

# Morphometric Study of Myocardial Changes During Puromycin Aminonucleoside Induced Nephropathy in Rats

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**Abstract.** Puromycin aminonucleoside (PAN)-treated rats developed a severe nephrotic syndrome. Morphometric analysis of the hearts revealed significant differences between treated and control rats regarding nuclear index, reticulin index, nuclear and myocellular transsectional area, capillary number and transsectional area of capillaries. These differences were comparable with those induced by adriamycin. It is suggested that PAN can induce morphometrically measurable abnormalities in hearts of rats comparable to the morphometric changes seen after adriamycin treatment. The question of whether nephropathy, a result of both DOX and PAN treatment of rats, adds to or perhaps even causes doxorubicin - induced cardiomyopathy remains unclear.

Rats are in widespread use as an animal model to study anthracycline induced cardiotoxicity (1,2). Besides changes in the histomorphological patterns seen in the hearts of anthracycline treated rats, several other side effects may be present, such as neurotoxicity, skin toxicity, G.I. toxicity, decreases of bone marrow function and nephrotoxicity. Cardiotoxicity induced by anthracyclines such as doxorubicin and daunomycin has been observed in several animals species (rabbits, rats, dogs, mice and monkeys). The rabbit and the monkey appeared to develop a dose-related cardiomyopathy with terminal heart failure (3). A qualitative or quantitative comparison of the histomorphological change in the hearts of these animals after anthracycline administration has not yet been made. It is noteworthy that toxic effects of anthracycline treatment, such as nephrotoxicity are quite different in different animals (4). Rats developed a more severe drug-related nephropathy, and thus it seemed possible that the severity of anthracycline-induced cardiac lesions may be

partly related to concomitant nephrotoxicity. Puromycin aminonucleoside (PAN) - induced nephrosis is an extensively studied experimental model in the rat. In a recent study the long term effects of doxorubicin and puromycin on the kidneys of rats were investigated. Although the course of the nephrotic syndrome was similar in both groups, the extent of focal and segmental glomerular hyalinosis and sclerosis (FSGHS) was different (5).

The purpose of this study was to investigate whether drug-induced nephropathy has an influence on morphometric parameters measured in heart tissues. Special attention was directed to the histomorphological changes in the heart, using morphometric methods which, according to the literature, are not so susceptible to subjective errors and are more reproducible. Morphological parameters constitute the earliest and most reliable changes for predicting drug-related cardiomyopathy (6).

## Material and Methods

*Animals, laboratory parameters and heart preparation.* Male Wistar rats (Central Institute for Experimental Animals, Zeist, The Netherlands), weighing 200 grams at the start of the experiments, were used. 30 rats received a single injection into the tail vein of PAN (Sigma Chemicals, St. Louis, Mo., USA), 150 mg/kg as a 2% solution in saline (7). 12 control animals were injected with an equal volume of saline. In the fifth week after the initial injection, 10 rats received a second injection of 150 mg/kg PAN iv and 6 of the control rats an equal volume of saline. Treated animals were sacrificed for histological examination after 2, 4, 6 and 7 weeks of treatment (group I-IV); control animals were sacrificed after 2, 6 and 7 weeks.

The animals were kept in metabolic cages. Food and water were supplied *ad libitum*. The 24 hours urine was collected daily and the excreted proteins determined with the Bio-Rad protein assay (Bio - Rad Laboratories, Richmond, Cal., USA) using bovine serum albumin standards. Terminal blood samples were taken just before the removal of hearts and kidneys. Hb, Ht, WBC, RBC, total protein, albumin, urea and creatinine levels were determined routinely.

In the group of PAN treated rats, 6 hearts in group I, 3 hearts in group II, 5 hearts in group III and 2 hearts in group IV were analysed. The three control groups consisted of 12 animals. Hearts were removed under anaesthesia with 10 mg/kg Nembutal and were fixed by retrograde perfusion with 2% glutaraldehyde in phosphate buffer 0.1 M at pH 7.4, with a perfusion pressure of 100 mm Hg. After 20 min of perfusion, the

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*Key Words:* Anthracyclines, nephropathy, morphometry.

hearts were taken off and postfixed by immersion in the same solution for 48 hours. All hearts were cut three times in a transverse plane, perpendicular to the ventricular septum, with the first transection 2 mm below the cardiac skeleton. The first slice with a thickness of 1 mm of the apical side of the first plane was processed for paraffin embedding with the standard technique. A second slice of 0.5 mm thickness was cut on the skeletal side of the first plane and processed in Epon in routine fashion. After embedding, semi-thin sections of 1  $\mu$ M were cut and stained with toluidine blue or PAS or Gomori's silver stain. Paraffin sections of 4  $\mu$ M were cut and stained with Haematoxylin and Eosin. Gomori's silver stain was used for the determination of reticulin (8).

