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Biochemical and histopathological profiling of Wistar rat treated with *Brassica napus* as a supplementary feed

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Abstract

Metabolic changes together with cardiovascular and hepatic factors are related to the development of diseases like myocardial lipidosis, heart disease, and profound toxicity. The aim of this animal study is to determine the effects of high erucic acid containing rapeseed oil (*Brassica napus* L.) varieties on liver, kidney and heart muscles in Wistar rats. Male Wistar rats were divided into three groups where each group containing four rats. Group A was considered as control diet group, while Group B rapeseed wild oil group and Group C rapeseed hybrid oil group were considered as experimental diet groups. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT),alkaline phosphatase(ALP), creatine kinase-MB (CK-MB) and creatinine of two experimental groups were significantly elevated while compared to the control groups ($p < 0.05$–0.001). Nevertheless, an increment in weight retardation ($p < 0.05$) was also observed in rapeseed hybrid oil treated groups. No significant weight retardation found in other two groups ($p > 0.05$). Noticeable tissue injury observed in this study is a sign of the relative toxicity of erucic acid containing rapeseed oil to mammalian species. The use of *Brassica napus* as a supplementary feed ingredient should be, therefore, thoroughly considered

Keywords: Rapeseed oil; *Rattus norvegicus*; Serum enzymes; Erucic acid; Tissue profiling

1. Introduction

The utilization of dietary fats in human and animal nutrition is an ongoing research aspires. Their high quantities may be found in plant seeds distributed in many parts of the world. Rapeseed (*Brassica napus*) especially known as Rai seed in the Indian subcontinent is one of them. It looks like bright yellow flowering plant belonging to the *Brassicaceae* family. As an agricultural product, rapeseed is important for its oil and protein contents. Rapeseed oil is the third most important vegetable oil after palm and soybean oils in the world [1]. Oilseeds of the Brassicaceae family are major sources of vegetable oil for nutritional purposes all over the world [2]. The high quality of the edible rapeseed oil is now a matter of great concern because of its high erucic acid content. Rats fed with high erucic acid doses for several weeks are correlated with the development of myocardial necrosis [3]. Rapeseed oil typically has a main fatty acid composition of 3.6% palmitic acid (16:0), 61.6% oleic acid (18:1), 21.7% linoleic acid (18:2) and 9.6% α-linolenic acid (18:3) [4]. Generally, rapeseed oil with high levels (C18:1; >60%) of oleic acid and low levels (C22:1; <1%) of erucic acid is considered to be of high nutritional quality [5].

Erucic acid is a long chain monounsaturated omega-9 fatty acid and is responsible for defective oxidation by the mitochondrial β-oxidation system [6]. Cardiac muscle looks significantly poor at oxidizing erucic acid. Previous animal studies based on short term or long term oral exposure to oils containing erucic acid. Regular outcome associated with short term and long term exposures to these oils are myocardial lipidosis. Erucic acid at

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a dose level of 1500 mg/kg body weight/day in rats related to myocardial lipidosis [7].

