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PROTECTIVE ROLE OF LIV.52 ON THE TOXICITY OF ACETAMINOPHEN DURING EMBRYOGENESIS AND POSTNATAL DEVELOPMENT IN WISTAR RATS

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ABSTRACT

Acetaminophen induced hepatic injury may also mediate its developmental toxic effects. The present study was carried out to evaluate the protective role of herbal hepato-protective formulation (Liv.52) against acetaminophen induced developmental toxicity in Wistar rats. Acetaminophen induced toxicity during embryogenesis and postnatal development was evaluated by administering daily doses of 500 or 1000 mg/kg/day through oral gavage starting from gestation Day 0 and up to lactation Day 21. Dose-dependent decrease in maternal body weights and food intake both during gestation and lactation in association with hepatic and renal toxicity was observed in dams exposed to 1000 mg/kg/day of acetaminophen. These changes were associated with higher post-implantation loss, lower litter size and live birth index. At 500 mg/kg/day dose-dependent decrease in maternal body weights and food intake were observed during gestation and lactation, but these changes were not associated with any changes in litter parameters evaluated. The administration of Liv.52 formulation did not induce any toxic effects during embryogenesis and postnatal development. However, co-administration of Liv.52 (1000 mg/kg/day) with acetaminophen induced partial or complete reversal of acetaminophen induced developmental toxic effects. In summary, Liv.52 an herbal hepato-protective formulation shown a significant protection against acetaminophen induced developmental toxicity in Wistar rats.

KEYWORDS: Liv.52, Acetaminophen, Toxicity, Developmental, Rat

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INTRODUCTION

Acetaminophen, also known as Paracetamol is one of the most widely used over-the- counter medication in the world[1-3]. Acetaminophen was first introduced as a prescription drug in the United States in 1955 and was approved by the Food and Drug Administration for sale as a non-prescription drug in 1960[4]. Acetaminophen has been reported as the most commonly recommended analgesic during pregnancy[5]. Based on US National Birth Defect Prevention Study and the Boston University Slone Epidemiology Center Birth Defects Study, acetaminophen is the most commonly administered OTC analgesic during pregnancy[6]. Acetaminophen is primarily metabolized by the liver and excreted by the kidneys; it is safe and effective when we use lower dose of acetaminophen, but excessive usage of acetaminophen can damage liver and the toxicity is not only associated with drug but also from one of its metabolite N-acetyl-p-benzoquinone imine (NAPQI) which is conjugated by hepatic glutathione to yield a product called mercapturic acid. Due to overdose of paracetamol, the glucuronidation and sulfation capacity is exceeded with formation of excess NAPQI. Liver damage is associated with depletion of glutathione, at this condition excessive NAPQI will bind with hepatic cell proteins and causes liver injury[7-9]. Acetaminophen overdose has been reported as the primary cause of acute liver failure in many countries[10-12] with more than 70,000 hospitalizations each year in the U.S. alone[13]. Liver disease is one of the major health problems worldwide because liver is a vital organ and has a wide range of functions in the body, including biotransformation and detoxification of endogenous and exogenous harmful substances, plasma protein synthesis, and glycogen storage[14]. There is evidence that overdoses of acetaminophen during pregnancy increases the risk for adverse reproductive outcomes, e.g. spontaneous abortions, a variety of malformations, fetal distress and hepatic and renal toxicity in infants[15]. Burdan et al[16] reported impairment in rat fetal liver without any macroscopic malformation at the higher dose of acetaminophen. In addition, prenatal exposure to the combination of paracetamol and caffeine in rats has been reported to cause intrauterine growth retardation and teratogenic effects (reduced fetal body weight/growth and placenta weight) in rats[17]. Herbal products and traditional medicines with better effectiveness and fewer side effects favoured against synthetically derived drugs in modern allopathic medication system[18]. Liv.52 is an herbal hepatoprotective formulation introduced in 1955 by Himalaya Herbal Health Care Company, since then, it has been sold worldwide and has been recognized by thousands of health professionals. Liv.52 is known to improve the functional efficiency of the liver by promoting detoxification and thus protecting from harmful food and medication toxins, maintaining healthy levels of liver enzymes. Liv.52 is also known to support liver's normal ability to burn fat and maintain body's metabolic homeostasis. Hepatoprotective effect of Liv.52 has been reported by several researchers in multiple hepatic diseases, including chronic liver diseases[19-21]. Hepatotoxins such as alcohols[22], heavy metals[23], paracetamol[24] induced liver damage. Mechanistically, Liv.52 is known to protect hepatocellular membrane damage by lowering lipid peroxidation[25-26]. Even though hepatoprotective effect of Liv.52 has been reported by several researchers, protective role of Liv.52 in acetaminophen induced maternal and developmental toxicity is not been tested. There is no information available regarding protection of acetaminophen induced hepatotoxicity will also protect its developmental toxic effects in a mammalian species. Hence, in the present experiment, we evaluated protective role of Liv.52 on acetaminophen induced effects on pregnant/lactating rats and their developing fetuses when administered orally to rats from gestation day 0 through weaning.

MATERIALS AND METHODS

(1) Materials

Acetaminophen was procured from Sigma Aldrich Co., 3050 Spruce Street, Saint Louis, MO

63103, USA along with the certificate of analysis with the purity of 99.8%. Liv.52 was procured commercially (The Himalaya Drug Company, INDIA).

(ii) Animals and Methodology

The study was conducted in an AAALAC (www.aaalac.org) accredited facility (Association for Assessment and Accreditation of Laboratory Animal Care International, 2001). The experimental project was approved by the Institutional Animal Ethics Committee (Proposal No. 023, dated 21 March, 2012). Wistar rats, in-house bred at Department of Safety Assessment, Advinus Therapeutics Limited, Plot 21 & 22, II Phase, Peenya Industrial Area, Bangalore – 560058, India were used for this experiment. A total number of 36 females of 11 to 12 weeks age (day '0' pregnant rats) confirmed by vaginal smear examination with weight ranging from 181 to 249 grams were divided into 6 groups of 6 females each. Rats selected for this study were checked for health status and were housed in a barrier facility with standard laboratory condition of 12 – 15 filtered fresh air changes, temperature range of 20 to 24 °C, relative humidity of 30 to 70 % with 12 hours fluorescent light and 12 hours dark cycle. The rats were provided *ad libitum* pelleted feed (Teklad Global 14 % Protein Rodent Maintenance Diet - Pellet (Certified) manufactured by Harlan Laboratories B.V. Maasheseweg 87c PO Box 553, 5800, AN Venray, The Netherlands) and deep bore-well water (passed through activated charcoal filter and exposed to UV rays in 'Aquaguard' on-line water filter-cum-purifier manufactured by Eureka Forbes Ltd., Mumbai 400 001, India) *ad libitum.*

(iii) Experimental Design and Treatment

The experimental design is summarized below

Groups	Group Name	Dose (mg/kg/day)	
		Acetaminophen	Liv.52
Group 1	Control	0	0
Group 2	Low dose acetaminophen	500	Not Applicable
Group 3	High dose acetaminophen	1000	Not Applicable
Group 4	Liv.52	Not Applicable	1000
Group 5	Low dose acetaminophen + Liv.52	500	1000
Group 6	High dose acetaminophen + Liv.52	1000	1000

The test item/s were suspended in vehicle i.e., 0.5 % w/v carboxymethylcellulose sodium salt in Milli-Q water. The dose formulations were prepared daily and administered as oral gavage. All groups comprised 6 pregnant females each. Group I received only the vehicle at 10 mL/kg body weight through oral gavage. Groups 2 and 3 received acetaminophen suspensions at the doses of 500 and 1000 mg/kg/day, respectively at 10 mL/kg body weight. Group 4 received only the Liv.52 suspensions at 10 mL/kg body weight. Groups 5 and 6 received acetaminophen suspensions at the doses of 500 and 1000 mg/kg/day, respectively, but received in addition Liv.52 suspensions at 1000 mg/kg body weight and the dose volume administered was 5 mL/kg body weights for each of test items. All the presumed pregnant females were continuously dosed from Day '0' of gestation until weaning sacrifice (Lactation Day 21).

