
49. McIntyre, G. I. Increased cell hydration promotes both tumor growth and metastasis: A biochemical mechanism consistent with genetic signatures. *Med. Hypotheses*, **69**, 1127–1130 (2007).
50. Minton, A. P. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. *J. Biol. Chem*, **276**, 10577–10580 (2001).
51. Ritz, P. *et al.* Effects of changes in water compartments on hysiology and metabolism. *Eur. J. Clin. Nutr*, **57** (Suppl 2), S2–S5 (2003).
52. Burg, M. B., Ferraris, J. D. & Dmitrieva N. I. Cellular response to hyperosmotic stresses. *Physiol. Rev*, **87**, 1441–1474 (2007).
53. Zhou, H. X., Rivas, G. & Minton, A. P. Macromolecular crowding and confinement: biochemical, biophysical, and potential physiological consequences. *Annu. Rev. Biophys*, **37**, 375–397 (2008).
54. Akabayov, B., Akabayov, S. R., Lee, S. J., Wagner, G. & Richardson, C. C. Impact of macromolecular crowding on DNA replication. *Nat. Commun*, **4**, 1615 (2013).

55. Matsuda, H., Putzel, G. G., Szleifer, I. & Szleifer, I. Macromolecular crowding as a regulator of gene transcription. *Biophys. J*, **106**, 1801–1810 (2014).
56. Mourao, M. A., Hakim, J. B. & Schnell, S. Connecting the dots: the effects of macromolecular crowding on cell physiology. *Biophys. J*, **107**, 2761–2766 (2014).
57. Wang, R., Ferraris, J. D., Izumi, Y., Dmitrieva, N. & Ramkissoon, K. Global discovery of high-NaCl-induced changes of protein phosphorylation. *Am. J. Physiol. Cell. Physiol*, **307**, C442–C454 (2014).
58. Lang, F. *et al.* Functional significance of cell volume regulatory mechanisms. *Physiol. Rev*, **78**, 247–306 (1998).
59. Burg, M. B. Macromolecular crowding as a cell volume sensor. *Cell. Physiol. Biochem*, **10**, 251–256 (2000).
60. Hoffmann, E. K. & Pedersen, S. F. Cell volume homeostatic mechanisms: effectors and signalling pathways. *Acta Physiol (Oxf)*, **202**, 465–485 (2011).
61. Rana, P. S., Kurokawa, M. & Model, M. A. Evidence for macromolecular crowding as a direct apoptotic stimulus. *J Cell Sci*, **133**, 243931 (2020).
62. Model, M. A., Hollembeak, J. T. & Kurokawa, M. Macromolecular crowding: a hidden link between cell volume and everything else. *Cell. Physiol. Biochem*, **55**, 25–40 (2021).
63. Minton, A. P. Water loss in aging erythrocytes provides a clue to a general mechanism of cellular senescence. *Biophys. J*, **119**, 2039–2044 (2020).
64. Alekseenko, L. L. *et al.* Heat shock induces apoptosis in human embryonic stem cells but a premature senescence phenotype in their differentiated progeny., **11**, 3260–3269 (2012).
65. Alekseenko, L. L. *et al.* Sublethal heat shock induces premature senescence rather than apoptosis in human mesenchymal stem cells. *Cell Stress Chaperones*, **19**, 355–366 (2014).
66. Lin, Y. T. *et al.* Elimination of undifferentiated human embryonic stem cells by cardiac glycosides. *Sci Rep*, **7**, 5289 (2017).
67. Huang, W. W. *et al.* Bufalin induces G0/G1 phase arrest through inhibiting the levels of cyclin D, cyclin E, CDK2 and CDK4, and triggers apoptosis via mitochondrial signaling pathway in T24 human bladder cancer cells. *Mutat Res*, **732**, 26–33 (2012).
68. Chou, W. H. *et al.* Ouabain induces apoptotic cell death through caspase- and mitochondria-dependent pathways in human osteosarcoma U-2 OS cells. *Anticancer Res*, **38**, 169–178 (2018).
69. Chang, Y. M. *et al.* Ouabain induces apoptotic cell death in human prostate DU 145 cancer cells through DNA damage and TRAIL pathways. *Environ. Toxicol*, **34**, 1329–1339 (2019).
70. Su, E. Y. *et al.* Bufalin induces apoptotic cell death in human nasopharyngeal carcinoma cells through mitochondrial ROS and TRAIL pathways. *Am. J. Chin. Med*, **47**, 237–257 (2019).
71. Gan, H. *et al.* Digitoxin inhibits HeLa cell growth through the induction of G2/M cell cycle arrest and apoptosis in vitro and in vivo. *Int. J. Oncol*, **57**, 562–573 (2020).
72. Geng, X. *et al.* Cardiac glycosides inhibit cancer through Na/K-ATPase-dependent cell death induction. *Biochem. Pharmacol*, **182**, 114226 (2020).

