



Protective Effect of D-003 on Renal Ischemia-Reperfusion in Rats

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SUMMARY. The effects of D-003, a mixture of higher aliphatic acids purified from sugarcane wax with antioxidant effects, and a grape seed extracts (GSE) on serum creatinine levels and on histological damage, were evaluated in a rat model of renal ischemia-reperfusion (I/R). Rats were subjected to sham operation or renal I/R. One group was treated orally with the vehicle, two were treated with D-003 (250 and 500 mg/kg) and other with GSE (200 mg/kg). Renal morphological alterations were assessed by histopathological examination of haematoxylin-eosin stained. After 24 h of post-ischemic acute renal failure, serum creatinine levels was increased, effect that was significantly decreased by about 30 % and 60 % with D-003 and with GSE, respectively. Compared with sham rats, ischemic controls demonstrated severe damage of renal function and morphology, while both D-003 and GSE treatments protected against these I/R-induced histological abnormalities too. D-003 protected moderately against the increase of serum creatinine and histological findings of I/R-induced renal injury in rats.

INTRODUCTION

Discontinuation of renal blood in renal ischemia reperfusion (I/R) injury constitutes the most common pathogenic factor for acute renal failure, depending on the duration of oxygen deprivation. It produces organ damage in diverse clinical circumstances and contributes to renal dysfunction in episodes of severe hemorrhagic shock, aortic aneurysm surgery, endotoxin sepsis, thermal burns, or transplantation surgery¹⁻³.

The mechanisms proposed to explain the I/R-induced injury include anoxia, release of oxygen-derived free radicals and neutrophil accumulation during reperfusion⁴⁻⁶. Different studies have demonstrated that reactive oxygen species (ROS) generated during I/R injury play a key role in the damage of both ischemic and reperfused tissues, causing oxidative damage of cellular macromolecules including membrane lipids, proteins and nucleic acids⁷⁻⁹. Elevated ROS generation depress glomerular filtration,

and renal function resulting in cell necrosis as a whole, and apoptosis in renal cells^{10,12}.

Different studies have shown that antioxidants protect renal, heart and liver cells against cellular injury induced by I/R^{13,14}, confirming the important role of free oxygen radicals in these physiopathologic event¹⁵.

D-003 is a mixture of higher primary aliphatic acids purified from sugar cane wax, containing octacosanoic (C₂₈) acid as the most abundant component, and C₃₀, C₃₂ and C₃₄, while C₂₄, C₂₅, C₂₆, C₂₇, C₂₉, C₃₁, C₃₃, C₃₅ and C₃₆ acids are present at lower concentrations¹⁶. D-003 has shown to produce *in vivo* antioxidant effects in animal and clinical studies¹⁷⁻¹⁹. D-003 orally given to rats for 4 weeks reduced plasma levels of thiobarbituric acid reactive substances (TBARS), carbonyl groups and lysine reactivity¹⁷, and the extent of lipid peroxidation (LP) as well¹⁸. Oral administration of D-003 for 60 days to healthy volunteers has shown to inhibit copper-induced LP of low-density lipoprotein (LDL)¹⁹.

KEY WORDS: Creatinine, D-003, Grape seed extract, Ischemia-reperfusion, Renal failure, Sugarcane wax.

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On the other hand, protective effects of D-003 on I/R-induced brain ischemia in Mongolian gerbils, myocardial infarct in rats and spinal cord ischemia in rabbits²⁰⁻²² have been reported. Although the mechanism whereby D-003 protects against I/R-induced damage in these models has not been fully elucidated, the antioxidant effect of D-003 could explain such results, at least partially.

Flavonoids, a group of polyphenolic compounds present in fruits and vegetables, produce several benefits, most linked to their antioxidant and anti-inflammatory properties^{23,24}. Since grape seed is rich (95%) in flavonoids²⁵, grape seed extracts (GSE) have shown effective antioxidant effects in experimental²⁶⁻²⁸ and clinical studies²⁹ that have been related to the enhancement of the endogenous antioxidant system³⁰. In particular, a proanthocyanidin-rich extract from grape seeds lowered serum urea nitrogen and creatinine in I/R-induced renal damage in rats, an injury associated with increased oxidative stress³¹.

