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Thermally Oxidized Coconut Oil as Fat Source in High-Fat Diet Induces Hepatic Fibrosis in Diabetic Rat Model

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ORIGINAL PAPER

Thermally Oxidized Coconut Oil as Fat Source in High-Fat Diet Induces Hepatic Fibrosis in Diabetic Rat Model

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Abstract

In the present study, HFD/STZ-mediated type 2 diabetic rodent model was used to comparatively evaluate coconut oil (CO) and thermally oxidized CO (TCO) as fat sources for the development of NAFLD. Female Wistar rats (six in each group; average bwt 200 g) fed HFD containing either CO or TCO for 2 months along with an intraperitoneal injection of streptozotocin (30 mg/kg bwt) at the end of 1-month feeding were found to develop fatty liver and subsequent inflammatory changes when compared to the normal laboratory diet-fed animals over 2-month period. Dyslipidemia as well as enhanced activities of serum hepatic marker enzymes (e.g., AST, ALT, and ALP) were prominent in TCO-fed animals. Further, HFD-fed animals showed alterations in their hepatic redox equilibrium. Hepatic GSH and antioxidant enzyme activities that form the part of a protective mechanism against oxidative/carbonyl stress were found to be increased in HFD-fed rats. Supporting this, CO- and TCO-containing-HFD-fed animals had enhanced lipid peroxidation (increased TBARs). Thus, fatty liver with heightened antioxidant defense, lipid peroxidation, and inflammation indicate hepatosteatosis. Histological details of the hepatic tissues corroborated sufficiently with these observations and showed an increased incidence of macrovesicles, inflammation, and hepatocyte ballooning in the TCO-fed rats than in CO-fed animals. Further, in support of this proposition, hydroxyproline, an index of collagen formation, was found to be significantly increased in TCO-fed rats than in the CO-fed group. Overall, the study shows that the formulation of HFD incorporated with TCO as a fat source, combined with STZ injection, is an efficient dietary model for developing hepatosteatosis with fibrotic stage in rats within 2 months. Administration of this modified diet for a more extended period may be a good model for cirrhotic and hepatocellular carcinoma studies, which need to be further assessed.

Keywords High-fat diet · Type 2 diabetes · Oxidative stress · Thermally treated fats · Fatty liver disease · Hepatocellular carcinoma

Abbreviations

ALP	alkaline phosphatase
ALT	alanine aminotransferase (serum glutamic pyruvic transaminase)
AST	aspartate aminotransferase (serum glutamic pyruvic transaminase)
bwt	bodyweight
CO	coconut oil
CPCSEA	Committee for the Purpose of Control And Supervision of Experiments on Animals
HCC	hepatocellular carcinoma
HFA	high fat area
GSH	reduced glutathione
GR	glutathione reductase
GPx	glutathione peroxidase
GST	glutathione S-transferase
GT	glucose tolerance
HFD	high-fat diet
HP	hydroxyproline
IAEC	Institutional Animal Ethical Committee

Supplementary information The online version contains supplementary material available at https://doi.org/10.1007/s12013-021-01009-5.

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LDL	low-density lipoprotein
LFA	low-fat area
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
PL	phospholipid
SOD	superoxide dismutase
STZ	streptozotocin
TBARs	thiobarbituric acid-reactive substances
TCO	thermally oxidized CO
TC	total cholesterol
TG	triglyceride
Tris	tris(hydroxymethyl)amine
VLDL	very-low-density lipoprotein

Introduction

Nonalcoholic fatty liver diseases (NAFLD) are a group of diseases characterized by excessive accumulation of triglycerides (TGs) and progressive inflammatory changes in the hepatic tissues that are associated with obesity, insulin resistance (diabetes mellitus, type 2), and hyperlipidemia. The condition is manifested by elevated liver enzyme activities in the blood, altered liver architecture, disruption of metabolism, secretion, and excretion of lipids. Hepatic steatosis (accumulation of more than 5.5% TGs in the hepatic tissue) is the first stage of NAFLD, which in turn causes liver injury and inflammation, called nonalcoholic steatohepatitis (NASH). Cytokine production is altered in the NASH stage, which is further manifested by lobular inflammation, hepatocyte ballooning, and fibrosis. Factors, such as oxidative injury and oxidative stress, are required for these progressive changes to liver cirrhosis before finally leading to hepatocellular carcinoma (HCC). Moreover, with the increasing incidence and prevalence of 9–40% in Asian countries and 9–32% in India [1, 2], the perception of NAFLD being a benign condition of little clinical significance is rapidly changing.

Lifestyle changes are involved in the development of hepatosteatosis wherein dietary habits, sedentary lifestyle, certain drugs, and alcoholism are predominant. Obesity, diabetes, insulin resistance, and dyslipidemia make the conditions even worse [3]. Excess caloric and fat intake (consumption of junk foods) along with sedentary lifestyle are the primary cause of the increasing incidence of obesity, metabolic syndrome, and associated NAFLD. High-calorie and fat consumption, which is nonnutritious, have been implicated in NAFLD's progression and severity by promoting de novo lipogenesis and increasing insulin resistance, oxidative stress, inflammation, fibrosis, and finally liver failure. The types and amount of dietary fat intake determine the development of hepatic steatosis. Consumption of saturated fats, especially long-chain fatty acids, is reported to be responsible for developing hepatic steatosis, while medium-chain saturated fats are considered beneficial [4]. Coconut oil (CO), a source of dietary medium-chain fats, extracted from mature coconut (*Cocos nucifera*) kernel, is being used in many Asian countries and south India.

However, the repeated heating of oils is known to generate a wide array of mutagenic, genotoxic, and carcinogenic compounds. Several studies have shown the influence of dietary components on the development of certain degenerative diseases, among which the role of fried foods, especially thermally oxidized oils, is being highlighted [5]. Narayanankutty et al. [6] have recently shown that thermally oxidized CO (TCO) exacerbates high-fructose-induced fatty liver disease over a 6-month feeding period.