Serum enzymes such as aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) are useful biomarkers of liver injury. These are the enzymes found mainly in the liver, red blood cells, heart, pancreas, kidneys and biliary ducts of the liver. The levels of AST and ALT in serum are used to diagnose body tissues especially the heart and the liver is injured or not [8]. Research suggests that, when body tissues are damaged, additional AST and ALT are released into the bloodstream and raise the serum enzyme level. As a result, the amount of AST and ALT in the blood is directly associated with the amount of tissue damage. High AST and ALT ratio (>1.5) in acute viral hepatitis may give rise to severe condition [9]. The ALP test is used to detect blocked bile ducts, liver damages or bone disorders. When liver cells damaged it releases increased amounts of ALP into the blood. ALP levels in plasma also rise with large bile duct obstruction, intrahepatic cholestasis, or infiltrative diseases of the liver. The normal range of ALP is 44 to 147 IU/L [10]. Various infections may increase the level of enzymes. While the enzyme CK is known as creatine kinase. The level of CK can vary according to age, gender, race and physical activity and its level are higher amongst black males, as well as newborns [11]. Blood levels of CK rise when skeletal muscles or heart cells are injured. CK is made up of three enzyme forms. According to lab tests online there are three types of CK: CK-MB, CK-MM, and CK-BB. CK-MB primarily found in heart muscle, CK-MM found primarily in skeletal muscle and CK-BB found primarily in the brain [12]. Excess physical activities are the most common source of high CK in the blood because it increases the flow of creatine kinase in the blood stream for several days [13]. Furthermore, this high CK in the blood stream may couple with elevated intracellular CK [14] that in turns creates a wide range of clinical conditions such as neuroleptic malignant syndrome, skeletal muscle diseases, malignant hyperthermia and severe muscle contractions [15]. Creatine phosphate generates creatinine in muscle tissue which carried via the bloodstream and eliminated by the kidneys. Abnormal kidney function increase the amount of creatinine level in blood stream. Each day, roughly 2% of creatine is converted to creatinine [16]. A recent Japanese study showed that, Japanese men are more porn to develop type-2 diabetes due to lower serum creatinine level in blood [17]. Therefore, it is of particular interest to investigate the adverse effects of high erucic acid containing rapeseed oil supplementation on liver, heart, and kidney muscles of rats via analyzing the biomarkers: AST, ALT, ALP, CK-MB, and creatinine.

2. Materials and Methods

2.1. Chemicals and assay kits

AST, ALT and ALP kits were purchased from CRESCENT Diagnostics, Jeddah, K.S.A. CK-MB kit was procured from Life Diagnostics, Inc., Pennsylvania, USA where serum creatinine kit was imported from Wako Pure Chemical Industries, Osaka, Japan. Bouin solution, ethanol and chloroform were obtained from Sigma-Aldrich Company, USA.

2.2. Collection, preparation, and storage of rapeseed varieties

Fresh and good quality wild and hybrid rapeseed varieties were collected from the local market of Kushtia, Bangladesh. To prepare the experimental oil, stored rapeseeds were taken to an oil mill. Freshly prepared refined oil was used during the study. The oil sample was preserved in dry, amber bottles at −28 °C to prevent photo-oxidation.

2.3. Experimental animals

For scientific research, male Wistar rats (*Rattus norvegicus*) with an average weight of about 35–40 g and 4 to 5 weeks of age were purchased from the Animal House Laboratory, Jahangirnagar University, Dhaka, Bangladesh. Experimental animals were housed in stainless steel cages with free access to drinking water and diet. The room temperature was controlled at 24 to 26 °C in a 12 h light/dark cycle. All the procedures were conducted under the strict guidelines and regulations approved by the Biotechnology and Genetic Engineering Department, Islamic University, Kushtia, Bangladesh.

2.4. Animal diet and dosing of oil

The animals were fed standard diet purchased from, Jahangirnagar University animal house, containing 7% protein source, 20% rice polish, 30% wheat bran and flour, 1.5% soybean oil and common salt, 0.5% vitamin mixture and 2.5% molasses. Then the rats were randomly distributed into three groups. Each group contained four rats and marked as control group A, rapeseed wild group B and rapeseed hybrid group C. Group A was fed with 15gm of standard diet/day and considered as the control group while group B and group C was fed with14.4gm of standard diet with 0.6gm wild rapeseed oil/rat/day and 0.6gm hybrid wild oil/rat/day respectively. Feeding was continued for 8 weeks (56 days). The amount of feed consumed and the individual body weight was recorded daily throughout the experiment.

2.5. Sample collection and preservation

Before sterilization, all the materials were cleaned with detergent and then washed with 95% ethanol. After 56 days of feeding, the 14 hours fasted rats were first subjected to light anesthesia using chloroform and sacrificed. Blood samples were collected from abdominal aorta with 3 ml syringe and transferred into 1.5 ml eppendorf tube. For coagulation, blood was kept about 10 minutes at room temperature. After centrifugation at 3000 r.p.m for 15 minutes at 4 °C using a thermo scientific centrifuge, serum was placed in a 1.5 ml eppendorf tube and preserved at −80 °C until the experiments were performed. Organs (heart, liver, and kidney) were collected and put into petridish and washed in 0.9% saline and preserved in formaldehyde for further investigation. Heart sample was collected and preserved in Bouin's Fluid. Bouin's fluid contains 71.43% saturated picric acid, 4.76% glacial acetic acid and 23.81% formaldehyde, which is known as a strong preservative.

