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Trophic effect of a methanol yeast extract on 3T3 fibroblast cells

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Synopsis

With regard to the increase of human life expectancy, interest for topical treatments aimed to counteract skin aging is still growing. Hence, research for bioactive compounds able to stimulate skin fibroblast activity is an attractive topic. Having previously described the effects of a new methanol yeast extract on growth and metabolic activity of *Saccharomyces cerevisiae*, we studied its effects on 3T3 fibroblasts to evaluate its potential antiaging property. This investigation demonstrates that this extract increases proliferation as well as migration of 3T3 cells and decreases their entrance in senescence and apoptosis phases. Altogether, these results open new perspectives for the formulation of innovative antiaging topical treatments.

INTRODUCTION

Skin is an organ the function of which is mainly to protect an organism against the external environment. In one way, skin impedes physical, chemical, and microbiological hazards and in the other, it regulates loss of water, electrolytes, and heat (1). Moreover, known to contain a large number of Langerhans cells, skin is considered a real sentry with regard to body immunity (2). In addition, the extensive nerve network present in skin makes an effective sensory link between organism and its environment. Epidermis, dermis, and hypodermis are the three main layers of the skin. It should be stressed that these layers derive from different embryonic origins. While epidermis derives from ectoderm, dermis and hypodermis are both of mesodermal origin. Thus, by contrast to epidermis, which is mostly composed of epithelial cells, dermis and hypodermis are mainly composed of mesenchymal cells (i.e., fibroblasts and adiposities, respectively).

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Besides its biological and physiological functions, skin is a predominant factor in terms of physical appearance, like a "social interface," it is an essential beauty criterion (3). Skin structure and appearance alterations caused, for instance, by aging or by sun exposure are often not well accepted. Hence, use of cosmetic treatments formulated to slow down skin damages is extremely widespread. Furthermore, progresses made in the field of medicine increase life expectancy (4); we can easily assume that demand of this type of topical treatment will constantly increase (5).

By contrast to most internal organs that mainly age through intrinsic mechanisms (chronological aging), aging of the skin is also subjected to extrinsic factors (environmental stresses) (6). Together, these two types of alterations often result in well-known characteristics: skin undergoes pigmentation changes and becomes wrinkled, saggy, and dry. Actually, most physical damages have their origin in dermis thickness reduction induced by fibroblast number and activity decreases. Hence, preservation or restoration of dermal fibroblast function is a goal that many anti-aging cosmetics claim to reach.

It is usually considered that free radicals are largely responsible for skin aging. Thus, most cosmetic treatments contain natural (i.e., retinol, ascorbic acid, tocopherol, etc.) or modified (i.e., tetra-isopalmitoyl ascorbate, tocopherol acetate, etc.) antioxidant compounds (7,8). It is also generally known that extracts from yeast *S. cerevisiae* may also be beneficial with regard to skin texture and appearance (8). To date, mainly two types of bioactive yeast extracts have emerged: chromium- and β-glucan-rich extracts (9–15).

Meanwhile, another type of yeast extract (MYE for Methanol Yeast Extract), has been described in a different context which is that of the alcoholic fermentation. This extract differs from traditional yeast extracts by its mode of production. Our group and others have shown that it had a positive effect on *S. cerevisiae* (16–19). Actually, MYE has been shown to accelerate the metabolism of carbohydrates via an increase in glucose transport, glycolytic flux, and intracellular content of fructose-2,6-bisphosphate and via the activation of the pyruvate decarboxylase. It was also shown that this extract was able to increase *S. cerevisiae* resistance to various stresses such as oxidative, alcoholic, osmotic, and UV-induced. In addition, MYE increases longevity of *S. cerevisiae*, a reference model for studying genetic and physiological modulations regulating longevity in higher organisms (20). Thus, all these observations led us to study the effect of this extract on higher eukaryotic cells and particularly on fibroblasts that play a major role in dermis structure.

MATERIAL AND METHODS

YEAST EXTRACTION METHOD

Two kilograms of pressed yeast *S. cerevisiae* (Algist Bruggeman, Gent, Belgium) were extracted with 2 l of pure methanol at room temperature and stirred for 24 h. After centrifugation and filtration, the bioactive fraction contained in the supernatant was precipitated by addition of 4 volumes of acetone. The precipitate was then recovered in 500 ml of distilled water and the bioactive fraction was isolated by differential precipitation with ethanol. Briefly, after addition of 35% v/v ethanol, precipitated fraction was discarded and after adding 50% ethanol, precipitated fraction was, this time, recovered (viscous precipitate). Consecutive additions of ethanol at 70% and 100% induced the precipitation of inactive compounds. Note that all fractions were tested with regard to

cell proliferation (data not shown). As a result, we obtained a viscous brownish and water soluble bioactive complex (MYE for Methanol Yeast Extract).

