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RESEARCH ARTICLE

Preclinical Study Exploring the Effects of Creatine Supplementation on Renal Antioxidant Enzymes Following Doxorubicin Treatment

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ABSTRACT

Introduction: Doxorubicin is an effective chemotherapy drug, but its use is limited by its cytotoxicity. One of doxorubicin's anticancer mechanisms is generation of reactive oxygen species which may lead to oxidative stress. The kidney, however, is very vulnerable to oxidative stress, and one way to manage oxidative stress is to scavenge reactive oxygen species via antioxidant enzymes. Although doxorubicin-induced oxidative stress has been extensively studied, a viable treatment to attenuate doxorubcin side effects has yet to be found. This study investigated the effect of creatine feeding on catalase, glutathione peroxide, and superoxide dismutase-1 expression in the kidney following doxorubicin treatment.

Methods: Twenty-eight male Sprague-Dawley rats were randomly assigned to four groups, control saline (C+SAL, n=7), control doxorubicin (C+DOX, n=7), creatine saline (Cr+SAL, n=6) and creatine doxorubicin (Cr+DOX, n=8). Control groups were fed normal chow, and creatine groups were fed chow supplemented with 3% creatine. After two weeks of feeding, doxorubicin groups received15 mg/kg doxorubicin whereas saline groups received saline as a placebo. Western blotting was used to access antioxidant enzyme expression in renal tissue.

Results: A significant between group difference was observed with catalase expression, but *post hoc* testing did not reveal where differences existed. A trend existed toward doxorubicin treatment increasing catalase expression and creatine attenuated this trend. Glutathione peroxidase and superoxide dismutase-1 presented a similar profile as catalase; however, no significant between group differences were observed. There was a trend, however, toward increased expression of glutathione peroxidase and superoxide dismutase-1 in doxorubicin-treated animals that seemed to be attenuated with creatine supplementation.

Conclusion: To our knowledge there are no studies exploring the antioxidant properties of creatine supplementation in the kidney with doxorubicin, and it is possible that creatine may enhance antioxidant properties that can attenuate the negative effects doxorubicin in the kidney. A trend towards antioxidant enzyme normalization promoted by creatine with doxorubicin suggests that creatine might have similar effect to that observed in previous studies using antioxidant drugs.

Keywords: Doxorubicin, Kidney, Antioxidant, Creatine.

Introduction

Doxorubicin (DOX, trade name Adriamycin®) is an anticancer drug first introduced in the 1970's, and to this date, it is commonly used in the treatment of a wide variety of cancers including, but not limited to, breast cancers, sarcomas, carcinomas, neuroblastoma, non-Hodgkins lymphoma, Hodgkin's lymphoma, and acute leukemia.[1] In spite of DOX's efficiency in combating cancer cells, its use is limited due to its severe cytotoxicity to, among others, cardiac, skeletal muscle, and renal cells with the latter being an important limiting factor to DOX treatment. Sternberg[2] first reported renal injury caused by DOX in the same year the drug was first extracted; however, the first description of DOX nephrotoxicity in rats was published later in 1976.[3]

One of the mechanisms by which DOX combats cancer cells is generation of free radicals. When the oxidized form of DOX (semiquinone) is converted back to DOX, the process releases reactive oxygen species (ROS), specifically superoxide, causing a disruption in the pro-oxidant-antioxidant balance that may lead to lipid peroxidation and protein oxidation which are capable of promoting membrane damage. This oxidative stress (OS) might also trigger apoptotic pathways leading to cellular death.[1,4]

The kidney is highly vulnerable to OS and many pathological conditions may result in a greater generation of ROS and depletion of antioxidants. Both increased ROS and decreased antioxidants may negatively affect normal functions of the kidneys, but one way to manage OS is to scavenge ROS via antioxidant enzymes. Studies have investigated the profile of antioxidant enzymes both in animals subjected to cisplatin and DOX injections, and both anticancer drugs promoted a decrease in antioxidants.[5] Bertani et al.[6] found that the nephrotic syndrome in rats subjected to a single dose of DOX arises soon after injection, and by the fifth day post-intravenous injection, animals presented elevated proteinuria suggesting that DOX has a direct toxic effect on the kidney.

To this date, the nephropathy associated with DOX treatment is still poorly understood although more recent studies corroborate with earlier findings that attribute the development of renal damage during DOX administration to increased ROS generation. A study using the temporary clamping of one renal artery during DOX injection showed partial protection of the clamped kidney while the unclamped kidney with normal blood flow suffered more intensely from the action of DOX.[7] Another study suggested that DOX might stimulate nitric oxide (NO) production by either endothelial nitric oxide synthase (eNOS) or inducible nitric oxide synthase (iNOS). These findings are important because in large quantities, NO has a cytotoxic effect and can cause nephrotoxicity.[4]

Morphological changes have also been observed following DOX treatment showing focal podocyte proliferation with epithelial adhesions to the Bowman capsule. It has been hypothesized that DOX affects endothelial progenitor cells (EPCs) causing impairment of the kidney's regeneration abilities. Infusion of EPCs in DOX-treated mice led to an increased vascular endothelial growth factor concomitant with decreased cellular apoptosis.[8] Immunohistochemical analysis showed increased number of B lymphocytes and T lymphocytes as well as macrophages cells in the tubulointerstitial area suggesting that an immunological response plays a role modulating DOX-induced renal injury.[9]

