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RESEARCH ARTICLE

PROTECTIVE ROLE OF LIV.52 ON THE TOXICITY OF ACETAMINOPHEN DURING EMBRYOGENESIS 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 (embryo-fetal toxicity and teratogenic potential) in Wistar rats. Acetaminophen induced toxicity during embryogenesis was evaluated by administering daily doses of 500 or 1000 mg/kg/day through oral gavage starting from gestation Day 0 pregnancy until Day 19 of gestation. Dose-dependent decrease in maternal body weights and food intake, gravid uterine weight and total and male/female fetal weights as well as fetal length during gestation were observed in dams exposed to 500 and 1000 mg/kg/dayof acetaminophen. At 1000 mg/kg/day of acetaminophen, dose-dependent decrease in red blood cells, haematocrit, red blood cell distribution width, total number of fetuses and mean litter size were observed. The administration of Liv.52 formulation did not induce any toxic effects during embryogenesis in Wistar rats. 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.

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INTRODUCTION

Teratology, the study of abnormal prenatal development and congenital malformations induced by exogenous chemical or physical agents, is a growing area of research for the possible prevention of effects both on mother and fetus. Birth defects are known to occur in children which requires proper medical care to diagnose or treat a birth defect; this compromises the quality of life of millions of people worldwide (O'Rahilly, 2001). Millions of people worldwide use over-the-counter medicines as analgesics and antipyretics on a regular basis during pregnancy. Acetaminophen, also known as Paracetamol is one of the most widely used over-the-counter medication in the world (Zimmerman, 1998; Black, 1984 and Frank *et al*., 1997).

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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 non-prescription drug in 1960 (National Toxicology Program, 1993). Acetaminophen has been reported as the most commonly recommended analgesic during pregnancy (Rayburn *et al*., 1982). 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 (Glover *et al*., 2003). Acetaminophen is primarily metabolized by the liver and excreted by the kidneys. Acetaminophen 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 (Bj¨ornsson and Olsson, 2006; Cover, 2005; Jaeschke *et al*., 2003). Acetaminophen overdose has been reported as the primary cause of acute liver failure in many countries (Bernal, 2003; Gow *et al*., 2004; Larson *et al*., 2005) with more than 70,000 hospitalizations each year in the U.S. alone (Budnitz *et al*., 2011). 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 (Cemek *et al*., 2010).

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 (Friedman *et al*., 1994). Impairment in rat fetal liver without any macroscopic malformation was observed by higher dose of acetaminophen (Burdan *et al*., 2001). 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 (Burdan, 2004).

Herbal products and traditional medicines with better effectiveness and fewer side effects favored against synthetically derived drugs in modern allopathic medication system (Sakthivel *et al*., 2012). 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 (Majumdar *et al*., 1977; Subramonium *et al*., 1998; Buwa *et al*., 2001). Hepatotoxins such as alcohols (Souza *et al*., 1990), heavy metals (Rathore and Nandi, 1992), paracetamol (Kapoor *et al*., 1994) induced liver damage. Mechanistically, Liv.52 is known to protect hepatocellular membrane damage by lowering lipid peroxidation (Saxena and Garg, 1981; Shivani Pandey *et al*., 1994).

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 embryo fetal developmental toxicity (teratogenicity) when administered orally to pregnant rats from '0' day of pregnancy and up to 19 day of gestation.

MATERIAL AND METHODS

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).

AnimalsandMethodology

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 and 22, II Phase, Peenya Industrial Area, Bangalore – 560058, India were used for this experiment. A total number of 36 femalesof 11 to 12 weeks age (day '0' pregnant rats) confirmed by vaginal smear examination with weight ranging from 181 to 230 grams were divided into6 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 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.* The test item/s were suspended in vehicle i.e., 0.5 % w/v Carboxymethylcellulose sodium salt in Milli-Q water and administered as oral gavage. All groups comprised 6 females each. Group 1 received only the vehicle at 10 mL/kg body weight through oral gavage. Groups2 and Group 3 received acetaminophen suspensions at the doses of 500 and 1000 mg/kg/day respectively,each group 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 acetaminophensuspensions 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 Day19 of gestation.

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.

