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The effects of levamisole on urinary enzyme measurements and proximal tubule cell inclusions in male rats

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Summary. A markedly increased incidence of large angular secondary lysosomes was observed in the proximal tubular cells of male Wistar rats dosed orally with levamisole, 75 mg/kg body weight for 15 days. These inclusions were similar in appearance to those previously observed in male rats treated with decahydronaphthalene. Urinary enzymes were measured throughout the study, and of these enzymes lactate dehydrogenase and *N*-acetyl-β-D-glucosaminidase activities were higher on days 9 and 13 for rats dosed with levamisole in comparison with control animals. Urine volumes increased for the levamisole treatment group, but no treatment related changes of urine protein output were found.

Keywords: levamisole, male rat, proximal tubules, hyaline droplets, angular inclusions, urine enzymes

Hyaline droplet inclusions in rat renal proximal tubule cells and their proteinic nature have been recognized for over 30 years (Oliver *et al.* 1954).

The male rat appears particularly prone to hyaline droplet formation in the proximal tubular cells of the kidney. When accumulation of hyaline droplets is marked cell necrosis may take place (Glaister 1986).

There is a growing list of chemicals, predominantly volatile hydrocarbons, e.g. decahydronaphthalene, which cause a specific type of acute and chronic renal toxicity in the male rat termed 'Light Hydrocarbon Nephropathy' (LHN) (Trump *et al.* 1985; Alden *et al.* 1984). Similar renal toxicity has not been noted in other mammals including female rats, mice, guinea-pigs, dogs and monkeys (Alden 1986). The primary response associated with LHN appears to be the induction and exacerbation of hyaline droplet formation in the proximal tubular cells. Further droplet formation results in cell necrosis and the shedding of necrotic cells into the tubular lumens.

The mechanism behind this increased incidence of hyaline droplets in the proximal tubular cells of male rats dosed with light hydrocarbons has not been established. Studies with decahydronaphthalene (Alden *et al.* 1984; Kanerva *et al.* 1987), 2,2,4-trimethylpentane (Stonard *et al.* 1986) and isoparaffinic solvent (Viau *et al.* 1986) have shown that the droplets which accumulate during exposure to these agents contain large amounts of alpha$_{2U}$ globulin, an androgen-dependent protein (Alden *et al.* 1984; Phillips & Cockrell 1984; Stonard *et al.* 1986).

Urinary excretion of enzymes act as a

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useful marker of proximal tubular damage, and enzyme changes have been reported in rat studies where compounds such as aminoglycosides and antimalarials produce lamellar (or myeloid) bodies within these cells (Luft & Patel 1978; Price 1982; Mackenzie 1983). Increases in the urinary enzymes, aspartate aminotransferase (AST), lactate dehydrogenase (LDH) and *N*-acetyl-β-D-glucosaminidase (NAG) were observed in male rats treated with decahydronaphthalene in conjunction with corresponding increases of cytoplasmic droplets and angular inclusions containing crystalloid bodies (Evans & Morgan 1986).

It is the experience of this laboratory that certain chemically unrelated pharmacological agents cause an increase in the incidence of hyaline droplet formation in male rats only (Read *et al.* 1987). One such agent is levamisole 'the levoisomer of tetramisole' an established anthelmintic and immunopotentiator.

This study was designed to measure urine enzymes in male rats given levamisole by oral gavage, and to examine renal tissue by light and electron microscopy. Previous studies had shown that rats tolerated the chosen dosage of 75 mg/kg body weight administered over a 14 day period.

Materials and methods

Animals and drug administration. Two groups of 10 male Wistar rats aged 5 to 6 weeks were obtained from Charles River (UK) Ltd, Manston. The rats were housed under controlled environmental conditions with a room temperature of 20 ± 2°C, and alternating 12 h periods of dark and light. The rats were fed *ad libitum* Porton Combined Diet (Special Diet Services, Witham) except during the scheduled urine collection periods when food was removed. Tap water was provided *ad libitum* throughout the study.

For 14 days, 10 rats were dosed once daily by oral gavage with levamisole, 75 mg/kg (Aldrich Chemical Co. Ltd, Poole) dissolved in 5% w/v dextrose (Boots Hospital Products). Ten control rats were similarly dosed with the dextrose vehicle for the corresponding period.

