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Toxicity profile of honey and ghee, when taken together in equal ratio

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ABSTRACT

Honey and ghee are an essential component of our diet. They play an important role like anti-inflammatory, antioxidative, antimicrobial, etc. It is written in Charak Samhita that an equal mixture of honey and ghee turn into a harmful component for health. This study was designed to explore the mechanism of toxicity through the biochemical and histological parameters in Charles foster rats (24 rats were used). We have divided these rats into four groups (n = 6) - normal, honey (0.7 ml/100 g bw), ghee (0.7 ml/100 g bw), and honey + ghee (1:1) (1.5 ml/100 g bw). Treatment was given orally for 60 days. All rats were sacrificed on 61 days. Biochemical parameters like liver function test, kidney function test, Oxidative stress, Glycemic, and some protein modification parameters were done in blood plasma. We found weight loss, hair loss, red patches on ear, and increased liver function test, oxidative stress, Amadori product formation, advanced glycation end-product formation, dipeptidyl protease (DPP-4) and decreased incretins (glucagon-like peptide-1(GLP-1) and gastric inhibitory polypeptide (GIP)) in honey + ghee group. H&E and immunohistochemistry results showed mild inflammation in liver tissue but no changes in the kidney, intestine and, pancreas. Thus it concluded that the increased formation of Amadori product, DPP-4 activity and low incretins (GLP-1, GIP) activity resulting high postprandial hyperglycemic response could be collectively responsible for oxidative stress-mediated toxicity of honey and ghee in the equal mixture.

1. Introduction

Honey and ghee are important constituents of the normal diet. Honey is a thick, sweet, yellowish liquid prepared by honey bees mostly by sugar-rich nectar of the flower. It is a complex mixture having about 200 substances [1]. According to Codex Alimentarius “Honey is the natural sweet substance, produced by honeybees from the nectar of plants or from secretions of living parts of plants, or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature”. The consistency of honey varies depending on the type of flower nectar used by the honey bee. Apart from these flower nectar sources, the consistency of honey varies due to some external sources like way and time of processing, seasonal and environmental factor, time of packing and storage [2]. Honey is a supersaturated solution of sugars, of which fructose (38%) and glucose (31%) are the important contributors. Some minor constituent is also present in a wide range which includes phenolic acid [3], certain enzymes like glucose oxidase, catalase, ascorbic acid [3], carotenoid like compound [4], organic acids [4], amino acids and proteins, malliard reaction [4], minerals, vitamins [5]. In addition, honey is bestowed with many beneficial properties which ranges from its high nutritive value to its potential antioxidant [6,7], anti-inflammatory and antimicrobial properties [8]; [9], along with its wound and sunburn healing capabilities [10,11].

Ghee is analogous to drawn butter fat, which is produced by heating butter to get rid of milk solids and water. It is prepared from cow’s milk, buffalo’s milk and the mixed milk’s as well. Ghee contains fats (99%), cholesterol, vitamins, tocopherols, lanosterol, free fatty acids, carotenes, ubiquinones, etc. [12]. Ghee is prepared from butter but the impurities and the milk solids have been removed, so most people having casein and lactose intolerance have not any problem with ghee.

Abbreviations: LFT, Liver function test; RFT, Renal function test; SGOT, Serum glutamate oxaloacetate transaminases; SGPT, Serum glutamate pyruvate transaminases; ALP, Alkaline phosphatases; BUN, Blood urea nitrogen; DPP-4, Dipeptidyl protease; GLP-1, Glucagon-like peptide-1; GIP, Gastric inhibitory polypeptide; GPPN, gly-pro-p-nitroanilide; ABTS, 2,2’-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid; NBT, Nitroblue tetrazolium chloride; TCA, Tricholoro acetic acid; GAA, Glacial acetic acid; ACB, Albumin Cobalt Binding; AGEs, Advanced glycation end products; SOD, Superoxide dismutases; LPO, Lipid peroxidation; GSH, Reduced glutathione; HB, Hemoglobin; TG, Triglycerides; bw, body weight

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Ghee is a rich source of omega-3 fatty acids, which aid in brain development [13]. These omega-3 and omega-6 fatty acids have been reported to alleviate cardiovascular diseases and other chronic diseases [14]. Besides, since antiquity Ghee is in vogue in Ayurveda to treat allergy, skin and respiratory diseases [15].

Although both are rich in nutrients but as per Ayurvedic literature its regular use in equal quantity, in one food, may be toxic [16]. As documented in Charak Samhita, when we take this incompatible diet for a short time then it may not be dangerous for health but when we take it regularly for longer time it becomes dangerous and maybe cause several sorts of diseases like sterility, herpes, eye diseases, skin eruptions, ascites, fistula, leprosy, sprue, edema, fever, rhinitis, fetal distress, and may even cause death [17]. However, this claim has not been scientifically validated till date, though some research papers are available [18,19].

Here we have tried to explore the effect of this mixture on the physical appearance, anatomical and physiological changes on GIT with special reference to oxidative stress, incretins secretion, structural modification in blood component i.e. circulating protein and carbohydrates.

Some food mediated toxicity happens due to oxidative stress [20–25] that's why we assayed oxidative stress parameters like antioxidant enzymes activity (Superoxide dismutase (SOD), catalase), reduced glutathione level (GSH), lipid peroxidation (LPO), and $ABTS^+$ radical scavenging assay. Honey is a rich source of fructose and glucose, while ghee is a rich in fats, so we checked the glycemic parameters like glucose, incretins (glucagon-like peptide-1(GLP-1) and gastric inhibitory polypeptide (GIP)), and dipeptidyl protease (DPP-4) and lipid-based parameters like triglycerides (TG), cholesterol and lipases enzyme activity. These sugars, fats, and proteins in diet are known to induce AGEs (advanced glycation end-products) through Amadori formation, protein unfolding and fragmentation culminating in a hyperchromic shift at 280 nm and decrease in albumin-cobalt binding (ACB).

In addition, we also checked the toxicity of equal ratio of honey and ghee by performing liver and kidney function tests with subsequent validation by histopathological screenings.

2. Method and materials

2.1. Materials

Honey was purchased from Patanjali. Ghee was purchased from Anik. SGOT, SGPT, ALP, urea, Creatinine, BUN, glucose estimation kits were purchased from Accurex Biomedical PVT LTD., Biosar, Thane. Lipase substrate, 4-nitrophenyl butyrate, DPP-4 substrate gly-pro-p-nitroanilide, and $ABTS^+$ tablet were purchased from Sigma-Aldrich. GLP-1 and GIP EIA kit were purchased from Sigma-Aldrich. Nitroblue tetrazolium chloride, metaphosphoric acid (HPO3)n, Methionine, Riboflavin, Bradford reagent, thiobarbituric acid, Bromocresol green (BCG) reagent, Trichloro-acetic acid were purchased from Hi-media Ltd, Calcutta, Hemocord-D, a hemoglobin reagent was purchased from Coral Clinical System, Goa.

2.2. Method

Experimental protocol of the study was approved by the animal ethical committee of the Institute of Medical Sciences, Banaras Hindu University, Varanasi (ethical committee letter # No Dean/2017/CAEC/720). 60 days old 24 healthy Charles Foster strain male rats from an inbreed colony were arbitrarily selected from the central animal house of our institute (Institute of Medical Sciences) between 100–200 g body weight range. The purpose of choosing a male rat is only to avoid the variation in female inherited characteristics like different metabolic rates, sex hormones, pregnancy and lactation. All rats were kept independently in propylene cage with the maintained hygienic condition and standard diet. All the animals were kept at 25° C with the standard condition of 12 h light/dark cycle. Before treatment start, rats were kept under the standard condition for 1 week with free access to standard chow diet and tap water for acclimatization. Nutritive information of normal rat diet, honey and ghee were given in Table 1 (Table 1: Nutritive information of normal rat diet, honey, and ghee).

Table 1
Nutrient information of normal rat diet, honey and ghee (per 100 g).

