Chapter 3

Effect of Antimalarials Treatment on Rat Liver Lysosomal Function- An In Vivo Study

Introduction

A variety of quinoline based antimalarial drugs are used for the treatment of malaria as well as for prophylaxis (1). Most common currently used antimalarials include: chloroquine (CQ), primaquine (PQ) and quinine (Q) (1,2). The mechanism of action of these antimalarials in general is less clearly understood. However, various explanations have been proposed for their antimalarial action as well as for their action on the biomolecules (1,2). These include: inhibition of enzymes of antioxidant defense mechanism and of mitochondrial respiratory activity, cytochrome oxidase and several dehydrogenases (3-7). The drugs accumulate in lysosomes and mitochondria (2,8). Thus intralysosomal concentration of CQ is reported to be about 1000 times higher compared to that in the plasma (1,9). Data in chapter 1 suggest that under *in vitro* conditions, concentrations of CQ, PQ and Q simulating therapeutic plasma levels showed stabilizing effect in the rat liver lysosome membranes. Hence it was of interest to find out whether *in vivo* antimalarials treatment also has similar effects. Interestingly the effects of *in vivo* treatment with antimalarials on lysosomal membrane functions have not been examined thus far. This aspect assumes importance since antimalarials accumulate in the lysosomes in high concentrations as cited above (1,9). Hence, the *in vivo* effects of the three antimalarial on the lysosomal function in terms of the marker enzyme activities i. e. acid phosphatase, cathepsin D, DNase II, RNase II were examined. Acid phosphatase activity was measured with β glycerophosphate (BGP) and *p*-nitrophenylphosphate (PNPP) as the substrates The results of these studies are summarized in this chapter.

Materials and methods

Chloroquine phosphate, primaquine phosphate and quinine hydrochloride were purchased from Sigma-Aldrich. Yeast RNA, Calf thymus DNA and bovine serum albumin (BSA) fraction V were purchased from Sigma Chemical Company, USA. Sodium β glycerophosphate (BGP) was purchased from Koch-Light Ltd. UK. Hemoglobin was from British Drug Houses, Poole, Dorset, UK, and *p*-nitrophenylphosphate (PNPP) was obtained from SRL, India. All other chemicals were of analytical reagent grade and were purchased locally.

Animals and treatment with antimalarials

Male albino rats of Charles-Foster strain weighing between 250-300 gm were used. The animals received daily injection of antimalarials intraperitoneally (I.P) for 7 or 14 days. Doses of the three antimalarials CQ, PQ and Q were: 10 mg, 0.21 mg and 30 mg /kg body weight respectively (10). The antimalarial solutions were prepared in saline. The controls received only the saline vehicle. The animals were killed by decapitation on 8^{th} and 15^{th} day of treatment. In the control groups the results for 7 or 14 day saline injected rats were in close agreement and hence the data were pooled. Thus the control is given only as one group in all the figures.

Enzyme assays

The animals were killed by decapitation and their livers were quickly removed and placed in beakers containing chilled (0 – 4^0 C) 0.25 M sucrose. The tissue was minced, was repeatedly washed with 0.25 M sucrose, and then homogenized using a Potter-Elvehjem type glass-Teflon homogenizer to obtain 10 % (w/v) homogenate. The homogenate was

centrifuged at 650 × g for 10 min to obtain nuclei-free supernatant which was used as the source of enzyme for determination of β glycerophosphatase (BGPase), *p*-nitrophenyl phosphatase (PNPPase), RNase II, DNase II and cathepsin D activities (11,12).

Measurement of activity of B-glycerophosphatase and p-nitro phenyl phosphatase.

The procedure is already described in chapter 2.

Measurement of activity of Cathepsin – D.

Reagents:

1) Buffered Substrate:

5mg of hemoglobin was dissolved in 1.0 ml of 0.2M sodium acetate-acetic acid buffer, pH 3.8. pH of substrate was adjusted with acetic acid.

2) Modified alkaline copper reagent:

Stock (a) 1 0 gm of trisodium citrate dihydrate with 0.50 gm of $CuSO_4 \cdot 5H_2O$ in 100 ml D/W.

Stock (b): 16.0 gm NaOH and 50 gm of Na_2CO_3 in 500 ml of D/W.

Working solution is prepared by adding 1.0 ml of stock (a) and 79.0 ml D/W then 20 ml of Stock (b) is introduced.

3) Diluted Folin –Ciocalteau Reagent:

The commercially available reagent was diluted 1:3 times in D/W.