(iv) Observations

All rats were observed for clinical signs and mortality throughout the experimental period. Rats were weighed on gestation days 0, 3, 5, 8, 11, 14, 17 and 20. The food intake was recorded on days 0-3, 3-5, 5-8, 8-11, 11-14, 14-17 and 17-20. Rats were weighed on lactation days 1, 4, 7, 11, 14, 18 and 21. The food intake was recorded on lactation days 1-4, 4-7, 7-11, 11-14, 14-18 and 18-21. Rats were evaluated for the duration of gestation, litter size and pup viability during lactation period. At birth, all the pups (both dead and alive) in a litter from each dam were observed for any external deformities. The number of pups born (litter size), sex and body weight of male and female pups on different days of lactation were recorded individually. The standardization of litter size to 8 pups was made on Day 4 of lactation. All the culled, dead and sacrificed pups were examined for external and

visceral variation/malformation and subjected to gross pathological examination. During the different days of lactation period, each pup was observed for postnatal developmental changes[27-31]. The postnatal developmental changes such as pinna unfolding (the point of bilateral pinna detached from the head from postnatal Day 1), incisor eruption (eruption of upper incisor through the gums from postnatal Day 7), ear opening (opening of ear canal on postnatal day 10) and eye opening (the total separation of the upper and lower eye lids and the complete opening of both eyes from postnatal Day 13) were observed. After completion of the 21-day postpartum period, female rats were anaesthetized under isoflurane and retro-orbital sinus was punctured to collect blood using a fine capillary tube. Blood sample was collected in tubes containing K_2EDTA and lithium heparinized tubes for determination of haematology and clinical chemistry respectively. The haematological parameters were determined using ADVIA 2120 haematology system (Bayer Health Care LLC, NY, USA). The plasma was separated and analysed for clinical chemistry parameters using Roche/Hitachi 902 (Hitachi High-Technologies Corporation, Tokyo, Japan) Automatic Analyzer. At necropsy, the maternal viscera, number of corpora lutea and distribution of implantation sites were observed in dams at necropsy. Liver and kidneys were collected from each dams, preserved in 10 % neutral buffered formalin and subjected for microscopic evaluation.

(v) Statistical Analysis

The statistical analyses was done using Dunnett's method following one way analysis of variance (ANOVA) for groups comparison and student's t-test was applied for single group comparison for parameters related to maternal body weight, food consumption, weight of pups, number of pups, number of corpora lutea, number of implantations, mean litter size and haematological and clinical chemistry parameters. *Z*-test was performed for testing the differences in proportions of the characters namely mating and fertility indices.

RESULTS

(i) Clinical Signs and Mortality

All rats tolerated daily oral doses of either test item alone or of both test articles together throughout the gestation period and there were no clinical signs or mortalities at any of the doses tested. In addition, no gross abnormalities were detected in the dams at necropsy in any of the groups.

(ii) Maternal Body Weights, Body Weight Gains and Food Intake during Gestation Period

A dose-related decrease in maternal body weights and weight gains and food intake was observed in acetaminophen treated groups at 500 and 1000 mg/kg/day when compared to the control group and these decreases were statistically significant during the gestation period. The maternal body weights (Table 1) and body weight gains (Table 2) were unaffected when Liv.52 was administered alone at 1000 mg/kg/day and these parameters were comparable to the control group. An increased body weight gains and food intake were observed in group 5 (Liv.52 at 1000 mg/kg/day+acetaminophen at 500 mg/kg/day) during the gestation period (Table 3).

Table 1
Mean Maternal Body Weights (g) during Gestation

Group and Dose	Treatment in Days [c]							
	0	3	5	8	11	14	17	20
G1 0 mg/kg/day	226.23 ±22.33	235.43 ±24.35	243.51 ±27.59	250.64 ±27.82	263.76 ±27.20	275.41 ±25.79	299.92 ±24.50	326.80 ±28.16
G2 500 [a] mg/kg/day	211.68 ±21.19	213.99 ±21.17	217.91 ±22.87	224.50 ±20.88	232.16* ±21.43	238.60* ±18.71	255.96* ±27.49	279.21* ±27.57
G3 1000 [a] mg/kg/day	210.30 ±13.78	204.58* ±10.83	209.36* ±13.16	216.18* ±12.91	221.33* ±11.71	229.63* ±14.36	240.79* ±15.22	256.96* ±17.24
G4 1000 [b] mg/kg/day	209.69 ±11.35	217.64 ±9.34	222.07 ±10.26	232.66 ±9.11	240.95 ±6.44	251.27* ±8.30	276.80 ±10.73	304.77 ±10.03
G5 500 [a]+1000 [b] mg/kg/day	209.73 ±11.79	212.11 ±13.08	216.90 ±12.95	224.66 ±15.96	233.38 ±15.24	243.03 ±11.69	262.26 ±5.84	297.29 ±10.91
G6 1000 [a]+1000 [b] mg/kg/day	208.66 ±13.90	202.07 ±19.81	208.29 ±17.42	216.41 ±15.07	220.86 ±13.73	228.46 ±12.21	241.67 ±12.53	258.74 ±23.06

*[a]: Acetaminophen; [b]: Liv.52; [c]: Mean±SD; *: Significantly different from control, P≤0.05*

Table 2
Mean Maternal Body Weight Change (g) During Gestation

Group and Dose	Treatment in Days [c]							
	0-3	3-5	5-8	8-11	11-14	14-17	17-20	0-20
G1 0 mg/kg/day	9.20 ±3.45	8.08 ±5.55	7.13 ±3.87	13.12 ±4.09	11.65 ±4.36	24.51 ±3.31	26.88 ±6.11	100.56 ±9.27
G2 500 [a] mg/kg/day	2.30* ±3.15	3.92 ±2.83	6.59 ±4.08	7.66 ±2.39	6.44 ±7.56	17.36 ±16.74	23.25 ±0.86	67.53* ±24.06
G3 1000 [a] mg/kg/day	-5.73* ±5.07	4.79 ±4.02	6.82 ±7.16	5.15* ±5.72	8.30 ±4.97	11.16 ±7.24	16.17* ±4.83	46.65* ±20.50
G4 1000 [b] mg/kg/day	7.95 ±3.03	4.43 ±2.45	10.59 ±3.82	8.30 ±4.27	10.32 ±3.94	25.53 ±5.95	27.97 ±2.70	95.07 ±10.08
G5500 [a]+1000 [b] mg/kg/day	2.39 ±2.10	4.79 ±2.73	7.76 ±4.03	8.72 ±5.59	9.65 ±4.09	19.23 ±10.46	35.03** ±8.37	87.57 ±5.56
G61000[a]+1000 [b] mg/kg/day	-6.59 ±8.29	6.22 ±3.03	8.12 ±4.61	4.44 ±2.86	7.60 ±6.21	13.21 ±11.60	17.07 ±14.95	50.08 ±31.55

*[a]: Acetaminophen; [b]: Liv.52; [c]: Mean±SD; *: Significantly different from control, P≤0.05*
***: Significantly different from group 2, P≤0.05*