So far, there are very few preclinical models available to study NAFLD. A high-fat diet (HFD)/streptozotocin (STZ) diabetic model in rodents and high-fructose-diet-induced diabetic rats can promote early fatty liver changes in animals within 2 months. However, no experimental models are available to induce progressive changes of fatty liver to cirrhosis and HCC. HFD/STZ model is more close to type 2 diabetes and has the potential to develop more advanced fatty liver conditions that may provide more insight into the link between chronic diabetes and the development of liver cirrhosis and HCC. In the present study, a cost-effective diabetic model with a high-calorie diet and HFD has been used to induce experimental NAFLD. Here, we use normal CO and TCO as fat sources for a comparative evaluation of its potential to develop NAFLD.

Materials and Methods

Animals and Experimental Design

Female Wistar rats weighing 180–220 g were purchased from the Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala. The animals were quarantined for 2 weeks at the animal house facility of Amala Cancer Research Centre, Thrissur, in polypropylene cages with proper bedding under standardized environmental conditions (22–28 °C, 60–70% relative humidity, 12 h light/dark cycle). They were fed on a nonpurified rat chow from Sai Durga Feeds (Bangalore, India) and filtered water during acclimatization. All the experimental procedures had prior permission from the Institutional Animal Ethical Committee (approval no: ACRC/IEAC/19(1) P-1) and strictly followed the guidelines of the CPCSEA Government of India.

The animals were divided into three groups (six each). Group 1 was kept as control and fed normal rat chow (Sai Durga feeds, Bangalore; Supplementary Information, Table S1). The other two groups (Groups 2 and 3) were fed on a HFD of 18% protein, 41% carbohydrate, and 40% fat (Supplementary Information, Table S2), in which CO or TCO as a fat source was incorporated. All animals were fed on their respective diets for 2 months. STZ (30 mg/kg/bwt) was injected intraperitoneally to induce diabetes in Groups 2 and 3 animals after 1 month of dietary regimen. At the start and end of the experimental period, glucose tolerance (GT) was determined following overnight fasting. Glucose levels of all the animals were monitored at weekly intervals till the end of the experimental period. The animals were then sacrificed following overnight fasting, and blood was collected by heart puncture. The serum was separated by centrifugation at 2000 rpm for 10 min. Liver, pancreas, and adipose tissues were collected and kept at −20 °C till assayed. A portion of liver tissue was fixed in 10% formalin for histological analysis.

Biochemical Assays

Activities of various serum parameters such as lipid profiling, liver marker enzymes, and hepatic tissue lipid profiling were determined using commercially available kits (Euro Diagnostic Systems Pvt. Ltd., Chennai, India), according to the manufacturer's instructions.

Estimation of Phospholipid (PL) and Total Cholesterol (TC) in Liver Tissue

Liver lipids were extracted following Folch's method [7]. These lipid extracts were used to estimate PL and cholesterol content present in the liver. TC was estimated as per Zak's method [8] and the PL content was determined [9].

Analysis of Oxidative Stress and Antioxidant Activity

Tissue homogenate (10%, w/v) was prepared in 0.1 M Tris HCl buffer, pH 7.0. The homogenate was centrifuged at 12,000 rpm for 30 min in a cold centrifuge. The clarified supernatant was collected and stored under −20 °C for biochemical analysis.

The extent of lipid peroxidation was assessed using the thiobarbituric acid-reactive substances (TBARs) method [10] and fibrosis by hydroxyproline (HP) assay [11]. Assays for reduced glutathione [12], activities of glutathione peroxidase (GPx) [13], glutathione reductase (GR) [14], superoxide dismutase (SOD) [15], catalase [16], and glutathione S-transferase (GST) [17] were carried out using hepatic tissue. Total protein in serum, liver, pancreas, and adipose tissues was estimated using the Lowry method [18].

Estimation of Tissue Lipase in the Liver, Pancreas, and Adipose Tissue

Approximately 100 µL of tissue homogenate (10% in PBS, pH 7.0) was taken in test tubes. To this, 100 µL of 200 mg/mL TG solution was added. The reaction mixture was made up to 1 mL with phosphate buffer, pH 7.4, and incubated at 37 °C for 1 h. About 50 µL of the reaction mixture was withdrawn at 0 and 1 h intervals into test tubes containing 100 µL of 1 N H_2SO_4 and 100 µL of 0.05 M sodium periodate. The test tubes were incubated at room temperature for 10 min. Following this, 100 µL of 0.5 M sodium arsenate was added. Further incubation was then carried out at room temperature for 5 min. Added 5 mL of chronotropic acid reagent and then placed in a boiling water bath (*ca.* 100 °C) for 30 min. The tubes were then taken out, cooled to room temperature, and measured absorbance at 570 nm. One unit of lipoprotein lipase activity was expressed as 1 mole of glycerol released per hour per mg of protein [19].

Histopathological Analysis

A portion of tissue was formalin (10 %) fixed and embedded in wax. Serial sections were made using an automatic microtome set at a thickness of 6 µm. H&E staining was performed on the tissue sections to evaluate structural integrity and picrosirius red staining to know the extent of fibrosis. The stained sections were observed under a Magnus INVI microscope (New Delhi, India), and images were taken at ×200 magnification. The histopathological examination was carried out by an experienced pathologist, who was unknown to the investigators of this study.

Statistical Analysis

Animal's experimentation was done only once. Individual biochemical assays using individual animal's serum/tissue were done in duplicate. The values are expressed as mean ± standard deviation (SD) of six animals per group. Statistical evaluation of the data was done by one-way ANOVA followed by Dunnett post hoc test using Graph Pad Instat 3 software. Results were considered statistically significant when the p value was <0.01.

