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The Energy-Corrective and Antioxidative Effect of Cytoflavin in the Postischemical Period of Human Dermal Fibroblasts in vitro

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Abstract—The effect of the metabolic drug Cytoflavin (CF) with antihypoxic and antioxidative properties on human dermal fibroblasts in the model of ischemia/reoxygenation in vitro was studied. It was revealed that, during the reoxygenation period after ischemia, the cell death rate in culture with CF appeared to be 2–2.7 times lower than in control cultures. Recovery of ATP synthesis after ischemia was 2.1 times faster in culture with CF. The drug effectively reduced the level of reactive oxygen species (ROS) in fibroblasts after H_2O_2 treatment, which allowed their survival to be maintained at the level of control cells. Pretreatment of cells with CF for one day ensured the maintenance of a normal level of ROS during the investigated time period in fibroblasts subjected to H_2O_2 treatment and reduced H_2O_2-induced cell death by almost one-third as compared to non-pretreatment control cells. Addition of CF into the culture medium in the postischemic period showed no effect on Hsp70 synthesis, but led to a decrease in GRP78 synthesis, raised after ischemia, to the control level, indicating a resolution of unfolded protein response in these cells and normalization of the functioning of their endoplasmic reticulum.

Keywords: dermal fibroblasts, cytoflavin, ischemia/reoxygenation, reactive oxygen species, ROS, Hsp70, GRP78

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INTRODUCTION

Hypoxia or ischemia play a crucial role in the pathogenesis of many acute and chronic diseases. A deficiency of oxygen inhibits the mitochondrial respiratory chain. As a result, oxidative phosphorylation—the main source of ATP—is substituted by the significantly less productive anaerobic glycolyis, which leads to a deficit of the macroergic phosphates and to acidosis. Due to a deficit of energy, the activity of antioxidative enzymes (catalase, glutathione peroxidase, and superoxide dismutase), as well as the level of nonenzymatic antioxidants (vitamins E and C, beta-carotene, ubiquinone, and others), decreases (Nordberg, Arner, 2001). Restoration of blood supply (reoxygenation) occurring spontaneously or induced therapeutically causes a second wave of disturbances in cells due to a rise of free radicals (oxidative stress), which produces activation of lipid peroxidation and damage to the integrity of cell membranes and DNA and leads to irreversible injury and death of cells, often to an even greater degree than ischemia (Li, Jackson, 2002). Such a cascade of disturbances has been described in ischemia/reoxygenation for cardiomyocytes (Doenst et al., 2008; Murphy, Steenbergen, 2008), neurons (Choe, 1996; Samoilov, 1999), glial cells (Ouyang et al., 2007), cells of renal tubules (Paller, Neumann, 1991), and several other cell types (Li, Jackson, 2002).

The protective response of cell to stresses (a decrease of concentration of macroergic phosphate, acidosis, and changes of redox balance) includes synthesis of heat shock proteins (HSP); accumulation of unfolded or misfolded proteins in a cell is a common stimulus for activation of HSP expression during ischemia/reoxygenation (Lipton, 1999; Chang et al., 2001; Latchman, 2001; Chi, Karliner, 2004). Many HSP function as molecular chaperones and are responsible for folding of the newly synthesized polypeptides and correction of misfolded proteins, prevent protein aggregation, and order "irreparable" proteins to degrade into proteosomes. In addition, HSPs participate in regulation of apoptosis at all its stages and have a direct antiapoptotic effect by interfering with the action of the key proapoptotc proteins. Of the HSP, the best studied is the protective role of two proteins of the Hsp70 family: Hsp70 located in the cytoplasm and GRP78 located in the endoplasmic reticulum (ER). The cytoprotective properties of these two chaperones have been observed in various models of ischemic disturbances in vitro and in vivo (Marber et al., 1995; Bush et al., 1999; Meissner et al., 2000; Zhang et al., 2004; Martindale et al., 2006; Thuerauf et al., 2006; Goel et al., 2010). The activation and efficiency of stress response are connected with the cell's energy status (Chang et al., 2001).

One of the approaches to metabolic correction of cell disturbances after ischemia and reoxygenation is searching for pharmacological agents that can help stimulate energy formation; decrease ROS; activate metabolic processes; and, therefore, prevent cell death. One such agent is Cytoflavin (CF), the combined antihypoxant and antioxidant actions of which are believed to be its important properties due to the complex of its constituents—succinic acid, nicotinamide, inosine, and riboflavin.