Table 3
Mean Maternal Food Intake (g) during Gestation

Group and Dose	Treatment in Days [c]							
	0-3	3-5	5-8	8-11	11-14	14-17	17-20	0-20
G1 0 mg/kg/day	17.90 ±1.25	21.53 ±2.78	20.38 ±2.67	22.38 ±2.56	23.64 ±2.65	24.05 ±2.05	22.87 ±3.51	21.83 ±2.05
G2 500 [a] mg/kg/day	14.80* ±2.13	17.95 ±5.04	17.74 ±3.61	18.81 ±2.47	19.79 ±2.55	18.72 ±6.33	18.24 ±5.94	18.01* ±3.04
G3 1000 [a] mg/kg/day	11.28* ±1.40	14.41* ±3.09	14.54* ±2.56	16.68* ±3.58	18.16* ±4.08	17.44* ±3.50	19.49 ±3.38	16.08* ±2.81
G4 1000 [b] mg/kg/day	17.48 ±1.86	19.88 ±2.25	20.54 ±2.03	22.21 ±2.69	22.71 ±2.11	23.69 ±1.96	23.64 ±2.69	21.53 ±1.72
G5 500 [a]+1000 [b] mg/kg/day	14.53 ±2.75	17.48 ±1.76	17.70 ±2.39	19.55 ±2.67	20.79 ±1.31	20.22 ±2.52	21.36 ±3.23	18.87 ±0.97
G6 1000 [a]+1000 [b] mg/kg/day	11.25 ±3.56	17.51 ±2.08	16.78 ±1.95	17.50 ±1.22	18.12 ±1.54	18.96 ±2.72	19.87 ±3.24	17.12 ±1.30

*[a]: Acetaminophen; [b]: Liv.52; [c]: Mean±SD; *: Significantly different from control, P≤0.05*

(iii) Maternal Body Weights, Body Weight Gains and Food Intake during Lactation Period

No statistically significant changes were observed in maternal body weights and body weights gains in all the doses tested except for the lower body weights on Day 4 of lactation period in acetaminophen treated group at 500 mg/kg/day. A dose-related decrease in maternal food intake was observed in acetaminophen treated groups at 500 and 1000 mg/kg/day when compared to the control group.

The maternal body weights (Table 4) and body weight gains (Table 5) were unaffected in Liv.52 treated group at 1000 mg/kg/day and these were comparable to the control group. An increased maternal food intake was observed in group 6 (Liv.52 at 1000 mg/kg/day+acetaminophen at 1000 mg/kg/day) during the lactation period (Tables 6 and 7).

Table 4
Mean Maternal Body Weights (g) during Lactation

Group and Dose	Treatment in Days [c]						
	1	4	7	11	14	18	21
G1 0 mg/kg/day	236.20 ±29.92	251.14 ±25.74	254.46 ±22.05	256.77 ±19.01	260.20 ±17.51	255.39 ±16.38	257.01 ±16.93
G2 500 [a] mg/kg/day	205.88 ±26.21	218.25* ±23.81	225.44 ±22.73	226.43 ±23.85	232.79 ±22.94	234.86 ±25.04	232.73 ±21.28
G3 1000 [a] mg/kg/day	221.44 ±19.95	221.77 ±17.63	232.39 ±17.78	241.43 ±5.17	241.22 ±2.33	247.48 ±6.48	243.70 ±9.72
G4 1000 [b] mg/kg/day	220.02 ±14.44	233.03 ±13.42	242.56 ±10.28	248.72 ±10.29	247.79 ±10.16	250.55 ±10.98	254.53 ±13.53
G5 500 [a]+1000 [b] mg/kg/day	207.59 ±11.60	220.96 12.01	228.78 ±13.78	230.49 ±14.54	232.66 ±19.09	241.19 ±21.46	240.53 ±20.41
G6 1000 [a]+1000 [b] mg/kg/day	216.36 ±14.62	222.05 ±13.48	221.60 ±14.57	214.61 ±25.66	219.22 ±22.18	217.33 ±25.29	222.36 ±19.90

*[a]: Acetaminophen; [b]: Liv.52; [c]: Mean±SD; *: Significantly different from control, P≤0.05*

Table 5
Mean Maternal Body Weight Change (g) During Lactation

Group and Dose	Treatment in Days [c]						
	1-4	4- 7	7-11	11-14	14-18	18-21	1-21
G1 0 mg/kg/day	14.94 ±12.42	3.32 ±9.65	2.31 ±9.38	3.43 ±6.67	-4.81 ±7.92	1.62 ±5.17	20.81 ±21.26
G2 500 [a] mg/kg/day	11.24 ±4.62	4.35 ±5.84	1.00 ±2.64	6.36 ±8.92	2.07 ±7.42	-2.13 ±4.05	22.88 ±11.00
G3 1000 [a] mg/kg/day	0.33 ±10.27	8.53 ±6.31	9.04 ±13.52	-0.21 ±6.33	6.26 ±6.19	-3.78 ±5.10	20.93 ±28.62
G4 1000 [b] mg/kg/day	13.01 ±1.79	9.53 ±9.11	6.16 ±5.51	-0.94 ±7.73	2.76 ±11.80	3.98 ±4.55	34.51 ±8.90
G5 500 [a]+1000 [b] mg/kg/day	13.37 ±5.09	7.82 ±6.08	1.71 ±4.50	2.17 ±9.57	8.53 ±3.84	-0.66 ±5.31	32.94 ±18.10
G6 1000 [a]+1000 [b] mg/kg/day	5.70 ±6.34	-0.46 ±8.67	-6.99 ±12.49	4.61 ±4.14	-1.88 ±5.39	5.02 ±6.78	6.00 ±16.97

[a]: Acetaminophen; [b]: Liv.52; [c]: Mean±SD

Table 6
Summary of Total Food Intake (g) During Lactation

Group and Dose	Treatment in Days [c]						
	1-4	4-7	7-11	11-14	14-18	18-21	1-21
G1 0 mg/kg/day	89.15 ±15.75	114.33 ±16.29	186.63 ±7.20	153.37 ±9.54	215.58 ±25.24	172.25 ±23.54	931.30 ±74.09
G2 500 [a] mg/kg/day	84.83 18.88	109.86 ±4.74	158.84* ±24.63	140.23 ±15.26	201.03 ±26.26	151.63 ±24.48	719.48 ±329.14
G3 1000 [a] mg/kg/day	37.45* 19.10	68.09* ±5.68	104.84* ±11.61	114.20* ±41.50	121.38* ±17.10	71.51* ±60.33	421.46* ±246.45
G4 1000 [b] mg/kg/day	87.44 ±7.60	110.84 ±10.54	178.61 ±14.91	144.85 ±23.91	217.10 ±12.96	170.24 ±9.90	909.08 ±38.78
G5 500 [a]+1000 [b] mg/kg/day	84.02 ±10.42	112.54 ±9.71	164.13 ±17.06	132.33 ±21.36	208.11 ±28.80	161.72 ±18.54	862.85 ±87.57
G6 1000 [a]+1000 [b] mg/kg/day	65.92 ±18.84	94.19 ±9.94	126.30 ±34.94	113.32 ±14.32	192.42*** ±32.67	134.03 ±14.18	726.17*** ±88.76

*[a]: Acetaminophen; [b]: Liv.52; [c]: Mean±SD; *: Significantly different from control, P≤0.05*
****: Significantly different from group 3, P≤0.05*

Table 7
Summary of Food Intake (g/rat/day) During Lactation

Group and Dose	Treatment in Days [c]						
	1-4	4-7	7-11	11-14	14-18	18-21	1-21
G1 0 mg/kg/day	29.72 ±5.25	38.11 ±5.43	46.66 ±1.80	51.12 ±3.18	53.89 ±6.31	57.42 ±7.85	46.56 ±3.70
G2 500 [a] mg/kg/day	28.28 ±6.29	36.62 ±1.58	39.71* ±6.16	46.74 ±5.09	50.26 ±6.57	50.54 ±8.16	35.97 ±16.46
G3 1000 [a] mg/kg/day	12.48* ±6.37	22.70* ±1.89	26.21* ±2.90	38.07 ±13.83	30.35* ±4.28	23.84* ±20.11	21.07* ±12.32
G4 1000 [b] mg/kg/day	29.15 ±2.53	36.95 ±3.51	44.65 ±3.73	48.28 ±7.97	54.28 ±3.24	56.75 ±3.30	45.45 ±1.94
G5 500 [a]+1000 [b] mg/kg/day	28.01 ±3.47	37.51 ±3.24	41.03 ±4.26	44.11 ±7.12	52.03 ±7.20	53.91 ±6.18	43.14 ±4.38
G6 1000 [a]+1000 [b] mg/kg/day	21.97 ±6.28	31.40*** ±3.31	31.58 ±8.73	37.77 ±4.77	48.10*** ±8.17	44.68 ±4.73	36.31*** ±4.44