Urines. Specimens (5h; 09.00 h to 14.00 h) were collected over ice from rats kept individually in metabolism cages. Urea, creatinine and all enzyme determinations (except NAG) were analysed using a Roche CobasBio centrifugal analyser. Alkaline phosphatase (ALP), LDH, and gamma glutamyl transpeptidase (GGT) were determined using reagent kits obtained from Boehringer Mannheim. Urinary enzyme and protein values were calculated as activity/5h or concentration units /5h respectively. Creatinine (CRE) was determined using an alkaline picrate method, leucine arylamidase (LAA) was measured by a kit method (EM Diagnostics, Poole) and alanine aminopeptidase (AAP) was determined using alanine-4-nitroanilide (Evans 1985). Urea was measured by a urease method (Beckman-RIIC, High Wycombe).

Urine total protein (UTP) was determined with a Coomassie Brilliant Blue dye-binding method (Evans & Parsons 1986), and NAG was measured using a ω-nitrostyryl substrate (Yuen *et al.* 1982); a Coulter Kem-o-Mat discrete analyser was used for both of these methods. Urine osmolality was measured with a Micro-osmometer (Allchin & Evans 1986).

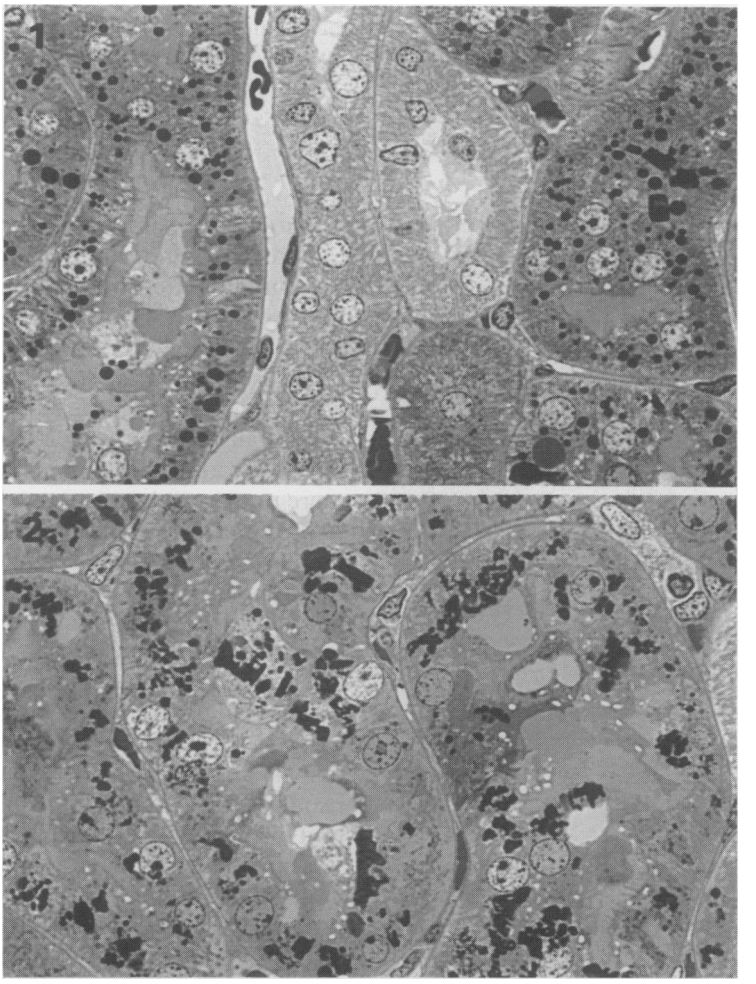
Microscopy. Kidney samples were fixed by immersion in neutral buffered formalin for 1–3 h before trimming and immersion in a storage fixative consisting of 4% formaldehyde and 1% glutaraldehyde in phosphate buffer. Slices ($\simeq 6 \times 4$ mm square and 1 mm thick) were processed for plastic histology, employing post-fixation in buffered osmium tetroxide, acetone dehydration and embedding in Spurr resin. Semi-thin (1–2 μm) sections were cut, mounted on slides, stained with methylene blue-azure II-basic fuchsin and examined with a Leitz Orthoplan light microscope. Following light microscopy of the semi-thin sections, the tissue blocks were trimmed and thin sections (70–90 nm) of selected areas were cut. The sections were

collected on copper/palladium G200 grids, stained with aqueous uranyl acetate and lead citrate, and examined at an accelerating voltage of 80kV in a Philips 301 transmission electron microscope.