Another study investigated the biochemistry of antioxidant enzymes and showed significantly reduced expression of catalase (CAT), glutathione peroxidase (GPx) and glutathione (GSH) in groups exposed to DOX as well as increased protein oxidation which is recognized as one of the mechanisms involved in DOX-induced nephrotoxicity.[4] It should be noted that chronic kidney disease (CKD) is often associated with OS that is usually evidenced by elevated concentrations of lipid peroxidation along with a significant reduction of superoxide dismutase (SOD), CAT, and GPx.[10] Szalay et al.[11] demonstrated that after eight weeks, rats exposed to DOX had significantly less weight gain compared to control animals. Proteinuria and fibrosis were greater in DOX animals, and neutrophil gelatinase-associated lipocalin (N-GAL) excretion was also increased in DOX animals indicating tubular epithelial damage. Also, DOX animals presented a severe inflammatory response showing a large infiltration of lymphocytes and macrophages as well as increased collagen type I expression (COL I), an indicator of fibrosis.

Although the oxidative stress induced by DOX has been extensively studied, new approaches aimed at battling DOX toxicities may contribute to a better understanding and lead to a viable treatment that will attenuate DOX-induced oxidative stress. One such approach involves the use of Creatine (Cr) which is a natural substance, mostly supplied by the diet, with the majority found in skeletal muscle.[12] For a few decades, supplementation with Cr has become common among athletes and sports enthusiasts because many studies have shown that exogenous Cr supplementation can increase performance,[13] and more recently, it has been

reported that Cr may have antioxidant properties. Mathews et al.[14] showed that Cr protected rats in a model of Huntington's disease in a similar way as antioxidant treatment. Lawler and Powers[15] made similar assumptions suggesting that the antioxidant capacity of Cr could explain the higher tolerance to fatigue and the protein turnover seen in skeletal muscle as free radicals are known to affect those parameters. In 2002, the same group reported a direct dose-response relationship between Cr concentrations and antioxidant scavanging capacity with an ability to quench superoxide and peroxynitrite ($OONO^-$).[16]

Sestili et al.[17] concluded that Cr may exert a direct antioxidant effect in cultured mammalian cells acutely injuried with a variety of compounds that generate ROS. Other studies have shown that Cr is capable of inhibiting lipid peroxidation markers induced by exercise in skeletal muscle. Rats receiving Cr supplementation also showed increased antioxidant capacity compared to control animals and reduced GSH and GSH/oxidazed gluthatione ratio (GSSG) with Cr supplementation.[18] These findings corroborate with a study that found a decrease in hydrogen peroxide (H_2O_2) production in skeletal muscle from rats that received Cr supplementation. Because the liver, an organ very susceptible to oxidative stress due to ROS generation, is responsible for synthesizing high amounts of Cr, Araujo et al.[19] investigated the effects of Cr supplementation on oxidative balance and liver antioxidant profile during exercise. The authors reported an indirect antioxidant effect of Cr in rat liver as it was capable of increasing the activity of CAT and GPx despite normalizing the concentration of H_2O_2 induced by exercise. Another study showed that Cr supplementation along with eight weeks of resistance training was able to reduce oxidative stress and positively influence SOD activity in the heart, liver, and gastrocnemius.[20]

Regardless of the beneficial properties of Cr that have been reported within the past few years, the fact that Cr is synthesized in the kidneys and converted to creatinine, a marker of impaired renal function[21] has falsely led studies to suggest that Cr might have a nephrotoxic effect. The effect of Cr in the kidney has been intensely discussed in the literature and findings are very controversial. It has been shown that Cr has a deleterious effect in rats with cystic fibrosis but no collateral effect in exercise trained animals.[22] In addition, no deleterious effects were found in sedentary rats within pre-existing renal failure.[23] Gualano et al.[24] also reported no signs of impaired renal functions in healthy subjects undergoing Cr supplementation.

To our knowledge there are no studies exploring Cr antioxidant properties in the kidney with DOX treatment, and the possibility of a naturally-occurring substance such as Cr with antioxidant properties that could be supplemented to cancer patients undergoing chemotherapy is indeed worth exploring. Therefore, the purpose of this study was to explore the effects of Cr supplementation on kidney antioxidant expression in rats receiving DOX treatment. It was hypothesized that Cr supplementation would attenuate OS in the kidney by altering antioxidant enzyme expression.

Methodology

ANIMAL CARE, DIET, AND DOXORUBICIN TREATMENT

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Northern Colorado and carried out in accordance with the Animal Welfare Act. Twenty-eight male Sprague-Dawley rats were obtained from Harlan (Indianaplois, IN) and housed in an environmentally controlled facility on a 12:12 hour light:dark cycle. Animals were singly housed, and food intake was measured daily in order to ensure animals were eating. 150 g of chow was made available at day one, and whenever less than 40 g was remaining, more chow was added to sum 150 g. All food and distilled water was provided *ad libitum*. Creatine fed animals received regular food (Teklad 2016) supplemented with 3% creatine, and control diet animals (C) received regular food (Teklad 2016) not supplemented with Cr.