Cathepsin D activity was measured in a total volume of 0 75 ml. 0.25 ml of nuclei-free homogenate was incubated at 37 °C for 2-3 minutes and the reaction was started by the addition of 0.5 ml of buffered substrate. After addition of the substrate the reaction was carried out at 37 °C for 10 min. The reaction was terminated with 2 0 ml of 5%(w/v) TCA and the tubes were kept in ice for 15 mins. The tubes were centrifuged at 2000 rpm for 10 mins. To 1ml of supernatant 2ml of modified copper reagent and 0.6 ml diluted FC Reagent was added. A blue color developed which was estimated colorimetrically at 660nm (11)

Measurement of activity of Ribonuclease II

Reagents

1) Buffered substrate:

RNA (2-mg/ml) was dissolved in 0.2M sodium acetate acetic acid buffer, pH 5.0

2) PCA-uranyl acetate reagent·

10% w/v PCA (Per Chloric Acid) + 0.25 gm or uranyl acetate per 100 ml of 10% PCA.

Ribonuclease II activity was measured in a total volume of 0.8 ml. 0.2 ml of nuclei-free homogenate was incubated at 37 °C for 2-3 minutes and the reaction was started by the addition of 0 6 ml of buffered substrate. After addition of the substrate the reaction was carried out at 37 °C for 10 min The reaction was terminated with 2.0 ml of PCA-uranyl acetate reagent and the tubes were kept in ice for 40 mins. The tubes were then centrifuged at 2000 rpm for 10 mins. The supernatant was appropriately diluted and read at 260nm. The enzyme activity was expressed as µmole of nucleotide released in supernatant of the reaction mixture.

Measurement of activity of Deoxyribonuclease II

Reagents

1) Buffered Substrate

DNA (1mg/ml) dissolved in 0.2M sodium acetate acetic acid buffer, pH 5.0 containing 0.2M KCl KCl was included in incubation mixture to permit full activity of DNase and to block alkaline deoxyribonuclease activity.

2) PCA Reagent

10% (w/. v) aqueous $HClO_4$ not containing Uranyl Acetate.

The procedure of the Assay are almost the same as described for Ribonuclease except that the termination was with PCA reagent only and after terminating the reaction and the tubes should stand on ice for 20 minutes at 4°C before centrifuging.

The 'free' activities were determined within 15 min of making the nuclie-free supernatant. The nuclei-free supernatants was diluted 1:5 times with distilled water and subjected to 5 cycles of freezing and thawing and then used for measurement of the 'total' activities (13,14). The ratio of Total activity / Free activity (T/ F ratio) was used as the measure of the membrane integrity (13,14).

For determination of the Pi content in the acid soluble pool known aliquots (0.1 ml) of nuclei-free supernatant were deproteinized with 2.0 ml cold (0-4^0 C) 5 % (w/v) trichloroacetic acid (TCA) as described previously (13). Determination of Pi content was by the method of Fiske and Subba Row (15).

For determination of the nucleotide pool 0.2 ml aliquots of nuclei-free supernatant were deproteinized with 2.0 ml of 10 % (w/v) perchloric acid containing 0.25% uranyl acetate. The tubes were kept on ice for 20 min and then subjected to centrifugation. After suitable dilution, the optical density of the supernatant was determined at 260 nm from which the nucleotide pool content was calculated using the molar extinction coefficient of 8.5×10^6 (11).

Tyrosine positive materials in the tissue were measured after deproteinization of measured aliquots (0.25 ml) of the nuclei-free supernatants with cold (0-4^0 C) 5 % (w/v) TCA as described earlier (13).

Protein estimation was by the method of Lowry *et al.*(16) with bovine serum albumin used as the standard.

The results are expressed as mean ± S.E.M.

