Evaluation of potential synergistic action of a combined treatment with alpha-methyl-prednisolone and taurine on the mdx mouse model of Duchenne muscular dystrophy

A. Cozzoli*, J.-F. Rolland*, R. F. Capogrosso*, V. T. Sblendorio*, V. Longo†, S. Simonetti‡, B. Nico† and A. De Luca*

Unit of Pharmacology, Department of Pharmaco-biology, Faculty of Pharmacy, †Department of Human Anatomy and Histology, Faculty of Medicine, University of Bari, and ‡Metabolic Disease Unit, Paediatric Hospital Giovanni XXIII, Bari, Italy

Aims: Glucocorticoids are the sole drugs clinically used in Duchenne muscular dystrophy, in spite of the relevant side effects. Combination of glucocorticoids with synergistic drugs may be one strategy to lower doses and control side effects, meanwhile providing wider control of the complex pathology. This study is a preclinical evaluation of the effect of a combined treatment of α-methyl-prednisolone (PDN) with taurine, a safe aminoacid with positive effects on some pathology-related events. **Methods:** PDN (1 mg/kg/day i.p.) and taurine (1 g/kg/day orally) were administered either alone or in combination, for 4–8 weeks to male dystrophic mdx mice chronically exercised on a treadmill. Effects were assessed *in vivo* and *ex vivo* with a variety of methodological approaches. **Results:** *In vivo,* each treatment significantly increased fore limb strength, a marked synergistic effect being observed with the combination PDN + taurine. *Ex vivo,* PDN + taurine completely restored the mechanical threshold, an electrophysiological index of calcium homeostasis, of extensor digitorum longus myofibres and the benefit was greater than for PDN alone. In parallel, the overactivity of voltage-independent cation channels in dystrophic myofibres was reduced. No effects were observed on plasma levels of creatine kinase, while lactate dehydrogenase was decreased by taurine and, to a minor extent, by PDN + taurine. A similar histology profile was observed in PDN and PDN + taurine-treated muscles. PDN + taurine significantly increased taurine level in fast-twitch muscle and brain, by high-pressure liquid chromatography analysis. **Conclusions:** The combination PDN + taurine has additive actions on *in vivo* and *ex vivo* functional end points, with less evident advantages on histopathology and biochemical markers of the disease.

Keywords: animal model, Duchenne muscular dystrophy, markers of disease, preclinical tests, synergistic drugs association, prednisolone and taurine

Introduction

X-chromosome gene mutations resulting in the absence of the protein dystrophin cause the severe Duchenne muscular dystrophy (DMD) in humans and dystrophic conditions

Correspondence: Annamaria De Luca, Sezione di Farmacologia, Dipartimento Farmacobiologico, Via Orabona 4, Campus, 70125 Bari, Italy. Tel: +39 080 5442245; Fax: +39 080 5442801; E-mail: adeluca@farmbiol.uniba.it

in animals, such as the mdx mouse [1,2], characterized by progressive muscle weakness and wasting.

Dystrophin is a subsarcolemmal component of a multimolecular network (the dystrophin–glycoprotein complex) that ensures a physical linkage between the intracellular cytoskeleton and the extracellular matrix, providing mechanical stability to myofibres during contraction [1]. The absence of dystrophin triggers a complex and still unclear sequence of events that finally lead to progressive myofibre degeneration, failing regeneration and fibrosis. Dystrophin-deficient myofibres show changes in calcium homeostasis, mainly sustained by the increased sarcolemmal influx of calcium ions through voltage-insensitive calcium channels [3–7]. Such changes contribute to modification in excitation-contraction coupling as well as to degeneration through the activation of proteolytic enzymes and/or apoptotic pathways [8–11]. There is also evidence of an early and self-sustained inflammatory response contributing to muscle degeneration and late fibrosis [12–16].

The complexity of the causal and temporal sequence of the pathological events complicate the identification of specific acting drugs to effectively control the disease, and justify the effort towards therapeutic strategies aimed at replacing or correcting the gene defect [17–19]. Glucocorticoids are the sole drugs of clinical interest for DMD patients. The mechanism for their beneficial action is not completely understood yet and may involve multiple effects, beside the classical anti-inflammatory and immunosuppressive ones. These include an improvement of regeneration and an increased expression of utrophin, the homologue-surrogate for dystrophin [20–22]. However, the clinical use of glucocorticoids in DMD children is limited by severe side effects over long-term use; this compels the search of safer drugs or of strategies to limit their side toxicity [23].

As for other complex disorders, one feasible strategy is to find compounds with relevant synergistic interactions: thus glucocorticoids in combination with a synergistic drug, may exert greater effects and/or have less side effects as a result of dose lowering. This rationale is reinforced by the anecdotal report that DMD patients often take various food and drink supplements or herbal remedies along with the classical glucocorticoids and it is important to develop a more systematic preclinical evaluation of the outcome of drug combinations, both *in vitro* and *in vivo* [23,24]. For instance, the combination of deflazacort with the food supplement L-arginine has been reported to produce an improved functional benefit in dystrophic mdx mice [25].

We therefore aimed to investigate the effects of a combined treatment of α-methyl-prednisolone (PDN), a clinically used glucocorticoid, with taurine. Taurine is a sulphonic amino acid normally present in skeletal muscle, able to modulate sarcolemmal excitability and calcium homeostasis [26]. It is used as a soft-drink supplement for its claimed ability to stimulate metabolism and provide energy. Little, if any, toxicity has been reported for taurine at the generally assumed quantities [27]. Complex fluctuations in tissue taurine content occur in mdx mouse in the different phases of muscle degeneration/regeneration, suggesting that the amino acid levels may be influenced by myofibre state and may in turn contribute to cellular and tissue dysfunction and/or repair; taurine increases seem to be generally associated with muscle regeneration and membrane stabilization [28–30]. In addition, taurine exerts anti-inflammatory and antioxidant actions [31], with potential beneficial outcomes on the pathology progression. We have previously found that taurine either applied *in vitro* or administered *in vivo* exerts beneficial effects on the altered excitation-contraction coupling mechanism of mdx myofibres [8,29]. Also the amino acid administration enhances mdx mouse strength impaired by a chronic exercise on treadmill, a protocol that is able to exacerbate *in vivo* and *ex vivo* markers of the murine pathology [2,8].

