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Chronic (90 days) oral toxicity assessment of ethephon, a commercially available fruit ripener in male wistar rats

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Abstract
Use of artificial fruit rippers are increasing worldwide. Ethrel is one fruit rippners that is in the market. The present study investigated the effects of ethrel on organ weights and haematological parameters of male rats. The rats were (n=9) treated with 100, 250, 500mg/kg of ethrel or distilled water (DW) for 90 days. Another two sets of animals (n=6) were orally treated with 500mg/kg of ethrel and DW for 90 days and were kept for another 28 days without treatment (recovery) to evaluate the reversibility. Food intake and body weight changes were recorded weekly. Upon autopsy, haematological parameters and sperm parameters were measured. Treatment did not exhibit any overt toxicity. Similarly, haematological and sperm parameters or relative organ weight did not change significantly. However, liver/body weight increased significantly at 500mg/kg. In conclusion, exposed to higher doses of ethrel may lead to liver enlargement. Further studies on liver toxicity is required.

Keywords: fruit ripening, Ethrel, liver toxicity

1. Introduction
Recently use of synthetic chemicals such as pesticides [Ratnasooriya *et al*., 1996] [1], and industrial chemicals [Peiris and Moore, 2001a and 2001b] [2, 3] for various purposes have increased immensely. Usage of artificial fruit ripening has increased lately to accommodate increasing customer needs. Among different ripening agents, calcium carbide, acetylene, ethylene, propylene and ethephon are the widely used ripening agents today [Bahodoria *et al*., 2018] [4]. Unsaturated hydrocarbons, epically ethylene, acetylene could promote ripening and induce colour changes effectively [Asif, 2012] [5]. Ethrel of which the active ingredient is ethephon, was first registered as a liquid plant growth regulator in the United States in 1973 [USEPA, 1995] [5], is currently being widely used as an artificial ripening agent in Sri Lanka. Ethrel contains 240g/L of the active ingredient, ethephon of which the chemical structure is 2-chloroethyl attached to phosphorous atom [National Center for Biotechnology Information, 2019] [7]. Ethrel is stable in solution at low pH and if the pH is raised, ethrel decomposes to ethylene, phosphate and chloride ions [Hakim *et al*., 2012] [8]. Extensive use of artificial ripening agents can lead to potential health risks [Fattah and Ali, 2010] [9]. It was reported by National Center for Biotechnology Information [7] that residues of ethephon can be found in food commodities and the tolerance vary among different fruits.
Throughout the world, the active ingredient of ethrel (ethephon) is widely used as an organophosphorous insecticide Similar to other organophosphorus compounds, ethrel can inhibit the plasma cholinesterase activity in mammals [10] hauxe and cause liver damage [Bhadoria *et al*., 2018] [4]. Ethylene, the metabolic product of ethrel found to increase tumours, increased kidney weight, necrosis in the stomach [Henwood, 1989] [11] and fibrosis of heart [Klonne, 1994] [12]. Ethephon can also have direct and indirect cholinergic effects on intestinal muscle contractions [Cetinkaya and Baydan, 2010] [13]. It is known to cause mutagenic, teratogenic, biochemical alterations and increase in all types of structural chromosomal aberrations in mice Measurement of intracellular calcium levels in various mammalian cell lines revealed that ethylene, produced by ethrel caused a significant up-regulation of calcium in these cells [El Raouf and Girgis, 2011] [14].
Recommended application of ethrel dose varies from 1.75 – 3.5L/ha and the said concentrations should be dissolved in 2000 – 4000L of water. Though recommended doses and method of use have been highlighted by the Consumer Affairs Authority in Sri Lanka, vendors in the country do not follow those instructions laid by the authority. Recently, it was reported that Consumer Affairs Authority destroyed large number of fruit from vendors while they were spraying ethrel directly from the bottle onto unripen fruits to accelerate ripening. Moreover, most vendors in Sri Lanka, directly dip unripen fruits in pure ethereal solutions. Use of ethrel for fruit ripening by vendors is increasing and there is a serious lack of data on long term exposure. Therefore, this study was aimed to investigate the toxicity of commercially available ethrel using male Wistar rats.

2. Materials and Methods
2.1 Chemicals and Animal Treatment
Commercially available ETF (chemical name: 2 chloroethylphosphonic acid; trade name Ethrel- Ethephon 480 g/l, SL; class- plant growth regulator, 98% pure) purchased from Harrisons chemicals (Pvt) Ltd Sri Lanka, available as liquid formulation.
Male Wistar male (*Rattus norvegicus*), aged between 8-12 weeks, and weighing 250 -330g were obtained from the Medical Research Institute (MRI), Sri Lanka. Animals were housed in plastic cages in well ventilated, standard conditions (temperature 25 ±2°C; photoperiod approximately 12 hours dark and 12 hours light; relative humidity 55%) animal house at Faculty of Medical Sciences, University of Sri Jayewardenepura. They were fed with pelleted food, and tap water was given *ad libitum*. Research was conducted in accordance with the internationally accepted principles for

care and use of laboratory animals and guidelines. Ethical approval for the present study was obtained from the institutional Ethics Review Committee (ethical number 16/2).

