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TESTOSTERONE INDUCES NEUROPROTECTION FROM OXIDATIVE STRESS. EFFECTS ON CATALASE ACTIVITY AND 3-NITRO-L-TYROSINE INCORPORATION INTO α-TUBULIN IN A MOUSE NEUROBLASTOMA CELL LINE

V. CHISU, P. MANCA, G. LEPORE, S. GADAU, M. ZEDDA AND V. FARINA

Department of Animal Biology, University of Sassari, Via Vienna 2, 07100 Sassari

INTRODUCTION

Neurons are highly polarized cells where microtubules are required for the formation and stability of axons and dendrites (4). Cyclic tyrosination/detyrosination of the C-terminus in α-tubulin subunit is one of the most studied posttranslational modifications of tubulin in dynamic microtubules (5). As is well known, tyrosinated α-tubulin is mainly expressed during neuronal plasticity events (12, 13). Tyrosine analogues can be incorporated into the C-terminus of α-tubulin with consequent possible alteration of the biochemical properties of tubulin, and formation of anomalous microtubules that affect normal cell functions (10). This is the case of 3-nitro-L-tyrosine (3NT), formed by nitration of tyrosine by nitric oxide-derived species following activation of glutamate NMDA receptors. Neural cells show a different sensitivity to nitrosative/oxidative stress agents with subsequent modification in many cellular compartments such as cytoplasmic membrane, nucleus and microtubular network. 3NT increases in many human diseases (7) and both nitrosative and oxidative insults may contribute to neuronal degeneration in various disorders (16). The severe effects of free radicals are kept under check by a delicate balance between the rate of their production and elimination by different antioxidant systems. Any shift in this critical balance could result in an increase in the peroxidative stress leading to cellular damage. Generation of several reactive oxygen species in neurons by glutamate action seems to play a relevant role in cell damage and death. In neurons, the deleterious effects of oxidant species can be controlled and prevented by enzymatic and non-enzymatic antioxidant defence systems. These include enzymes like catalase (2), which breaks down hydrogen peroxide, an oxidizing agent that gives rise to hydroxyl radicals, the most reactive oxygen intermediates.

Gonadal steroids are known to be of primary importance in the normal maintenance of brain functions. The brain seems to be an important target of sexual hormones, since these are able to cross the blood-brain barrier due to their high lipid solubility (25). There is a substantial evidence indicating that sexual hormones can modify cell proliferation, neuroplasticity and vulnerability to neural insults including oxidative stress (20). Sexual hormones exhibit neuroprotective and neurotherapeutic effects on many different populations of neurons in the peripheral and central nervous systems (11). Estrogens and their derivatives have shown

Corresponding Author: prof. Marco Zedda, Department of Animal Biology, University of Sassari, via Vienna 2, 07100 Sassari, Italy - Tel. + 39 079 229583 - Fax + 39 079 229432 - E-mail: mzedda@uniss.it

to protect neurons from oxidative stress-induced death. Indeed, *in vitro* studies have indicated that 17β-estradiol reduces neural damage caused by serum deprivation, β-amyloid treatment, and exposure to glutamate (15). Similarly, testosterone (T), like other androgens, may exert biological effects on neural cells, leading to changes in cell shape, survival and axonal regeneration of motor neurons *in vivo* (17). It is well-known that T mediates its cellular effects through both androgen and estrogen receptors, following aromatisation to estradiol, with multiple possible mechanisms of action (24). In particular, a role of T is known to provide a neuroprotective effect on motoneuron injury via an androgen receptor mechanism (25). Although the action of T *in vitro* is not well understood, data from cell culture studies have shown that T can exert neuroprotective effects against specific oxidative insults (3, 14).

In order to gain further insight into the extent and mechanism of neuroprotection induced by T, an *in vitro* model is here proposed in which 3NT-exposed undifferentiated mouse neuroblastoma cells were supplemented with T. Thus, the present study is directed to evaluate the possibility of hormone protection in this cell line from oxidative/nitrosative stress-induced damage.