	Normal rat diet	Honey (Patanjali)	Ghee (Anik)
Carbohydrate(including Starch, Sucrose, Cellulose)	48.8 g	80 g	0.0g
Natural sugar	-	80 g	0.0g
Added sugar	-	0 g	
Protein	~21 g	1 g	0.0g
Fat	~3 g	0 g	-
Sodium	-	20 mg	-
Potassium	-	130 mg	-
Calcium	0.8 g	12 mg	-
Phosphorus	0.4 g	5 mg	-
Iron	-	1.6 mg	-
Water	13 g	-	0.3g
Fiber	5 g	-	0.0g
Cholesterol	-	-	275-325mg
Saturated fatty acids	-	-	58g
Monounsaturated fatty acids	-	-	28g
Polyunsaturated fatty acids	-	-	2g
Trans fatty acids	-	-	5 g
Vitamin A	1 g	-	2000-3500IU
Ash	8 g		
Energy	306.2 kcal	320 kcal	879 kcal

2.3. Treatment

After acclimatization, the rats were divided into four groups (n = 6). Group-1 received a normal diet. Group-2 received honey along with the normal diet. Group-3 received ghee along with the normal diet. Group-4 received an equal mixture of honey and ghee (Honey + ghee) along with the normal diet. For finalization of dose, we have taken the basis of earlier publications where 2.5 g/kg/day dose of honey have been used [26]. As per Ayurvedic practice, the recommended dose of honey is 48 g/day and the recommended dose of ghee is 30 ml/day for a healthy 70 kg men. Based on these literatures we have decided the dose. Honey and ghee were given 0.7 ml/100 g body weight for 60 days. Equal ratio of honey and ghee was mixed (1.5 ml/100 g body weight) and given to rats for 60 days. Just before given, the mixture was vigorously vortex for emulsion the mixture and immediately given by the oral gavages. The weights of all the rats in a given group were read and their blood was collected on day 1 before the treatments started. These experiments were carried out for 60 days and weight, physical appearance, food intake was recorded. Blood was collected in EDTA vials on every 15^{th} day for 60 days. Hemolysate was formed by adding 25 µl blood in 975 µl cold distilled water. Plasma was isolated by centrifuging the blood at 5000 rpm for 15 min in the cooling centrifuge. Rats were sacrificed on 61^{th} day by using ethyl ether and liver, kidney, intestine, pancreas were collected and cleaned with 1x PBS and preserved in 10 % formaldehyde for histological studies.

2.4. Biochemical study

2.4.1. Liver function test

Serum glutamate oxaloacetate transaminases (SGOT), Serum glutamate pyruvate transaminases (SGPT), alkaline phosphatases (ALP) test were done by using the commercial kit available (Accurex Biomedical). Albumin level was estimated by BCG reagent (Hi-media). Protein estimation was done by Bradford's reagent (Hi-media).

Table 2
Weight (g) of all groups rat on different days.

Days	Normal		Honey		Ghee		Honey + Ghee	
	Weight	% change	Weight	% change	Weight	% change	Weight	% change
1 day	120 ± 17.3	—	158 ± 17.5	—	115 ± 15	—	161 ± 27	—
15 day	130 ± 20.3	7.69	179 ± 10.3	11.18	136 ± 25	15.85	166 ± 24	3.6
30 day	180 ± 28.4	33.33	206 ± 11.5	23.15	150 ± 14	23.33	193 ± 23	16.59
45 day	210 ± 25.3	42.85	230 ± 22.5	30.97	185 ± 21	37.83	178 ± 25	9.58
60 day	250 ± 30.4	52	245 ± 27.8	35.2	190 ± 28	39.47	160 ± 20	−0.78

% change was calculated between 1st day and present day.

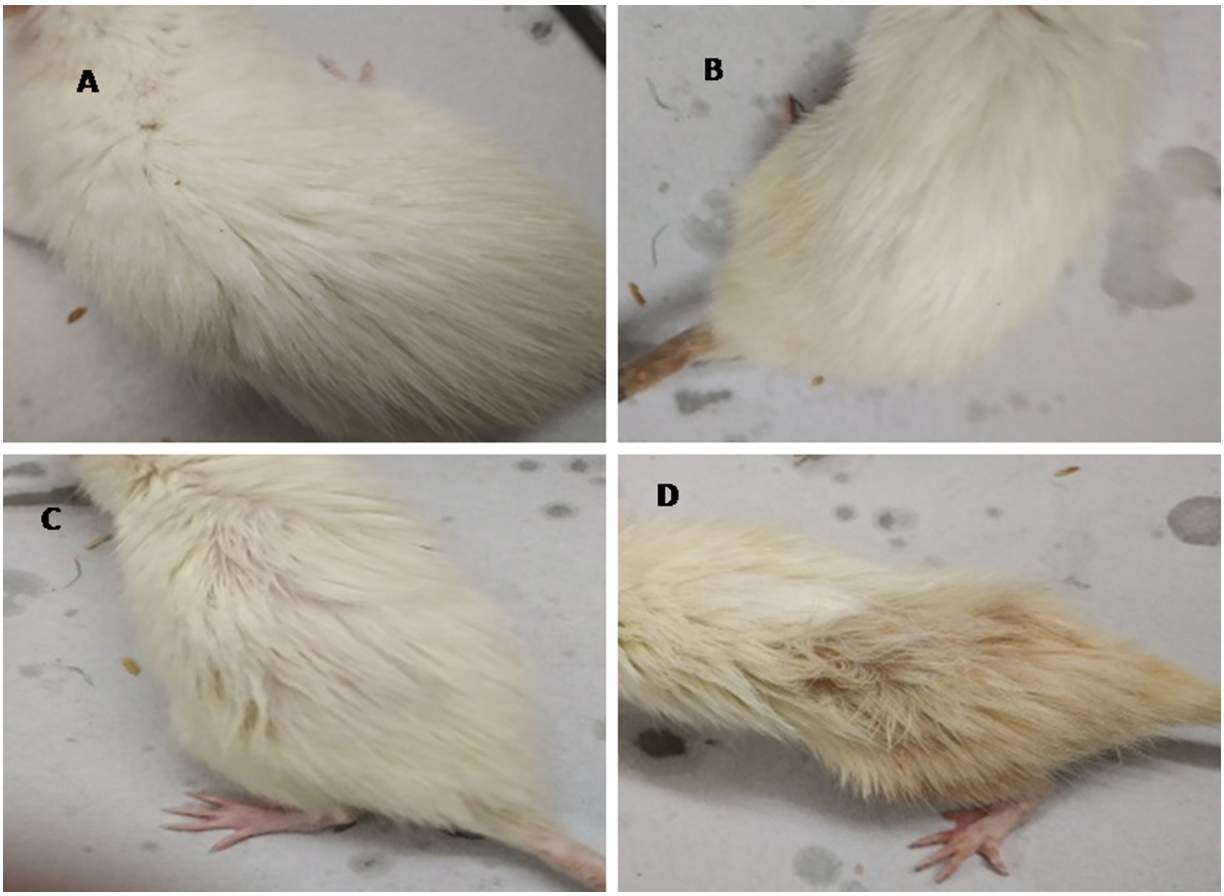

Fig. 1. Hair loss and yellowish on skin of rats (n = 6). A (Normal), B (Honey), C (Ghee), D (Honey + Ghee). Honey + Ghee group showed some loss of hair and yellowish on back skin.

2.4.2. Kidney function test

Urea, blood urea nitrogen (BUN), and Creatinine test were done by using a commercial kit available (Accurex Biomedical).

2.4.3. Oxidative stress parameter

2.4.3.1. Superoxide dismutase:. Superoxide dismutase enzyme activity was calculated according to Beauchamp and Fridovich method [27] with minor modification [28]. Briefly, we prepared three tubes blank, control, and sample tubes. In sample tubes, the reaction mixture contained SOD buffer (pH 7.8), 250 µl hemolysate (10 times diluted), 300 µl L-methionine (130 mM), 150 µl NBT (750 µM), and 100 µl riboflavin (60 µM). Riboflavin and hemolysate were absent in blank and control tubes respectively. Then these tubes were kept in front of fluorescent light for 10 min. NBT reduced in blue color formazan in the attendance of riboflavin through a photochemical reaction. The reaction was inhibited in the presence of enzymes. The color produced was read on 560 nm.

2.4.3.2. Catalase:. Catalase enzyme activity was calculated by Aebi's method via following the H_2O_2 go down at 240 nm [29]. Briefly, the reaction was started by adding 10 µl hemolysate (10 times diluted) in 900 µl of 30 mM H_2O_2 and absorbance was observed for 3 min at 240 nm. Enzyme activity was manifested in U/mg.

2.4.3.3. Reduced glutathione:. GSH was done according to described by Beutler et.al. [30] with slight modification [31]. To 0.2 ml EDTA blood 3 ml of precipitating reagent (metaphosphoric acid (HPO3)n 1.67 g, EDTA di-sodium salt 0.2 g, and sodium chloride 30 g in 100 ml of distilled water) was added and incubated for 5 min at room temperature and then centrifuged. Then 4 ml 0.3 M sodium hydrogen phosphate solution was added in 2 ml of supernatant. Then 1 ml of 0.01% DTNB reagent was added and incubated for 5 min. The absorbance was taken at 412 nm. GSH content was calculated with the standard curve of GSH.