*[a]: Acetaminophen; [b]: Liv.52; [c]: Mean±SD; *: Significantly different from control, P≤0.05*
****: Significantly different from group 3, P≤0.05*

(iv) Number of Pups during Lactation Period

No statistically significant changes were observed in mean number of male, female and mean total pups per litter in acetaminophen treated group at 500 mg/kg/day. However, there was statistical significant decrease in mean number of male, female and mean total pups per litter in acetaminophen treated group at 1000 mg/kg/day when compared to control group. The mean number of male, female and mean total pups per litter was unaffected when Liv.52 alone was administered at 1000 mg/kg/day. A statistical significant increase in mean number of male, female and mean total pups per litter was observed in group 6 (Liv.52 at 1000 mg/kg/day+acetaminophen at 1000 mg/kg/day) during the lactation period (Table 8).

Table 8
Summary of Mean Number of Pups during Lactation Period

Group and Dose	Mean No. of male pups on day					Mean No. of female pups on day					Mean No. of pups for combined sex on day				
	1	4	7	14	21	1	4	7	14	21	1	4	7	14	21
G1 0 mg/kg/day	5.83 ±1.72	3.83 ±0.41	4.00 ±0.00	4.00 ±0.00	4.00 ±0.00	7.00 ±2.00	4.00 ±0.00	4.00 ±0.00	4.00 ±0.00	4.00 ±0.00	12.83 ±1.47	7.83 ±0.41	8.00 ±0.00	8.00 ±0.00	8.00 ±0.00
G2 500[a]mg/kg/day	4.67 ±1.21	4.00 ±0.00	4.00 ±0.00	4.00 ±0.00	4.00 ±0.00	6.67 ±1.51	4.00 ±0.00	4.00 ±0.00	4.00 ±0.00	4.00 ±0.00	11.33 ±1.97	8.00 ±0.00	8.00 ±0.00	8.00 ±0.00	8.00 ±0.00
G3 1000[a] mg/kg/day	2.80* ±1.64	1.75* ±0.50	1.75* ±0.50	1.75* ±0.50	1.75* ±0.50	1.80* ±1.30	1.25* ±0.50	1.25* ±0.50	1.25* ±0.50	1.25* ±0.50	4.60* ±2.41	3.00* ±0.82	3.00* ±0.82	3.00* ±0.82	3.00 ±0.82
G4 1000[b] mg/kg/day	5.00 ±1.67	3.83 ±0.41	4.00 ±0.63	4.00 ±0.63	4.00 ±0.63	5.50 ±2.66	3.50 ±0.84	3.50 ±0.84	3.50 ±0.84	3.50 ±0.84	10.50 ±3.02	7.33 ±1.21	7.50 ±1.22	7.50 ±1.22	7.50 ±1.22
G5500[a]+1000[b] mg/kg/day	6.00 ±2.37	3.83 ±0.41	3.83 ±0.41	3.83 ±0.41	3.83 ±0.41	6.67 ±1.37	4.17 ±0.41	4.17 ±0.41	4.17 ±0.41	4.17 ±0.41	12.67 ±2.25	8.00 ±0.00	8.00 ±0.00	8.00 ±0.00	8.00 ±0.00
G61000[a]+1000[b] mg/kg/day	4.00 ±1.41	3.75*** ±0.96	3.75*** ±0.96	3.75*** ±0.96	3.75*** ±0.96	4.75 ±2.63	3.50*** ±1.29	3.50*** ±1.29	3.50*** ±1.29	3.50*** ±1.29	8.75 ±2.63	7.25*** ±1.50	7.25*** ±1.50	7.25*** ±1.50	7.25*** ±1.50

*[a]: Acetaminophen; [b]: Liv.52; [c:] Mean±SD; *: Significantly different from control, P≤0.05*
****: Significantly different from group 3, P≤0.05*

(v) Body Weight of Pups during Lactation Period

Body weight of pups indicated that, there was decrease in mean weight of male, female and total weight of pups per litter in acetaminophen treated groups at 500 and 1000 mg/kg/day without statistical significance. The similar trend was observed when Liv.52 at 1000 mg/kg/day was co-administered with acetaminophen at 500 or 1000 mg/kg/day. The mean number of male, female and mean total pups per litter was unaffected when Liv.52 alone was administered at 1000 mg/kg/day (Table 9).

Table 9
Summary of Mean Weight of Pups during Lactation Period

Group and Dose	Mean wt. of male pups on day					Mean wt. of female pups on day					Mean wt. of pups for combined sex on day				
	1	4	7	14	21	1	4	7	14	21	1	4	7	14	21
G1 0 mg/kg/day	5.70 ±0.96	8.31 ±1.49	13.11 ±1.60	25.22 ±1.28	35.92 ±2.41	5.68 ±1.35	8.11 ±0.84	13.39 ±1.64	25.24 ±1.59	35.75 ±3.07	5.69 ±1.16	8.18 ±1.02	13.25 ±1.58	25.23 ±1.32	35.84 ±2.69
G2 500[a] mg/kg/day	5.78 ±1.08	8.27 ±1.29	12.88 ±1.52	22.12 ±3.15	31.65 ±4.71	5.54 ±1.17	8.22 ±1.24	12.51 ±1.68	21.95 ±3.00	31.81 ±4.92	5.64 ±1.14	8.24 ±1.25	12.70 ±1.53	22.04 ±3.04	31.73 ±4.71
G3 1000[a] mg/kg/day	5.62 ±0.62	7.58 ±1.92	10.65 ±3.64	21.43 ±7.68	31.24 ±10.10	5.45 ±0.55	7.29 ±2.19	10.44 ±3.39	20.74 ±6.53	32.44 ±8.06	5.53 ±0.55	7.44 ±1.95	10.54 ±3.38	21.10 ±7.05	31.72 ±8.83
G4 1000[b] mg/kg/day	6.15 ±0.70	9.19 ±1.99	13.42 ±2.27	24.11 ±5.11	35.60 ±8.98	5.82 ±0.51	8.66 ±1.52	12.92 ±1.85	23.58 ±4.77	35.18 ±7.42	5.99 ±0.64	8.96 ±1.77	13.20 ±2.09	23.87 ±4.94	35.43 ±8.30
G5 500[a]+1000[b] mg/kg/day	5.78 ±0.80	8.24 ±1.13	12.45 ±1.20	21.46 ±1.61	31.62 ±2.79	5.56 ±0.89	7.94 ±1.17	12.07 ±1.22	20.94 ±1.14	31.33 ±2.55	5.68 ±0.83	8.09 ±1.14	12.26 ±1.19	21.19 ±1.35	31.45 ±2.66
G6 1000[a]+1000[b] mg/kg/day	6.39 ±1.02	9.04 ±2.21	12.81 ±4.01	20.77 ±9.02	29.69 ±14.46	6.22 ±0.69	9.06 ±1.72	13.03 ±3.17	21.15 ±8.32	30.27 ±13.15	6.36 ±0.79	9.12 ±1.89	13.05 ±3.45	21.13 ±8.53	30.18 ±13.69

