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Degree of ethoxyquin-induced nephrotoxicity in rat is dependent on age and sex

Margaret M. Manson, Jonathan A. Green, Brian J. Wright, and Philip Carthew

MRC Toxicology Unit, Woodmansterne Road, Carshalton, Surrey SM5 4EF, UK

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Abstract. The toxicity of ethoxyquin (EQ) to rat kidney was examined in males which were either weanling or adult at the beginning of the experiment, and also in adult females. Female rats were much less susceptible to the toxic effects of EQ than males of the same age. In males damage to the cortex, mainly as an acceleration of the normal ageing process, was similar in both age groups, but rats exposed to EQ as weanlings also suffered from extensive papillary necrosis. Male rats were more prone than females to proteinuria, which was greatly exacerbated by EQ in both age groups. Thus there is very little evidence of nephrotoxicity in adult female rats on exposure to EQ at 0.5% in the diet for 26 weeks. In males, the initial age of the animal, as well as the length of treatment, influences the extent of damage.

Key words: Ethoxyquin(6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline) – Nephrotoxicity – Rat – Proteinuria

Introduction

It is well established that antioxidants can modify the carcinogenic potential of certain chemicals (Chung et al. 1986; Hagiwara et al. 1986; Kahl 1986; Kensler et al. 1986; Preat et al. 1986; Takahashi et al. 1986; Williams et al. 1986; Mandel et al. 1987; Ito and Hirose 1987; Imaida et al. 1988; Mizumoto et al. 1989; Rao et al. 1989) or can cause cancer in their own right (Ito et al. 1985; Inai et al. 1988; Hirose et al. 1990). However, in a previous study (Manson et al. 1987) to examine the protective effect of ethoxyquin (EQ) in rat liver treated with aflatoxin B_1 (AFB_1), we found that by 23 weeks, while EQ completely prevented the formation of preneoplastic liver lesions, it caused severe kidney damage. EQ appeared to accelerate greatly the chronic progressive nephrosis seen in normal ageing, with an increase in the incidence of basophilic and normal staining hyperplastic tubules. The latter were of interest because previously an increased incidence of adenomas was observed in kidneys of rats which had been fed a combination of EQ and AFB_1, compared with AFB_1 alone, for 8 weeks followed by 1 year on control diet (Cabral and Neal 1987).

More recently, a study using weanling rats reported that EQ caused papillary necrosis and ascending pyelonephritis in male rats (Hard and Neal 1990). Reexamination of the data from our previous study (Manson et al. 1987) which had used 7–8 week-old rats confirmed that these lesions were not present. If susceptibility to papillary necrosis was peculiar to weanling rats, this might explain why this lesion had also not been noted by other workers in EQ-treated animals.

The present study was designed to investigate age-related kidney damage by EQ and the resistance shown by females to such toxic effects.

Materials and methods

Diet. Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4 trimethylquinoline) was obtained from Sigma Chemical Co., Poole, Dorset at 90% purity. The purity was checked by gas chromatography both before mixing with the diet and after several weeks storage in the diet (E. Bailey, personal communication). EQ at 0.5% and Arachis oil at 2% (BDH, Poole, Dorset) were mixed with powdered MRC 41B diet as described previously (Manson et al. 1987).

Animals. Male and female Fischer 344 rats were fed a diet containing 0.5% ethoxyquin as described previously (Manson et al. 1987). Animals of different ages at the start of treatment received diet for different periods of time as detailed in Table 1. Age matched control animals were included.

Urine and serum samples. Urine samples were collected at room temperature by placing individual animals in a metabolism cage for a period of 24 h. Samples were collected from male and female rats in groups 5 and 6 after 24 h, 7 days, 12 weeks and 26 weeks on EQ diet. Samples were also collected from males in groups 1–4 after 20 and 30 weeks on the diet. Samples from age-matched controls were also obtained at all time points. Urines were stored at –20° C until required.