All the presumed pregnant females were euthanized under isoflurane anesthesia on Day 20 of gestation 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 blood samples were centrifuged for about 10 minutes at refrigerated conditions at 5000 rpm and plasma was separated and analysed for clinical chemistry parameters using Roche/Hitachi 902 (Hitachi High-Technologies Corporation, Tokyo, Japan) Automatic Analyzer. Then the maternal viscera were examined macroscopically. The ovaries were removed and placed in a pre-labelled container and the corpora lutea were counted immediately under a dissecting microscope. The gravid uterus was cut open along the ante-mesometrial side exposing the amniotic sacs. The sacs were ruptured and the number and position of implantation, early or late resorptions and dead or live fetuses were recorded. The umbilical cord of each fetus was cut and fetuses removed in a sequential order as present in the uterus, blotted dry and placed in a tray. The fetuses were then sexed, individually weighed and the crown-rump length measured using a digital vernier caliper. External examination of fetuses for morphological abnormalities under an illuminated magnifying lens at a magnification of 5X/10X was made. All the live fetuses are euthanized under isoflurane anesthesia. Following which 50 % of fetuses are transferred into 70 % ethyl alcohol for visceral/soft tissue evaluation under an illuminated magnifying lens at a magnification of 5X/10X (Staples, 1974) and the remaining 50 % fetuses are skinned, eviscerated and transferred into 70 % ethyl alcohol for skeletal abnormalities (Staples and Schnell, 1964). The specimens for skeletal observations was processed and stained using alizarin red stain for the ossified parts and evaluated under a Stereoscopic Zoom microscope with typical magnification levels of 8X to 80X.

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, gravid uterine weight, food consumption, number of corpora lutea, number of implantations, litter size, litter weight, length and fetus number and haematological and clinical chemistry parameters. The incidences of pre and post-implantation loss, number of early and late resorptions were analyzed using Kruskal Wallis test. The percentages of skeletal malformations, sex ratio, dams with any resorptions were analyzed using 2X2 contingency table. A probability of 0.05 was accepted as statistically significant for all the applied tests.

RESULTS

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 caesarean section in any of the groups.

Maternal body weights, bodyweight 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.

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 food intake was 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

End Point	Treatment [c]					
	G1	G2	G3	G4	G5	G6
	0 mg/kg/day	500 [a] mg/kg/day	1000[a] mg/kg/day	1000[b] mg/kg/day	500 [a] +1000 [b] mg/kg/day	1000 [a] +1000 [b] mg/kg/day
0	197.89	199.13	199.86	201.47	202.27	202.93
	±11.67	±9.44	±8.78	±10.18	±12.16	±14.34
3	204.84	200.54	195.26	208.42	204.60	198.45
	±12.75	±8.88	±9.30	±14.86	±15.88	±23.48
5	209.43	206.21	198.45	213.23	210.96	202.44
	±11.64	±7.96	±10.84	±12.58	±15.44	±22.93
8	209.88	208.82	202.50	221.04	217.61	210.51
	±13.51	±8.51	±9.26	±13.13	±15.73	±23.46
11	229.36	217.70	214.52	234.90	229.88	219.21
	±15.52	±11.04	±9.77	±13.53	±16.11	±22.96
14	239.50	228.93	221.62	245.16	237.04	226.44
	±15.48	±10.54	±8.32	±18.20	±16.43	±23.45
17	261.39	250.19	237.73*	265.78	258.68	240.74
	±17.53	±12.66	±10.84	±18.69	±19.62	±27.39
20	287.76	278.59	258.46*	295.03	293.87	261.21
	±17.15	±12.96	±13.69	±27.37	±26.15	±34.72

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

Haematology and clinical chemistry

Haematological parameters Table 4 indicated decreased 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 4.

A dose-related reduction in total and male/female fetal weights as well as fetal length (crown-rump) in acetaminophen treated groups at 500 and 1000 mg/kg/day.