[a]: Acetaminophen; [b]: Liv.52; [c:] Mean±SD

(vi) Survival Data of Pups

An increase in gestation length (average days to litter) was observed in acetaminophen treated groups at 500 and 1000 mg/kg/day and in Liv.52 treated group at 1000 mg/kg/day. There were no external abnormalities observed in live and dead pups in all the groups tested. The total number of pups born and mean litter size was lower in acetaminophen treated group at 1000 mg/kg/day as compared to the control group. Higher number of dead/cannibalized pups up to Day 4 and lower Day 4 survival index was observed in acetaminophen treated groups at 500 and 1000 mg/kg/day. However, Day 4 survival index was higher in group 5 (Liv.52 at 1000 mg/kg/day+acetaminophen at 500 mg/kg/day) and group 6 (Liv.52 at 1000 mg/kg/day+acetaminophen at 1000 mg/kg/day). The total number of pups alive on lactation days 4, 7, 14 and 21 were less in acetaminophen treated group at 1000 mg/kg/day as compared to the control group. The mean viable litter size, live birth index and 24 hour survival index were lower in acetaminophen treated group at 1000 mg/kg/day while these indices were higher in group 6 (Liv.52 at 1000 mg/kg/day+ acetaminophen at 1000 mg/kg/day). The mean number of implantations was lower in acetaminophen treated group at 1000 mg/kg/day and Liv.52 treated group at 1000 mg/kg/day. Higher pre-implantation loss was observed in acetaminophen treated group at 500 mg/kg/day. Higher post-implantation loss was observed in acetaminophen treated group at 1000 mg/kg/day and lower post-implantation loss was observed when Liv.52 was co-administered with acetaminophen at 1000 mg/kg/day (Table 10).

Table 10
Survival Data of Pups

Groups	G1	G2	G3	G4	G5	G6
Dose (mg/kg/day)	0	500[a]	1000[a]	1000[b]	500[a]+1000[b]	1000[a]+1000[b]
No. of females	6	6	6	6	6	6
No. of pregnancies	6	6	5	6	6	5
Gestation Length (Days) $	22.17 ±0.41	23.00* ±0.00	24.20* ±0.45	22.83* ±0.41	22.83 ±0.41	23.00 ±0.00
No. littered	6	6	5	6	6	4
No. of live litters	6	6	5	6	6	4
Total No. of pups born	78	72	26	63	77	36
Mean litter size $	12.8	12.0	5.2*	10.5	12.8	9.0
No. of pups dead at first observation	1	4	3	0	1	1
No. of pups alive on day 1	77	68	23	63	76	35
Observation of live pups - NAD	77	68	23	63	76	35
No. of pups dead/cannibalized on day 2	0	2	6	0	1	0
No. of pups alive on day 2	77	66	17	63	75	35
No. of pups dead/cannibalized up to day 4	1	11	11	0	2	0
No. of pups alive on day 4	76	57	12	63	74	35
No. of pups discarded	28	17	0	18	26	6
No. of pups alive after standardization on day 4	48	40	12	45	48	29
No. of pups dead/cannibalized from day 5-7	0	0	0	0	0	0
No. of pups alive on day 7	48	40	12	45	48	29
No. of pups dead/cannibalized from day 8-14	0	0	0	0	0	0
No. of pups alive on day 14	48	40	12	45	48	29
No. of pups dead/cannibalized from day 15-21	0	0	0	0	0	0
No. of pups alive on day 21	48	40	12	45	48	29
Mean viable litter size	12.7	11.3	4.6	10.5	12.7	8.8
Live birth index (%) #	98.7	94.4	88.5*	100.0	98.7	97.2***
24 hour survival index (%) #	100.0	97.1	73.9*	100.0	98.7	100.0***
Day 4 survival index (%) #	98.7	83.8*	52.2*	100.0	97.4**	100.0***
Day 7 survival index (%) #	100.0	100.0	100.0	100.0	100.0	100.0
Day 14 survival index (%) #	100.0	100.0	100.0	100.0	100.0	100.0
Day 21 survival index (%) #	100.0	100.0	100.0	100.0	100.0	100.0
No. of females conceived/pregnant (confirmed at necropsy)	6	6	5	6	6	5
No. of dams used for corpora lutea and implantation count	6	6	6	6	6	5
No. of corpora lutea	93	95	87	77	91	66
No. of implantations	85	79	74	67	81	56
Mean No. of corpora lutea $	15.5	15.8	14.5	12.8*	15.2	13.2
Mean No. of implantations $	14.2	13.2	12.3	11.2*	13.5	11.2
Gestation index	100.0	100.0	83.3	100.0	100.0	80.0
Implantation index	91.40	83.16	85.06	87.01	89.01	84.85
Percentage of pre-implantation loss #	6.4	16.2*	14.6	12.4	10.4	15.4
Percentage of post-implantation loss #	10.2	13.3	63.7*	7.6	6.3	26.6***

[a]: Acetaminophen; [b]: Liv.52; #:Compared by 'Z' test
$:Compared by Bartlett, ANOVA and Dunnett's test after transformation (Arc sine, √ x + ½)
**: Significantly different from control, P≤0.05; **: Significantly different from group 2, P≤0.05*
****: Significantly different from group 3, P≤0.05*

(vii) Postnatal Developmental Observation in Pups

Pinna Detachment

The appearance of pinna detachment was observed during days 2-5 in the control and in group 4 (Liv.52 at 1000 mg/kg/day) and group 6 (Liv.52 at 1000 mg/kg/day+acetaminophen at 1000 mg/kg/day) and during postnatal development (PND) 2-4 in group 2 (acetaminophen at 500 mg/kg/day, group 3 (acetaminophen at 1000 mg/kg/day) and group 5 (Liv.52 at 1000 mg/kg/day+ acetaminophen at 500 mg/kg/day). There were few statistical significant differences (increase or decrease) were observed among all groups of the pups in the pinna detachment, however pinna detachment was observed between days 2-5 (Table 11).

Table 11

Postnatal Developmental Observation in Pups (Pinna detachment and Incisor eruption)

Group and Dose (mg/kg/day)	Post Natal Days											
	Pinna detachment (%)					Incisor Eruption (%)						
	1	2	3	4	5	7	8	9	10	11	12	13
G1 0 mg/kg/day	0	14	33	95	100	0	0	6	21	69	100	100
G2 500[a] mg/kg/day	0	7	68*	100*	100	0	0	18*	65*	100*	100	100
G3 1000[a] mg/kg/day	0	17	75*	100*	100	0	0	0*	42*	75	92*	100
G4 1000[b] mg/kg/day	0	33*	78*	92	100	0	2	24*	89*	100*	100	100
G5500[a]+1000[b] mg/kg/day	0	3	49**	100	100	0	0	15	60	92	100	100
G61000[a]+1000[b]mg/kg/day	0	3***	18***	91***	100	0	0	4***	11***	64	100***	100

[a]: Acetaminophen; [b]: Liv.52; Note: The data is compared by Z-test
**: Significantly different from control, P≤0.05; **: Significantly different from group 2, P≤0.05*
****: Significantly different from group 3, P≤0.05*

Incisor Eruption

Incisor eruption was observed during PND 9-12 in the control and in group 5 (Liv.52 at 1000 mg/kg/day+acetaminophen at 500 mg/kg/day) and group 6 (Liv.52 at 1000 mg/kg/day+acetaminophen at 1000 mg/kg/day). Incisor eruption was observed during PND 9-11 in group 2 (acetaminophen at 500 mg/kg/day), PND 10-13 in group 3 (acetaminophen at 1000 mg/kg/day) and PND 8-11 in group 4 (Liv.52 at 500 mg/kg/day). There were few statistical significant differences (increase or decrease) were observed among all groups of the pups in the incisor eruption but the incisor eruption was observed between days 8-13 (Table 11).

Ear Opening

The ear opening was observed during PND 13-16 in control group. The ear opening was observed during PND 13-15 in group 2 (acetaminophen at 500 mg/kg/day), group 4 (Liv.52 at 1000 mg/kg/day) and group 5 (Liv.52 at 1000 mg/kg/day+and acetaminophen at 500 mg/kg/day). The ear opening was observed during PND 13-17 in group 3 (acetaminophen at 1000 mg/kg/day) and group 6 (Liv.52 at 1000 mg/kg/day+ acetaminophen at 1000 mg/kg/day). The average days on which ear opening occurred was significantly delayed in group 3 (acetaminophen at 1000 mg/kg/day) and similar trend was seen in group 6 (Liv.52 at 1000 mg/kg/day+acetaminophen at 1000 mg/kg/day) (Table 12).

