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Estimation of vitamin C human protective dose for acetaminophen toxicity, using acute animal toxicity study

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Abstract: ***Acetaminophen (APAP) is the main cause of fulminant hepatic failure. Vitamin C is a natural antioxidant with protective potentials against APAP toxic damage. In this animal study, and after an LD50 determination and selection of suitable lethal dose, the investigation was done to select a proper protective dose of vitamin C against lethal APAP dose. All 6 animal groups received a lethal dose of APAP (3250 mg/kg), group II, III and IV received 500, 1000 and 1500 mg/kg vitamin C respectively, group V received 1200 mg/kg N-Acetylcysteine (NAC), and group VI receive 1000 mg/kg vitamin C and 1200 mg/kg NAC. Mortality was recorded and liver histopathology was carried out. The results showed, the mortality rate in the group I was 68.75% and 37.5%, 31.25% in group II and III respectively, while group IV Showed a higher mortality rate and in group V and VI it was 25%. There was also a gradual reduction in the grade of histopathological damage in all groups, ranging from 2.4 ± 0.55 in group I to 0.4 ± 0.55 in group V and VI. In conclusion, vitamin C showed an increasing reduction in mortality and more histopathological protection, and it was more significant at 1000 mg/kg. NAC adds no more protection or reduction in mortality. The estimated protective dose of vitamin C was 700 to1127 mg for each gram of APAP. Incorporation of this dose of vitamin C with APAP preparations may be considered as a promising method for reducing mortality or severity of APAP intoxication.***

Keywords: Acetaminophen, Vitamin C, liver, protective dose, in vivo study

1. INTRODUCTION

Acetaminophen (Paracetamol, APAP) is an effective, well tolerated and widely used analgesic and antipyretic agent, but its safety is greatly linked to an appropriate dose [1]. Fulminant hepatic failure was reported after acute and chronic use of APAP, with increasing number of reported cases [2-3]. It's the main cause of fulminant hepatic failure in many countries [4-5]. APAP overdose represents 50% of self-poisoning hospital admission and it is the commonest cause of acute liver failure [5-6]. In Jordan, drug poisoning accounted for more than 42% of poisoning cases, and APAP products were responsible for 13.4% of all drug poisoning cases [7].

APAP is metabolized in the liver to inactive glucuronid and sulfate conjugated metabolites that are eliminated through the kidneys. 5% of APAP is excreted unchanged in the urine and only 5-10% of it is converted to the reactive toxic intermediate metabolite, N-acetyl-p-benzoquinone imine (NAPQI) through oxidation by hepatic cytochrome P450 enzymes. NAPQI is inactivated by conjugation to glutathione, and eliminated through the urine [8]. NAPQI will be in high level and binds to cellular nucleophil proteins leading to hepatocelluar toxicity following overdose, or when glutathione depletion occurs and if Cytochrome P450 enzyme is induced [9]. According to previous studies, oxidative stress with increased generation of reactive oxygen species, depletion of reduced glutathione (GSH) and lipid peroxidation play a crucial role in the development of APAP-induced hepatic and renal damage [9-11].

N-acetylcysteine (NAC) is a cysteine prodrug and a glutathione precursor molecule with antioxidant properties that has free access to intracellular compartments. NAC is used in clinical practice as an acceptable antidote and antioxidant because it's reduced thiol groups and has the ability to scavenge oxygen free radicals. The indirect vasodilatory effect of NAC by increasing local nitric oxide concentration, also contributes to its antioxidant characteristic. As well as, NAC prevents APAP toxicity by increasing sulfate conjugation. In addition, NAC has a safe profile with minimum adverse reactions [12-15].

Vitamin C (ascorbic acid) is one of the nonenzymatic cell protective natural antioxidant. A number of animal studies have investigated the potential role of vitamin C in the prevention of APAP induced hepatotoxicity. But only Romero-Ferret et al[16] showed no reduction in mortality, while others showed significant protective effects [17-19].

Prevention of APAP poisoning remains globally an important issue. Several measures have been considered to reduce APAP overdoses, including, adding of APAP antidote to paracetamol tablets, reducing the therapeutic dose, inclusion of warnings on packs and reducing publicity [20-22]. Many agents that have been investigated for modulation of liver toxicity of APAP are of little value in clinical practice as they are to be administered before (chemoprevention) or concomitantly with APAP (chemoprotection) [9] and still options for the APAP toxicity and associated complications prophylaxis are limited [23].Therefore chemoprotective

agents for APAP related hepatic complications become of great importance because most of previously used measures were of little or no significance [22].

The present animal study was design to study the protective effect of simultaneous administration of vitamin C to a lethal dose of APAP and to select a protective dose of vitamin C, then extrapolation of the proper human protective dose of vitamin C from the animal study. No previous study designed to extrapolate the vitamin C protective dose for APAP lethal doses was published.

2. MATERIALS AND METHOD

2.1. Drugs and Chemicals

Paracetamol tablet (contain 500 mg Paracetamol) was purchased from the local market as Panadol®, and prepared as suspension in distilled water to simulate normal human conditions of poisoning. NAC was purchased from Sigma Chemicals Company, St. Louis, while Vitamin C from Acrose ® Company and were added to the Paracetamol suspension at different concentration according to the experiment.

