Brain aging and testosterone-induced neuroprotection: studies on cultured sheep cortical neurons

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Abstract

OBJECTIVE: This research reports the expression of topoisomerase βII in fetal sheep neuronal cells. The β isoform of DNA topoisomerase II plays a role in DNA repair process in non proliferating cells as neurons and its expression tends to be downregulated with senescence.

METHODS: Cortical neurons from 60-day-old sheep embryos underwent two protocols: the former based on rising time of culture (10, 20 and 30 days); the latter based on the 72hrs exposure to 3-nitro-L-tyrosine (oxidative/nitrosative stressor) and/or testosterone.

RESULTS: Our results showed an increase in β-galactosidase activity and, in contrast, a reduction in topoisomerase βII expression with time (first protocol). The exposure of sheep primary neurons to 3-nitro-L-tyrosine led to an upregulation of βII topoisomerase expression to be likely seen as a reaction to nitrosative stress. Testosterone addition to 3-nitro-L-tyrosine-exposed cells results in topoisomerase βII decrease possibly due to the neuroprotective properties of testosterone (second protocol). No significant variations in the marker of aging β-galactosidase were observed in the cells exposed to 3-nitro-L-tyrosine and testosterone.

CONCLUSION: The protocol based on time could be of some interest as a model of neuronal senescence in vitro. Topoisomerase βII decrease with aging likely indicates a reduced ability to repair DNA during neuronal senescence.

In contrast, the second protocol may not be seen as a reliable model of aging since 3-nitro-L-tyrosine does not lead to a topoisomerase βII decrease. Testosterone was able to cope with oxidative/nitrosative damage, allowing cells to reduce their needs in DNA repair which in turn leads to a downregulation of topoisomerase IIβ expression.

ORIGINAL ARTICLE

INTRODUCTION

Brain aging indicates the biological, morphological, and functional changes neurons undergo with time. This process can limit their capacity to replicate and culminate in an arrested state in which cells remain viable. During brain aging, increased oxidative stress and accumulation of damaged molecules promote dysfunction of different metabolic and signaling pathways (LeBel & Bondy 1992). During mitochondrial respiration, cells are known to suffer oxidative damage as a result of an unregulated production of reactive oxygen species (ROS) and/or reactive nitrosative species (RNS) (Chance *et al.* 1979; Halliwell 1992; 2001; 2006). ROS and many other DNA-damaging agents can cause cells to enter a state of irreversible cell-cycle arrest, accompanied by characteristic morphological and functional alterations, referred to as senescence (Ben-Porath & Weinberg 2004; Wang *et al.* 2009).

Typically, senescent cells become enlarged and express a pH-dependent β-galactosidase activity (Dimri *et al.* 1995) and the corresponding assay has become one of the most commonly used markers of cell aging. In order to cope with the potentially dangerous ROS and RNS, cells have developed endogenous protective enzyme systems (e.g., catalase, glutathione peroxidase, superoxide dismutase), whose defeat leads to cell dysfunction or death. The central nervous system is particularly exposed to oxidative injury because of the high rate of oxygen consumption, the large amount of easily peroxidizable fatty acids, and the relatively poor concentration of antioxidant defense systems (Andersen 2004; Emerit *et al.* 2004; Sayre *et al.* 2008).

In response to oxidative injuries, the brain is able to produce neuroprotectant steroids such as androgens and estrogens that act paracrinally in many cellular functions. Indeed, the brain of vertebrates can produce steroids (Baulieu 1991) and can convert androgens into estrogens by P450 aromatase (Naftolin *et al.* 1971). Because of its localization in various cellular compartments, aromatase is supposed to lead to the formation of neuroprotective estrogen, which acts not only through the classical receptors but also by direct and rapid effects on neuronal membranes (Roselli 2007).

The aging process is associated with an increase in cellular stress and the induction of stress response pathways (Haigis & Yanker 2010).