In the present study, we checked the potential energy corrective and antioxidant activities of CF in human dermal fibroblasts in a model of ischemia/reoxygenation in vitro and studied the effect of CF on the level of synthesis of the chaperones Hsp70 and GRP78 in these cells.

MATERIALS AND METHODS

Cell culture. Human fibroblasts isolated from forearm skin were cultured in MEM (Biolot, Russia) supplemented with 12% fetal bovine serum (FBS, Gibco, United States), 100 U/mL penicillin, 100 μg/ml streptomycin (Gibco), and 0.3 mg/mL L-glutamine (Biolot). The culture medium was changed every six or seven days. Cells of the fourth to ninth passage were used.

Ischemia in vitro. Fibroblasts were grown in Carrel glass flasks (unlike plastic, glass does not bind oxygen) and, at 70–80% confluence, were exposed to oxygen and glucose deprivation (Chen et al., 2007; Guo et al., 2007; Osorio-Fuentealba et al., 2009). For this, the culture medium was replaced by glucose-free DMEM (Gibco) that had been saturated with N_2 for 15 min and the air atmosphere was replaced with N_2. After 1-h hypoxia at 37°C, fibroblasts were reoxygenated by replacing glucose-free medium with culture medium under normoxic conditions for the indicated times with or without of 1.6 μL/mL CF (NTTF Polycan, Russia). After 24 h of reoxygenation, the percentage of dead cells was assessed by Trypan blue staining.

Intracellular ATP content was determined by the luceferin/luciferase method with a bioluminescence-based ATF determination kit (Molecular Probes, United States) according to the manufacturer's protocol. The cells were lysed with lytic buffer, and 10 μL of each sample were added to 90 μL of the luciferase reagent. Luminescence was measured for an integration time of 10 s in a TD-20/20 luminometer (Turner Designs, United States). The background value of luminescence (no ATP or no cellular lysate) was subtracted. The obtained data were normalized to protein concentration as determined by Lowry. The ATP levels were expressed as percentages of the control values (intact cells under normoxic conditions). Data from three independent experiments were pooled.

To determine the intracellular reactive oxygen species, the redox-sensitive fluorescent marker 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA, Molecular Probes) was used. Carboxy-H_2DCFDA diffuses into cells, in which the acetate groups are removed by intracellular esterases. Oxidation of the modified carboxy-H_2DCFDA converts it into a fluorescent adduct. The cells grown in glass flasks (for subsequent ischemia/reoxygenation) or in 35-mm plastic dishes (for exposure of cells to H_2O_2) were washed with warm phosphate-buffered saline (PBS). The cells were incubated with 5 μM carboxy-H_2DCFDA in Hepes-buffered Hanks' balanced salt solution, pH 7.4, and incubated for 1 h at 37°C. Then, the dye was washed from the cells twice with PBS and the cells either were exposed to ischemia or to 500 μM H_2O_2 in the culture medium for 30 min, after which the cells were washed and incubated in Hepes-buffered Hanks' balanced salt solution with or without addition of CF. The cell fluorescence was revealed with an Axiovert 200M microscope (Carl Zeiss, Germany) supplied with a Leica DFC 420c camera (Leica, Germany), at the excitation and emission wavelengths of 480 and 520 nm, respectively. The intensity of fluorescence was estimated with the aid of the ImageJ 1.35r software (National Institutes of Health, United States). The data (all reproduced) were obtained in five experiments (three for cell ischemia and two with H_2O_2).

Cell survival and proliferation were measured with a CellTiter 96 Aqueous One Cell Proliferation Assay (MTS-test, Promega, United States). The active component of the reagent—tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS)—is converted by cells into a colored formazan product dissolved in the culture medium. The optical density of the medium in the wells is directly proportional to the number of living cells. Fibroblasts exposed to ischemia/reoxygenation were removed after 24 h from the bottom of the culture flask by a trypsin-EDTA, washed, and seeded in a 96-well flat-bottom plate in an amount of 10^4 cells per well in 100 μL of culture medium. Then, the cells were incubated at 37°C and 5% CO_2 under normoxic conditions with or without CF. After 24 h, 20 μL of the reagent were added into each well and, 2 h later, the optical density of the medium was measured at 492 nm with a 96-well plate reader (Titertek Multiskan, Finland). In experiments with H_2O_2 exposure, all manipulations were performed in 96-well flat-bottom plates. The experiments were carried out two to three times in repeats.

