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To cite this article: Maria Fernanda C. Carvalho, Rosa M. Viero & Vitor A. Scares (1999) Laboratory Study: Effect of Allopurinol in the Course of Adriamycin Induced Nephropathy, Renal Failure, 21:2, 147-154, DOI: 10.3109/08860229909066979

To link to this article: https://doi.org/10.3109/08860229909066979

Published online: 07 Jul 2009.
LABORATORY STUDY

Effect of Allopurinol in the Course of Adriamycin Induced Nephropathy

Maria Fernanda C. Carvalho,1 Rosa M. Viero,2 and Vitor A. Soares1

1Division of Nephrology
Department of Internal Medicine
2Department of Pathology,
Botucatu Medical School-UNESP
Botucatu, SP, Brazil

ABSTRACT

The role of superoxide in adriamycin-induced nephropathy (single dose; i.v. 3 mg/kg) has been studied by blocking superoxide synthesis through the administration of allopurinol (500 mg/L in drinking water). In Experiment I (EI), allopurinol administration was started 3 days prior to nephropathy induction and continued until day 14. In Experiment II (EII), allopurinol administration was started 2 weeks after nephropathy induction and was maintained until the end of the experiment (26 weeks). Affected glomeruli frequency and tubulointerstitial lesion index (TILI) were determined at Weeks 2 and 4 (EI) and Week 26 (EII). In EI, the 24 h mean proteinuria in the nephrotic control group (NCG-I) differed from that of the treated nephrotic group (TNG-I) at Week 1 (TNG = 33.3 ± 6.39 mg/24 h; NCG = 59.8 ± 6.3 mg/24 h; p < 0.05) and 2 (NCG-I = 80.0 ± 17.5 mg/24h; TNG-I = 49.1 ± 8.4 mg/24 h; p < 0.05). No glomerular alterations were observed and TILI medians were not different in both nephrotic groups at week 2 (NCG-I = 4++; TNG = 1+) and 4 (NCG = 4++; TNG = 4+). In EII, NCG-II and TNG-II presented different 24 h
proteinuria values only at Week 6, (136.91 ± 22.23 mg/24 h and 72.66 ± 10.72 mg/24 h, respectively; p < 0.05). Between nephrotic groups, there was no statistical difference in the median of affected glomeruli (CNG-II = 56%; TNG-II = 48%) and TILI (NCG-II = 8+; TNG-II = 9+). Thus, allopurinol was associated with a transient reduction in proteinuria and it did not alter the progression of the nephropathy.

Key Words: Adriamycin nephropathy; Proteinuria; Superoxide; Allopurinol; Glomerulosclerosis.

INTRODUCTION

Adriamycin (doxorubicin) is an antibiotic that has been used for the treatment of various neoplasms. One of the mechanisms proposed for its anti-tumor action is the formation of free radicals during its metabolism (1).

In the rat, Adriamycin induces the appearance of renal lesions characterized by the early development of persistent proteinuria and late glomerulosclerosis due to unknown mechanisms (2). Several investigations have suggested that oxygen free radicals, mainly superoxide, may mediate adriamycin-induced nephropathy. Thus, Adriamycin increases the production of superoxide anions by renal cells (3) and this increase coincides with the onset of proteinuria (4). Either the administration of superoxide scavengers (4,5) or the inhibition of superoxide synthesis (6) reduce proteinuria in this model.

Purine metabolism is one of the pathways by which superoxide is generated and drugs such as allopurinol, which inhibit purine metabolism, reduce the production of this anion. There have been several investigations on the role of the superoxide anion in the genesis of proteinuria (4,5,6). However, they were short-term investigations and neither the effects upon tissue alterations nor the course of glomerulosclerosis were analyzed. The aim of this study was to evaluate the effect of the allopurinol-induced inhibition of free radicals formation upon parenchymatous lesions and the development of adriamycin-induced nephropathy.

MATERIAL AND METHODS

Animals

Male 200–250g Wistar rats (provided by the Central Vivarium of Sao Paulo State University (UNESP)) fed a standard 25% - protein diet was used.