After two weeks of C or Cr feeding, animals received intraperitoneal injections (i.p) with either 15 mg/kg doxorubicin hydrochloride (Cr+DOX, C+DOX) or saline at an equivalent volume (Cr+SAL, C+SAL) as a placebo. Five days following injections, each animal was anesthetized using heparinized (100U) sodium pentobarbital (50 mg/kg), and when a tail pinch reflex was absent, the heart was removed and the right kidney was excised. The excised kidney was weighed, cut into three pieces from the renal hilum, frozen in liquid nitrogen, and stored at -80°C for biochemical analysis.

BIOCHEMICAL ANALYSIS

Kidney samples were homogenized with RIPA lysis buffer (Santa Cruz Biotechnology) and protease inhibitor cocktail (Santa Cruz Biotechnology) and total protein concentration was analyzed using the Bradford method.[25] A LPO-586TM Assay kit (OXIS International, Inc.) was used to assess lipid peroxidation of samples, and MDA+HAE

concentration was calculated according to manufacturer's instructions.

Protein concentrations of remaining homogenized solutions were standardized and prepared with Laemmli buffer (Sigma) for Western blotting. PVDF membranes were blocked with milk solution and first incubated overnight with primary antibody for β-Actin (Santa Cruz Biotechnology). The next morning, the primary antibody was removed and membranes were then incubated with a secondary mouse antibody (Santa Cruz Biotechnology) for one hour. An imaging apparatus (Li-Cor) was used to develop probed membranes, and the clearest image was chosen. ImageJ (Wayne Rasband National Institute of Health, USA) was used to quantify protein concentration of bands. β-Actin was used as a loading control and all proteins investigated in the study were corrected to values relative to β-Actin values. After images were collected, membranes were stripped with stripping buffer (Thermo Fischer Scientific) and protocol was repeated for CAT (Abcam), GPx (Abcam) and SOD-1 (Abcam).

STATISTICAL ANALYSIS

All data are presented as mean ± SEM, and parameters were analyzed using a one-way analysis of variance (ANOVA) to determine group differences. When a significant *F*-value was observed, Tukey *post hoc* testing was performed to determine where differences existed. Significance was set at $\alpha=0.05$.

Results

All animals started the study with similar body masses (Figure 1), and at the end of the two and a half week protocol, Cr+DOX body mass was significant lower than Cr+SAL (Figure 2). To evaluate whether DOX animals had lost weight or simply gained less weight than C animals, the change in body mass was calculated (Figure 3). Figure 3 shows a significant decrease in C+DOX and Cr+DOX body masses compared to Cr+SAL. Cr+SAL also gained significantly less weight than C+SAL animals. In addition, the change in body mass in the Cr+DOX group was significantly different than C+SAL with the former displaying a decrease in body mass and the latter showing an increase body mass (Figure 3).

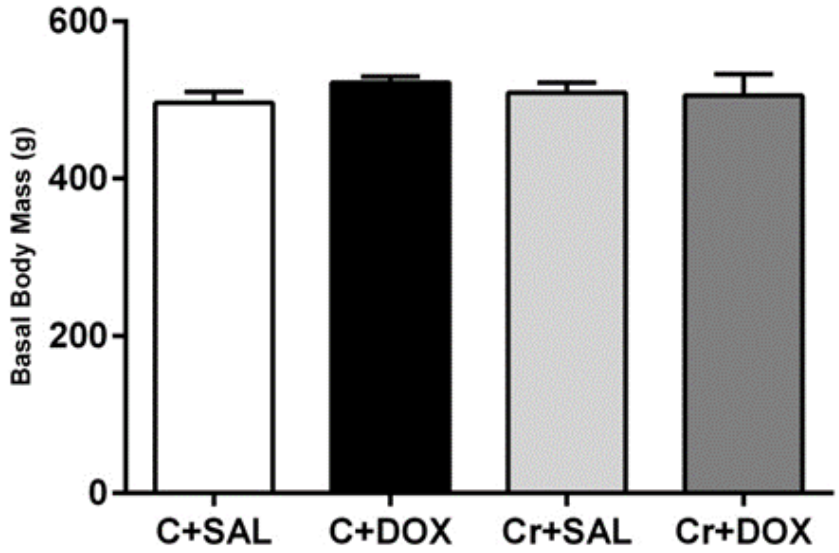

***Figure* 1.** Basal body mass. C+SAL, control+saline; C+DOX, control+DOX; Cr+SAL, creatine+saline and Cr+DOX, creatine+DOX. No significant difference between groups ($p>0.05$).