Offprint requests to: M. Manson

Table 1. Treatment schedules and effect of EQ on kidney and body weight

	Initial age and treatment time	Body weight (g)	Kidney weight (g)	Kidney weight as percentage of body weight
Group 1	C ♂ 3 week ($n=4$) 20 week	381.3±11.1	2.90±0.13	0.78±0.02
	EQ ♂ 3 week ($n=4$) 20 week	342.0±2.4*	3.60±0.14	1.05±0.05**
2	C ♂ 8 week ($n=4$) 20 week	406.3±7.8	3.11±0.81	0.73±0.02
	EQ ♂ 8 week ($n=4$) 20 week	373.8±12.8	3.58±0.11	0.98±0.02***
3	C ♂ 3 week ($n=5$) 30 week	403.8±9.5	2.94±0.24	0.74±0.07
	EQ ♂ 3 week ($n=5$) 30 week	363.6±10.9*	4.22±0.12	1.18±0.02**
4	C ♂ 8 week ($n=5$) 30 week	435.8±12.4	2.80±0.03	0.65±0.03
	EQ ♂ 8 week ($n=5$) 30 week	368.6±13.4**	4.30±0.15	1.16±0.07***
5	C ♂ 8 week ($n=8$) 26 week	398.5±3.4	2.36±0.04	0.59±0.01
	EQ ♂ 8 week ($n=8$) 26 week	341.1±9.7***	2.78±0.10	0.81±0.02***
6	C ♀ 8 week ($n=8$) 26 week	222.9±3.9	1.51±0.04	0.68±0.01
	EQ ♀ 8 week ($n=8$) 26 week	193.9±3.1***	1.51±0.05	0.78±0.02***

The number of animals per group is shown in brackets. Values shown ± SE
* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Serum samples were prepared from blood taken from some animals in each of groups 3 and 4 at the time of sacrifice.

BrdU incorporation. In order to determine the level of hyperplasia in kidney tubules, two animals in each of the groups 1–4 were given an injection (i.p.) of BrdU (Sigma Chemical Co., Poole, Dorset) (50 μg/g body weight in 0.9% saline) approximately 1 h before sacrifice.

Tissue preparation. Animals were killed by CO_2, kidneys removed immediately and 2–3 mm slices cut for fixation in acetone or neutral buffered formalin. For those animals which received an injection of BrdU, slices were also fixed in Carnoys, along with a piece of gut as positive control. Care was taken to section kidney in order to obtain the tip of the papilla.

Histochemistry and immunocytochemistry. Acetone fixed sections of kidney were stained histochemically for gamma glutamyl transpeptidase (Manson et al. 1981).

BrdU incorporation into DNA was detected immunocytochemically using a rat monoclonal antibody to BrdU (Sera-Lab., Crawley Down, Sussex) as described previously (Green et al. in press). Formalin fixed sections were stained routinely with H and E and by the alizarin red S method to detect calcium deposits.

Immunoblotting of urine and serum samples. Neat urine samples (10 μl) were loaded in an equal volume of sample buffer on to an 11% SDS-PAGE gel and blotted on to nitrocellulose. Blots were probed with polyclonal antisera against albumin (Nordic Immunological Labs, Maidenhead, Berks), or α2μ-globulin (kindly supplied by Dr. J Foster, ICI, Alderley Edge, Cheshire) as described previously (Green et al. 1990). These two proteins were chosen as it was suspected they would represent most of the total protein excreted by male rats (Roy and Neuhaus 1966).

Serum samples were run as described above, using 1 μl serum plus 25 μl sample buffer. The immunoblot was probed with antibody against α2μ-globulin.

Results

Effect of EQ on tissue and body weight

EQ caused a significant depression in body weight in all treated groups. In both males and females the total kidney weight expressed as a percentage of body weight was significantly greater in treated animals. However, the difference was more obvious in males (Table 1), there being no difference in absolute kidney weight between control and treated females.