After 1 week of acclimatization, 36 male rats were randomly divided into four groups: three treated groups and one control group. In the treated groups, rats were exposed to ethrel dissolved in distilled water by gavaging orally at doses of 100, 250, 500 mg/kg of ethrel daily for 90 consecutive days. The control group was administered with equal volume of distilled water.

To study the recovery of animals following Ethrel treatment, another12 animals were randomly divided into two groups (recovery test) and treated either with 500 mg/kg of ethrel or distilled water for 90 consecutive days. The animals were kept for a further period of 28 days without treatment to determine the reversibility, persistence or delayed occurrence of toxic effects [Parasuraman, 2011] [15]. Throughout the study period, animals were checked twice a day for mortality and any clinical sign of toxicity following Ethrel treatment.

2.2 Body weight, food consumption and relative organ weight

Body weight and food consumption were recorded from the date of commencement and at weekly intervals in all animals including recovery test groups. Just prior to autopsy, animals were fasted overnight and were scarified by an overdose of ether. Upon scarification, blood samples were collected from cardiac puncture and the vital organs (liver, spleen, kidney and testis) were excised, cleared of fat and connective tissue, blotted dried and weighed. Relative weight of each organ was calculated by dividing the weight of each organ by the body weight and multiplying by hundred. Same procedure was followed for the animals in the recovery test groups at the end of 28 days.

2.3 Sperm Counts and Motility

Immediately after sacrifice, the left cauda epididymis of each rat was excised and an incision (about 1 mm) was made to liberate sperm into a Petri dish containing 5 ml of mammalian saline. After homogenizing, the samples were further diluted (1/200) and the sperm counts were measured using an improved Nebaur's haemocytometer. Similarly, right cauda epididymis of each rat was excised and a drop of sperm was squeezed onto a pre-cleaned microscopic slide. Subsequently, 2 drops of mammalian saline were added to mobilize the sperm and sperm motility was assessed calculating motile sperm per unit area and was expressed in percentage.

2.4 Determination of DNA Damage in Sperm:

Sperm smears were prepared on pre- cleaned microscopic slides and air dried for 5 min. The smears were fixed in carnoy's solution (methanol: acetic acid 1: 3) for at least 3 h. Then slides were washed in distilled water and air dried. Subsequently, slides were stained with acridine orange for 5 min. Smears were evaluated with the aid of fluorescent microscope with excitation of 490nm.Two hundred sperm from each staining protocol were scored and graded. All sperm exhibiting yellow to red was scored as denatured DNA and sperm exhibiting green colour was scored as native DNA [Tejada *et al*., 1984] [16].

2.5 Haematological Studies

Immediately after collecting blood from the cardiac puncture, about one ml of blood was added in to micro tubes containing EDTA. Subsequently, blood parameters; red blood cell count (RBC), total and differential leukocyte count, platelet count, haematocrit (HCT) and hemoglobin (Hb) was determined.

2.6 Statistical Analysis

Difference between control, treated and recovery groups were evaluated statistically using one-way analysis of variance (ANOVA) and two sample *t*- test. The data was expressed as the mean± standard error (SEM). Significance was set at $P< 0.05$. Minitab software package (Minitab Co., USA) was used for statistical analysis.

3. Results & Discussion

There was no mortality and no apparent signs of clinical toxicity were observed in the rats treated with ethrel for 90 days. As shown in table 1, there was no significant difference in body weight and food consumption between ethrel exposure groups and the control groups. Relative weights in all treated groups (100 mg/kg, 250 mg/kg and 500 mg/kg and 500 mg/kg - recovery) did not show any significant difference ($p > 0.05$) from the control (DW). However, during the recovery period the relative liver weight in animals treated with 500 mg/kg BW of ethrel increased significantly ($p < 0.05$) when compared to control (Table 1).

The sperm count and sperm motility in all Ethrel treated groups did not show a significantly different ($p > 0.05$) from the control (Table 3). Similarly, spermatozoa stained with acridine orange indicated that ethrel had no significant effect on the sperm DNA integrity compared to their respective control groups (Figure 1) indicating absence of reproductive toxicology unlike other commonly used organophosphorus pesticides [Peiris *et al*., 2017 and 1995] [17, 18]. Haematological indices are shown in table 3. Compared to the control animals exposed to Ethrel did not show any significant differences in haematological indices such as RBC count, total and differential leukocyte count, platelet count, HCT and Hb. The present study showed that ethrel showed liver enlargement in rats treated with the highest dose (800 mg/kg ethrel). Similar results were reported by Bandoria *et al*. [4] with the active ingredient ethephon.