Results

Marginal Weight Loss Observed in CO and TCO Incorporated HFD-fed Rats Following STZ Injection

There were only minimal variations observed in the body-weight of normal animals fed with normal chow. However, a marginal decline in weight was observed in animals fed

Table 1 Bodyweight of the experimental animals (g)

Duration of feeding	Bodyweight (g)		
	Normal	CO	TCO
Week 0	207.5 ± 1.2	211.2 ± 0.7	212.5 ± 1.1
Week 1	207.7 ± 1.2	211.2 ± 0.7	212.5 ± 1.1
Week 2	216.5 ± 1.8	198.8 ± 1.5	209.7 ± 0.7
Week 3	211.7 ± 1.1	196.8 ± 1.4	208.7 ± 1.0
Week 4	210.5 ± 1.3	191.5 ± 1.8	201.6 ± 1.3
Week 5	205 ± 1.5	174 ± 0.9	186 ± 0.9
Week 6	205 ± 1.2	173.5 ± 1.0	192 ± 1.5
Week 7	207.3 ± 1.0	177.7 ± 1.0	193 ± 1.3

Bodyweight of TCO- and CO-containing-diet-fed group animals did not vary but started to decline following streptozotocin injection on the 4th week period. Values are given as mean ± SD of six animals

CO coconut oil, *TCO* thermally oxidized coconut oil, *SD* standard deviation

Table 2 Blood sugar level of experimental animals

Duration of feeding	Blood glucose (mg/dL)		
	Normal	CO	TCO
Week 0	87.0 ± 2.8	109 ± 3.2	93.5 ± 2.1
Week 1	87 .0 ± 2.8	105 ± 2.2	94.3 ± 2.0
Week 2	89.3 ± 1.9	102.3 ± 3.5	117 ± 0.7
Week 3	89.5 ± 2.8	105.2 ± 1.5	110 ± 1.4
Week 4	93 ± 0.7	103.1 ± 1.4	98.7 ± 1.4
Week 5	91.7 ± 0.6	226 ± 1.2	286 ± 1.0
Week 6	90 ± 1.4	287.7 ± 1.8	407 ± 1.4
Week 7	94 ± 1.3	290 ± 1.4	361 ± 1.2

For the initial 4-week period, the glucose level gradually and moderately increased in CO- and TCO-containing-HFD-fed animals and following streptozotocin injection a drastic increment documented. Values are given as mean ± SD of six animals

CO coconut oil, *TCO* thermally oxidized coconut oil, *SD* standard deviation

with CO- and TCO-containing diet following STZ injection. At the end of the experiment, the weight loss documented in Groups 2 and 3 was approximately 34 and 19 g (Table 1).

CO and TCO Incorporated HFD-fed Rats Experienced Higher Fasting Blood Glucose and Loss in GT

An increase of 7 mg/dL blood glucose was noticed in the normal group animals throughout the experimental period. While in CO- and TCO-containing-HFD-fed animals, blood glucose level gradually and moderately increased for the initial 4-week period, and thereafter following STZ injection, a drastic increment was documented with 181 and 268 mg/dL at the end of the experimental period with respect to the starting glucose level (Table 2).

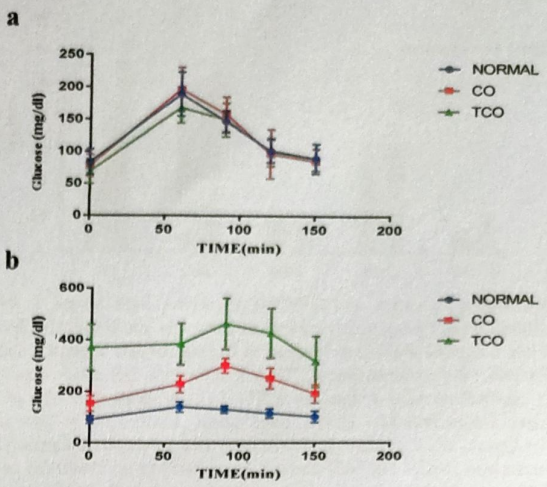

Fig. 1 Changes in glucose tolerance capacity of experimental animals. Six Wistar rats in average were grouped into three groups, Group 1 was fed with normal rat chow and Groups 2 and 3 were fed with CO and TCO for 2 months. Initial and final glucose values from 30 to 150 min have been depicted in (**a**) and (**b**), respectively

Table 3 Glucose tolerance expressed as area under curve (AUC)

	Normal	CO	TCO
Initial glucose tolerance			
AUC	17463.9 ± 550.3	16376.6 ± 1454.4	17053.8 ± 2078.8
Final glucose tolerance			
AUC	17463.9 ± 673.9	24577.5 ± 1399.5*	51199.54 ± 2544.9*

Statistical evaluation of the data was done by one-way ANOVA followed by Dunnett post hoc test using Graph Pad Instat 3 software. Results were considered statistically significant when the p value was <0.01. Values are given as mean ± SD of data done in duplicates of six animals

CO coconut oil, *TCO* thermally oxidized coconut oil, *SD* standard deviation

*$p < 0.01$ compared with Group 1 rats fed with normal rat chow

Before the start of the experiment, the glucose clearance capability of all the animals was similar in all the groups (Fig. 1a). On the other hand, at the end of the experiment, when the GT level was monitored again, the GT level in the normal group remained unaffected. However, in CO- and TCO-containing-HFD-fed animals, the GT levels were found to be reduced. In CO and TCO group animals, elevated glucose levels following 30 min of glucose administration remained the same till the end of 150 min (Fig. 1b). GT test expressed as area under curve has been depicted in Table 3.

Elevated Liver Marker Enzymes in the Blood were Documented in HFD-fed Rats

Compared to the normal group animals, a significant increase in liver marker enzyme activities was observed in

Table 4 Serum liver marker enzymes of experimental animals

Group	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	Bilirubin (mg/dL)
Normal	66.7 ± 1.5	63.7 ± 3.5	105.3 ± 3.5	0.3 ± 0.00
CO	185 ± 4.0*	84.3 ± 4.0*	208.3 ± 6.7*	0.33 ± 0.06
TCO	260 ± 5.0*	180.0 ± 4.6*	463.3 ± 15.3*	0.37 ± 0.06

Liver marker enzymes SGOT, SGPT, and ALP levels have been found to be increased in CO- and TCO-fed rats. Statistical evaluation of the data was done by one-way ANOVA followed by Dunnett post hoc test using Graph Pad Instat 3 software. Results were considered statistically significant when the p value was <0.01. Values are given as mean ± SD of data done in duplicates of six animals

CO coconut oil, *TCO* thermally oxidized coconut oil, *SD* standard deviation

*$p < 0.01$ compared with Group 1 rats fed with normal rat chow

the CO- and TCO-containing-HFD-fed animals. The value of SGOT was 66.7 ± 1.5 IU/L in normal rats, which was found significantly increased in CO (185 ± 4.0 IU/L) and TCO (260 ± 5.0 IU/L) containing HFD-fed animals ($p < 0.01$). Similarly, in normal groups of rats, the SGPT activity was 63.7 ± 3.5 IU/L, and the other two groups (CO and TCO) exhibited enhanced SGPT activity ($p < 0.01$) than normal group animals. Herein, CO-fed animals documented 84.3 ± 4.0 IU/L and TCO-fed animals had 180.0 ± 4.6 IU/L. Higher ALP activities ($p < 0.01$) were also documented in the HFD-fed animals compared to the normal rats (105.3 ± 3.5 IU/L). CO-containing-diet-fed animals had 208.3 ± 6.7 IU/L and TCO-fed animals had 463.3 ± 15.3 IU/L. On the other hand, the bilirubin level did not change much in different experimental groups, and the values documented were ~0.3 mg/dL in all the groups (Table 4).