2.4.3.4. $ABTS^{\cdot+}$ radical scavenging activity:. $ABTS^{\cdot+}$ radical scavenging

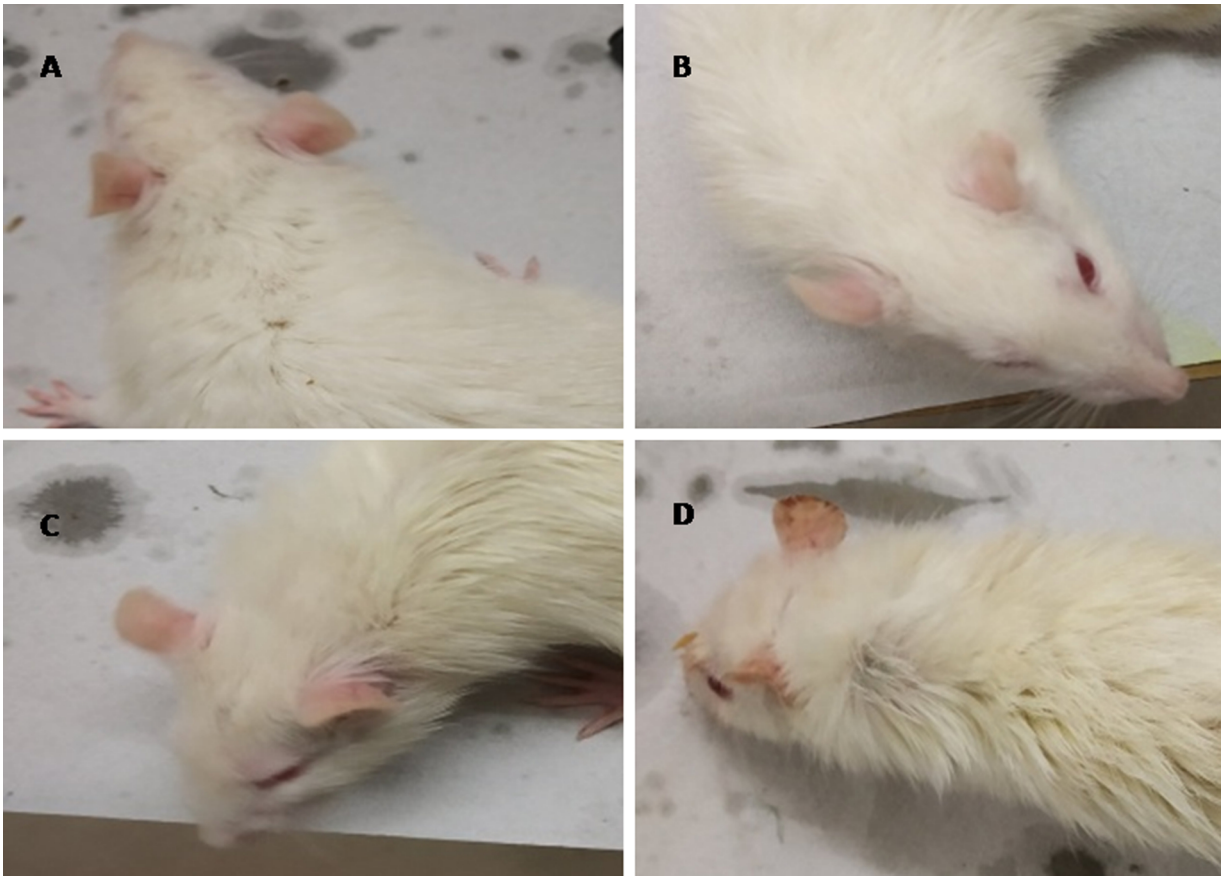

Fig. 2. Appearance of red patches on ear of rats (n = 6). A (Normal), B (Honey), C (Ghee), D (Honey + Ghee). Honey + Ghee group showed appearance of red patches on the ear.

Table 3
Physical appearance of different group's rat on different time interval.

	Normal		Honey		ghee		Honey + ghee	
	30day	60day	30day	60day	30day	60day	30day	60day
Hair loss	NC	NC	NC	NC	NC	NC	NC	C
Red patches on ear	NC	NC	NC	NC	NC	NC	NC	C

(NC means no change and C means change found on different time interval).

Table 4
Effect of honey, ghee and equal ratio of honey and ghee on liver function test and kidney function test (in plasma).

	1day	60 days			
		Normal	Honey	Ghee	Honey + Ghee
LFT					
SGOT(IU/L)	63 ± 5.4	65.5 ± 4.5	69 ± 2.9	68.7 ± 5.1	70.5 ± 9.9*
SGPT(IU/L)	43.2 ± 4.3	44 ± 3.9	48.7 ± 1.7	47.8 ± 2.9	58 ± 2.61*
ALP(IU/L)	405 ± 62	410 ± 47.7	438 ± 41.6	449 ± 27	548 ± 35*
Albumin(g/dl)	3.5 ± .14	3.6 ± .15	3.8 ± .17	3.9 ± .31	5.1 ± .39*,#,@
Protein (mg/ml)	7.1 ± .31	7.3 ± .26	7.2 ± .31	7.44 ± .17	8.1 ± .72*,#
RFT					
Urea (mg%)	47 ± 3.1	48 ± 2.8	45.1 ± 2.9	45.36 ± 5.2	45.9 ± 4.08
Creatinine (mg%)	0.35 ± .015	0.34 ± .013	0.37 ± .012	0.35 ± .01	0.39 ± .014
BUN (mg%)	23 ± .56	24.2 ± 1.7	22.97 ± .66	21.15 ± 2.3	24.29 ± 1.4

* $p < 0.05$, comparison of normal group with honey, ghee and honey + ghee group.
# $p < 0.05$, comparison of honey group with ghee and honey + ghee group.
@ $p < 0.05$, comparison of ghee group with honey and honey + ghee group.

Table 5
Effect of honey, ghee and equal mixture of honey and ghee on oxidative stress parameters (in blood, plasma and hemolysate).

	1day	60 days			
		Normal	Honey	Ghee	Honey + Ghee
SOD(U/mg) in hemolysate	0.78 ± 0.02	0.77 ± 0.01	0.78 ± 0.01[@]	0.73 ± 0.02[*,#]	0.63 ± 0.05[*,#,@]
Catalase(U/mg) in hemolysate	1.12 ± 0.05	1.04 ± 0.05	0.99 ± 0.01[@]	0.87 ± .01[*,#]	0.46 ± .03[*,#,@]
GSH(μmol) in blood	11.24 ± 0.31	11.18 ± 0.26	10.49 ± 0.35[*,@]	9.925 ± 0.53[*,#]	6.02 ± 0.62[*,#,@]
LPO(nmol/mg) in liver tissue homogenate	0.83 ± 0.03	0.85 ± 0.02	0.92 ± .05[*,@]	1.09 ± .06[*,#]	1.42 ± .05[*,#,@]
LPO(nmol/mg) in plasma	0.44 ± .04	0.45 ± .05	0.47 ± .03[@]	0.64 ± .04[*,#]	0.7 ± .03[*,#,@]
$ABTS^{\cdot+}$(%inhibition) in plasma	67.4 ± 4.5	66.95 ± 1.8	64.61 ± 7.1	64.12 ± 6.8	56.48 ± 6.5[*]

[*] $p < 0.05$, comparison of normal group with honey, ghee and honey + ghee group.
[#] $p < 0.05$, comparison of honey group with ghee and honey + ghee group.
[@] $p < 0.05$, comparison of ghee group with honey and honey + ghee group.

Table 6
Effect of honey, ghee and equal ratio of honey and ghee on glycemic parameters, protein modification parameters and some of the lipid profiles (in plasma).

	1day	60 days			
		Normal	Honey	Ghee	Honey + Ghee
Glycemic Parameters					
Glucose (mg%)	83 ± 2.3	85.13 ± 3.15	99.65 ± 7.45[*]	102.8 ± 8.45[*]	129.9 ± 3.77[*,#,@]
DPP-4(units/ml)	3.3 ± 0.46	3.4 ± 0.58	4.65 ± 0.47[*]	4.32 ± 0.6[*]	5.29 ± 0.64[*,#,@]
GLP-1(pg/ml)	784 ± 39.4	755 ± 42.9	566 ± 82.8[*]	586 ± 94.4[*]	398 ± 51.1[*,#,@]
GIP(pg/ml)	9001 ± 452	8983 ± 540	6023 ± 644[*]	6887 ± 1132[*]	4196 ± 1098[*,#,@]
Protein modification parameters					
Hemoglobin(g/dl)	11.4 ± .62	11.6 ± .71	11.3 ± .93	10.8 ± .44	8.39 ± .41[*,#]
ACB (% decreases)	1.24 ± 0.08	1.27 ± 0.05	1.19 ± 0.04	1.17 ± 0.09	0.84 ± 1[*]
UV (280 nm)	0.451 ± 0.04	0.469 ± 0.04	0.458 ± 0.05	0.521 ± 0.07	0.70 ± 0.05[*]
Amadori(μmol/mg)	1.2 ± 0.36	1.21 ± 0.55	1.48 ± 0.78[*]	1.24 ± 0.33[#]	1.68 ± 0.71[*,#,@]
AGE content Florescence intensity (Ex390/Em460))	56.4 ± 5.9	58.42 ± 6.2	63.96 ± 4.06	61.76 ± 1.73	72.05 ± 2.64[*]
Some Lipid Profile					
TG (mg%)	48 ± 2.4	50 ± 2.9	62 ± 5.2[*]	60 ± 2.1[*]	63 ± 2.9[*]
Cholesterol (mg%)	54.3 ± 2.7	57.7 ± 2.38	62.7 ± 1.63	67 ± 2[*]	73.3 ± 4.51[*]
Lipases (units/ml)	11.54 ± .78	11.78 ± .6	14.6 ± 1.3[*]	14.3 ± 1.12[*]	14.82 ± 1.75[*]

[*] $p < 0.05$, comparison of normal group with honey, ghee and honey + ghee group.
[#] $p < 0.05$, comparison of honey group with ghee and honey + ghee group.
[@] $p < 0.05$, comparison of ghee group with honey and honey + ghee group.

activity was determined according to described by Re et al. [32].