EVALUATION OF THE ANTIOXIDANT PROPERTY OF MYE

The antioxidant capacity of MYE was evaluated by the DPPH method (1,1 diphenyl pycril 2 hydrazil) following a protocol adapted from Szabo *et al.* (21). Briefly, a methanol solution of 1 mM DPPH was incubated in the presence or absence (control) of MYE at 0.001%, 0.01%, 0.1%, and 1% v/v. After 15 min incubation at room temperature, absorbance at 517 nm was measured using an Oasis UVM340 spectrophotometer. Antioxidant effect of the yeast extract was compared to the one of ascorbic acid at concentrations ranging from 0.001 to 1mM.

MEASUREMENT OF YEAST PROLIFERATION

About 5×10^6 cells of *S. cerevisiae* previously grown on rich medium were used to inoculate 50 ml of glycerol minimal medium (4% glycerol/0.17% yeast nitrogen base/0.5% ammonium sulphate; Difco, Detroit, MI) (22) supplemented or not (control) with MYE at 0.01%, 0.1%, and 1%. After 24, 48, and 96 h of incubation at 30°C with shaking, growth was assessed by measuring the absorbance at 660 nm using a Unicam UV1 spectrophotometer.

ANIMAL CELL CULTURE

3T3 cells (mouse embryonic fibroblasts) were from DSMZ bank (Braunschweig, Germany). Cells were cultured in DMEM medium supplemented with 4 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (all reagents from Invitrogen/Gibco, Carlsbad, CA). Cells were maintained at 37°C in a humid atmosphere containing 5% CO_2.

MTT CYTOTOXICITY ASSAY

3T3 cells were seeded in 96-well plates (3000 cells/well). Twenty four hours after seeding, cells were treated or not (control) with MYE at 0.01%, 0.1%, and 1% for a period of 48 h. Cell viability was then assayed by exposure to 0.03% MTT w/v. After removal of medium, produced formazan was dissolved in DMSO (1 h, room temperature, under agitation) for measurement by spectrometry at 550 nm using an Oasis UVM340 spectrophotometer.

3T3 CELL GROWTH MEASUREMENT

3T3 cells were seeded in 96-well plates (3000 cells/well). Twenty four hours after seeding, cells were treated or not (control) with MYE at 0.01% and 0.1% for a period

of 72 h. Cell growth was then measured by crystal violet staining as previously described (23). Briefly, cells were washed with PBS, fixed for 15 min in glutaraldehyde (1% in PBS), and stained for 30 min with crystal violet (0.1% w/v in distilled water). After removal of dye excess, cell-bound crystal violet was extracted with 1% v/v Triton X-100 and absorbance was measured at 550 nm using an Oasis UVM340 spectrophotometer.

CLONOGENICITY STUDY

3T3 cells were seeded in 6-well dishes at low concentration (100 cells/well). Twenty four hours after seeding, cells were treated or not (control) with MYE at 0.01% and 0.1% for 72 h. Cells were then fixed and stained with crystal violet as described above. After staining, visible clones (at least about 20 cells) were counted and diameter of the 10 largest colonies was recorded.

SENESCENCE ASSAY

3T3 cells were seeded in 6-well plates (10^5 cells/well) and incubated for 96 h in DMEM containing 2% FBS. Cells were then treated or not (control) with MYE at 0.01% and 0.1% and incubated for an additional 72 h in DMEM containing 2% FBS. Senescence was evaluated via the detection of β-galactosidase activity using the Senescence Detection Kit from Merck Biosciences (Schwalbach, Germany) according to the manufacturer's instructions.

TUNEL ASSAY FOR APOPTOSIS DETECTION

3T3 cells were seeded in 6-well plates (10^5 cells/well) and allowed to grow for 24 h. Medium was then replaced by serum-free DMEM containing or not (control) MYE at 0.1% for 48 h. Fragmented DNA was then biotinilated by using the DeadEnd tunnel assay system from Promega (Madison, WI), labeled with streptavidin-coupled Dylight 594 (Invitrogen) and visualized by fluorescence microscopy.