We have performed a chronic (4–8 weeks treatment) *in vivo* treatment with α-methyl-prednisolone (1 mg/kg i.p.) and taurine (1 g/kg/day per os) either alone or in combination, in treadmill exercised adult mdx animals. A multidisciplinary *in vivo* and *ex vivo* approach has been used to evaluate the general outcome of the treatment on disease-sensitive indices. The final aim was to evaluate the possible presence of a synergistic action between the two compounds that may justify their combined use in patients.

Methods

All experiments were conducted in accordance with the Italian Guidelines for the use of laboratory animals, which conform with the European Community Directive published in 1986 (86/609/EEC). Most of the experimental procedures used conform the standard operating procedures for preclinical test in mdx mice available on http://www.treat-nmd.eu/research/preclinical/SOPs/ [2,32].

In vivo experiments

Animal groups, treadmill running and drug treatment Male mdx and wild type (WT, C57/BL10ScSn) mice of 4–5 weeks of age (Charles River, Italy for Jackson Laboratories, USA), homogeneous for body weight were assigned to 'exercised' and 'sedentary' groups. The groups of exercised mice underwent a 30 min running on an horizontal treadmill (Columbus Instruments, USA) at 12 m/min, twice a week, for 4–8 weeks [8,33] and were composed by seven vehicle-treated and six prednisolone-taurine-treated mdx mice. Based on previous results [8], we chose the dose of 1 mg/kg i.p. for PDN, while taurine was administered orally in chow-enriched pellets at the maximal dose of 1 g/kg/day. Both compounds have been already tested singularly in exercised mdx mice [8]. However, in order to avoid any bias due to variability of experimental conditions, two additional groups of exercised mdx mice were used. One group was made of five animals treated only with 1 mg/kg PDN i.p. while the other group of four animals received only taurine-enriched food up to 1 g/kg/day. The treatment started 1 day before the beginning of the exercise protocol, and continued until the day of sacrifice. When necessary, age-matched untreated exercised WT mice were also used. 'Sedentary' mdx (vehicle-treated or not) and WT mice were left free to move in the cage, without additional exercise and monitored at the same time points of exercised counterparts, according to the experimental need. Every week all mice were monitored for body weight and fore limb force by means of a grip strength meter (Columbus Instruments, USA); the end of the 4th week was considered for statistical analysis [8,34]. At the end of the 4th week of exercise/treatment the *ex vivo* experiments were also started. The animals continued to be exercised/treated until the day of sacrifice and were used for the *ex vivo* experiment within the 8th week.

In vitro studies

Muscle preparations Animals of 8–12 weeks belonging to the different groups were anesthetized with 1.2 g/kg urethane i.p. Extensor digitorum longus (EDL) muscle of one hind limb was removed and rapidly placed in the recording chamber for the electrophysiological recordings. Gastrocnemius (GC) muscles were removed and processed for histology procedures, while tibialis anterior (TA) and slow soleus muscles, the heart and the brain were removed for biochemical experiments. These tissues were washed in PBS and rapidly frozen in liquid nitrogen-cooled isopentane and stored at −80°C until use. The right half side of diaphragm was placed in the recording chamber for intracellular microelectrode recordings. Flexor digitorum brevis (FDB) muscle was used for patch clamp recordings.

Electrophysiological recordings EDL muscles were bathed at 30 ± 1°C in the following normal physiological solution (in mM): NaCl 148; KCl 4.5; CaCl$_2$ 2.0; MgCl$_2$ 1.0; NaHCO$_3$ 12.0; NaH$_2$PO$_4$ 0.44 and glucose 5.55, continuously gassed with 95% O$_2$ and 5% CO$_2$ (pH = 7.2–7.4).

The mechanical threshold (MT) was determined in the presence of tetrodotoxin (3 μM) using a two microelectrode 'point' voltage clamp method [8,29]. Depolarizing command pulses of duration ranging from 500 to 5 msec (0.3 Hz) were progressively increased in amplitude from the holding potential (H) of −90 mV until visible contraction. The threshold membrane potential (V, in mV) was read on a digital sample-and-hold millivoltmeter for each fibre at the various pulse durations t (in msec); mean values at each t allowed to construct a 'strength-duration' curve. The pulse duration range allowed to reach a constant rheobase voltage in each experimental condition, thus minimizing the potential effect of time as additional variable. The rheobase voltage (R, in mV) and the time constant (τ, msec) to reach the rheobase were obtained by non-linear least square algorithm using the following equation: V = [H − R exp (t/τ))/(1 − exp (t/τ)] [8,29].

Patch clamp recordings were performed on enzymatically isolated FDB muscle fibres (2.5 mg/ml collagenase type XI-S, Sigma, St. Louis, MO) prepared as described in [7], then washed with bath solution and transferred into the chamber (RC-22C; Harvard Apparatus, Edenbridge, UK).

Cell-attached patch clamp recordings were performed with 4–5 MΩ patch pipettes in borosilicate glass, at room temperature, using an Axopatch200B patch clamp amplifier (Axon Instruments, Foster City, CA) and pClamp8 software. Pipette solution contains 110 mM CaCl$_2$, 10 mM HEPES and 0.01 mM DIDS. A depolarizing 'bath' solution containing 150 mM potassium aspartate, 5 mM MgCl$_2$ and 10 mM EGTA ensured a close to 0 mV membrane potential; transmembrane patch potential was imposed by intrapipette potential. Channel conductance was estimated during construction of I/V, while channel occurrence was qualitatively estimated as the number of patches displaying channel activity over the normal

number of patches sampled. Accordingly, patches were subdivided in silent patches (without detectable channel activity), patches with analysable channel activity (with clearly detectable and analysable single channel events, as previously described) and patches with channel overactivity (with many overlapping events not allowing a detailed analysis) [7].

Histology GC muscles were dissected from surrounding tissue, fixed in a modified acetate-free Bouin solution for 4 h and then routinely processed and paraffin wax embedded. Six micron transversally cut sections was stained by haematoxyl-eosin or toluidine blue to calculate the percent of both healthy myofibres with peripheral nuclei (peripherally nucleated fibres) and regenerating/regenerated myofibres, showing central nuclei (centrally nucleated fibres), as well as the area of necrosis and of non-muscle tissue. Morphometric analysis was performed on 10 cross sections from each experimental group by means of 3–4 animals per group, by using an Image Analysis software (Olympus Italia, Rozzano, Italy) [15,35]. A high inter-individual variability is generally observed in the histology profile of mdx mouse muscles; this implies the need of a greater number of animals for a detailed morphometric analysis. However, the number of mice used in the present study allowed a general estimation of the presence of the typical signs of dystro-pathology in both untreated and drug-treated muscles.