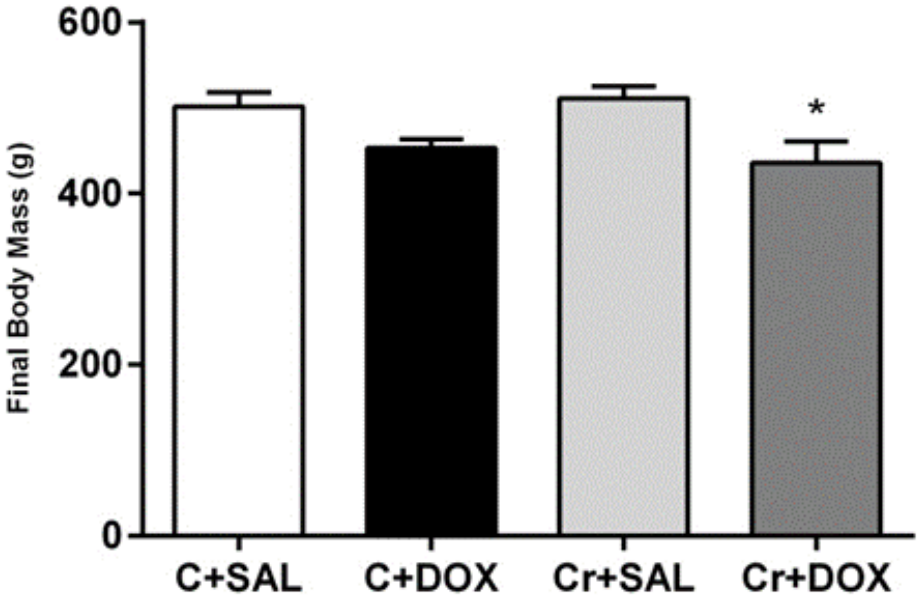

Figure 2. Final body mass. C+SAL, control+saline; C+DOX, control+DOX; Cr+SAL, creatine+saline and Cr+DOX, creatine+DOX. * Significantly lower than Cr+SAL ($p<0.05$).

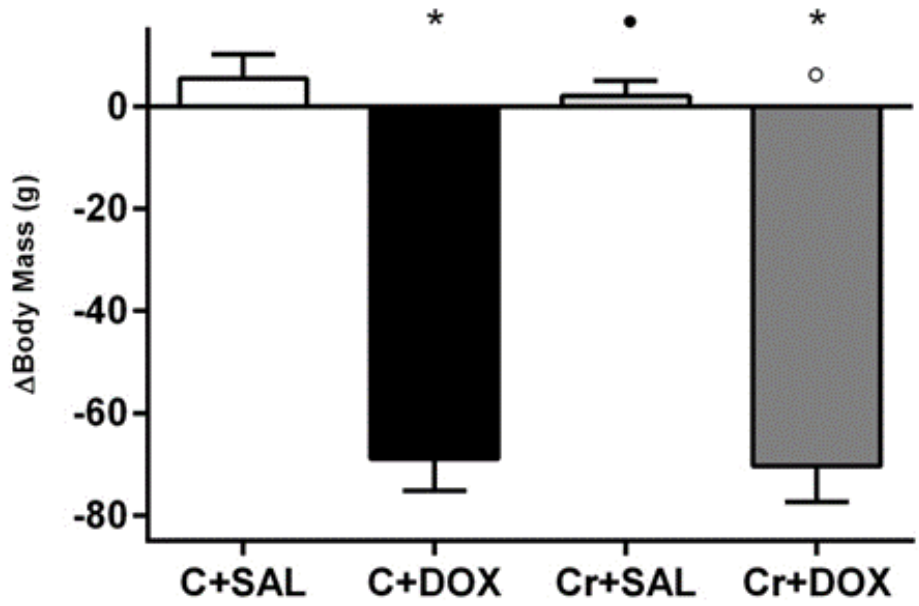

Figure 3. Change in body mass. C+SAL, control+saline; C+DOX, control+DOX; Cr+SAL, creatine+saline and Cr+DOX, creatine+DOX.

* Significantly different than Cr+SAL ($p<0.05$).
• Significantly different than C+DOX ($p<0.05$).
° Significantly different than Cr+SAL ($p<0.0$).

On the last day of the *in vivo* protocol, animals were sacrificed and kidney mass was measured following kidney excision. Figure 4 shows that Cr+DOX had significantly lower kidney mass than Cr+SAL. No significant differences were found in kidney mass relative to body mass among the groups as shown in Figure 5. Lipid peroxidation was measured using a commercial kit, and results showed that it was decreased in all groups compared to C+SAL as demonstrated in Figure 6. Antioxidant enzyme profiles were investigated using a Western blot protocol. Figure 7 shows the expression of CAT, and ANOVA detected a significant between group differences, *post hoc* testing, however, did not reveal where the differences existed. GPx and SOD-1 are illustrated in Figures 8 and 9, respectively, and their profile look similar; however, no significant differences were observed in these enzymes. There was a trend, however toward an increased expression in DOX treated animals that seemed to be attenuated with Cr supplementation.