Experimental Design

Experiment I

Seventy-two rats were distributed into 4 groups, with 18 animals each. Control groups (HCG-I and HTG-I) were inoculated with isotonic saline (IS) (0.9 g/100 mL NaCl); 3 mL/kg body weight, i.v.) and nephrotic groups (NCG-I and TNG-I) received 3 mg/kg, i.v. of Adriamycin (Adriblastina ®, Farmitalia Carlo Erba S.A., Brazil). Adriamycin and IS were injected on day zero. The treated groups (HTG-I and TNG-I) received allopurinol
(Zyloric® WelIcome Foundation Limited - Inglaterra Coopers Brazil S.A.) in drinking water (500 mg/L) from day 3 through day 14.

Two weeks after adriamycin administration, a renal biopsy was carried out on 9 animals from each group. Twenty-four-hour urine was collected weekly for proteinuria. After a 4-week observation period the animals were sacrificed.

Experiment II

Seventy-two rats were distributed into four groups, with 18 animals each. Control groups (HCG-II and HTG-II) received IS (3 mL/kg, i.v.) and nephrotic groups received adriamycin (3 mg/kg, i.v.). From two weeks after inoculation adriamycin or IS until the end of the experiment HTG-II and TNG-II daily received allopurinol (500 mg/L) in drinking water.

After a 26-week observation period the rats were sacrificed and samples of renal parenchyma were collected. Twenty-four-hour urine was collected every 4 weeks for proteinuria.

Evaluation of the Nephropathy Intensity

Proteinuria

The animals were placed in metabolic cages with free access to water, but not to food, for 24-h urine collection. Proteinuria was determined by the sulfo-salicylic acid method.

Microscopy

Samples of the renal parenchyma were collected 2, 4 (experiment I) and 26 (experiment II) weeks after adriamycin administration.

For examination by light microscopy kidney fragments were fixed in Helly solution, embedded in paraffin, cut into 4 mm and stained with hematoxylin-eosin, Schiff periodic acid and silver methenamine.

The renal parenchyma was examined by two independent investigators, without prior knowledge on the origin of the renal sample. One slide per animal was randomly chosen for evaluation.

A semiquantitative method was used to assess the glomerular lesion in a sample of 100 sequential glomeruli and the percent of the glomerular presenting lesions were determined (GLI). The following glomerular lesions were considered: focal or global sclerosis, expansion of the mesangial matrix, and synchia between Bowmans capsule and the glomeruli. Interstitial fibrosis, cellular infiltration, tubular atrophy and atrophy were also analyzed in a semiquantitative manner and scored as 0 to 3+. The tubular-interstitial lesion index (TILI) is the sum of + signals attributed to each lesion.

Small pieces of kidney cortex were fixed in 2.5% glutaraldehyde for electron microscopy.

Statistical Methods

Weight and proteinuria are expressed as means ± SEM and histological data as medians. Comparison of sequential data (weight and proteinuria) was done by profile analysis (7). Only the animals that survived until the end of the observation period were used. At the end of the experiment, survival rates were compared by the $\chi^2$ test. The non-parametric data (TILI and GLI) were compared by the Kruskall-Wallis test for independent samples. Statistical significance was set at $p < 0.05$. 
RESULTS

Experiment 1

Proteinuria

The 24-h proteinuria in HCG-I and HTG-I remained unchanged throughout the experiment (range, 2.6 ± 0.4 mg/24 h to 11.5 ± 1.1 mg/24 h and 2.9 ± 0.3 mg/24 h to 11.3 ± 1.1 mg/24 h, respectively).

The mean proteinuria of TNG-I was significantly different from that of NCG-I on the first (TNG-I = 33.2 ± 6.4 mg/24 h NCG-I = 59.8 ± 6.3 mg/24 h; p < 0.05) and second weeks (TNG-I = 49.1 ± 8.4 mg/24 h, NCG-I = 80.01 ± 17.48 mg/24 h; p < 0.05). After the cessation of allopurinol no statistical difference was observed in the proteinuria of both nephrotic groups (week 3: NCG-I = 89.7 ± 15.7, TNG = 136.8 ± 20.8 mg/24 h; p > 0.05, week 4: NCG-I = 128.4 ± 23.5 mg/24 h, TNG-I = 119.1 ± 18.5 mg/24 h; p > 0.05).

From week 1 to 4, mean proteinuria was higher in NCG-I and TNG-I than in the control groups (HCG-I and HTG-I) (p < 0.05) (Figure 1).