Plasma level of creatine kinase (CK) and lactate dehydrogenase (LDH) Blood was collected by heart puncture soon after animal death in EDTA/heparin rinsed centrifuged tubes. The blood was centrifuged at 3000 g for 10 min and plasma was separated and stored at −20°C. The relative activity of CK (a marker of sarcolemmal fragility) and lactate dehydrogenase (a marker of metabolic distress, especially in exercised animals) was estimated by standard spectrophotometric analysis by using diagnostic kits (Sentinel, Farmalab – Italy) within 7 days from plasma preparation. Briefly, CK activity is determined with the CK-NAC liquid kit (Sentinel diagnostic) in a three-step reaction. This includes the formation of ATP from the dephosphorylation of creatine phosphate and its use by hexokinase in the conversion of glucose in glucose-6-phosphate. This latter is then finally transformed into 6-phosphogluconate by the glucose-6-phosphate-dehydrogenase with the formation of NADPH. Thus, the time-dependent variation of absorbance at 340 nm due to NADPH production is a direct measure of CK activity in the sample. For the activity of LDH, the kit (LDH liquid – Sentinel Diagnostic) allows to measure the time-dependent variation of absorbance at 340 nm due to the degradation of NADH in the reaction of transformation of pyruvate into lactate.

High-pressure liquid chromatography determination of taurine levels TA muscles, soleus, heart and brain were weighed and homogenized with 10 ml of $HClO_4$ (0.4 N) per g of tissue. The homogenized muscles were buffered with 80 µl K_2CO_3 (5.5 g/10 ml) for each millilitre of $HClO_4$ used. The homogenates were centrifuged at 600 g for 10 min at 4°C. The supernatants were stored at −80°C until assay. This latter consisted in a high-pressure liquid chromatography determination [29]. In particular, the Biochrom Amino Acid Analyser, a PC-controlled automatic liquid chromatograph with post-column detection system and the Ultra Physiological Fluid Chemical Kit (Biochrom Ltd), which allows quantitative analysis of amino acids in biological fluids and extracts, have been used.

The sample is injected onto a column of cation exchange resin and derivatized with o-phthalaldehyde. The reaction with the amino acids present in the eluent forms conjugated compounds whose quantity is then established by spectrophotometric analysis. The amount of each reaction product is directly proportional to the quantity of amino acid present. The retention time of peak identifies the amino acid, the area under the peak indicating the quality of amino acid present. The required calibration analysis has been performed by using nor-leucine as internal standard.

Statistics

All data are expressed as mean ± standard error of the mean (SEM) or ± standard deviation (SD). The SE estimate for the fitted rheobase (R) and time constant (τ) values (and relative independent statistical analysis) were obtained as previously described. Independent one-way ANOVA analysis for multiple comparison of drug efficacy was performed on the two fitted values [8,29].

Statistical analysis for direct comparison between two means was performed by unpaired Student's *t*-test. Multiple statistical comparison between groups was performed by one-way ANOVA, with Bonferroni's *t*-test post

hoc correction for allowing a better evaluation of intra- and inter-group variability and avoiding false positive.

Results

Effect of PDN + taurine treatment on body weight and fore limb strength

Animal groups were homogenous for body weight and fore limb strength at the beginning of the study (Table 1). As expected, a typical reduction in fore limb strength was observed after 4 weeks of exercise in the mdx animals [8]. The three groups of drug-treated mdx mice showed an amelioration of the exercise-induced decrease of fore limb strength, detectable on both the absolute strength value and its 4-week increment (Table 1). However, the effect was remarkable and significant only with the combination PDN + taurine, which exerted a greater effect than either of the two drugs administered alone. A difference in body weight gain was observed between the drug-treated groups, with PDN- and PDN + taurine-treated mice showing the less increment. To take into account the inter-individual influence of body weight, for each mouse the fore limb strength has been normalized to body weight both at the beginning (time 0) and at the end of 4 weeks of exercise (time 4) and the normalized force increment over the 4 weeks of treatment was calculated (Figure 1). In agreement with previous findings [8], both PDN and taurine significantly contrasted the exercise-induced impairment of normalized force increment. The incre-

ment presently observed with PDN was greater than that previously found, likely in relation to the different administration route used (i.p. *vs.* oral [8];). The combination PDN + taurine produced a remarkable synergistic anabolic action; in fact the 4-week increment of normalized force was significantly higher with respect to that observed with the two drugs singularly administered as well as to that observed in WT animals.

Effect of PDN + taurine treatment on MT and calcium permeable channels

A shift of the voltage threshold for contraction (MT) towards more negative potentials is a typical hallmark of EDL muscle fibres of mdx mice [8,29]. The threshold potential values of PDN + taurine-treated exercised mdx fibres were significantly shifted towards more positive potentials *vs.* those of untreated ones, at each pulse duration (Table 2). Thus, the strength-duration curve almost overlapped that of WT muscle fibres and the value of rheobase was restored to the WT ones (Figure 2A,B). The effects of the combination PDN + taurine on MT was similar, although slightly greater, to those of taurine alone, both treatments being significantly more effective than PDN alone. A significant amelioration of the fitted value of the time constant to reach the rheobase was also observed as it was 10 ± 0.7 msec in exercised mdx and 6.5 ± 0.4 msec in PDN + taurine treated myofibres ($P < 0.003$ by Bonferroni's *t*-test after ANOVA), a value similar to that of WT myofibres (7.35 ± 0.4 msec). Again,