#### Quantitation of histomorphological parameters

**Nuclear Index.** From the paraffin sections in each heart three subendocardial areas – left ventricular septum subendocardial, left ventricular papillary muscle and left ventricular free wall – were photographed twice on 35 mm diapositive film using a 200 x microscope magnification. Colour slides were projected on a 120 x 80 cm projection screen divided into 10 x 10 cm squares by black lines. Colour slides were analysed by counting non-myocyte nuclei *versus* myocyte nuclei per square. The slides – two per area – were randomly arranged and the origin of the specimen was not known to the analyser. Dividing the number of non-myocyte nuclei by the number of myocyte nuclei resulted in the nuclear index, which was calculated for all three locations in every heart. The number of measurements per intracardiac site was two and the number of measurements per heart six. From these data the average nuclear index for each group of animals was calculated.

**Reticulin index.** Paraffin sections of 4  $\mu$ M thickness were stained with Gomori's silver stain. Microphotographs with a microscopic magnification of 400 were made on 35 mm Agfa Ortho 25 ISO film. Again, the three areas in each heart were analysed twice. The negatives were magnified to prints of 6x9 cm. The nuclei visible in the prints were cut out by a scalpel. Reticulin areas were measured by the threshold setting. The reticulin index was calculated as the ratio of reticulin area to number of myocellular transections. The average reticulin index was determined for each group of animals.

**Nuclear and myocellular transectional area.** Photographs of PAS stained semi-thin sections were enlarged to 30x40 cm with a microscopic magnification of 400. Cells transected in the nuclear zone were identified by number. These cells were also outlined on a digitising tablet as were their nuclei. Two photographs per area were necessary in order to obtain the minimum sample size. From these data the mean myocyte transectional area and the mean myocyte nuclear transectional area were calculated for the different groups. Both parameters were corrected for cardiac dilatation by the use of the left ventricular lumen circumference (8).

**Capillary number and transectional area.** The same photographs were used for the determination of the nuclear and myocellular transectional area and were analyzed with regard to capillary number and transectional area. The capillaries were outlined on a digitising tablet. The mean capillary transectional area and the mean number of capillaries were calculated for each group.

**Statistical methods.** Differences between the groups were analysed by the two-sided rank sum test of Wilcoxon, Mann and Whitney. Reproducibility and other statistical parameters related to the reliability of the morphometric measurements have been published separately (8).

## Results

**Clinical chemistry.** All rats treated with PAN developed a severe nephrotic syndrome characterized by proteinuria, polyuria, hypalbuminemia, oedema and *ascites*. During the experiments we lost several PAN-treated rats by death. After

the first treatment with PAN, body weight decreased from  $207.0 \pm 6.3$  g to  $186.7 \pm 41.3$  g within two weeks, whereas the body weight of the control rats increased from  $207.5 \pm 6.2$  g to  $250.0 \pm 17.9$  g. At the start of the experiment all animals excreted about 5 mg protein / 24 hours in the urine. Four days after the first PAN injection, the level of the urinary protein excretion increased and reached a first maximum value of 590 mg / 24 hours after two weeks. After the first peak a delayed drop was observed. Up to the fifth week after the first PAN treatment protein excretion slowed down to 134 mg / 24 hours. Eleven days after the second PAN injection, the urinary protein excretion reached a second maximum value of 696 mg / hours (Fig. 1).

The course of the polyuria is shown in Fig. 2. Polyuria started after one week and reached a peak at day 9 of 18 ml/24 hours. After three weeks the urine volume had returned to a normal value of 9 ml/24 hours. Five days after the second PAN injection the urine volume started to increase, reaching a maximum of 35 ml/24 hours three days later. The urine volume recovered to 14ml/24 hours two weeks after the second PAN administration. The urine volumes of the control rats ranged from 5 to 10 ml / 24 hours.