Cell cycle phase distributions were determined by flow cytometry. Fibroblasts after 24-h reoxygenation were washed and resuspended in PBS. Then, the cells were treated with 0.1% Triton X-100 and stained with a mixture of two dyes—olivomycin and ethidium bro-

mide—in the presence of 15 mM $MgCl_2$ for 24 h at 5–7°C. The final concentration of the dyes amounted to 40 μg/mL for olivomycin and 20 μg/mL for ethidium bromide. Prior to measurement of fluorescence, the cells were incubated in 0.05% RNase for 30 min at 37°C. DNA histograms were obtained with a laboratory model of the flow cytometer on the basis of a LUMAM-I microscope. The source of light was a DRSh-250-2 gas-discharge mercury lamp (excitation 380–450 nm, emission >520 nm).

Electrophoresis and immunoblotting. Fibroblasts that were intact, after ischemia, and at different periods of reoxygenation with or without CF were washed with cold PBS and lysed with 50 μL of RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 1% NP-40, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA, 1 mM PMSF, and Protease inhibitor cocktail (Sigma)) on ice for 5 min. A one-quarter part of the buffer Laemmli (300 mM Tris, pH 6.8, 10% SDS, 25% 2-mercaptoethanol and 50% glycerol) was added to lysate and incubated in a water bath at 100°C for 5 min. The concentration of protein was determined by Bradford's method by using ovalbuin to construct a calibration curve.

Electrophoretic separation of proteins was performed in 10% polyacrylamide gel in the presence of SDS at a current strength of 30 mA for 2.5–3 h. The proteins separated in polyacrylamide gel were transferred onto a nitrocellulose membrane (BioRad, United States) at a voltage of 100 V and current strength not more than 300 mA for 1.5–2 h in the transfer buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, 15% methanol). The membrane was stained with Ponceau S (Sigma) to visualize protein bands. Immunoblotting was performed according to the ECL procedure (Western Blotting Protocols, Amersham, United States) following the recommendations of the manufacturer of the antibodies. After transfer, the membrane was washed several times with TTBS buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20). Then, sites of unspecific binding were blocked in TTBS containing 5% dry defatted milk (Valio, Finland) for 1 h at room temperature. Following one wash (15 min) in TTBS, the membrane was incubated overnight with the primary antibodies in TTBS with 3% BSA at 4°C: a polyclonal rabbit antibody against Hsp70 ((K-20)-R, Santa Cruz Biotech., Inc., United States) or a polyclonal rabbit antibody against GRP78 (Abcam, United Kingdom) diluted 1 : 1000. Then, the membrane was washed and incubated for 1 h with horseradish peroxidase-conjugated goat antirabbit IgG secondary antibodies (Sigma) diluted 1 : 10000 in TTBS containing 5% milk. Peroxidase activity product was revealed with the enhanced chemoluminescence (ECL) method. For this, the membrane was washed once with water (10–15 s), after which it was incubated in the ECL solution for 1 min and placed between two layers of the film. The chemiluminescence was recorded by exposure onto a CEA RP NEW X-ray film (CEA AB, Sweden).

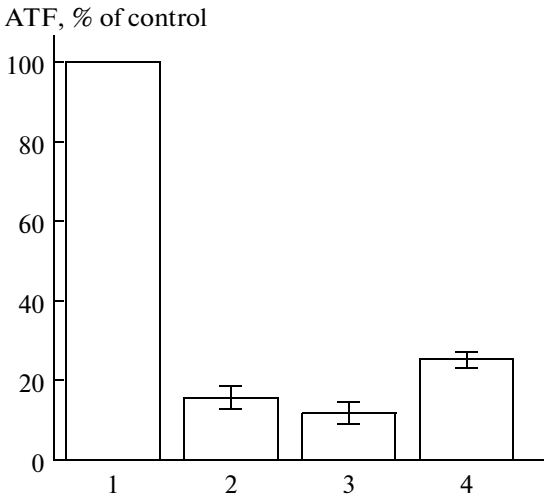

Fig. 1. Effect of Cytoflavin (CF) on ATP level in cells after ischemia and reoxygenation.
1—intact cells, 2—after 1 h of ischemia, 3—1 h of reoxygenation, 4—1 h of reoxygenation with CF.