Table 1. Effect of PDN, taurine and PDN + taurine treatments on body weight and fore limb strength of mdx mice

	n	*Body weight (g)* *Time 0*	*Body weight (g)* *Time 4*	*ΔBW*	*Force (kg)* *Time 0*	*Force (kg)* *Time 4*	*ΔF*
Wild type	6	18.4 ± 0.56	23.7 ± 0.53	5.3 ± 0.81	0.098 ± 0.08	0.152 ± 0.009	0.055 ± 0.013
Sed Mdx	6	18.3 ± 0.61	24.6 ± 0.78	6.4 ± 0.4	0.095 ± 0.006	0.159 ± 0.008	0.064 ± 0.012
Exer mdx	7	19.9 ± 0.60	25.4 ± 0.6	5.54 ± 0.54	0.100 ± 0.009	0.137 ± 0.004*	0.037 ± 0.01
Exer mdx PDN	5	20.7 ± 1.67	23.9 ± 0.53	3.20 ± 1.22*†	0.103 ± 0.01	0.149 ± 0.008	0.045 ± 0.011
Exer mdx Taurine	4	19.1 ± 0.31	23.9 ± 0.64	4.76 ± 0.51	0.098 ± 0.001	0.146 ± 0.006	0.047 ± 0.007
Exer mdx PDN+Taurine	6	19.7 ± 0.58	22.6 ± 0.57*†	2.90 ± 0.34*†‡	0.084 ± 0.005	0.158 ± 0.006†	0.074 ± 0.005†§

Columns are as follows: groups of mice used; *n*: number of mice per group; Body weight, in g at the beginning (time 0) and after 4 weeks (Time 4) of exercise and/or drug treatment; ΔBW, difference between body weights at time 4 and time 0; Force: fore limb strength, in kg at the beginning (Time 0) and after 4 weeks of exercise and/or drug treatment (Time 4); ΔF: Fore limb strength increment between time 4 and time 0. Each value is the mean ± SEM from the number of animals indicated in the column. For each parameter, the statistical significance between groups was evaluated by ANOVA test for multiple comparison (*F*-values) and Bonferroni's *t*-test post hoc correction. Significance at ANOVA test was found for the following parameters BW T4 ($3.77 < F < 4.33$; $0.02 < P < 0.05$); ΔBW ($3.57 < F < 7.1$; $0.002 < P < 0.05$); ΔF4 ($F = 3.61$; $P < 0.05$). The Bonferroni's test showed statistical significance as shown in the table with respect to the following groups: *Sed Mdx ($0.002 < P < 0.05$), †Exer Mdx ($0.005 < P < 0.05$), ‡WT ($P < 0.01$), §Exer Mdx PDN ($p < 0.04$).
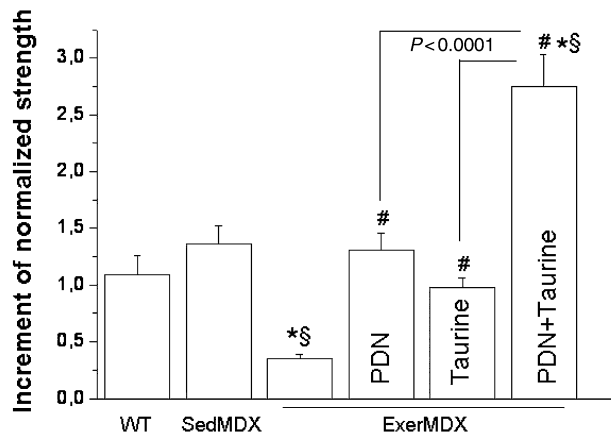
Figure 1. Effect of drug treatments on fore limb force of exercised mdx mice. The bars show the normalized force increment (Δ Normalized Force) for wild-type (WT) and mdx mice undergoing or not the exercise protocol (SedMDX and ExerMDX) with or without a concomitant treatment with a-methylprednisolone (PDN), taurine and/or PDN + taurine. Δ Normalized Force has been calculated as follows: for each mouse the fore limb strength has been normalized to the respective body weight both at the beginning of exercise/treatment protocol (Time 0) and after 4 weeks (Time 4). The difference between the normalized strength values at the two time points allowed to calculate for each mouse of each group the increment and to evaluate the effect of either exercise, treatment or both on it. Each bar is the mean ± SE from the number of mice in brackets above each bar. The size of the SE of ΔNF has been calculated from the standard error of the mean (SEM) values of normalized strength at time 0 and time 4, taking into account the size, as percent of the mean, of each SEM and considering the sum of them as indicative of the maximal size of the calculated SE. This approach allowed to minimize the unpredictable variability due to differences in absolute values of the individual differences [15]. Statistical significance between groups was evaluated by ANOVA test for multiple comparison (*F*-values) and Bonferroni's *t*-test post hoc correction and was as follows: $10 < F < 40$; $P < 0.005$; Significantly different with respect to *WT ($P < 0.001$); §sedentary mdx ($P < 0.0001$); #untreated exercised mdx mice ($P < 0.0001$). The statistical significance of PDN + Taurine-treated mdx mice *vs.* the single-drug-treated groups is also shown in the figure.

the effect of the combined treatment was greater than that observed for taurine (8.2 ± 0.4 msec) and PDN (8.6 ± 1.2 msec) alone. The time constant values of the two individual drug treatments were not significantly different with respect to those of WT and untreated exercised mdx values by ANOVA test. The alteration of the MT in dystrophic myofibre is correlated with the alteration of calcium homeostasis; the latter is mostly related to the enhanced sarcolemmal permeability to calcium via voltage-independent channel pathways [6,7]. Thus, we verified the potential ability of the combined treatment to act on the overactivity of voltage-independent and mecha-

nosensitive cationic channel in mdx myofibres by patch clamp recordings on freshly isolated myofibres. Due to the complexity of recordings in native myofibres, we focused only on the outcome of the combined treatment in comparison with untreated exercised mdx and WT myofibres. Cell-attached patch clamp recordings were performed in FDB muscle fibres with calcium as the sole cation in the pipette solution. The fibres from PDN + taurine-treated animals showed a significant reduction of channel openings with respect to untreated counterparts, showing a profile of activity similar to that of WT myofibres (Figure 2C). In fact, active patches from treated fibres had brief channel openings often occurring as singular events, in contrast with the longer and superimposed openings observed in untreated ones. No differences were observed in single channel conductance, this latter being around 30 pS in any experimental condition (value in WT myofibres: 32 ± 1.6 pS; 30 fibres/4 preparations), while main differences were observed in channel density/occurrence and kinetic. In particular, the decreased activity in myofibres from treated animals was paralleled by a decrease in channel occurrence, that is briefly summarized in Figure 2D. According to previous data [7], the main feature in myofibres from untreated exercised mdx animals was a marked decrease in the number of silent patches *vs.* patches with a channel activity; these latter were also characterized by a large presence of patches with channel overactivity. Interestingly, the fibres from mice treated with the combination PDN + taurine had a close-to-normal ratio between silent and active patches; also a marked increase of normally active *vs.* overactive patches was observed (Figure 2D).

