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Telopodes of telocytes are influenced in vitro by redox conditions and ageing

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Abstract Telocytes (TCs) are a novel cell type identified among interstitial cells in various organs. TCs are characterized by very long cell processes (tens to hundreds micrometres) named telopodes (Tps) with uneven calibre: dilations (podoms) and very thin segments (podomers). However, little is known about the factors which influence Tps conformation. Recently, extracellular matrix proteins were found to influence Tps extension, adherence and spreading. Here, we show that oxidative stress and ageing influence formation of new Tps of TCs cultivated from human non-pregnant myometrium. Using real-time videomicroscopy, we found that ageing the TCs to passage 21 increased the ratio of Tps/TC number with about 50 %, whereas oxidative stress hindered formation of new Tps in both aged and young TCs (passage 7). Under oxidative stress, newly formed cell processes were up to 25 % shorter. Migration pathway length was decreased by 30–40 % for both young and aged cells in an oxidative stress environment. Contrary, addition of *N*-acetyl cysteine in cell culture medium shifted TCs morphology to a long and slender profile. In conclusion, we showed that TCs specific morphology in vitro is influenced by oxidative status balance, as well as ageing.

Keywords Telocytes · Telopodes · Ageing · Oxidative stress · Cell prolongations · *N*-acetyl cysteine · Cell migration

Introduction

Telocytes (TCs) were identified as a new cell type among interstitial cells [1]. The main feature that distinguishes TCs from any other cell is their long and thin prolongations named telopodes (Tps), ranging from tens to hundreds μm. Therefore, TCs are shortly defined as 'cells with Tps' [2]. TCs were found in heart [3–5], including cardiac valves [6], gastrointestinal tract [7, 8], liver [9], bone marrow [10], blood vessels [11], kidney [12, 13], lung [14, 15], uterus [16, 17], fallopian tubes [18] and even in the eye [19]. The existence and spatial conformation of Tps were confirmed by focused ion beam scanning electron microscope (FIB-SEM) [20, 21]. Several reports presented the chromosomal gene expression profiles [22–25], the microRNA profile [26], proteomic profile [14, 27] and the secretome of TCs [28]. Recent reviews on TCs are available [29–31].

The influence of extracellular matrix proteins [32] and low level laser stimulation [33] on Tps formation were reported. Since a recent paper identified TCs as interstitial cells with a very high expression of mitochondrial superoxide dismutase (10 times over fibroblasts) [27], we were interested to test the consequences of overwhelming their antioxidant protection. There are data showing the effect of altered SOD2 expression in various cell types: Wang et al. indicated that overexpression of SOD2 in vascular smooth muscle cells inhibited cell migration and proliferation [34], while Zhang et al. reported that knockdown of SOD2 in mouse embryonic fibroblasts leads to a senescence-like phenotype, with decreased signals cell growth and

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proliferation [35]. Oxidative stress has been under intense scrutiny as it has been shown to negatively influence the cellular milieu and cell behaviour in a broad range of pathologies [36], as well as in normal ageing [37]. Oxidative stress-linked cellular effects were thoroughly investigated, from activation of cell signalling [38, 39], oncogenic transformation [40], to cellular senescence [41], cell cycle progression [42] and telomere length [43]. Cytoskeleton modifications and cell morphology changes were also reported [44]. However, most studies involved end-point analysis, which could overlook early cellular events. We propose here time-lapse analysis of cell morphology under oxidative stress in an attempt to identify a morphological response pattern to oxidative stress. We used TCs isolated from human non-pregnant myometrium, which were thoroughly described in a previous report [17]. In conclusion, we show here that balance between oxidative and reducing conditions, as well as ageing impact Tps formation and morphology in cell culture.

Materials and methods

Cell cultures and cell treatments

Myometrium TCs were isolated as previously described from human non-pregnant myometrium [17]. In brief, biopsy pieces were stereo-dissected and minced, treated with 10 mg/ml collagenase Ia in complete cell medium. Dissociated cells were separated from non-digested tissue by filtration through a cell strainer (100 μm), collected by centrifugation at 250×*g* for 10 min at room temperature and seeded in 25-cm^2 plastic culture flasks at a density of 5×10^4 cells/cm^2.