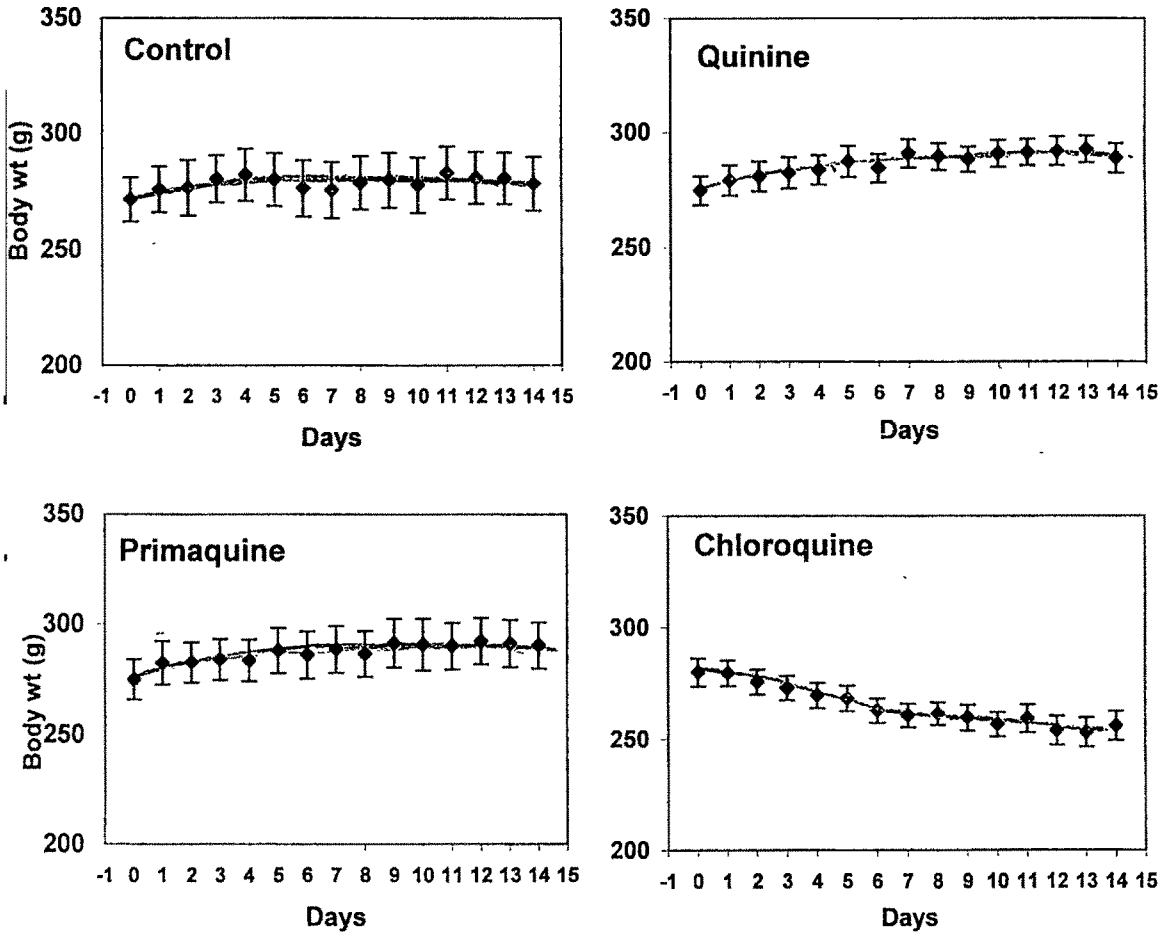
Students' t–test was used to calculate the level of significance.

Results

The effects of antimalarials treatment on the body weights are shown in Fig. 1. It can be noted that in the control group the body weight increased marginally during the 14 day period. A similar pattern was seen in the PQ and Q treated animals. By contrast, by the end of the first week, CQ treatment resulted in significant decrease in the body weights which persisted through the second week of treatment. Following CQ treatment, the liver

Fig. 1. Effect of antimalarials treatment on the body weights. Each data point represents mean of body weights of eight rats. The error bars represent the S.E.M.

Fig.1


Control
Quinine
Primaquine
Chloroquine
Body wt (g)
350
300
250
200
-1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days

weight also decreased significantly (34 % decrease) in the 7 day treatment group. However a partial recovery (22 % decrease) was observed at the end of 14 day treatment. PQ and Q treatments had no effect on the liver weight (Chapter 4).

The tissue protein content did not change following antimalarials treatment for 1 week. However, PQ and Q treatment for 2 weeks resulted in 21 % and 14 % decrease in the tissue protein content (Table 1).

The effects of antimalarials treatment on lysosomal enzyme activities are depicted in Fig. 2-4 Thus following CQ treatment (Fig. 2) it was noted that both 'free' and 'total' PNPPase activities increased in both 7 and 14 day groups. The T/ F ratio increased by 16 % in the case of CQ-7 group whereas no change was seen for CQ-14 group. A similar pattern was seen also for the BGPase activities. The T/ F ratio almost doubled in the 7 day treatment group. The 'free' and 'total' RNase II activities increased progressively with duration of treatment with the magnitude of increase being much higher in the case of the 'total' activity Thus the T/ F ratio increased progressively. The DNase II activity was not detectable in the control group However, CQ treatment caused a transient increase in the 'free' activity on day 7 of the treatment. The 'total' DNase II activity was significantly elevated resulting in higher T/ F ratios. CQ treatment caused a significant increase in both 'free' and 'total' cathepsin D activities in the 7 day treatment group and the T/ F ratio was significantly elevated. Increased cathepsin D levels following CQ treatment has also been reported by Korolenko *et al.* (17).

Following PQ treatment the 'free' PNPPase activity decreased significantly while the 'total' activity was somewhat low in 7 day group; in the 14 day group the activity was

Table 1. Effect of antimalarials treatment on tissue protein content.