Statistics. Urine volume, osmolality, creatinine and protein values were analysed by one-way ANOVA. The enzyme data were all log transformed prior to analyses; one-way ANOVA was used for LDH, NAG and ALP. The variables LAA, AAP and GGT were adjusted for baseline values by using analysis of covariance.

Results

No significant difference was observed between kidney weights of untreated and treated animals, and there was no evidence



Figs 1 & 2. Light micrographs of renal cortex illustrating the inclusion load within the proximal tubular cells of vehicle control (Fig. 1) and levamisole treated (Fig. 2) rats. × 200. The majority of inclusions in the vehicle control consist of the globular type, and only a few angular inclusions are present. In the levamisole treated rat the situation is reversed i.e., the majority of the inclusions are of the angular type some of which are quite large.

Table 1. Semi-quantitative estimation of inclusions in the proximal tubular cells

	Control rats ($n=10$)			Levamisole treatment group ($n=10$)		
Inclusion load grading*	Globular inclusions	Angular inclusions	Large vacuoles	Globular inclusions	Angular inclusions	Large vacuoles
++++	—	—	—	—	5	—
+++	1	—	—	—	5	—
++	9	1	—	1	—	9
+	—	9	8	9	—	1

* The + to ++++ scale indicates the number of inclusions or vacuoles with + denoting occasional, ++ moderate, +++ many and ++++ very numerous. The numbers recorded in the columns indicate the number of animals in the group showing a particular inclusion load.

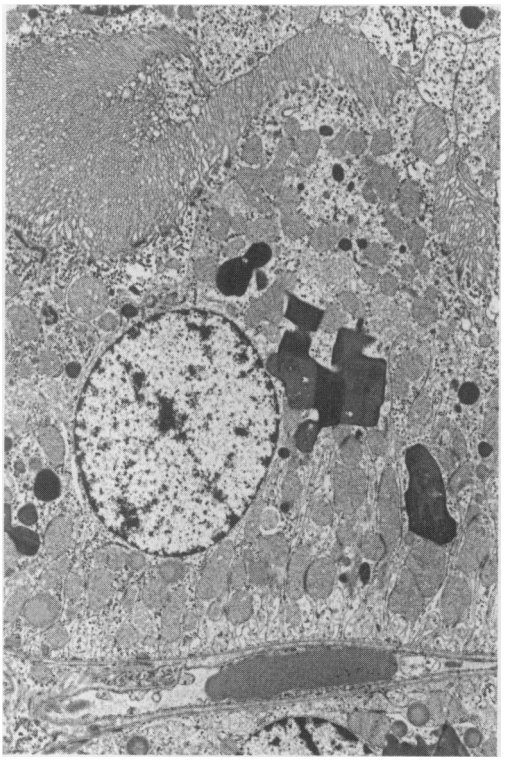


Fig. 3. Electron micrograph of proximal tubule cells of rats dosed with levamisole. Note although the inclusion loading is heavy the ultrastructural appearance of the cellular organelles appears normal. ×6120.

of increased proximal tubular cell necrosis in rats treated with levamisole.

Examination of the semi-thin plastic sections revealed varying numbers of globular and angular inclusions in the proximal tubular cells of kidneys from rats dosed with levamisole or the vehicle alone. These inclusions stained densely with methylene blue (Figs 1 & 2). The angular inclusions were more numerous in rats treated with levamisole whereas globular inclusions were more numerous in proximal tubular cells of control animals (Table 1). Electron microscopy showed that both types of inclusion were bound by a single triple layered membrane approximately 9.0 nm thick. Many of the globular inclusions simply had a homogenous electron dense matrix, others had crystalloid bodies. The angular inclusions usually had a matrix of similar density to that of the globular inclusions; they possessed crystalloids which were often much longer than those seen in the globular bodies. The majority of the heavily loaded cells apart from numerous inclusions showed normal ultrastructural features (Fig. 3). Occasionally, the angular inclusions were found to contain vesicles and other membranous material indicating that these inclusions were lysosomal bodies. Varying numbers of