Statistical analysis was performed with the aid of the Origin 6 software (Microcal Software Inc., United States). The data are presented as the mean and the standard deviation. Student's *t*-test was used for comparing the differences between the means ($p < 0.05$).

RESULTS

After 1-h simulated ischemia, the ATP content in fibroblasts decreased down to 15.7% of the initial level in intact cells (Fig. 1). One hour after normoxic incubation of cells in the complete medium with glucose, the ATP level in cells continued decreasing (to 12%). The decrease can be explained by a prevalence of ATP utilization by energy-dependent systems at the initial reoxygenation periods over ATP synthesis. Addition of CF to the culture medium during reoxygenation increases cellular ATP content, on average, by 2.1 times (up to 25.5%).

To study cell survival 24 h after the beginning of reoxygenation, we removed the cells from the flask bottom and counted the percentage of dead cells by using Trypan blue. Ischemia for 1 h and subsequent reoxygenation for 24 h led to death of around 6% of cells, whereas reoxygenation in the presence of CF reduced the cell death rate down to 2.2% (the percentage of dead cells in the control amounted to about 1%). The cell survival and proliferation 48 h after ischemia were estimated with the MTS-test (table). After 24 h of reoxygenation, the cells were seeded in equal concentrations into a 96-well plate and culti-

Survival and proliferative activity of fibroblasts for the second day of reoxygenation

Culture medium	Cells;		
	Intact (control)	After ischemia/reoxygenation (Isch/R)	After ischemia and reoxygenation with CF
Without FBS	779.0 ± 31.4	699.3 ± 39.3[a]	781.0 ± 28.2[c]
With FBS	911.3 ± 24.5	999.3 ± 46.1[b]	922.5 ± 29.5[d]

Note: Differences from control are significant at [a] $p < 0.1$ and [b] $p < 0.05$; differences from Isch/R are significant at [c] $p < 0.05$, [d] $p < 0.1$.

vated for 24 h in media either with FBS (cell proliferation analysis) or without FBS in order to inhibit proliferation (cell survival analysis). We found that fibroblasts after 48 h of reoxygenation had an increased proliferative activity (almost 10% higher than in control cells), whereas CF addition during reoxygenation retained proliferation at the level of control cells. Cell death was observed only among the cells cultivated after ischemia without CF.

To confirm differences in the proliferative activity of postischemic fibroblasts cultivated with and without CF, we analyzed the distribution of cell cycle phases using DNA flow cytometry (Fig. 2). As soon as 24 h after ischemia, induction of fibroblast proliferation was observed that compensated for the cell loss: the difference in proliferative activity between cells cultivated with and without CF amounted to 7% (the cells in the S phase were 15.7 and 22.5%, respectively). At the same time, reoxygenation with CF led to accumulation of tetraploid cells (the differences between the G_2 phase cell counts amounted to 5%). As was assumed by Ermis et al. (1998) and Obberinger et al. (1999), the increase of tetraploid cells among wound fibroblasts can lead to more successful healing of tissue injury. The difference in cell death rates can be evaluated using the amount of revealed nuclear fragments in cytometry histograms: upon cultivation of cells during the postischemic period with CF, this amount is almost twice as small as without CF (6.9 and 13.5%, respectively).

Measurement of intracellular ROS in cultivated dermal fibroblasts has shown that reoxygenation following 1-h ischemia does not lead to an increase in the ROS level compared to intact cells. Moreover, a decrease in ROS production initially occurred and, then, the ROS level gradual returned to the control level (Fig. 3a). This decrease of the ROS level was not associated with leakage or inactivation of the fluorescent dye, which was confirmed by an increase of the fluorescence intensity in cells after addition of H_2O_2.

To test the antioxidative efficiency of CF, we induced oxidative stress in cells by the addition of 500 μM H_2O_2 for 30 min (Figs. 3b, 3c). Following 30-min incubation of cells with CF effectively decreased the ROS level. When the dermal fibroblasts exposed to H_2O_2 for 30-min were incubated with CF, the ROS level decreased, with this resulting in an increase of cell survival up to the level of the intact cells in a 24-h period (Fig. 3d). Pretreatment of dermal fibroblasts with CF during 24 h before H_2O_2 exposure (30 min) significantly inhibited intracellular ROS production down to the level of the control cells in the studied time period. The survival of fibroblasts pretreated with CF after 24 h was one-third higher than in cells not pretreated with CF.