Redox status was modified by treatment with 200 μM H_2O_2 and *N*-acetylcysteine (NAc), in concentrations ranging from 200 to 10 mM.

Cell viability analysis

5000 cells were plated in each well and left to adhere overnight. The following day serial dilutions of H_2O_2 or NAc were added in triplicates for 24 h. Then, MTS was added to each well, according to manufacturer's protocol (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) After 3 h at 37 °C in a humidified, 5 % CO_2 atmosphere, the absorbance at 490 nm was recorded using an ELISA plate reader.

Time-lapse videomicroscopy

Cells were seeded at a density of 5000 cells/chamber in 4 chamber 35 mm bottom glass dishes (HIQ4 glass dishes, Ibdi), allowed to adhere overnight, and then treated with H_2O_2 or NAc at the indicated concentrations for at least 24 h. Treated cells were recorded every 10 min in a Nikon BioStation IM (EU, Amsterdam) compact cell incubation and monitoring unit. Data were collected from at least nine different microscopic fields from each chamber. Analysis of Tps and cell migration was performed using NIS-Elements BR3.0 Microscope Imaging Software (EU, Amsterdam).

Measurement of intracellular oxidative stress

5000 cells were plated in 4 chamber 35 mm bottom glass dishes (HIQ4 glass dishes, Ibdi) and incubated with 5 μM CellROX green (Life Technologies, C10444) in complete media and treated with H_2O_2 200 μM. Cell staining was documented in real-time, every 20 min, using the Nikon BioStation cell incubator. Data were collected for 20 h.

Tps length and cell migration analysis

Tp lengths used for statistical analysis represented the average of three measurements taken at three successive time points, between 2 and 8 h of cell treatment. Cell migration was assessed as the sum of all linear trajectories of the nucleolar-associated heterochromatin. Migration was assessed for the same duration for all cases (e.g. 4 h) during the first 8 h of cell treatment.

Statistical analysis

Statistical analysis was performed using two-tailed unpaired *t* test using SPSSv19 software. Statistical significance for Tps length ($n = 15$) and migration pathway length ($n = 10$) was reached for $^*p < 0.05$.

Ethical statement

The study was performed in accordance to the declaration of Helsinki and it was approved by the local Bioethics Committee of the University of Medicine and Pharmacy, Bucharest. Written informed consent was given by all patients.

Results

Ageing the TCs cell culture to passage 21 increased Tps formation

Given the extensive data accumulated on the relationship between ageing and oxidative stress, including SOD2 involvement in cellular senescence [45], we were interested

to see if ageing our cell culture (20 passages) would influence Tps formation. We found that the aged cell culture exhibited increased number of Tps with specific, moniliform aspect (Fig. 1b), versus earlier passage (passage 7, Fig. 1a). By quantifying the ratio between Tps/TCs in aged and young cell cultures, we observed that ageing increased Tps formation with about 50 % (Fig. 1).

Time-lapse videomicroscopy showed that oxidative stress decreased formation of Tps in a time-dependant manner, as well as the migration pathway length of TCs

We exposed TCs culture (passage 7) to 200 μM H_2O_2 for 24 h to observe the effect on Tps formation in real time. Dosage was chosen lower than the literature reports on H_2O_2-induced apoptosis, ranging from 3 to 500 μM [46–48]. Non-apoptotic effects were reported with concentrations up to 300 μM [49]. We did perform, however, a toxicity curve on TCs culture to establish that the chosen concentration is not cytotoxic in this particular case.

We observed that formation of Tps is hindered and the effect is time-dependant, being most obvious during the first 8–10 h (Fig. 2). Also, the effect persists throughout various passages. Length measurement of new Tps formed during the first 10 h of oxidative stress yielded a 25 % decrease versus control.

Migration was also affected, as migration pathway length decreased up to 40 % under oxidative stress versus control.