ACTIN CYTOSKELETON REARRANGEMENT STUDY

3T3 cells were seeded in 8-chamber Lab-Tek slides and allowed to grow for 48 h before treatment with or without (control) MYE at 0.1%. After 90 min of incubation, monolayers were washed twice with PBS, fixed in a 3% paraformaldehyde–PBS solution (20 min, room temperature), permeabilized with a 0.1% Triton X-100-PBS solution (5 min, room temperature, under agitation), and nonspecific binding side were blocked with a 2% BSA–PBS solution (overnight, 4°C). Cells were then exposed for 1 h to 1 µg/ml Texas red-labeled phalloidin (for F-actin) or 10 µg/ml FITC-labeled DNAse I (for G-actin), both diluted in a 2% BSA–PBS solution. After several washes with a 0.05% Tween 20-PBS solution, F- and G-actin were visualized by fluorescence microscopy. In addition,

fluorescence of Texas red-labeled phalloidin associated to F-actin was quantified using Image J software (National Institute of Health).

MIGRATION STUDY BY SCRATCH ASSAY

Migration of 3T3 cells was assessed by the technique of the scratch assay (24) using a protocol adapted for cells having a high migration rate (wound of 2.2 mm). 3T3 cells, seeded 6-well plates (5×10^5 cells/well), were incubated for 48 h without any treatment. Cells were then treated with mitomycin C at a concentration of 5 µg/ml for a period of 90 min in order to inhibit cell proliferation. After wound formation using a 2.2-mm width scraper (t_0), cells were washed 3 times with PBS and incubated for a period of 24 hours (t_{24}) in the absence (control) or presence of MYE at 0.01 and 0.1%. Migration rate was then calculated (difference of the wound width at t_0 and t_{24} normalized by the width at t_0). Note that marks made on the back of the plate were done in order to identify the exact location of measurements.

RESULTS

MAIN CHARACTERISTICS OF MYE EXTRACT

MYE is obtained by methanol extraction of pressed yeast *S. cerevisiae*. Various stages of precipitation are then performed to purify this extract (see Materials and Methods for details). Brownish and liquid at room temperature, this extract has a specific gravity of 1.41 and a pH value of 7.5. Preliminary studies carried out by G-25 exclusion chromatography have shown that the molecular mass of the MYE is about 1500 Da and it shows an absorption peak at 260 nm (16,17).

The bioactive properties of yeast extracts containing chromium or soluble β-glucans being established (9–15), we attempted to detect the presence of these compounds in our extract. Concerning chromium, analysis carried out by ICP (inductively coupled plasma) showed that this element is only present in trace amounts (less than 10 ppm; data not shown). Similarly, enzymatic assays (Megazyme, Wicklow, Ireland) made on the MYE failed to demonstrate the presence of β-glucans (data not shown). Hence, according to these experiments, one may assume that the extract described here differs from chromium- or β-glucans-rich yeast complexes. Note that presence of amines, probably from peptidic origin, was detected by both ninhydrin and BCA (Pierce, Rockford, IL) colorimetric assays (data not shown).

ANTIOXIDANT PROPERTIES OF MYE

It is a general agreement that antioxidant compounds are beneficial with regard to skin aging. Hence, we evaluated the potential antioxidant property of MYE using DPPH (Figure 1). In this assay, MYE exhibits antioxidant property with an IC50 of 1.16×10^3 ppm. Tested in parallel as a positive control, ascorbic acid has an IC50 of 0.034 mM (= 5.9 ppm). Thus, although MYE is about 200 times less potent than ascorbic acid in this assay, this extract appears to possess significant antioxidant properties.