Table 1
Effect of the two varieties of rapeseed oil on body weight retardation in rats fed with high erucic acid diet.

Duration (week)	Group A Control (diet)	Group B Rapeseed oil (Wild)	Group C Rapeseed oil (Hybrid)
1st week	10.04 ± 0.05	9.12 ± 0.08	7.98 ± 0.08
2nd week	12.97 ± 0.08	10.15 ± 0.03	9.06 ± 0.05
3rd week	14.02 ± 0.06	12.07 ± 0.14	9.92 ± 0.12
4th week	17.12 ± 0.04	12.95 ± 0.12	11.16 ± 0.17
5th week	21.07 ± 0.05	15.98 ± 0.08	12.02 ± 0.07
6th week	25.07 ± 0.09	17.06 ± 0.09	13.21 ± 0.12
7th week	29.03 ± 0.06	22.15 ± 0.19	14.89 ± 0.14
8th week	34.70 ± 0.11	25.03 ± 0.07	17.85 ± 0.26

Average weight gain ± SE (Standard Error). No significant variation observed in between Group A and Group B; Group B and Group C ($p > 0.05$) but Group A and Group C results significantly differ ($p < 0.05$) from each other.

2.6. Biochemical analysis of serum

The level of clinical biochemistry such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine, Creatine kinase (CK) isoenzyme CK-MB was evaluated to determine the enzymatic activities of the livers, kidneys and heart muscles of the control groups and the experimental groups. The activity of all serum enzymes was measured using commercially available kits according to the manufacturer's instructions.

2.7. Histopathological studies

The fixed tissues of rats were dehydrated with ethanol. Then the tissues were passed through xylene solution to remove the ethanol and facilitate molten paraffin wax infiltration at 55 °C. After that, they were embedded in a wax block. Paraffin sections of 6 micron (μ) thickness were cut with the rotary microtome and placed on cleaned glass slides. Finally, the sections were stained with hematoxylin and eosin. The stained slides were examined using a light microscope where the photomicrographs of the tissue samples were recorded.

2.8. Statistical analysis

The results of this study were expressed as mean ± standard error (Mean ± SE). To assess the significance of the differences between the control group and the two experimental rat groups (A and B), a statistical analysis was performed using one-way analysis of variance (ANOVA) for repeated measurements with the significance assessed at the 5% significance level ($p < 0.05$).

3. Results and Discussion

Body weight was measured every week (Table 1) where it was observed that rapeseed hybrid oil group rats (Group C) exhibit a significant decrease in body weight as compared to the control group rats (Group A) fed with normal standard diet ($p < 0.05$). No statistical significance was found between Group B and C rats ($p > 0.05$) (Fig. 1).

Previous studies showed that micronutrients in rapeseed exert a potential benefit to hepatoprotection via reducing excessive hepatic fat accumulation and oxidative stress [1]. In another study, it was found that the native composition of fatty acids of rapeseed oil induces many physiological responses in inflammatory cytokines, carbohydrate metabolism, adipose tissue, and adipokines [18]. In the present study, the average body weights of the rats were measured for 8 weeks. When the average weight of these experimental groups plotted against time, it was observed that average weight of the rats in control group A linearly increased from the first week to last week at each time point in Fig. 1. From 5th to 8th-week duration, control group A showed highest weight gain while the other two groups (Group B and Group C) showed weight retardation. As a result, significant evidence of growth retardation found amongst these groups ($p < 0.05$) as compared to control group rats by means of the presence of high amount of erucic acid (Table 1). For this reason, rats tend to consume lower amount of diet, leading to ultimate weight loss. It might also happen that due to this metabolic disorder, they could not able to digest it.