DNA topoisomerase II is known to be essential for cell proliferation, since some mitotic events such as condensation and segregation of daughter chromosomes are entirely dependent on its activity (Nitiss 2009). In addition, it is likely involved in other DNA transactions, like DNA replication, transcription, and recombination.

The two isoforms of DNA topoisomerase II, the 170 kD topo IIα and the 180 kD topo IIβ, present in mammals belong to different gene products, having conserved catalytic activities. The α-isoform is present in proliferating cells, while the β-isoform is predominantly present in non-proliferating cells, namely neurons, suggesting its role in non-replicating functions of DNA (Tsutsui *et al.* 2001, Kondapi *et al.* 2004).

The decreasing activity of topo IIβ with aging (Kondapi *et al.* 2004) points out its possible role in DNA repair activity in neurons during aging.

In the present investigation, topo IIβ expression was evaluated in an aging neuronal model consisting in primary fetal sheep neurons undergone three different times of culture. In addition, cells were tested with the oxidant 3-nitro L-tyrosine (3NT) in order to insight into the mechanisms of neuroprotection and anti-aging effects induced by testosterone in cells exposed to stress conditions.

MATERIALS AND METHOD

Sample collection

Twelve 60-day-old fetuses were obtained from sheep slaughtered in local abattoirs when accidentally pregnant. The period of gestation was established by measuring fetal crown-rump length, following the tables reported in the literature (McGeady *et al.* 2006). Fetal brains were collected, the cerebral cortex isolated and fragmented under sterile conditions, and the blocks were suspended in ice-cold cell freezing medium composed of Dulbecco's Modified Eagle's Medium (DMEM) with 1% HEPES (pH 7.4), 10% fetal calf serum, and 10% dimethylsulfoxide (DMSO), following the cryopreservation method reported for bovine neural tissue (Hashimoto *et al.* 2000). Cryotubes were slowly frozen to −80 °C and transferred to liquid nitrogen the following day.

Cell culture

Frozen tissue blocks were rapidly thawed in water bath at 39 °C and minced in smaller fragments under sterile conditions. A papain dissociation kit (Worthington Biochemical, Lakewood, NJ, USA) was used to dissociate cells. Cells were then suspended in basal medium consisting of a 1:1 mixture of DMEM and Ham's F12, without phenol red, supplemented with penicillin (30 mg/l), streptomycin (50 mg/l), sodium bicarbonate (2.4 g/l), insulin (10 μg/ml), transferrin (10 μg/ml), sodium selenite (10^{-8} M) and 10% fetal calf serum. Serum was heat-inactivated dextran-coated charcoal-stripped in order to deprive it of endogenous steroids. Cell suspensions were incubated at 39 °C in a 5% CO_2 humidified atmosphere.

Cells were plated at the rate of 5×10^5 on glass coverslips previously coated with poly-L-lysine (Sigma, St. Louis, MO, USA).

Cell characterization

One monolayer per brain was used to characterize cells by double immunofluorescence with monoclonal anti-class III β-tubulin and polyclonal anti-glial fibrillary

acidic protein (GFAP) antibodies, widely-recognized markers of neuronal and glial cells respectively, diluted 1:200 and then incubated for 1hr at 37 °C with anti-mouse TRITC and anti-rabbit FITC conjugated antibodies diluted 1:100. All antibodies were from Sigma. One additional monolayer per brain was used as negative control by substituting the primary antibody with non-immune mouse and rabbit sera.

Percentages were determined by counting stained and unstained cells under confocal microscope at 40× magnification in 4 random fields. A minimum of 200 cells were counted per field.

Aging and neuroprotection protocols

Monolayers underwent two different protocols:

- cells were cultured for 10, 20 or 30 days with the medium changed every 3 days.

This protocol was aimed at analyzing time-dependent changes

- cells were treated 3 days after seeding in according to the following scheme:
  1. basal medium (control cells).
  2. 72 hr-exposure to 360 μM 3NT
  3. 72 hr-exposure to 360 μM 3NT and 50 nM testosterone
  4. 72 hr-exposure to 50 nM testosterone.

