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Jackfruit Seed Powder Supplementation Attenuates High Sugar Diet-Induced Hyperphagia and Hyperglycemia in Mice †

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Abstract: Intake of high sugar diets (HSD) is strongly associated with the development of obesity, diabetes and other metabolic diseases. Diets that are rich in dietary fiber have been reported to have substantial health benefits. Jackfruit seed powder (JSP) is a good source of dietary fiber and can be a possible candidate to fight against metabolic diseases. JSP supplementation showed a significant reduction in HSD-induced hyperphagia and also in body weight gain. The addition of JSP significantly improved glucose tolerance and reduced LDL cholesterol. Overall, JSP consumption could play a vital role in the management of metabolic disorders caused by HSD.

Keywords: jackfruit seeds; hyperphagia; hyperglycemia; hyperlipidemia; high sugar diet

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1. Introduction

Artocarpus heterophyllus Lam., which is commonly known as jackfruit is a tropical climacteric fruit, belonging to Moraceae family [1]. This fruit is grown in different parts of Asia, Africa, and South America [2]. Jackfruit tree grows in warm and moist regions [3]. It is most widely cultivated in Bangladesh, Burma, Malaysia, Indonesia, Thailand, and on a smaller scale in Brazil and Australia [4]. Jackfruit is the national fruit of Bangladesh, and is known as "kathal". The jackfruit ranks third in area of cultivation and second in production among the fruits of Bangladesh. Jackfruit has been reported as an abundant source of protein, potassium, thiamine, niacin, calcium, sodium, magnesium and vitamin B_6 [5]. Commonly, the pulps of the mature and ripen jackfruits are eaten by the people. Jackfruit seeds are normally discarded or sometimes kept for consumption. As jackfruit is highly seasonal and seeds have a shorter shelf life, hence go waste during the seasonal glut. In rural areas, seeds are dried and roasted to consume as snacks. Though the nutritional properties of jackfruit seed have not yet been fully explored, it is a good source of protein, starch and dietary fiber. The protein concentration of the jackfruit seeds may vary from 5.3 to 6.8% [6]. Jackfruit seed contains lignans, isoflavones, saponins, other phytonutrients and they have wide-range of health benefits. Jackfruit seed is also a rich source of many minerals such as N, P, K, Ca, Mg, S, Zn, Cu, etc. [7]. Large number of fruits and seeds are produced in a part of the year in Bangladesh. As the seeds are recalcitrant, they germinate immediately after maturity. Therefore, fresh seeds cannot be kept for long time. As a result, a large amount of the total seeds remains unused. However, seed flour can be an alternative product to be used in some food stuffs [8]. To best of our knowledge, no study has yet been done to evaluate the physiological significance of jackfruit seed consumption.

Diabetes mellitus is a disease of modern times which is characterized by a disorder of carbohydrate, fat and protein metabolism [9,10]. Whereas, obesity is known as the accumulation of excess body fat resulting from a chronic imbalance of energy due to excess consumption of nutrients, inadequate physical activity or other factors. Excess energy intake combined with low energy expenditure induces lipid accumulation not only in adipose tissue but also in liver, muscle, and other internal tissues. High sugar diet (HSD) intake accelerates body weight gain [11–13]. Previous study reported that consumption of high sugar diet develops insulin resistance and hyperglycemia in rats [14]. Furthermore, hepatic steatosis (grade 1) and increase in triglyceride concentration due to the intake of HSD have also been reported [14]. The phytonutrients such as lignans, saponins, and isoflavones present in the jackfruit seeds, plays beneficial role in human health [15]. Seeds make up around 10% to 15% of the total fruit weight and are a good source of dietary fiber [16]. Dietary fibers have been shown to improve blood glucose control by trapping ingested carbohydrates inside the viscous gel formed after digestion. The proteolytic activities of different animal pancreatic preparations were reported to be inhibited effectively by jackfruit seed extract [17]. Consumption of soluble dietary fiber reduces postprandial glucose responses after carbohydrate-rich meals, as well as lowering total and LDL cholesterol levels [18]. In a previous report, authors mentioned that resistant starch present in jackfruit seeds may control blood sugar and keep the gut healthy [19]. Therefore, we hypothesize that supplementation of jackfruit seed flour will ensure the food security and may contribute to metabolic disorders through glucose and lipid homeostasis.