	Protein content (mg/ g tissue)			
Treatment	Control	CQ	PQ	Q
7 Day	112.1±2 90	110.7±2.94	114.3±3.14	119.7±2.27
14 Day	115.3±4.69	114.7±2.42	90.4±0.83[b]	103.5±2.13[a]

The results are expressed as mean ± S.E.M. of eight independent observations.

[a] $p< 0.01$ and [b] $p<0.001$ compared with the corresponding control.

Fig. 2. Effect of treatment with CQ on lysosomal enzyme activities. The left hand panel depicts the enzyme activities. The right hand panel indicates the T/F ratio. The units (on ordinate) are expressed in: nmole PNP formed/ 10 min / mg protein for PNPPase; μ mole of Pi liberated / 10 min /mg protein for BGPase; n mole nucleotides formed / 10 min / mg protein for RNase II and DNase II and μg tyrosine positive materials liberated / 10 min / mg protein. The error bars represent the S.E.M.

a, $p< 0.05$; b, $p<0.02$; c, $p<0.01$; d, $p<0.002$ and e, $p<0.001$ compared with the corresponding control.

Fig. 2

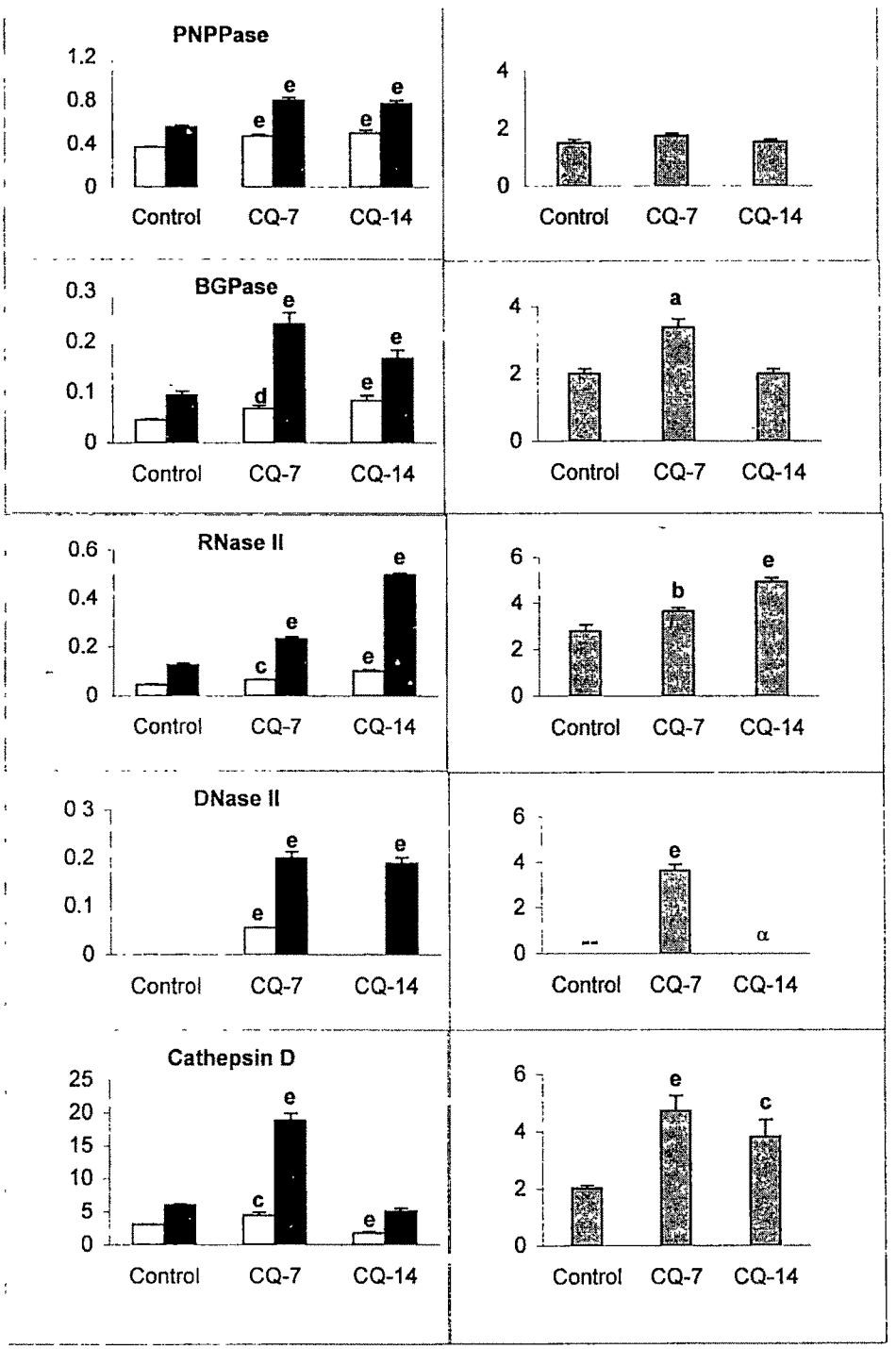

Activity
T/F Ratio
PNPPase
BGPase
RNase II
DNase II
Cathepsin D
Control
CQ-7
CQ-14

Fig. 3. Effect of treatment with PQ on lysosomal enzyme activities. The details are as described in the text and in Fig.1.

a, $p<0.02$; b, $p<0.01$; c, $p<0.002$ and d, $p<0.001$ compared with the corresponding control.