73. Vereninov, A. A. *et al.* Differential transcription of ion transporters, NHE1, ATP1B1, NKCC1 in human peripheral blood lymphocytes activated to proliferation. *Cell. Physiol. Biochem*, **11**, 19–26 (2001).
74. Karitskaya, I., Aksenov, N., Vassilieva, I., Zenin, V. & Marakhova, I. Long-term regulation of Na,K-ATPase pump during T-cell proliferation. *Pflugers Arch*, **460**, 777–789 (2010).
75. Skou, J. C. The Na,K-pump. *Methods Enzymol*, **156**, 1–25 (1988).
76. Glynn, I. M. & Karlish S.J.D. **The sodium pump..** *Ann. Rev.Physiol*, **37**, 13–55 (1975).
77. Therien, A. G. & Blostein, R. Mechanisms of sodium pump regulation. *Am. J. Physiol. Cell. Physiol*, **279**, C541–C566 (2000).
78. Lingrel, J. B. The physiological significance of the cardiotonic steroid/ouabain-binding site of the Na,K-ATPase. *Annu. Rev. Physiol*, **72**, 395–412 (2010).
79. Xiao, A. Y., Wei, L., Xia, S., Rothman, S. & Yu, S. P. Ionic mechanism of ouabain induced concurrent apoptosis and necrosis in individual cultured cortical neurons. *J. Neurosci*, **22**, 1350–1362 (2002).
80. Alonso, E. *et al.* Nanomolar ouabain elicits apoptosis through a direct action on HeLa cell mitochondria. *Steroids*, **78**, 1110–1118 (2013).
81. Chen, D., Song, M., Mohamad, O. & Yu, S. P. Inhibition of Na⁺/K⁺-ATPase induces hybrid cell death and enhanced sensitivity to chemotherapy in human glioblastoma cell. *BMC Cancer*, **14**, 716 (2014).
82. Eskiocak, U. *et al.* Synergistic effects of ion transporter and MAP kinase pathway inhibitors in melanoma. *Nature Comm*, **7**, 12336 (2016).
83. Lee, C. H. *et al.* Bufalin induces apoptosis of human osteosarcoma U-2 OS cells through endoplasmic reticulum stress, caspase- and mitochondria-dependent signaling pathways. *Molecules*, **22**, 437 (2017).
84. Lei, Y. *et al.* Digitoxin inhibits proliferation of multidrug-resistant HepG2 cells through G. *Oncol. Lett*, **20**, 71 (2020).
85. Garlid, K. D. & Paucek, P. Mitochondrial potassium transport: the K⁺ cycle. *BBA-Bioenergetics*, **1606**, 23–41 (2003).
86. Kaasik, A., Safiulina, D., Zharkovsky, A. & Veksler, V. Regulation of mitochondrial matrix volume. *Am. J. Physiol. Cell Physiol*, **292**, C157–C163 (2007).
87. Nowikovsky, K., Schweyen, R. J. & Bernardi, P. Pathophysiology of mitochondrial volume homeostasis: Potassium transport and permeability transition. *Biochim. Biophys. Acta*, **1787**, 345–350 (2009).
88. Szabò, I. & Zoratti, M. Mitochondrial channels: ion fluxes and more. *Physiol. Rev*, **94**, 519–608 (2014).
89. Szabò, I. & Gulbins, E. Voltage-gated potassium channels as regulators of cell death. *Front. Cell Dev. Biol*, **8**, 611853 (2020).
90. Urbani, A., Prosdociami, E., Carrer, A., Checchetto, V. & Szabò, I. Mitochondrial ion channels of the inner membrane and their regulation in cell death signaling. *Front.Cell Dev. Biol*, **8**, 620081 (2021).