Table 2. Mean Maternal Body Weight Gains

End Point	Treatment [c]					
	G1	G2	G3	G4	G5	G6
	0 mg/kg/day	500 [a] mg/kg/day	1000 [a] mg/kg/day	1000 [b] mg/kg/day	500 [a] +1000 [b] mg/kg/day	1000 [a] +1000 [b] mg/kg/day
0-3	6.95	1.41*	-4.60*	6.96	2.33	-4.49
	±3.78	±2.35	±5.15	±6.32	±4.56	±9.23
3-5	4.58	5.67	3.19	4.81	6.36	3.99
	±3.51	±2.37	±3.98	±3.85	±1.35	±3.27
5-8	0.45	2.60	4.06	7.81	6.65**	8.07
	±11.20	±2.81	±5.67	±2.30	±1.84	±3.08
8-11	19.48	8.89	12.01	13.86	12.28	8.70
	±14.58	±2.89	±4.40	±2.93	±3.01	±2.49
11-14	10.15	11.23	7.11	10.26	7.16**	7.22
	±1.86	±2.47	±4.18	±6.54	±1.99	±4.75
14-17	21.88	21.26	16.11	20.62	21.64	14.31
	±3.55	±4.20	±4.63	±4.03	±4.66	±7.47
17-20	26.37	28.40	20.72	29.25	35.19	20.47
	±2.96	±5.87	±9.43	±9.43	±7.52	±9.22

[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. Summary of Food Intake (g/rat/day)

End Point	Treatment [c]					
	G1	G2	G3	G4	G5	G6
	0 mg/kg/day	500 [a] mg/kg/day	1000 [a] mg/kg/day	1000 [b] mg/kg/day	500 [a] +1000 [b] mg/kg/day	1000 [a] +1000 [b] mg/kg/day
0-3	15.18	12.83	10.05*	16.57	13.07	8.26
	±1.92	±2.19	±1.99	±2.90	±2.56	±2.76
3-5	19.29	17.12	16.03	19.45	20.17	16.41
	±2.60	±2.93	±2.69	±2.43	±2.44	±2.12
5-8	18.91	15.45*	14.10*	18.88	19.19**	16.51
	±3.07	±1.86	±1.93	±2.91	±2.42	±2.32
8-11	19.49	16.88	17.61	20.26	20.08**	16.79
	±2.72	±1.33	±1.87	±1.78	±2.04	±1.71
11-14	20.85	19.05	18.32*	22.29	21.45**	18.66
	±2.13	±1.43	±1.44	±3.28	±2.08	±2.52
14-17	21.25	19.75	19.44	22.39	21.64	19.16
	±1.69	±2.11	±2.04	±2.28	±1.71	±2.61
17-20	20.24	20.08	20.84	22.56	24.27**	20.35
	±2.35	±1.79	±1.93	±3.38	±2.04	±2.56
0-20	19.32	17.31*	16.66*	20.39	19.97**	16.60
	±1.33	±1.65	±1.33	±2.33	±1.74	±1.68

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

The clinical chemistry parameters were unaffected by the treatment at any of the doses tested Table 5.

Maternal parameters

A dose-related decrease in gravid uterine weight was observed in acetaminophen treated groups at 500 and 1000 mg/kg/day when compared to the control group. The decreased gravid uterine weight was statistically significant in acetaminophen treated group at 1000 mg/kg/day. The mean gravid uterine weights, mean number of corpora lutea, implantations, early and late resorptions, pre and post-implantation loss was unaffected when Liv.52 alone was administered at 1000 mg/kg/day. An increased mean gravid uterine weightwas observed in group 5 when Liv.52 at 1000 mg/kg/day was co-administered with acetaminophen at 500 mg/kg/day Table 6.

Litter parameters

Litter data indicated reduction in total number of fetuses and mean litter size in acetaminophen treated group at 1000 mg/kg/day.

The statistical significance was observed in acetaminophen treated group at 1000 mg/kg/day for total and male/female fetal weights as well as fetal length.A statistical significant increase in fetal weight (combined sex) was observed when Liv.52 was administered alone in group 4 when compared with the control group.An increase in total and male/female fetal weights as well as fetal length was observed in group 5 when Liv.52 at 1000 mg/kg/day was co-administered with acetaminophen at 500 mg/kg/day Table 7.

Fetal morphologicalobservations

The fetal external observations showed one incidence of thread like tail along with omphalocele in acetaminophen treated group at 1000 mg/kg/day. The visceral examinations did not reveal any significant abnormalities in any of the groups. The skeletal system of fetus stained with alizarin red stain also did not show any major malformations Table 8.