In light of these facts, this study was undertaken to evaluate the protective effect of D-003 in a renal failure model induced by I/R in rats.

MATERIALS AND METHODS

Animals

Male Sprague Dawley rats (200-250 g) from the National Centre for Laboratory Animal Production (CENPALAB Havana, Cuba) were adapted for 14 days to experimental conditions that were maintained during the study: temperature 25 ± 2 °C, humidity 55-60% and light/dark cycles of 12 h. Rats were housed 3/cage. Access to water and standard chow (rodent pellets from CENPALAB) was freely allowed. Animal health was monitored daily during the adaptation period.

Animal handling followed Cuban Guidelines for Good Laboratory Practices (GLP). An independent Ethical Board approved the study protocol and the use of the animals in the study.

Administration and dosage

D-003 batch (Chemistry Department, Centre of Natural Products, Havana City, Cuba) was used after confirm its quality specifications, including composition and purity. For dosing, D-003 was suspended in Tween 20/water 2% and GSE (Blackmores, Australia) was dissolved in distillate water.

Rats were randomized into five groups of 8 rats each: One group was false-operated (sham)

and 4 were submitted to surgical procedure for inducing I/R-induced renal damage. Of these, one was a positive control group treated orally with the vehicle, two groups were treated with D-003 (250 and 500 mg/kg), other with GSE (200 mg/kg). Treatments were given by oral gastric gavage for 15 days prior to induce I/R renal injury.

The doses of D-003 selected had proven to be effective in models of I/R-induced organ damages²⁴⁻²⁶, and those of GSE as antioxidant.

Induction of ischemia-reperfusion renal injury

The animals were subjected to left renal warm ischemia for 45 min, and reperfusion for 24 h. Briefly, under intraperitoneal sodium thiopental (Cuban Pharmaceutical Industry –IMEFA–, Havana City, Cuba) anaesthesia (40 mg/kg) and through a midline incision; the abdominal contents were displaced to the right side. The left renal artery and vein were dissected and the vascular pedicle was temporarily ligated with 2-0 silk. Right nephrectomy was performed, the abdominal contents were replaced and the incision was sutured. At the end of the ischemic period (45 min) the abdominal cavity was re-entered, the ligature was removed and reperfusion was supplied. At the 24 h of reperfusion, blood was collected to perform the creatinine assay and the left kidney was removed for histological analysis³².

Measurement of biochemical parameters

Blood samples were centrifuged (3000 r.p.m. for 10 min) to separate the serum. Serum concentrations of creatinine were measured with spectrophotometer Ultrascopic 2000 (Pharmacia, Biotech) as indicators of impaired glomerular function.

Histological examinations

The kidneys were fixed in a 10% neutral buffered formalin solution and embedded in paraffin. Five micrometer thick sections were cut, and stained with haematoxylin and eosin for histopathological examination in an Olympus BH2 microscope (Olympus Optical Co., Ltd, Tokyo, Japan).

The renal sections from all treatments were examined for tubular cell swelling, interstitial edema, tubular dilation, moderate to severe necrosis of epithelium, hyaline casts. The mean was calculated in a minimum of 10 fields for each kidney slide that were examined and as-

Groups	Doses (mg/kg)	Creatinine ($\mu\text{mol/L}$) (X \pm EE)	Inhibition (%)
Sham (false-operated)	0	43.18 \pm 4.1	
Positive Control (I/R + vehicle)	0	247.6 \pm 15.2 +++	-
I/R + D-003	250	182.2 \pm 22.6 *	32
I/R + D-003	500	187.8 \pm 17.3 *	29
GSE	200	127.1 \pm 9.9 **	59

Table 1. Effect of D003 on serum creatinine in rats with I/R-induced renal injury. GSE grape seed extract, I/R ischemia/reperfusion. +++ $p < 0.001$. Comparisons with sham, * $p < 0.05$ ** $p < 0.01$ Comparisons with positive control (Mann Whitney U Test).

sessed for severity of changes using scores on a scale of none (0): no damage, (1): mild damage with tubular cell swelling and dilated tubular lumen; moderate (2): moderate necrosis of epithelium, and dilated lumen; and severe damage (3): severe necrosis of epithelium and several hyaline casts.