Table 12

Postnatal Developmental Observation in Pups (Ear opening and Eye opening)

Group and Dose (mg/kg/day)	Postnatal days													
	Ear opening (%)								Eye opening (%)					
	10	11	12	13	14	15	16	17	13	14	15	16	17	18
G1 0 mg/kg/day	0	0	0	17	79	94	100	100	0	4	33	88	100	100
G2 500[a] mg/kg/day	0	0	0	15	63*	100*	100	100	0	0*	35	78	100	100
G3 1000[a] mg/kg/day	0	0	0	8*	50*	83*	83*	100	0	8	25	75*	92*	100
G4 1000[b] mg/kg/day	0	0	0	16	71	100*	100	100	0	11	53*	78	100	100
G5 500[a]+1000[b] mg/kg/day	0	0	0	10	65	100	100	100	0	0	17**	69	100	100
G6 1000[a]+1000[b] mg/kg/day	0	0	0	17***	20***	37***	80	100	0	0***	14***	17***	66***	100

[a]: Acetaminophen; [b]: Liv.52; [c]:Mean±SD; Note: The data is compared by Z-test
**: Significantly different from control, P≤0.05; **: Significantly different from group 2, P≤0.05*
****: Significantly different from group 3, P≤0.05*

Eye Opening

The eye opening was observed during PND 14-17 in control and group 4 (Liv.52 at 1000 mg/kg/day). The eye opening was observed during PND 15-17 in group 2 (acetaminophen at 500 mg/kg/day) and group 5 (Liv.52 at 1000 mg/kg/day+acetaminophen at 500 mg/kg/day). The eye opening was observed during PND 14-18 in group 3 (acetaminophen at 1000 mg/kg/day) and PND 15-18 in group 6 (Liv.52 at 1000 mg/kg/day+acetaminophen at 1000 mg/kg/day). There were few statistical significant differences (increase or decrease) among all groups of the pups in eye opening but the eye opening was observed between days 14-18 (Table 12).

(viii) Haematology

Haematological parameters indicated decrease in red blood cells, hemoglobin, haematocrit and red cell distribution width in the acetaminophen treated group alone at 1000 mg/kg/day. The haematological parameters were unaffected in all the other groups (Table 13).

Table 13
Summary of Haematology Parameters

Group and Dose	WBC G/L	RBC T/L	HGB g/L	HCT L/L	MCV fL	MCH pg	MCHC g/L	NEUT %	LYM %	MONO %	EOS %	BASO %
G1 0 mg/kg/day	5.41 ±1.68	8.19 ±0.36	143.83 ±2.93	0.485 ±0.012	59.42 ±3.47	17.60 ±0.82	296.67 ±6.44	37.65 ±7.82	56.92 ±8.97	3.50 ±2.03	0.95 ±0.68	0.32 ±0.08
G2 500[a] mg/kg/day	6.18 ±3.02	7.12 ±0.85	141.00 ±22.03	0.460 ±0.078	64.23 ±4.99	19.72* ±1.51	307.67 ±12.71	41.42 ±9.53	52.67 ±11.30	4.22 ±1.90	0.65 ±0.49	0.28 ±0.10
G3 1000[a] mg/kg/day	5.93 ±1.25	5.86* ±1.61	123.17 ±32.79	0.412 ±0.115	70.42* ±3.42	21.17* ±0.94	301.00 ±8.10	44.10 ±6.49	52.23 ±6.99	2.03 ±1.25	0.68 ±0.57	0.30 ±0.24
G4 1000[b] mg/kg/day	6.61 ±2.08	8.33 ±0.32	145.50 ±11.26	0.502 ±0.030	60.18 ±2.59	17.48 ±1.05	290.00 ±7.18	38.10 ±5.66	56.37 ±5.40	4.14 ±0.86	0.53 ±0.70	0.28 ±0.17
G5 500[a]+1000[b] mg/kg/day	6.12 ±2.53	7.77 ±0.33	147.00 ±6.66	0.490 ±0.027	63.05 ±2.59	18.90 ±0.54	300.00 ±9.25	54.92** ±11.37	41.05 ±11.05	2.80 ±1.19	0.43 ±0.29	0.20 ±0.09
G6 1000[a]+1000[b] mg/kg/day	5.46 ±2.13	7.13 ±0.33	145.33 ±6.35	0.477 ±0.022	67.10 ±5.35	20.43 ±1.05	305.33 ±17.95	44.02 ±20.27	48.88 ±21.26	5.65 ±6.40	0.55 ±0.22	0.18 ±0.08

[a]: Acetaminophen; [b]: Liv.52; [c]:Mean±SD;
**: Significantly different from control, P≤0.05; **: Significantly different from group 2, P≤0.05*

Table 13 contd
Summary of Haematology Parameters

Group and Dose (mg/kg/day)	Abs neuts G/L	Abs lymph G/L	Abs mono G/L	Abs eos G/L	Abs baso G/L	PLT G/L	MPV fL	RDW %	HDW g/L
G1 0 mg/kg/day	2.04 ±0.80	3.07 ±0.95	0.20 ±0.18	0.05 ±0.03	0.02 ±0.01	1006.67 ±74.02	9.20 ±1.61	13.15 ±0.61	18.48 ±1.66
G2 500[a] mg/kg/day	2.73 ±1.89	3.05 ±1.16	0.29 ±0.26	0.05 ±0.05	0.01 ±0.01	910.33 ±337.30	9.17 ±1.62	14.37 ±1.90	19.23 ±3.96
G3 1000[a] mg/kg/day	2.57 ±0.45	3.14 ±0.90	0.13 ±0.10	0.04 ±0.03	0.02 ±0.01	945.33 ±182.48	11.12* ±0.70	21.70* ±7.56	23.33 ±6.57
G4 1000[b] mg/kg/day	2.56 ±0.89	3.74 ±1.14	0.28 ±0.13	0.03 ±0.04	0.02 ±0.02	859.67* ±118.04	8.67 ±1.45	13.13 ±1.64	18.27 ±1.13
G5 500[a]+1000[b] mg/kg/day	3.49 ±2.01	2.39 ±0.72	0.18 ±0.13	0.02 ±0.01	0.01 ±0.01	923.67 ±93.82	9.85 ±2.00	14.65 ±1.13	18.65 ±1.74
G6 1000[a]+1000[b] mg/kg/day	2.57 ±1.78	2.55 ±1.44	0.25 ±0.21	0.03 ±0.02	0.01 ±0.01	936.33 ±133.67	9.63 ±1.58	16.00 ±1.30	18.20 ±1.90

*[a]: Acetaminophen; [b]: Liv.52; [c]:Mean±SD; *: Significantly different from control, P≤0.05*

(ix) Clinical Chemistry

Clinical chemistry parameters showed increase in alanine amino transferase and aspartate amino transferase levels in the acetaminophen treated group alone at 1000 mg/kg/day indicative of hepatic injury. The clinical chemistry parameters were unaffected in all the other groups (Table 14).