Table 4. Summary of Haematology Parameters

End Point	Treatment [c]					
	G1	G2	G3	G4	G5	G6
	0 mg/kg/day	500 [a] mg/kg/day	1000 [a] mg/kg/day	1000 [b] mg/kg/day	500 [a] +1000 [b] mg/kg/day	1000 [a] +1000 [b] mg/kg/day
WBC	5.58	5.34	6.23	5.93	5.29	4.82***
(G/L)	±0.52	±1.52	±1.43	±1.18	±0.74	±0.49
RBC	7.32	6.84	6.24*	7.22	6.81	6.62
(T/L)	±0.22	±0.43	±0.35	±0.17	±0.14	±0.49
HGB	130.33	124.17	117.67*	129.17	125.00	123.00
(g/L)	±4.89	±4.22	±8.16	±2.32	±1.41	±4.77
HCT	0.437	0.419	0.388*	0.424	0.416	0.408
(L/L)	±0.015	±0.011	±0.030	±0.011	±0.003	±0.021
MCV	59.68	61.32	62.03	58.70	61.10	61.70
(fL)	±2.32	±2.62	±2.28	±0.44	±1.07	±1.70
MCH	17.80	18.18	18.82	17.90	18.40	18.65
(pg)	±0.95	±0.74	±0.62	±0.56	±0.54	±0.87
MCHC	298.17	296.50	303.33	305.00	300.67	302.17
(g/L)	±9.60	±4.18	±3.83	±9.47	±4.23	±6.49
NEUT	34.55	35.69	36.23	33.10	37.59	36.22
(%)	±4.31	±3.86	±2.68	±5.54	±6.26	±4.84
LYM	57.88	57.51	58.07	59.27	55.21	56.93
(%)	±4.79	±4.51	±3.28	±5.47	±6.88	±5.54
MONO	6.38	4.84	3.95*	6.43	5.55	5.00
(%)	±1.55	±1.27	±1.77	±0.88	±2.07	±1.00
EOS	0.43	0.96	0.65	0.42	0.91	0.83
(%)	±0.38	±1.17	±0.62	±0.23	±1.56	±0.83
BASO	0.18	0.26	0.25	0.20	0.13**	0.18
(%)	±0.10	±0.08	±0.15	±0.06	±0.12	±0.04
PLT	1145.67	1090.83	1017.17	1187.67	1073.17	1104.83
(G/L)	±135.31	±88.44	±62.10	±124.25	±294.46	±134.46
RDW	11.23	13.92*	16.80*	11.25	13.18	15.67
(%)	±0.23	±0.74	±1.36	±0.41	±0.58	±1.38
HDW	19.98	19.95	20.82	19.12	19.53	20.62
(g/L)	±1.32	±2.02	±2.11	±1.40	±0.94	±1.73

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

Table 5. Summary of Clinical Chemistry Parameters

End Point	Treatment [c]					
	G1	G2	G3	G4	G5	G6
	0 mg/kg/day	500 [a] mg/kg/day	1000 [a] mg/kg/day	1000 [b] mg/kg/day	500 [a] +1000 [b] mg/kg/day	1000 [a] +1000 [b] mg/kg/day
Glu	4.21	4.16	4.53	4.52	4.94	6.50
(mmol/L)	±0.37	±0.76	±1.05	±1.13	±1.72	±2.06
BUN	6.00	6.44	7.51	5.80	7.04	7.29
(mmol/L)	±0.47	±0.56	±2.10	±0.96	±1.58	±1.03
T.Pro	58.83	59.08	61.15	63.98	63.20	67.17***
(g/L)	±4.12	±4.10	±2.18	±4.19	±6.81	±4.06
AST	61.33	53.50	60.00	59.17	56.00	60.83
(U/L)	±8.38	±15.24	±5.48	±5.00	±6.03	±8.38
ALT	60.33	71.33	73.83	68.83	71.00	71.00
(U/L)	±15.29	±12.24	±14.89	±8.18	±8.34	±8.29
ALP	43.33	45.50	56.00	42.00	53.83	62.17
(U/L)	±11.27	±11.22	±11.49	±7.92	±13.99	±10.01
GGT	1.33	1.50	0.83	1.17	1.33	1.17
(U/L)	±1.03	±0.84	±0.75	±0.98	±1.03	±1.17
T.Bil	2.68	2.55	2.83	2.56	2.09	2.29
(µmol/L)	±0.51	±0.43	±0.49	±0.42	±0.70	±0.58
Creat	44.17	39.17	40.17	40.67	39.50	38.00
(µmol/L)	±3.60	±4.36	±3.92	±3.44	±4.68	±4.00
Alb	38.95	40.97	41.93	42.90	39.60	45.63
(g/L)	±3.12	±3.68	±1.74	±5.23	±2.65	±4.43
Ca	2.21	2.26	2.34	2.24	2.13	2.23
(mmol/L)	±0.08	±0.20	±0.18	±0.23	±0.32	±0.23
Chol	1.88	2.12	1.83	2.20*	2.02	2.07
(mmol/L)	±0.26	±0.38	±0.31	±0.14	±0.33	±0.49
Na	145.07	146.80	144.22	144.57	151.80	149.38
(mEq/L)	±4.81	±2.71	±5.43	±5.56	±7.81	±5.40
K	4.62	4.36	4.75	4.60	5.16**	5.03
(mEq/L)	±0.37	±0.32	±0.35	±0.44	±0.31	±0.34
Cl	103.77	102.07	104.18	104.43	97.22	100.83
(mEq/L)	±5.78	±3.13	±6.56	±7.08	±6.53	±5.51
A/G	1.97	2.27	2.22	2.07	1.76**	2.29
Ratio	±0.22	±0.24	±0.36	±0.48	±0.37	±0.74
Glob	19.88	18.12	19.22	21.08	23.60	21.53
(g/L)	±1.90	±1.22	±2.65	±2.30	±6.36	±6.20