ABTS reagent was formed by adding 2.7 ml $ABTS^{\cdot+}$ solution (1 tablet in 3 ml) in 0.3 ml of potassium persulphate solution (24 mM) and left for 16 hours in the dark. Next day it was diluted with water to get absorbance 0.7 at 734 nm. To 1 ml of $ABTS^{\cdot+}$ solution add 10 μl of plasma and absorbance was measured at 734 nm. The absorbance of only $ABTS^{\cdot+}$ reagent was used as control. Present inhibition was calculated according to: Present inhibition = (OD of control-OD of test / OD of control)*100

2.4.3.5. Lipid Peroxidation:. Estimation of lipid peroxidation level was done by the thiobarbituric acid method [33]. In this method, 0.5 ml of hemolysate (0.5 ml tissue homogenate) was taken and 0.5 ml of 10% TCA was added and then centrifuged at 1000 g for 10 minutes. Then 1.5 ml of 0.67% thiobarbituric acid (made in 50% GAA) was added in 0.5 ml supernatant and test tubes were kept in a boiling water bath for 20-30 min. Absorbance was taken on 532 nm after cooling the test tubes.

2.4.4. Glycemic parameters

The glucose level was estimated by using the Accurex Biomedical kit. GLP-1 and GIP enzyme activity were done by using the EIA kit (Sigma-Aldrich). DPP-4 (dipeptidyl peptidase-4) activity was done by adding 95 μl GPPN (0.2 mM) as a substrate in the mixture of 65 μl Tris-HCl (50 mM, 7.5 pH) and 10 μl plasma. Absorbance was taken immediately and after 20 min at 405 nm.

Enzyme unit (U/ml) = (((ΔAbs/min (sample)- (ΔAbs/min(blank))* reaction volume*dilution factor)/molar extinction coefficient * path length*sample volume

2.4.5. Protein modification parameter

2.4.5.1. Amadori test:. The formation of Amadori products was assessed using the method of Johnson and Baker [34], with nitroblue tetrazolium (NBT). 100 μl of the sample per well were transferred to a 96-well plate. Then in each well 100 μl of NBT reagent (250 μM NBT in 0.1 M carbonate buffer, pH 10.35) was added and the plate was incubated for 2 h at 37 °C. Absorbance was measured at 525 nm. An absorption coefficient of 12,640 $cm^{-1}\,mol^{-1}$ was used.

2.4.5.2. UV Absorbance Spectroscopy:. Absorption profiles of normal and treated plasma were recorded on Perkin Elmer Lambda 25 UV/VIS spectrometer (Department of Physics, BHU) in 200 to 400 nm wavelength range using quartz cuvette of 1 cm path length. An increase in absorbance at 280 nm indicated the increase in hyperchromic shift due to protein unfolding/fragmentation [35,36].

2.4.5.3. Fluorescence Study for AGEs:. AGE-specific fluorescence was recorded by exciting the samples (1:1000) at 390 nm and keeping the emission range of 400 to 600 nm in Perkin Elmer LS 45 fluorescence spectrometer (Department of Physics, BHU) [36].

2.4.5.4. Albumin Cobalt Binding Assay:. The 100 μl of serum was mixed

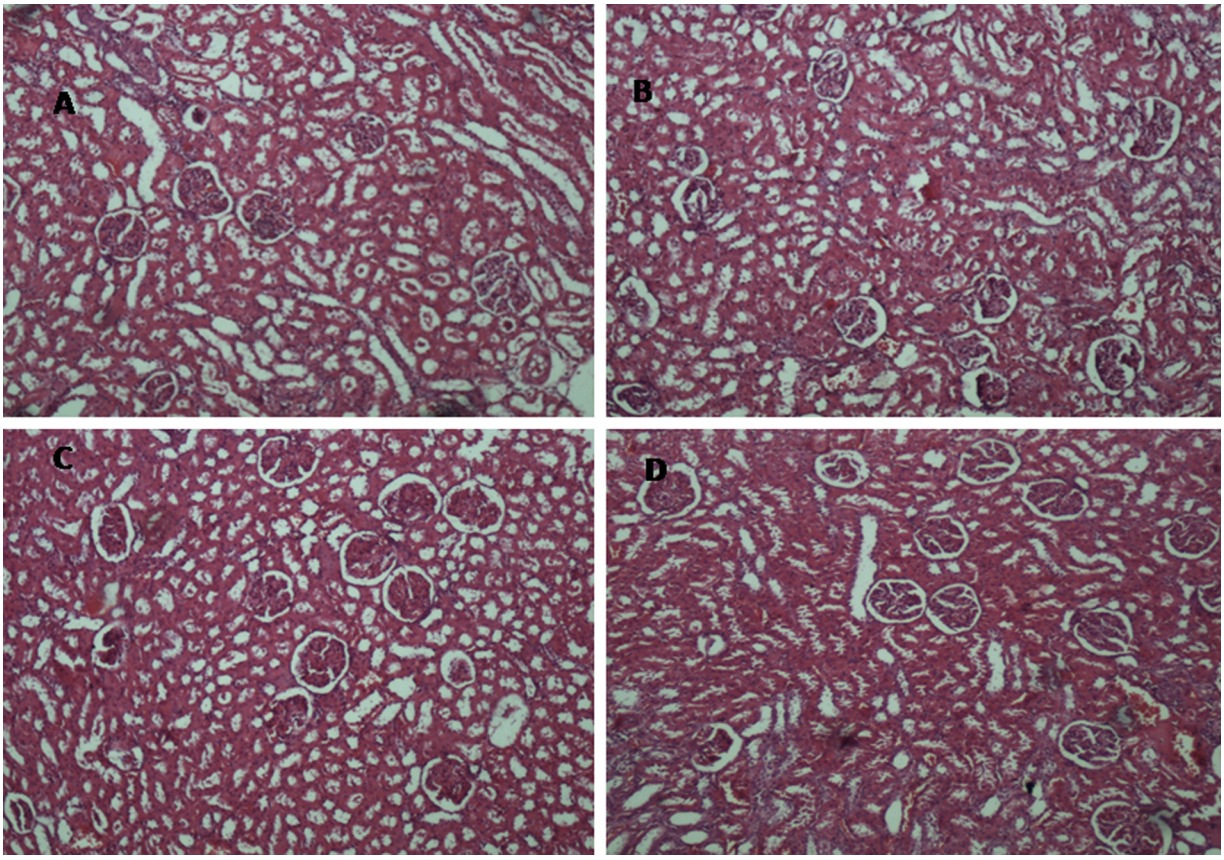

Fig. 3. H&E image of Kidney tissue (10X), A (Normal), B (Honey), C (Ghee), D (Honey + Ghee). This image showed the normal morphology of kidney tissue in all 4 groups (n = 6).

with 25 µl cobalt chloride ($CoCl_2$, 1 mg/ml) in a 96-well plate and incubated at room temperature for 10 minutes. Then 25 µl dithiothreitol (DTT, 1.5 mg/ml) was added, followed by 2-minute incubation to allow the reaction with free cobalt salt. Then, 150 µl saline was added to stop the reaction. Absorbance was taken at 470 nm [37]. % decrease was calculated which indicates the albumin to cobalt binding.

2.4.5.5. Hemoglobin:. Hemoglobin (HB) estimation was done by using Hemocord-D hemoglobin reagent (Coral Clinical System).

2.4.6. Triglycerides, cholesterol level and Lipase activity in plasma

Triglycerides and cholesterol level was estimated by Accurex Biomedical kit. Lipase activity was done according to Winkler and Stuckmann [38]. Briefly, the reaction mixture was prepared by mixing one volume of a solution containing 10 mM of PNPB into isopropanol with 18 parts of the solution of phosphate buffer 100 mM, pH-7, and 7.0% ± 0.5% w/v triton x100. Ten µl of the enzyme was added in appropriate dilution and 190 µl of the reaction mixture was placed in ELISA plate and read absorbance at 415 nm for 10 min and time interval 30 seconds at 30°C.