Lesser-known and underutilized agricultural commodities which have beneficial effects on human health have been focused by research community in recent years. Jackfruit seeds also contain resistant starch, which helps to maintain blood glucose homeostasis and keep a healthy gut. Jackfruit seeds contain a number of phytochemicals which have antioxidant and anticancer activity [20]. However, no study exists on the impact of jackfruit seed powder supplementation on glucose and lipid homeostasis. Therefore, this study was undertaken to evaluate the potential benefits of jackfruit seed powder supplementation to maintain glucose and lipid homeostasis.

2. Materials and Methods

2.1. Collection of Jackfruit Seeds and Powder Preparation

Mature and ripen Jackfruits were collected from the local market of Mymensingh, Bangladesh. The fruits were opened and the required seeds inside the pulps were collected. The collected seeds were washed properly, sliced and dried under the sun. After proper sun drying, the seeds were kept in an oven at 60 °C for 24 h to remove the moisture completely. Dried pieces were finely ground by a grinding machine and stored in polythene bag until further use.

2.2. Food Formulation and Diet Paradigms

Normal food formulation (ND) includes Wheat, Wheat bran, Rice Polishing, Fish meal, Oil cake, Gram, Pulses, Milk, Soybean Oil, Molasses, Salt and Embavit (vitamin) at different proportions as shown in Table 1 [21]. Three diet paradigms deployed in this study were normal diet (ND), 30% (*w*/*w*) sucrose (HSD), and HSD in combination with 20% (*w*/*w*) of JSP (HSD + JSP). The justification of the dose referred to previous studies [22,23]. The diets were provided ad libitum for animals and changed daily to ensure its quality. Each group of treatment consisted of at least four mice housed in individual cages. The treatment using diet paradigms was carried out for 8 weeks continuously.

Table Composition of normal food formulation used in this study (for 100 g).

Ingredients of Normal Lab Diet	Percent
Wheat	40%
Wheat bran	20%
Rice Polishing	5.5%
Fish meal	10.0%
Oil cake	6.0%
Gram	0.39%
Pulses	0.39%
Milk	0.38%
Soybean Oil	1.5%
Molasses	0.095%
Salt	0.095%
Embavit (vitamin)	0.1%

2.3. Experimental Animals

Six weeks-old Swiss albino male mice were obtained from the Animal Resources Facility of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR, B) and adapted for 10 days in order to acclimatize them with the new environment. Animals were housed in a well-ventilated room at 28± 2 °C and a relative humidity of 70–80% with natural day and light. Normal food and water were available ad libitum before the starting of feeding experiments. Animals were divided into three groups and each group contained at least 4 mice. During the rearing period, animals were also habituated for handling every day to minimize the stress response that may occur in the experiment. All protocols used in this study were approved (AWEEC/BAU/2020_30) by the Animal Welfare and Experimentation Ethics Committee of Bangladesh Agricultural University, Bangladesh guided by the Council for International Organizations of Medical Sciences international guiding principles of biomedical research involving animals.

2.4. Measurement of Food Intake

Food intake by the individual mouse was measured weekly at 10:00 am for 8 weeks according to the following formula:

$$\text{Food intake} = \text{Initial food weight} - \text{remaining food weight}$$

3.3.2. Measurement of Body Weight

The body weight of each mouse was measured with the help of an electric balance (eki300-2n electronic scale, A&D company Ltd., Korea) at 7 days interval up to the end of the experiment. Change in body weight (ΔBW) of each mouse was also calculated at the end of the feeding experiment.

