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Oxandrolone protects against the development of multiorgan failure, modulates the systemic inflammatory response and promotes wound healing during burn injury

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ABSTRACT

Oxandrolone is a synthetic oral non-aromatizable testosterone derivative. This drug has been used successfully for several decades to safely treat growth delays in various diseases including Turner's syndrome. Currently the use of oxandrolone is under clinical testing in children with burn injury; the available data indicate that the anabolic steroid increases net muscle protein balance, maintains lean body mass, and reduces intensive care unit stay. Although oxandrolone is already in clinical trials in burn patients, preclinical burn-related studies with oxandrolone– especially those that go beyond muscle-related parameters and focus on burn-associated organ dysfunction, inflammatory response and wound healing– remain to be conducted. In the current project, using a well-characterized murine model of third-degree burn, we have tested the effect of oxandrolone on indices of organ injury, clinical chemistry parameters and plasma levels of inflammatory mediators. In oxandrolone-treated mice (1 mg/kg/day for up to 21 days) there was a significant amelioration of burn-induced accumulation of myeloperoxidase levels in heart and lung (but not the liver and kidney) and significantly lower degree of malon dialdehyde accumulation in the liver (but not the heart, lung and kidney). Oxandrolone-treated mice showed a significant attenuation of the burn-induced elevation in circulating alkaline aminotransferase and amylase levels, while blood urea nitrogen and creatinine levels remained unaffected, indicative of protective effects of the anabolic hormone against burn-induced hepatic and pancreatic (but not renal) functional impairment. Multiple burn-induced inflammatory mediators (TNF-α, IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-12, IP-10, G-CSF, GM-CSF and interferon-γ) were significantly lower in the plasma of oxandrolone-treated animals after burn injury than

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, one-way analysis of variance; BUN, blood urea nitrogen; H_2S, hydrogen sulfide; IL, interleukin; IFN, interferon; MDA, malon dialdehyde; MPO, myeloperoxidase; PBS, phosphate buffered saline; TNF, tumor necrosis factor.

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in the plasma of controls subjected to burns. Finally, oxandrolone significantly accelerated burn wound healing. We conclude that oxandrolone improves organ function, modulates the systemic inflammatory response and accelerates wound healing in a murine model of burn injury.

1. Introduction

Oxandrolone is a synthetic oral non-aromatizable testosterone derivative. When compared to testosterone, this drug possesses significantly lower virilizing activity. Stimulation of protein synthesis and anabolism is viewed as the primary mode of oxandrolone's action. This action takes place mainly through an action on androgen receptors in the skeletal muscle. Oxandrolone is viewed as a safe therapeutic agent in pediatric patient populations, and it is clinically approved to counteract growth delays in Turner's syndrome and several other growth-related conditions. Oxandrolone is also used clinically to stimulate anabolism in patients suffering from various muscle wasting disorders (including infections, neuromuscular disorders and AIDS) [1,2].

An accumulating body of clinical data supports the efficacy and safety of oxandrolone in adult burn patients [3–6]. Moreover, in pediatric burn patients, oxandrolone was also found to be safe and efficacious: it increases the net muscle protein balance, sustains lean body mass, improves muscle strength, and reduces the length of stay in the intensive care units [7–15].

Most of the clinical studies published so far focused on anabolic and muscle-related actions of oxandrolone, while less attention has been paid on the effects of oxandrolone on the function of parenchymal organs, or on the inflammatory response, or on wound healing. In fact, not only clinical, but also preclinical studies with oxandrolone are rather limited on these areas. Therefore, in the current project, using a well-characterized murine model of third-degree burn, we have tested the effect of oxandrolone on

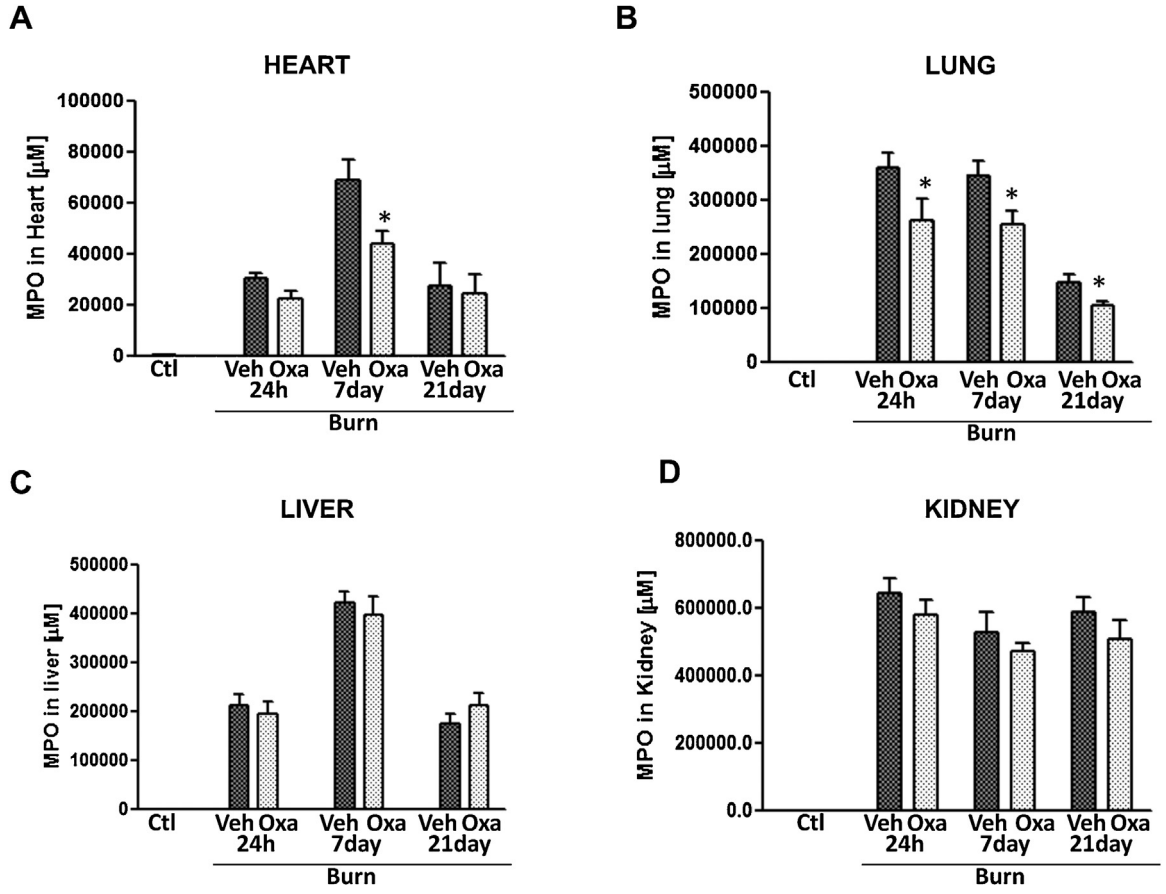

Fig. 1 – Effect of oxandrolone treatment in burn-induced increases in heart, lung, liver and kidney MPO levels. (A) heart, (B) lung, (C) liver and (D) kidney MPO levels are shown in sham-control mice, in mice subjected to burn injury for and in burn mice treated with oxandrolone (1 mg/kg., i.p.) once a day for 1 day, 7 days and 21 days. Data are shown as mean ± SEM of 10 animals/group; *$p < 0.05$ shows a significant effect of oxandrolone in burn animals compared to burn alone.