Enzyme unit (U/ml) = (((ΔAbs/min (sample) - ΔAbs/min (blank))* reaction volume*dilution factor)/molar extinction coefficient * sample volume

2.5. Histological study

2.5.1. Hematoxylin and Eosin staining (H&E)

H&E staining was done in formalin-fixed liver, kidney, pancreas, and intestine (jejunum) tissue followed by rehydration, dehydration, block preparation, slide preparation, staining and mounting described in the early procedure [39]. The image was examined under a microscope (Eclipse 50i; Nikon, Kanagawa, Japan) encumbered with imaging software (NIS Elements Basic Research; Nikon).

2.5.2. Immunohistochemistry (IHC)

Immunohistochemistry was done on the formalin-fixed liver section by using a specific antibody for CD45 (AM111-5 M, BioGenex Fremont, CA 94538, USA) and CD15 (AM302-5 M, BioGenex Fremont, CA 94538, USA) in Department of Pathology, IMS, BHU to detect inflammatory cell infiltration.

2.6. Statistical study

Statistical analysis was carried out using one way ANOVA test followed by Post Hoc analysis with Tukey's test by using SPSS software. All the results were expressed in mean ± SD. The statistically significant difference was considered as p-value less than or equal to 0.05.

3. Results

3.1. Effect on Physical appearances

The weight of normal group rats was 52% increased, in honey group rats weight was 35.2 % increased, in ghee group rats weight was 39.47 % increased on 60^{th} day comparison to 1^{st} day. We found that the weight of the honey + ghee group rats was firstly increased 16.59% on 30^{th} day. After 30 days weight was found to decrease in the honey + ghee group. On 60^{th} day weight was -0.78 % decreased in Honey + Ghee group rats comparison to 1^{st} day (Table 2). The bodyweight of rats taken for this study was ranging between 100-200 g. The bodyweight of each group was varied at the starting point due to the

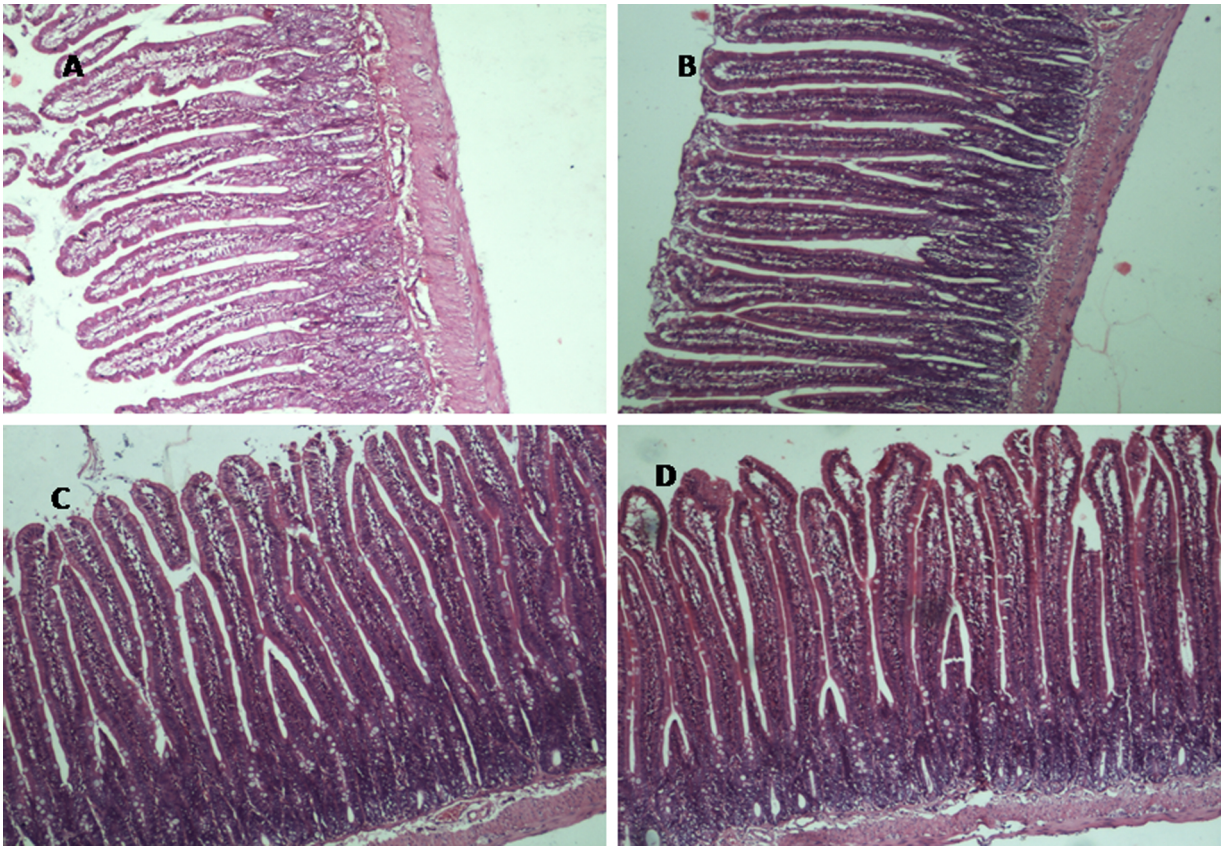

Fig. 4. H&E image of intestine (jejunum) tissue (10X), A (Normal), B (Honey), C (Ghee), D (Honey + Ghee). This image showed the normal morphology with normal villi length in all four groups (n = 6).

random selection of rats for grouping. We found normal skin in all 3 groups till 60 days. But in equal ratio of honey and ghee fed rats (honey + ghee) we found the hair loss on 60th day (Fig. 1). We also found the red patches on the ears of honey + ghee group rats on 60th days but there were no any red patches found on normal, honey and ghee group rats (Fig. 2). The result of physical appearance on different days was shown in Table 3.

3.2. Effect on Biochemical parameter

3.2.1. Liver function test

Plasma SGPT, SGOT, ALP, Protein, albumin, HB level was found similar in honey, ghee and the normal group. SGOT, SGPT, ALP, Protein, and albumin level was found 7%, 24%, 25%, 9.8% and 25% increased respectively in the honey + ghee group in assessment with the normal group (Table 4).

3.2.2. Kidney function test

Plasma urea, Creatinine, and blood urea nitrogen level were found similar in all groups (Table 4).

3.2.3. Oxidative stress parameters

Activity of blood superoxide dismutase and catalase was found 14% and 50% decreased respectively in honey + ghee group comparison to normal group. Level of reduced glutathione (GSH) was found 46% decreased in honey + ghee group. No significant changes were found in honey group in comparison to normal. Level of lipid peroxidation was 40% increased in honey + ghee group with comparison to the normal group. Decreased % inhibition of $ABTS^{\cdot+}$ indicated decreased free radical scavenging activity in the honey + ghee group (Table 5).

3.2.4. Glycemic parameters

Plasma glucose level was found to be 14%, 16% and 34% increased in honey, ghee and honey + ghee group respectively in comparison to the normal group. Plasma DPP-4 level was found to be 29%, 23% and 37% increased in honey, ghee and honey + ghee group respectively in comparison to the normal group. Plasma GLP-1 level was found to be 27%, 24%, and 49% decreased in honey, ghee and honey + ghee group respectively in comparison to the normal group. Plasma GIP level was found to be 31%, 21% and 51% decreased in honey, ghee and honey + ghee groups respectively in comparison to the normal group (Table 6).

3.2.5. Protein modification parameters

Amadori product formation in plasma was found to increase by 18% in honey and 27% in the honey + ghee groups. UV absorption at 280 nm was 33% increased in the honey + ghee group plasma sample. Albumin cobalt binding capacity was found significantly decreased (34%) in honey + ghee group. Increased fluorescence intensity (18%) showed increased advance glycation ends product formation in honey + ghee group. Hemoglobin level was found 27% decreased in the honey + ghee group (Table 6).

3.2.6. Triglycerides, cholesterol and Lipases activity level

Triglycerides level was found increased 19%, 16%, and 20% in honey, ghee and honey + ghee treated groups respectively in comparison to the normal group. Cholesterol level was found to be increased by 14.3% and 21.2% in ghee and honey + ghee group respectively. Lipases level was found increased by 18.5%, 17%, and 20% in honey, ghee and honey + ghee treated groups respectively in comparison to the normal group (Table 6).