Morphological Analysis

At the time of the first biopsy (Week 2) there were no glomerular lesions in the four studied groups, so the median of GLI was 0 in all of them. Some animals of the nephrotic groups showed a mild inflammatory lesion in the tubulointerstitial region but there was no statistical difference among the median of TIL1 of the four groups (HCG-I = 0, HTG-I = 1, NCG =1 and TNG= 1).
At the time of sacrifice (week 4), at light microscopy, most animals of HCG-I and HTG-I showed no glomerular lesions (median of GLI = 0 in both groups) or tubulointerstitial abnormalities (median of TILI = 0 in both groups)

All the animals from the nephrotic groups (NCG-I and TNG-I) presented mild histological alterations characterized by tubular dilatation and atrophy, and interstitial inflammatory infiltrate, of little intensity, in most cases. TILI in NCG-I (median 4+, range from 0 to 11+) did not statistically differ from that of TNG-I (median 4+, range from 2 to 12+).

Electron Microscopy

The examination of the samples obtained by renal biopsy on week 2 revealed that the animals of the control groups (HCG-I and HTG-I) did not present lesions.

The rats of the nephrotic groups (NCG-I and TNG-I) presented podocytic lesions characterized by retraction of foot processes, vacuoles of several sizes sometimes confluent, and osmiophilic inclusions. Detachment of the podocytes from the glomerular basement membrane and destruction of the podocytes were frequently seen. No difference was observed between nephrotic groups.

The examination of the samples obtained after 4 weeks, at sacrifice, revealed the same alterations described above with the same pattern within the groups.

Experiment II

Survival Rates

At the end of the experiment, the control groups survival rates HCG-II 100%; HTG-II = 89%) were higher than those of the nephrotic groups (NCG-II = 66.7%, TNG-II = 66.7%), (p < 0.05). There was no difference between NCG-II and TNG-II (Figure 2).

Proteinuria

The control groups (HCG-II and HTG-II) presented, throughout the experiment, proteinuria mean values ranging from 5.4 ± 0.6 mg/24 h to 10.7 ± 1.3 mg/24 h; and from 5.1 ± 0.6 mg/24 h to 12.9 ± 1.3 mg/24 h, respectively; (p > 0.05).

Both nephrotic groups presented heavy proteinuria from week 2 (NCG-II: 96.0 ± 23.4 mg/24 h; TNG-II = 70.1 ± 11.9 mg/24 h) until the end of the experiment, at week 26. (NCG-I = 222.3 ± 27.2 mg/24 h; TNG-II = 188.6 ± 17.1 mg/24 h) (Figure 3). At week 6, 4 weeks after the beginning of allopurinol administration, there was a significant difference between the proteinuria of NCG-II (136.9 ± 22.2 mg/24 h) and that of TNG-II (136.9 ± 22.2 mg/24 h; p < 0.01). After that there was no significant difference between the two groups until the end of the experiment.

Light Microscopy

HTG-II and HCG-II showed only mild tubulointerstitial lesions, with discrete tubular atrophy and mild interstitial inflammation; the TILI of HCG-II was 0, and that of HTG-II was 1+. There were no glomerular lesions in the healthy groups.

The analysis of the renal parenchyma of the nephrotic groups showed severe tubular atrophy, interstitial inflammatory process, tubular dilatation and interstitial fibrosis. There was no significant difference between the TILI of NCG-II (ranged from 3 to 12+, median 8+) and that of TNG-II (ranged from 3 to 12+, with median 9+).
Figure 2. Survival of the four studied groups in experiment II. HCG-II = Health control group II; HTG-II = Health treated group II; NCG-II = Nephrotic control group II; TNG-II = Treated nephrotic group II.

Figure 3. Proteinuria associated with adriamycin-induced nephropathy in experiment II. Data are reported as means ± SEM. HCG-II = Health control group II; HTG-II = Health treated group II; NCG-II = Nephrotic control group II; TNG-II = Treated nephrotic group II.
Both nephrotic groups showed glomerulosclerosis either focal or global. There was no statistical difference between the GLI of NCG-II (median = 56%, range from 4 to 90%) and that of TNG-II (median 48%, range from 2 to 98%).