Fig. 3

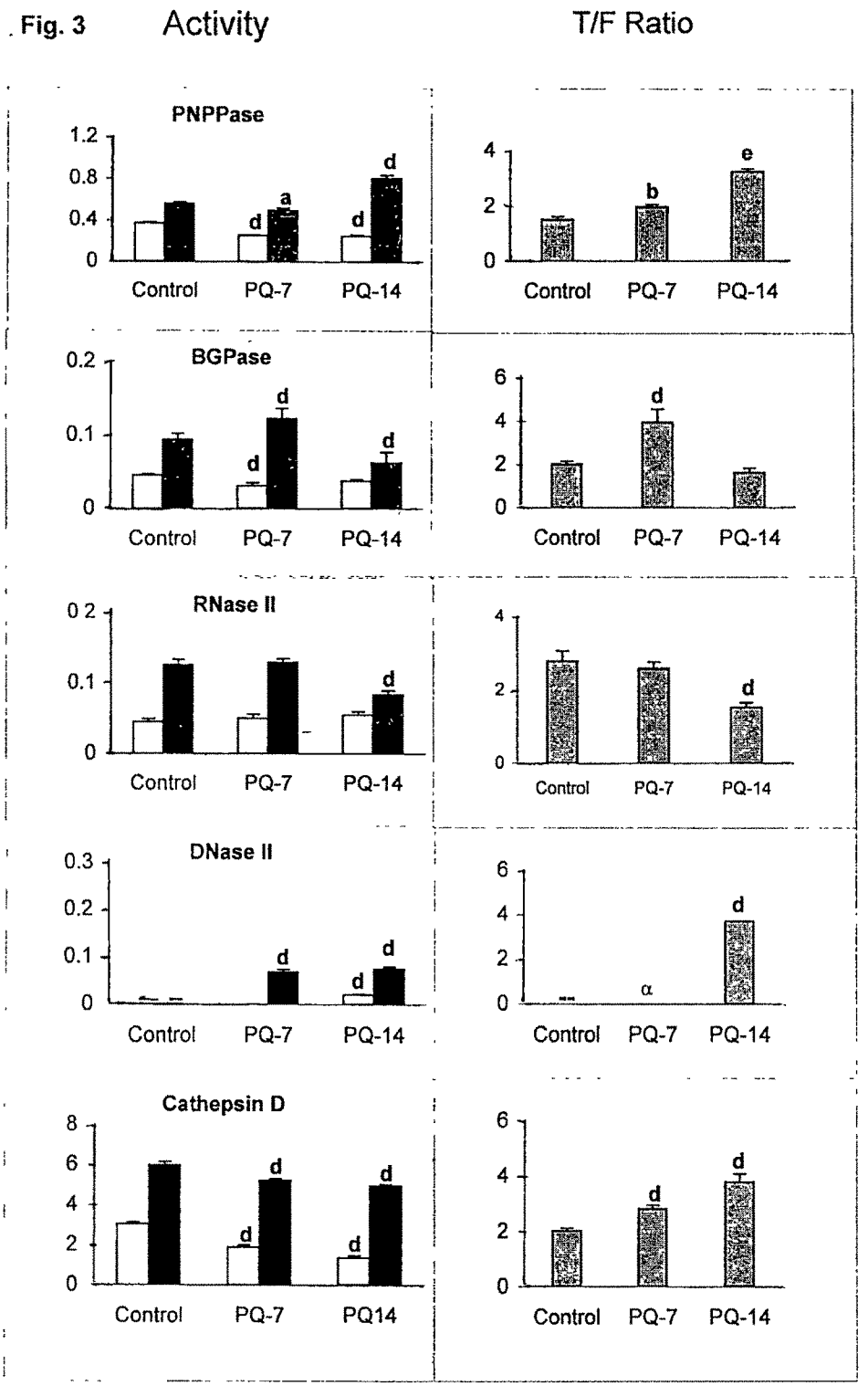