Aged cells exhibit increased vulnerability to hydrogen peroxide treatment, in terms of Tps formation

We assessed whether hindered formation of Tps under oxidative stress is influenced by ageing. The aged cell culture (passage 21) was exposed to the same experimental

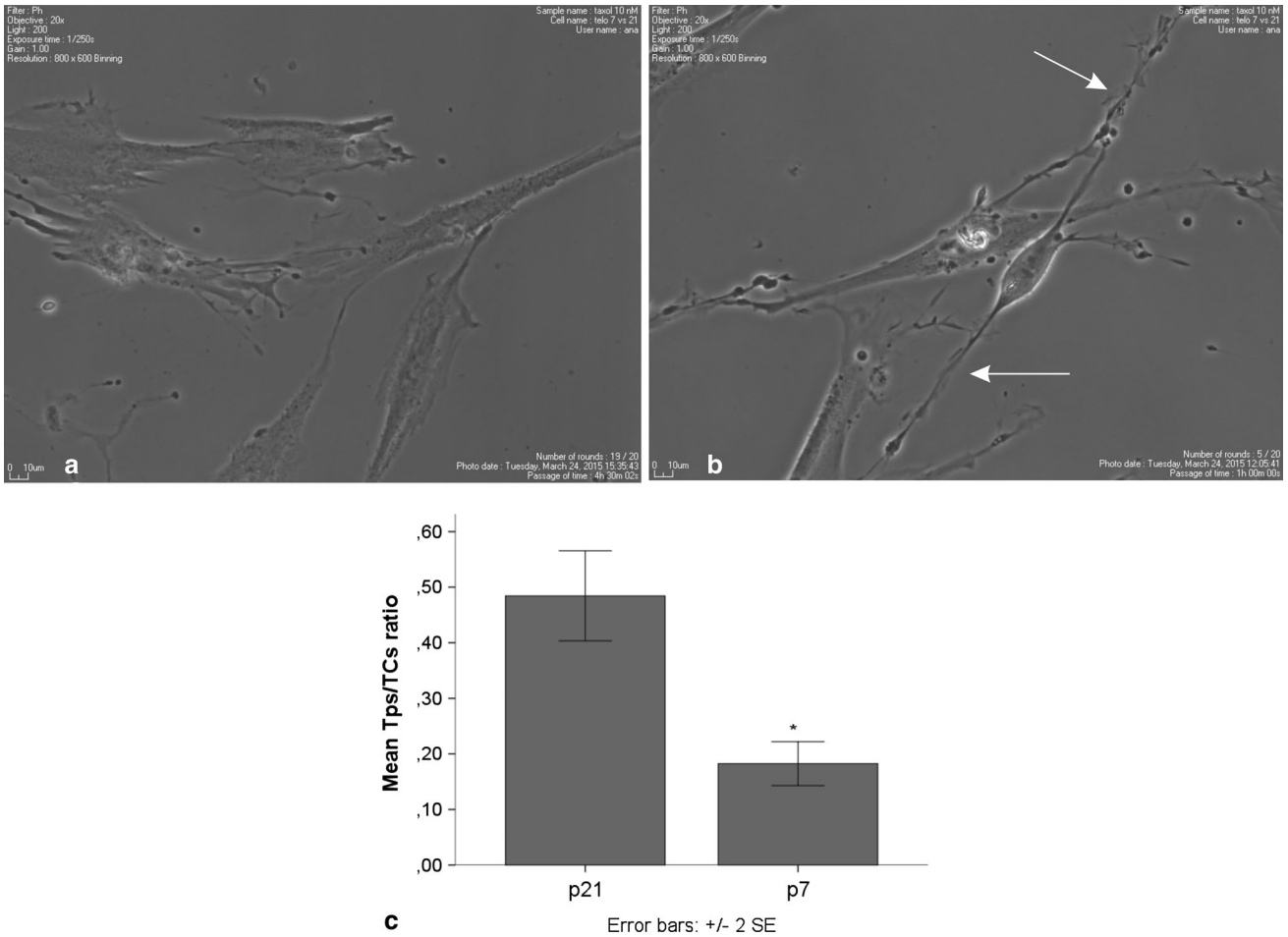

Fig. 1 Human non-pregnant myometrium. TCs cell culture passage 7 (**a**) and passage 21(**b**). Ageing increased the number of Tps with specific moniliform aspect (*white arrows*). The ratio of Tps/TCs number on a given field was increased in aged cells (p = passage). Statistical significance was performed using two-tailed, unpaired t test ($^*p < 0.05$) (**c**)