[a]: Acetaminophen; [b]: Liv.52; [c]: Mean±SD;
*: Significantly different from control, P≤0.05; **: Significantly different from group 2, P≤0.05
***: Significantly different from group 3, P≤0.05

Table 6. Summary of Maternal Parameters

End Point	Treatment [c]					
	G1	G2	G3	G4	G5	G6
	0 mg/kg/day	500 [a] mg/kg/day	1000 [a] mg/kg/day	1000 [b] mg/kg/day	500 [a] +1000 [b] mg/kg/day	1000 [a] +1000 [b] mg/kg/day
Gravid uterine weight (g)	60.83 ±13.59	50.59 ±8.31	33.36* ±16.76	54.29 ±14.33	58.64 ±14.95	30.89 ±20.19
Number of Corpora lutea	14.50 ±2.81	11.67* ±1.03	13.17 ±0.75	13.17 ±1.83	14.17** ±2.23	14.33 ±1.37
Number of Implantations	12.17 ±2.64	10.50 ±1.38	12.83 ±2.40	9.83 ±2.93	11.67 ±2.80	10.33 ±2.80
Early Resorptions	0.50 ±0.55 4.11%	0.67 ±0.82 6.35%	1.67 ±1.03 12.99%	0.00 ±0.00 0.00%	0.83 ±0.98 7.14%	3.00 ±2.37 29.03%
Late Resorptions	0.00 ±0.00 0.00%	0.00 ±0.00 0.00%	2.00* ±1.90 15.58%	0.00 ±0.00 0.00%	0.00 ±0.00 0.00%	1.00 ±1.26 9.68%
Pre-implantation Loss	2.33 ±0.82 16.09%	1.17 ±0.98 10.00%	0.33 ±2.07 2.53%	3.33 ±3.83 25.32%	2.50 ±2.26 17.65%	4.00 ±2.19 27.91%
Post-implantation Loss	0.50 ±0.55 4.11%	0.67 ±0.82 6.35%	3.67* ±1.21 28.57%	0.00 ±0.00 0.00%	0.83 ±0.98 7.14%	4.00 ±2.76 38.71%
Dams with any Resorption	3 ±50.00	3 ±50.00	6* ±100.00	0* ±0.00	3 ±50.00	4 ±66.67
Dams with all Resorption	0 ±0.00	0 ±0.00	0 ±0.00	0 ±0.00	0 ±0.00	1 ±16.67