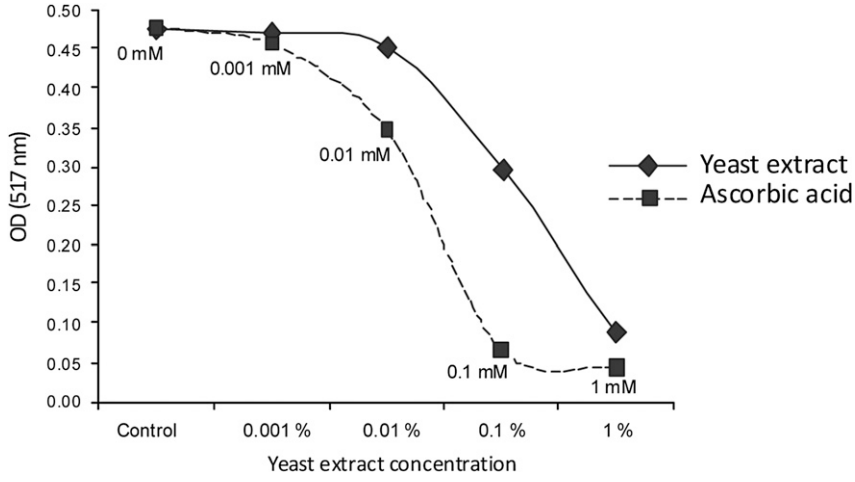

Figure 1. Antioxidant activity of MYE. Yeast extract at concentrations ranging from 0.001% to 1% is tested by the DPPH (1,1 Diphenyl Pycril Hydrazil 2) method. Effect is compared to that of ascorbic acid (concentrations ranging from 0.001 to 1mM; concentrations given on the plot). Antioxidant capacity is correlated with the decrease in absorbance (O.D.; optical density) of DPPH at 517 nm. Data are representative of three independent experiments.

EFFECT OF MYE ON RESPIRATORY-DEPENDENT YEAST GROWTH

Experiments with classical yeast culture media, that is, YPD (glucose) and wort (maltose in major part) have demonstrated that proliferation of *S. cerevisiae* is significantly increased by MYE (16,19). Remarkably, we show here that this increased proliferation is exacerbated in respiratory conditions (glycerol-containing minimal medium) (Figure 2). It should be noted that HPLC analyses, which demonstrated the absence of fermentable sugars in MYE, exclude a potential trivial effect of such substrates (data not shown). Hence, it appears that this extract is more effective on respiratory system.

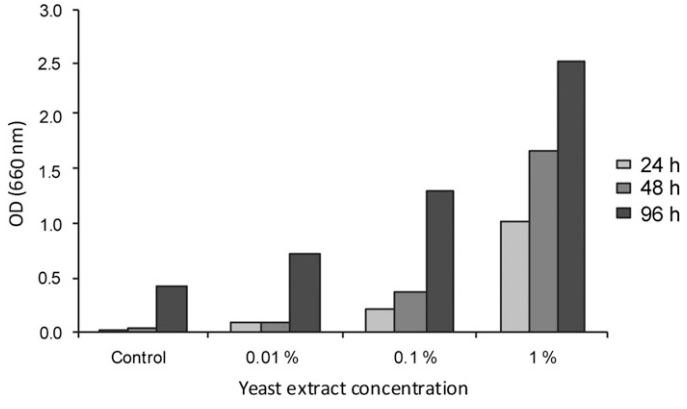

Figure 2. Effect of MYE on the proliferation of *S. cerevisiae* in respiratory medium. Yeast cells were inoculated into minimal medium containing 4% glycerol. After 24, 48, and 96 h of incubation in the absence (control) or presence of MYE at 0.01%, 0.1%, and 1%, growth is evaluated by measuring the absorbance (O.D.; optical density) at 660 nm.

TROPHIC EFFECTS OF MYE ON 3T3 FIBROBLAST CELL LINE

MTT cytotoxicity assays were performed on 3T3 cells in order to determine if deleterious side effects are observed and to establish the usable concentration limit (Figure 3). These assays revealed that MYE can be used up to 0.1% without inducing any decrease of 3T3 metabolic activity. On the contrary, at this concentration, it appears that MYE induces a noticeable increase of mitochondrial activity that could be relevant to growth stimulation. In agreement with this view, proliferation assays carried out by crystal violet staining show that addition of MYE at 0.1% induces a significant increase of 3T3 cell proliferation (264% as compared to untreated cells) after a 72 h incubation period (Figure 4). Of note, additional experiments carried out in antibiotic-free medium exclude any bias potentially induced by penicillin and/or streptomycin (see insert of Figure 4).

Actually, the proliferation induction we observe here may be the result of two phenomena: an increase of cell number entering in cell cycle or a decrease of the generation time. As shown by clonogenicity experiments (Figure 5), it appears that the yeast extract plays on both these behaviors. Indeed, MYE is able to increase the number of clones (increased number of cells entering into the cycle) and, on the other hand, to increase their size (more division within colonies).