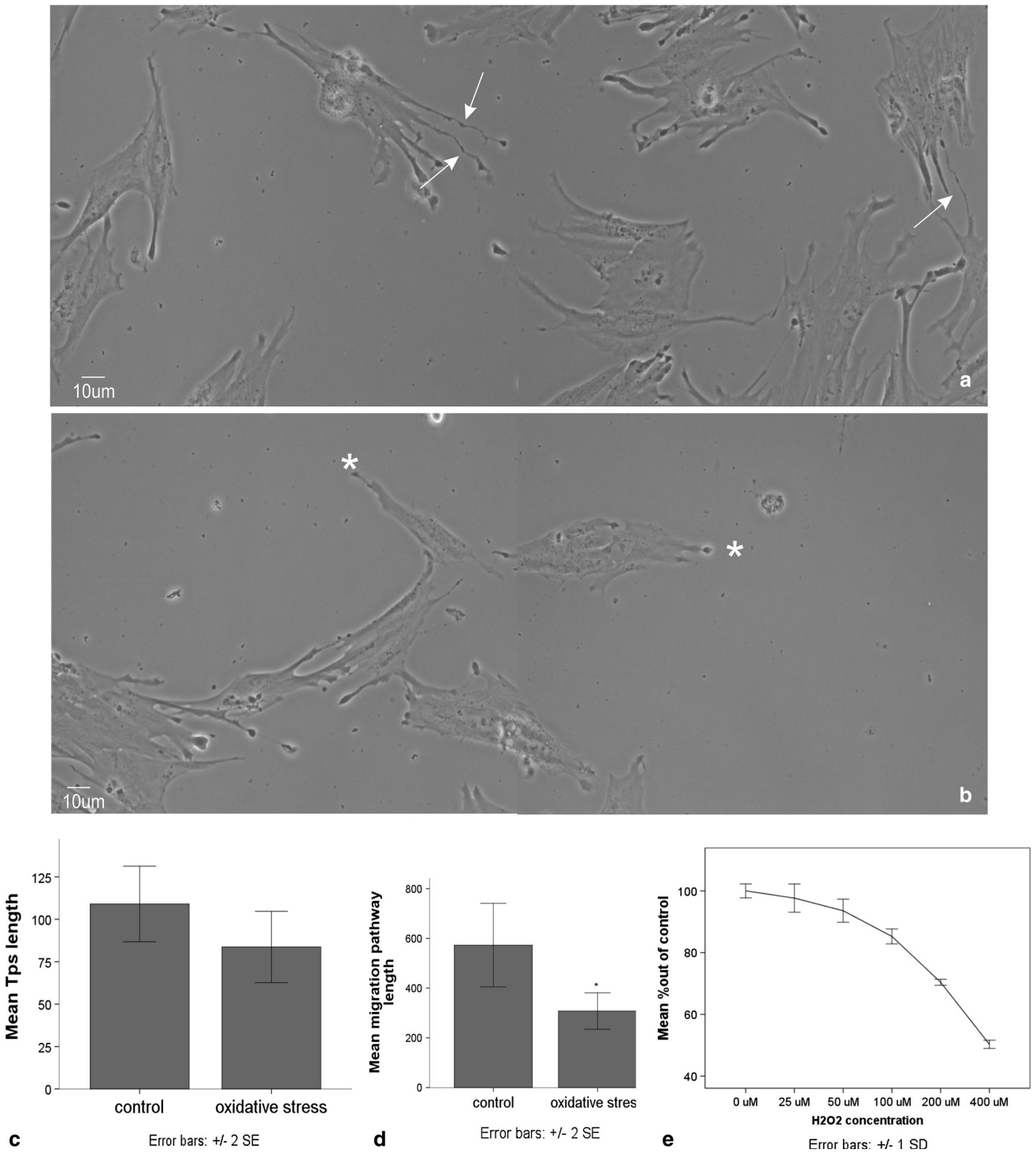

Fig. 2 Assessment of Tps length and TCs migration under oxidative stress by videomicroscopy in human non-pregnant myometrium TCs. Myometrium TCs were recorded for 24 h under standard cell culture conditions (**a**) and oxidative stress (200 μM H_2O_2) (**b**). Oxidative stress hindered cells' ability to form Tps (*white asterisk*). Cell prolongation's mean length was assessed by measuring length at different time points. The mean value was further used for statistical analysis ($n = 10$) (**c**). Migration length was assessed by following migration pathway of nucleoli as cell marker ($n = 10$) (**d**). H_2O_2 toxicity was assessed using an MTS assay. Each point represents the mean ± SD of 3 replicates (**e**). Statistical significance was performed using two-tailed, unpaired *t* test ($^*p < 0.05$)