Parameters of blood chemistry, as measured at the time the animals were killed, are shown in Table I. Two weeks after the first PAN injection blood urea increased from  $6.4 \pm 1.8$  mmol/L to  $15.0 \pm 2.6$  mmol/L. No striking increase of creatinine was observed (from  $46 \pm 7$  mmol/L to  $51 \pm 6$  mmol/L). Total protein and albumin levels decreased during this time (total protein from  $5.5 \pm 0.3$  g/L to  $3.4 \pm 1.7$  g/L; albumin from  $2.8 \pm 0.3$  g/L to  $0.5 \pm 0.4$  g/L). After four weeks blood urea and total protein levels returned to regular values, except for the albumin level. The second administration of PAN induced changes comparable to those that followed the first injection. These data indicate that a severe nephrotic syndrome had developed in PAN-treated rats.

**Morphometric analysis.** The results obtained after the measurement of the morphometric parameters in heart tissue of PAN-treated rats and controls are summarized in Table II. All six parameters examined in this study changed as a result of treatment with PAN. The nuclear index calculated as the ratio of the number of non-myocyte nuclei to the number of myocyte nuclei increased with time and number of treatments from 2.33 to 3.25. This rise was significantly different ( $p$ -value  $< 0.01$ ) from controls in all four PAN-treated groups. The increase of the index is caused by an increase of the number of non-myocyte nuclei and not by a decrease of the number of myocyte nuclei, which remains constant. A similar change was observed from the reticulin index. This index increased with time and number of treatments from 2.20 up to 4.37, and this increase was significantly different ( $p$ -value  $< 0.01$ ) from controls in all four PAN-treated groups. The nuclear transectional area of myocytes from treated animals was larger than in control animals (increase from 69 up to 171 arbitrary units). The results in PAN group I and II did not

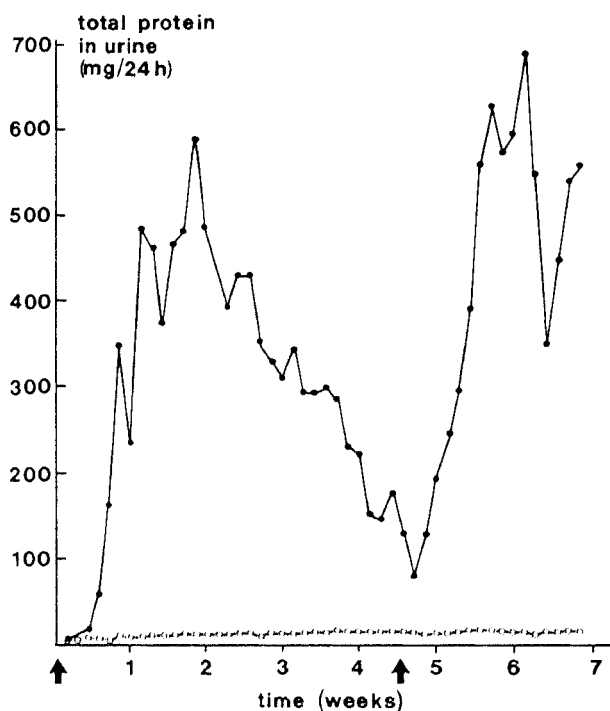


Figure 1. Course of the total protein excretion in urine during seven weeks. The arrows indicate PAN injections. Dots indicate PAN rats, circles indicate control rats.

differ significantly from the controls, although the mean values were quite different. Only groups III and IV (after the second PAN administration) were significantly different ( $p$ -value  $< 0.05$ ) from the controls. Nearly the same observation was made when the myocyte transsectional area was evaluated. There was a marked increase of this parameter with the time and number of treatments from 1949 up to 4514 arbitrary units. PAN groups II to IV differed significantly ( $p$ -values  $< 0.05$ ) from the controls. The number of capillaries increased with time and number of treatments from 60 up to 84 / area. Evaluating the size distribution of the capillaries and the density of capillaries by histograms (not shown as figure), it was observed that the newly developed population of capillaries had a smaller caliber. The capillary transsectional area decreased in the PAN groups from 451 to 338 arbitrary units. PAN groups III and IV differed significantly from the controls ( $p$ -value  $< 0.05$ ).