Blood and Hepatic Lipid Profile of HFD-fed Rats Indicated Pronounced Dyslipidemia

TC level was significantly ($p < 0.01$) higher in CO- and TCO-containing HFD given animals (103.99 ± 2.04 and 107.77 ± 4.67 mg/dL respectively) when compared to the level in the normal diet-fed animals (96.07 ± 3.12 mg/dL). Similarly, an increased level of TGs ($p < 0.01$) was found in TCO (114.59 ± 5.00 mg/dL) and CO (79.60 ± 3.92 mg/dL) containing HFD-fed animals when compared to the normal diet-fed ones (51.72 ± 4.86 mg/dL) animals. In the case of HDL cholesterol, animals fed with TCO-containing HFD documented lower levels (19.35 ± 1.00 mg/dL) than normal diet-fed animals (20.31 ± 2.50 mg/dL). At the same time, a slightly increased level was seen in animals fed with HFD-containing CO (22.70 ± 3.21 mg/dL). However, calculated values for LDL remained more or less the same in all the experimental groups. The normal diet-fed animals had a mean LDL value of 65.42 ± 2.91 mg/dL, whereas CO and TCO incorporated HFD-fed animals documented LDL

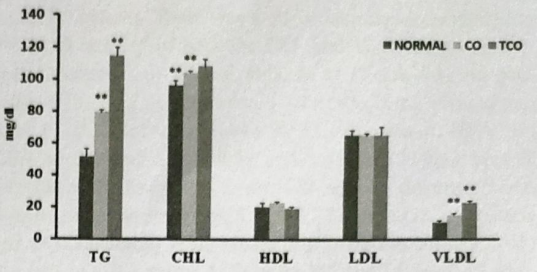

Fig. 2 Serum lipid profile of HFD-fed rats indicated pronounced dyslipidemia. Wistar rats were divided into three groups of six animals each. Group 1 was fed normal laboratory diet, while Groups 2 and 3 animals were fed with CO- and TCO-containing high-fat diet for 2 months with single dose of STZ at the end of 1-month feeding. At the end of experiment, serum lipid profile was determined using commercially available kits (EUORO Diagnostics). LDL cholesterol was calculated using Friedewald formula [23] and VLDL was calculated as TG/5. Statistical evaluation of the data was done by one-way ANOVA followed by Dunnett post hoc test using Graph Pad Instat 3 software. Results were considered statistically significant when the p value was <0.01. Values are given as mean ± SD of data done in duplicates of six animals. **$p < 0.01$ compared with Group 1 rats fed with normal rat chow

levels of 65.38 ± 3.01 and 65.50 ± 5.18 mg/dL. Compared to VLDL level calculated for normal groups (10.77 ± 1.14 mg/dL), increased level ($p < 0.01$) was found in animals fed with TCO (22.97 ± 1.00 mg/dl) and CO (15.33 ± 0.78 mg/dL) (Fig. 2).

At least 50% of the hepatic tissue was found to be infiltrated with fats within this 2-month experimental period and a clear demarcation of high-fat area (HFA) and low-fat area (LFA) were possible. Therefore, the lipid profile, antioxidant status, and histological details of these two different areas were separately analyzed.

Compared to the hepatic TG level documented in normal group animals (3.92 ± 0.15 mg/g tissue), HFA of animals fed with CO and TCO showed a significant increase in TG (6.02 ± 1.40 and 7.03 ± 0.22 mg/g tissue) ($p < 0.01$), respectively. However, an increase in the TCO-fed group was marginal compared to CO-fed animals. There was a significant ($p < 0.01$) increase in hepatic HDL level in CO and TCO incorporated HFD-fed animals were observed when compared to normal diet-fed groups (0.47 ± 0.09 mg/g tissue). The HDL level of CO from HFA and LFA were 1.64 ± 0.14 and 2.12 ± 0.14 mg/g tissue and HFA and LFA of TCO were 1.78 ± 0.50 and 1.78 ± 0.16 mg/g tissue. The PL level observed in the hepatic tissue of normal animals was 3.92 ± 1.28 mg/g tissue. In HFA and LFA of CO incorporated HFD-fed animals were documented with 5.40 ± 3.40 and 5.12 ± 2.68 mg/g tissue, respectively. While in TCO incorporated HFD-fed groups had PL level of 6.99 ± 2.97 and 5.38 ± 0.82 mg/g tissue in the HFA and LFA (Fig. 3a).

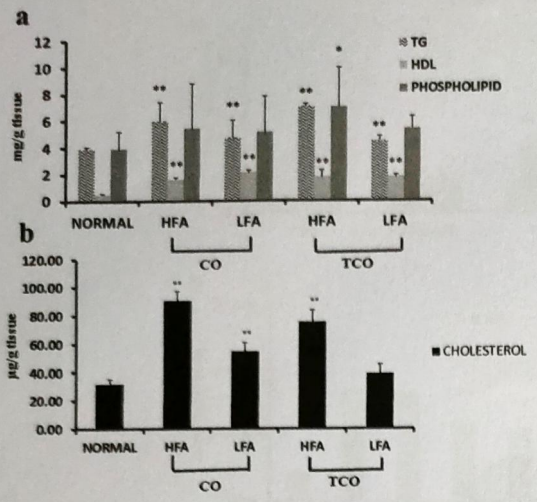

Fig. 3 Hepatic lipid profile of HFD-fed rats indicated pronounced dyslipidemia. Wistar rats were divided into three groups of six animals each. Animals in Group 1 (normal group) were fed normal laboratory diet, while those in Groups 2 and 3 were fed (respectively) with CO- and TCO-containing high-fat diets for 2 months with single dose of STZ at the end of 1-month feeding. At the end of experiment, hepatic tissue was excised extracted with chloroform/methanol and the lipid profile was determined as described in the kit from EUORO Diagnostic, which is depicted in (**a**) and that of cholesterol in (**b**). Statistical evaluation of the data was done by one-way ANOVA followed by Dunnett post hoc test using Graph Pad Instat 3 software. Results were considered statistically significant when the p value was <0.01. Values are given as mean ± SD of data done in duplicates of six animals. *$p <$ 0.05, **$p < 0.01$ compared with Group 1 rats fed with normal rat chow