METHODS

Cell culture and treatments

C1300 cells (*American Type Culture Collection*, Rockville, MD, USA), an undifferentiated mouse neuroblastoma cell line at passage 60, were seeded at the concentration of $5.6X10^5$ per ml and grown in phenol-red-free medium supplemented with 10% heat inactivated dextran-coated charcoal-stripped newborn calf serum RPMI-1640 medium, 2 μM L-glutamine, 100 units/ml penicillin G and 100 μg/ml streptomycin sulphate (basal medium). Cells at 37 °C in a 5% CO_2 humidified atmosphere were cultured according to the following protocols:

i) basal medium for 96 h (ctrl cells);
ii) 280 μM 3NT for 96 h (3NT cells);
iii) 50 μM T and 280 μM 3NT for 96 h (3NT + T cells);
iv) 280 μM 3NT for 48 h and 50 nM T for the following 48 h (3NT/T cells);
v) 280 μM 3NT for 48 h and basal medium for the following 48 h (3NT/bm cells);
vi) 100 μM flutamide (F), a well known T-antagonist via androgen receptor, 50 μM T and 280 μM 3NT for 96 h (3NT + T + F cells);
vii) 280 μM 3NT for 48 h and 50 μM T plus 100 nM F for the following 48 h (3NT/T + F cells);
viii) 50 μM T for 96 h (T cells).

The concentration of 280 μM 3NT was chosen after establishing LD50 after 48 h exposure which was 180 μM. As to T concentration, a preliminary study carried out in our labs revealed that 30 μM after 48 h exposure did not significantly alter neuroblastoma cell proliferation rate. Moreover, T concentration of 100 and 250 μM (48 h exposure) led to a progressive decrease in the proliferation rate and cell viability and IC50 value was estimated at a concentration of 500 μM (48 h exposure). Hence, in the present investigation 50 μM T was used in agreement with a previous study (9). IC50 value after 48 h exposure for F was estimated at 200 μM and a concentration of 100 μM was used. T and F were dissolved in absolute ethanol and then diluted in basal medium to the final concentrations. To check possible ethanol toxic effects, a further control was performed by incubating cells for 96 h in basal medium containing absolute ethanol at the same 0.0001% v/v concentration used to dissolve T and F (etOH ctrl cells).

Microscopic examination and cell proliferation assay

In order to evaluate morphological features, monolayers were observed under phase contrast optics at the living state. Cell proliferation assay was performed in 96-well microtiter plates con-

taining $5x10^3$ cells in 200 µl medium per well. Cells undergone all protocols above indicated and after 96 h were incubated in 3-(4,5-dimethilthiazolyl-2)-2,5-diphenyltetrazolium bromide assay (MTT, Cell Titer® 96, *Promega*, Madison, WI, USA) for 1 h. MTT was bioreduced by living cells into a colored formazan product that is soluble in tissue culture medium. Absorption values were read setting the automatic microtiter reader at 492 µm (Uniskan II, *Labsystem*, Helsinki, Finland).

Detection of apoptotic cells and viability test

Cells were stained with the fluorescent DNA-binding dye 4'-6-diamidino-2-phenylindole (DAPI, *Sigma*, Saint Louis, MO, USA) for nuclear visualization. DAPI is known to form complexes with natural double-stranded DNA, showing a fluorescence specific to AT.

Bis-benzimide (Hoechst 33342, *Sigma*, Saint Louis, MO, USA) and propidium iodide (PI, *Sigma*) were used for cell viability estimation. The cell death rate was calculated as dead cells *vs.* total cell number X 100 and intra-observer and inter-observer variability were each < 2%.

Western blot

Cells were suspended in stabilizing solution (5 µM TRIS HCl, 2 µM EGTA, 0.1 µM phenylmethyl-sulfonyl fluoride, pH 8.0), supplemented with protease inhibitors (Complete-mini, *Roche*, Basel, Switzerland). Cells were homogenized mechanically and then sonicated for 1 h. Protein content was determined (DC Protein Assay, *BioRad*, CA, USA) and aliquots of 15 µg proteins each were fractionated in SDS-PAGE 12% gel and then transferred onto 0.45 mm nitrocellulose membranes. Non-specific protein binding was blocked incubating membranes with 0.1% Tween 20-containing PBS and 5% skim milk. Nitrocellulose membranes were incubated with primary antibodies against tyrosinated α-tubulin (monoclonal TUB-1A2, *Sigma*), 3NT (monoclonal 1A6, *Upstate*, Lake Placid, NY, USA) and androgen receptor (polyclonal, *Chemicon*, Temecula, CA, USA), overnight at 4 °C at 1:1,000, 1:250 and 1:1,000 dilution respectively. As secondary antibodies, an anti-mouse or anti-rabbit IgG-alkaline phosphatase-conjugated (*Sigma*) were used at 1:1,000 dilution (1:30,000 for anti-rabbit antibody) for 2 h at room temperature. The immunoreactive bands were visualized by incubating the membrane with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) and heat fixed.