This protocol was based on the use of 3NT, an oxidative stress-inducing agent, and testosterone.

Afterwards, coverslips were fixed in 4% paraformaldehyde at room temperature for 30 min. and stored at +4 °C.

Topoisomerase II β immunoreactivity

Cells underwent double immunofluorescence procedure according to the same protocol indicated in the *Cell characterization* paragraph using as primary antibodies polyclonal anti-topo IIβ (Santa Cruz Biotech., Dallas, TX, USA), diluted 1:100, and monoclonal anti-class III β-tubulin. As secondary antibodies, anti-rabbit FITC and anti-mouse TRITC antibodies, diluted 1:100, were used. Cells were viewed under the confocal microscope Leica TCS SP5. Optical density was performed by means of Image J 1.42q software. All data were expressed as means ±SD and tested by ANOVA. A probability of 0.05 or less was considered as being significant.

β-galactosidase assay

Cultured cells grown on glass coverslips were fixed with 2% formaldehyde 0.2% glutharaldehyde at room temperature for 15 min, washed three times and then treated with *Senescence β-galactosidase staining kit* pH 6 (Cell Signaling Technol., Danvers, MA, USA), for 10 hr at 37 °C in the dark. After rinsing, cells were viewed under a bright field microscope.

RESULTS

First protocol (10, 20, 30 days of culture)

The methodology used to obtain neuronal primary cultures was the same as that described previously (Lepore *et al.* 2009). Preliminary tests performed at our laboratories have ascertained that the most suitable period to grow viable neurons is around day 60 of sheep gestation. Cells from 60-day embryos grew quickly giving 70%-confluent monolayers within 10 days. In baseline conditions, cells were numerous and viable. Their cell bodies were spindle-like, oval- or triangular-shaped with long processes that tended to have contacts with those of contiguous cells. Immunofluorescent method revealed that more than 90% of cultured cells could be identified as neurons since they were class III β-tubulin-positive, whereas the remaining cells were GFAP-immunopositive astrocytes (Figure 1). Because of the marked discrepancy in the percentage of the two cell types in favor of neurons, a diagram has been believed unnecessary.

When cells were observed in the living state with phase contrast optics after 10 days *in vitro*, neither were detectable appreciable signs of suffering nor any morphological changes. After 20 and 30 days of culture, cells appeared enlarged and flattened with reduced cytoplasmic processes, as can be seen by means of immunofluorescence (Figure 2B, C). Topo IIβ expression decreased with time. Indeed, its immunoreactivity was to the

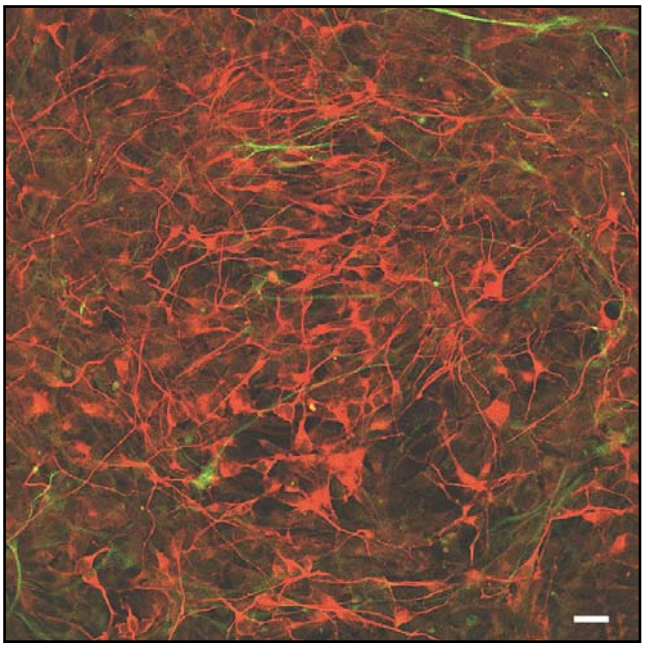

Fig. 1. Characterization of cultured cells. The double immunofluorescence staining revealed that most cells were III β-tubulin-positive neurons (red) whereas only a tiny minority were GFAP-positive astrocytes (green). Note that the cell bodies of neurons were spindle-like, oval- or triangular-shaped with long processes that tended to have contacts with those of contiguous cells. Scale bar: 30 μm