Similarly, a high TC ($p < 0.01$) value was found in animals fed with HFD-containing CO (90.62 ± 6.98 µg/g tissue) and TCO (75.31 ± 8.30 µg/g tissue) when compared to that of the normal group (31.88 ± 3.57 µg/g tissue). In the case of CO-fed animals, a marginally increased TC level was observed compared to the TCO-fed animal group (Fig. 3b).

Heightened Antioxidant Enzyme Activities do not Prevent a Hike in TBARs in the HF Area of HFD-fed Rats

The animals fed CO and TCO included in HFD had elevated activity of antioxidant enzymes in hepatic tissue compared to the normal rats. In the case of catalase activity, the normal diet-fed animals had 1.82 ± 0.46 U/mg proteins and the activity was elevated in CO incorporated HFD-fed rats; however, no significant change was observed between the HFA and LFA of CO-containing-HFD-fed animals (2.72 ± 0.71 and 2.33 ± 0.51 U/mg protein). Similarly, compared to normal animals, the. Catalase enzyme activity had a marginal increase and HFA and LFA of TCO-containing-HFD-fed animals were found to have 2.38 ± 0.51 and 2.44 ± 0.12 U/mg protein activities. SOD activity was also followed a similar pattern. There was a slight increase documented in the SOD activity of both the CO- and TCO-fed group animals compared to normal animals (1.12 ± 0.19 U/mg protein). HFA of TCO recorded 1.74 ± 0.03 U/mg protein and LFA of TCO recorded 1.44 ± 0.33 U/mg protein. HFA and LFA of CO had 1.54 ± 0.48 and 1.51 ± 0.33 U/mg protein enzyme activities, respectively. GR activity documented in normal group animals was 5.01 ± 0.7 U/mg protein. Animals fed CO-containing HFD resulted in a significant ($p < 0.01$) increase in GR activity, both in the HF and LF areas (9.66 ± 0.96 and 9.94 ± 1.25 U/mg protein). On the other hand, GR activity in the HF area of TCO-fed animals was 5.62 ± 0.57 U/mg protein, which is close to the level of normal group animals, and in LF area was found to be 6.7 ± 1.35 U/mg protein. GPx activity in the normal animals was 1.87 ± 0.3 U/mg protein. The enzyme activity in the HFA and LFA of CO-containing-diet-fed animals were 4.27 ± 0.48 and 3.44 ± 0.31 U/mg protein ($p < 0.01$), whereas HFA and LFA of TCO-containing-diet-fed animals were 3.44 ± 0.31 and 2.48 ± 0.18 U/mg protein ($p < 0.01$). GST activity was found to be 2.38 ± 0.61 U/mg protein in normal rats. Animals fed CO- and TCO-containing HFD had elevated GST activity ($p < 0.01$) mainly in HFA (6.78 ± 1.11 and 6.54 ± 1.17 U/mg protein) and only a slight increase in LFA (4.91 ± 0.84 and 3.28 ± 1.16 U/mg protein), respectively (Fig. 4a).

Reduced glutathione (GSH) is a tripeptide, serves as a key antioxidant molecule in the body. Significantly increased ($p < 0.01$) level of this tripeptide was seen in HFA of the liver in CO- and TCO-containing-diet-fed animals (10.85 ± 1.88 and 9.53 ± 0.67 µmol/mg protein) when compared to normal rats (4.73 ± 0.66 µmol/mg protein). There observed a lower level of GSH in LFA of the liver in CO (7.15 ± 1.04 µmol/mg protein) and TCO (5.52 ± 1.86 µmol/mg protein) containing diet-fed animals (Fig. 4b).

The level of TBARs in the hepatic tissue of normal group animals was 1.64 ± 0.32 nmol/mg protein. The level was found significantly increased ($p < 0.01$) in HFAs of CO (4.98 ± 1.47 nmol/mg protein) and TCO (4.42 ± 0.30 nmol/mg protein) containing-HFD-fed animals. LFA of these animals documented a relatively less amount of TBARs, which were 2.89 and 3.67 nmol/mg protein, respectively. Here, LFA of TCO-containing diet animals had still a higher amount of TBARs than CO-fed animals (Fig. 4c).

Elevated Lipase Activity in HFD-fed Rats

A significant increase in the hepatic lipase was observed in the HFA of the TCO-fed group (0.89 ± 0.17 U/g protein $p <$ 0.01) compared to the other groups (Fig. 5).

The glycerol liberated in the reaction was higher in both CO and TCO treated groups than the normal animals in the pancreas and adipose tissues (Table 5). Pancreatic lipase activity in CO and TCO-containing-HFD-fed animals were