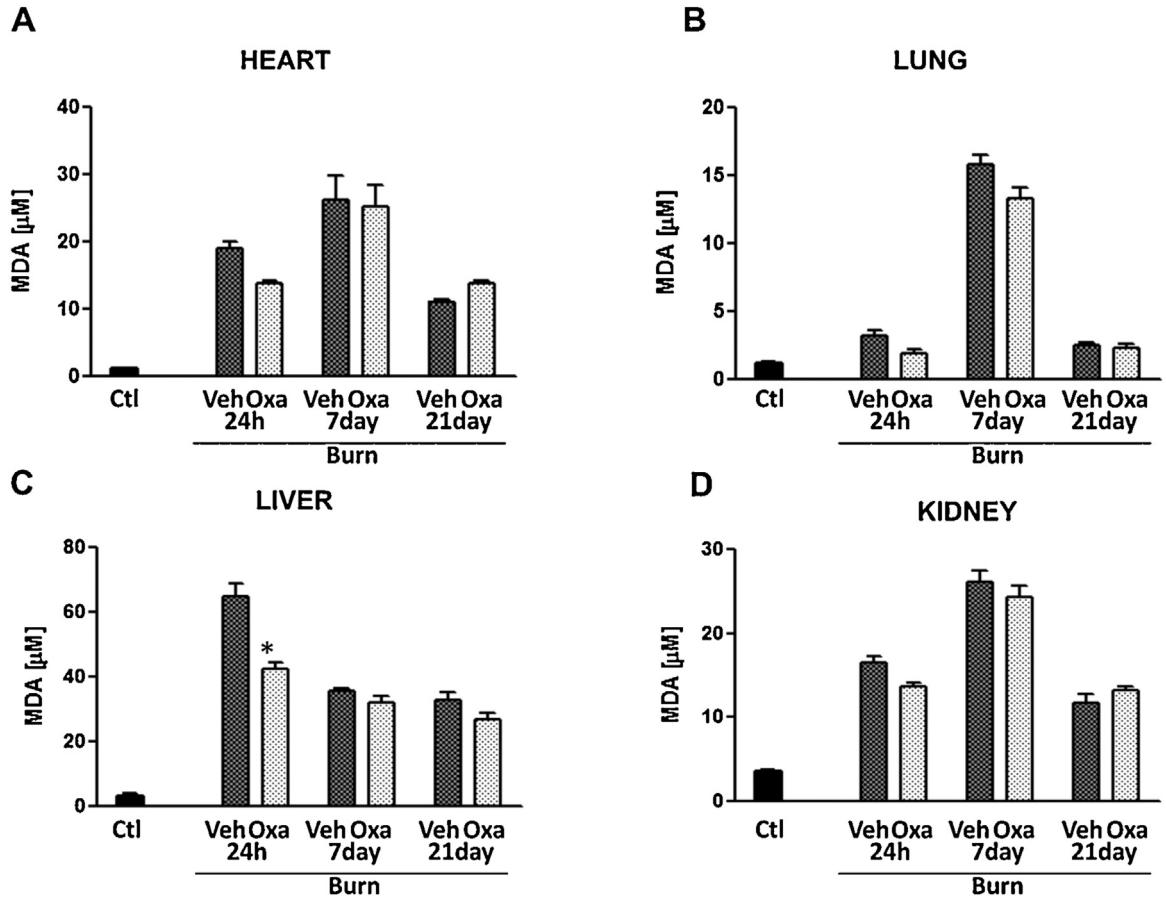

Fig. 2 – Effect of oxandrolone treatment in burn-induced increases in heart, lung, liver and kidney MDA levels. (A) heart, (B) lung, (C) liver and (D) kidney MDA levels are shown in sham-control mice, in mice subjected to burn injury for and in burn mice treated with oxandrolone (1 mg/kg., i.p.) once a day for 1 day, 7 days and 21 days. Data are shown as mean ± SEM of 10 animals/group; *$p < 0.05$ shows a significant effect of oxandrolone in burn animals compared to burn alone.

indices of organ injury, clinical chemistry parameter, plasma levels of inflammatory mediators and the rate of wound healing. The data presented in the current report support the safety and efficacy of oxandrolone, because they show that oxandrolone improves the function of the liver, attenuates the production of multiple inflammatory mediators and accelerates wound healing.

2. Materials and methods

2.1. *Materials*

The chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. *Animals and experimental design*

Ten-twelve weeks old BALB/c mice (male) were housed at 24–26 °C on a 12:12 light: dark cycle. Animals were subjected to burn injury as previously described [16]. Sham and burned mice were subjected to the same experimental procedures (with the exception of injury). After an intraperitoneal (i.p.) injection of buprenorphine (0.1 mg/kg), mice were anesthetized by isoflurane inhalation. Forty percent of the dorsum was shaved and ~1cc of lactated Ringer's solution was injected under the skin along the spinal column. The dorsum of the animals was then subjected to ~95 °C water (duration: 10 s) to induce a full-thickness scald wound covering ~30% of the total body surface area. Resuscitation followed using 2 cc of lactated Ringer's solution. Animals were individually housed during the experiment. In order to reduce suffering, pain or distress, mice were scored twice daily during the experiment. The scoring utilized an IACUC-approved Rodent Intervention Score Sheet. Buprenorphine was administered when indicated, in order to reduce pain and distress of the animals.

N=60 animals subjected to burn were treated with either vehicle or oxandrolone (1 mg/kg/day i.p.) once a day. Animals (n=10/group) were sacrificed at 24 h or on the 7th day or on the 21st day under anesthesia. In turn, blood, heart, lung, liver and kidneys were collected for subsequent analysis. On Day 21, the size of the burn wound (relative the original wound size) was also quantified.