highest degree after 10 days of culture, whereas it was very low after 20 days until it almost disappeared after 30 days (Figure 2).

β-galactosidase activity increased with time. It was indeed weak after 10 days of culture to become very marked after 20 days remaining high up to 30 days, when almost all cells were strongly positive (Figure 3).

Second protocol (nitrosative stress and neuroprotection)

As to phase contrast observations, no morphological changes could be detected after 72-hr exposure to 3NT, 3NT plus testosterone, and testosterone.

Topo IIβ expression increased of about 50% after 3NT exposure when compared to untreated cells. In addition, the exposure to both 3NT and testosterone resulted in an up-regulation of topo IIβ expression of about 35%. In contrast, the exposure to testosterone only did not induce any significant changes (Figure 4).

β-galactosidase activity was not clearly apparent after the different treatments above described.

DISCUSSION

The present work based on sheep neuronal cells *in vitro* may provide a useful tool in the studies on aging processes. The sheep was chosen as an experimental model on account of two considerations. The former is that sheep fetuses allow considerable amounts of brain tissue to be rapidly collected (Lepore *et al.* 2009). The latter is the interest elicited by sheep in the investigations of neurodegenerative processes as that species is susceptible of Scrapie and Maedi-Visna diseases. In neurodegenerative disorders an outstanding role is played by oxidative stress, which also deals with the mechanisms underlying brain senescence processes (Salminem *et al.* 2011; Sycora *et al.* 2013).

The fetal age of 60 days allowed to obtain primary cultures of around 90% viable neurons, in agreement with Kay *et al.* (2006) who set up cortical and cerebellar neurons from 9 to 15-week old fetal sheep brains. According to those Authors, nearly all the cells cultured from the youngest fetuses (9 weeks-old) adhered as neurons, whereas the proportion of glial cells tended to increase with age.

First protocol (10, 20, 30 days of culture)

The cell shape detected after 20 and 30 days of culture, i.e. flat morphology, is a feature found in other *in vitro* aging models, like astrocytes and cerebellar granule neurons (Bhanu *et al.* 2010; Salminem *et al.*