Table 2. Urine volume, osmolality creatinine and protein mean ± s.e. values

	Volume (ml)		Osmolality (mosmol/kg)		Creatinine (mmol/l)		Total protein (mg/5h)	
Day	Control group	Levamisole group	Control group	Levamisole group	Control group	Levamisole group	Control group	Levamisole group
−5	1.60±0.27	1.67±0.19*	1222±166	1188±144*	3.89±0.70†	4.66±0.61	0.31±0.02†	0.36±0.07
−2	1.55±0.19	1.45±0.12	1068±92†	1175±138†	3.81±0.44*	4.90±0.55	0.39±0.05*	0.44±0.05
3	1.85±0.32	6.25±0.55		360±39	4.35±0.42	1.26±0.17	0.81±0.16*	0.51±0.09
6	2.30±0.22	7.35±1.45	1072±105	426±52	3.83±0.36	1.34±0.20	1.24±0.26	1.60±0.14
9	3.00±0.27	5.40±0.65	836±80	508±50	3.44±0.34	1.82±0.23	2.66±0.26	3.51±0.44
13	3.05±0.38	4.95±0.52	826±77	609±44	3.67±0.32	2.45±0.23	2.90±0.29	3.70±0.28

$n = 10$ except where indicated.
* $n = 9$.
† $n = 7$.

Table 3. Urine enzyme activity per 5h collection period, means ± s.e.

	Control group (day)						Levamisole group (day)					
Enzyme (units)	−5	−2	3	6	9	13	−5	−2	3	6	9	13
LDH (iu/l × 10^{-3})	51.8±7.9	49.5±4.9	67.4±7.5	40.9±3.4	43.9±4.9	51.9±5.6	63.4±11.7	55.9±5.8	55.2±5.8	64.6±15.2	274.6±65.6	291.2±34.3
ALP (iu/l × 10^{-3})	541.4±64.6	493.5±68.1	609.9±85.3	793.5±92.8	1162.9±112.9	1173.9±113.7	488.2±83.8	435.8±35.8	751.1±101.8	1025.9±117.6	1291.2±118.9	1382.6±108.1
AAP (iu/l × 10^{-3})	55.3±6.3	61.5±9.5	126.8±11.3	177.4±20.3	240.0±24.1	258.2±29.6	58.4±10.1	54.5±4.5	144.4±17.1	179.4±19.8	187.0±25.9	210.3±15.8
LAA (iu/l × 10^{-3})	58.3±6.8	67.0±11.5	131.5±11.9	182.1±21.9	243.5±27.1	252.0±31.2	56.8±9.4	58.2±4.5	137.4±17.0	159.1±16.9	177.6±24.3	222.6±21.4
GGT (iu/l × 10^{-3})	773±141.0	830.0±161.4	1646±155.4	2365±344.9	3336±382.9	3293±359.8	719±131	717±67.9	1750±218.0	2114±200.0	2314±318.0	2789±286.4
NAG (μmol/l/h)	1817±231.0	1614±217.4	2267±242.1	2892±344.9	2267±233.5	3897±457.9	2285±331	1833±184.5	3760±389.6	4665±644.0	4338±660.0	7127±872.2

$n = 10$ except for Levamisole group on day −5 when $n = 9$.

large vacuoles with amorphous content were also seen in the proximal tubule cells and their incidence was higher in the levamisole treatment group.

In urines from the levamisole treatment group, the mean volumes increased, whilst the creatinine and osmolality values decreased on days 3, 6, and 9 ($P<0.005$, Table 2). The increases of mean urinary protein values were similar in both control and levamisole treatment groups. There were no significant treatment related changes for urinary ALP, AAP, LAA or GGT (Table 3). Urinary LDH was markedly increased ($P<0.001$) on days 9 and 13, and NAG levels were higher ($P<0.01$) on days 9 and 13 in rats treated with levamisole.