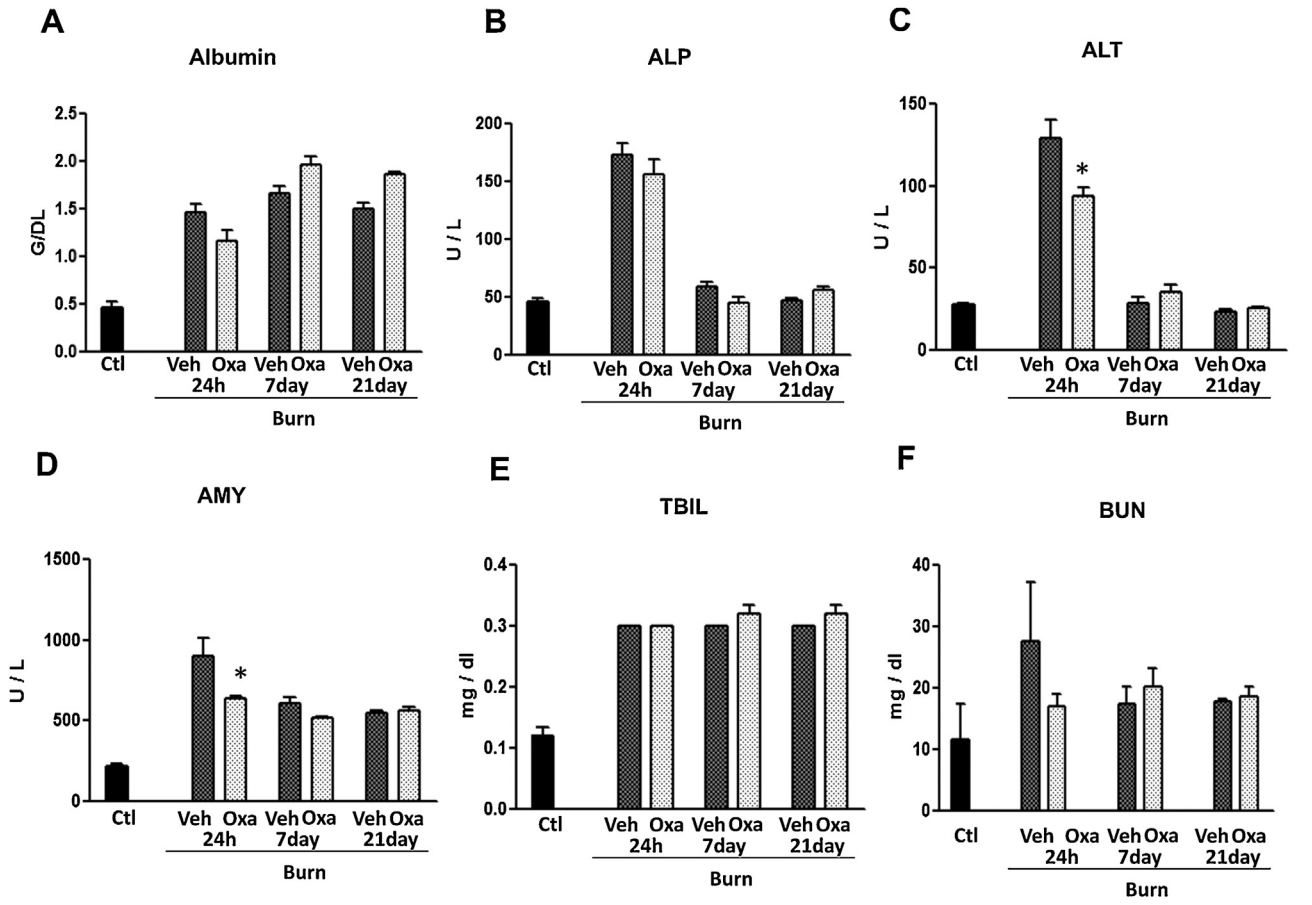

Fig. 3 – Oxandrolone reduces the burn-induced increases in selected parameters of organ injury. Various physiological and organ injury marker levels (albumin, alkaline phosphatase [ALP], alanine aminotransferase [ALT], amylase [AMY], total bilirubin (TBIL), plasma globulin [GLOB]) measured by Vetscan analysis, are shown in sham-control mice, in mice subjected to burn injury and in burn mice treated with oxandrolone (1 mg/kg., i.p.) once a day for 1 day, 7 days and 21 days. Data are shown as mean ± SEM of 10 animals/group; *$p < 0.05$ shows a significant effect of oxandrolone in burn animals compared to burn alone.

All investigations confirm to the Guide for the Care and Use of Laboratory Animals published by the NIH and was conducted after the protocol was approved by the local IACUC.

2.3. *Myeloperoxidase (MPO) assay*

The activity of MPO (an indicator of polymorphonuclear cell infiltration into the tissues) was measured in tissue homogenates using Enzo Life Sciences' fluorometric detection kit [16].

2.4. *Malondialdehyde (MDA) assay*

MDA levels in tissue homogenates were quantified and used to assess the degree of oxidative stress using Enzo Life Sciences' fluorometric detection kit [16].

2.5. *Measurement of biochemical parameters of organ dysfunction*

Blood was collected via cardiac puncture. Within one hour of collection, the samples were subjected to analysis via the Vetscan apparatus [16] which measures various biochemical parameters and indices of organ function. Troponin-I levels in the plasma (used as an indicator of skeletal muscle damage) were measured by Life Diagnostics, Inc.'s ELISA kit.

2.6. *Plasma cytokine quantification*

Blood was centrifuged at 4 °C for 10 min at 1000 g. The Luminex system and Invitrogen's Mouse Cytokine Magnetic 10-plex Panel kit was used as described [16] to quantify the plasma levels of TNF-α, IL- α, IL-β, IL-2, IL-3, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, KC, MIP-1 α, IP-10, INF-γ, G-CSF, GM-CSF and VEGF.

2.7. *Statistical analysis*

Numerical values were expressed as mean values ± SEM. Student's t-test, one-way and two-way ANOVA with Tukey's post-hoc test were utilized to detect difference between groups. Statistical analysis was conducted with the Graphpad software. Statistical significance was accepted when $p < 0.05$.