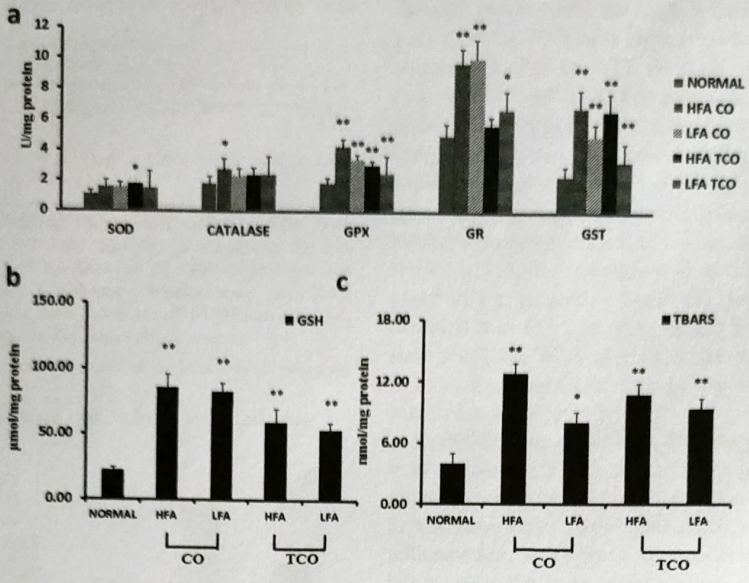

Fig. 4 Heightened antioxidant enzyme activities do not prevent a hike in TBARs in the HF area of HFD-fed rats. Wistar rats were divided into three groups of six animals each. Group 1(normal group) was fed normal laboratory diet, while Groups 2 and 3 animals were fed with CO- and TCO-containing high-fat diet for 2 months with single dose of STZ at the end of 1-month feeding. At the end of the experiment, hepatic tissue antioxidant enzymes was calculated from OD values using proteins values as described by Habig et al. [17] (**a**). Reduced glutathione level was calculated as described by Moron et al. [12], which is depicted in (**b**). Hepatic lipid peroxidation was determined using TBARs method (**c**). Statistical evaluation of the data was done by one-way ANOVA followed by Dunnett post hoc test using Graph Pad Instat 3 software. Results were considered statistically significant when the p value was <0.01. Values are given as mean ± SD of data done in duplicates of six animals. $*p < 0.05$, $**p < 0.01$ compared with Group 1 rats fed with normal rat chow

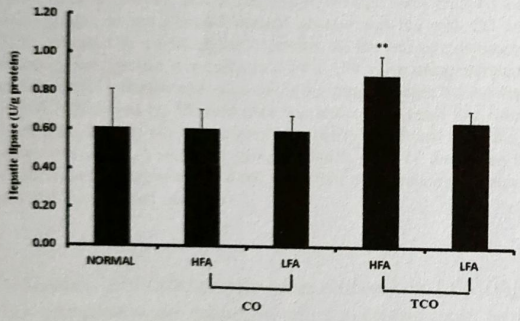

Fig. 5 Elevated hepatic lipase activity in HFD-fed rats. Wistar rats were divided into three groups of six animals each. Group 1 (normal group) was fed normal laboratory diet, while Groups 2 and 3 animals were fed with CO- and TCO-containing high-fat diet for 2 months with single dose of STZ at the end of 1-month feeding. At the end of experiment, the hepatic tissue lipase activity was determined using direct method [19]. Statistical evaluation of the data was done by one-way ANOVA followed by Dunnett post hoc test using Graph Pad Instat 3 software. Results were considered statistically significant when the p value was <0.01. Values are given as mean ± SD of data done in duplicates of six animals. $**p < 0.01$ compared with Group 1 rats fed with normal rat chow

2.31 ± 0.04 U/g protein and 2.79 ± 0.11U/g protein, $p <$ 0.01, and the normal rats had 1.04 ± 0.04 U/g protein. Adipose tissue lipase activity was higher in TCO-containing-HFD-fed animals (2.62 ± 0.05 U/g protein, $p <$ 0.01), while CO-fed rats have 1.89 ± 0.09 U/g protein, $p <$ 0.01. The normal rats documented 1.07 ± 0.04 U/g protein lipase activity.

Increased HP Content Documented in the Hepatic Tissue of HFD-fed Rats

Hepatic HP measured as an index of collagen content was significantly higher in HFA of CO- and TCO-containing-diet-fed animals (2.07 ± 0.24 and 2.57 ± 0.3 µg/g tissue, $p <$ 0.01), respectively, when compared to the normal hepatic level (1.10 ± 0.27 µg/g tissue). LFA of both CO- and TCO-containing-diet-fed animals documented 1.48 ± 0.24 and 1.73 ± 0.1 µg/g tissue, respectively ($p < 0.05$) (Fig. 6).

Histological Analysis

Histological analysis using H&E staining showed normal hepatic architecture in the normal diet-fed animals (Fig. 7a). In CO-fed animals, microvesicles affluent areas were observed in HFA (Fig. 7b) but not in LFA (Fig. 7c). However, HFA of TCO-containing-diet-fed animals showed progressed hepatosteatosis (Fig. 7d) as evidenced by the increased incidence of macrovesicles, hepatocellular

Table 5 Pancreatic and adipose tissue lipase activity of experimental animals

Tissue/Organ	Normal	CO	TCO
Pancreatic lipase (U/g Protein)	1.04 ± 0.04	2.31 ± 0.04*	2.79 ± 0.11*
Adipose tissue lipase (U/g protein)	1.07 ± 0.04	1.89 ± 0.09*	2.62 ± 0.05*

Higher pancreatic and adipose tissue lipase activities have been observed in CO- and TCO-fed rats. Statistical evaluation of the data was done by one-way ANOVA followed by Dunnett post hoc test using Graph Pad Instat 3 software. Results were considered statistically significant when the p value was <0.01. Values are given as mean ± SD of data done in duplicates of six animals

CO coconut oil, *TCO* thermally oxidized coconut oil, *SD* standard deviation

*$p < 0.01$ compared with Group 1 rats fed with normal rat chow

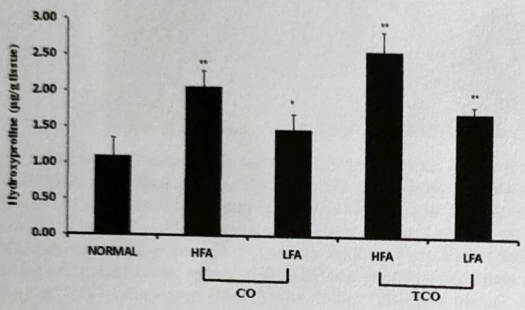

Fig. 6 Increased hydroxyproline content documented in the hepatic tissue of HFD-fed rats. Six Wistar rats in average were grouped into three groups, except Group 1 normal groups were fed with CO and TCO indicated in the figure for 2 months. At the end of experiment, tissue hydroxyproline was calculated from OD value using Bergman and Loxle [11]. Statistical evaluation of the data was done by one-way ANOVA followed by Dunnett post hoc test using Graph Pad Instat 3 software. Results were considered statistically significant when the p value was <0.01. Values are given as mean ± SD of data done in duplicates of six animals *$p < 0.05$, **$p < 0.01$ compared with Group 1 rats fed with normal rat chow

ballooning, and inflammation than observed in LFA (Fig. 7e). Hepatosteatosis stages of all the groups have been scored and represented in Table 6.