Histopathological changes in the kidney

Male rats 20 week study. All four animals in group 1, started on EQ as weanlings and fed for 20 weeks, showed substantial papillary necrosis (Fig. 1 *a*) involving up to one third of the papilla in the most severe case. Small amounts of Ca^{2+} deposit were identified in serial sections stained by alizarin red S. In the cortex there were eosinophilic cytoplasmic inclusions in the tubular epithelial cells and protein accumulation in the lumina of some tubules. Foci of regenerating basophilic tubules (characteristic of normal ageing in much older animals) were evident, with some cells containing brown pigmented intracytoplasmic inclusions. There was some thickening of basement membranes around tubules and Bowman's capsules and hyperplasia of the pelvic transitional epithelium. Only one animal showed some evidence of limited pyelonephritis, which was considered to be an incidental lesion due to bacterial infection as polymorphonuclear cells were visible in the lumina of affected tubules.

Of those animals started on the diet at 8 weeks of age (group 2), two had no damage to the papilla and two had very small amounts of necrosis of the interstitial cells at the very tip of the papilla. The lesions in the cortex described above, which are typical of EQ treatment (Manson et al. 1987), were, however, very evident. GGT histochemistry revealed many tubules where enzyme activity was reduced or absent. Age matched controls for both groups showed none of the above changes (Fig. 1 *b*).

Male rats, 30 week study. All animals started as weanlings and receiving EQ for 30 weeks (group 3), showed extensive papillary necrosis from one third to complete involvement. One animal had severe calcification in the papilla (Fig. 1 *c*) and this and one other animal showed segmental scarring due to pyelonephritis, (Fig. 2). However, this was