Catalase test

Ice-cold cells were collected and sonicated to rupture cell membranes. Catalase activity was measured as the rate of disappearance of hydrogen peroxide monitoring absorbance setting the spectrophotometer at 240 µm (1).

Statistical analysis

All data are expressed as mean ± SD. Cell proliferation and catalase activity were analyzed by one-way ANOVA or Student *t* test. A probability of 0.05 or less was considered significant.

RESULTS

The morphological features in the living state of the cells undergone the different procedures are illustrated in Figure 1. Prior to confluence, ctrl cells appeared irregularly shaped and displayed 4-5 cytoplasmic processes up to 50 µm long, which gave rise to a number of short branches. Cells exposed to 3NT were reduced in number and showed morphological changes in comparison with controls, such as globular shape, and reduction or absence of cell processes. In addition, vacuoles could be frequently observed in the cytoplasm. In 3NT + T and, to a greater extent, in 3NT/T cells, these changes were less evident. In 3NT/bm cells morphological pictures overlapped with those detected in 3NT cells. T alone induced elongation of cytoplasmic processes raising from irregularly-shaped cells.

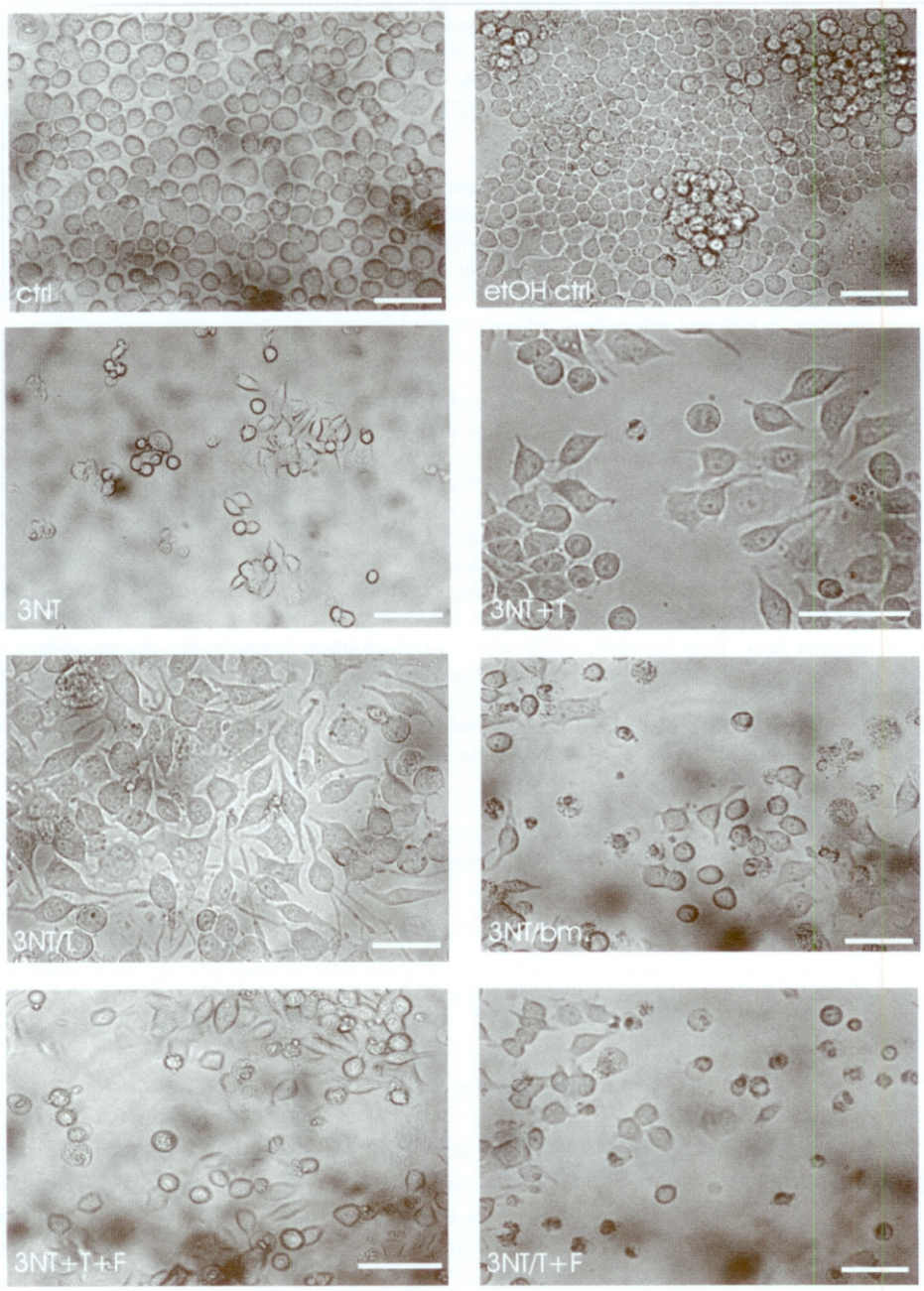

Fig. 1. - *T treatment protects neuroblastoma cells against 3NT toxicity.*

3NT, 3NT/bm, 3NT + T + F and 3NT/T + F cells are reduced in number and show globular shape and shorter cytoplasmic processes in comparison with ctrl cells. In contrast, 3NT + T and 3NT/T cells are relatively undamaged. Cells exposed to etOH (etOH ctrl) showed the same morphological characteristics of ctrl cells. Phase contrast. Bar = 100 μm.

Briefly, the morphological pictures were similar to those found in ctrl cells prior to confluence. Biological effects of F were investigated in 3NT + T + F and 3NT/T + F cells and morphological changes were very similar to those detected in 3NT cells.

All the results from ctrl and etOH ctrl cells are fully corresponding. Data concerning the latter are therefore omitted onward.

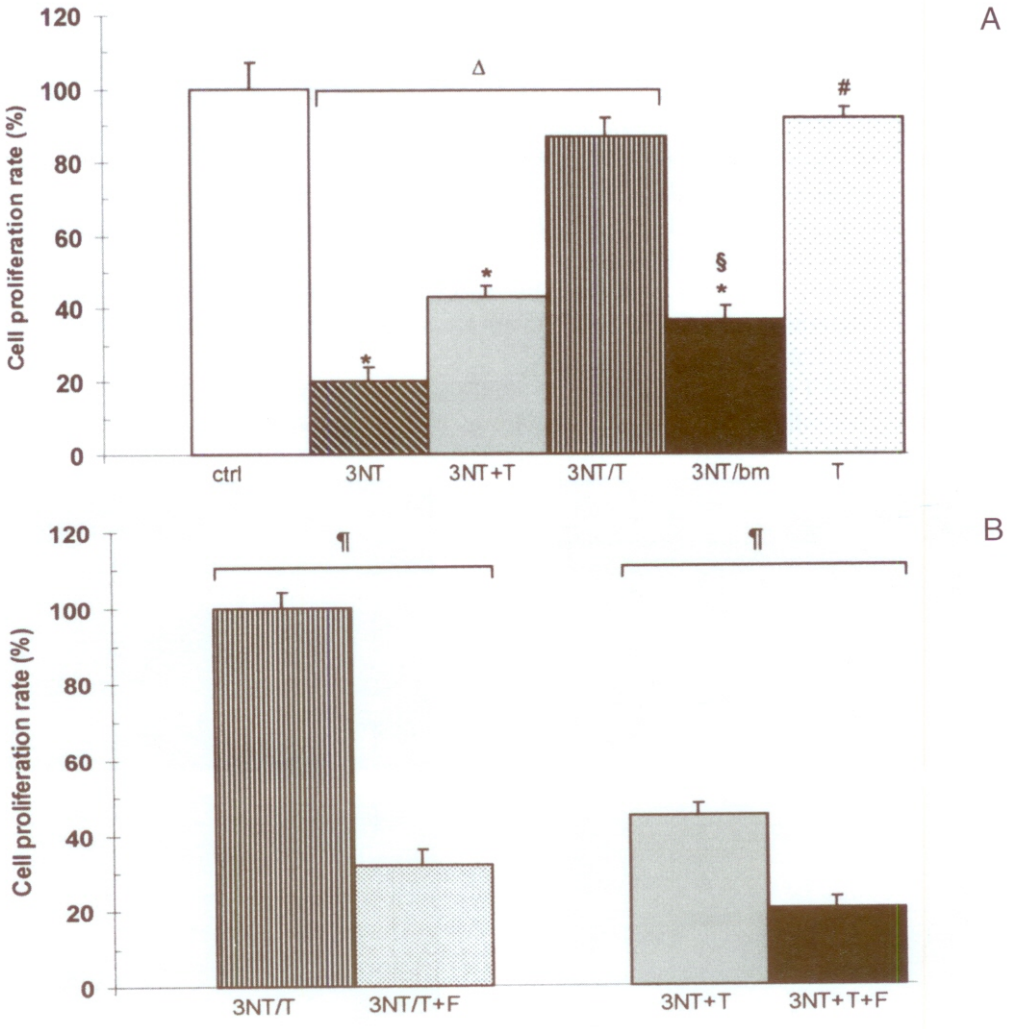

Fig. 2. - *Diagram A shows cell proliferation rate which declines in cells exposed to 3NT.*