As proliferation and clonogenicity experiments show that MYE favors cell mitosis, there is suspicion that it could also impede their entry into senescence phase. Thus, analysis of β-galactosidase activity of 3T3 cells cultured in low amount (2%) serum-containing medium was achieved in order to evaluate the potential ability of MYE to reverse the effect of serum impoverishment on senescence induction (Figure 6). As expected, a 3-day treatment with MYE at 0.1% reduces over 80% the number of β-galactosidase-positive senescent cells.

Likewise, effect of MYE was evaluated on apoptosis induced by serum starvation (Figure 7). Analyses carried out by TUNEL assay show that addition of 0.1% of yeast extract significantly decreases number of apoptotic cells (45% as compared to untreated cells) after 2 days of treatment. Note that these results agree with 7-AAD permeability experiments which also showed a significant decrease of apoptotic cells (64% as compared to untreated cells; data not shown).

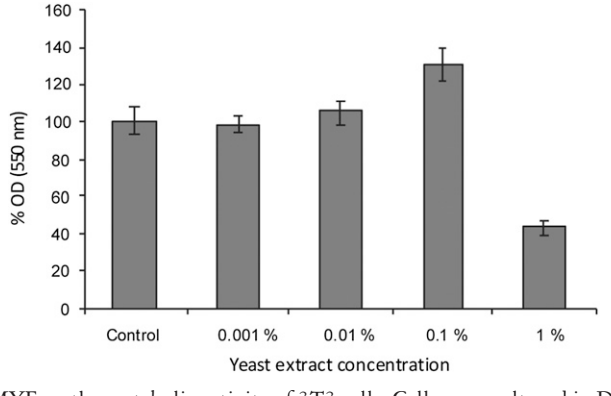

Figure 3. Effect of MYE on the metabolic activity of 3T3 cells. Cells were cultured in DMEM in the absence (control) or presence of MYE at concentration ranging from 0.001% to 1%. Metabolic activity was assessed by MTT assay after 48 h of incubation. Results are expressed in percentage absorbance (mean ± S.D.) of the control. Measurements were performed in sixplicate and the figure refers to an experiment performed twice.

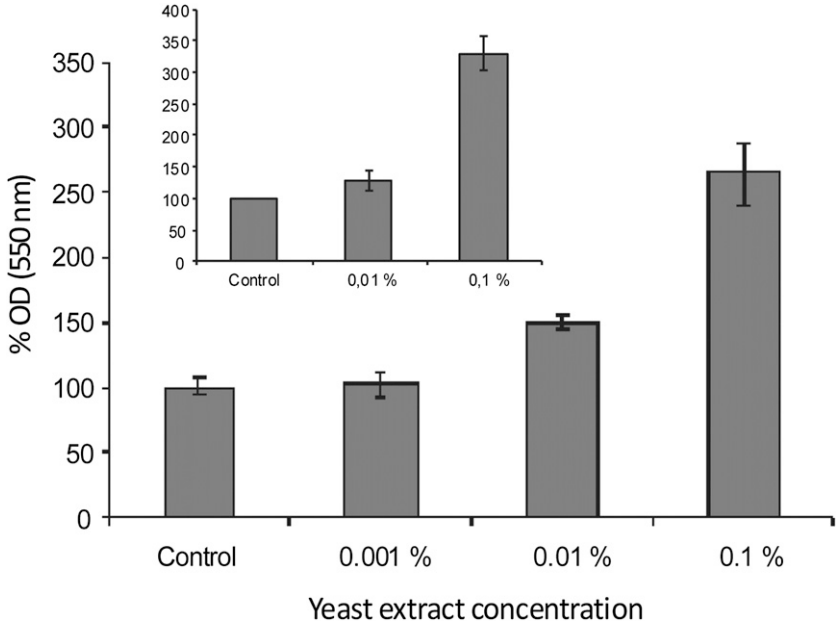

Figure 4. Effect of MYE on the proliferation of 3T3 cells. Cells were cultured in DMEM in the absence (control) or presence of MYE at concentration ranging from 0.001% to 0.1%. Proliferation was assessed by incorporation of crystal violet after 72 h of incubation. Results are expressed in percentage absorbance (mean ± S.D.) of the control. The figure is representative of three experiments performed in sixplicate. Insert refers to the mean values of two experiments carried out in sixplicate in the absence of antibiotics.