Effect of PDN + taurine treatment on histological markers of muscle damage

For the histology analysis on exercised mdx animals, either treated or not, muscles were sampled between 48 and 72 h after the last bout of exercise. In line with previous results [15,33,35], the GC muscles of exercised mdx mice showed marked structural alterations with extensive areas of degeneration, the presence of necrotic fibres and of non-muscle tissue (Figure 3A – panel a). Around 70% of the fibres were centronucleated; these fibres were of variable size and isolated or in clusters often nearby necrotic fibres. This is a clear marker of ongoing degeneration-regeneration cycles. Infiltrates, resembling mononuclear inflammatory cells described in dystrophic

Table 2. Effect of PDN, taurine and PDN + taurine treatment on voltage threshold for mechanical activation of EDL muscle fibres of exercised mdx mice

Experimental conditions	Duration (msec)						
	5	10	20	50	100	200	500
Exer MDX	−53.9 ± 0.8 (23)	−64.1 ± 0.5 (26)	−72.2 ± 0.4 (27)	−75.7 ± 0.3 (27)	−75.4 ± 0.3 (32)	−75.6 ± 0.25 (31)	−75.9 ± 0.24 (33)
Exer MDX PDN	−53.3 ± 0.4 (31)	−59.8 ± 0.3 (29)	−66.6 ± 0.2 (30)	−73.4 ± 0.2 (33)	−73.6 ± 0.2 (33)	−73.4 ± 0.23 (32)	−73.9 ± 0.24 (34)
Exer MDX Taurine	−47.2 ± 0.8 (22)	−59.8 ± 0.9 (22)	−67.0 ± 0.6 (23)	−70.3 ± 0.7 (23)	−71.3 ± 0.8 (29)	−70.3 ± 0.7 (25)	−70.0 ± 0.6 (24)
Exer MDX PDN + Taurine	−52.2 ± 1.1 (21)	−60.9 ± 0.7 (18)	−66.8 ± 0.8 (23)	−69.2 ± 0.5 (30)	−69.2 ± 0.8 (23)	−69.6 ± 0.7 (28)	−69.5 ± 0.5 (35)

The columns from left to right are as follows. Experimental conditions, the fibres sampled are from extensor digitorum longus (EDL) muscles from exercise mdx mice untreated or treated with the test compounds: α-methylprednisolone (PDN); Taurine or the combination PDN + Taurine. For each experimental group are shown the threshold membrane potential values obtained with depolarizing command pulse of duration ranging from 5 up to 500 msec. The values are expressed as mean ± SEM from the number of fibres shown in parentheses below each value.

All values from each treatment, with the exception of the threshold values of PDN and PDN + taurine-treated muscle at 5 msec pulse durations were significantly more positive with respect to the values of untreated exercised by ANOVA and Bonferroni's *t*-test post hoc correction ($P < 0.05$ and less). The possible role of time in the overall phenomenon has been evaluated by the calculation of the time constant (τ) and the consequent one-way ANOVA analysis, as described in the text.

muscle, were also present (Figure 3A – panel a). In order to evaluate the ability of taurine to exert synergistic effects with the gold standard PDN, we focused on the comparison of the histological profile of the PDN + taurine-treated animals *vs.* that of PDN-treated ones. For both treated groups the muscles showed a more regular architecture and a significant reduction of areas of necrosis *vs.* untreated ones (Figure 3A – panels b and c).

In fact, the area of necrosis was reduced by about 60–70% by both treatments. A 20% reduction in the percentage of centronucleated fibres in favour of normal ones was also observed in PDN- but not PDN + taurine-treated muscles. In this latter group a slight 20% decrease in the non-muscle area was observed (from 6.3 ± 3.5 % to 4.7 ± 3.2%; 10 sections/3 muscles per group), an effect not detected in PDN-treated muscles. However, the morphology of the PDN-treated muscles appeared to be more homogeneous than that of PDN + taurine-treated ones, with a greater symmetry of fibre size and less presence of infiltrates. It is important to underline that a more representative comparison of the effects of the two treatments would have benefited by analysis of a greater number of animals to reduce the high inter-individual variability typical of histology profile of dystrophic animals. Thus, the present evaluation is mainly indicative of a similar trend of activity of PDN, either alone or in combination with taurine, on the histology profile.

Effect of PDN + taurine treatment on CK and LDH

The effect of exercise and drug treatment on CK and LDH is shown in Figure 3**B,C**. An increase in enzyme activity was observed in exercised *vs.* sedentary animals. No significant decrease in the level of CK enzyme was observed with any of the treatments used (Figure 3**B**). A significant decrease in LDH was observed in plasma from taurine-treated animals; however, this effect was less evident in the animals treated with the drug combination (Figure 3**C**).