Statistical analysis revealed that the nephrotic groups presented TILI and GLI higher than those of the control groups ($p < 0.05$). No significant difference was observed between NCG-II and TNG-II.

Electron Microscopy

The examination of the renal parenchyma fragments collected at sacrifice showed no lesions in the control groups (HCG-II and HTG-II). In NCG-II and TNG-II there were severe podocytes retraction and vacuoles and osmiophilic inclusions in the podocytes cytoplasm. There was no qualitative difference between NCG-II and TNG-II.

DISCUSSION

The data presented in this study show that the animals inoculated i.v. with a single 3 mg/kg dose of adriamycin (NCG-I and NCG-II) had early, heavy, and progressive proteinuria as previously reported (2,8,9).

The use of allopurinol in the adriamycin-induced nephropathy was associated with reduction, but not suppression, of urinary protein excretion in the initial stage. However, its anti-proteinuric effect did not last throughout the experiment. Reduction occurred even when allopurinol was administered after the nephropathy was established.

Adriamycin enhances the generation of the superoxide anion in the kidney (3), and the inhibition of superoxide synthesis reduces proteinuria at early stages of the adriamycin-induced nephropathy (4,5,6,10). Since allopurinol reduces the synthesis of free radicals by the renal parenchyma (6), the proteinuria reduction observed in this study might be a consequence of this effect.

Allopurinol was able to reduce proteinuria even when the treatment was started 2 weeks after the nephropathy induction, but such a reduction did not last for long. Wu et al. (10) reported that the adriamycin-induced synthesis of free radicals increased over the first weeks and disappeared thereafter.

The fact that allopurinol administration reduced proteinuria even after the nephropathy was established lead to the assumption that the superoxide increased production lasted for at least 2 weeks.

Our results support those found in the literature (4,5,6,10), which report that the block of superoxide synthesis ameliorates, but does not suppress, proteinuria in rats with adriamycin-induced nephropathy. These results suggest that other mediators of the increased glomerular basement membrane permeability should be involved in the genesis of proteinuria in this model.

Histological examination of the renal parenchyma of the nephrotic animals showed that as early as 4 weeks after the induction of nephritis, interstitial tubular lesions occurred with mild inflammatory infiltrate, mild tubular dilatation, and tubular atrophy. By electron microscopy, 2 and 4 weeks after inoculation, osmiophilic inclusions and podocytic vacuolization were observed with intense degeneration of the epithelial cells, as previously reported (2,8,9). No difference was observed in neither the intensity nor the type of histological lesions among the animals of both the allopurinol treated and non-treated groups. On a short-term basis, allopurinol has been reported to reduce the intensity of tubulointerstitial and podocytic lesions (5,10). However, these investigators did not undertake semi-quantitative studies.
The nephrotic animals showed severe tubulointerstitial lesions. The importance of these lesions in the progression of adriamycin-induced nephropathy has been pointed out by Bertani et al. (8) nevertheless, the tubulointerstitial lesion mechanism is still not clearly understood. Some evidence suggests that free radicals may play a role in tubulointerstitial lesions. According to Schrier et al. (11) the increased active transportation of sodium by the nephron, which occurs in some nephropathies, may lead to a larger consumption of oxygen, and increase the production of free radicals with a consequent cellular lesion. Furthermore, heavy proteinuria increases energy consumption because protein reabsorption by the tubular epithelial cells, over the physiological range, is energy-dependent (12) Moreover, Alfrey and Hammond (13) have shown that in animals with adriamycin-induced nephropathy, the iron concentration is increased and the copper and selenium concentration is reduced in the intratubular fluid, and this enhances the free radicals cytotoxic action.

In the present work, allopurinol did not attenuate glomerular and/or tubulointerstitial lesions, neither at a short, nor at a long-term basis, even when the treatment was maintained throughout the observation period. This suggests that in this model, the formation of the superoxide anion may not be important in the progression of the renal lesion.

In conclusion, in the adriamycin-induced nephropathy, the increase of the permeability in the glomerular basement membrane depends initially on superoxide formation. However, its maintenance does not depend on this radical, and the course of the nephropathy is not affected by superoxide anion.

ACKNOWLEDGMENT

The authors are indebted to Professor Paulo Curi for the statistical study.

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