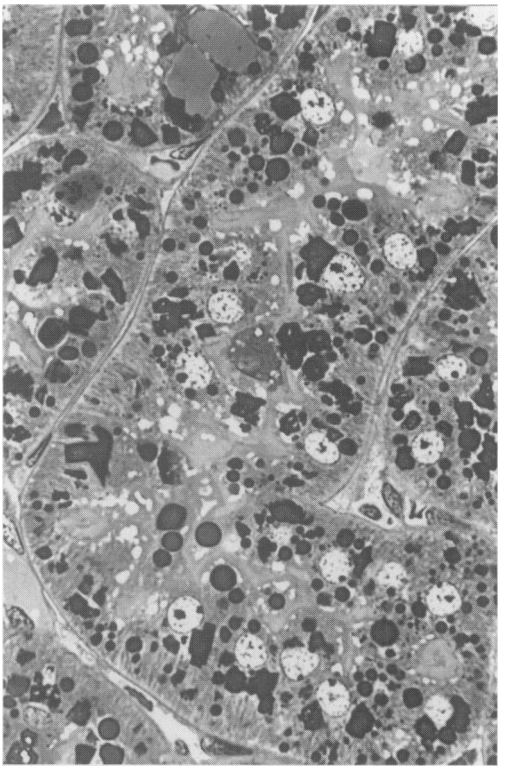


Fig. 4. Light micrograph of renal cortex illustrating the inclusion load within the proximal tubular cells of a rat dosed with decahydronapthalene (1000 mg/kg/day) for 14 days. Note the inclusion load is extremely heavy consisting of both the angular and globular types. Also a single necrotic cell is present in the tubule lumen. × 200.

Discussion

In rats and humans, diuresis has been shown to increase the excretion rates of the urinary enzymes LDH, AAP, GGT and ALP which were measured in this study (Josch & Dubach 1967; Jung *et al.* 1986). Increased urine output occurred in the levamisole treatment group before the enzyme excretion rates changed. There were no treatment-related changes for the enzyme ALP from the endoplasmic reticulum or the brush border enzymes GGT, AAP and LAA; these findings together with the morphological evidence suggest that the membranes remained functional. The increased excretion of ALP, GGT, AAP and LAA in both the control and levamisole treatment groups probably reflects the maturation of renal nephron function in rats of this age (Stoykova *et al.* 1983). Treatment related changes of urinary ALP may not have been detected as levamisole is known to inhibit this enzyme (Van Belle 1976).

The four- to five-fold increase in urinary LDH excretion suggests a change in renal cell membrane permeability with a consequent release of this cytosolic enzyme, but if changes of the cell membrane have occurred, then the effects are not sufficiently severe to increase the excretion of the other enzymes, e.g. GGT or AAP. Displacement of the cytosolic LDH from the intact cells due to the increased mass and number of cytoplasmic inclusions may be another possible explanation. The increase of urinary NAG on Day 13 is approximately two-fold in rats given levamisole, and this change may reflect increased lysosomal enzyme activity or expulsion of lysosomes by exocytosis or cytoplasmic 'blebbing' rather than cell degeneration.

There were strong similarities between the morphology of the angular inclusions seen in this study and those previously seen in rats dosed orally with decahydronaphthalene

(Fig. 4) where changes in the urinary excretion of LDH and NAG were also found (Evans & Morgan 1986).

Other studies using immunocytochemical procedures have shown that both the globular and angular inclusions stain strongly for the male specific protein alpha$_{2U}$ globulin in rats treated with levamisole or decanaphthalene. The staining reaction at the ultrastructural level was uniformly distributed over the matrix and crystalloids of the angular inclusions. On closer examination the crystalloids were shown to possess a highly ordered pattern of internal organisation consisting of several dense strial patterns (Read *et al.* 1987).

Unlike the investigations looking at light hydrocarbon nephropathy' increased proximal tubular cell necrosis was not a feature of this study with levamisole.

Failure to detect significant changes of urine protein suggest that further studies are required to explain the accumulation of alpha$_{2U}$ globulin and the increased incidence of hyaline droplets in male rats dosed with levamisole. It is not clear whether the processes involved are similar to those associated with the phenomenon described in 'light hydrocarbon nephropathy'.

There is very little evidence to suggest that levamisole is nephrotoxic in man (Symöens *et al.* 1979). In a study of anti-rheumatic drugs where urinary NAG was used as a marker of renal tubular damage, NAG values fell in patients given 150 mg levamisole per day for 26 weeks (Dieppe *et al.* 1978).

It would seem therefore, that the increased incidence of hyaline droplets including the angular bodies seen with certain compounds accompanied by increased enzymuria is unique to the post-pubertal male rat and may have little relevance to man.

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