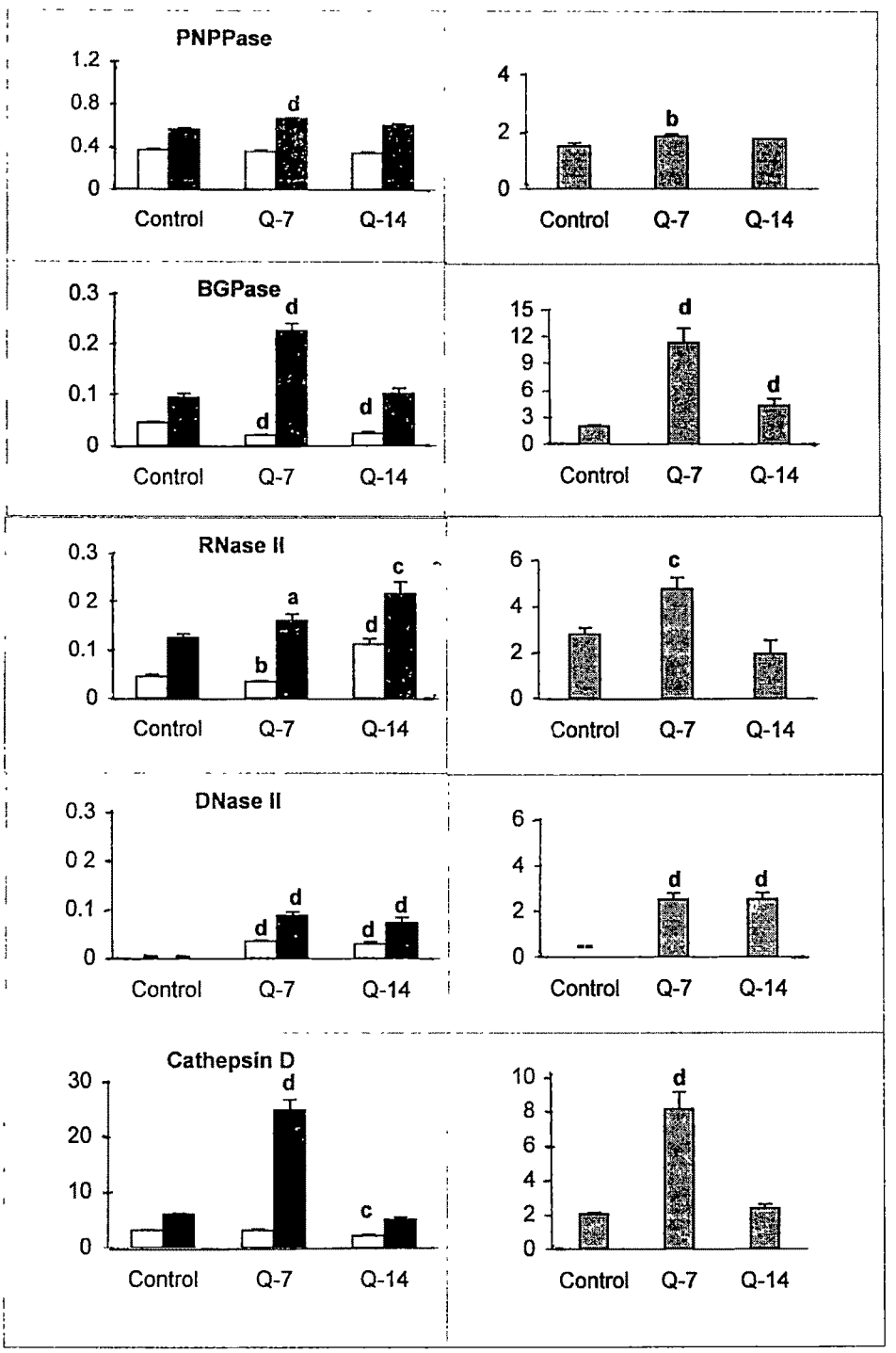