3.3.3. Intraperitoneal Glucose Tolerance Test (ipGTT)

The intraperitoneal glucose tolerance test (ipGTT) was conducted at the end of treatment by following the standard procedure as described in another report [24]. Mice were fasted for approximately 4 h by transferring mice to clean cages with no food or feces in the hopper or bottom of the cage. Access to drinking water was ensured at all times. The tip of the tail was scored using a fresh or sterilized scalpel blade. The first small drop of blood was discarded. A small drop of blood (<5 μL) was placed on the test strip of the blood glucose meter. Blood glucose level was measured using a standardized automated blood glucose test meter (GlucoleaderTM Enhance Blood Glucose Meter, HMD Biomedical Inc., Hsinchu County, Taiwan). A single dose of glucose (2 g/kg BW) was injected intraperitoneally for each mouse. The concentration of blood glucose was recorded for each mouse at 0, 15, 30, 60, and 120 min after ip glucose administration. The area under curve (AUC) data was subsequently calculated from the blood glucose levels in ipGTT.

3.4.4. Blood Samples Collection and Preparation of Serum

At the end of 8 weeks, blood samples were collected from the Posterior Vena Cava by the method described previously [25]. The mice were placed inside the air tight container one by one containing cotton soaked with chloroform. The abdominal cavity of anesthetized mouse was opened by making a V-cut through the skin and abdominal wall 1 cm caudal to the rib cage. The intestines were shifted over to the left and the liver was pushed forward. The widest part of the posterior vena cava (between the kidneys) was located. A 26-gauge needle and a 1 ml syringe were used. The needle was carefully inserted into the vein and blood was drawn slowly until the vessel wall collapses. The blood was collected in a 1.5 ml Eppendorf tube containing EDTA which acts as anticoagulant. Then the blood containing tubes were centrifuged at 4000 rpm for 10 min at 4 °C (Gyrozen 1580R Multi-Purpose High-Speed Refrigerated Centrifuge, Gangnam-gu, Seoul, Korea). After centrifugation, the supernatant serum without unwanted blood cells was collected in a new tube. Serum samples were stored at −20 °C until lipid profile assay.

3.4.6. Measurement of Organ Weight

After collecting the blood samples, the internal organs like liver, heart, kidney, white adipose tissue (WAT) and brown adipose tissue (BAT) were harvested and trimmed to removes additional tissues. The organs were cleaned in saline solution and placed on a filter paper to remove the saline on the surface. Then the organ weights were measured using a digital balance (eki300-2n electronic scale, A&D company Ltd., Korea).

3.4.5. Determination of Lipid Profile Parameters

Lipid profile studies involved analysis of parameters such as total cholesterol (TC) level determined by CHOD-PAP method [26]; triglyceride (TG) level determined by GPO-PAP method [27] and HDL cholesterol level determined by CHOD-PAP method [28]. HumaTex febrile antigen test kit (Human Diagnostic, Wiesbaden, Germany) was used and the absorbance of all the tests was determined using Humalyzer, Model No-3000 (Human GmbH, Wiesbaden, Germany). Serum LDL cholesterol concentrations were calculated using the Friedewald equation [29] as follows:

$$\text{LDL cholesterol (mg/dl)} = \text{Total cholesterol} - \text{HDL cholesterol} - (\text{Triglyceride}/5)$$

3.5. Statistical Analysis

All statistical analyses were performed using Prism 5 (GraphPad Software 7.0, CA). All data were displayed as mean ± SE. An analysis of variance (ANOVA) followed by Tukey's post-hoc test was employed to justify the significant differences among groups of treatment. The $p < 0.05$ was set as a significant value for all analysis.

3. Results

3.1. Effect of Jackfruit Seed Powder (JSP) on Food Intake of Mice

We carried out food intake measurement throughout the experimental period of 8 weeks. There was no significant difference in weekly food intake among the groups at the beginning of the experiment (Figure 1). However, Supplementation of 30% sucrose and 30% sucrose & 20% Jackfruit seed powder supplementation in the food influenced the food intake per mouse from the 2nd week of the treatment (38.00 ± 3.34 g for ND, 42.50 ± 4.57 g for HSD, 36.00 ± 1.73 g for HSD + JSP). Although high sugar diet increased the food intake as compared to normal diet, it was significant only after 6th week of the treatment. High sugar diet (HSD) supplementation showed an increase in food intake than the normal diet (control) which was reversed by the addition of jackfruit seed powder (JSP). Jackfruit seed powder (JSP) supplementation significantly reduced food intake in comparison to high sugar diet group (30% sucrose) from 3rd (40.00 ± 3.51g for ND, 48.25 ± 4.39 g for HSD, 36.25 ± 3.99g for HSD + JSP) to 8th (42.50 ± 3.95g for ND, 57.00± 1.22 g for HSD, 40.25 ± 3.01 g for HSD + JSP) week of the experiment (Figure 1). The food intake was comparable between control group and HSD + JSP fed group.