Effect of PDN-taurine treatment on tissue taurine level

The PDN and the combined PDN + taurine treatments have a similar effect on both histology and plasma enzyme profile. Then, we verified the real occurrence
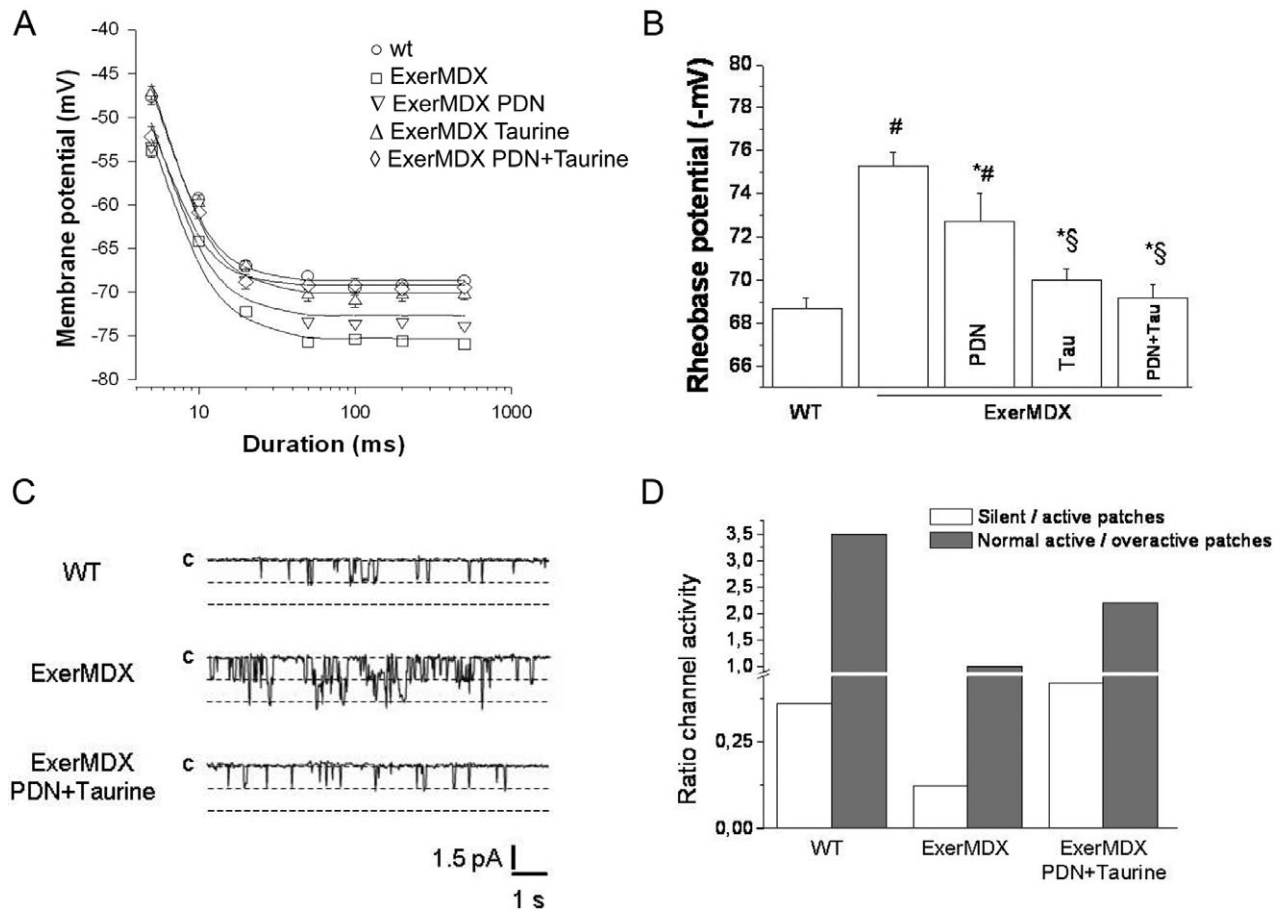
Figure 2. Effect of drug treatment on markers of calcium homeostasis in exercised mdx myofibres. In (**A**) Effect of treatment with alpha-methyl prednisolone (PDN), taurine and with PDN + taurine on mechanical threshold of extensor digitorum longus muscle fibres. The data, expressed as mean ± SEM from 18–38 values from 3–5 preparations (see also Table 2), show the voltages for fibre contraction (mechanical threshold) at each pulse duration in different experimental conditions and in particular in WT (open circles), untreated exercised mdx mice (Exer MDX, open squares), and PDN, taurine or PDN + taurine-treated exercised mdx mice. All threshold values of Exer-MDX untreated or PDN-treated muscle fibres were significantly more negative with respect to those of WT ones ($P < 0.001$), while the strength-duration curve obtained in taurine and PDN + taurine-treated mdx muscle fibres almost fully overlapped that of normal WT. For some data point the standard error bar is not visible being smaller than symbol size. (**B**) Rheobase voltages obtained from the fit of the strength-duration curves in (**A**) to the equation described in the method section. Statistical significance has been evaluated by ANOVA and Bonferroni's *t*-test post hoc correction and is as follows #*vs.* WT ($P < 0.05$ and less); **vs.* untreated exercised ($P < 0.03$ for PDN-treated and $P < 0.0001$ for taurine and PDN + taurine-treated muscle); §*vs.* PDN-treated ($P < 0.03$ for taurine-treated and $P > 0.006$ for PDN + taurine-treated muscles). (**C**) Representative traces of single channel inward currents recorded in the cell-attached configuration of the patch clamp technique at −40 mV in FDB fibres from mice belonging to different groups: WT; exercised mdx (mdx exer) and exercised mdx treated with PDN + Taurine (mdx exer PTX). Due to the mechanosensitive nature of the current, FDB WT myofibres were obtained from animals undergoing a similar exercise protocol than mdx mice [7]. The closed state of the channel is indicated by c. The traces shown are representative of 10–12 experiments. (**D**) Schematic description of the channel occurrence in the different experimental condition. The white bars indicate the ratio of sampled patches showing no channel activity *vs.* those showing activity, while the grey bars show the distribution within active patches as a ratio of those with normal 'analysable' channel opening events and those characterized by excessive overactive events. Exer mdx myofibres show a marked reduction of silent and normally active patches *vs.* overactive ones while the PDN + taurine myofibres showed a clear trend towards a WT-like occurrence, mostly related to the increase in silent patches and a reduction of overactive ones. The ratios have been calculated from 6–8 fibres/muscle from 3–4 muscle per experimental group, with a total estimate of 18–24 fibre sampled per each experimental condition.

of a modification in taurine content in target tissues of animals undergoing the combined treatment. The results are shown in Figure 4. A significant increase was found in the fast-twitch muscle TA, while no effects were observed in the slow soleus muscle, likely in relation to its higher basal level of the amino acid. Also a marked significant increase in taurine content has been observed in the brain, while little, if any, effect was observed in the heart.

Discussion

Duchenne muscular dystrophy is a complex disease, with several pathways contributing to the progressive muscle degeneration and final fibrosis; so far the temporal and causal sequences of different events are poorly understood. From a pharmacological point of view, a feasible approach is to use combination of drugs able to target different aspects of the pathology cascade, so as to have positive additive effects on disease course and symptoms.

We presently performed a preclinical test of a drug combination clinically relevant for DMD patients. In fact, PDN belongs to the glucocorticoids, the class of drugs clinically used in DMD patients, while taurine is an amino acid commonly used as food and drink supplement, with a claimed energizing activity [27]. The study evaluated if the combination could be an advantage in terms of synergistic action, which could help to reduce steroid dose and in turn the side effects. Also, the outcome of this preclinical study may help to understand the possible variable response to steroids between patients in relation to empirical consumption of taurine as supplement.