Statistical analysis

Data are presented as means \pm SE. Comparisons between control and treated group were performed using the Mann Whitney U test. Values of $P < 0.05$ were considered to be statistically significant. All analyses were performed using statistics software (Release 4.2; StatSoft Inc, USA).

RESULTS

Table 1 summarizes the effects of treatment on serum creatinine levels. Positive controls had increased levels (5.7-fold) of serum creatinine compared with sham-operated rats. Oral treatment with repeat doses of D-003 (250 and 500 mg/kg) significantly and moderately reduced serum creatinine by 32% and 29% respectively, while GSE at 200 mg/kg decreased serum creatinine levels by 59%. The effects of D-003, however, did not increase with the doses, and were inferior that those induced with GSE. The histopathological changes observed were graded. The sham operated group did not show morphological features typical of renal damage (Fig. 1A), whereas the kidneys of the positive control group were severely damaged (Fig. 1 B,C) showing marked tubular cell swelling and dilation, interstitial edema, hyaline cyst and necrosis of the epithelium. Groups treated with D-003 (500 mg/kg) or GSE (200 mg/kg) exhibited kidneys with moderate histological damage (Fig. 1 D-F), while curiously the damage of the group treated with the lowest dose of D-003 (250 mg/kg) was only mild (Table 2).

DISCUSSION

This study demonstrates that D-003 that orally administered for 15 days significantly protected against the increase of serum creatinine levels and kidney morphology changes following I/R events. The effects of both doses of D-003 on the increase of serum creatinine elicited by I/R were practically the same, while their effects on morphological changes were apparently paradoxical, since the lowest dose rendered a better protection than the highest one.

Positive control rats exhibited the characteristic pattern of I/R-induced renal injury in the rat, like a marked increase of serum creatinine and typical histopathological findings that indicate that I/R produced a severe renal damage, accompanied by glomerular dysfunction, as has been reported by other authors^{33,34}. These results confirm the validity of this model in our experimental conditions and support that the protective effects here reported were treatment-related.

Oral treatment with GSE (200 mg/kg) markedly (59%) reduced the increase of serum creatinine and also attenuated the morphological changes induced by I/R, consistently with the powerful antioxidant effect of GSE and the protective effects of a proanthocyanidin-rich ex-

Groups	Doses (mg/kg)	Histological damage
Sham (false-operated)	-	0
Positive Control (I/R + vehicle)	-	3
I/R + D-003	250	1.42 \pm 0.2 **
I/R + D-003	500	2.25 \pm 0.16 *
GSE	200	2.28 \pm 0.18 *

Table 2. Effect of D-003 on histological damage of kidneys in rats with I/R-induced renal injury. GSE grape seed extract, I/R ischemia/reperfusion. These are the values of media of animals by group with a scale that ranged from 0 to 3 as described in Materials and Methods.