Activity
T/F Ratio
PNPPase
BGPase
RNase II
DNase II
Cathepsin D
Control
PQ-7
PQ-14
PQ14

Fig. 4


Activity
T/F Ratio
PNPPase
1.2
0.8
0.4
0
d
Control
Q-7
Q-14
4
2
0
b
Control
Q-7
Q-14
BGPase
0.3
0.2
0.1
0
d
d
d
Control
Q-7
Q-14
15
12
9
6
3
0
d
d
Control
Q-7
Q-14
RNase II
0.3
0 2
0.1
0
b
a
d
c
Control
Q-7
Q-14
6
4
2
0
c
Control
Q-7
Q-14
DNase II
0.3
0 2
0.1
0
d
d
d
d
Control
Q-7
Q-14
6
4
2
0
--
d
d
Control
Q-7
Q-14
Cathepsin D
30
20
10
0
d
c
Control
Q-7
Q-14
10
8
6
4
2
0
d
Control
Q-7
Q-14

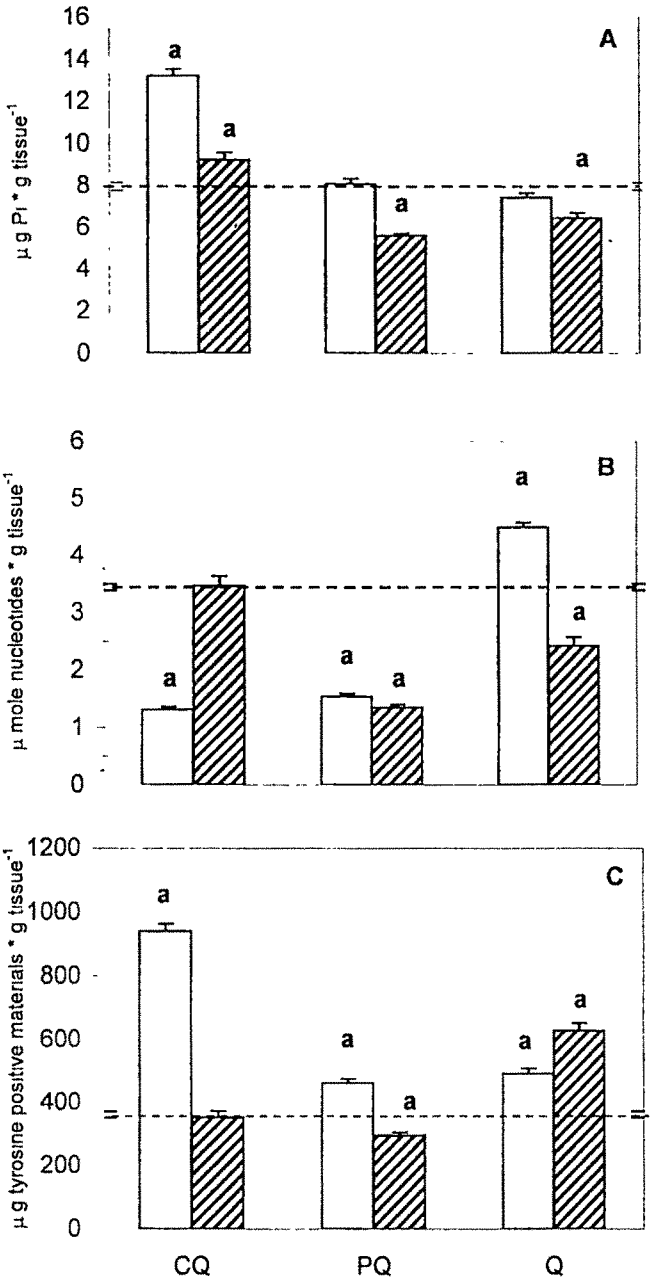
significantly high. The latter group also showed significantly high T/ F ratio. For BGPase the 'free' activity decreased significantly in both the groups, while the 'total' activity decreased only in 2 week group. The 'free' RNase II activity was unchanged but the 'total' activity decreased after 2 week treatment. The 'free' DNase II activity increased in 2 week treatment group while the 'total' DNase activity was elevated in both the groups. The 'free' cathepsin D activity showed a transient pattern with initial significant reduction followed by 40% increase whereas the 'total' activity increased by 3.2 fold in the 2 week treatment group.

Q treatment resulted in a small but reproducible increase in the 'total' PNPPase activity in the 7 day group while this treatment had significant lowering effect on 'free' BGPase activity; the 'total' BGPase activity increased only after 7 day treatment 'Free' RNase II activity was somewhat low after 7 day treatment but more than doubled following 14 day treatment. The 'total' RNase II activity increased progressively (29 and 37 % increase respectively). Q treatment also resulted in stimulation of both 'free' as well as 'total' DNase II activities almost to the same extent in both the treatment group. 'Free' cathepsin D activity was low following 14 day treatment with Q whereas the 'total' activity increased by 4 fold in the 7 day treatment group.

In view of the significant changes in the lysosomal enzyme profiles, (Fig. 2-4) it was of interest to find out if the antimalarials treatment also affected the pool size of Pi, nucleotides and amino acids. The results are shown in Fig. 5. As can be seen, the content of Pi in the pool increased by 67% after treatment with CQ for 7 days; with 14 day treatment the increase was 55% 7 day treatment with PQ and Q had no effect on the pool size, but 14 day treatment resulted in decreased pool size (19 to 30% decrease).

Fig. 5 Effect of antimalarials treatment on the size of: A. Pi pool, B. nucleotide pool and C tyrosine positive materials in rat liver. The dashed lines indicate the values for control which are 8.00 ± 0.24 μg Pi, 3.47 ± 0.08 μ mole nucleotide and 368.4 ± 8.14 μg tyrosine positive materials/ g tissue. The error bars represent the S.E.M.

a. $p < 0.001$ compared with the corresponding control.