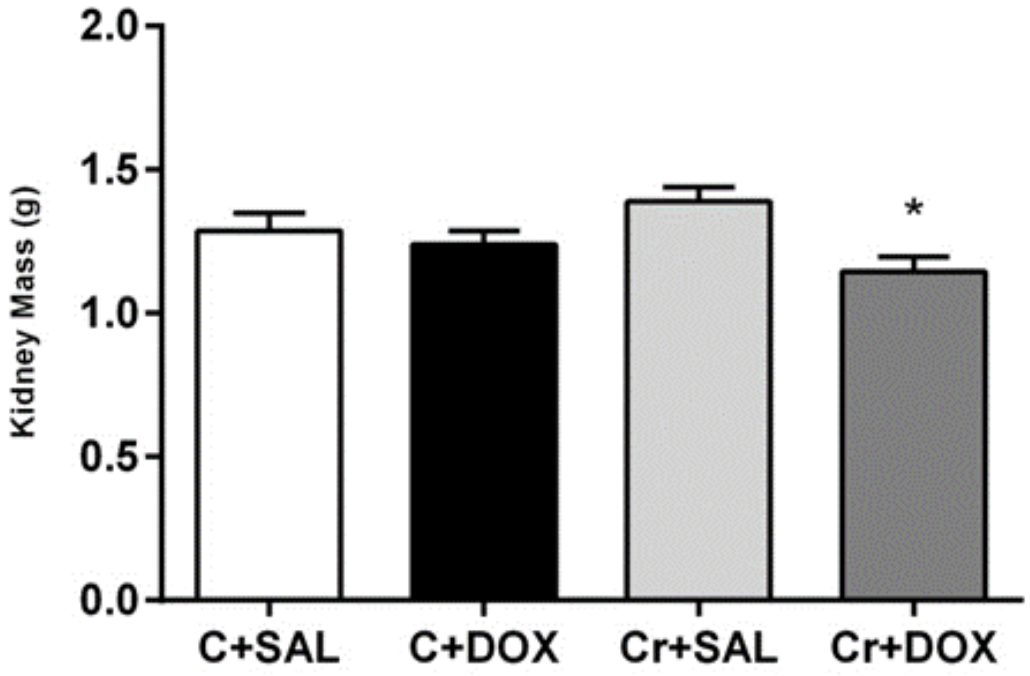

Figure 4. Kidney mass. C+SAL, control+saline; C+DOX, control+DOX; Cr+SAL, creatine+saline and Cr+DOX, creatine+DOX. * Significantly lower than Cr+SAL ($p<0.05$).

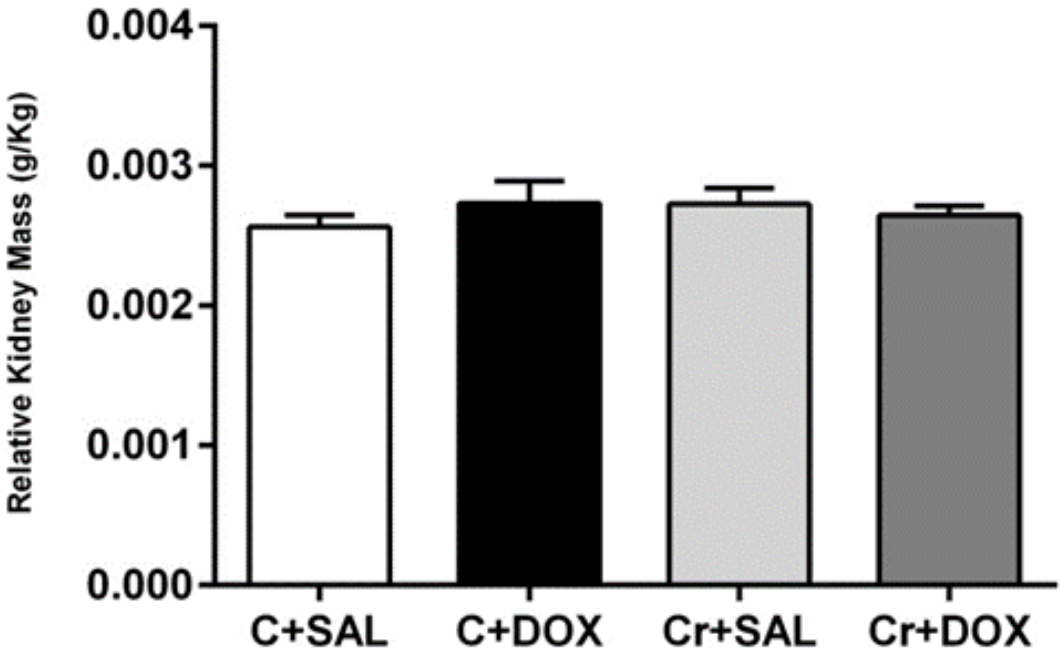

Figure 5. Relative kidney mass. C+SAL, control+saline; C+DOX, control+DOX; Cr+SAL, creatine+saline and Cr+DOX, creatine+DOX. No significant difference between groups ($p>0.05$).