The results clearly showed that the combination has significant advantages *vs.* the two drugs alone on *in vivo* animal strength, showing a remarkable synergistic anabolic action. The increase in animal strength is indicative of an ameliorative action on the muscular system. However, this *in vivo* outcome cannot rule out the action of the drugs on other systems, i.e. the peripheral and/or the central nervous system and/or the cardiovascular system, which also have important influences on muscle performance [2]. Thus, it was important to verify the muscle-based effects of the drug association. We have previously described the ability of taurine and, to a lesser extent of PDN, to ameliorate the excitation-contraction coupling of mdx myofibres, determining a shift of the MT towards the more positive potentials typical of WT muscles [8]. This effect of taurine can also be observed upon acute *in vitro* application to dystrophic myofibres, in line with direct

action on mechanisms dealing with calcium handling [29]. The observation that the combined treatment was able to exert a full restoration of the MT, strongly supports the supposition that taurine supplementation to PDN therapy has a beneficial effect on excitation-contraction coupling and that this effect can play a role in the anabolic action *in vivo*.

Interestingly, the combination also counteracted the overactivity of the subset of calcium channels that has been previously found to contribute to the altered calcium homeostasis in dystrophic myofibres [7]. The effects of PDN + taurine on calcium-dependent MT and ion channel activity closely resemble those recently observed with pentoxifylline [34], being rather different from what was observed with anti-cytokine and anti-inflammatory drugs that had little if any effect on calcium homeostasis [15,33]. These results corroborate that the altered calcium homeostasis and the entities possibly involved in abnormal calcium permeability, likely belonging to transient receptor potential (TRP) channel family, can be directly targeted by specific pharmacological interventions. This drug effect may have a possible positive outcome on animal strength and muscle performance, as toxins able to inhibit mechanosensitive channels or genetic silencing of specific TRP channel subsets may protect dystrophic muscle from eccentric-contraction induced deficit [36,37]. A detailed analysis of the effect of each treatment on the molecular mechanism related to calcium handling was beyond the aim of the present study. Thus, the patch clamp investigation was restricted only to the muscles from mdx mice treated with the PDN + taurine combination, also in consideration of the complexity of these recordings on native myofibres. However, no evidence is available about the possible effects of PDN or taurine on mechanosensitive TRP-like channels and the obtained results push towards further investigations with the two drugs alone, and especially taurine, on calcium entry pathways.

In general, the results support the important role of taurine in different pathophysiological condition of skeletal muscle. In fact, an active transport system concentrates taurine against its gradient and the muscle level depends on muscle fibre phenotype and function, as also demonstrated in the present study [38,39]. Experiments performed on isolated vesicles of rat muscle SR showed that taurine is able to directly stimulate the calcium reuptake by the Ca^{2+}-ATPase pump [40], a mechanism that may in turn modulate the activity of store-operated sarcolemmal channels [41]. The action on calcium stores
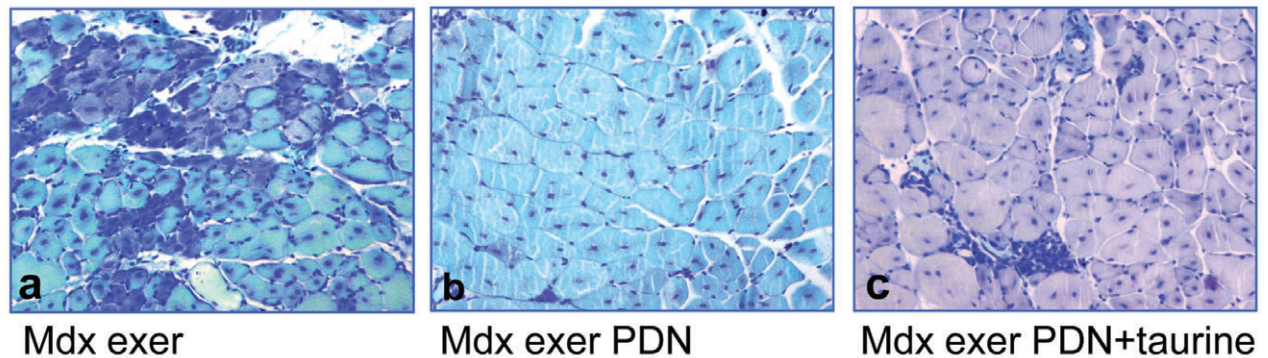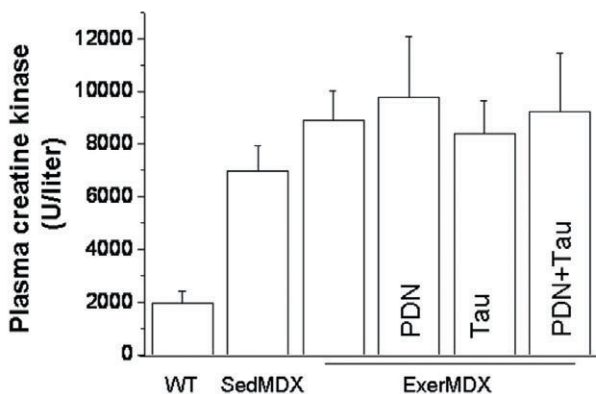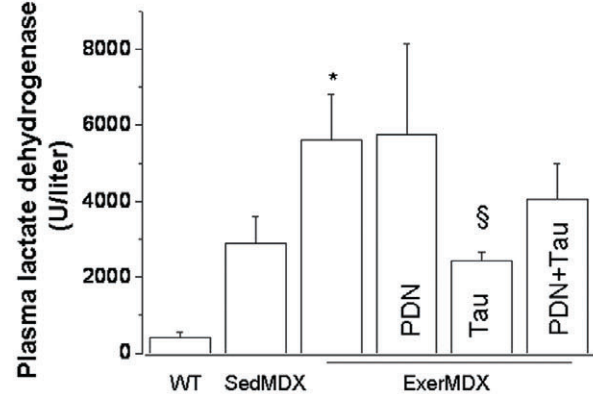
Figure 3. Effect of drug treatments on morphological and biochemical markers of dystro-pathology. (**A**) Morphological features of gastrocnemius from untreated exercised (a), PDN-treated exercised (b) and PDN + taurine-treated exercised mdx mice (c), by toluindine blue staining. The sections from untreated exercised muscles showed a less homogenous structure, with greater variability in fibre dimension, larger areas of necrosis accompanied by mononuclear infiltrates and/or small regenerating fibres. A larger area of non-muscle tissue is also present. PDN-treated muscles showed a more homogenous architecture with respect to both untreated and PDN + taurine-treated muscle. Pictures at × 20 magnification. (**B**) Plasma creatine kinase levels measured by standard spectrophotometric analysis. Each column is the mean ± SEM from 4–7 animals. Statistical significant differences were only found between values of mdx mice (either exercised or not and treated or not) and WT ones. (**C**) Plasma Lactate dehydrogenase levels measured by standard spectrophotometric analysis. Each column is the mean ± SEM from 4–7 animals. Statistical significant difference was found between groups by mean of ANOVA test ($F > 4$; $P < 0.01$). Bonferroni's post hoc *t*-test showed that all values from exercised mdx group, with the exception of that from taurine-treated group, were significantly different from WT values ($P < 0\ 003$). Other statistical differences are as follows: significantly different *vs.* sedentary mdx mice ($P < 0.03$) and §*vs.* exercised mdx mice ($P < 0.02$).