Analysis of changes in the synthesis of the cell chaperones Hsp70 and GRP78 revealed an increase of GRP78 after 15 h of reoxygenation in both variants of cell cultivation with and without CF in comparison with the control (Fig. 4). However, after 24 h of reoxygenation, GRP78 was decreased in cells cultivated with CF and approached the level of the control cells. This finding indicates that success in the endoplasmic reticulum has been achieved. At the same time, no significant changes were observe in the Hsp70 synthesis level for 24 h of reoxygenation in cells cultivated with or without CF as compared with control intact cells.

The obtained results show that CF has an effective influence on the recovery of ATP production; on the decrease of intracellular ROSs; and, thereby, on the viability of the dermal fibroblasts exposed to ischemia/reoxygenation or H_2O_2.

DISCUSSION

Fibroblasts are cells with peculiar properties. On one hand, fibroblasts of different tissues have universal characteristics: they all are motile spindle-shaped cells of mesenchymal origin, synthesize components of the extracellular matrix (ECM), cytokines, and growth factors. On the other hand, fibroblasts of differing tissue localization differ from each other and, within each tissue, are heterogeneous in composition, phenotype, repertoire of expressed genes, surface markers, proliferative activity, level of secreted products, and response to various stresses (Das et al., 2002; Sorrell and Caplan, 2004; Souders et al., 2009). Such a

plasticity of fibroblasts is determined by their peculiar role in setting and maintaining the organ functions carried out by finely regulated synthesis and degradation of ECM, auto- and paracrine signaling, proliferation, migration, and differentiation into myofibroblasts. Fibroblasts play a key role in reparation of tissue injuries via ECM production and scar formation. Disregulation of processes of synthesis of ECM components and their degradation by matrix metalloproteinases, proliferation of fibroblasts at the initial stages, and apoptosis at final stages of tissue reparation can lead to delay (failure) or excess of wound repair with the development of fibrosis. Therefore, a reduction of necrotic sites and surrounding apoptosis zones, a decrease of inflammatory response, and an acceleration of normal cell metabolism recovery during the ischemic and postischemic periods are important factors facilitating normal functioning of a tissue and organ.

The differences in the wound healing process between normal or ischemic skin are described using a mathematical model (Xue et al., 2009) consistent with the experimental results (Roy et al., 2009). According to this model, a normal wound with a radius of 4 mm closes in about 13 days, whereas an ischemic wound of equal size heals much more slowly, the healing after 20 days being about 25%.

The beneficial effect of CF on the fibroblast's reparative function was observed in experiments in vivo using a model of ischemic heart injury in rats (Bul'on et al., 2002). The introduction of CF eliminated the progression of necrotic zones in myocardium with replacement of necrotic areas by a connective tissue followed by subsequent scar formation that matured into macrofocal postinfarction sclerosis as an outcome of myocardium infarction.

The CF metabolic preparation has been successfully used for more than ten years in the practice of emergency therapy and in treatment of chronic diseases (Afanas'ev and Lukianova, 2010). This complex preparation composed of two metabolites (succinic acid and inosine) and two coenzymes (riboflavin mononucleotide and nicotinamide). The pharmacological effects of each component are widely known in separation. Inosine is an ATP precursor, provides intracellular energy transport, increases the activity of several enzymes of Krebs' cycle, and activates anaerobic glycolysis under conditions of hypoxia in the absence of ATP. Succinic acid is the most important Krebs' cycle substrate; upon its oxidation, a maximum energy amount is produced; it increases the circulation of the cycle; and decreases the concentrations of lactate, pyruvate, and citrate that were accumulated at the earlier hypoxia stages. Nicotinamide is a precursor of coenzymes (NAD+ and NADP+) that are hydrogen carriers and provide oxidation-reduction processes; it improves microcirculation and activates the antioxidixing system of ubiquinone reductases. Riboflavin mononucleotide is included in the composition of flavin enzymes that participate in the degradation of succinic acid and other acids and maintains glutathione (cofactor of glutathione peroxidase) in a reduced form, protecting the cell from ROS and determining the intracellular redox status. The combination of all these components in CF produces a synergic action on the cell metabolism and gives it the properties of an effective antihypoxant.