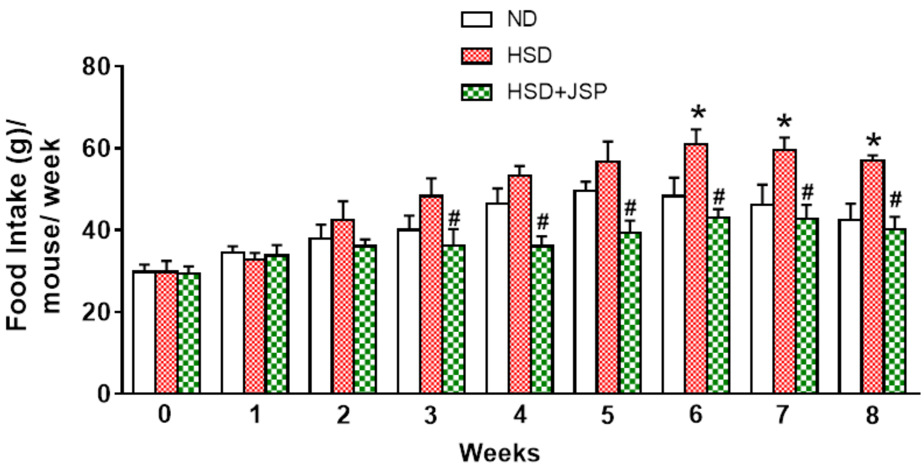


Figure 1. JSP supplementation attenuated HSD-induced hyperphagia. Mice were allowed to ad libitum access to food. Food intake by mice was measured weekly for a period of 8 weeks. ND: Normal Diet; HSD: High Sugar Diet and JSP: Jackfruit Seed Powder. * $p < 0.05$ vs ND; # $p < 0.05$ vs. HSD by one way ANOVA followed by Tukey's post-hoc test. Bars represent mean±SEM. $n \geq 3$ for each group.

3.2. Effect of Jackfruit Seed Powder (JSP) on Body Weight of Mice

We also measured the body weight of each mouse to reveal the effectivity of jackfruit seed powder (JSP) in mitigating the development of HSD-induced obesity. The result showed that body weight tended to be decreased in JSP supplemented group (37.50 ± 1.55 g for ND, 41.75 ± 3.94 g for HSD, 33.25 ± 0.75g for HSD + JSP at 2nd week) as compared with HSD group (Figure 2A), but it was statistically insignificant ($p > 0.05$) until 2nd Week of the treatment. However, the body weight was significantly lower in 30% HSD+ 20% JSP group as compared with HSD group from 3rd to 8th week of the treatment (Figure 2A). The body weight gain in HSD + JSP mice was statistically insignificant ($p > 0.05$) with ND group but significant with HSD group (Figure 2B).