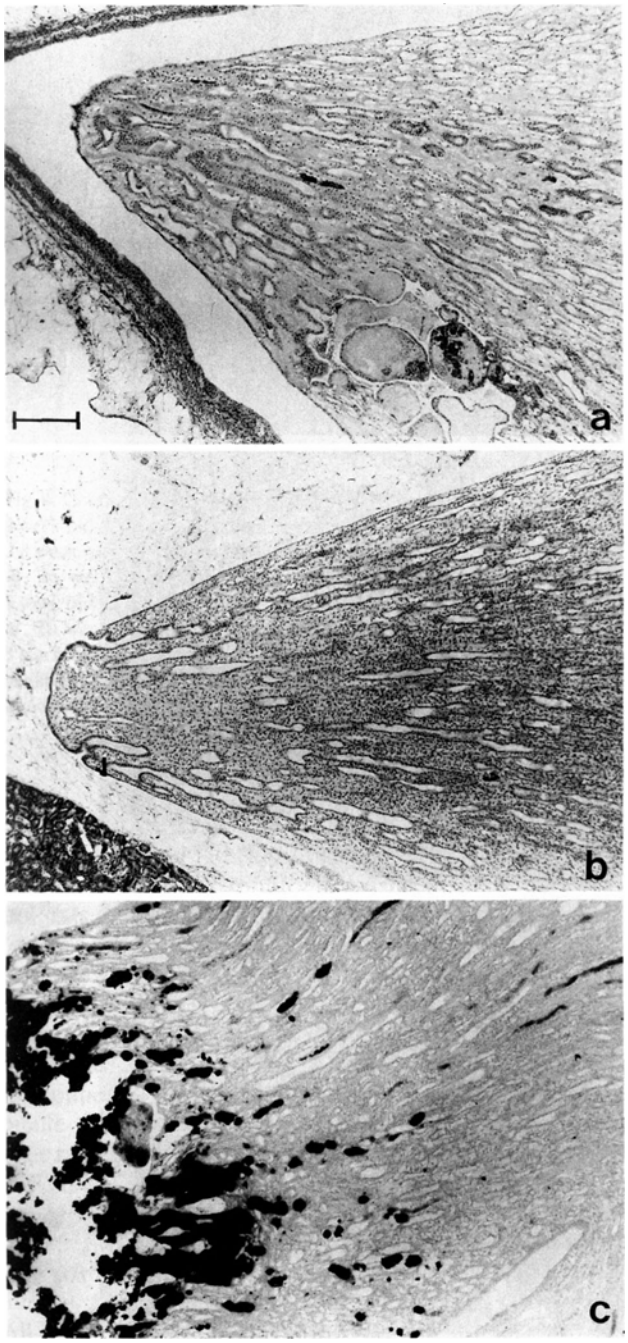

Fig. 1. Effect of EQ on the papilla. Formalin fixed sections were stained with H and E (**a, b**) or alizarin red S (**c**). **a** 3-week-old male, fed on EQ diet for 20 weeks, showing extensive papillary necrosis, **b** 3-week-old control male after 20 weeks, **c** 3-week-old male fed on EQ diet for 30 weeks showing severe calcification in the papilla (bar = 300 μm for all photographs)

not seen in other animals in this group and was considered an incidental lesion due to intercurrent bacterial infection.

Of the rats started on EQ at 8 weeks of age (group 4), one had papillary necrosis involving the bottom one third and one had loss of interstitial cells at the very tip.

All animals in groups 3 and 4 had extensive damage in the cortex as described for the 20 week study. Control animals of both age groups had a very small number of

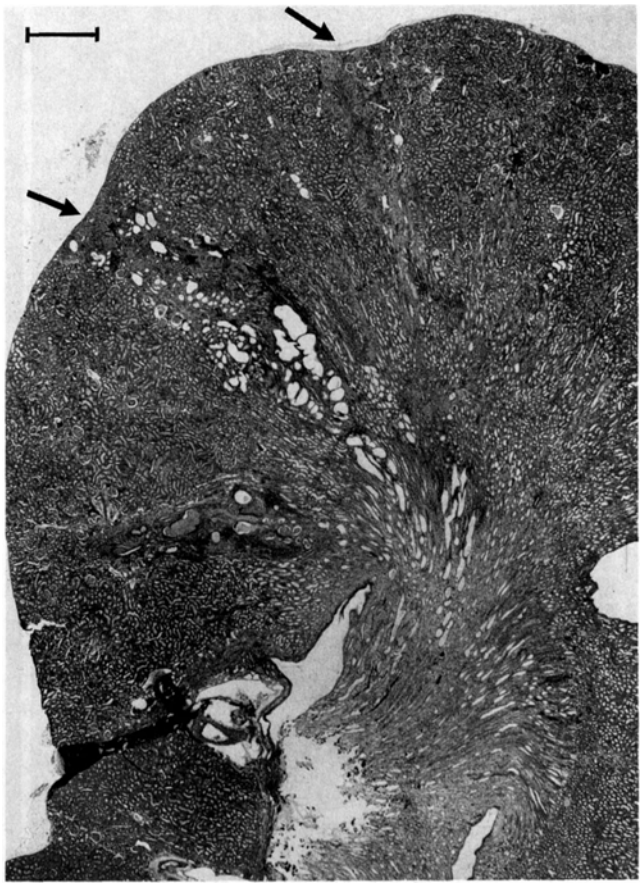

Fig. 2. Section of kidney from a 3-week-old male fed on EQ diet for 30 weeks showing segmental pyelonephritis (*arrows*) (*bar = 1 mm*)

regenerating basophilic tubules, typical of the normal ageing lesions for animals 33–38 weeks old.

BrdU labelling

Two animals from each of groups 1–4 received an injection of BrdU about 1 h before sacrifice, in order to examine the degree and localisation of hyperplasia in kidney caused by administration of EQ. BrdU labelling was greater in kidneys of EQ fed animals after both 20 and 30 weeks on the diet compared with age matched controls. There was, however, considerable variation between treated animals particularly after 30 weeks on diet. Labelled nuclei in epi-

Table 2. BrdU labelled nuclei in epithelial tubule cells

	Initial age and treatment time	Control : treated
Group 1	(3 week, 20 week)	1 : 3.5, 1 : 3.6
2	(8 week, 20 week)	1 : 3.8, 1 : 4.0
3	(3 week, 30 week)	1 : 1.5, 1 : 2.5
4	(8 week, 30 week)	1 : 3.0, 1 : 7.0