Table 1: Body weight gain, Food consumption and relative organ weight of male rats exposed to ethrel for 90 days

Parameter	Control	100 mg/kg	250 mg/kg	500 mg/kg	Recovery Test	
					Control	500 mg/kg
Body weight (g)						
Initial	293.33±7.10	303.33±6.95	298.11±7.60	303.33±6.17	299.33±5.42	301.00±9.67
30 days	318.22±6.14	327.44±5.40	321.11±6.85	327.00±5.75	321.17±5.35	320.20±8.33
60 days	342.89±5.15	347.67±4.39	342.67±4.97	348.11±4.40	342.67±4.95	342.00±6.04
90 days	367.11±4.07	373.33±3.73	366.00±4.93	369.22±4.45	381.60±4.06	383.50±2.67
Final Body weight gain (%)	25.15	23.08	22.77	21.72	27.48	27.41

Food consumption (per 100 g of BW)						
Food intake (g/day)	11.47±0.22	11.32±0.14	11.86±0.47	11.21±0.46	11.24±0.15	10.88±0.33
Relative organ weight						
Liver	3.256 ± 0.074	3.234 ± 0.113	3.260 ± 0.028	3.511± 0.120*	3.022 ± 0.216	3.282 ± 0.093
Kidney	0.613 ± 0.020	0.624 ± 0.063	0.646 ± 0.052	0.714 ± 0.047	0.654 ± 0.043	0.632 ± 0.056
Spleen	0.213 ± 0.008	0.208 ± 0.012	0.225 ± 0.021	0.216 ± 0.006	0.303 ± 0.015	0.300 ± 0.008
Testis	0.776 ± 0.029	0.802 ± 0.025	0.825 ± 0.048	0.822 ± 0.050	0.792 ± 0.036	0.821 ± 0.039

Data are mean ± SEM; *p <0.05 compared to control

Table 2: Effect of ethrel (treated for 90 days and the recovery group) on haematological parameters

Parameter	Control	100 mg/kg	250 mg/kg	500 mg/kg	Recovery Test	
					Control	500 mg/kg
RBC ($\times 10^6$ mm^{-3})	8.55±0.66	8.56±0.59	8.52±0.40	7.82±0.52	8.71±0.22	7.58±0.48
Platelets ($\times 10^3$ mm^{-3})	371.20 ±4.53	372.34± 8.78	370.24 ± 6.22	375.92 ± 5.60	384.42±10.32	405.65±12.66
HCT (%)	48.56±1.47	49.17±1.25	47.86±1.98	45.22±2.88	48.28±2.12	45.16±2.56
Hb (g/dl)	15.50±0.47	14.50±0.63	14.25±0.44	13.78±0.70	14.62±0.66	13.55±0.88
WBC ($\times 10^3$mm^{-3})	11.10±1.99	11.56±2.50	11.43±2.99	12.42±2.51	11.30±1.84	12.12±2.66
N (%)	29.90±2.10	27.09±2.20	28.55±2.10	31.08±1.50	29.10±1.26	35.25±0.98
L (%)	67.28±1.44	70.27±1.02	68.95±1.54	66.22±1.22	68.22±1.52	61.61±1.23
M (%)	2.50±0.72	2.40±1.10	2.20±0.42	2.00±0.00	2.20±0.52	2.50±0.75
E (%)	0.32±0.11	0.24±0.15	0.30±0.22	0.70±0.30	0.36±0.22	0.64±0.22
B (%)	0.22±0.10	0	0	0.13±0.20	0.12±0.10	0

Data are mean ± SEM; *p <0.05 compared to control

Table 3: Sperm parameters of male rats exposed to ethrel for 90 days

Parameter	Control	100 mg/kg	250 mg/kg	500 mg/kg	Recovery	
					Control	500 mg/kg
Sperm count ($\times 10^6$/ml)	48.33 ±1.56	48.26 ± 2.66	48.23 ± 2.41	48.54 ±1.66	49.44 ± 2.63	48.66 ± 2.31
Sperm motility (%)	73.00 ± 2.10	70.10 ± 2.65	72.25 ± 2.59	72.50 ± 2.46	70.25 ± 2.72	70.50 ± 2.31

Data are mean ± SEM; p <0.05 and **p < 0.01 compared to control

4. Conclusion

Daily exposure of humans to ethrel is unknown, however, few efforts have been undertaken to evaluate ethrel effects on rat liver. There was no mortality and no apparent signs of clinical toxicity observed in the rats treated with ethrel for 90 days indicating minimal toxicity on animal's general wellbeing. Though the percentage body weight gain was slightly reduced in the highest dosage, the weight was recovered during the recovery period. From the results it can be concluded that ethrel induce liver enlargement but did induce either haematological or testicular toxicity. However, it is important to study the effects over a longer period of time. Detail studies on liver toxicity and its mechanism of action is recommended.

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