The staining for collagen by picrosirius red in the hepatic tissues of TCO-containing-diet-fed animals was higher than the CO-fed group. These observations confirm that TCO-induced hepatic damage gets advanced to the stage of fibrosis (Fig. 8).

Discussion

HFD feeding in experimental animals is known to induce insulin resistance and STZ injection induces pancreatic damage providing additional hit to make the animals diabetic. In this study, the animals fed with HFD containing both CO and TCO have shown a drastic decrement in the bodyweight after the STZ injection. The observed weight loss in CO- and TCO-fed groups indirectly indicates a diabetic condition in these animals. Food and water consumption has also been found higher in both groups. In addition, the glucose levels shoot up in both these groups following STZ injection. Corroborating to this, the final GT level determined at the end of the experiment is significantly lower in TCO-fed animals than CO-given animals compared to the respective initial GT level. Therefore, it is assumed that CO and TCO might have reduced insulin sensitivity and TCO animals could be more sensitive to STZ-induced pancreatic damage due to its higher level of loss in insulin sensitivity.

Under diabetic conditions, free fatty acids in the body tend to increase and lipids as triacylglycerol often accumulate in tissues as lipid patches, which is the primary event in the fatty liver. These lipid patches may cause injury, inflammation, and fibrosis (lymphogranuloma), an underlying cause for liver cirrhosis and cancer (HCC) [20]. Insulin resistance is manifested by dyslipidemia, especially hypertriglyceridemia and low HDLc. Also, TCO-containing-diet-fed groups have shown an increased TC, TG, and VLDL level in serum compared to normal and CO treated animals. The serum levels of LDL are similar in all experimental groups. However, HDLc is found higher in CO and not in TCO included HFD-fed animals. These results show that TCO-fed animals induce pronounced dyslipidemia than CO-fed animals. In addition, the serum AST, ALT, and ALP activities are more prominent in the TCO-fed groups. Elevated levels of AST, ALT, and ALP in the blood are clinically considered as important markers of hepatic damage and indicate liver dysfunction. It has been noticed that at least 50% of the hepatic tissue of the HFD-containing CO- and TCO-fed animals have fatty infiltration within this short experimental period. Higher fatty infiltration is seen in TCO-fed animals.

Therefore, the lipid profile of hepatic tissue from the HFA and LFA has been analyzed. Increased PL and TG are documented in the HFA of TCO-containing-diet-fed animals, while TC is found comparatively higher in the HFA of CO-fed animals. The increase in PLs and TG and decrease in HDL of TCO-fed group animals indicate the tendency to accumulate lipids in the hepatic tissue. Another factor influencing the TG levels is the increase in hepatic lipoprotein lipase activity in HFA of TCO-fed animals. Hepatic lipase produces free fatty acids from lipoproteins, which are transported into the hepatocyte [21]. In insulin-resistant states, hepatic lipase activity further increases and promotes the amount of free fatty acids available for uptake into hepatocytes, allowing for increased oxidation and/or esterification to TG [22]. The excess TG, which has not

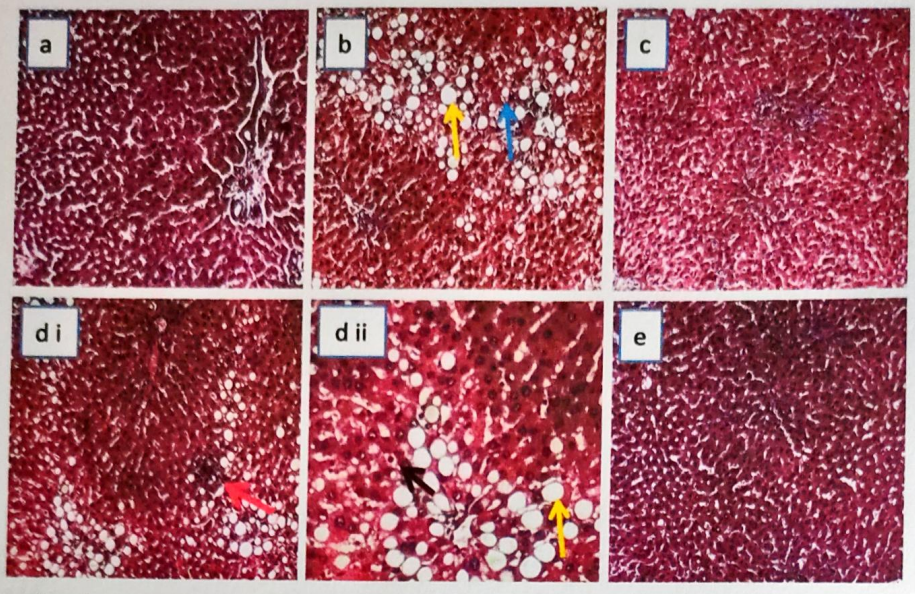

Fig. 7 Haematoxylin and eosin staining of hepatic tissues. Wistar rats were divided into three groups of six animals each. Group 1 (normal group) was fed normal laboratory diet, while Groups 2 and 3 animals were fed with CO- and TCO-containing high-fat diet for 2 months with single dose of STZ at the end of 1-month feeding. Tissues were sectioned using microtome, stained with H&E and photographed under a Magnus INVI microscope (New Delhi, India) (200×): **a** normal, **b** high-fat area of CO-containing diet, **c** low-fat area of CO-containing diet, **d** i high-fat area of TCO-containing diet, **d** ii zoomed image of **d** i, and **e** low-fat area of TCO-containing diet. Arrow symbols of different colors are used to indicate the presence of (red) inflammation, (blue) microvesicles, (yellow) macrovesicles, and (black) hepatocyte ballooning

Table 6 Hepatosteatosis stages in rats fed on normal feed and diet containing CO and TCO

Characteristics	Normal	CO (LFA)	CO (HFA)	TCO (LFA)	TCO (HFA)
Macrovesicular steatosis	0	0–1	1	1–2	2–3
Hepatocyte ballooning	0	0	1	0	2–3
Portal tract inflammation	0	0	1	0	1–2