Such decrease is not detectable in T, and 3NT/T cells. In 3NT + T proliferation rate is about 40%. In diagram B, antagonistic effects of F on T-induced proliferation are evident in 3NT + T + F and 3NT/T + F cells. Data are shown as percentage of maximum values and expressed as mean ± SD. Asterisk indicates significant difference from ctrl cells (Student t test); Δ significant differences among 3NT, 3NT + T and 3NT/T cells (ANOVA); # significant difference from 3NT, 3NT + T and 3NT/bm cells, Student t test; § significant difference from 3NT and 3NT/T cells (Student t test); ¶ significant difference, Student t test (B). Each column represents the average of seven single experiments, $p < 0.05$.

The same experimental conditions were used to test the effect of 50 μM T on cellular proliferation. When compared to ctrl cells, 3NT cells inhibited significantly cell proliferation. In contrast, T increased cell growth rate which was approx. 40% in 3NT + T cells and 90% in 3NT/T cells, when growth rate was fixed at 100% in ctrl cells. T cells displayed a growth rate approximately similar to that found in ctrl cells (Fig. 2A). Moreover, in 3NT/T + F and 3NT + T + F cells the growth rate was lower when compared to that detected in 3NT/T and 3NT + T cells respectively (Fig. 2B).

About 80% of 3NT cells exhibited characteristic apoptotic pictures, i.e. condensed and hyperchromatic DAPI-stained nuclei, with chromatin gathered at the periphery of the nuclei. These features were not evident in 3NT + T and 3NT/T cells, where nuclei showed the normal phenotype and were pale and homogenous. Indeed, the percentage of apoptotic nuclei in 3NT/T cells was about 10% in average. A parallel trend was displayed by the cell death rate after Hoechst 33342/PI staining. About 80% of 3NT cells showed the red PI staining, whereas in 3NT + T and 3NT/T cells almost 95% nuclei were stained in blue by the Hoechst dye, i.e. were viable (Fig. 3).

By Western blot analysis a band was observed with anti-androgen receptor antibody in C1300 cells undergone each treatment (data not shown). The band was approx. 110kDa and could be referred to the androgen receptor.

The effects of T on the expression of α-tubulin are shown in Figure 4. The anti-α-tubulin antibody visualized the α-tubulin subunit as a single band located at approximately

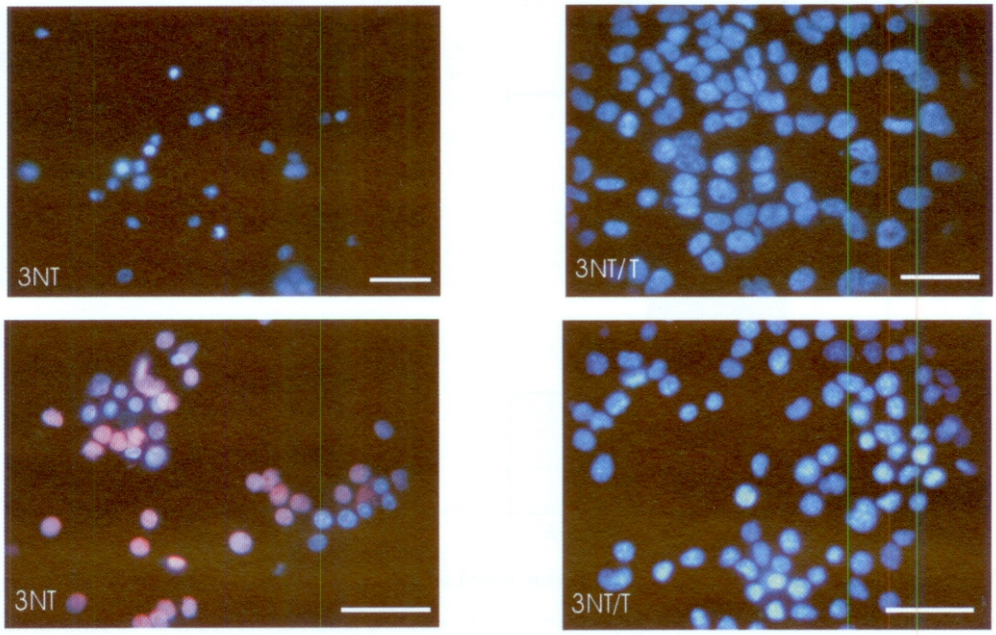

Fig. 3. - *DAPI staining shows shrunk and hyperchromatic nuclei in 3NT cells.*

These pictures are not evident in 3NT/T cells (upper). Hoechst 33342/PI staining displays a high number of dead cells (red nuclei) in 3NT cells. By contrast, a great deal of living cells (blue nuclei) are present in 3NT/T cells (lower). Bar = 100 μm.