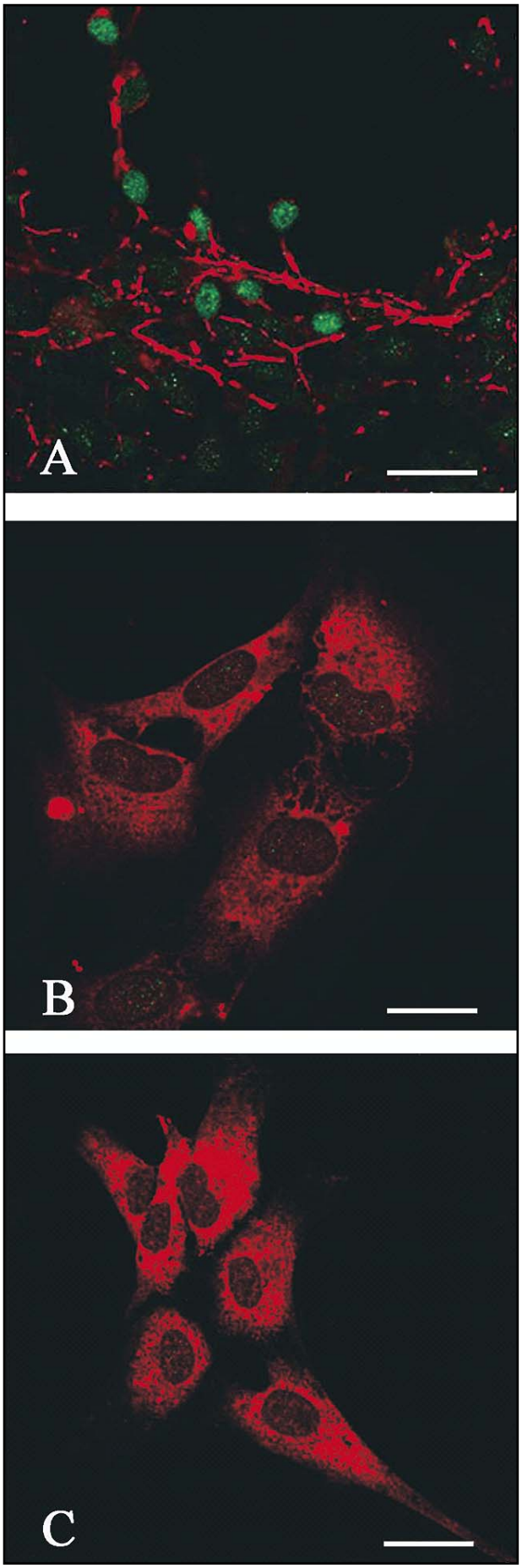

Fig. 2. Topo IIβ expression decreases with time. ts immunoreactivity was to the highest degree after 10 days of culture (A), whereas it was very low after 20 days (B) until it almost disappeared after 30 days (C). Note that after 20 and 30 days of culture, cells appeared enlarged and flattened with reduced cytoplasmic processes (B, C). Scale bar: 60μm (A); 20μm (B, C).