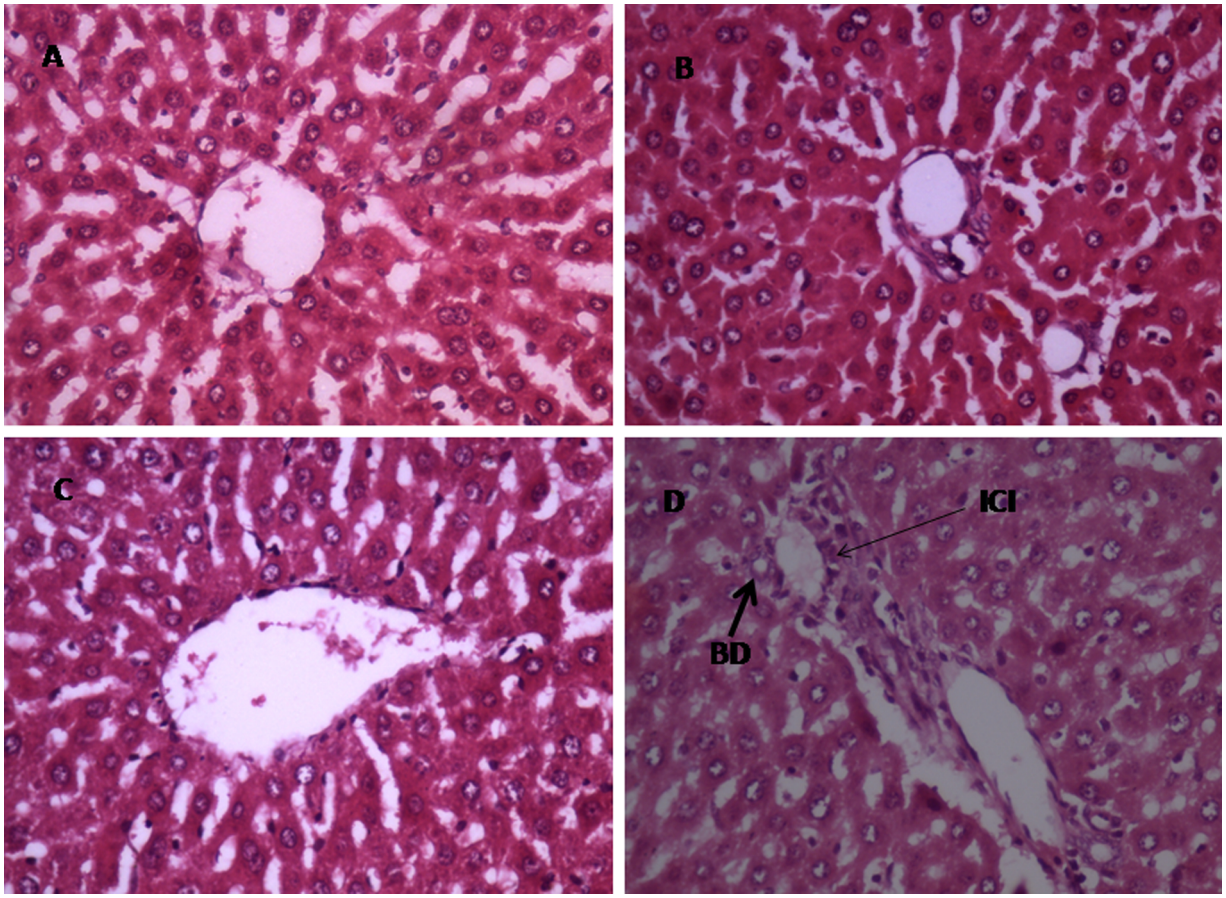

Fig. 5. H&E image of liver tissue (40X). A (Normal), B (Honey), C (Ghee), D (Honey + Ghee). In honey + ghee showed some amount of inflammatory cell infiltrations (ICI), bile duct dilation (BD) comparison than the normal, honey and ghee group(n = 6).

3.3. Histological study

H&E staining of kidney tissues showed normal morphology in all groups (Fig. 3). H&E staining of intestine (jejunum) tissue showed normal morphology with normal villi length in all four groups (Fig. 4). H&E staining of liver tissue of normal, honey and ghee group rat showed normal morphology and no inflammation. Only in honey + ghee treated group liver tissue showed inflammatory cell infiltration, some amount of bile duct dilation, and mild inflammation in the portal tract which indicates initiation of inflammation in liver tissue (Fig. 5). H&E staining of pancreas tissue showed normal morphology with normal Islet of Langerhans in all groups (Fig. 6). Immunohistochemical staining revealed the initiation of CD45- positive cell (Fig. 7) and CD15-positive cells (Fig. 8) accumulation in liver tissue of honey + ghee group, which showed inflammatory cell infiltration (infiltration in leukocyte, lymphocyte and granulocytes).

4. Discussion

As Ayurveda mentions, an equal mixture of honey and ghee become toxic after long term use, so, the present study focused on the scientific validation of this toxicity. The weight of rats selected for this study of ranging between 100-200 g. These rats were randomly selected for the grouping. Due to this random selection, the weight of rats in each group was varied at the starting point. In this study, the weight of rats, treated with an equal mixture of honey and ghee was significantly decreased in comparison to normal, honey and ghee groups. Weight of ghee group was also found to be decreased in comparison to the normal group and the diet intake of ghee group was also found decreased because omega-3 fatty acids present in ghee is responsible for lower huger and weight gain [40–42]. We found less weight gain in honey group in comparison to normal group. Honey is also responsible for the lower weight gain after long term use [43]. These rats also showed hair loss and skin patches. Such effects have been reported in patients going under chemotherapy, immune check point inhibitor, program protein inhibitor (PD1) at the latter stage of treatment even after discontinuing the medicine. In these patients, the symptoms of Alopecia (hair loss), skin patches, dermatitis eczema are observed [44]. The genes like Tnfrs19, Ercc2, Lama5, Ctsl, Per1 are shown to be involved in etiology of skin disorders, these genes regulate various biological process involved in hair follicle development, hair follicle maturation, hair follicle morphogenesis and regulation of hair cycle [45]. Thus our observations showing the toxicity of honey and ghee are supported by other toxins reported here. The biochemical mechanism behind this toxicity could be associated with oxidative stress as it has been reported in the case of hair loss and alopecia [46]. Literature document that the AGEs is responsible for the glycation of many extracellular membrane proteins like collagen, elastin [47]. Then the glycated extracellular protein is no longer responsible for the tightness of the skin, so the skin becomes aged and loses. This looseness of the skin may be responsible for the loss of hair and weight as well. This could be possibly caused behind the hair loss and weight loss in rats of the honey + ghee group in our experiments.

The increased formation of Amadori product and advanced glycation end-product leads to direct inflammation in the liver by various types of signalling pathways [48,49]. The possible cause of the liver inflammation in honey + ghee group rats could be the formation of Amadori product, advanced glycation end-product and oxidative stress. Earlier literature showed that the honey protects liver against toxicity [50]. We found that the liver function test enzymes serum glutamate