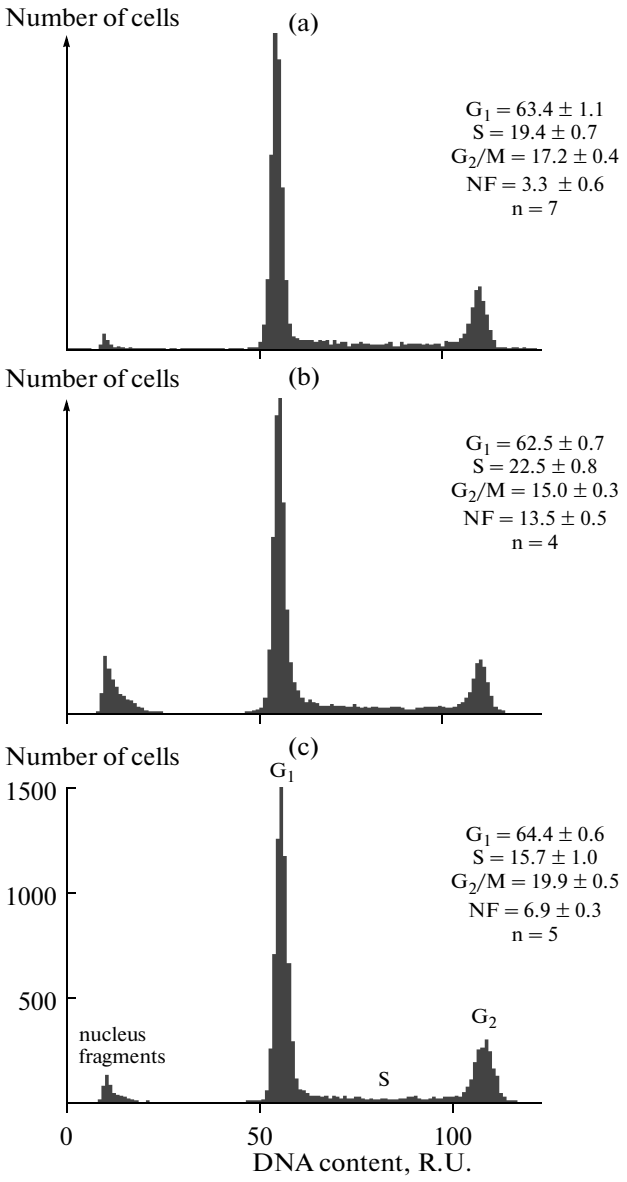

Fig. 2. Cell distribution for the cell cycle phases. Flow cytometry.

a—intact fibroblasts, b—fibroblasts after ischemia and 24-h reoxygenation with CF. NF—nuclear fragments.

Means and confidence intervals are presented.