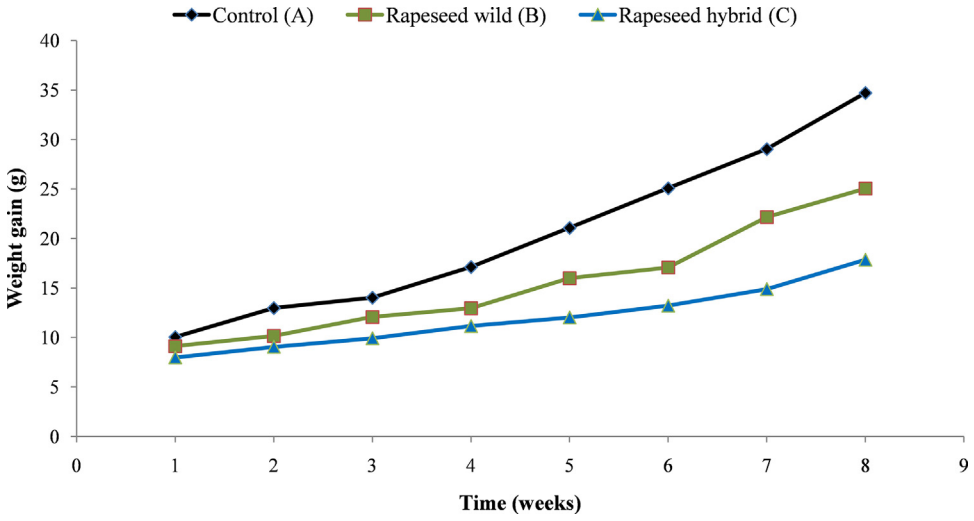

Fig. 1. Potential effect of rapeseed oil against body weight retardation in rats fed with high erucic acid diet.