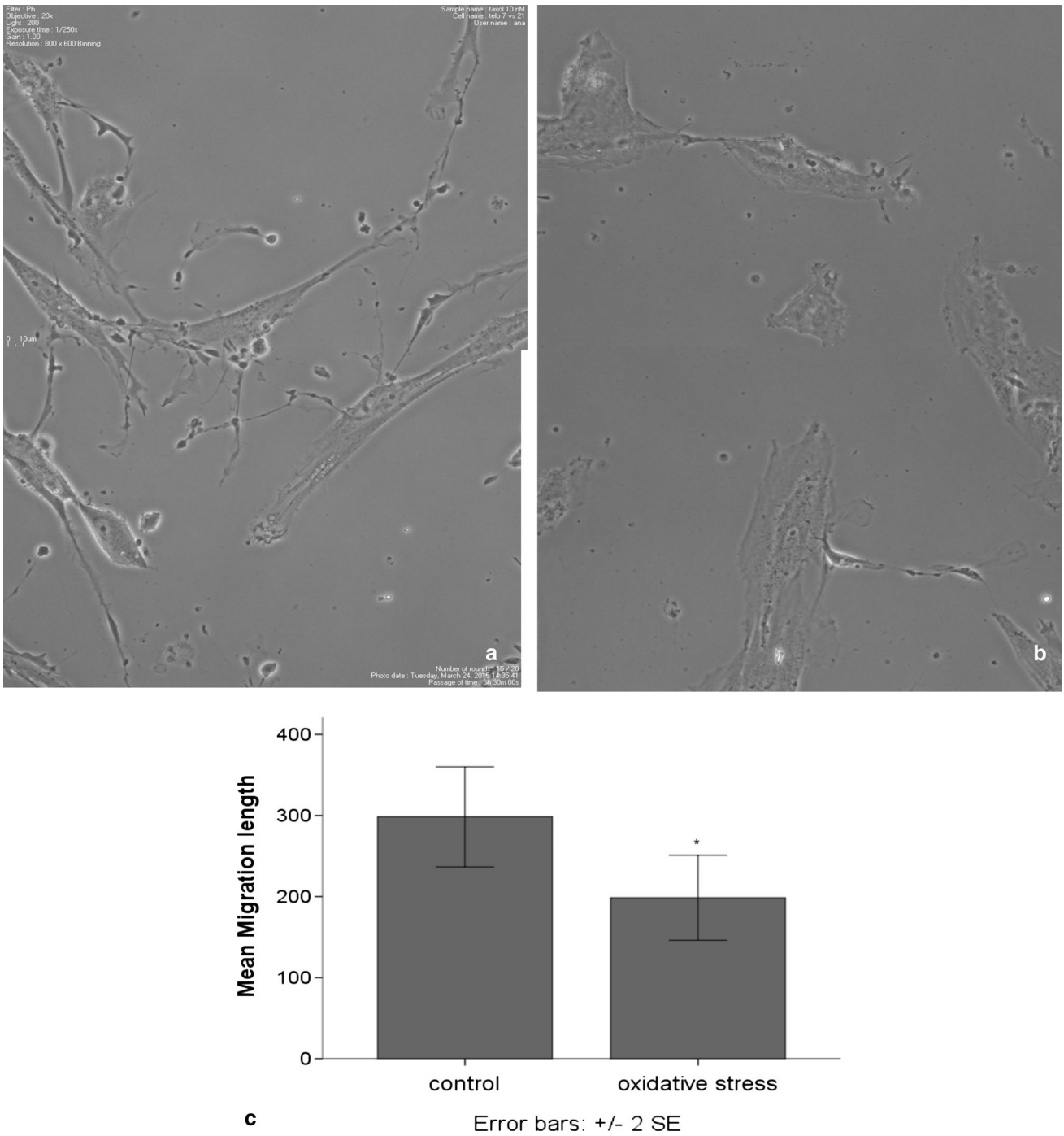

Fig. 3 Impairment of Tps formation under oxidative stress in aged TCs. Aged TCs were recorded for 24 h under standard cell culture conditions versus oxidative stress (200 μM H_2O_2) in a time-lapse cell culture setup. At t_0, TCs exhibited normal morphology, with long, moniliform Tps (**a**), whereas after 13 h of oxidative stress, formation of such prolongations was still severely impaired (**b**). Migration length was decreased under oxidative stress (**c**). Statistical significance was performed using two-tailed, unpaired *t* test (*$p < 0.05$)

conditions as passage 7, in order to analyse the length of Tps and migration pathway.

Oxidative stress produced the same effect on aged cells as it did on young cell culture—impairment of Tps formation, but with a greater impact. Aged TCs failed almost completely to extend new Tps during the first 8–10 h of H_2O_2 exposure (supplementary movie 1) and the effect extended up to 15 h (Fig. 3b). Migration pathway was decreased with approximately 30 % under oxidative stress versus control (c).

Next, we stained cells with a marker for intracellular reactive oxygen species, compatible cu cell culturing, which allowed us to observe in real-time generation of cytoplasmic oxidative stress. Thus, we noticed that aged cells showed increased basal levels of oxidative stress when compared to young cells (Fig. 4).

High doses of *N*-acetyl cysteine preserve cellular morphology to the point of inertness

We were interested to see whether an antioxidant treatment would produce opposite changes of cell morphology and migration. Therefore, after MTS evaluation of cell toxicity, we treated TCs with 200 mM NAc. However, at this dosage, cells were practically "frozen": they did not modify their shape, nor migrated or divided