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

Table 7. Summary of Litter Parameters

End Point	Treatment [c]					
	G1	G2	G3	G4	G5	G6
	0 mg/kg/day	500 [a] mg/kg/day	1000 [a] mg/kg/day	1000 [b] mg/kg/day	500 [a] +1000 [b] mg/kg/day	1000 [a] +1000 [b] mg/kg/day
No. of litters	6	6	6	6	6	6
Total no. of fetuses	70	59	45	59	65	38
Mean litter size	12	10	8	10	11	6
Dead fetuses	0	0	0	0	0	0
Total live fetuses						
a. Number	70	59	45	59	65	38
b. Weight (g)	3.33 ±0.13	2.98 ±0.23	2.34* ±0.41	3.52* ±0.17	3.39** ±0.15	2.47 ±0.35
c. Length (mm)	34.26 ±0.91	33.17 ±1.35	29.19* ±2.84	34.90 ±1.18	34.27 ±1.15	29.89 ±2.49
Live male fetuses						
a. Number	35	24	26	31	30	18
b. Weight (g)	3.43 ±0.25	3.06 ±0.36	2.53* ±0.27	3.62 ±0.18	3.56** ±0.18	2.68 ±0.29
c. Length (mm)	34.53 ±1.02	33.18 ±1.92	30.17* ±1.62	35.53 ±1.06	35.17 ±1.38	31.03 ±1.67
Live female fetuses						
a. Number	35	35	19	28	35	20
b. Weight (g)	3.29 ±0.13	2.91 ±0.20	2.10* ±0.55	3.42 ±0.19	3.24* ±0.26	2.29 ±0.37
c. Length (mm)	34.04 ±1.01	32.95 ±1.29	28.10* ±4.05	34.27 ±1.26	33.31 ±1.83	29.06 ±2.61
Sex Ratio - Male : Female	1:1	1:1.46	1:0.73	1:0.9	1:1.17	1:1.11

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

The main findings were normal variations (related to the ossification of some bone components like delayed/incomplete/poor ossification) and some minor anomalies (hypoplastic sternum, dumbbell/split vertebra centra, and rudimentary/accessory/extra ribs) seen across the treated groups. However, majority of these normal variants and minor anomalies were significantly increased in group 3 (acetaminophen at 1000 mg/kg/day) and in group 6 (Liv.52 at 1000 mg/kg/day+acetaminophen at 1000 mg/kg/day).

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 (Mitchell *et al*., 1973).There are number of reports on fatal complication of acetaminophen overdose for both mother and fetus (McElhatton *et al*., 1997; Wang *et al*., 1997; Wilkes *et al*., 2005).