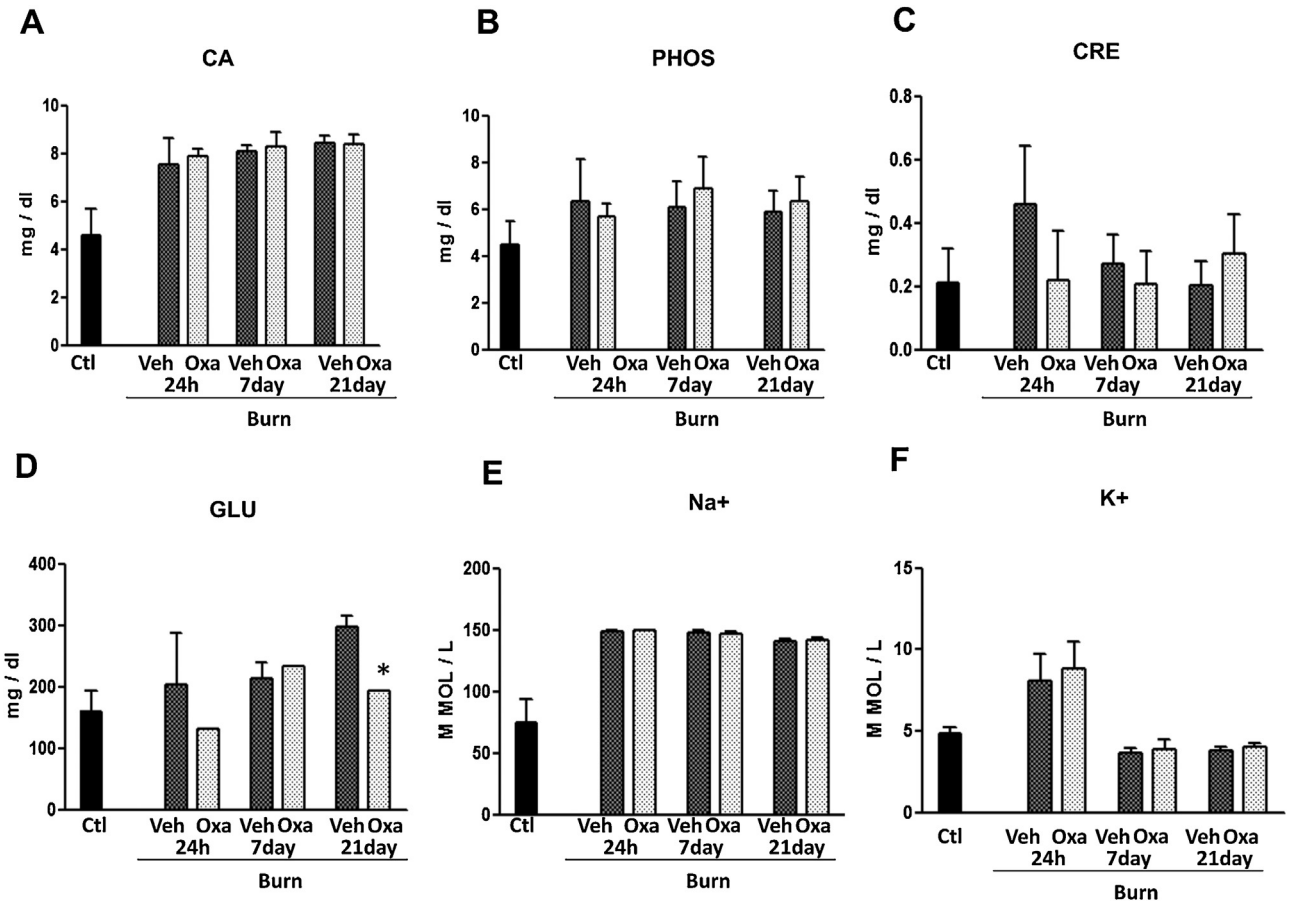

Fig. 4 – Oxandrolone reduces the burn-induced increases in selected parameters of organ injury. Various physiological and organ injury marker levels (plasma calcium [CA], plasma phosphate [PHOS], plasma creatinine [CRE], plasma glucose [GLU], plasma sodium [Na+] and plasma potassium [K+]) measured by Vetscan analysis, are shown in sham-control mice, in mice subjected to burn injury and in burn mice treated with oxandrolone (1 mg/kg., i.p.) once a day for 1 day, 7 days and 21 days. Data are shown as mean ± SEM of 10 animals/group; *$p < 0.05$ shows a significant effect of oxandrolone in burn animals compared to burn alone.

3. Results

Burn induced a marked increase in the levels of MDO and MDA in all tissues studied (lung, liver, heart and kidney), indicating the infiltration of these tissues with inflammatory cells, and the presence of increased oxidative stress in these tissues, respectively (Figs. 1 and 2). The burn-induced increases in heart MPO levels were lower in the oxandrolone-treated mice than in the vehicle-treated mice both at 24 h after-burn (Fig. 1), while the burn-induced increases in lung MPO levels were lower in the oxandrolone-treated mice than in the vehicle-treated mice at all three time points studied (24 h, 7 days, 21 days). The increases in liver and kidney MPO levels were not affected by oxandrolone (Fig. 1). The burn-induced increases in heart, lung and kidney MDA levels were not affected by oxandrolone; however, at 24 h after-burn, oxandrolone reduced MDA levels in the liver (Fig. 1).

Burn also induced significant alterations in the clinical chemistry parameters and organ injury markers. Increases were noted in the levels of the hepatic/bone injury marker ALP, the hepatic injury marker ALT, the pancreatic injury marker amylase, and the renal dysfunction markers creatinine and blood urea nitrogen (BUN) (Figs. 3 and 4). Many of these parameters peaked at 24 h. Oxandrolone treatment attenuated plasma ALT and amylase levels at 24 h (Fig. 3). The other clinical chemistry parameters remained largely unaffected by oxandrolone. Importantly, oxandrolone did not induce any worsening of any of the clinical chemistry parameters, indicating that the drug – at least at the dose level applied in the current study – does not exert any observable adverse effects on organ function (Fig. 3,4).

In line with the well-known development of the systemic inflammatory response during burns, we have detected marked increases in the plasma levels of TNF-α, IL-1α IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, KC, MIP-1α, IP-10 and IFN-γ. Oxandrolone treatment reduced the plasma levels of several of these mediators; these inhibitory effects were the most pronounced at 24 h (Figs. 5–7). Interestingly, oxandrolone also attenuated the