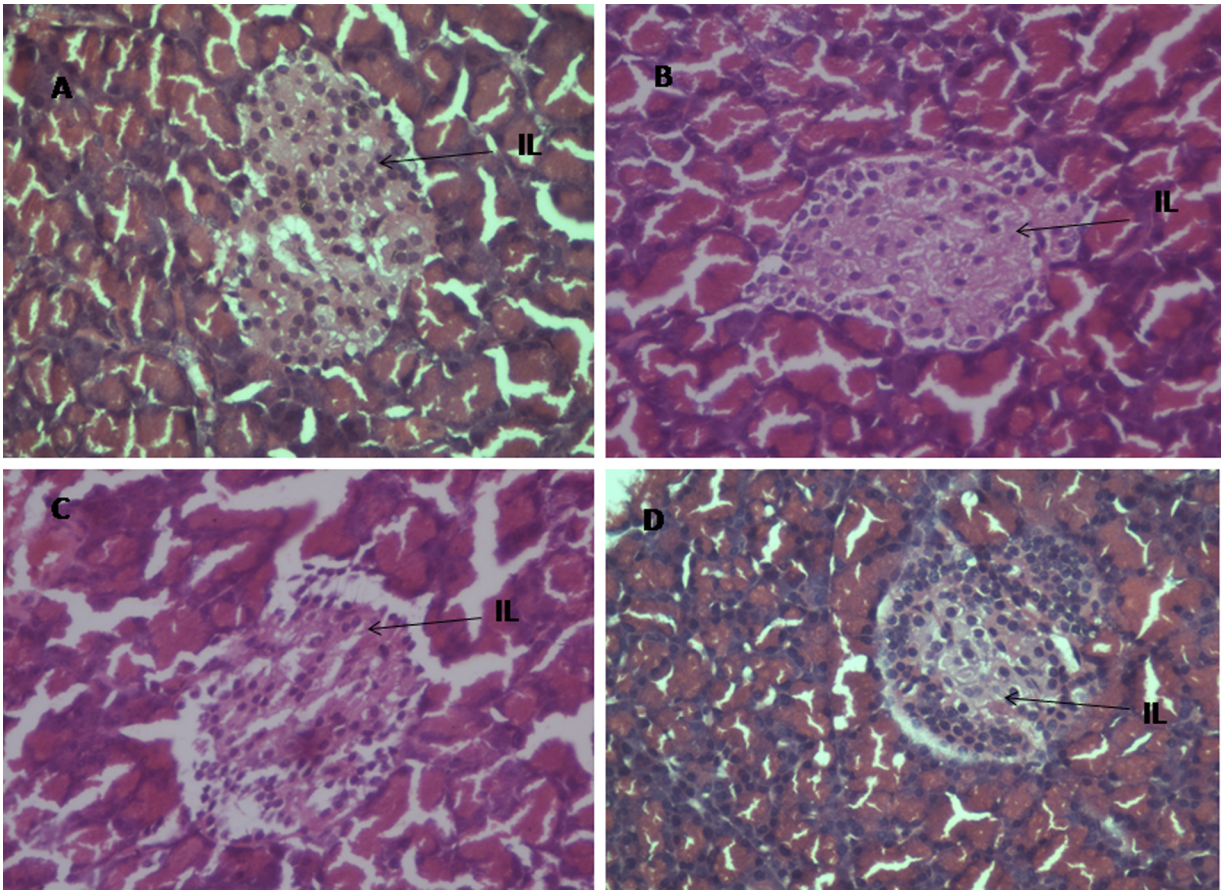

Fig. 6. H&E image of Pancreas tissue (40X), A (Normal), B (Honey), C (Ghee), D (Honey + Ghee) (Arrow shows the normal islet of Langerhans (IL) (n = 6). This image showed the normal architecture of Pancreas tissue.

oxaloacetate transaminases (SGOT), serum glutamate pyruvate transaminases (SGPT), alkaline phosphatases(ALP) activity were significantly augmented in honey + ghee group in comparison to normal, honey and ghee group. These enzymes are the initial marker of liver inflammation. The increased amount of protein, albumin in honey + ghee group in comparison to the other three groups also indicates the early sign of liver inflammation. Protein, albumin and ALP results were also found increased in a similar manner in early reported literature [18] in honey mixed with ghee group comparison to normal group. The given dose of honey and ghee was used in early literature was 102 mg/150 g/day and 64 µl/150 g/day respectively and for 6 weeks.

In this study, we did not find any significant differences in blood urea, Creatinine, blood urea nitrogen and kidney histology between all groups, which indicates that the equal mixture of honey and ghee has no effect on kidney till 60 day.

In our study, we found a decreased level of SOD, catalase, GSH, % inhibition of ABTS and increased level of LPO. These results agree with other earlier investigations [18] where LPO level was high and SOD, catalase and GSH level was low in honey mixed with ghee group in comparison to normal group. It is known that honey is rich in a polyphenolic compound like flavonoids (e.g., quercetin, luteolin, kaempferol, apigenin, chrysin, galangin) and phenolic compound (like caffeic and β-coumaric acids) [51] vitamins and proteins which give honey antioxidant properties [6]. As well as ghee also contains some vitamins which give antioxidative properties to ghee. Superoxide anion is the primary oxygen radical species generated by mitochondria which is converted to hydrogen peroxide (H_2O_2) by spontaneous dismutation or by superoxide dismutase (SOD), present both within the mitochondria and in the cytosol. This hydrogen peroxide, in turn, is transformed into the water by glutathione peroxidase or catalase; otherwise, in the attendance of divalent cations such as iron, H_2O_2 can undergo Fenton's reaction to produce hydroxyl radical ·OH. Due to increased production of ROS, the activity of these enzymes like SOD, catalase are increased in the beginning to overcome the oxidative stress indicating the adaptive step but when ROS productions increases continuously then these antioxidative enzymes are unable to overcome oxidative stress and then level decreases. One of the studies showed that the furfural and 5-hydroxy methyl furfural create oxidative stress by acting thiol-reactive electrophiles [52], which relates to decreases GSH level.

Albumin is the most abundantly present protein in the plasma of a healthy person. It has three structurally similar domains (I, II and, III) for its stability and binding and transport of different molecules, both endogenous and exogenous [53]. It has free cysteine at 34-position, a main molecular site for thiolation, nitrosylation and oxidation and other cysteine molecules are involved in internal disulfide bond formation [54]. Albumin has different sites for the binding of cobalt and other metal ions but due to free radical actions and some modifications on different sites of albumin occur, then the capacity of albumin to cobalt binding is decreased which is the sign of oxidative stress. In addition, an equal ratio of honey and ghee may block free ^{34}SH of albumin and reducing its antioxidant properties. In this study, we found an increased level of albumin but decreased level of albumin cobalt binding which may be due to oxidative stress. The decrease in % inhibition of $ABTS^{\cdot+}$ indicates the decreased free radical scavenging activity and increased lipid peroxidation also indicates the oxidative stress.

Honey is reported as an antidiabetic agent but when we take it in higher doses and for a longer time the fructose and glucose present in it may responsible for the augmentation in glucose level because fructose is responsible for the creation of insulin resistant and obesity

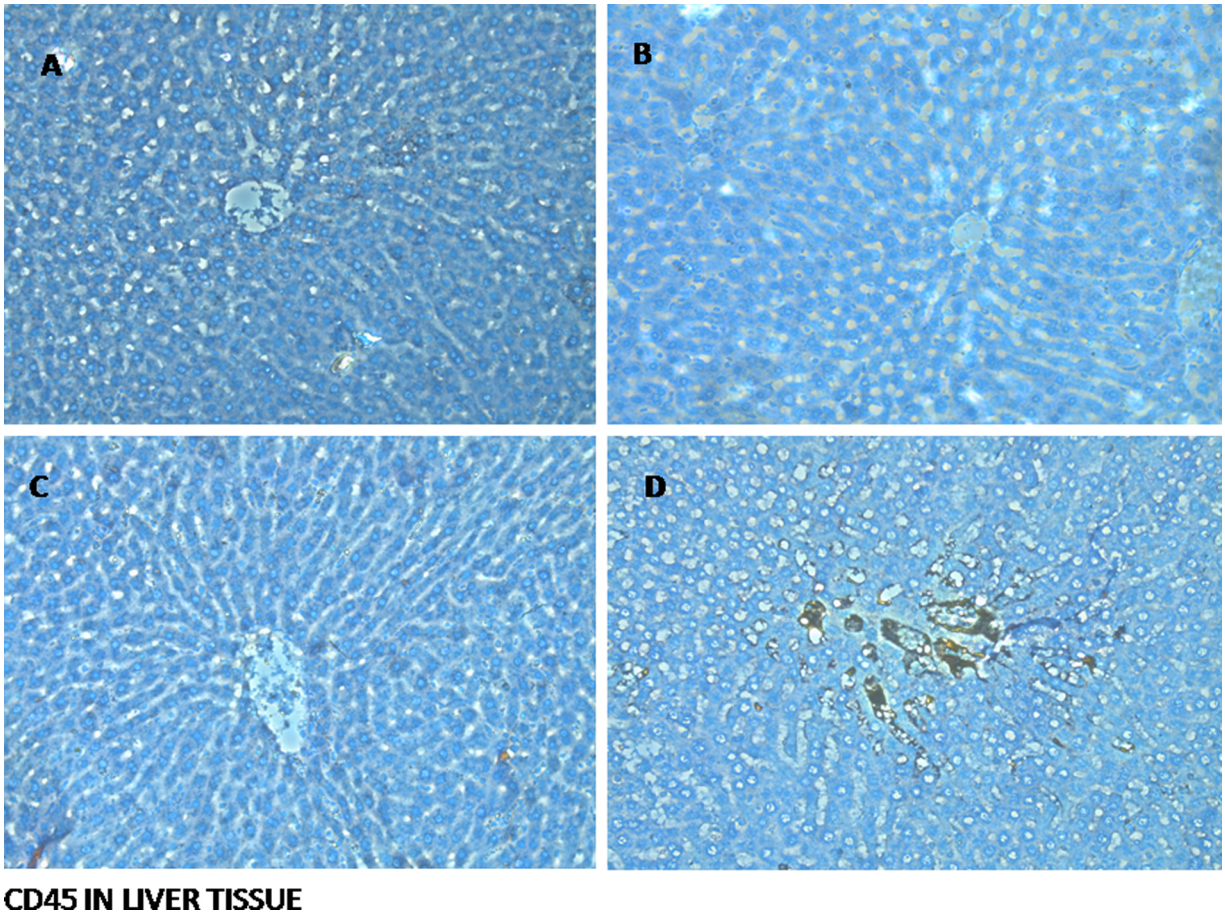

Fig. 7. Immunohistochemical staining image (40X) of liver tissue with CD45 for detection of infiltrations in inflammatory cells (40X). A (Normal), B (Honey), C (Ghee), D (Honey + Ghee). Honey + Ghee group image showed the initiation of CD45 positive cells accumulation in liver tissue.

development [55]. Long term carbohydrate and fat-rich diet feeding are linked to the hyperglycemia condition. Increased glucose level is responsible for the activation of DPP-4 enzyme. DPP-4 is an intestinal enzyme responsible for the inactivation of GLP-1 because the sustained activity of GLP-1 is not recommended for normal physiology. The normal life for GLP-1 is 3-4 minute thus the optimum activity of DPP-4 is desired for a healthy person but increases in DPP-4 is injurious to health because it damages the normal level of GLP-1 and GIP also [56]. Glucagon-like peptide-1 (GLP-1) is a remarkable antidiabetic gut hormone with combinatorial actions of stimulating insulin secretion, inhibiting glucagon secretion, increasing beta-cell mass, reducing the rate of gastric emptying and inducing satiety. GLP-1 also plays anti-inflammatory roles beyond glucose control [57–59]. Gastric inhibitory polypeptide (GIP) inhibits gastric acid secretion and stimulates insulin secretion in healthy volunteers. 3-deoxyglucosone is an Amadori product. Accumulation of 3-deoxyglucosone in intestinal tissue is responsible for the attenuation of GLP-1 secretion which causes the insulinotropic effect in rats [60]. For the first time, we are reporting the changes in incretins hormones by using an equal amount of honey and ghee which are not reported earlier. Further study is required to linked this to toxicity.