during 24 h of treatment. We repeated the experiment with 100 mM and obtained the same result (supplementary movie 2). Furthermore, cells did not recover motility after we replaced NAc-containing media with normal media. At 10 mM NAc, cells regain motility; but after 30 h, they begun to change shape to an elongated cell body, extending long and stable cell prolongations (Fig. 4b). Migration pathway length was not significantly modified under reducing conditions versus control (Figs. 4c, 5).

Discussion

Our main observations are that oxidative stress hindered Tps formation, while reducing environment promoted the stabilization of cellular processes. We aimed to achieve an experimental setup, simple, reliable, reproducible, starting from previously reported and widely accepted experimental conditions that would allow us to study TCs morphology. We chose external oxidative stress with H_2O_2 for the following reasons: (i) H_2O_2 has been used for quite some time in oxidative stress studies (comprehensively reviewed in [50] and (ii) its effects on apoptosis [51], senescence [52], cell signalling [53–55] and cell cycle progression [42] are thoroughly investigated. The downside of most of earlier studies with H_2O_2 is the end-point type of analysis, sometime extended to several days post-treatment.

Although intensely studied in cell and molecular biology, little is known about oxidative stress effects on cell morphology and even less regarding cell prolongations. Most data are collected from neuronal biology and there are several recent reports to highlight the involvement of oxidative stress in dendrite and axon formation. Gioran et al. showed that mutations of mitochondrial complex I subunits in C. elegans and subsequent impaired oxidative phosphorylation caused an unexpected outgrowth of