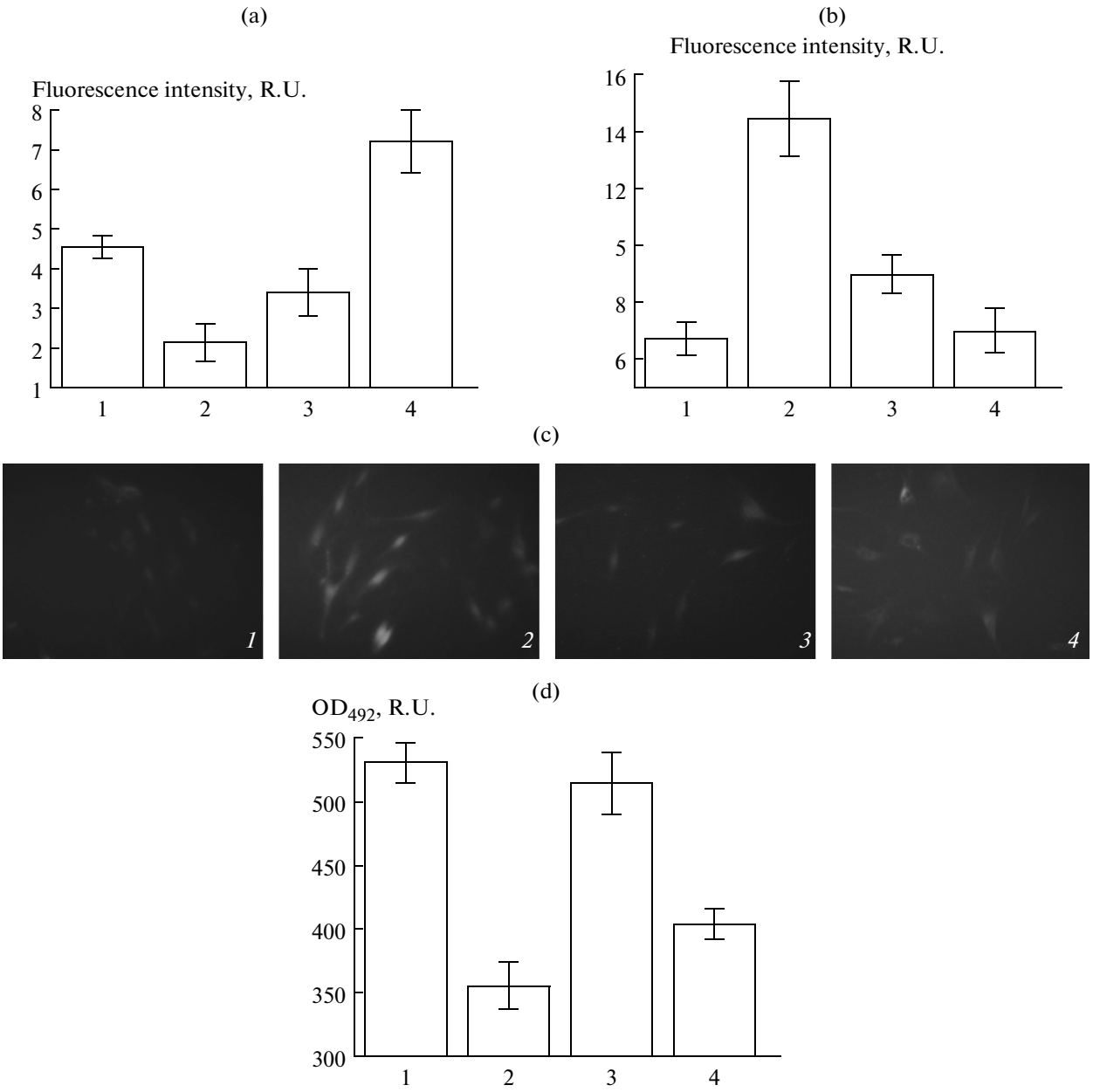

Fig. 3. ROS level in human fibroblasts cultivated under different conditions.

a—ROS levels in cells after hypoxia and subsequent reoxygenation: 1—intact cells; 2 and 3—30 and 60 min of reoxygenation, respectively; 4—H_2O_2 is added after 60 min of reoxygenation, an increase of fluorescence indicates preservation of dye activity; b—effect of CF on the ROS level during oxidative stress caused by H_2O_2 exposure: 1—intact cells, 2—30 min of H_2O_2 exposure, 3—CF is added after H_2O_2 exposure (30 min), 4—24 h of pretreatment with CF, then the medium was replaced by medium with H_2O_2 for 30 min; c—fluorescent microscopy (obj. 20×): *1*—intact cells, *2*—30 min of H_2O_2 exposure, *3* – CF is added after H_2O_2 exposure (30 min), *4* – 24 h of pretreatment with CF, then the medium was replaced by the medium with H_2O_2 for 30 min; d—survival of fibroblasts 24 h after H_2O_2 exposure (MTS-analysis): 1—intact cells, 2—30 min in medium with H_2O_2, 3—24 h with CF after 30-min H_2O_2 exposure, 4—24 h of pretreatment with CF, then H_2O_2 exposure.

According to our results, the addition of CF into the culture medium during early periods (1 h) of reoxygenation after ischemia in fibroblasts allows to increase the ATP content by 2.1 times, which indicates a fast effect of CF on energy production.