2.2. Animals

Adult male Wistar rats weighing 180-200 g were housed in metal cages at 23^0C and fed *ad libitum* control pellet diet. All animals were maintained on a 12/12 hour light/dark cycle (lights on at 8 am). They were allowed one week to acclimate before experimental manipulations began. Experiments were performed between 9-10 am after overnight fasting.

The research was approved by Animal Care and Use Committee of Jordan University of Science and Technology.

2.3. Experimental Design

Because the LD_{50} is well known to vary according to animal species, route, type of preparation and many environmental factors [24], the LD50 was determined. Also, since the mortality rate was utilized in this study as an indicator for vitamin C protective effect, therefore the selection of suitable dose with a known mortality rate is essential to avoid unexpected high or low variation in mortality.

Animals were randomly divided into two groups, the first one used to extrapolate the oral acute toxicity (LD50) of APAP and the second, to test the protective effect of simultaneous oral administration of vitamin C and lethal dose of APAP.

2.4. Determination of LD50

Extrapolation of the LD50 for the APAP was done by using 5 groups 10 animals each. Animals were given orally an increasing dose of aqueous suspension of APAP started with 2500 mg/kg after an overnight fasting.

2.5. Protective effect of vitamin C against Paracetamol acute toxicity

LD_{50} was calculated as previously described [25-26]. A dose of 3250 mg/kg was selected (above LD50) to get higher mortality rates, so the protective effect of vitamin C can be observed. To test this hypothesis; animals were allocated randomly into groups, each of eight animals. All experimental groups received PAPA dose of 3250 mg/kg orally and increasing dose of vitamin C started with 500 mg/kg with or without ANC after an overnight fasting and as follows:

Group I: received only APAP.

Group II: APAP + 500 mg/Kg Vitamin C.

Group III: APAP + 1000 mg/kg vitamin C.

Group IV: APAP + 1500 mg/kg vitamin C.

Group V: APAP + 1200 mg/kg NAC alone.

Group VI: APAP + 1000 mg/kg, Vitamin C and 1200 mg/kg NAC.

Death response was recorded after 24 hours, lived animals were scarified and livers were removed, 3-4 small pieces were fixed immediately in 10% formalin solution for 24 hours and processed routinely for histological examination. The examination was performed by a pathologist who had no prior knowledge of the treatment groups. The grades of histopathological damage were ranged from 0 to III as follows: 0, no centrilobular degeneration and necrosis; I, up to 30% of the lobules involved with necrosis; II, 30- 60% of the lobules affected; and III, more than 60% of the lobules affected.

It is worth to mention that, 16 animals were used for group received 3250mg/kg PAPA to have sufficient survival (group I), while the best protective dose of vitamin C was repeated twice for validation of the results. While the results of other groups were not validated due to logistic difficulties

3. STATISTICAL ANALYSIS

SSPS® 16.0 for windows statistical software was used for statistical analysis of the data. Chi square test was used to find the significance between different groups. The Data was expressed as a mean ± SD. P value < 0.05 was considered statistically significant.

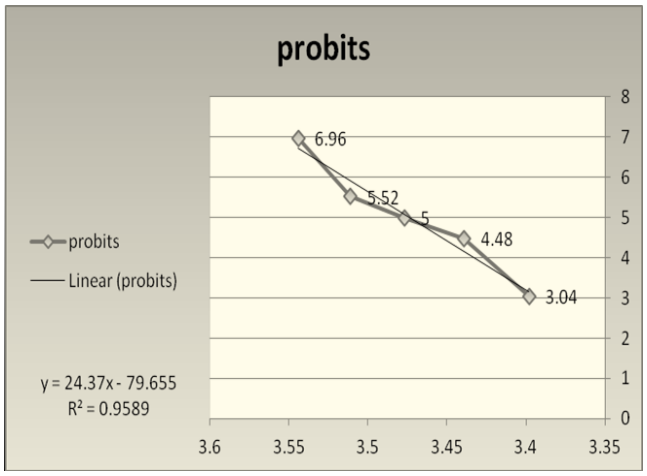
4. RESULT AND DISCUSSION

The LD50 for APAP was estimated to be 2976.7 ± 124.239mg/kg (Table 1 and Figure 1). The mortality for group received 3250mg APAP/kg (group I) was 68.75% as expected since the dose was greater than LD50.But when 500mg vitamin C was administrated simultaneously with APAP, the mortality rate was reduced to 37.5% (3/8) (group I vs. III, P=0.142) and 1000mg vitamin C significantly (P=0.034) reduced the mortality rate of acute APAP toxicity to 31.25% (5/16) (group I vs. III).The beneficial effect of vitamin C in reduction the mortality rate of acute APAP toxicity was not observed at a dose of 1500mg vitamin C/kg body weight (group 1 vs. IV) (Table 2). Simultaneous

administration of ANC along with 1000mg Vitamin C did not show any additive or synergistic effect in reduction of mortality rate of acute APAP toxicity (group V and VI vs. group I). Although simultaneous administration of 1200mg/kg ANC and 3250mg APAP showed statistically significant reduction in mortality rate (group V vs. I p=0.043) (Table 2).