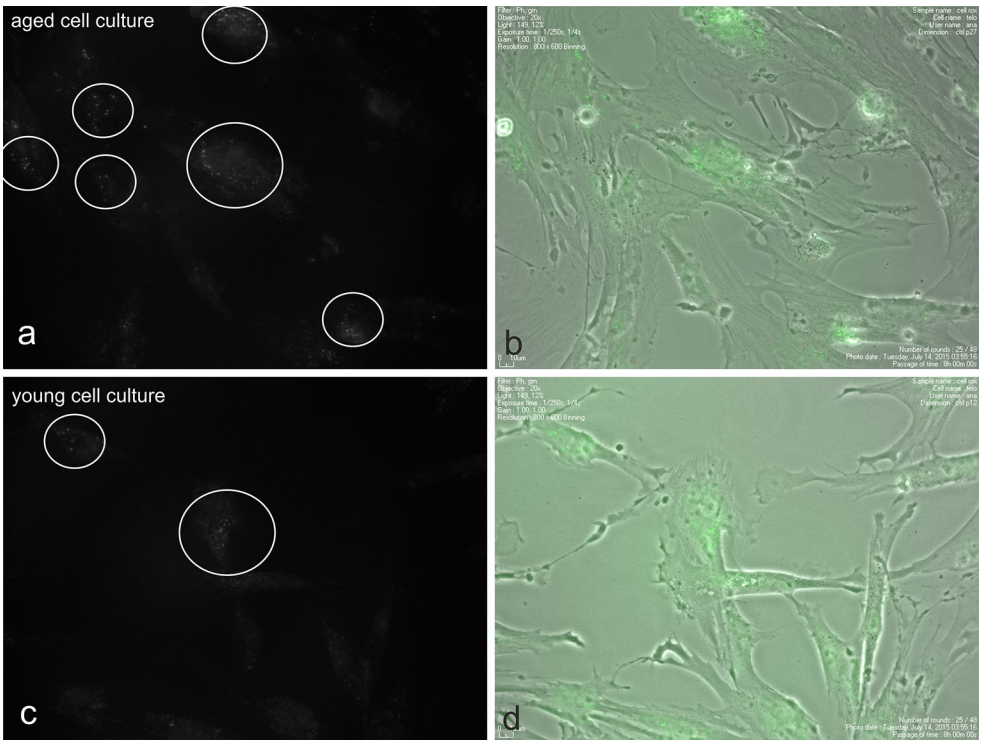

Fig. 4 Cell staining with reactive oxygen species-dependant dye. Both aged cells (*upper panel*) and young cells (*lowe panel*) were incubated with 5 μM CellROX *green* and recorded for 20 h, every 20 min, with both *green* filter (**a**, **c**) and phase contrast. Fluorescent spots (*white circles*) were detected in basal conditions more frequently in aged than young cells. Overlapping of phase-contrast and fluorescent images of aged (**b**) and young (**d**) cell culture