Table 14
Summary of Clinical Chemistry Parameters

Group and Dose	Glu mmol/L	BUN mmol/L	T.Pro g/L	AST U/L	ALT U/L	ALP U/L	GGT U/L	T.Bil µmol/L	Creat µmol/L
G1	4.59	8.96	58.22	111.67	110.50	90.67	0.00	6.88	37.67
0 mg/kg/day	±0.59	±0.60	±4.48	±12.13	±7.40	±17.47	±0.00	±0.63	±1.75
G2	5.34	7.91	58.08	124.33	135.50	94.33	0.50	6.52	33.33
500[a] mg/kg/day	±1.19	±1.19	±4.48	±27.59	±23.30	±30.40	±0.84	±1.50	±3.83
G3	5.58	8.39	65.45*	142.83	145.83	102.83	1.67*	7.12	37.83
1000[a] mg/kg/day	±0.36	±1.70	±3.95	±50.82	±33.19	±55.16	±1.37	±1.96	±6.55
G4	5.26	8.70	60.43	119.83	119.00	122.17	0.83	7.24	37.00
1000[b] mg/kg/day	±1.00	±0.91	±3.38	±10.21	±12.85	±35.41	±0.41	±0.73	±3.79
G5	5.04	8.29	60.08	120.00	123.00	123.17	1.00	7.36	34.83
500[a]+1000[b] mg/kg/day	±0.72	±0.44	±2.26	±14.48	±13.87	±32.15	±0.00	±0.58	±3.31
G6	5.61	7.98	63.85	103.00	114.17	107.33	0.83	6.34	36.17
1000[a]+1000[b] mg/kg/day	±1.12	±1.32	±5.54	±17.75	±17.61	±28.18	±0.98	±1.18	±7.94

[a]: Acetaminophen; [b]: Liv.52; [c]:Mean±SD; *: Significantly different from control, P≤0.05

Table 14 contd
Summary of Clinical Chemistry Parameters

Group and Dose	Alb g/L	Ca mmol/L	Chol mmol/L	Na mEq/L	K mEq/L	Cl mEq/L	A/G Ratio	Glob g/L
G1 0 mg/kg/day	35.95 ±2.43	2.35 ±0.07	2.19 ±0.42	148.18 ±11.49	4.32 ±0.38	99.05 ±5.47	1.62 ±0.10	22.27 ±2.28
G2 500[a] mg/kg/day	38.12 ±4.25	2.29 ±0.11	2.64 ±0.47	145.37 ±8.41	4.40 ±0.55	104.10 ±5.62	1.92* ±0.27	19.97 ±1.59
G3 1000[a] mg/kg/day	40.00 ±3.23	2.40 ±0.11	2.19 ±0.40	150.32 ±11.14	4.13 ±0.59	105.88 ±3.39	1.58 ±0.19	25.45* ±2.28
G4 1000[b] mg/kg/day	38.13 ±2.77	2.39 ±0.10	2.53 ±0.45	147.63 ±9.94	4.39 ±0.40	111.80* ±8.61	1.72 ±0.20	22.30 ±2.10
G5 500[a]+1000[b] mg/kg/day	36.97 ±1.25	2.40 ±0.08	2.65 ±0.38	151.60 ±8.22	4.55 ±0.53	110.92** ±3.65	1.60** ±0.11	23.12** ±1.57
1000[a]+1000[b] mg/kg/day	40.58 ±3.87	2.34 ±0.14	2.37 ±0.27	149.12 ±7.54	4.55 ±0.58	111.32 ±7.74	1.75 ±0.16	23.27 ±2.26

[a]: Acetaminophen; [b]: Liv.52; [c]:Mean±SD; *: Significantly different from control, P≤0.05
*****: Significantly different from Group 2, P≤0.05***

(x) Gross Pathology

No gross pathological changes were observed in any of the dams and/or pups during weaning sacrifice (Day 21 lactation).

(xi) Histopathology

No histopathological changes were observed in liver and kidneys in group 2 (acetaminophen at 500 mg/kg/day), group 4 (Liv.52 at 1000 mg/kg/day) and group 5 (Liv.52 at 1000 mg/kg/day+acetaminophen at 500 mg/kg/day). However, microscopic evaluation of liver showed three incidences of hepatocellular necrosis and kidneys showed two incidences of dilated tubules and one incidence of protein material in tubules in acetaminophen treated group at 1000 mg/kg/day. One incidence of dilated tubules was persisted ingroup 6 (Liv.52 at 1000 mg/kg/day+acetaminophen at 1000 mg/kg/day) (Table 15).

Table 15
Summary of Histopathological Findings

							21st day Lactation	
Group No.			G1	G2	G3	G4	G5	G6
TISSUE AND	Dose (mg/kg/day)		0	500[a]	1000[a]	1000[b]	500[a]+1000[b]	1000[a]+1000[b]
OBSERVATION	No. of rats		6	6	6	6	6	6
No. of rats examined			6	6	6	6	6	6
1.	LIVER		(6)	(6)	(6)	(6)	(6)	(6)
	Hepatocellular necrosis		0	0	3	0	0	0
2.	KIDNEYS		(6)	(6)	(6)	(6)	(6)	(6)
	Dilated tubules		0	0	2	0	0	1
	Protein material in tubules		0	0	1	0	0	0

[a]: Acetaminophen; [b]: Liv.52

DISCUSSION

Acetaminophen is a common antipyretic agent which is safe in therapeutic doses but can produce fatal hepatic necrosis in humans and animals with higher doses. Liver damage induced by the acetaminophen is a classical model for screening hepatoprotective activity[32]. There are number of reports on fatal complication of acetaminophen overdose for both mother and fetus[33-35]. The adverse reproductive outcomes such as maternal growth and developmental toxicity are considered to be significant due to higher doses of Acetaminophen during pregnancy. Hence, understanding the effect of repeated administration of acetaminophen in pregnant animals is important for determining the risk on to the dam and fetus. In this study, we suspect acetaminophen induced hepatic injury may also mediate its developmental reproductive effects in the dams. Liv.52, an herbal medicine is recognized by thousands of health professionals as one of the most effective liver formulas. Liv.52 has been proved to be evident as hepatoprotective agents in various liver disorders[20-21]. However, there is no information available regarding protection of acetaminophen induced hepatotoxicity will also protect its developmental toxic effects in a mammalian species. Hence, the present study attempted to investigate the protective effect of Liv.52 on acetaminophen induced maternal and or other abnormalities during embryogenesis in Wistar rats when administered orally to pregnant rats from '0' day of pregnancy till 21st day lactation. Rat is considered a standard laboratory rodent species and widely used for developmental toxicity testing and is also recommended by various regulatory authorities for toxicity assessment. The oral gavage route was used to administer both acetaminophen and Liv.52, as it is the intended route of exposure in human populations. The graded doses selected for acetaminophen were 500 mg/kg/day as low dose and 1000 mg/kg/day as high dose. The dose selected for Liv.52 was 1000 mg/kg/day. In addition, the high doses selected for both the test item/s which are also referred to as the limit dose by regulatory toxicity guidelines related to reproduction toxicity testing[36-37]. The concurrent control group rats received vehicle containing 0.5 % w/v Sodiumcarboxymethyl cellulose in Milli-Q water and the same vehicle was also used as acetaminophen and Liv.52 suspensions. This vehicle is common and widely used in toxicology studies. All rats tolerated daily oral doses of either test item alone or of both test items together throughout the gestation and lactation period and there were no clinical signs or mortalities at any of the doses tested. A dose-related decrease in maternal body weights was observed in acetaminophen treated groups at the dose levels of 500 and 1000 mg/kg/day, so that the maternal body weights and body weight gains were lower at the end of 20th day gestation period. The decreased body weight gains in these dose groups were correlated to the reduced food intake during the same time frame of the treatment period. Daily oral doses of Liv.52 at 1000 mg/kg/day did not affect maternal body weights and body weight gains and these were similar to the control group during the gestation period. The decrease observed in maternal body weights or body weight gains and food intake was completely reversed when Liv.52 was co-administered in group 5 (acetaminophen at 500 mg/kg/day) and not reversible in group 6 (acetaminophen at 1000 mg/kg/day) during the gestation period. A clear dose-related difference was not observed in maternal body weights and body weight gains in acetaminophen treated groups at the dose levels of 500 and 1000 mg/kg/day as compared to the control group. This is because of the data of one rat at 500 mg/kg/day and two rats at 1000 mg/kg/day were not considered for the mean values and these rats were sacrificed prior to schedule as pups born were either found dead or cannibalized during early period of the lactation period. A dose-related decrease in maternal food intake was observed in acetaminophen treated groups at the dose levels of 500 and 1000 mg/kg/day doses during the lactation period. Daily oral doses of Liv.52 at 1000 mg/kg/day did not affect maternal food