Table 8. Fetal Morphological Observations

End Point	Treatment – (No.) (Fetus%) (Litter%) [c]					
	G1	G2	G3	G4	G5	G6
	0 mg/kg/day	500 [a] mg/kg/day	1000 [a] mg/kg/day	1000 [b] mg/kg/day	500 [a] +1000 [b] mg/kg/day	1000 [a] +1000 [b] mg/kg/day
No. of litters	6	6	6	6	6	6
Total no. of fetuses	70	59	45	59	65	38
External Examination[d]	70 (6)	59 (6)	45(6)	59 (6)	65 (6)	38 (5)
Small fetus	0(0)(0)	0(0)(0)	1(4.55)(8.33)	0(0)(0)	0(0)(0)	0(0)(0)
Thread like tail and omphalocele	0(0)(0)	0(0)(0)	1(4.55)(1.67)	0(0)(0)	0(0)(0)	0(0)(0)
Visceral observations[d]	35 (6)	30 (6)	23 (6)	29 (6)	32 (6)	19 (5)
Soft tissue observations	e	e	e	e	e	e
Skeletal observations[d]	35(6)	29(6)	22(6)	30 (6)	33 (6)	19 (5)
Hypo Stern No. 1	0(0)(0)	0(0)(0)	1(4.55*)(0.17)	0(0)(0)	0(0)(0)	0(0***)(0)
Hypo Stern No. 2	0(0)(0)	0(0)(0)	2(9.09* (0.33)	0(0)(0)	0(0)(0)	1(5.26)(0.20)
Hypo Stern No. 3	0(0)(0)	0(0)(0)	1(4.55*)(0.17)	0(0)(0)	0(0)(0)	1(5.26)(0.20)
Hypo Stern No. 4	0(0)(0)	0(0)(0)	1(4.55*)(0.17)	0(0)(0)	0(0)(0)	0(0***)(0)
Hypo Stern No. 5	0(0)(0)	0(0)(0)	2(9.09*)(0.33)	0(0)(0)	1(3.03)(0.17)	1(5.26)(0.20)
Hypo TV: centra:1/1	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	1(5.26***)(0.20)
Hypo Scapaula	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	1(5.26***)(0.20)
Hypo Clavicle	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	1(5.26***)(0.20)
Split Stern No.1	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	1(5.26***)(0.20)
Split Stern No.3	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	2(10.53***)(0.40)
Split Stern No.4	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	1(5.26***)(0.20)
Split Stern No.5	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	1(5.26***)(0.20)
Split TV: centra:1/13	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	1(5.26***)(0.20)
Split LV: centra:1/6	0(0)(0)	0(0)(0)	1(4.55*)(18.18)	0(0)(0)	0(0)(0)	0(0)(0)
DB: TV: centra:1/13	3(8.57)(0.50)	4(13.79)(0.67)	4(18.18*)(0.67)	1(3.33)(0.17)	2(6.06)(0.33)	2(10.53)(0.40)
DB: TV: centra:2/13	1(2.86)(0.17)	1(3.45)(0.17)	2(9.09)(0.33)	0(0)(0)	1(3.03)(0.17)	2(10.53)(0.40)
DB: TV: centra:3/13	0(0)(0)	0(0)(0)	1(4.55*)(0.17)	0(0)(0)	1(3.03)(0.17)	1(5.26)(0.20)
DB: TV: centra:4/13	0(0)(0)	0(0)(0)	1(4.55*)(0.17)	0(0)(0)	0(0)(0)	0(0***)(0)
DB: LV: centra:1/6	0(0)(0)	0(0)(0)	2(9.09*)(0.33)	1(3.33)(0.17)	0(0)(0)	0(0***)(0)
Asy DB: LV:centra:1/6	0(0)(0)	0(0)(0)	3(13.64*)(0.50)	0(0)(0)	0(0)(0)	0(0***)(0)
UO:TV:centra:1/13	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	1(5.26***)(0.20)
Wavy: Rib(Rt/Lt/B):5/13	0(0)(0)	0(0)(0)	1(4.55*)(0.17)	0(0)(0)	0(0)(0)	0(0***)(0)
Wavy: Rib(Rt/Lt/B):7/13	0(0)(0)	0(0)(0)	1(4.55*)(0.17)	0(0)(0)	0(0)(0)	0(0***)(0)
Wavy: Rib(Rt/Lt/B):8/13	0(0)(0)	0(0)(0)	1(4.55*)(0.17)	0(0)(0)	0(0)(0)	0(0***)(0)
Wavy: Rib(Rt/Lt/B):9/13	0(0)(0)	0(0)(0)	1(4.55*)(0.17)	0(0)(0)	0(0)(0)	0(0***)(0)
Rud: Rib(Rt/Lt/B):15	0(0)(0)	0(0)(0)	3(13.64*)(0.50)	0(0)(0)	0(0)(0)	1(5.26***)(0.20)
Acc.: Rib(Rt/Lt/B):14	2(5.71)(0.33)	5(17.24*)(0.83)	3(13.64)(0.50)	2(6.67)(0.33)	3(9.09)(0.50)	1(5.26***)(0.20)
Acc.: Rib(Rt/Lt/B):15	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	0(0)(0)	2(10.53***)(0.40)
Extra Rib(Rt/Lt/B):14	0(0)(0)	4* (13.79*)(0.67)	6* (27.27*)(1.00)	1(3.33)(0.17)	5 (15.15)(0.83)	5(26.32)(1.00)
Extra LV:centra,arch 7	0(0)(0)	1(3.45)(0.17)	3* (13.64*)(0.50)	0(0)(0)	2 (6.06)(0.33)	4(21.05)(0.80)
FL(Rt/Lt/B): flexed at wrist	0(0)(0)	0(0)(0)	1(4.55*)(0.17)	0(0)(0)	0(0)(0)	0(0***)(0)
Major malformations	Nil	Nil	Nil	Nil	Nil	Nil

[a]: Acetaminophen; [b]: Liv.52; [c]: Mean±SD; [d]: Number of fetuses/litters *: Significantly different from control, P≤0.05;
[e]: No Major observations; [f]: Total number of fetuses (litters) exhibiting variations.malformations ***: Significantly different from group 3, P≤0.05