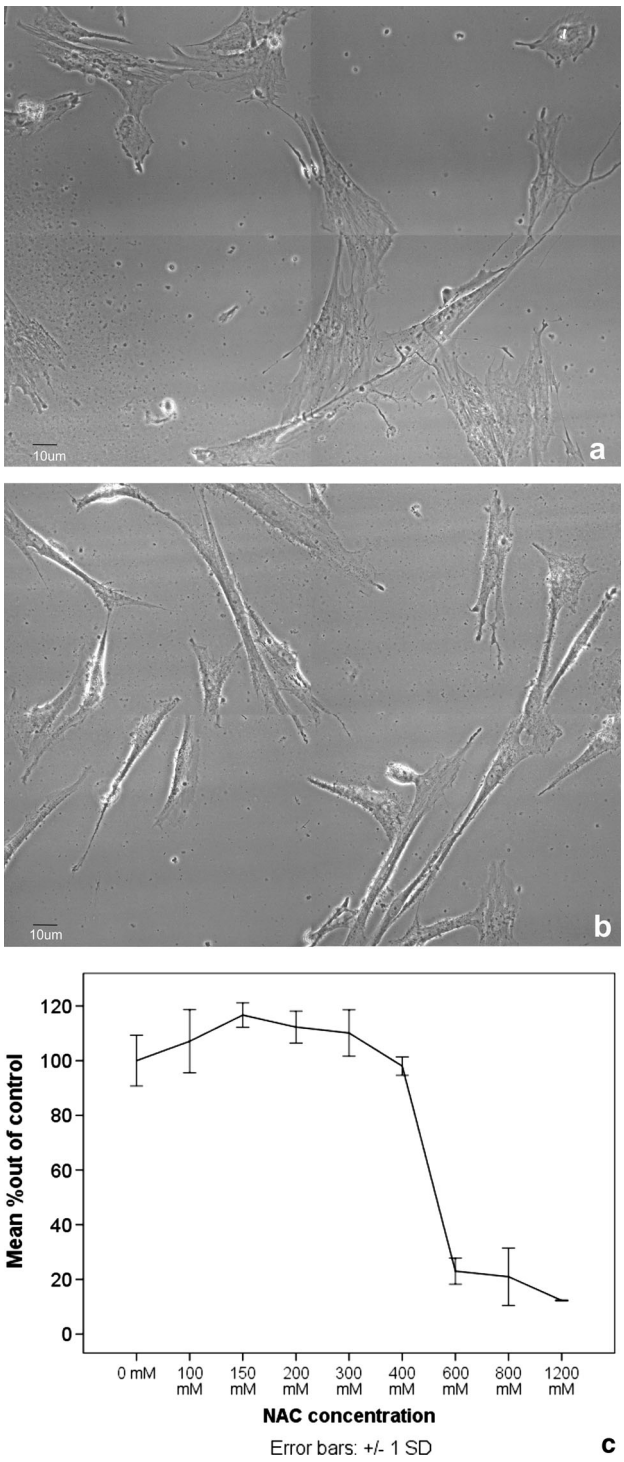

Fig. 5 TCs morphology changes under 10 mM *N*-acetyl cysteine treatment (control (**a**) versus treated cells (**b**)). Visible modifications of cell morphology appear after 30 h and become distinct after 48 h of cell treatment. Under reducing conditions, cell shape becomes more elongated and cell prolongations more stable (**b**). NAc toxicity was assessed by MTS assay. Each point represents the mean ± SD of 3 replicates (**c**)

dendritic arbors and ectopic structures [56]. Zou et al. used a bigenic mouse model, which overexpressed SOD only in Ca/calmodulin-dependent protein kinase-(CaMKII) positive neurons in an otherwise EC-SOD-deficient environment and reported an increased dendritic arborisation [57]. Jackson et al. argued that rabies-infected dorsal root ganglia developed axonal injury via oxidative stress; this mechanism "may explain previous observations of the degeneration of neuronal processes" [58].

We investigated cell behaviour under oxidative stress in a time-lapse videomicroscopy setup which allowed us to observe early modifications. We noticed that a 24-h endpoint study would overlook important cell behaviour and morphology alterations. Also, the time window to observe significant changes differs between young and old cells. Oxidative stress hindered the cells' ability to form Tps. Notably, the cells do not lose the already established cell processes, just the ability to generate new ones. Contrary, when treated with NAC, cells tend to extend their bodies and form long cell processes. The effect is, however, visible after at least 30 h. Pretreatment with NAC for 1–24 h, as reported in most studies [59–61], is insufficient for occurrence of significant cell morphology changes. Importantly, cell migration was impaired in both oxidative and extreme reducing environments. Time-lapse analysis of cell migration could provide more accurate results than scratch-wound assay, regarding cell motility. Scratch-wound assay evaluates the cover-up of a nude surface after a given period of time, disregarding the contribution of cells multiplication to the process. By time-lapse, we analysed only those cells that did not entered mitosis throughout the entire measured time interval. Also, most assays are evaluated for a 24-h period [62, 63], which extends beyond the optimum time for migration assessment under oxidative stress.

Evaluation of cell migration under different redox conditions may have putative important impact on therapeutics. For example, totally opposite pathological processes, such as chronic liver diseases and hepatocellular carcinoma [64], share an oxidative microenvironment. From such a perspective, oxidative balance emerges as a delicate equilibrium to be further tilted towards the desired effect—increased migration for stem cells and precursor cells in degenerative processes or decreased cell migration for tumours. It is possible that oxidative stress could influence the microvesicle release by TCs [65] or their secretome [28].

Conclusions

Our study showed that TCs morphology can be changed by ageing or by modifying the redox balance of the cell culture environment. Oxidative stress impaired the ability of TCs to form Tps and the migration pathway length. Ageing further aggravated this effect. Contrary, a reducing

environment promotes the elongation of the cell body and stabilization of cell processes, via a mechanism to be further determined.

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Compliance with ethical standards

Conflict of interest None to declare.

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