The Amadori product formation occurs when the protein and glucose level is increase in the blood which is the basis of the formation of advanced glycation end-products (AGEs). Here in the present study, we found that the significant increase in Amadori product formation in honey and honey + ghee group and advanced glycation end product formation in honey + ghee group. The formation of Amadori product (glycation and oxidation of sugar and protein) leads to the formation as well as accumulation of reactive carbonyl species through dehydration and rearrangement of Amadori product [61]. Glyoxal, methylglyoxal and 3-deoxyglucosaone are the major carbonyl intermediates [62] which are responsible for the intracellular oxidative stress and then cellular apoptosis. AGEs have long been measured as poisonous that promote cell death and cause organ damage in man. AGEs is not responsible only for diabetic complication as well as it contributes for the development of many others metabolic diseases such as cardiovascular diseases (CVDs) and neurodegenerative diseases and ageing [63]. We found in our study that the absorption of serum protein was notably increased in the group of an equal mixture of honey and ghee fed rats than the other groups. These changes in absorption could be due to the formation of AGEs and the unfolding /fragmentation of protein [35]. Here we found a decreased level of hemoglobin; this may be due to the glycation of hemoglobin. Increased Amadori product formation may be due to the glycation of hemoglobin. Glycation of protein may lead to protein unfolding and fragmentation which increases the hyperchromic shift at 280 nm [35,36].

When we take the carbohydrate-rich diet it is directly related to fatty acid synthesis and further triglycerides. A fat-rich diet contains many fats, triglycerides and cholesterol which are directly linked to the TG and cholesterol accumulation in liver and blood also. Enzyme lipase is responsible for the degradation of these triglycerides into fatty acids. The data obtained from the present study showed the similar results. One of the previous publication also showed the results in similar manner where the level of TG and cholesterol is high in honey mixed with ghee group in comparison to normal group [18].

H&E staining of any tissue is used for looking the normal architecture of the tissue. In the present study, we found the normal architecture of kidney, pancreas, and intestine but we found some amount of

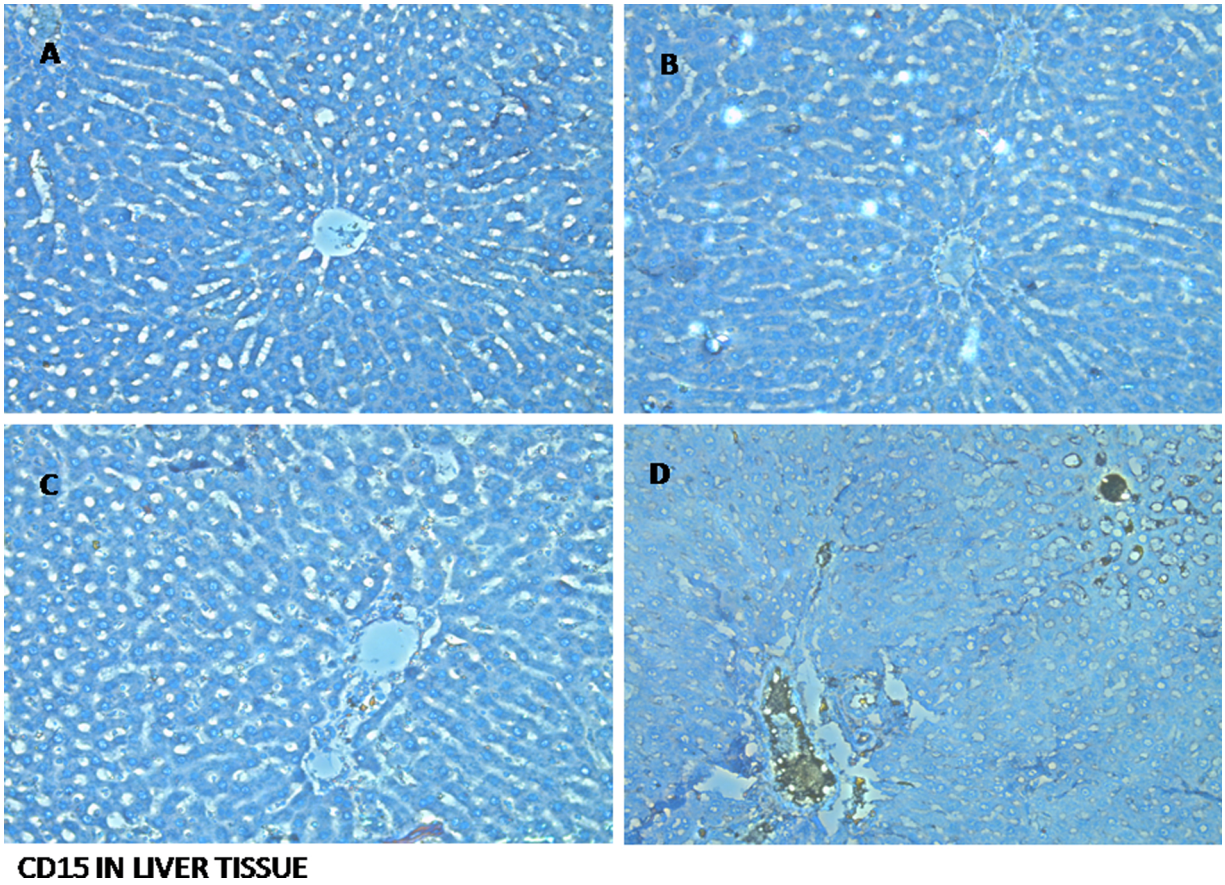

Fig. 8. Immunohistochemical staining image (40X) of liver tissue with CD15 for detection of infiltrations in inflammatory cells (40X). A (Normal), B (Honey), C (Ghee), D (Honey + Ghee). Honey + Ghee group image showed initiation of CD15 positive cells accumulation in liver tissue.
All the experiment was repeated thrice.

inflammatory cell infiltration in liver tissue. For the demonstration of this infiltration, we did the immunohistochemistry of CD45 and CD15 in liver tissue. CD45 is commonly known as leucocyte-common antigen, is a family of high molecular weight transmembrane protein tyrosine phosphatase (PTPase) which is expressed on all nucleated hematopoietic cells [64,65]. CD15 antigen is expressed on mature neutrophils and myeloid cells [65–67]. We found positive expressions of CD45 and CD15 in honey + ghee treated groups and negative expression in normal, honey and ghee treated groups, which indicates the initiation of inflammatory cell infiltration (leucocytes, lymphocytes and granulocytes) in honey + ghee group on 60th day.

5. Conclusion

As discussed above, increased oxidative stress generation, decreased albumin cobalt binding, increased Amadori product formation, advance glycation end-product formation, glucose and DPP-4 augmentations which relate to GLP-1 and GIP attenuation, liver function test enzyme elevation, liver tissue inflammation, inflammatory cell infiltration, bile duct dilation, rise in TG, cholesterol and lipases level are the evidence which can say that the formation of Amadori product, decreased antioxidant enzymes and, increased advanced glycation end product could be the possible cause of toxicity of equal ratio of honey and ghee. These may be responsible for weight and hair loss as well as the appearance of red patches on ears.

Author's contributions

Prof. Yamini Bhusan Tripathi and Prerana Aditi planned the experiments on incompatible diet and selected equal mixture of honey and ghee. Prerana Aditi carried out the *in vivo* experiments and, was involved in data interpretation and preparation of manuscript. Shivani Srivastava had done the experiment on incretins hormones (GLP-1 and GIP). Harsh Pandey helped in carried out the histology experiments and taking images of all slides. All authors read and approved the manuscripts.

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Declaration of Competing Interest

The authors declare that they have no competing interests.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.toxrep.2020.04.002.

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