In general, myocardial damage increased with time and number of PAN injections. The significance of the change varied among the six parameters used in this study. The nuclear index and the reticulindex were most sensitive to changes in the heart even at an early stage of toxic heart damage. Parameters with the lowest value for predicting heart damage were capillary number and capillary transsectional area.

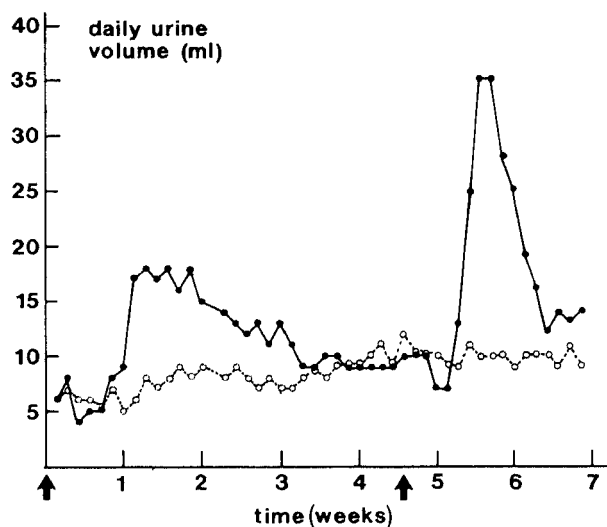


Figure 2. Course of the daily urine volume during seven weeks. The arrows indicate PAN injections. Solid line indicates PAN rats, dotted line indicates control rats.

### Discussion

It is obvious from the clinical chemistry data that PAN-treated rats developed a severe nephrotic syndrome. Changes in the parameters measured are in accordance with the literature using this drug schedule (7). Many aspects of PAN-induced nephropathy have been studied in the past. Clinical chemistry data and histomorphological changes in the kidneys have been extensively evaluated (9, 10), but the individual influence of the nephrotic syndrome or of puromycin on the heart have not been determined. In a recent paper (11), it was suggested that cardiomyopathy observed in rats after doxorubicin treatment is a phenomenon independent of nephropathy. We examined heart tissues by morphometric analysis to quantify changes in the histomorphometrical patterns of cardiac cells. In a study on doxorubicin (ADM) cardiotoxicity in Wistar rats (8), it was shown that morphometry could be used for the assessment of cardiotoxicity, which is an important and sometimes restrictive side-effect of anthracyclines used for the treatment of cancer (12). The extent of cardiotoxicity was measured by biochemical parameters, myocardial function and morphological pattern analysis (6, 13, 14, 15). Morphological changes are the earliest and most reliable parameters for predicting drug-related cardiotoxicity.

In experimental systems, ADM can induce a number of structural changes in the heart, such as cardiac dilation and diffuse interstitial fibrosis, which represent a delayed, cicatricial expression of ADM injury, vacuolization, myocellular atrophy and necrosis as well as a reduced number of myofibrils and mitochondria (3). In the rat the origin of the cardiac damage is complicated by the presence of an ADM-induced nephrotic syndrome, comparable to that induced by PAN.

Table I. Mean values ( $\pm$  SD) of body weight and blood chemistry data in PAN-treated rats and control rats.

Group	n	Body weight (g)	Total protein (g/L)	Albumin (g/L)	Urea (mmol/L)	Creatinine (mmol/L)
Controls	12	283 $\pm$ 37	55 $\pm$ 3	28.0 $\pm$ 3.2	6.4 $\pm$ 1.8	46 $\pm$ 7
PAN I	6	187 $\pm$ 41	34 $\pm$ 17	5.1 $\pm$ 4.4	15.0 $\pm$ 2.6	51 $\pm$ 6
PAN II	3	215 $\pm$ 13	52 $\pm$ 1	15.4 $\pm$ 1.2	7.0 $\pm$ 0.9	58 $\pm$ 13
PAN III	5	254 $\pm$ 36	27 $\pm$ 13	8.7 $\pm$ 3.8	15.2 $\pm$ 1.6	54 $\pm$ 5
PAN IV	2	215 $\pm$ 7	38 $\pm$ 3	10.3 $\pm$ 3.6	13.0 $\pm$ 1.6	34 $\pm$ 12

Table II. Mean values ( $\pm$  SD) of the histomorphometric parameters measured in heart tissue of PAN-treated rats and control rats.