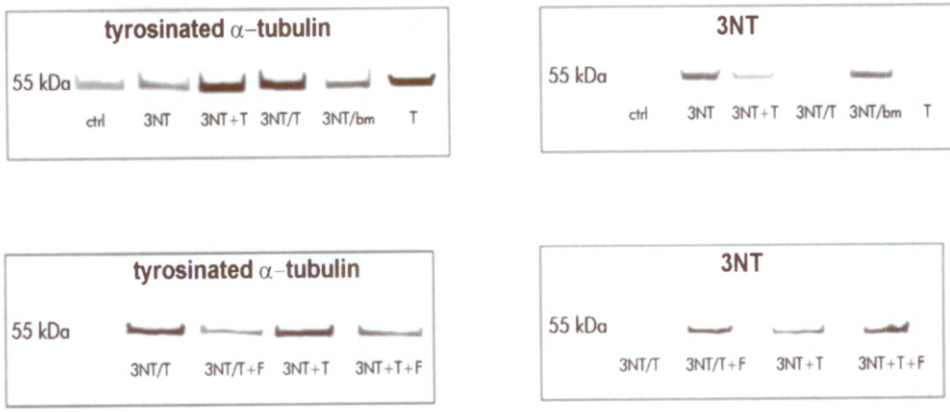

Fig. 4. - *T treatment induces upregulation of tyrosinated α-tubulin, whereas it significantly decreases after F exposure.*

A 3NT-incorporating protein which co-migrates with tubulin is poorly detectable in 3NT + T cells. F increases the amount of 3NT in 3NT/T + F and 3NT + T + cells.

55kDa. In T, 3NT + T and 3NT/T cells, the intensity of the band was more marked than in ctrl, 3NT and 3NT/bm cells. The presence of F attenuated tyrosinated α-tubulin expression as was evident in 3NT + T + F and 3NT/T + F cells. The antibody against 3NT revealed a single nitrated protein co-migrating with α-tubulin in 3NT and 3NT/bm cells, whereas that band was poorly detectable in 3NT + T cells and entirely absent in T cells. Finally, 3NT immunoreactivity was increased in 3NT/T + F and 3NT + T + F in comparison to 3NT/T and 3NT + T respectively.

With the aim at evaluating one of the most widespread antioxidant defence mechanisms, catalase activity was tested in all the experimental conditions. In 3NT cells, catalase activity strongly decreased as far as about 20%. Values tended towards those obtained from ctrl cells in 3NT + T, 3NT/T and T (Fig. 5A). This datum was not detected in the presence of F (3NT + T + F and 3NT/T + F cells), as shown in Figure 5B.

DISCUSSION

Our morphological observations show that T exposure makes C1300 cells less vulnerable to 280 μM 3NT-induced stress in comparison with control cells. A previous work carried out in our laboratories (28) has reported that these cells showed severe alterations at lower 3NT concentrations. Indeed, both morphological and functional changes were significant from 90 μM 3NT. Our current findings provide novel evidence that T has remarkable inhibitory effects on 3NT injuries. Both morphological pictures and growth rate support the hypothesis that T can attenuate the toxic effects of 3NT on neuroblastoma cells. This neuroprotective action was mainly effective when 3NT exposure did not exceed 48 h. In 3NT/T cells T biological effects appeared indeed stronger than in 3NT + T cells.