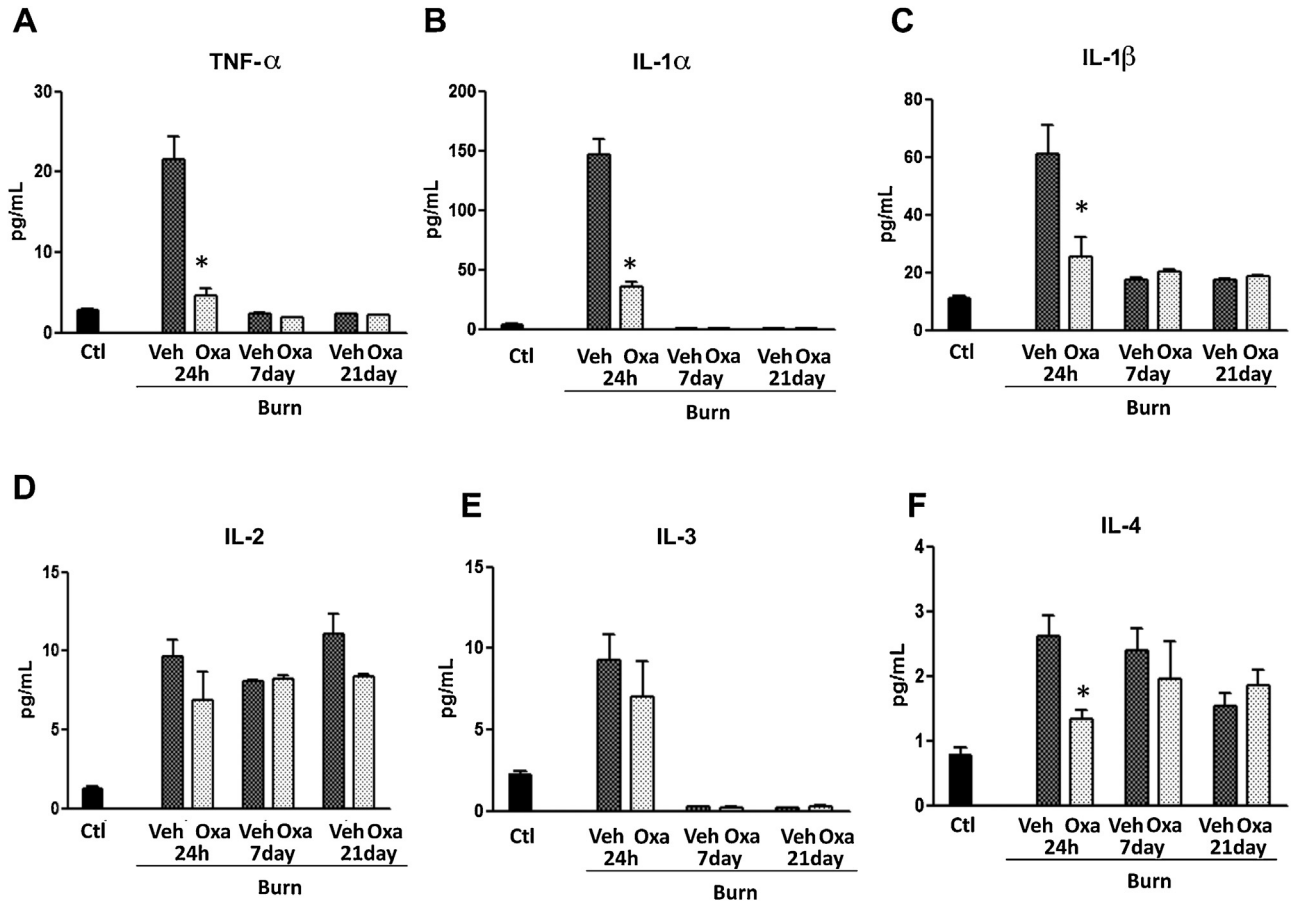

Fig. 5 – Oxandrolone reduces the burn-induced increases in plasma cytokine levels. Plasma cytokine levels, measured by Luminex analysis, are shown in sham-control mice, in mice subjected to burn injury and in burn mice treated with Oxandrolone (1 mg/kg., i.p) once a day for 1 day, 6 day and 20 days to three different set of groups. Burn markedly increased plasma TNF-α, IL-1α, IL-1β, IL-2, IL-3 and IL-4 levels; the increases in TNF-α, IL-1α, IL-1β and IL-4 were reduced by oxandrolone treatment. Data are shown as mean ± SEM of 10 animals/group; *$p < 0.05$ shows a significant effect of oxandrolone in burn animals compared to burn alone.

plasma G-CSF and GM-CSF levels after-burn, but did not significantly influence the plasma levels of the angiogenic hormone VEGF (Fig. 8).

Oxandrolone also exerted a slight, but statistically significant inhibitory effect on plasma levels of troponin-I (indicative of modest protective effect against the skeletal muscle damage) (Fig. 9). In addition, oxandrolone-treated mice exhibited a faster wound healing response than vehicle-treated animals, quantified at the termination of the experiments (21 days) (Fig. 10).

4. Discussion

Despite of its common clinical use, the mode of oxandrolone's action (as well as the action of the other anabolic steroids) remains incompletely understood. While it is generally accepted that the principal mode of oxandronole's pharmacological action occurs through binding to the androgen receptor (a nuclear receptor) [17], additional mechanisms may also be involved. For example, a competitive antagonism at the glucocorticoid receptor and/or a suppression of glucocorticoid action via crosstalk between the androgen and glucocorticoid receptors may also be involved [18]. Moreover, a recent study, using a phenotypic similarity screening approach, has identified the PKR2 (prokineticin receptor 2 protein) as another potential receptor target for oxandrolone [19].

Slight modifications in anabolic steroid structure can lead to drastically different biological effects in vitro and in vivo. This is well illustrated by Mendenhall's study, which compared the effect of testosterone, testosterone propionate, testolactone, oxandrolone, and stanozolol on the immune function in rats. This study clearly established that– while in the short term, all of the drugs tested produce immunosuppressive effects (and to a comparable degree)– later on (after 10 days of treatment), some of the tested drugs (including oxandrolone) revert to immunostimulatory actions, while other drugs remain immunusuppressive [20]. These data indicate that each anabolic steroid molecule should be studied on its own right: prior findings with other members of the