To estimate the antioxidant action of CF, we exposed dermal fibroblasts to ischemia and subsequent reoxygenation. As has been described for several cell types (Li, Jackson, 2002), there is an increase of ROS occurring at reoxygenation, which leads to increased

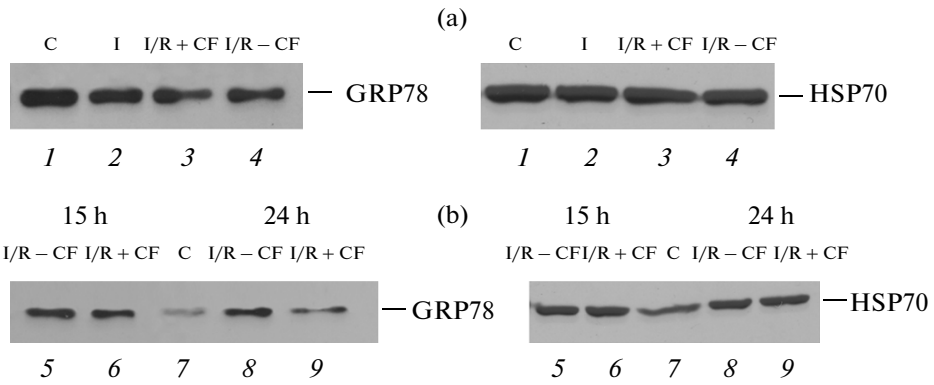

Fig. 4. Effect of CF on synthesis of GRP78 and Hsp70 by human fibroblasts at different terms.
a—6 h, b—15-24 h of reoxygenation (R) after ischemia (I); a: *1*—intact cells, *2*—1 h of ischemia, *3*—after 6 h of reoxygenation with CF, *4*—after 6 h of reoxygenation without CF; b: *5*—15 h without CF, *6*—15 h with CF, *7*—intact cells, *8*—24 h without CF, *9*—24 h with CF.

cell death. We observed that, after 30 and 60 min of reoxygenation, the ROS level in fibroblasts was lower than in control cells. This result is in accord with the study (Panchenko et al., 2000), the authors of which showed that dermal fibroblasts reduce ROS production during hypoxia and slowly restore it during the reoxygenation period up to the level of intact cells. Nevertheless, during reoxygenation, in vivo fibroblasts are subjected to the elevated ROSs released by endothelial cells (Terada, 1996), as well as of phagocyting cells (at respiratory burst) in the area of the wound and/or necrosis caused by chronic and acute ischemia. This contribution of endothelial cells to the enhanced ROS production has been suggested to be a main cause of cell death in ischemic tissues (Zweier et al., 1988). Moreover, the oxidative stress causes qualitative and quantitative changes of the ECM synthesized by cardiac fibroblasts, which may play an essential role in the pathogenesis of myocardial remodeling following myocardial infarction (Siwik et al., 2001; Sen et al., 2006; Roy et al., 2010; Jun et al., 2011).

We used 30-min H_2O_2 exposure for initiation oxidative stress in dermal fibroblasts. The addition of CF after H_2O_2 exposure decreased the elevated ROS level in cells by two-thirds. This antioxidant effect of CF resulted in a higher survival of dermal fibroblasts comparable to its level in intact cells. Pretreatment of cells with CF prevented an increase of ROS production in early stages after H_2O_2 exposure, which led to an increase in fibroblast survival after 24 h, i.e., increased resistance of these cells to oxidative stress.

One of the most important mechanisms of cell defense from ischemia/reoxygenation injury is the overexpression of the protective proteins Hsp70 and GRP78. Since CF is able to significantly enhance the protein synthetic function of cells after hypoxia (Bul'on et al., 2006), we studied whether increased synthesis of these chaperones makes a contribution to the cytoprotector properties of CF. After 15 and 24 h of reoxygenation, we observed an enhancement of Hsp70 synthesis, but use of CF did not lead to an increase of this synthesis. One of the known functions of Hsp70 is modulation of the activities of glutathione peroxidase and glutathione reductase, which regulate the cellular redox status in response to ischemic stress (Guo et al., 2007). It is possible that the absence of an increase in ROS production above its normal level revealed in our experiments during reoxygenation could be a factor that prevents the initiation of significant changes in Hsp70 expression.

At the same time, 24-h cell cultivation with CF during reoxygenation decreased GRP78 synthesis, which showed an increase after 15 h. Since overexpression of GRP78 is a marker of ER stress and activation of unfolded protein response (UPR), the progression of which leads to cell death, the decrease in GRP78 synthesis revealed in our study indicates a successful resolution of cellular response to unfolded proteins in ER, which is also confirmed by an increase in fibroblast survival during reoxygenation with CF.

In conclusion, our study shows that CF produces pronounced effects on dermal fibroblasts as a cytoprotector, energy corrector, and antioxidant. Based on this, we can suggest that CF will be useful for clinical application in complex therapy in diseases associated with skin injuries of traumatic and trophic genesis.

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