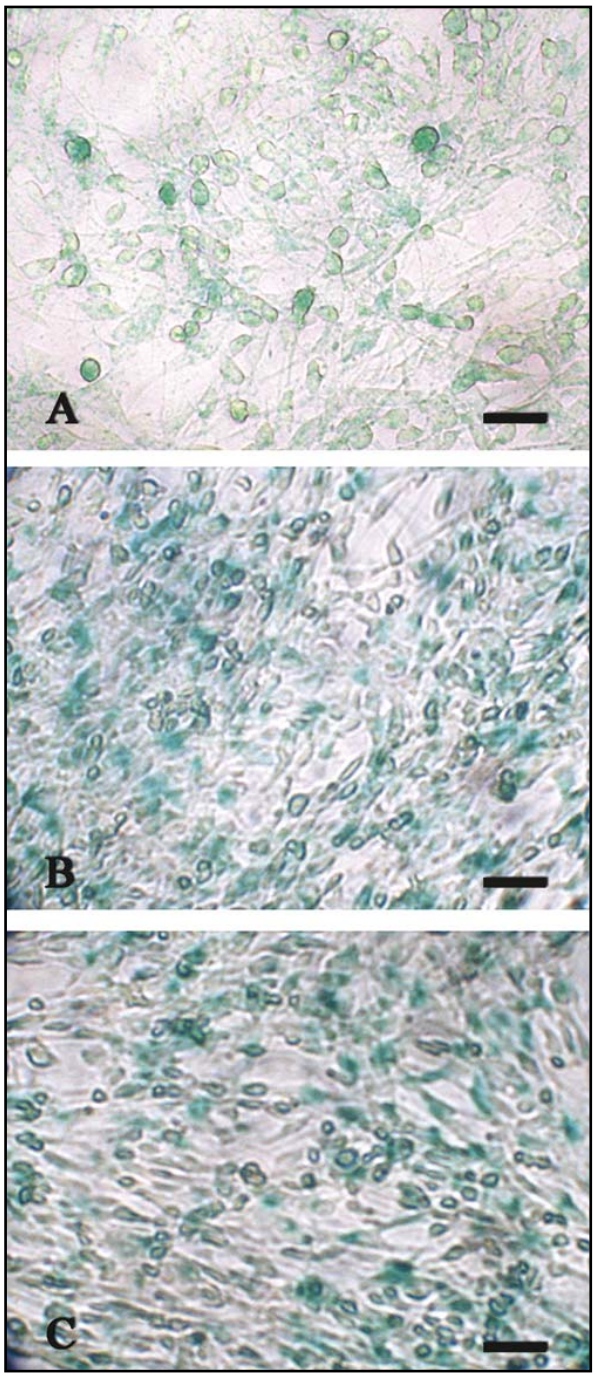

Fig. 3. β-galactosidase activity increases with time. It is indeed weak after 10 days of culture (A) to become very marked after 20 days (B) remaining high up to 30 days (C). Scale bar: 60 µm.

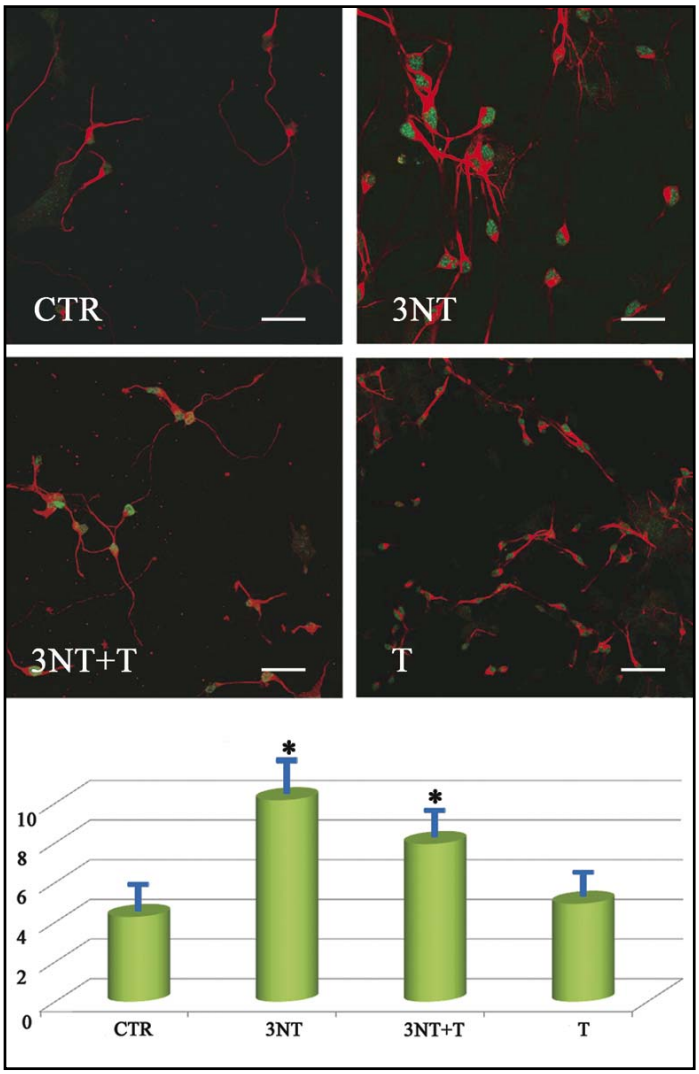

Fig. 4. Nitrosative stress and neuroprotection. Immunofluorescence staining shows that topo IIβ expression, after 3NT exposure, is higher than control cells (CTR). Cells treated with 3NT and testosterone (3NT+T) show a lesser rate of topo IIβ expression in comparison with 3NT exposed-cells.
Cells treated with testosterone only (T) do not show any qualitative variation in topo IIβ expression when compared to control cells.
In details, the graph of the optical density shows an increase in topo IIβ expression of about 50% after 3NT exposure in comparison with control cells. The addition of 3NT and testosterone results in an up-regulation of topo IIβ expression of about 35%. The exposure to testosterone does not induce any significant changes. Asterisks indicate significant difference in comparison with all data ($p < 0.05$). CTR = control cells; 3NT = cells exposed to 3NT; 3NT+T = cells exposed to 3NT and testosterone; T= cells exposed to testosterone. Scale bar: 60µm (CTR, 3NT, 3NT+T); 80µm (T).