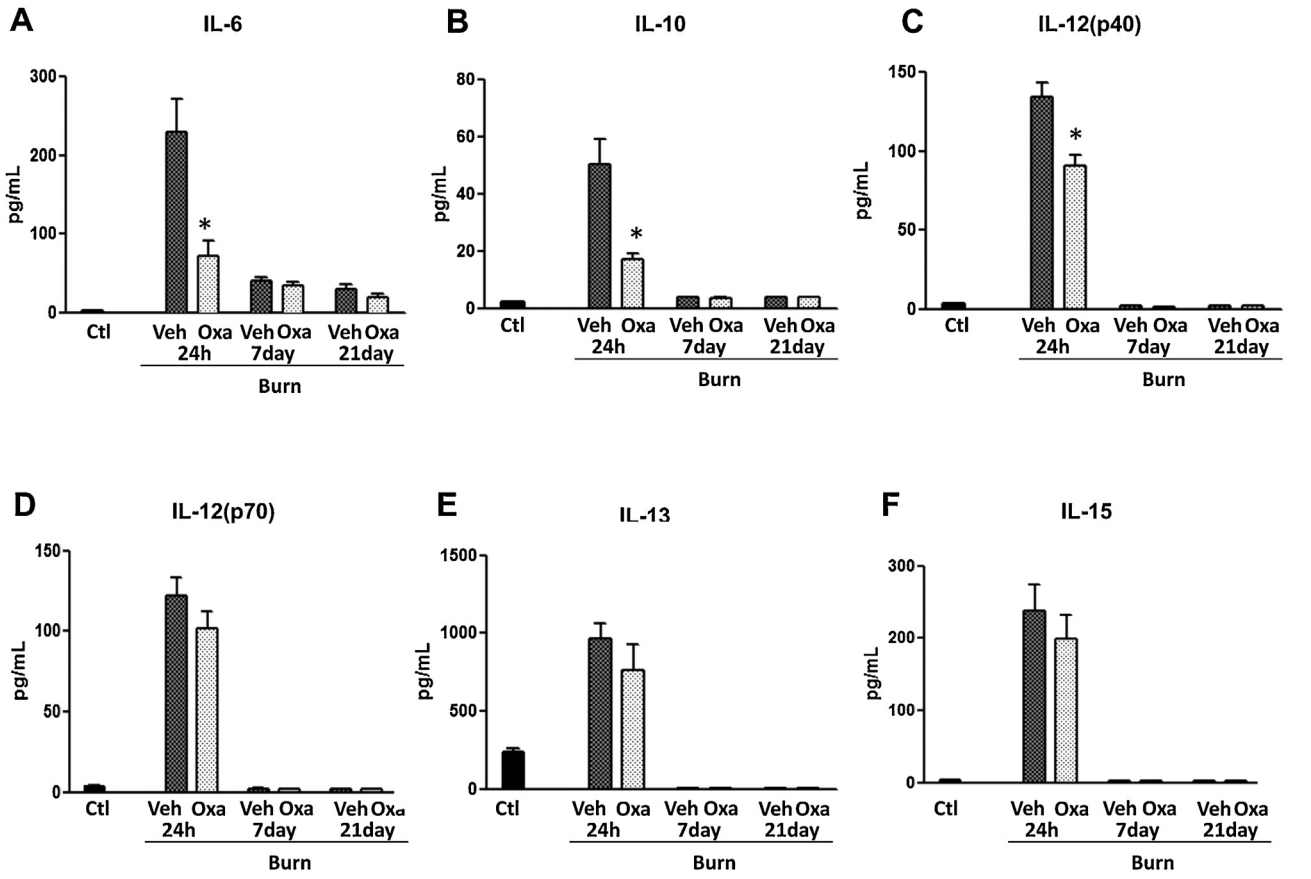

Fig. 6 – Oxandrolone reduces the burn-induced increases in plasma cytokine levels. **Plasma cytokine levels, measured by Luminex analysis, are shown in sham-control mice, in mice subjected to burn injury and in burn mice treated with oxandrolone (1 mg/kg., i.p) once a day for 1 day, 7 day and 21 days to three different set of groups. Burn markedly increased plasma IL-6, IL-10, IL-12(p40), IL-12(p70), IL-13 and IL-15 level; the increases in IL-6, IL-10 and IL-12(p40) were reduced by oxandrolone treatment. Data are shown as mean ± SEM of 10 animals/group; *$p < 0.05$ shows a significant effect of oxandrolone in burn animals compared to burn alone.**

anabolic steroid class may not be generally applicable to other members of the anabolic steroid class.

In this context, it is important to emphasize that – while there are many prior reports on some members of the anabolic steroid class, chiefly, testosterone – on organ function, inflammatory mediator production and wound healing, a similar evaluation of oxandrolone on the same parameters has been largely missing. This is even more surprising when we consider the fact that oxandrolone is widely used in various clinical conditions and is currently being evaluated in various forms of burn injury and may be on the way to become a standard of clinical therapy (see Section 1). With these considerations in mind, we have now evaluated the effect of oxandrolone in a well-characterized mouse model of burn-induced inflammation and multiple organ failure. The dose of oxandrolone (1 mg/kg/day) selected for the study is higher than the dose typically used in humans (0.1 mg/kg), but it is the correct one, when considering the mouse-to-human HED (Human Equivalency Dosing) calculations [21]. Similar to our study, prior studies in rodents that evaluated the effect of oxandrolone on various metabolic and immune parameters utilized doses of 1 mg/kg or higher (e.g. Refs. [20,22,23]).

The first conclusion of our study is that oxandrolone – at least at the dose used in the current study, and in the time frame of the current study (1 mg/kg/day, 21 days) – failed to induce any adverse effects on any of the clinical chemistry parameters and indices of organ injury. Thus, the findings indicate that the anabolic steroid, when applied at a reasonable dose, may be safely tolerated. The second conclusion is that oxandrolone exerts organ-specific effects in burn injury. While in some organs (e.g. kidney), oxandrolone failed to affect any of the related functional (e.g. BUN, creatinine) or biochemical (MPO, MDA) parameters, in other organs (e.g. the liver), we noted beneficial effects, both in terms of functional parameters (ALT plasma levels) and biochemical (MDA) parameters. The reduction in MDA (a marker of oxidative damage) is independent of a reduction to MPO (which indicates that oxandrolone suppresses oxidative stress in this organ through mechanisms other than inhibiting the accumulation of neutrophils in this organ). If the reduction in tissue MDA was secondary to a generalized reduction of pro-