intake and these were similar to the control group during the lactation period. The decrease observed in food intake was completely reversed when Liv.52 was co-administered in group 5 (acetaminophen at 500 mg/kg/day+Liv.52 at 1000 mg/kg/day) and partially reversible in group 6 (acetaminophen at 1000 mg/kg/day +Liv.52 at 1000 mg/kg/day) during the lactation period. The mean number of pups (male, female and combined sex) were unaffected by the treatment with Acetaminophen at 500 mg/kg/day. The treatment with acetaminophen at 1000 mg/kg/day resulted in decreased mean number of pups (male, female and combined sex) and was found to be reversible when Liv.52 (at 1000 mg/kg/day) was co-administered with acetaminophen (at 1000 mg/kg/day). The decrease in mean weight of pups per litter (male, female and combined) was observed at 500 and 1000 mg/kg/day acetaminophen treated groups and similar trend was continued when Liv.52 (at 1000 mg/kg/day) was co-administered either at 500 or 1000 mg/kg/day of acetaminophen. Daily oral doses of Liv.52 at 1000 mg/kg/day did not affect mean number of male, female and mean total pups per litter and mean weight of male, female and total weight of pups per litter and these were similar to the control group.The slight increase observed in gestation length observed in acetaminophen treated group at 500 mg/kg/day and Liv.52 treated group at 1000 mg/kg/day was considered to be within the biological variation and therefore was not considered toxicologically relevant. However, slightly longer gestation length observed in group 3 is attributed to the treatment of acetaminophen at 1000 mg/kg/day and reversible in group 6 when Liv.52 at 1000 mg/kg/day was co-administered with acetaminophen at 1000 mg/kg/day. The total number of pups born and mean litter size was unaffected in group 2 (acetaminophen at 500 mg/kg/day), group 4 (Liv.52 at 1000 mg/kg/day) and in group 5 (acetaminophen at 500 mg/kg/day+Liv.52 at 1000 mg/kg/day). The total number of pups born and mean litter size was reduced in group 3 (acetaminophen at 1000 mg/kg/day) and partially reversible in group 6 (acetaminophen at 1000 mg/kg/day+Liv.52 at 1000 mg/kg/day). The reduction in total number of pups born in acetaminophen treated group at 1000 mg/kg/day is correlated to lower mean litter size which is due to the higher post-implantation loss in the same group. There were no external abnormalities observed in live and dead pups in any of the groups tested. The mean viable litter size was reduced in group 3 (acetaminophen at 1000 mg/kg/day) and partially reversible in group 6 (acetaminophen at 1000 mg/kg/day+Liv.52 at 1000 mg/kg/day). The live birth index (%) was reduced in group 3 (acetaminophen at 1000 mg/kg/day) which is correlated to the post-implantation loss in the same group and reversible in group 6 (acetaminophen at 1000 mg/kg/day+ Liv.52 at 1000 mg/kg/day). The 24 hour and Day 4 survival index was reduced in group 3 (acetaminophen at 1000 mg/kg/day) which are correlated to the number of pups dead/cannibalized up to Day 4 lactation in the same group and considered reversible in group 6 (acetaminophen at 1000 mg/kg/day+Liv.52 at 1000 mg/kg/day). The slightly lower number of corpora lutea and implantations observed in group 4 (Liv.52 at 1000 mg/kg/day) was considered to be within the biological variation and therefore considered to be of no toxicological relevance. Postnatal developmental observation of pups indicated that, there were no significant differences among pups of all groups in pinna detachment, incisor eruption and eye opening. The average days on which ear opening occurred was significantly delayed in group 3 (acetaminophen at 1000 mg/kg/day) which could be due to maternal toxicity effects and similar trend was seen in group 6 (acetaminophen at 1000 mg/kg/day+Liv.52 at 1000 mg/kg/day). Haematological parameters were unaffected by acetaminophen treatment at 500 mg/kg/day, however, the acetaminophen treatment at 1000 mg/kg/day resulted in decreased red blood cells, hemoglobin, haematocrit and red cell distribution width levels. This finding corresponds to previous observations studied in rats wherein acetaminophen induced hematotoxicity was observed[38]. Daily oral doses of Liv.52 did not affect any of the hematology parameters at 1000 mg/kg/day. The decrease observed in red blood cells,

hemoglobin, haematocrit and red cell distribution width levels completely returned to normal when Liv.52 was co-administered in group 6 (acetaminophen at 1000 mg/kg/day + Liv.52 at 1000 mg/kg/day). Clinical chemistry parameters were unaffected by acetaminophen treatment at 500 mg/kg/day, however, acetaminophen treatment at 1000 mg/kg/day resulted in elevated liver enzymes such as ALT and AST levels which are correlated to the hepatocellular necrosis of liver microscopically. This finding was similar to findings of Dirgha K Patel et al.,[39] wherein acetaminophen treatment caused hepatotoxicity as evidenced by marked elevation in AST, ALT, Lactate dehydrogenase, and total bilirubin levels. This finding was similar to findings of Wankhade et al.,[40] wherein acetaminophen treatment resulted in increased in AST, ALT, Alkaline phosphatase, total bilirubin and decrease in total proteins. Daily oral doses of Liv.52 did not affect any of the clinical chemistry parameters at 1000 mg/kg/day. The increased liver enzymes AST and ALT returned to normal when Liv.52 at 1000 mg/kg/day was co-administered with acetaminophen at 1000 mg/kg/day. No gross pathological changes were observed in any of the dams and or pups during 21st weaning sacrifice. No histopathological changes were observed in liver and kidneys in all the rats of group 2 (acetaminophen at 500 mg/kg/day), group 4 (Liv.52 at 1000 mg/kg/day) and group 5 (acetaminophen at 500 mg/kg/day+ Liv.52 at 1000 mg/kg/day). Incidences of hepatocellular necrosis of liver and dilated tubules of kidneys were observed in group 3 rats treated with acetaminophen at 1000 mg/kg/day. Normal liver was seen in group 6 rats when acetaminophen at 1000 mg/kg/day was co-administered with Liv.52 at 1000 mg/kg/day. One incidence of dilated tubules of kidneys persisted in group 6 when acetaminophen at 1000 mg/kg/day was co-administered with Liv.52 at 1000 mg/kg/day.

CONCLUSION

The study indicated, dose-dependent decrease in maternal body weights and maternal food intake during gestation and lactation and mean weight of male, female and total pups per litter during lactation period in rats treated with acetaminophen at 500 or 1000 mg/kg/day. In addition, the treatment with acetaminophen at 1000 mg/kg/day resulted in lower mean litter size, viable litter size, live birth index, higher post-implantation loss, changes in haematological parameters (decreased red blood cells, hemoglobin hematocrit and red cell distribution width), changes in clinical chemistry parameters (increased alanine amino transferase and aspartate amino transferase) and microscopic evaluation in liver (hepatocellular necrosis) and kidneys (dilated tubules of kidneys). However, many of these affected parameters were found to be reversible or partially reversible when Liv.52 at 1000 mg/kg/day was co-administered with acetaminophen at 500 mg/kg/day or 1000 mg/kg/day. The treatment with Liv.52 did not induce any maternal and or developmental toxicity in Wistar rats when administered daily through oral gavage from gestation Day '0' and up to day 21 of lactation at the tested dose of 1000 mg/kg/day under the test conditions. In summary, acetaminophen can cause toxicity during embryogenesis and postnatal development at 500 or 1000 mg/kg/day. Our results show Liv.52 an herbal formulation could attenuate acetaminophen induced hepatotoxicity, thereby it may prevent toxic effects on the reproductive organs. However, we planned to identify the exact protective mechanism(s) of Liv.52 in the reproductive organs in future studies.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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