MYE MODIFIES ACTIN CYTOSKELETON AND INCREASES MIGRATION OF 3T3 CELLS

Besides modulation of cell proliferation and survival described above, it should be stressed that MYE may also induce cell shape changes such as a perceptible increase of spindle-shaped morphology (see Figure 8 below). Hence, modification of actin cytoskeleton

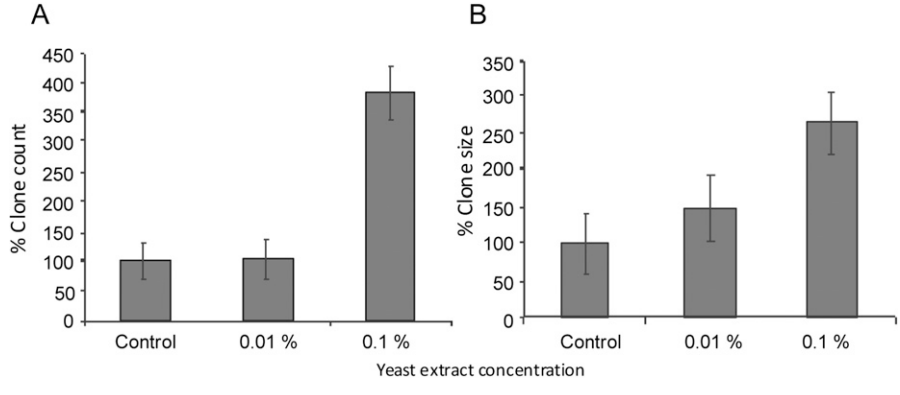

Figure 5. Effect of MYE on clonogenicity of 3T3 cells. Cells seeded at low density were incubated for 72 h in the absence (control) or presence of MYE at 0.01% and 0.1%. (A) Number of visible clones (at least approximately 20 cells). (B) Size of the 10 largest colonies. Results are expressed in percentage of the control (mean ± S.D.). The figure refers to an experiment performed in triplicate.

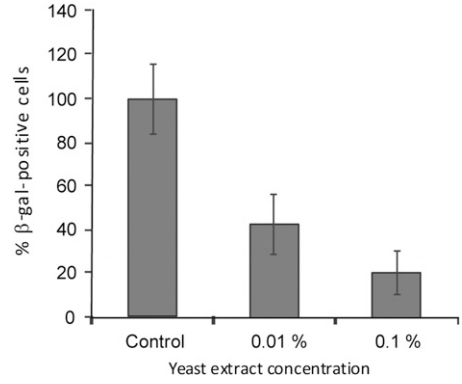

Figure 6. Effect of MYE on senescence of 3T3 cells. Senescence was evaluated by detection of β-galactosidase activity after 72 h of incubation in the absence (control) or presence of MYE at 0.01% and 0.1%. Senescent cells were counted by crossing wells in three directions (horizontal, vertical, and diagonal). Results are expressed in percentage of the control (mean ± S.D.). Figure refers to an experiment performed twice.

organization seems likely; a statement confirmed by fluorescence microscopy (Figure 8A). In these assays, Texas red-phalloidin was used to label filamentous actin (F-actin) and FITC-DNAse I for globular actin (G-actin). Results show that MYE at 0.1% increases F-actin pool at the expense of the one of G-actin. Note that these results are confirmed by densitometry analysis of F-actin level (Figure 8B) and by western blot analysis of the F-actin triton-insoluble pool (increase of 214% as compared to untreated cells; data not shown).

In view of the fact that the actin cytoskeleton rearrangement observed here could be associated with cell migration process, scratch assays were carried out on 3T3 cells in order to evaluate the ability of MYE to promote such a mechanism (Figure 9). Of note, mitomycin C, a mitosis inhibitor, was used to avoid bias that might be related to the effect of MYE on cell proliferation. Results show that, after 24 h of treatment with yeast extract at a concentration of 0.1%, scratch wound healing is strongly increased (473% as compared to untreated cells), confirming the effect of MYE on 3T3 fibroblast migration.