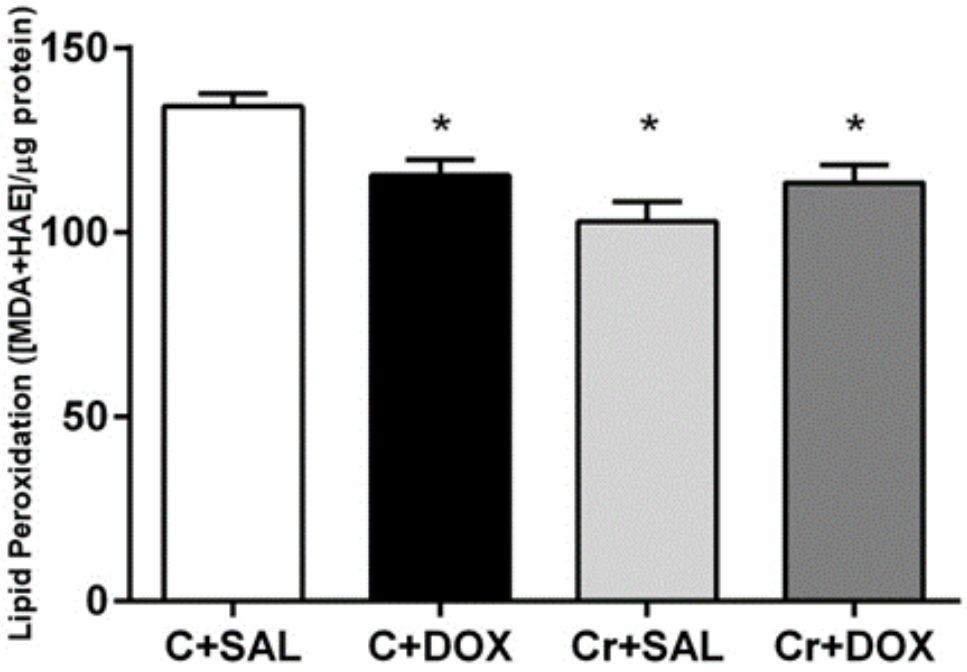

Figure 6. Lipid Peroxidation. C+SAL, control+saline; C+DOX, control+DOX; Cr+SAL, creatine+saline and Cr+DOX, creatine+DOX. * Significantly lower than C+SAL ($p<0.05$).

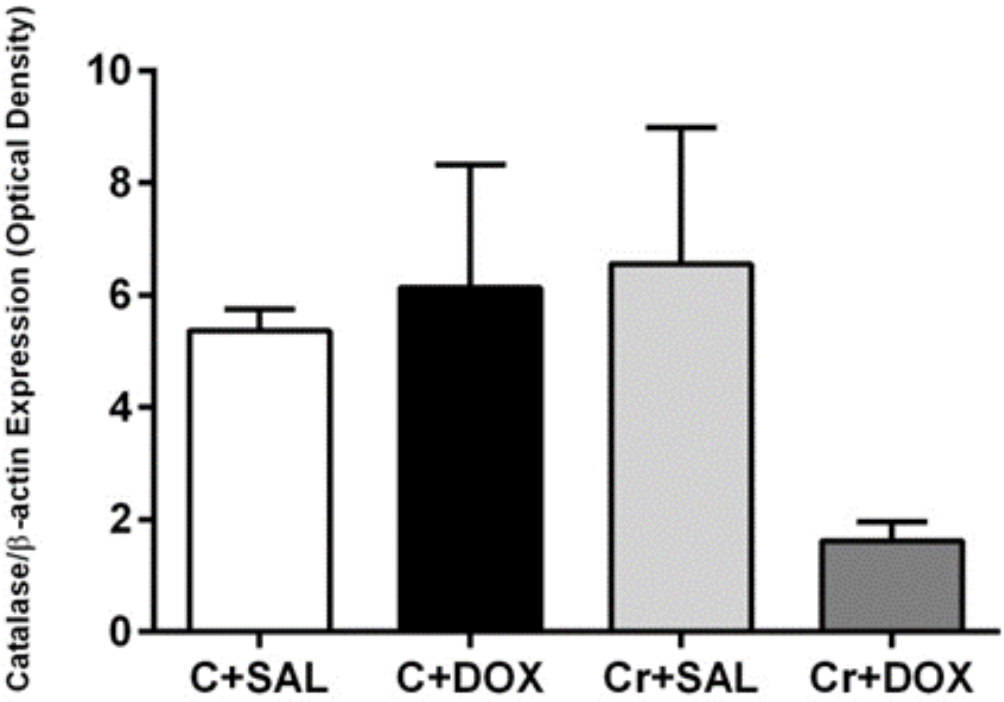

Figure 7. Catalase expression. C+SAL, control+saline; C+DOX, control+DOX; Cr+SAL, creatine+saline and Cr+DOX, creatine+DOX. Significance between group difference ($p<0.05$).