BrdU-labelled nuclei were counted in epithelial tubule cells after immunocytochemistry on Carnoys fixed sections of control and EQ treated rats. Results are shown for individual pairs of animals, and expressed relative to control values of 1

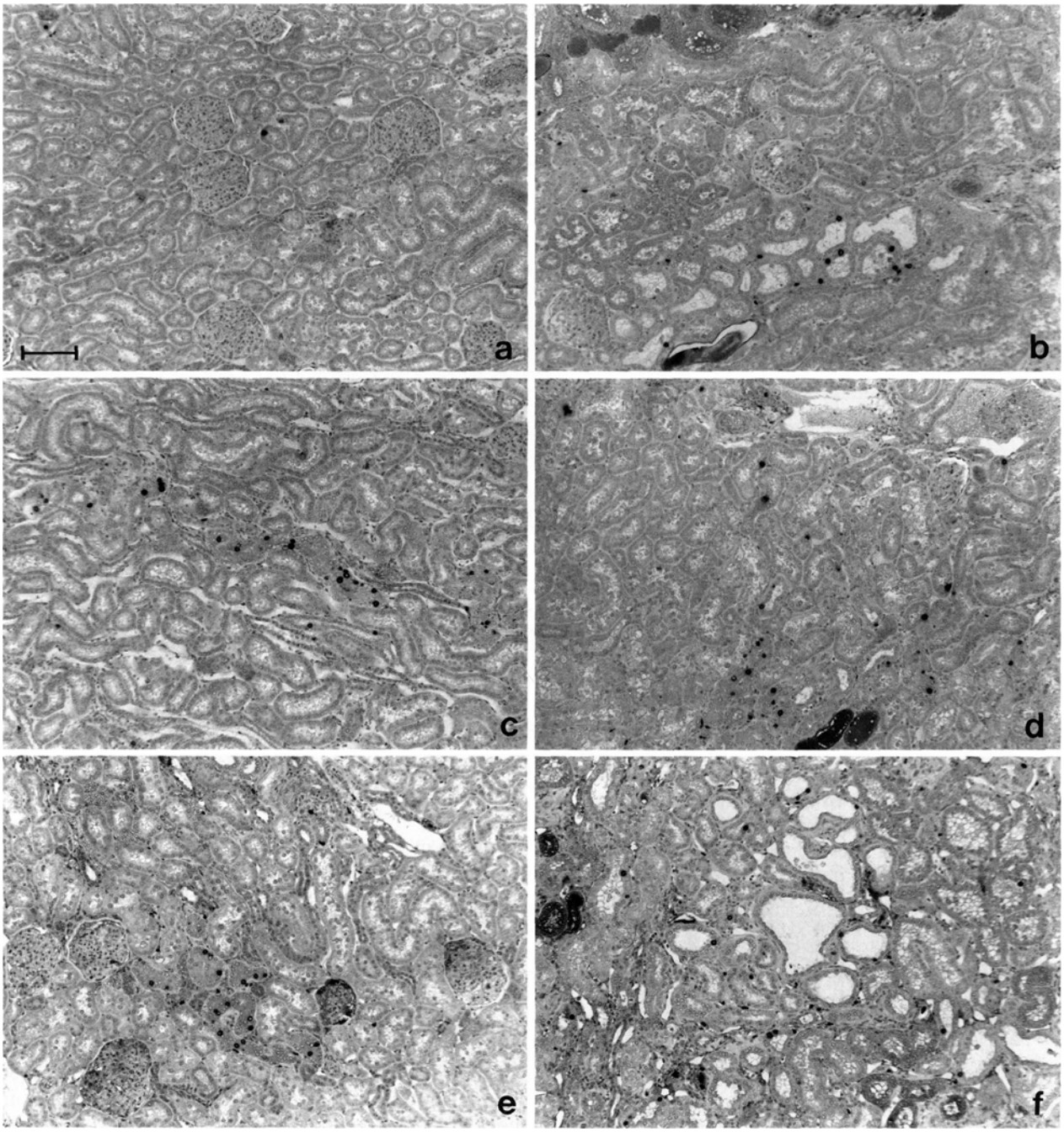

Fig. 3. BrdU labelling of tubular epithelial cells. Carnoys fixed sections from male rats were stained immunocytochemically using anti-BrdU monoclonal antibody and counterstained with H and E (bar = 100 μm for all photographs). **a** 8 weeks old, 20 weeks control diet. **b** 8 weeks old, 20 weeks EQ, showing labelling of nuclei in regenerative basophilic tubules. **c** 3 week old, 20 weeks EQ, showing labelling in tubules whic stain normally with H and E. **d** 8 weeks old, 20 weeks EQ, as in c. 3 weeks old, 30 weeks EQ, as in **c**. **f** 8 weeks old, 30 weeks EQ, showin labelling in basophilic tubules and normal staining tubules. Some tubule are enlarged due to hyperplasia (centre of field).

thelial cells were counted, after immunocytochemistry on Carnoys fixed sections, to give an idea of the level of labelling in each animal. A total of 40 sequential areas (in total approximately 150 mm^2) were scanned by light microscopy on two kidney sections for each animal using a 10× objective lens. Results for individual animals are shown in Table 2. Labelling was not exclusively confined to the regenerating basophilic tubules typical of normal ageing lesions, but significant numbers of mildly hyperplastic tubules which stained normally with H and E were also labelled (Fig. 3).