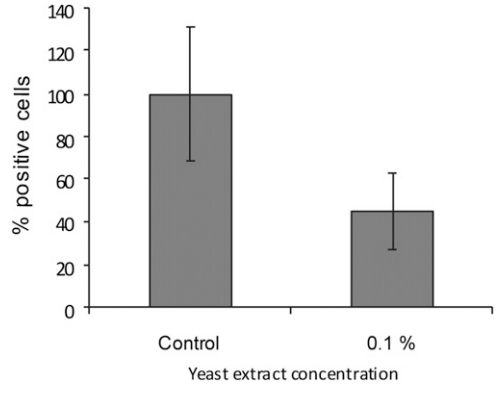

Figure 7. Effect of MYE on apoptosis of 3T3 cells. Apoptosis was evaluated by TUNEL assay after 48 h of incubation in serum-free DMEN in the absence (control) or presence of MYE at 0.1%. Positive cells were counted in four randomly selected microscope fields. Results are expressed in percentage of the control (mean ± S.D.).

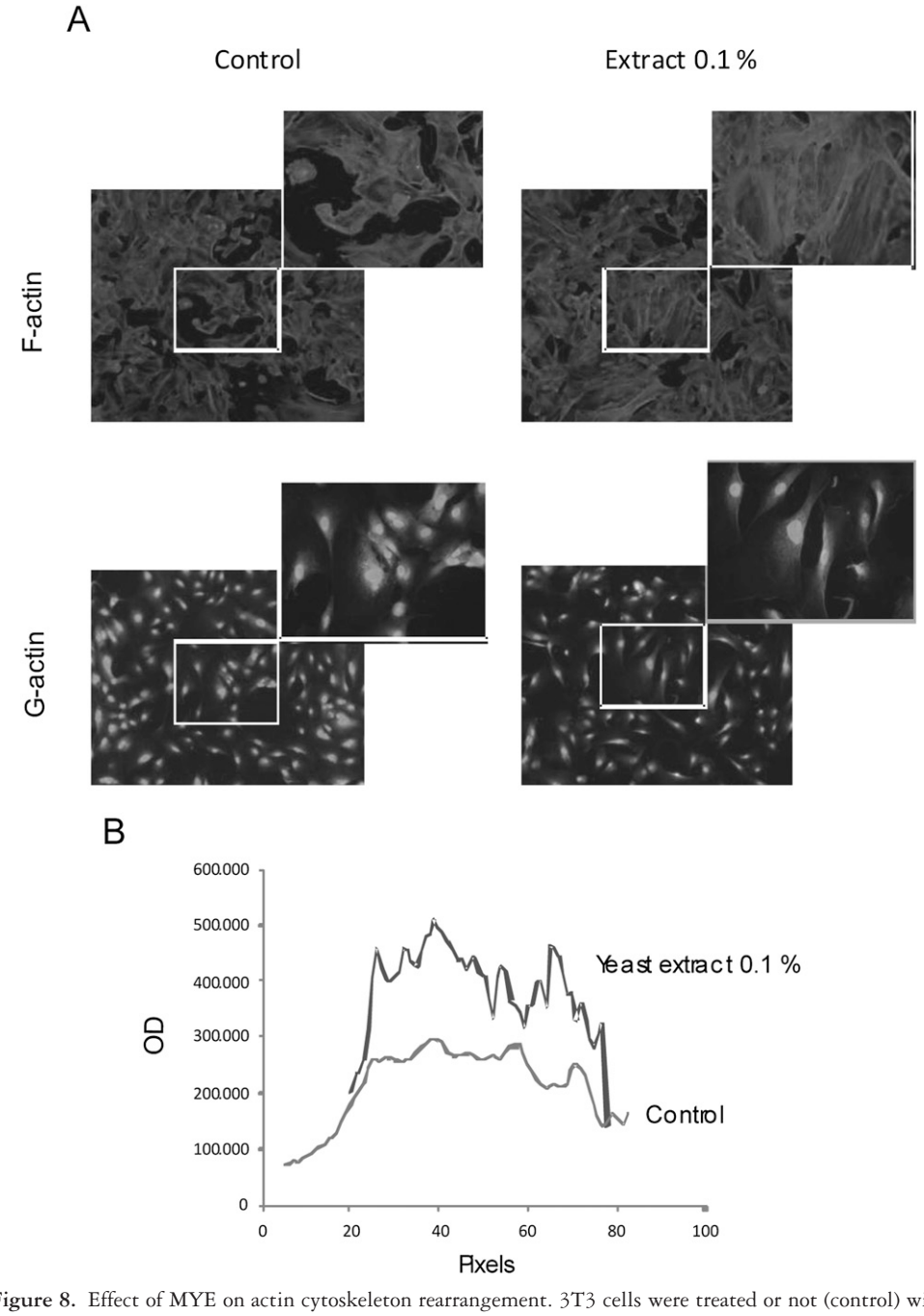

Figure 8. Effect of MYE on actin cytoskeleton rearrangement. 3T3 cells were treated or not (control) with MYE at 0.1% during 90 min. (A) F-actin and G-actin were demonstrated by fluorescence microscopy after labeling with Texas-red-phalloidin and DNAse I-FITC, respectively. The figure is representative of three independent experiments. (B) Densitometry analysis of F-actin-associated fluorescence. For both control and MYE-treated conditions, 10 cells were sampled by using Image J software.