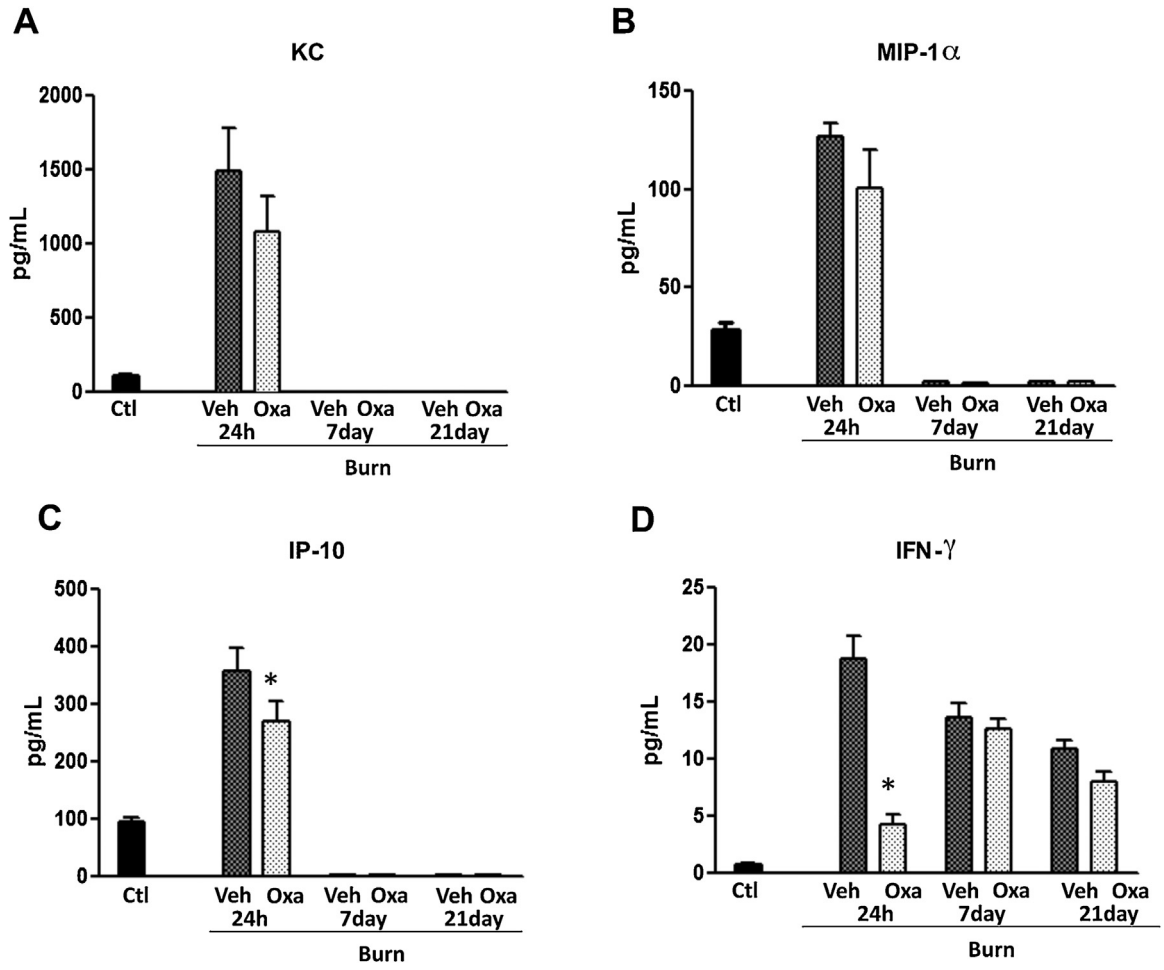

Fig. 7 – Oxandrolone reduces the burn-induced increases in plasma cytokine and chemokine levels. Plasma cytokine and chemokine levels, measured by Luminex analysis, are shown in sham-control mice, in mice subjected to burn injury and in burn mice treated with oxandrolone (1 mg/kg., i.p) once a day for 1 day, 7 day and 21 days to three different set of groups. Burn increased plasma KC, MIP-1α, IP-10 and IFN-γ, levels; the increases in IP-10 and IFN-γ were reduced by oxandrolone treatment. Data are shown as mean ± SEM of 10 animals/group; *$p < 0.05$ shows a significant effect of oxandrolone in burn animals compared to burn alone.

inflammatory mediator production (as suggested from the changes in plasma levels of multiple inflammatory mediators in oxandrolone-treated mice subjected to burn) then we would have expected a generalized effect on MPO and MDA levels in multiple organs studied. However, this was not the case: the beneficial effects of oxandrolone appear to be restricted to selected parameters in each of the tissues studied. We hypothesize that organ-specific effects of oxandrolone may reflect local actions of the hormone, perhaps driven by a differential expression of receptors that oxandrolone binds to in various tissues.

Oxandrolone also exerted significant inhibitory effects on the production of many (but not all) circulating inflammatory mediators. The effect of oxandrolone does not appear to be distinguish between pro-inflammatory (e.g. TNFα, IL-1β) and anti-inflammatory (e.g. IL-10) mediators, and, thus, it appears to be directionally different from the effect of glucocorticoids, which, in many models of critical illness/systemic inflammation, suppress pro-inflammatory mediators (e.g. TNF-α) while enhance anti-inflammatory ones (e.g. IL-10) (e.g. Refs. [24,25]). Circulating inflammatory mediators during burn injury are produced by many cells and tissues (not only circulating leukocytes but also parenchymal cells), and oxandrolone is likely to affect some (or many) of these cells and tissues. It is curious – and currently unexplained – how oxandrolone inhibits the production of some (but not other) mediators that are believed to be under the control of the exact same signaling pathway and transcriptional mechanism. For instance, the two subunits of IL-12 are controlled by the same transcription factors [26], and yet only one of them was significantly suppressed in our experiments.

The beneficial effect of oxandrolone on wound healing is interesting, both from the basic science point of view, as well as with respect to its potential clinical applicability. Similar to our findings, in a rat model of linear incision wound healing, Demling has previously reported the acceleration of the wound healing in response to oxandrolone treatment [27]. As far as we can determine, the current report and the paper by