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 (Subramonium *et al*., 1998; Buwa *et al*., 2001). 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 21stday 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 (OECD, 2001; ICH, 2005). 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. Decreased maternal body weights or body weight gains and food intake were found to be reversible in groups 5 (Liv.52 at 1000 mg/kg/day+acetaminophen at 500 mg/kg/day) and not reversible in group 6 (Liv.52 at 1000 mg/kg/day+acetaminophen 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 blood cell distribution width levels. This finding corresponds to previous observations studied in rats wherein acetaminophen induced hematotoxicity was observed (Yousef *et al*., 2010). Daily oral doses of Liv.52 did not affect any of the hematology parameters at 1000 mg/kg/day. Decreased red blood cells, hemoglobin, haematocrit and red blood cell distribution width levels were completely returned to normal when Liv.52 was co-administered in group 6 (Liv.52 at 1000 mg/kg/day+acetaminophen at 1000 mg/kg/day).Daily oral doses of either test article alone or of both test articles together did not clearly affect any of the clinical chemistry parameters and gross anomalies at caesarian section at either dose level. This finding corresponds to previous observations studied in rats wherein acetaminophen did not induce any toxicologically significant effects on clinical chemistry parameters (Venkatesan and Deecaraman, 2014). Maternal data indicated dose-related decrease in gravid uterine weight in acetaminophen treated groups at the dose levels of 500 and 1000 mg/kg which correlated with reduced fetal weights and considered due to acetaminophen treatment. The treatment with Liv.52 at 1000 mg/kg did not affect mean gravid uterine weights, mean number of corpora lutea, implantations, early and late resorptions, pre and post-implantation loss. Decreased gravid uterine weight was completely reversed when Liv.52 was co-administered in group 5 (Liv.52 at 1000 mg/kg/day+acetaminophenat 500 mg/kg/day) and not reversible in group 6 (Liv.52 at 1000 mg/kg/day+acetaminophen at 1000 mg/kg/day).

Litter data indicated reduction in total number of fetuses and mean litter size in acetaminophen treated group at 1000 mg/kg/day. In addition, there was reduction in total and male/female fetal weights as well as fetal lengthin both acetaminophen treated groups at 500 and 1000mg/kg/day. This finding was similar to the findings of Burdan *et al.* (2001) wherein, there was statistical significant decrease in fetal body length by acetaminophen treatment at 350 mg/kg/day. In addition, Burdan F. 2004 reported that, prenatal exposure to the combination of paracetamol and caffeine in rats resulted in dose-dependent effects on fetal body weight/growth and placental weight at the mid (35 mg/kg of paracetamol+7 mg/kg of caffeine) and high doses (350 mg/kg of paracetamol+70 mg/kg of caffeine).The treatment with Liv.52 alone resulted in increased fetal weight (combined sex) at 1000 mg/kg/day. Increased total and male/female fetal weights as well as fetal length in group 5 indicated normalcy when Liv.52 (1000 mg/kg/day) was co-administered with acetaminophen at 500 mg/kg/day. However, data of total and male/female fetal weights as well as fetal lengthin group 6 indicated partial recovery when Liv.52 (1000 mg/kg/day) was co-administered with acetaminophen at 1000 mg/kg/day.The fetal external observations showed one incidence of thread like tail along with omphalocele in acetaminophen treated group at 1000 mg/kg/daywhich was found to be normal when subjected to skeletal evaluation. The visceral examinations did not reveal any significant abnormalities in any of the groups. The skeletal system of fetus stained with alizarin red stain also did not show any major malformations. The main findings were normal variations (related to the ossification of some bone components like delayed/incomplete/poor ossification) and some minor anomalies (hypoplastic sternum, dumbbell/split vertebra centra, and rudimentary/accessory/extra ribs) seen across the treated groups. However, majority of these normal variants and minor anomalies were significantly increased 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) and these findings were attributed to decreased maternal growth and reduced fetal weight.

Conclusion

The study indicated dose-dependent decrease in maternal body weights, maternal food intake, gravid uterine weight and reduction in total and male/female fetal weights as well as fetal length in acetaminophen treatment groups at 500 and 1000 mg/kg /day. In addition, the treatment with acetaminophen at 1000 mg/kg/day resulted in decreased haematological parameters (red blood cells, hemoglobin hematocrit and red blood cell distribution width) and decreased total number of fetuses and mean litter size. However, co-administration of Liv.52 (1000 mg/kg/day) with acetaminophen induced partial or complete reversal of acetaminophen induced developmental toxic effects. The treatment with Liv.52 did not induce any maternal toxicity and fetal development toxicity in Wistar rats when administered daily through oral gavage from gestation day '0' and up to day '19' gestation at the tested dose of 1000 mg/kg/day under the test conditions. Liv.52 is considered to have no teratogenic potential at 1000 mg/kg/day. In summary, acetaminophen can cause toxicity during embryogenesis at 500 or 1000 mg/kg/day. Our results show Liv.52 an herbal formulation could attenuate acetaminophen induced developmental 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.

Conflict of interest statement

The authors do not have any conflict of interest to disclose.

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