Male and female rats, 26 week study

Male rats (group 5), treated with EQ from 8 weeks of age showed evidence of retained protein aggregates in the tubules (7% of the cortex), basophilic tubule hyperplasia

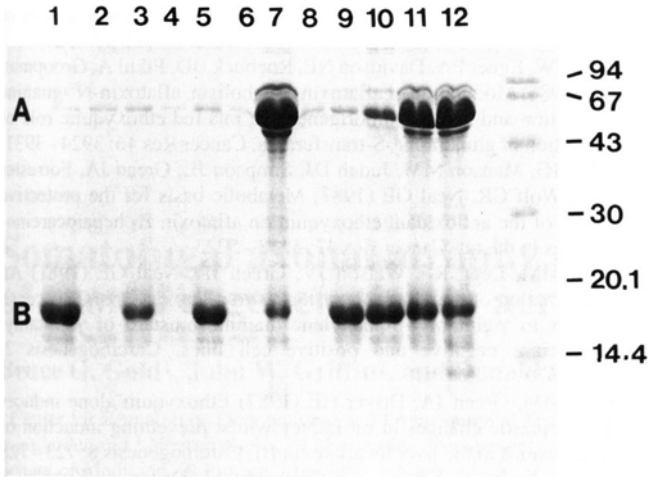

Fig. 4. Coomassie blue stained SDS-PAGE gel of urine samples. Lane *1* 8 week old ♂, 1 week control diet; *2* 8 week old ♀, 1 week control diet; *3* 8 week old ♂, 1 week EQ; *4* 8 week old ♀, 1 week EQ; *5* 8 week old ♂, 26 week control diet; *6* 8 week old ♀, 26 week control diet; *7* 8 week old ♂, 26 week EQ; *8* 8 week old ♀, 26 week EQ; *9* 3 week old ♂, 20 week control diet; *10* 8 week old ♂, 20 week control diet; *11* 3 week old ♂, 20 week EQ; *12* 8 week old ♂, 20 week EQ. A = albumin (m.wt 63.7 K daltons). B = α2μ-globulin (m.wt 18.7 K daltons). Molecular weight markers in K daltons are shown on the right hand side

(5% of the cortex) and thickening of basement membranes as described above at 20 and 30 weeks. However, there was no evidence of papillary necrosis or calcification. Control rats of both sexes and females treated with EQ (group 6) had a negligible incidence of these lesions. It has been shown in a separate study that females exposed to EQ as weanlings are also resistant to the nephrotoxic effects of this antioxidant (Hard and Neal 1990). However, it was of interest to note that both males and female rats receiving EQ contained significant amounts of brown pigment in the tubule cells which was identified in a previous study in male rats as being mainly composed of lipofuscin (Manson et al. 1987).