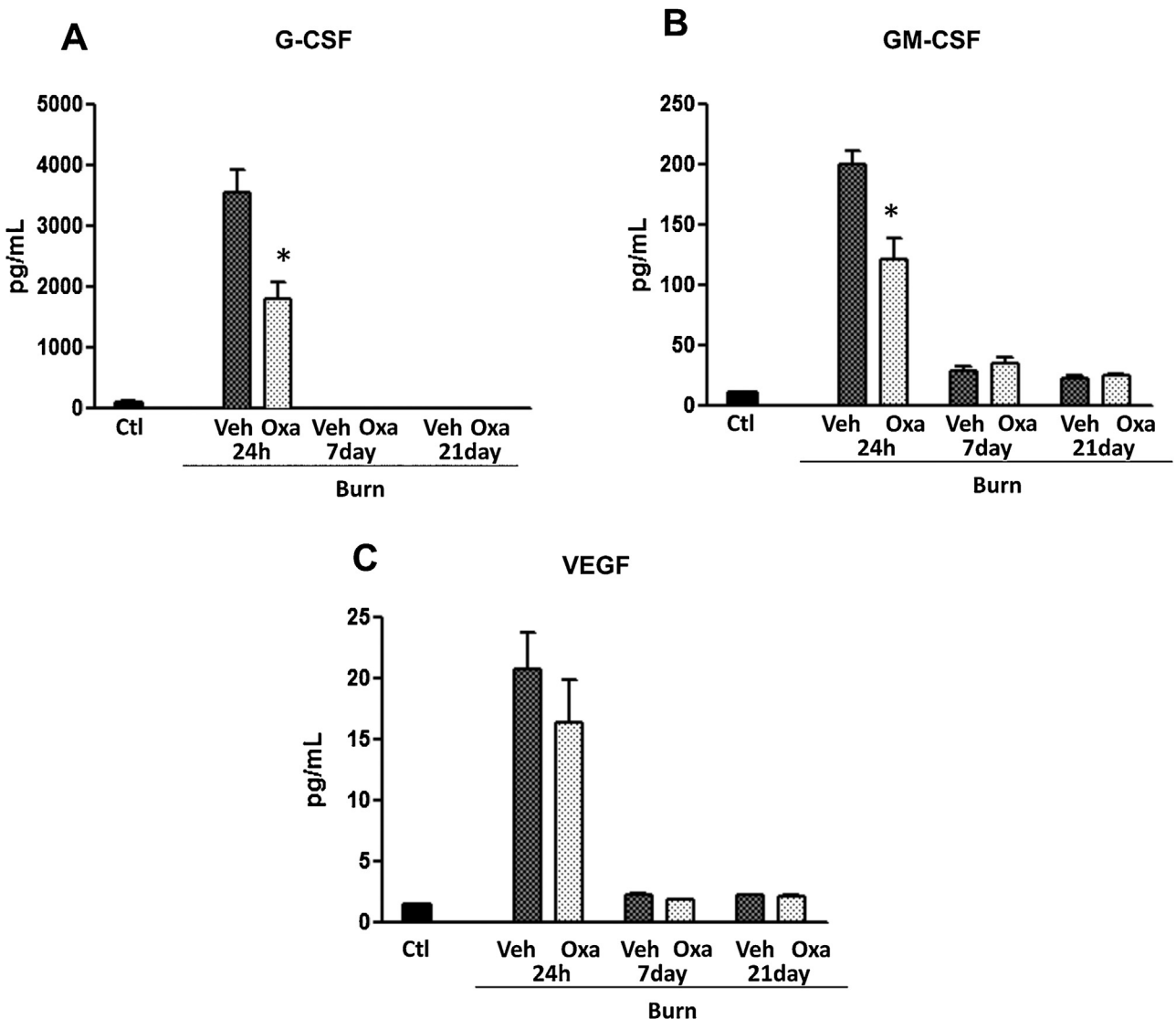

Fig. 8 – Oxandrolone reduces the burn-induced increases in plasma colony-stimulating factor and growth factor levels. Plasma cytokine and chemokine levels, measured by Luminex analysis, are shown in sham-control mice, in mice subjected to burn injury and in burn mice treated with oxandrolone (1 mg/kg., i.p) once a day for 1 day, 7 day and 21 days to three different set of groups. Burn increased plasma G-CSF, GM-CSF and VEGF levels; the increases in G-CSF and GM-CSF were reduced by oxandrolone treatment. Data are shown as mean ± SEM of 10 animals/group; *$p < 0.05$ shows a significant effect of oxandrolone in burn animals compared to burn alone.

Demling are only two preclinical studies evaluating the effect of oxandrolone on wound healing. In addition, there is some small-scale clinical evidence that oxandrolone can accelerate the healing of pressure ulcers [28] and burn wounds [29]. None of the studies currently available in the literature have determined the exact molecular mode of the steroid's action in these models; it is generally assumed that a generalized acceleration of protein synthesis may be – at least in part – responsible for the observed effects.

Whether the findings obtained in the current animal model have clinical/translational implications remains to be seen. There are insufficient data in the literature to answer the question as to whether oxandrolone in burn patients exerts beneficial effects on hepatic function. The improvement in biochemical parameters in the lung noted in the current study is consistent with a recent report showing that long-term treatment of pediatric burn patients with oxandrolone exerts beneficial effects on the lung function [14]. The effect of oxandrolone on pro-inflammatory mediator production has only been evaluated in one study in burn patients; this report failed to notice significant effects of the steroid, although a trend for a faster return to baseline was apparent for the plasma levels of TNF-α and IL-1β over the course of the 40 days after-burn [10]. As far as the effect of oxandrolone on the rate of wound healing, once again, further work will be needed to confirm and extend the existing small-scale clinical study [29],

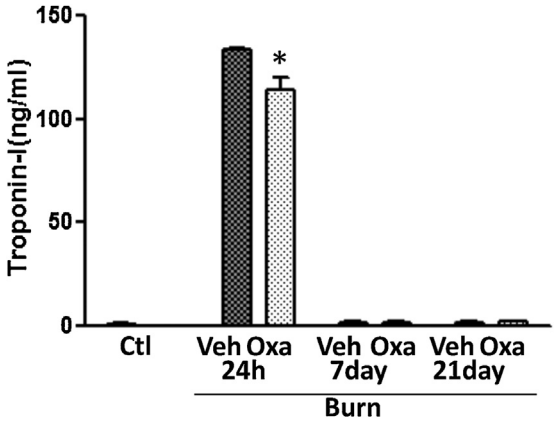

Fig. 9 – Oxandrolone reduces the burn-induced increases in plasma troponin-I levels. Plasma troponin-I levels, measured by commercial available kit, are shown in sham-control mice, in mice subjected to burn injury and in burn mice treated with oxandrolone (1mg/kg., i.p) once a day for 1day, 7day and 21 days. Burn markedly increased plasma troponin-I levels at 24h; this was reduced ($^{*}p<0.05$) by oxandrolone treatment. Data are shown as mean±SEM of 10 animals/group.

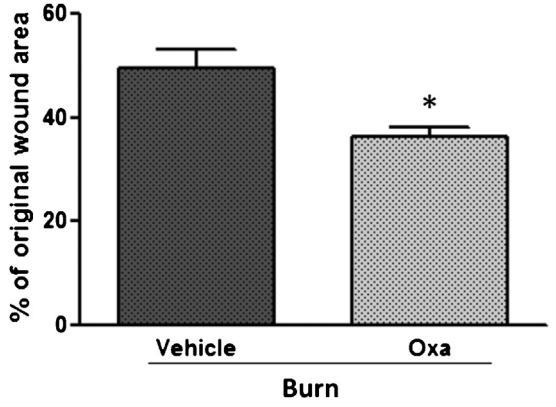

Fig. 10 – Oxandrolone accelerates the healing of the burn wound. % wound area is shown on Day 21 in the vehicle control group and in burn mice treated with oxandrolone (1mg/kg., i.p). Oxandrolone significantly ($^{*}p<0.05$) reduced the % wound area in animals, indicative of stimulation of the wound healing process. Data are shown as mean±SEM of 10 animals/group.

in order to determine whether oxandrolone has clinically significant beneficial effects on the rate of wound healing in the clinical scenario.

Although further work remains to be conducted to address the above points, the current study supports the safety of oxandrolone therapy in burns and supports the notion that this anabolic steroid– in addition to exerting beneficial effects on skeletal muscle mass and function – may also exert additional beneficial effects in terms of modulation of the inflammatory response, restoration of multiorgan function and acceleration of the wound healing process.

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Conflict of interests

The authors declare no conflicts of interest in relationship to this study.

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