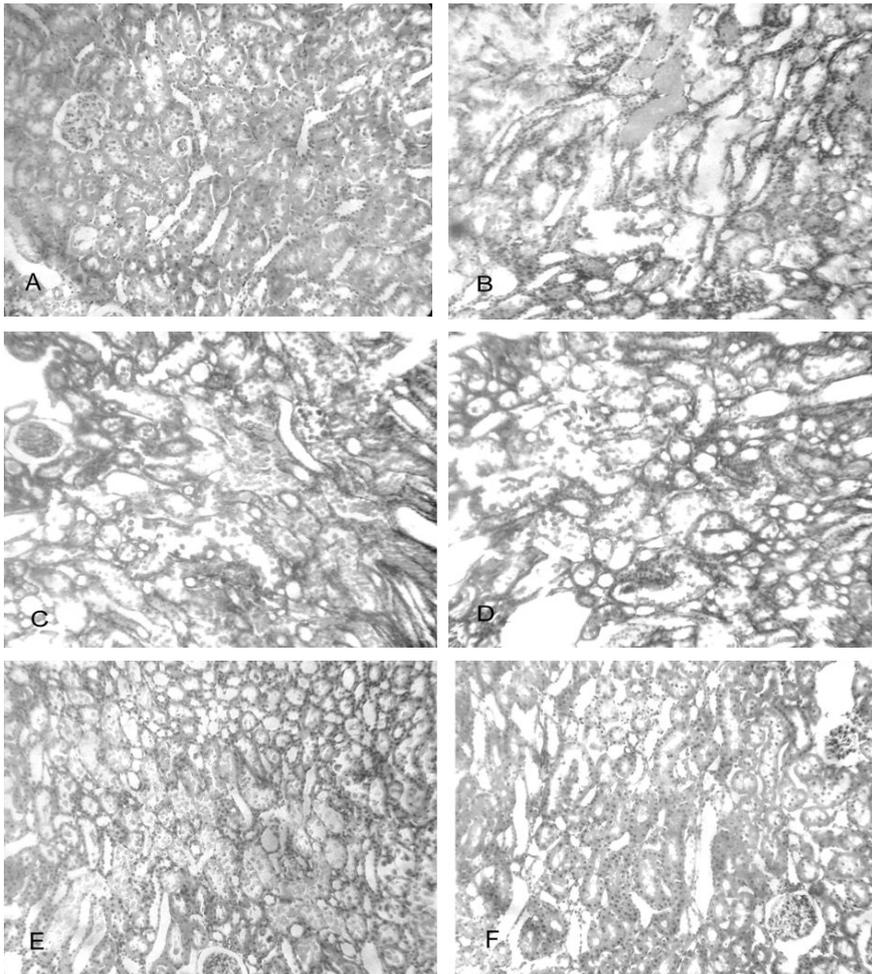


Figure 1. Kidney sections stained with haematoxylin and eosin (400 X). **A)** Sham-operated rat. **B)** and **C)** Rat exposed to renal I/R showing tubular cell swelling, medullary congestion, hyaline casts and moderate to severe necrosis. **D-F)** Rats exposed to I/R treated with D-003 (250 and 500 mg/kg) (1D and 1E, respectively) or with GSE (200 mg/kg) that show reduced morphological alterations.

tract from grape seeds against I/R-induced renal damage in rats previously reported ³². On the other hand, D-003 orally administered at 5, 25, 100 and 250 mg/kg for 4 weeks to rats significantly reduced markers of LP in plasma and hepatic tissue ³⁵.

Compared with GSE, D-003 was less effective for reducing serum creatinine, consistent with the results of a previous comparative study in which D-003 and GSE orally administered for 4 weeks reduced LP in rat plasma and liver. At the highest dose, the effects of both treatments on plasma malondialdehyde (MDA) were similar, while GSE was more effective to lower plasma total peroxides and liver LP ³⁶.

Clinical and experimental studies have provided evidence that I/R-injury is mediated by ROS ⁷⁻⁹. The damage caused by the lack of blood flow and deficient oxygen delivery to the tissues and by the further restoration of blood flow has been linked with the generation of free radicals ³⁷. These radicals can attack a wide variety of cellular components, like DNA, proteins, and membrane lipids and that can be considered as a major etiological component of acute

renal failure, these alterations eventually leading to the destruction of the renal tissue ^{14, 38}.

Disregarding the source of free radicals, since LP is the main pathway for tissue radical damage the reduction of LP appears to be an attractive strategy to protect the kidney from ROS-mediated damage. Substances like probucol ³⁹, carvediol ⁴⁰ polyphenols and α -tocopherol ^{14,41,42} among others, exert renoprotective effects probably by their radical free scavenging and antioxidant activities on I/R-induced damage. Therefore, the nephro-protective properties of D-003 in this model could be probably associated to its antioxidant effects.

Although renal I/R injury is the most common etiological factor, to speculate about the potential clinical relevance of these results is premature. Further experimental studies should corroborate the present data including wider doses of D-003 to explore dose-dependence and longer treatment duration as well. Also, other experimental models should be used before investigate whether a potential renoprotective effect is manifested in patients with potential of suffering acute renal failure.

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