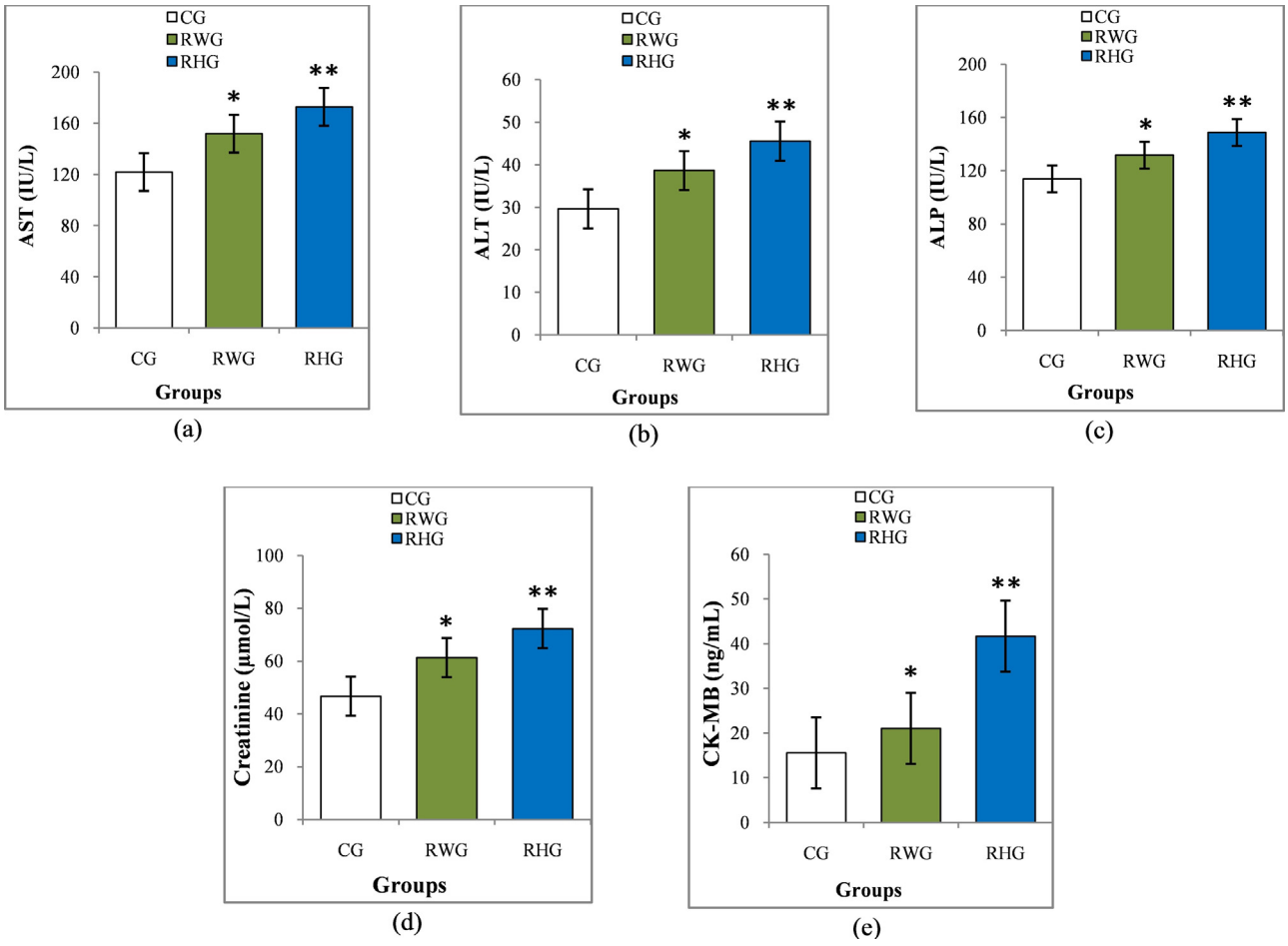

Fig. 2. Effects of rapeseed oil on serum AST (a); ALT (b); ALP (c); creatinine (d) and CK-MB (e) levels in Wistar rat model. Values are expressed in Mean ± SE. n = 4, *$p < 0.05$, **$p < 0.001$ compared with control group. CG = Control Group, RWG = Rapeseed Wild Group and RHG = Rapeseed Hybrid Group.

3.1. Biochemical findings

Serum AST, ALT, and ALP are the enzyme biomarkers to monitor the liver structural integrity and damage and aids in the clinical diagnosis of liver toxicity conditions [19]. The effects of rapeseed oil supplementation on the level of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) are illustrated in Fig. 2**(a–c).** When there is an injury to the organs due to any reason then these enzymes are spilled into the blood stream. Therefore, AST, ALT and ALP levels in rat serum were examined. The reference ranges of AST, ALT and ALP are 50 to 150 IU/L, 10 to 40 IU/L and 30 to 130 IU/L respectively [20]. The AST levels were 121.95 ± 5.16 IU in CG, 15.93 ± 11.49 IU in the RWG oil and 172.90 ± 16.61 IU in the RHG oil groups Fig. 2**(a)**. The two experimental groups had significantly ($p < 0.05$–0.001) higher AST levels than the control. The AST levels of the RHG were significantly higher than the RWG.

The serum ALT levels in the two test groups and control are shown in Fig. 2 **(b)**. The ALT levels were 29.66 ± 1.94 IU in control groups, 38.68 ± 4.61 IU in the RWG oils and 45.57 ± 4.95 IU in the RHG oils. Dietary supplementation significantly ($p < 0.05$–0.001) increased the ALT levels over the control group. However, there was no significant difference in the ALT levels between the two experimental groups.

Fig. 2(c) shows the mean levels of ALP enzymes for the rapeseed oil treated and control groups. The enzyme levels were 131.68 ± 9.70 I.U. for the rapeseed wild oil-fed group and 113.79 ± 4.73 I.U. for the control. The level for the rapeseed hybrid oil fed groups was 148.69 ± 9.10 I.U. A highly significant increase in the ALP enzyme level was noted in rats fed with the rapeseed hybrid oil diet compared with control ($p < 0.001$). There was no significant difference ($p > 0.05$) in the enzyme levels between the RWG oil and the RHG oil but rapeseed wild oil-fed group had a significant increase in the ALP enzyme level compared with control ($p < 0.05$) (Table 2).