Western blot likely indicates that T increases the expression of tyrosinated α-tubulin and modulates microtubular functions, suggesting a direct interaction of the hormone with

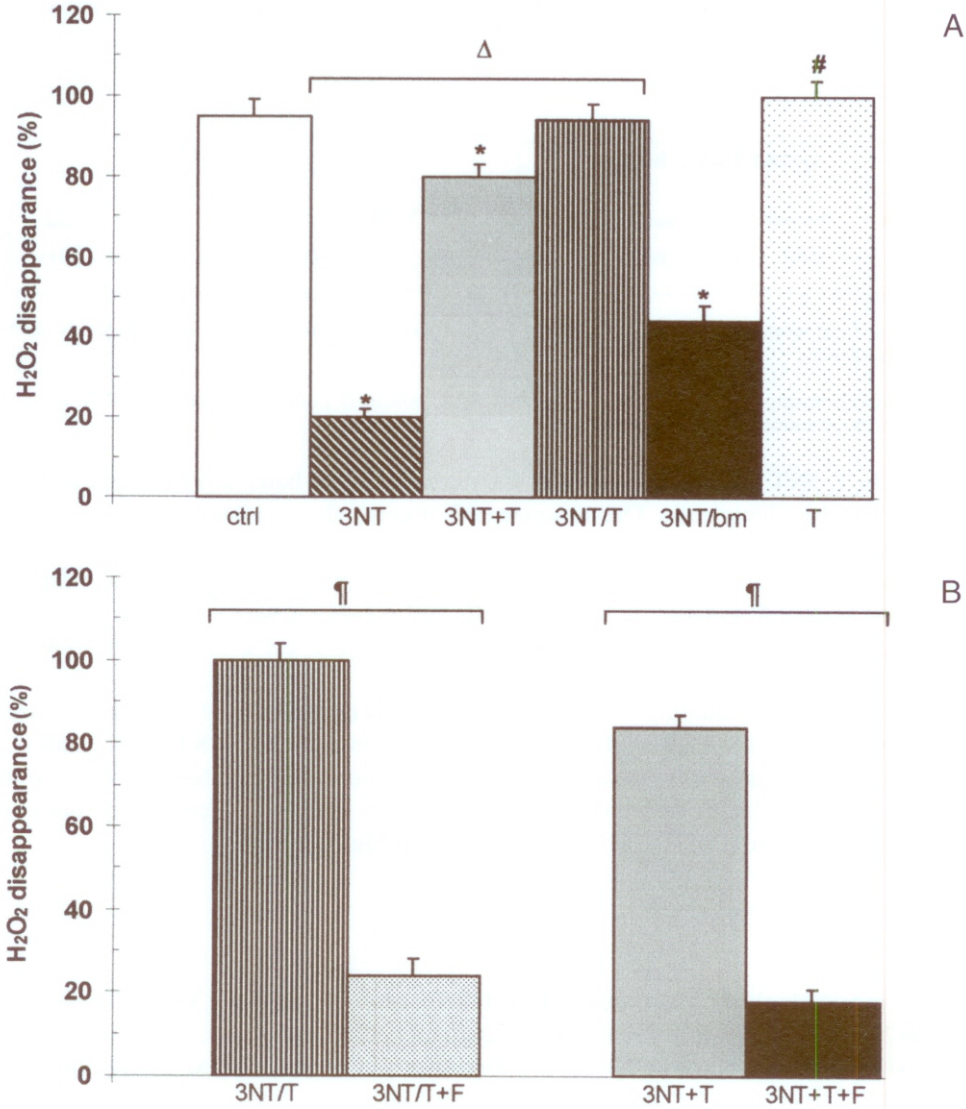

Fig. 5. - *Catalase activity, expressed as H_2O_2 disappearance, declines in 3NT and 3NT/bm cells, whereas it enhances after T exposure (T, 3NT + T and 3NT/T cells).*

Asterisk indicates significant difference from ctrl cells (Student t test); Δ significant differences among 3NT, 3NT + T and 3NT/T cells (ANOVA); # significant difference from 3NT, 3NT + T and 3NT/bm cells (Student t test) (A). When F is added (3NT/T + F and 3NT + T + F cells), catalase activity levels are lower than 30%; ¶ significant difference, Student t test (B). Data are shown as percentage of maximum values and expressed as mean ± SD. Each column represents the average of seven single experiments, $p < 0.05$.

tubulin in microtubular assembly dynamics (18). As tyrosinated α-tubulin is typical of dynamic microtubules and thereby is widely recognized as a marker of plasticity events, it can be concluded that testosterone can enhance neural plasticity. T has been observed to display a specific binding site to tubulin (23), and increases the amount of tubulin in different brain regions (26).

Neuronal plasticity, a typical structural adaptation of neurons to functional requirements, needs dynamic forms of microtubules. The α-subunit of tubulin, the major component of microtubules, undergoes posttranslational cyclic tyrosinaton-detyrosination process through the action of the enzymes tubuline tyrosine ligase and C-peptidase, and the tyrosinated form is considered a marker of dynamic microtubules (12, 13).