91. Bortner, C. D. & Cidlowski, J. A. The role of apoptotic volume decrease and ionic homeostasis in the activation and repression of apoptosis. *Pflügers Arch*, **448**, 313–318 (2004).
92. Bortner, C. D. & Cidlowski, J. A. Cell shrinkage and monovalent cation fluxes: role in apoptosis. *Arch. Biochem. Biophys*, **462**, 176–188 (2007).
93. Bortner, C. D. & Cidlowski, J. A. Ions, the movement of water and the apoptotic volume decrease. *Front. Cell Dev. Biol*, **8**, 611211 (2020).
94. Vereninov, A. A. *et al.* Pump and channel K⁺(Rb⁺) fluxes in apoptosis of human lymphoid cell line U937. *Cell. Physiol. Biochem*, **22**, 187–194 (2008).
95. Lang, F. & Hoffmann, E. K. Role of ion transport in control of apoptotic cell death. *Compr. Physiol*, **2**, 2037–2061 (2012).
96. Kondratskyi, A., Kondratska, K., Skryma, R. & Prevarskaya, N. Ion channels in the regulation of apoptosis. *Biochim. Biophys. Acta*, **1848**, 2531–2546 (2015).
97. Arrebola, F. *et al.* Changes in intracellular electrolyte concentrations during apoptosis induced by UV irradiation of human myeloblastic cells. *Am. J. Physiol. Cell Physiol*, **290**, C638–C649 (2006).
98. Arrebola, F. *et al.* Biphasic behavior of changes in elemental composition during staurosporine-induced apoptosis., **10**, 1317–1331 (2005).
99. Nolin, F. *et al.* Stage-specific changes in the water, Na⁺, Cl⁻ and K⁺ contents of organelles during apoptosis demonstrated by a targeted cryocorrelative analytical approach. *PLoS One*, **11**, e0148727 (2016).
100. Poulsen, K. A. *et al.* Deregulation of apoptotic volume decrease and ionic movements in multidrug-resistant tumor cells: role of chloride channels. *Am. J. Physiol. Cell. Physiol*, **298**, C14–C25 (2010).
101. Kulikov, A., Eva, A., Kirch, U., Boldyrev, A. & Schneider-Bobis, G. Ouabain activates signaling pathways associated with cell death in human neuroblastoma. *Biochim. Biophys. Acta*, **1768**, 1691–1702 (2007).
102. Trenti, A. *et al.* Cardiac glycoside ouabain induces autophagic cell death in non-small cell lung cancer cells via a JNK-dependent decrease of Bcl-2. *Biochem. Pharmacol*, **89**, 197–209 (2014).
103. Cerella, C. *et al.* Early downregulation of Mcl-1 regulates apoptosis triggered by cardiac glycoside UNBS1450. *Cell Death Dis*, **6**, e1782 (2015).
104. Yosef, R. *et al.* Directed elimination of senescent cells by inhibition of BCL-W and BCL-XL. *Nature Communications*, **7**, 11190 (2016).
105. Deryabin, P. I., Shatrova, A. N. & Borodkina, A. V. Apoptosis resistance of senescent cells is an intrinsic barrier for senolysis induced by cardiac glycosides. *Cell. Mol. Life Sci.* Oct29 (2021)
106. Vassilieva, I. O. *et al.* Senescence-messaging secretome factors trigger premature senescence in human endometrium-derived stem cells. *Biochem. Biophys. Res. Commun*, **496**, 1162–1168 (2018).
107. Domnina, A. *et al.* Three-dimensional compaction switches stress response programs and enhances therapeutic efficacy of endometrial mesenchymal stem/stromal cells. *Front. Cell Dev. Biol*, **8**, 473 (2020).

Tables

Table 1. Cell K (K_i) and Na (Na_i) content in hMSECs treated with trypsin-containing medium and seeded into new culture.