Creatinine is a chemical waste product that is carried via the bloodstream and eliminated by the kidneys. If the filtration in the kidney is deficient, creatinine blood levels rise. After 8 weeks of examination, creatinine levels of CG includes the value 46.70 ± 3.14 µmol/L whereas RWG oil and RHG oil includes the values 61.30 ± 6.69 µmol/L and 72.32 ± 7.48 µmol/L respectively shown in Fig. 2 **(d)**. The biochemical reference range of creatinine is 0.2–0.8 mg/dL or 17.68–70.72 µmol/L [21]. Our observed result indicated those hybrid rapeseed oil consuming Wistar rats groups were more

Table 2
Effects of serum enzymes on liver, heart, skeletal muscles and kidney.

Bio-markers with unit	Group A Control (diet)	Group B Rapeseed oil (Wild)	Group C Rapeseed oil (Hybrid)
AST (IU/L)	121.95 ± 5.16^{a}	15.93 ± 11.49^{b}	$172.90 \pm 16.61^{c¥}$
ALP (IU/L)	113.79 ± 4.73^{a}	131.68 ± 9.70^{b}	$148.69 \pm 9.10^{c¥}$
ALT (IU/L)	29.66 ± 1.94^{a}	38.68 ± 4.61^{b}	$45.57 \pm 4.95^{c¥}$
CK-MB (ng/mL)	15.58 ± 1.20^{a}	21.05 ± 2.55^{b}	$41.69 \pm 8.73^{c¥}$
Creatinine (μmol/L)	46.70 ± 3.14^{a}	61.30 ± 6.69^{b}	$72.32 \pm 7.48^{c¥}$

All values are expressed as mean ± SE. A different superscript letters within each row are significantly different ($p < 0.05$) whereas superscript with the symbol (¥) indicate that the results are highly significant ($p < 0.001$).