Table1. Experimental animal groups used to extrapolate the LD50.

Group	Dose mg/kg	Log dose	Dead %	Correcte %	Probits
1	2500	3.398	0	2.5	3.04
2	2750	3.439	30	30	4.48
3	3000	3.477	50	50	5
4	3250	3.511	70	70	5.52
5	3500	3.544	100	97.5	6.96



*Calculated LD50 = 2976.7 ± 124.239.

Figure 1: The correlation between the Log dose of APAP and probit units.

Regarding, the grade of histopathological changes, for group I the average changes were 2.4 ± 0.55 while 1.4 ± 1.34 for group II and this reduction was not statistically significant. Group III showed a greater reduction in average (0.4 ± 0.69) but it was significant at P value =0.053. No more significant protection from the histopathological point of view with an average of (0.4 ± 0.55) was observed in both groups V and VI. Concerning group IV, no histopathological investigation was done because of the high mortality rate (87.5%) and insufficient number of surviving animals were not obtained. Results are shown in table 2.

Table 2. Death response with average histopathological grade for the tested groups

	Group I	Group II	Group III	Group IV	Group V	Group VI
Mortality%	68.75	37.5	31.25	87.5	25	25
Grade of Histopathology	2.4 ± 0.55	1.4 ± 1.34	0.4 ± 0.69	-	0.4 ± 0.55	0.4 ± 0.55

Paracetamol (APAP) is the most widely used over-the-counter (OTC) analgesic and antipyretic agent in the world. Although it is considered a very safe drug, but it can induce liver damage after single large dose ingestion [27] and also through repeated ingestion of supra therapeutic doses [28] or through chronic low dose intake [29].Thus, finding a chemoprotective agent against APAP hepatotoxicity is of great importance.

In previous studied, vitamin C was investigated for its protective and preventive effect either at toxic doses of APAP or given in repeated doses, or used through non-oral routes [16,18]. Other studies were conducted using mixtures including vitamin C or NAC [19,30]. All previous studies showed significant preventive or protective effect of vitamin C except Romero-Ferret et al [16] who reported that, vitamin C did not cause any decrease in the mortality rate when administrated either simultaneously or two hours after the oral administration of 875 mg/kg of Paracetamol to mice. This could be explained by a high sensitivity of mice to hepatic toxic effect of APAP [9]. No reported study was designed to estimate the proper protective dose of vitamin C utilizing the normal poisoning scenario in human with a single dose APAP.

In this study, vitamin C showed an increased reduction in mortality, which was parallel to the presence of histological protection in treated groups with increasing vitamin C dose. Simultaneous administration of 1000 mg/kg and APAP showed a maximum protection against APAP toxicity, as compared to 500mg/kg vitamin C. The beneficial effect of vitamin C in reducing mortality rate is related to its function as powerful antioxidants, which may inhibit the generation of free radicals by APAP metabolites [31] reducing the intermediates back to the parent compound [32]. Besides, vitamin C can bind covalently to APAP reactive metabolites [33] disturbing the billiary excretion of its glutathione conjugates [34]. Using NAC alone or in combination with 1000 mg/kg vitamin C added no more great significant reductions in both mortality and

histopathological protection. In addition, neither high dose vitamin C nor NAC or both in combination have a high protection from APAP toxicity to be considered as a single dose antidote. High dose vitamin C (1500 mg/kg) showed a pro-oxidant effect [34-37] which lead to high mortality rate that's consistent with the results shown by Abraham et al [38] although it was investigated for nephrotoxic effect.

To extrapolate the proper and useful initial protective dose of vitamin C to be added to the APAP preparations that expected to reduce both toxicity and mortality in human, 1000 mg vitamin C was used. Extrapolation was done using the method described FDA guidance [39] by dividing the animal dose to a factor 10, or specific factor, according to the animal species used if conversion from animal to human dose based on surface area (6.2 for rodents). So, starting safe dose in human for further studies will range between 100 – 161 mg/kg. Since, a single dose of APAP in adult above 10 grams or 200 mg/kg of body weight, have a reasonable likelihood of causing toxicity [40]. By dividing the estimated safe dose of vitamin C to this toxic dose, then the recommended vitamin C dose to be added for each 1 gram paracetamol will be between 700 mg to 1127 mg. A lower dose is recommended since high doses carry a risk for pro-oxidant effect.

5. CONCLUSION

Combining APAP with a suitable dose vitamin C can be considered as a promising method to reduce severity and mortality that resulted from acute hepatic damage due to APAP lethal doses, and further studies will be required with large number of animals with monitoring of liver enzymes or on human who are at risk of acute liver injury due to high therapeutic doses. Adjusted doses of vitamin C will be required since very high dose, render the patient at risk of more serious injury due to its pro-oxidative effect. In addition, administration of vitamin C along with NAC will be a more effective antidote for APAP induced acute hepatotoxicity. The estimated safe dose of Vitamin C to be incorporated into APAP preparations according to this study was 700 – 1127 mg for each gram, and better to start with the lower dose for future human studies.

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