Group	n	Nuclear index	Reticulin index	Nuclear transectional area	Myocyte transectional area	Number of capillaries	Capillary transectional area
CONTROLS	12	2.33 $\pm$ 0.06	2.20 $\pm$ 0.18	69 $\pm$ 21.2	1949 $\pm$ 653	60.4 $\pm$ 4.3	451 $\pm$ 80.6
PAN I	6	2.75 $\pm$ 0.24**	3.68 $\pm$ 0.37**	145 $\pm$ 46.5 n.s.	2551 $\pm$ 841 n.s.	65.5 $\pm$ 5.2 n.s.	439 $\pm$ 96.4 n.s.
PAN II	3	2.80 $\pm$ 0.18**	4.25 $\pm$ 0.25**	197 $\pm$ 54.2 n.s.	4100 $\pm$ 836*	73.2 $\pm$ 6.7*	384 $\pm$ 104.5 n.s.
PAN III	5	3.18 $\pm$ 0.09**	4.56 $\pm$ 0.31**	168 $\pm$ 22.7*	4475 $\pm$ 924*	79.8 $\pm$ 7.8*	367 $\pm$ 111.3*
PAN IV	2	3.25 $\pm$ 0.15**	4.73 $\pm$ 0.04**	171 $\pm$ 18.4*	4514 $\pm$ 945*	84.2 $\pm$ 6.2*	338 $\pm$ 109.2*

n.s = not significant, \*p < 0.05, \*\*p < 0.01, nuclear transectional area, myocyte transectional area and capillary transectional area are expressed in arbitrary units (AU) calculated by the computer from digitized data.

Our results in PAN-treated rats clearly showed several changes in the morphometric pattern which are possibly related to cardiac damage. The observed alterations of the nuclear index, the nuclear and myocyte transectional areas and the transectional areas, as well as the numbers of capillaries, are very similar and in some cases identical to the alterations observed in rats after treatment with ADM (8). Both drugs – PAN and ADM – induce a severe nephrotic syndrome in the rat (10, 16). In ADM treated animals like the rabbit, the incidence of renal lesions parallels the incidence of myocardial lesions (17). The nephrotic syndrome in rats comprises severe hypo-proteinaemia and electrolyte (K<sup>+</sup>, Ca<sup>++</sup>, Mg<sup>++</sup>) changes affecting tissue (heart) micro circulation, perfusion and metabolism, which can be a significant co-factor for the development of the observed myocardial alterations measured with morphometric techniques. Moreover, the possible direct toxic effects of PAN on the heart have to be taken into account. Puromycin has a structure very similar to that of an aminoacyl derivative of the terminal adenylic residue of tRNA. Puromycin interrupts peptide-chain elongation by formation of a covalent peptidyl-puromycin derivative which dissociates from the ribosome, since it does not have the precise structure for it to be recognized by the translocation apparatus. Thus it is evident that this antibiotic acts as a strong inhibitor of protein synthesis (18). It is known that ADM binds to DNA and RNA, inhibiting myocardial DNA and RNA synthesis. The mRNA content in the myocardium is lowered as well as the polyribosome content. As a result, the myocar-

dial protein synthesis is reduced which implicates a loss of structural and functional integrity of the myocardium (19). These similarities between ADM and PAN with regard to their influence on the protein synthesis led to the suggestion that the effects of ADM or PAN administration on the heart, i.e. loss of structural and functional integrity, may be the same.

The influence of the nephrotic syndrome on the morphometric pattern of the heart cannot be established with this model. Theoretically, it might be possible that a severe disturbance of microcirculation and metabolism can affect the myocardium, but this has to be evaluated in further studies. The myocardium as an organ has only a limited pattern of morphologically visible reactions to different toxic events. The drug specificity of any of these cardiac changes should be considered with care, especially since the use of this morphometric model for evaluating drug-induced cardiac damage is only valid if all other possible influences can be ruled out.