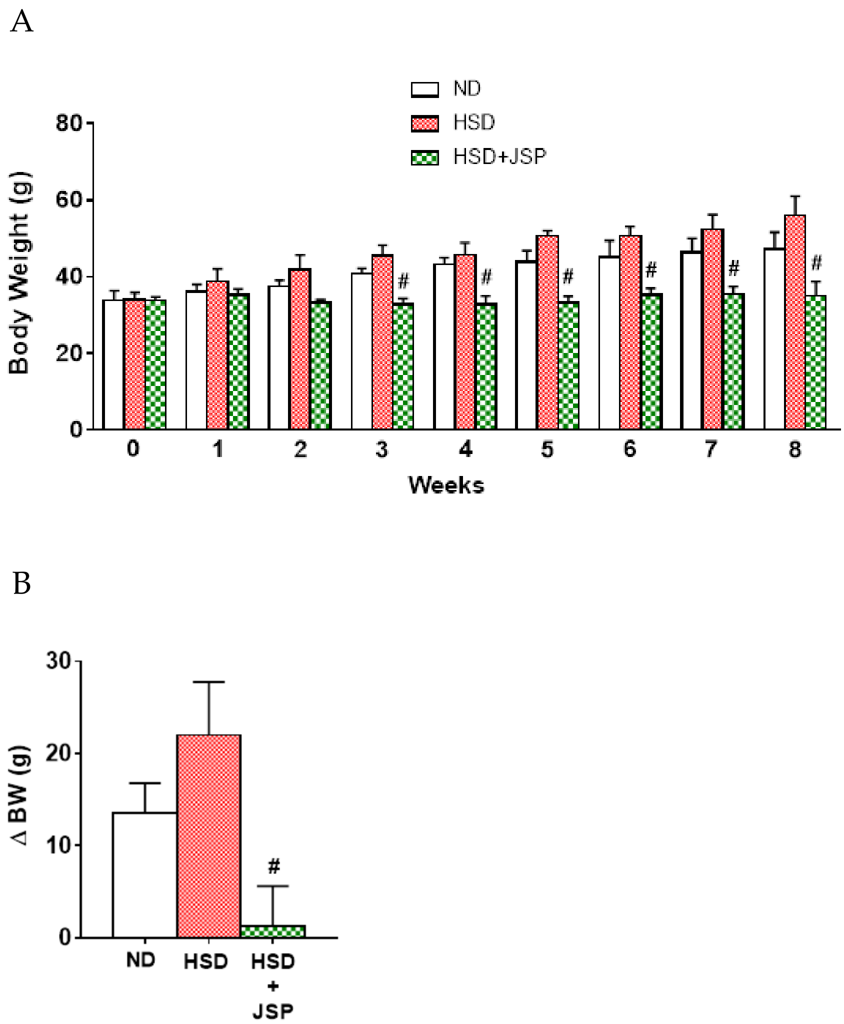
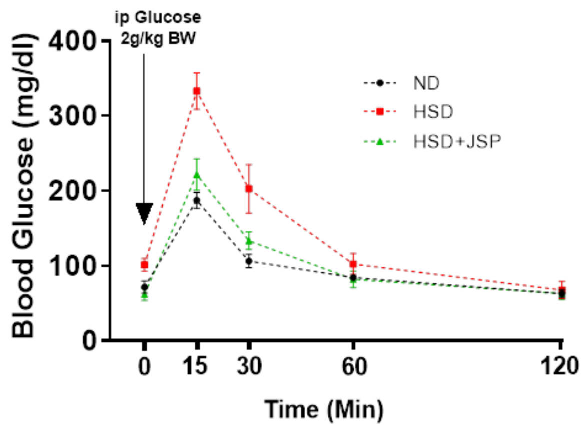


Figure 2. JSP supplementation counteracted the body weight gain in HSD-fed mice. (**A**) Body weight was measured weekly for 8 weeks, (**B**) Body weight gain was determined at the end of treatment. ND: Normal Diet; HSD: High Sugar Diet and JSP: Jackfruit Seed Powder. # $p < 0.05$ vs. HSD by one way ANOVA followed by Tukey's post-hoc test. Bars represent mean±SEM. $n \geq 3$ for each group.

3.3. Effect of Jackfruit Seed Powder (JSP) on Glucose Tolerance in Mice

Intraperitoneal Glucose Tolerance Test (ipGTT) was performed after 8 weeks of feeding experiment and results were presented in Figure After glucose (2 g/kg BW, ip) challenge, HSD-fed mice were unable to utilize glucose properly to establish homeostasis and develop glucose intolerance. There was an elevation in blood glucose concentration in HSD diet fed mice compared to the control mice (187.40 ± 10.85 vs. 333.25 ± 24.43 mg/dL) after 15 min of glucose (2 g/kg BW, ip) challenge. Jackfruit seed powder (JSP) supplementation in HSD diet fed mice (221.83 ± 20.94 mg/dL) showed remarkable reduction in blood glucose concentration as compared to HSD-fed mice. The blood glucose level in HSD + JSP supplemented mice also quickly return to the baseline in compariosn to that of HSD group. Increase in AUC of HSD group (vs. ND group) was reversed by the supplementaion of JSP and which was significantly different as compared with the HSD group (Figure 3B).