Values are given as mean ± SD. 0 indicates normal hepatic characteristics and 1, 2, and 3 indicate low, moderate, and severe hepatic damage, respectively

CO coconut oil, *TCO* thermally oxidized coconut oil, *LFA* low-fat area, *HFA* high-fat area, *SD* standard deviation

been secreted as very-low-density lipoproteins, is stored in large and more abundant lipid droplets, resulting in steatosis. On the other hand, pancreatic lipase is found to increase in CO- and TCO-containing-diet-fed animals. These diet-induced lipase activities might have augmented the formation of free fatty acid and glycerol in the intestinal milieu, thereby enhancing their uptake. This increased uptake might also be contributed to the accumulation at the liver site. Also, an increase in the hormone-dependent adipose lipase in HFD-fed animals might have reduced the TG accumulation in adipose tissue, instead mobilized. This mobilization, in turn, could have increased the TG level in the liver.

It is thus possible that a higher extend of hepatic tissue damage could be due to lipotoxicity. Histological details of hepatic tissue also show an increased incidence of microvesicles and hepatocyte ballooning in the HFA of TCO-fed rats than of CO-fed animals. Hepatic micro- and macrovesicular areas are characteristics of fatty liver and hepatic ballooning is indicative of inflammatory changes. Together it is assumed that fatty liver mediated lipotoxicity and inflammation (steatohepatitis) prevails more with TCO incorporated HFD-fed rats than CO. Further to this end, HP content, documented as an index of collagen formation in the liver, has significantly increased in the HFA of both CO- and TCO-fed animals. Here also, TCO-fed animals are

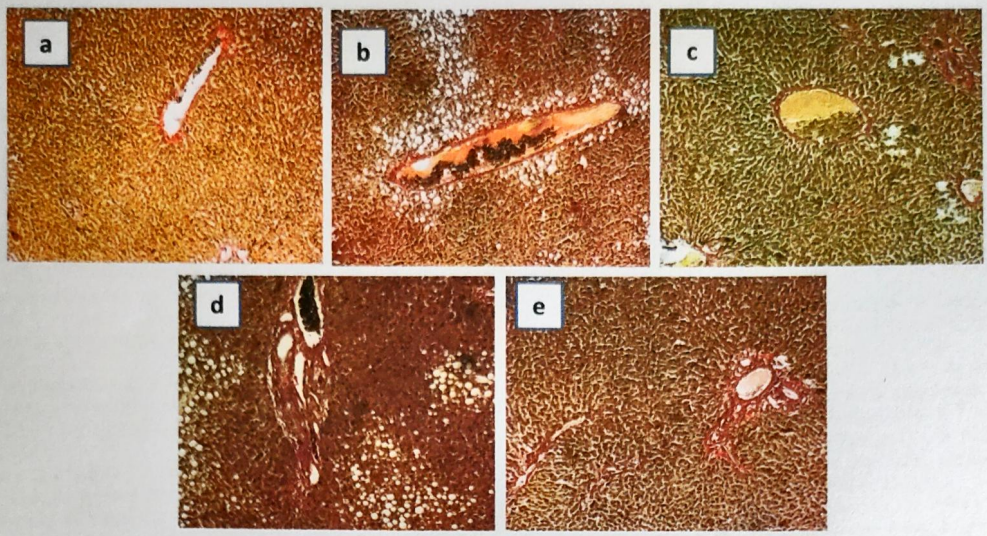

Fig. 8 Picrosirius red staining of hepatic tissues. Wistar rats were divided into three groups of six animals each. Group 1 (normal group) was fed normal laboratory diet, while Groups 2 and 3 animals were fed with CO- and TCO-containing high-fat diet for 2 months with single dose of STZ at the end of 1-month feeding. Tissues were sectioned using microtome, stained with picrosirius red stain and photographed under a Magnus INVI microscope (New Delhi, India) (200×): **a** normal, **b** high-fat area of CO-containing diet, **c** low-fat area of CO-containing diet, **d** high-fat area of TCO-containing diet, and **e** low-fat area of TCO-containing diet

observed with higher hydroxyl proline content than the CO-fed group. The staining for procollagen by picrosirius red stain confirms this fact. These findings suggest that TCO-induced hepatic damage advances to the level of fibrosis ignition. This observation is strongly supported by the report that TCO exacerbates high-fructose-induced liver damage and induces lipogranuloma [6].

Further, HFD-fed animals have shown alterations in their hepatic redox equilibrium. Reduced glutathione is the central antioxidant molecule in the intracellular system, which is found to be elevated in all groups compared to normal group animals. The elevated level in the HFA of TCO included diet-fed group is found lower than the HFA of CO-fed group animals. GPx, an enzyme involved in the detoxification of peroxidases, GST, which is involved in the conjugation of lipophilic xenobiotics with GSH and accelerating their elimination, catalase that depletes hydrogen peroxide, and SOD that catalase dismutation reaction of superoxide anion have shown a similar trend. This enhancement in enzyme activities may be a natural resistant mechanism against the overwhelming presence of lipophilic carbonyls in the hepatic tissue due to lipid peroxidation in TCO-containing-diet-fed rats. Supporting this, both HFA and LFA of hepatic tissues in TCO-fed animals show enhanced lipid peroxidation as revealed from the TBARs level. These results indicate that fatty liver experiences elevated antioxidant defense system and lipid peroxidative injury and inflammation, a condition called steatohepatitis.

Conclusion

Our results clearly show that TCO is more deleterious than CO in promoting HFD-induced hepatic damage. The HFD incorporated TCO/STZ could serve as a useful model for NASH within a short span of 2 months. Prolonged exposure to HFD–TCO may likely lead to hepatic cirrhosis and early stages of HCC, which needs to be assessed further to develop a rodent dietary model for HCC.

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Compliance with Ethical Standards

Conflict of Interest The authors declare no competing interests.

Ethical Approval All experiments involving animals conducted in this study were in accordance with the institution's ethical standards and had prior permission from the Institutional Animal Ethical Committee that follow the guidelines of CPCSEA, Government of India (approval no: ACRC/IEAC/19(1) P-1). The manuscript does not contain any studies involving human participants performed by any of the authors.

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