A
µ g Pi * g tissue-1
16
14
12
10
8
6
4
2
0
a
a
a
a
a
a
B
µ mole nucleotides * g tissue-1
6
5
4
3
2
1
0
a
a
a
a
a
a
C
µ g tyrosine positive materials * g tissue-1
1200
1000
800
600
400
200
0
a
a
a
a
a
a
CQ
PQ
Q

The nucleotide pool size decreased after one week treatment with CQ and PQ, the effect persisted also in PQ group after 14 day treatment. Quinine treatment resulted in an initial increase (30% increase) followed by 30% decrease.

Tyrosine positive material was always high in all the groups receiving antimalarials treatment for 7 days and especially so in those animals receiving CQ treatment. Prolonged treatment with PQ resulted in decrease (16 %) while that with Q caused a further increase (71 % increase).

Discussion

From the data presented (Fig. 2-4) it is clear that CQ treatment in general resulted in increase in the 'free' as well as 'total' activity of all the lysosomal enzymes. In contrast to CQ, treatment with PQ had practically no effect in general on either the 'free' or 'total' activities except for DNase II. In this instance 14 day treatment resulted in increase in both the 'free' as well as 'total' activities. Following Q treatment the pattern was intermediate Although the βGPase and cathepsin D activity decreased, there was an overall increase in general in the RNase II and DNase II activities Also, the 'total' activities increased substantially.

Thus the emerging picture is that the treatment with antimalarials resulted in an overall increase in the 'free' as well as 'total' activities. Also in general the ratios of T/F activities were higher implying that the lysosomal membranes were more intact. However, this parameter and or conclusion may be considered as somewhat deceptive in view of the fact that both 'free' and 'total' activities increased and the increase in the

'free' activities was already much higher compared to the control (e.g. see Fig 2-4) The increase in 'free' activities especially in the case of RNase II and DNase II is a matter of concern, since this can lead to indiscriminate degradation of RNA and DNA ultimately resulting in necrosis of the cells in the host tissue i e liver. Earlier we have reproted that in paracetamol-induced hepatotoxicity, the 'free' as well as 'total' RNase II and DNase II activities had increased (18). Necrosis associated with paracetamol-hepatotoxicity is well recognized (19). The 'free' cathepsin D activity increased substantially in experimental thyrotoxicosis has already been reported (20). Involvoment of lysosomes in cellular pathophysiology is well recognized (21).

It was also noted that the acid phosphatase activity increased substantially, at least in the early period following antimalarials treatment (Fig. 2-4). In other studies it was shown that lysosomal acid phosphatase preferentially acts on nucleotides and that AMP is the preferred substrate (22). The concerted action of activated nucleases and acid phosphatase would lead not only to the breakdown of nucleic acids but also to the further dephosphorylation of mononucleotides, thereby leading to the acceleration of the process of cell degeneration

Interestingly, antimalarials treatment also significantly affected the pool size. The changes in the Pi pool size correlated well with 'free' BGPase activity. However, no such correlation between nucleotide pool size and RNase II and DNase II or between the tyrosine pool size and cathepsin D activity was noted. Needless to say, at any given point the pool represents the balance between the anabolic and catabolic activity of the cell. Hence it may be suggested that the antimalarials treatment might affect not only the

lysosomal enzyme activities and membrane stability but also affect the overall metabolism of the cell as reflected in the pool size (Fig. 5)

In conclusion, results of the present studies suggest that prolonged exposure to antimalarials can have adverse effects on the host tissue i e. liver through activation of lysosomal enzymes especially the nucleases. Besides, the metabolic activity of the cells also may be severely affected as reflected in the alteration in the pool size. In related studies it was also found that the antimalarials treatment resulted in severe impairment in oxidative energy metabolism in rat liver mitochondria (next chapter).

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Summary

Effects of treatment in vivo with the antimalarials: CQ, PQ and Q on lysosomal enzymes and lysosomal membrane integrity were examined. Treatment with the three antimalarials showed an apparent increase in the membrane stability. CQ treatment resulted in increase in both the 'free' and 'total' activities of all the enzymes i.e. acid phosphatase, RNase II, DNase II and cathepsin D. PQ treatment lowered the 'free' and 'total' activities of acid phosphatase and cathepsin D, but the DNase II activities increased. Treatment with Q resulted in increased 'free' and 'total' activities of RNase II and DNase II while 'free' activities of acid phosphatase and cathepsin D were low; the 'total' activities increased significantly. Our results suggest that an overall increase in free nucleases activities following prolonged treatment with antimalarials may lead to cell damage and / or necrosis