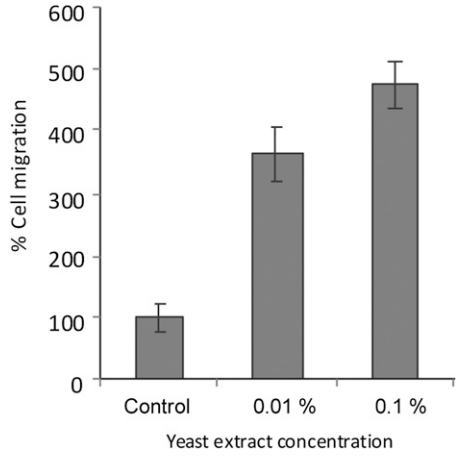

Figure 9. Effect of MYE on the migration of 3T3 cells. After scratching cell monolayer with 2.2-mm-wide scrapper and treatment with mitomycin C, cells were incubated 24 h in the absence (control) or presence of MYE to 0.01% and 0.1%. Data represent the increase of cell migration after 24 h (i.e., decrease of the wound size). The figure is representative of four independent experiments performed in triplicate.

CONCLUSION

Dermal connective tissue primarily comprises a matrix of hydrated protein fibers such as collagen, elastin, fibronectin, and glycosaminoglycans. This extracellular structure that is synthesized and colonized by fibroblasts serves both as mechanical support and exchange surface for the epidermis. Decrease of skin fibroblast activity caused by age and environmental stresses induces a dermal thickness reduction leading to the appearance of wrinkles and a loss of elasticity. On the other hand, progress made in the field of medical research, consistently increased our life expectancy often inducing a mismatch between health status and physical appearance. Hence, interest for cosmetics aimed to reduce time effect is still growing.

In order to counteract skin aging, development of effective treatments able to sustain or increase fibroblast activity and proliferation is essential. In this regard, use of yeast extracts appears to be a valuable and attractive approach. Indeed, despite the fact that it is a by-product of brewing industry, yeast is a natural and organic raw material rich in bioactive compounds. Research conducted by our group and others have highlighted the biological activity of a methanol extract able to stimulate *S. cerevisiae* metabolism and proliferation (16–19). Demonstration that this extract may also induce such a trophic effect on fibroblast cells will be a first step for the formulation of a new topic antiaging treatment. Hence, in this study, we analyzed the effect of this MYE on various biological processes such as mitochondrial activity, proliferation, senescence, apoptosis, cytoskeleton rearrangement, and migration of 3T3 fibroblast cells.

Results showed that, up to a concentration of 0.1%, MYE is not cytotoxic and induces, after 72 h of incubation, a significant increase of 3T3 cell proliferation. As suggested by clonogenicity experiments, the growth stimulation may be the result of both an increase of fibroblast number entering in cell cycle and a decrease of the generation time. Accordingly, we demonstrated that the yeast extract decreases both senescence and apoptosis when serum is depleted from culture medium (partially or totally, respectively). In

addition, demonstration of F- and G-actin by fluorescence microscopy revealed a significant modification of cytoskeleton rearrangement that is characterized by an increase of actin fibers. In agreement with this observation, we demonstrated that MYE induces a significant increase of 3T3 fibroblast cells migration.

Altogether, these results highlight the positive effect of MYE on fibroblast cells. In this view, one may assume that methanol extraction allows the recovery and the purification of interesting bioactive compound(s) present in yeast cells. Of note, MYE is liquid at room temperature and, then, appears more suitable for use in cosmetics than yeast whole cells or classical yeast extracts. On the other hand, it should be stressed that nature of the bioactive compound(s) contained in MYE remains unknown, even if experimental data suggest a peptidic profile (16–18). Up to now, LC-MS, IR, and NMR analyses failed to unambiguously identify this (or these) compound(s); ongoing preparative HPLC experiments are carried out in order to solve this question.

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