It may be concluded that the rat is not an unambiguous model for studying anthracycline-induced cardiomyopathy by using morphometric analysis, and this means that an alternative animal model has to be found without the aforementioned problems.

#### Acknowledgements

This study was supported by The Netherland Cancer Foundation (KWF), project no. THT 80-2. Klaus Mross is a recipient of an EORTC fellowship.

## References

- 1 Hu TS, Brandle EE and Zbinden G: Inhibition of cardiotoxic, nephrotic and neurotoxic effects of doxorubicin by ICRF-159. *Pharmacology* 26: 210-220, 1983.
- 2 Deprez-Decampeneere D, Jaenke R and Trouet A: Comparative cardiac and renal toxicity of daunorubicin in the rat and rabbit. *Cancer Treat Rep* 43: 395-397, 1982.
- 3 Young DM: Pathologic effects of adriamycin in experimental systems. *Cancer Chemotherap* 6: 159-175, 1975.
- 4 Jaenke RS and Fajarda LF: Adriamycin-induced myocardial lesions: Report of a workshop. *Am J Surg Pathol* 1: 55, 1977.
- 5 Grond J, Weening JJ and Elema JD: Glomerular sclerosis in nephrotic rats. *Lab Invest* 51: 277-285, 1984.
- 6 Iatropoulos LF: Anthracycline cardiomyopathy: Predictive value of animal models. *Cancer Treat Symp*. 3: 3-17, 1984.
- 7 Olson JL, Rennke HG and Vankatachalam MA: Alterations in the charge and size selectivity barrier of the glomerular filter in aminonucleoside nephrosis in rats. *Lab Invest* 44: 271 - 279, 1981.
- 8 Van Velzen D, Alons CL and Veldhuizen RW: Morphometric study of myocardial changes in adriamycin cardiomyopathy of the rat. *J Lab Clin Med* in press, 1986.
- 9 Frenk S, Antonowicz I, Craig JM and Metcalf J: Experimental nephrotic syndrome induced in rats by aminonucleoside. Renal lesions and body electrolyte composition. *Proc Soc Exp Biol (NY)* 89: 357-362, 1955.
- 10 Lannigan R, Kark R and Pollak VE: The effect of a single intravenous injection of aminonucleoside of puromycin on the rat kidney. *J Path Bact* 83: 357-362, 1962.
- 11 Van Hoesel QCCM, Steerenberg PA, Dormans JAMA, De Jong WH, De Wildt DJ and Vos JG: Time - course study on doxorubicin induced nephropathy and cardiomyopathy in male and female LOV / M / WsL rats: lack of evidence for a causal relationship. *J Nat Cancer Inst* 76 (2): 299 - 307, 1986.
- 12 Bristow MR: Anthracycline cardiotoxicity. *In: Drug-induced heart disease*. Bristow MR (ed). Elsevier / North Holland, 1980, pp. 191 - 215.
- 13 Zähringer J, Höfling B, Raum W and Kandolph R: Effect of adriamycin on the polyribosome and messenger RNA content of rat heart muscle. *Biochim Biophys Acta* 608: 315-323, 1980
- 14 Breed JGS: Studies on anthracycline cardiac toxicity. Thesis, Free University, Amsterdam, 1981.
- 15 Höfling B, Mendler N, Heimisch W, Boning L, Kolb HJ and Bolte HD: Tierexperimentelles Modell zur akuten und chronischen Adriamycin-Kardiotoxizität. *Beitr Onkol Karger*, Basel 9: 49-63, 1981.
- 16 Bertani T, Poggi A, Pozzoni R, Delaini F, Sacchi G, Thoua Y, Mecca G, Remuzzi G and Donati M: Adriamycin-induced nephrotic syndrome in rats. *Lab Invest* 46: 16-23, 1982.
- 17 Fajardo LF, Eltringham JR, Stewart JR and Klauber MK: Adriamycin nephrotoxicity. *Lab Invest* 43: 242-253, 1980
- 18 Lehninger AL: *Biochemistry*. Worth Pub Inc New York, 1981, pp. 941-944.
- 19 Zähringer J: The regulation of protein synthesis in heart muscle under normal conditions and in the adriamycin-cardiomyopathy. *Klin Wschr* 59: 1273-1287, 1981.

*Received September 8, 1987*

*Accepted October 22, 1987*