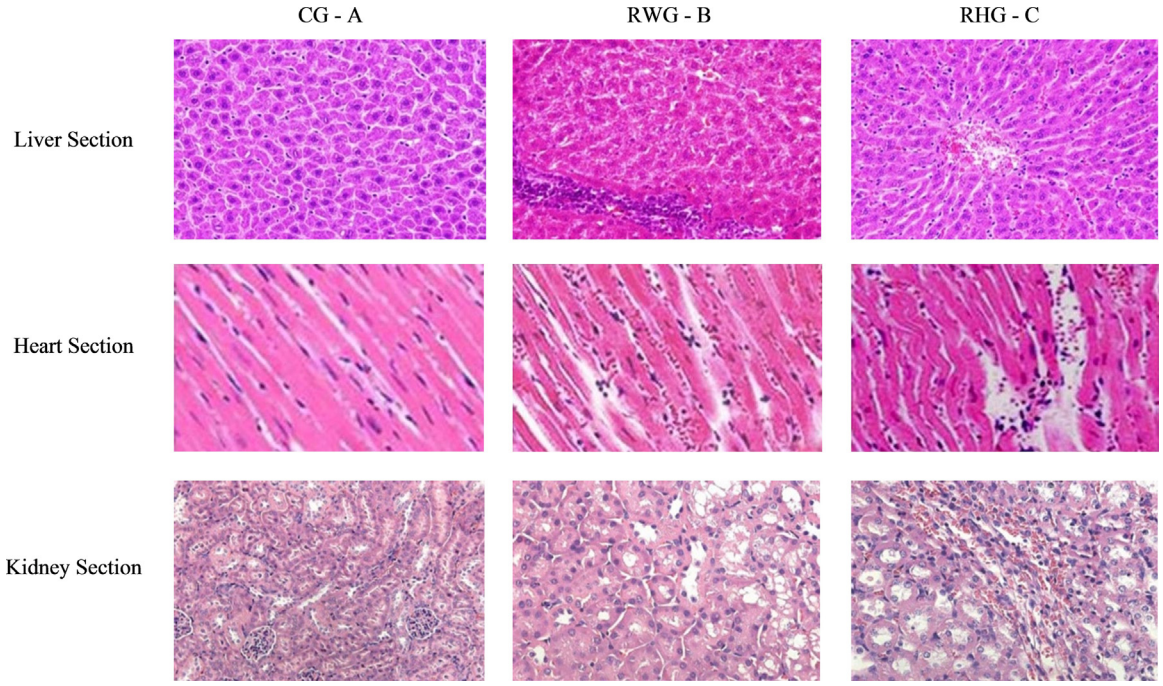

Fig. 3. Photomicrographs of rat liver, heart and kidney sections stained with haematoxylin and eosin (original magnification ×200) from each group: CG – A: control rapeseed oil group; RWG – B: Wild rapeseed oil group; RHG – C: Hybrid rapeseed oil group. CG – A showing normal microenvironment where RWG – B and RHG – C showing leukocyte infiltration and haemorrhage in all tissues of rats treated with *B. napus*. CG – A = Control Group A, RWG – B = Rapeseed Wild Group B and RHG – C = Rapeseed Hybrid Group C.

porn to tissue inflammation rather than the other two groups of animals.

A high CK-MB level suggests that there is disease or damage to the heart and skeletal muscle. On comparing with the CK-MB level of CG (15.58 ± 1.20 ng/mL), RWG (21.05 ± 2.55 ng/mL) oil and RHG (41.69 ± 8.73 ng/mL) oil had increased CK-MB values (Fig. 2 **(e)**). The detection level of CK-MB is 0.312–20 ng/mL [22]. RHG oil had highly significant values ($p < 0.001$) compared with the control group that may indicate damage to the heart and skeletal muscles.

3.2. Histopathological findings

The histological analysis revealed that the accumulation of high erucic acid was drastically increased in RHG animals as compared to CG oil treated rats shown in Fig. 3. Results highlighted that there was no remarkable difference between RWG and RHG animals in liver, heart and kidney histological sections. Microscopic analysis indicated that the percentage of erucic acid accumulation was significantly increased in RWG animals as compared to control group. Moreover, the accumulation of erucic acid in RHG was highly significant as compared to control group.

Previous experiment showed that feeding low erucic acid rapeseeds oil decreases creatinine level in the rat [23]. In our present study, it has been found that creatinine level increased in rat's blood. This increase may be due to fed high erucic acid rapeseeds oil in experimental groups. In the current investigation, creatinine level of RHG oil is highly significant ($p < 0.001$) compared to CG and RWG oil showed a significant increase ($p < 0.05$) compared to CG. The increase in creatinine level of RHG oil and RWG oil may indicate that kidney function is not normal. Hence, the elevated level of serum enzymes in the present study is probably due to the destructive effects of the hazardous erucic acid present in both forms of rapeseed oil.

The present study was not designed to study the mechanism of erucic acid. Future studies on a genetic level are required to improve the rapeseed oil quality and to introduce low erucic acid

containing rapeseed varieties for human and animal consumption. A recent study showed that the knockdown of BnFAE1 sharply decreased the levels of erucic acid (less than 3%) and largely increased the contents of oleic acid (more than 60%). This result demonstrated that BnFAE1 is a reliable target for genetic improvement of rapeseed in seed oil quality promotion [24].

In this study, it was found that the erucic acid from the rapeseed oil administered orally alters liver, heart and kidney functions in rats. It is noteworthy that the wild rapeseed oil variety has a less injurious impact on the examined organs than the hybrid one. The reason for the elevation of erucic acid level in the hybrid rapeseed oil variety is not readily perceptible from this study. This hypothesis remains to be elucidated. It could be due to the genetic variance of *Brassica napus*. Moreover, a significant alteration in biochemical and histological parameters obviously found in this study is a sign of toxic effects of this plant on mammalian species. Therefore, the use of rapeseed as supplementary diet should be thoroughly considered.

4. Conclusion

The outcome of the present study suggests that supplementation of high erucic acid containing rapeseed oil decreases the overall body weight, while increases the heart, liver, and kidney tissue enzyme biomarkers that lead to the development of inflammation and hemorrhage. In the past, most of the studies regarding *Brassica napus* were conducted on animals. As a result, further studies are required at the molecular level to investigate the amounts of the erucic acid present in different edible oils including *Brassica napus* for human consumption.

Conflicts of interest

The authors declare no conflicts of interest.

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