		Cation content $\mu\text{mol/g}$		K_i/Na_i	
		K_i	Na_i		
1	Control cells in monolayer confluent culture		631 ± 21	90 ± 3	7,01
2	Cells were detached with 0.05% trypsin-containing medium, washed and suspended in fresh culture medium				
	Time in suspension	2 min	624 ± 28	663 ± 23	0,94
		30 min	626 ± 20	586 ± 32	1,08
		1 h	630 ± 18	590 ± 25	1,08
3	Trypsin-treated, suspended cells are seeded in a new monolayer culture				
	Time after seeding	1 h (attached cells)	644 ± 40	108 ± 9	5,03
		24 h (proliferating cells)	810 ± 24	118 ± 18	6.86

Control cells (1) were from confluent proliferating culture of hMSECs. Cellular K and Na was measured by emission flame photometry as described in Methods.

Figures

Figure 1

Oxidative stress induces premature senescence in hMESC. Cells were seeded in 3-cm dishes (10x10⁴ cells per dish) and at the second day after plating, some cultures were subjected to 200 μ M H₂O₂ for 1 h with the following H₂O₂ replacement and cell cultivation under normal conditions. (A) Growth curves of control (1) and H₂O₂-treated (2) hMESC. (B) Forward scatter reflecting cell size change on the 5th day after the oxidative stress. (C) Lipofuscin accumulation estimated by autofluorescence measurement on the 5th days after the stress. (D) TMRM fluorescence reflects the mitochondrial membrane potential decrease after oxidative stress. (E, F) Representative microphotographs of SA- β -galactosidase staining in control (E) and stressed hMESC (F). Scale bar is 50 μ m. Data are shown as mean + SD (n =3), ***p < 0.005 vs. the control cells. Ctr, control cells; MFI, mean fluorescence intensity; TMRM, tetramethylrhodamine, methyl ester; SA- β -Gal, senescence associated β -galactosidase.

Figure 2

K⁺ and Na⁺ content and Rb⁺ influxes during premature senescence development in stress-induced hMESC. (A, C) Changes in cell K⁺ (A) and Na⁺ (C) content in hMESC after oxidative stress and during senescence progression. Cell content of K⁺ (A, circles) and Na⁺ (C, triangles up) in cycling hMESC during culture growth. Cell content of K⁺ (A, columns) and Na⁺ (C, columns) during senescence development. Dashed columns represent cell content of K⁺ (A) and Na⁺ (C) after treatment of cells with 200 μ M H₂O₂ for 1h. (B, D) Changes in ouabain-sensitive (B, squares) and ouabain-resistant (D, triangles down) Rb⁺ influxes in cycling hMESC during culture growth. Ouabain-sensitive (B, columns) and ouabain-resistant (D, columns) Rb⁺ influxes during senescence development. For the first 8 day, data are shown as means + SD of six independent experiments performed in triplicate; significant difference between stress-induced and control proliferating cells (Ctr) was calculated using one-way ANOVA with Tukey's post hoc tests, *p < 0.05. For late senescent cells (18-22 days) data are shown as means + SD (n=3-5).

Figure 3

Ouabain treatment lead to severe changes in cell K⁺, Na⁺ content and in Na⁺/K⁺ pump-mediated transport, but did not induce apoptosis in both young (proliferating) and senescent hMESC. (A, B) Ouabain (5x10⁻⁷ M) inhibits growth of hMESC cultures (A) and blocks cells in G₂/M of cell cycle (B). (C, D) Ouabain-induced changes in cell K⁺ (C) and Na⁺ (D) content in proliferating (young) and senescent hMESC. (E-G) Ouabain inhibits Rb⁺ influx in concentration-dependent manner in young and senescent hMESC (E, F) but did not affect the passive Rb⁺ influx (G). Rb⁺ flux is presented as μ mol/g, 30 min. Data are shown as mean + SD, n=3-5. *Significant difference relative to young cells treated with the same ouabain concentration in the same experiment (t-test, *p < 0.05). (H, I) Ouabain treatment (10⁻⁶ M, 2 days) increases the number of PI-positive "dead" cells in young hMESC (H) but did not induce apoptosis both in young and senescent hMESC (I). Flow cytometry assay for Annexin-V was performed on cells treated with 10⁻⁶ ouabain for 1 day. The bar-graph indicate the mean percentage \pm SD of PI⁺ cells, n=3, t-test, *p < 0.05 vs the control, ns – insignificant.