2011). Similar morphological patterns have also been described in *in vivo* models, where synapse loss, significant reduction of dendritic and axonal arborization, decrease in post-synaptic densities and dendritic spines were noted in the aged human brain (Raz & Rodrigue 2006; Esiri 2007; Yanker *et al.* 2008). Therefore, on account of cell morphological features, our protocol likely represents a reliable model of aging. This is also confirmed by the increase in the activity of β-galactosidase. Despite the fact that the role played by β-galactosidase in replicative senescence has been well investigated, studies on postmitotic cells like neurons are very few. In a recent paper (Geng *et al.* 2010), elevated activity of β-galactosidase by dependence on age has been reported in rat hippocampal neurons. As to *in vitro* experiments, our data on sheep primary cortical

neurons agrees with the previous finding of increased β-galactosidase activity in rat cultured cerebellar granule cells (Bhanu *et al.* 2010). Though the exact mechanism of elevated β-galactosidase activity in senescent cell is still unclear (Geng *et al.* 2010), our data showing a marked increase from 20 days of culture onwards likely support the idea that a prolonged culture period may result in stress accumulation leading to senescence. Our results show that topo IIβ expression decreases in a time-dependent way. Indeed, its immunoreactivity was to the highest degree after 10 days of culture, whereas it was very low after 20 days until it almost disappeared after 30 days. The two isoforms α and β of the nuclear enzyme DNA topoisomerase II play a key role in DNA replication, transcription, chromosome condensation, genetic recombination and repair (Wang 1996). The predominant expression of the isoform topo IIβ in differentiating neurons suggests its role in non-replicating functions of DNA (Tsutsui *et al.* 2001). Its decrease with aging is confirmed in our investigation and likely indicates a reduced ability to repair DNA during neuronal senescence.

Second protocol (nitrosative stress and neuroprotection)

The observed increase in topo IIβ expression consequent upon the exposure to 360μM 3NT may be seen as an attempt to cope with oxidative/nitrosative stress through enhanced DNA restoring processes by neurons. Such reactive response to stressors agrees with what reported in the literature. Indeed, Authors demonstrated that, in myotubes maintained in culture, elevated exposure to ROS results in an activation of mitochondrial topoisomerase (Medikayala *et al.* 2011). The present study also shows that the addition of testosterone slows down topo IIβ expression in stressed neurons consistently with the well-known neuroprotective properties of that neurosteroid (Hammond *et al.* 2001; Chisu *et al.* 2006). The rate of topo IIβ expression detected when testosterone is added to 3NT-stressed cells is not, however, as low as that observed when testosterone is added to neurons cultured under baseline conditions. Indeed, topo IIβ expression does not change significantly in this last case if compared to that found in control cells.

β galactosidase activity, a widely recognized marker of senescence (Bhanu *et al.* 2010), does not show any increase after 3NT exposure, so, such oxidative/nitrosative stress in our cells, cannot be seen as an experimental model of senescence. This finding does not seem to agree fully with what reported in the literature. Indeed, it is referred that ROS and RNS, among which 3NT may be included, can cause morphological and functional alterations, referred to as senescence (Ben-Porath & Weinberg 2004; Wang *et al.* 2009). On the other hand, we detected an increase in topo IIβ expression in 3NT-exposed neurons confirming that our second protocol does not lead to a reliable model of aging. Testosterone revealed its ability to counteract oxidative stress damage, allowing cells to reduce their needs in DNA repair which in turn leads to a downregulation of topo IIβ expression.

Therefore, it can be concluded that the protocol based on time is the sole in our investigation to represent a valid model of neuronal senescence *in vitro*.

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