Tyrosine could be replaced by 3NT leading to different biological effects such as cellular dysfunction, DNA damage and cell death (10). Our results let us speculate that T diminishes the capability of neural cells to incorporate 3NT into the C-terminus of tubulin. Such incorporation, according to previous studies (6, 21), entails an NMDA receptor mechanism. If so, it can be assumed that T plays a prominent role in influencing neuronal plasticity processes *in vitro*.

Even though 3NT was proved to be incorporated into tyrosinated α-tubulin irreversibly (10), a reversible incorporation would be supposed on account of T positive effects on 3NT-induced cell damage. Therefore, our data likely confirm what reported by Bisig *et al.* (8), who described a reversible incorporation. In our work, the amount of 3NT bound to tubulin would depend on the time of 3NT exposure, as is higher in 3NT + T than in 3NT/T cells.

To confirm that in neuroblastoma cells the classical mechanism of steroid action was involved in T effects, we used the anti-androgen F. This molecule determined a significant decrease in the expression of tyrosinated α-tubulin. The hypothesis that T and F compete with the same binding site is confirmed by the observation that F increased 3NT incorporation, whereas T inhibited it. Since T receptors bind to F (27), it inhibits T-induced effects on C1300 cells. This result indicates that T neuroprotection *in vitro* presupposes binding to specific receptors.

Can T attenuate 3NT stress exploiting the cellular antioxidant defence systems? In our work, when 3NT was added to the medium, catalase activity significantly decreased, but recovered after T exposure (3NT + T and 3NT/T cells). In the presence of F (3NT + T + F and 3NT/T + F cells), catalase activity returned undistinguishable from that showed by 3NT cells. A correlation between reduction of 3NT incorporation into tubulin and increase in catalase activity could be postulated. 3NT induces formation of H_2O_2 which exerts oxidative damages (22). Intracellular NO leads to tubulin degradation (19). By means of its positive effect on catalase activity, T could inhibit 3NT incorporation into tubulin so preventing abnormal microtubular dynamics.

In conclusion, the susceptibility to oxidative stress was reduced by the exposure of C1300 cells to T. This androgen likely explicates a neuroprotective role involving catalase activity, though this mechanism needs to be further tested. The possible neuroprotective role of T in neuroblastoma cells exposed to 3NT has led to the ascertainment that the hormone interacts with 3NT incorporation into tyrosinated α-tubulin. This is in agreement with the recovering of normal morphological features and proliferation rate which follows T exposure. T biological action in neural cells *in vitro* may be mediated through the classical androgen receptor mechanism, even though there could exist other pathways not explored so far.

SUMMARY

3-nitro-L-tyrosine is formed by nitric oxide following different pathways such as NADPH oxidase, xanthine oxidase or glutamate NMDA receptor activation and is involved in the pathology of different neurological disorders. Unlike estradiol, a neuroprotective role of androgens against oxidative cell injury has not been fully investigated.

This work targets the possible effects of testosterone on neuroblastoma cells exposed to 3-nitro-L-tyrosine. C1300 mouse undifferentiated neuroblastoma cells exposed to 3-nitro-L-tyrosine were cultured in the presence of testosterone. Morphological examination, proliferation and nuclear viability assays were performed. The expression of tyrosinated α-tubulin and incorporation of 3-nitro-L-tyrosine into protein were also estimated. Cells exposed to 3-nitro-L-tyrosine showed globular shape, reduced cytoplasmic processes and growth inhibition in comparison with controls. When testosterone was added to the medium, these changes were not evident. In addition, testosterone induced an upregulation of tyrosinated α-tubulin, a marker of neuronal plasticity, and a decrease in 3-nitro-L-tyrosine incorporation into tubulin.

Our results suggest that testosterone exposure can diminish 3-nitro-L-tyrosine toxic effects on the morphology and growth rate of neuroblastoma cells. The upregulation of tyrosinated α-tubulin in testosterone-exposed cells would be consistent with concurrent plasticity events. Failure in α-tubulin nitration detected in cells exposed to both 3-nitro-L-tyrosine and testosterone, may support the idea that testosterone interferes with 3-nitro-L-tyrosine protein incorporation. Moreover, testosterone-induced neuroprotection likely entails a linkage with the androgen receptor as is suggested by the flutamide-induced inhibition of the hormone activity. Finally, the neuroprotective effects of testosterone in neuroblastoma cells could deal with the cellular antioxidant defence system, as shown by testosterone-induced increase in catalase activity.

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