along with a modulation of sensitivity of the contractile filaments to calcium may also contribute to the anabolic action of taurine [42]. Accordingly, taurine physiologically works for modulating in excitation-contraction coupling mechanism of striated fibres. In fact, a shift of the MT towards more negative potentials is commonly observed in conditions of taurine depletion either naturally occurring (as in aged muscle) or induced pharmacologically [43,44]. In both conditions taurine supplementation, either *in vitro* or *in vivo*, has beneficial effects on MT, in line with the proposed ability of taurine to act as a protective agent in stressed muscle [45]. Interestingly, taurine depletion has been found to decrease muscle force output [46], corroborating the link between amino acid level and proper tissue function both *in vivo* and *ex vivo*. Accordingly, taurine levels fluctuate in mdx muscles in relation to the disease phase, with compensatory increases being suggested after acute degenerative phases and glucocorticoid treatment [28–30]. Future studies will further evaluate the role of taurine as a pathology modi-
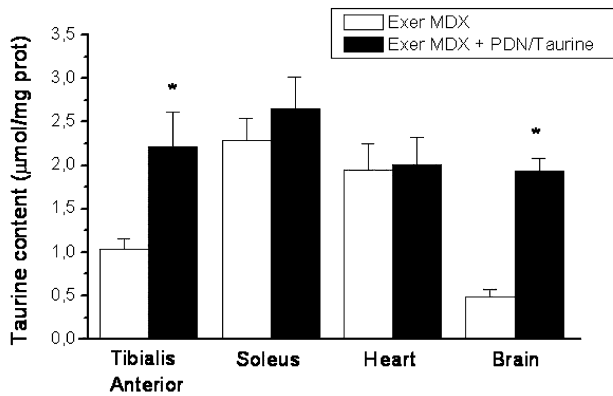
Figure 4. Tissue level of taurine after combined drug treatment. (**A**) HPLC determination of taurine content in tissue from untreated and PDN + taurine-treated animals. Each bar is the mean ± SEM from 3–6 samples. *Significantly different from untreated controls by means of Student's *t*-test.

fier as well as a biomarker. However, the significant increase in amino acid content presently observed on combined treatment shows that taurine can be effectively up-taken by fast-twitch muscle, in line with previous observations [45], and that this mechanism may account for the amelioration of excitation-contraction coupling. However, the possible muscle-type and organ-specific actions also have to be taken into account in the overall action of taurine.

The drug combination did not lead to any advantage in terms of plasma levels of CK *vs.* the two drugs alone, while the beneficial effect of taurine on LDH was attenuated. The lack of effect of PDN on muscular enzyme activity in dystrophic subjects has been described, but no data are available about taurine. However, taurine supplementation has been found to reduce plasma levels of LDH and CK in an isoprenaline-induced cardiomyopathy model [47]. Thus, our result suggests that taurine controls metabolic distress in exercised dystrophic animals, being less effective on a marker of sarcolemmal weakness such as CK. The correlation between muscle damage and level of muscular enzymes in the blood stream is puzzling. In fact, many drugs acting as anti-inflammatory and/or antioxidant, or strategies able to enhance dystrophin, may exert a membrane protective effect leading to a significant reduction of CK, in parallel with histological evidence of decreased dystro-pathology signs [15,33,35]. However, in the absence of a specific membrane effect of the drug, an increased muscular activity due to an improved muscle function may also maintain elevated levels of CK. Thus,

the evaluation of the histology profile was of importance to better verify the outcome of the present treatments. Interestingly, the combined drug treatment did not show any clear advantage on histology profile, with effects rather similar, if not smaller, than those observed by PDN alone. Thus, the results suggest that the amelioration of *in vivo* and *ex vivo* functional parameters are indeed related to the increased levels of the aminoacid and its action on calcium homeostasis, while the protection against dystrophic degeneration is mainly due to the action of PDN.

The lack of clear synergism on muscle morphology reinforces the interest of detailed studies for assessing the effect of taurine *per se* on histology profile, meanwhile opening general considerations about the real therapeutic advantage of the drug combination, also in relation to potential drug interactions. One such issue is related to drug-metabolizing enzymes. Glucocorticoids are mainly metabolized via phase I reaction involving the cytochrome P-450 3A4 and may also act as a potent inducer via glucocorticoid receptor [48,49]. Although little information is available on the effect of taurine on drug metabolism, it is worth-mentioning that it can act as a positive modulator of cytochrome P-450 3A4 induction [50]. Then, over a long term of combined treatment with both drugs, an acceleration of drug metabolism may occur, potentially leading to a reduction of the therapeutic level of steroids in the patients. This possibility is merely speculative in light of the present data and we cannot exclude that a longer treatment with both drugs would be actually required to observe a greater effect on muscle histology as well as on other parameters, such as the heart function. In fact, taurine supplementation might exert a long-term protection over prednisolone-aggravated dystrophic cardiomyopathy, an effect that could be observed in older mdx mice [23,40].

The present data provide promising early evidence about potential benefits in associating PDN with taurine to enhance muscular function in dystrophic subjects; however, longer protocols are required to better understand the therapeutic advantage over the long-term use and to rule out the occurrence of any adverse outcome.

Acknowledgements

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