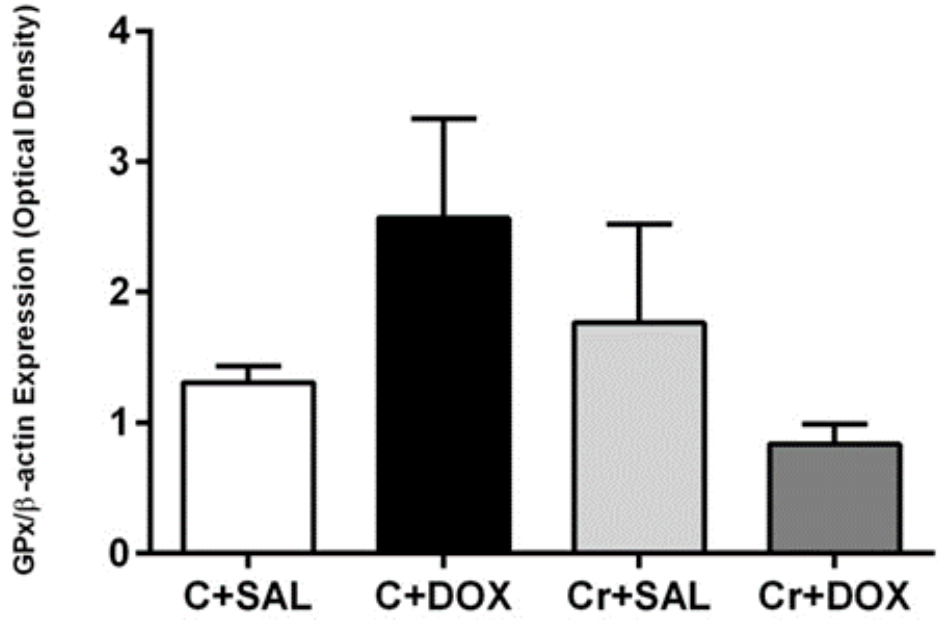

Figure 8. Glutathione Peroxidase expression. C+SAL, control+saline; C+DOX, control+DOX; Cr+SAL, creatine+saline and Cr+DOX, creatine+DOX. No significant between group differences (p>0.05).

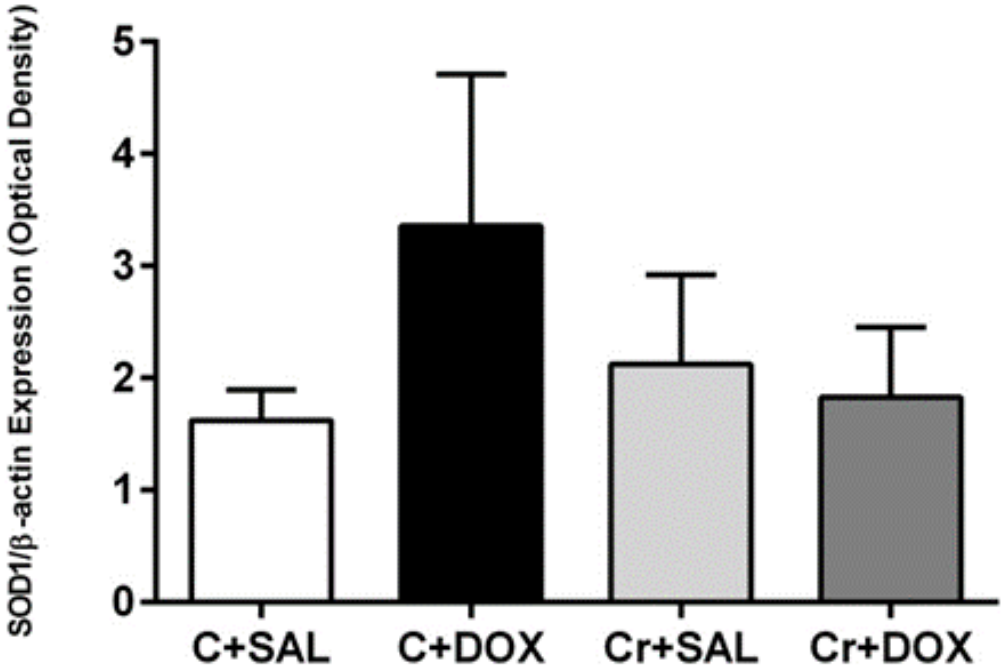

Figure 9. Superoxide Dismutase 1 expression. C+SAL, control+saline; C+DOX, control+DOX; Cr+SAL, creatine+saline and Cr+DOX, creatine+DOX. No significant between group differences (p>0.05).

Discussion

The cytotoxicity of DOX has been extensively investigated, and it is well known that this drug has the ability to generate ROS (specifically superoxide) that cause damage to healthy tissues in addition to destroying cancer cells. The elucidation of the yet unclear mechanisms involved in DOX action is important to the elaboration of an intervention that will attenuate its side effects and decrease the limitations in the use of this powerful anticancer drug.

The nephrotoxicity attributed to DOX has been reported to induce OS primarily by decreasing levels of antioxidant enzymes such as CAT and GPx and increasing levels of lipid peroxidation observed 10 days after DOX administration.[4] At 5 days after injections, the current study presented a different profile with no statistical differences in antioxidant expressions, but CAT, GPx and SOD-1 tended to be higher in DOX treated animals. However, it is known that an increase in H_2O_2 would induce an increase in antioxidant enzyme expression in attempt to reestablish and maintain

homeostasis to protect the systems from OS.[19] It is possible that results from the current study are not reflecting the most common pathway of DOX action, and antioxidant enzyme upregulation is a response to increased ROS. It has also been reported that cisplatin, another antineoplastic agent toxic to tubular cells, is said to be dose and time dependent.[26] We can speculate that the dose of DOX used in our experiment or the time of action we allowed for the drug to act was not enough to damage the kidney to the same extent that other studies have previously reported.