Analysis of urine and serum samples

All urine samples from both male and female rats, even after the shortest treatment time of 24 h, were dark brownish green compared with the normal yellowish colour of samples from control animals. Samples from both sexes after different times on the EQ diet were run on 11% SDS-PAGE gels (Fig. 4). Bands A and B were identified by immunoblotting as albumin and α2μ-globulin, respectively. Results showed that all male rats excreted α2μ-globulin in their urine, while female urine contained negligible amounts of this protein as previously reported (Roy et al. 1976; Vandoren et al. 1983). However, males treated with EQ (Fig. 4, lanes *3, 7, 11* and *12*) appeared to excrete slightly less of this protein than age matched controls (lanes *1, 5, 9* and *10*).

With respect to albumin excretion, control and EQ-treated samples from both sexes after 7 days (lanes 1–4) showed similar amounts of albumin in the urine and this was also the case for control and treated females (lanes 6 and 8) even after 26 weeks on the diet. However, males treated for 20 or 26 weeks excreted greatly increased amounts of albumin (lanes *7, 11* and *12*).

When serum proteins from control and treated males, after 20 weeks on the diet, were run out on SDS-PAGE gels no differences were observed in the levels of albumin (as determined by Coomassie blue staining), and an immunoblot probed with antibody to α2μ-globulin also showed no significant difference in this protein between control and treated animals (data not shown).

Discussion

The main conclusions of this study are that adult female rats are much less susceptible to the nephrotoxic effects of EQ than male rats and that the degree of toxicity in male rats is dependent on the age of the animals when they are started on the diet. Male adult rats treated with EQ suffered mainly from lesions in the cortex of the kidney, whereas weanling rats eventually (after 20 and 30 weeks) developed extensive papillary necrosis in addition to the cortical lesions.

Hyperplasia of the transitional epithelium of the kidney accompanying papillary necrosis has been reported previously (Hard and Neal 1990) in the response of young rats to EQ. The underlying cause of papillary necrosis is unknown.

Male rats are more prone than female rats to proteinuria, a condition which is exacerbated in normal animals with age (Sellers et al. 1950). From the SDS-PAGE analyses of urine and serum it is obvious that, in male rats, EQ treatment greatly enhances the urinary excretion of albumin. The levels of α2μ-globulin, a protein implicated in other conditions involving renal toxicity (Swenberg et al. 1989), were not increased and may even have been decreased slightly.

Despite the lack of toxicity of EQ to female rat kidneys this sex also showed a significant increase in kidney weight as a percentage of total body weight as well as accumulation of brown pigment in the renal tubules as described previously (Manson et al. 1987). A previous study using a single large dose of butylated hydroxytoluene also showed female rats to be less susceptible to nephrotoxicity and proteinuria than males (Natagawa and Tayama 1988).

BrdU incorporation into nuclei of epithelial cells showed that there was DNA synthesis in the regenerating, basophilic tubules as would be expected, but in addition, evidence of cell proliferation was also found in tubules which stained normally with H and E.

EQ is known to enhance the carcinogenic effect in the kidney when given in combination with, or following, some chemicals such as N-ethyl-N-hydroxyethyl-nitrosamine (Ito et al. 1986) and AFB_1 (Cabral and Neal 1987). However, the question as to whether EQ itself has any carcinogenic potential in this tissue, as suggested by the appearance of hyperplastic tubules with a different morphology to the regenerative basophilic tubules seen in ageing lesions (Manson et al. 1987), is still not resolved. What is clear from the present data is that if a lifetime study

is planned to look at the involvement of EQ in kidney tumour formation, then it would not be advisable to start with weanling male animals. This could increase the incidence of pyelonephritis, leading to confusion in interpretation of the histopathology in the kidney cortex, or even cut short the study. It would be preferable to use adult animals where the possible incidence of secondary pyelonephritis resulting from papillary necrosis would seem to be minimal.

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