As previously stated, Cr is a novel alternative to prevent oxidative stress since it has been reported to contain antioxidant properties via removing peroxide anions and peroxynitrite.[16] Therefore, the present study aimed to investigate the effect of Cr on renal antioxidant enzymes. Although not statistically significant, Cr supplementation tended to increase the expression of the investigated enzymes in SAL treated animals. If this is the case, these findings would corroborate early studies reporting that Cr could indirectly play a role as antioxidant via increasing the activity of GSH-GPx and CAT.[19] Extensive studies involving skeletal muscle have shown that Cr supplementation contributed to a decrease in OS markers in soleus and gastrocnemius muscles as well as plasma antioxidants following a moderate bout of aerobic exercise supporting the findings that Cr has the potential to quench ROS.[18]Another study with skeletal muscle did not find changes in the expression of antioxidant enzymes such as, SOD-1, SOD-2 and CAT, after Cr supplementation.[27] They did find, however, that Cr directly affects superoxide radicals thus suggesting a scavenging effect of Cr.

N-acetylcystein (NAC) is an antioxidant capable of increasing the cellular levels of GSH therefore acting as a free radical scavenger. In a study focusing on aluminum phosphide poisoning in humans, it was found that after five days of NAC treatment, SOD and glutathione reductase expression were two times higher compared to participants who did not get NAC treatment.[28] NAC also has the ability to decrease MDA levels in chronic hemodialysis patients.[29] Lead (Pb) generates ROS, and a study involving Pb-exposed human subjects used different doses of NAC to attenuate the reported oxidative stress involved in the toxicity of Pb. They reported increased SOD activity in subjects exposed to Pb compared to non-exposed controls. Since NAC could only decrease SOD activity in blood cells in a dose-dependent manner, they attributed the results to the H_2O_2 scavenger properties of NAC. Moreover, GPx expression and activity were reported to elevate in blood cells of Pb-exposed subjects with no significant difference observed in CAT expression.[30] Conversely, an animal model of Pb-toxicity demonstrated decreased activities of SOD, GPx and CAT in the kidney homogenates of rats, and treatment with NAC restored the activities of these enzymes.[31]

Other animal studies have focused on the effects of NAC, and one explored the effects of NAC administration in different tissues of rats. The results indicate that NAC is tissue-specific in regard to superoxide and may increase or decrease expression of CAT, GPx, SOD-1 and SOD-2 depending on the tissue and the mechanism causing OS. They found that NAC is capable of attenuating alterations in antioxidant enzyme expression caused by diabetes by attenuating SOD-1 expression decreases in kidney tissue. On the other hand, they reported that SOD-2 was increased in the kidney and lungs of diabetic rats, and NAC promoted a different response in those tissues prmoting a further decrease in the kidney and an increase in the lungs.[32]

NAC has been shown to maintain antioxidant defenses as well as improve hepatic antioxidant defenses. In biliary obstructed rats, CAT, cytosolic and mitochondrial SOD, and GPx are significantly reduced and NAC administration corrected the reduction in glutathione concentration. The treatment with NAC resulted in significant preservation of CAT, mitochondrial SOD and the different forms of GPx activities.[33]

The present study suggests a tendency of Cr to increase SOD-1 expression in healthy animals and possibly attenuate the exacerbated increase in SOD-1 promoted by DOX treatment. Moreover, CAT and GPx followed similar patterns that could be due to a similar effectiveness of Cr as NAC which varies according to the site of the OS and the mechanism behind it. Although Cr supplementation has been accused of being deleterious to renal function, it is important to clarify that no conclusive evidence has been presented on the matter. Studies on healthy untrained males show that Cr supplementation does not impair renal function after a period of moderate training.[24] However, the antioxidant properties of Cr have not yet been explored in the kidney, and in light of the previous findings from studies utilizing NAC to attenuate a decrease in antioxidant enzyme expression to counteract increased generation of ROS in different tissues, it is possible that the antioxidant properties of Cr have a similar mechanism of action to NAC. Studies exploring Cr have not yet fully elucidated

its mechanisms of action, and further investigation is required in the field. We hypothesized that changes in antioxidant enzyme profiles in the kidney would be attenuated with Cr supplementation, and possibly this is done in a similar way as described with other antioxidants such as NAC.

Conclusion

To our knowledge there are no studies that have explored Cr's antioxidant properties in the kidney and the possibility of using this natural substance with antioxidant properties as a nutritional supplement with. Results from the current preclinical study may act as a guide for future studies to see how to best take advantage of the antioxidant role that Cr may play in battling DOX-induced side effects. Previously, our laboratory reported that Cr supplementation minimizes skeletal muscle dysfunction[34-36] and liver damage [37] associated with DOX treatment, but these of course are merely two sites of DOX toxicities and side effects. The potential effects of Cr on DOX kidney side effects in the current study are promising which may allow for Cr to be used more extensively as a complimentary approach for minimizing DOX-induced toxicities and side effects and therefore improving cancer patient quality of life.

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