A



B

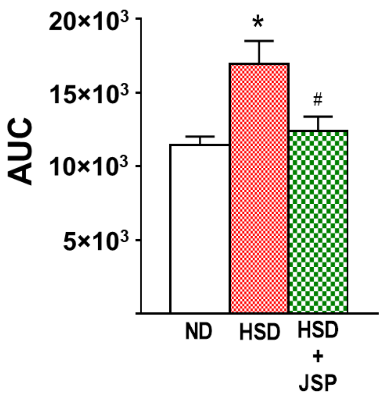
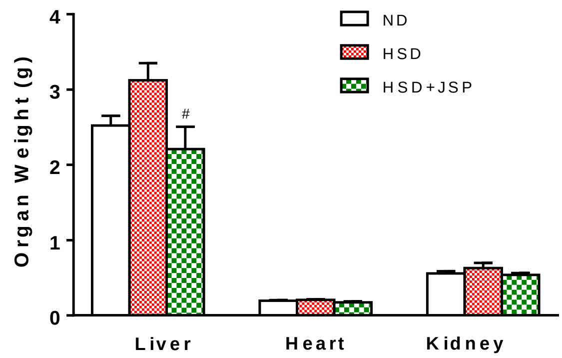


Figure 3. JSP supplementation with HSD improved glucose tolerance. After i.p. administration of glucose (2mg/kg BW), glucose tolerance test was performed at the end of the feeding trial. Blood glucose content was measured at 0, 15, 30, 60 and 120 min after i.p. glucose administration. The area under the curve (AUC) for glucose tolerance test was quantified. ND: Normal Diet; HSD: High Sugar Diet and JSP: Jackfruit Seed Powder. * $p < 0.05$ vs. ND; # $p < 0.05$ vs. HSD by one way ANOVA followed by Tukey's post-hoc test. Bars represent mean±SEM. $n \geq 3$ for each group.

3.4. Effect of Jackfruit Seed Powder (JSP) on Organs Weight of Mice

At the end of the feeding experiment, vital organs were obtained from euthanized animals and wet weights were measured. In comparison to the control group, the actual liver wet weight showed an insignificant increase ($p > 0.05$) in the HSD mice (Figure 4A). Jackfruit seed powder (JSP) supplementation significantly attenuated the weight of the liver in the HSD-treated mice (2.52 ± 0.13 g BW for ND, 3.12 ± 0.23 g for HSD and 2.21 ± 0.30 g for HSD + JSP). There were no significant different in heart and kidney weight of the mice (Figure 4A). As shown in Figure 4B, HSD-treated mice showed an increase in white adipose tissue (WAT) which was significantly reduced by JSP supplementation (0.50 ± 0.04 g BW for ND, 0.57 ± 0.02 g for HSD and 0.40 ± 0.02 g for HSD + JSP). Although it was statistically insignificant, the weight of brown adipose tissue (BAT) tends to increase in HSD diet fed mice as compared to the control group (Figure 4B). Jackfruit seed powder supplementation normalized the BAT weight in HSD diet fed mice.

A



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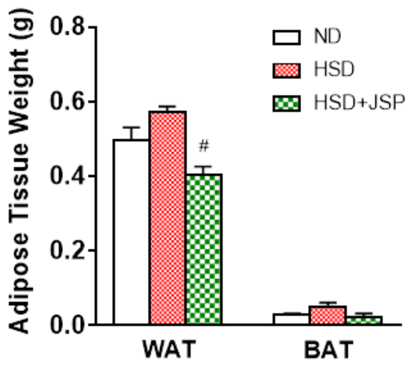

Figure 4. JSP supplementation significantly reduced liver weight and the weight of WAT**.** After 8 weeks of feeding experiment, animals were sacrificed and organs were isolated and weighed. (**A**) Weight of Liver, Heart and Kidney; (**B**) Weight of white adipose tissue (WAT) and brown adipose tissue (BAT). ND: Normal Diet; HSD: High Sugar Diet and JSP: Jackfruit Seed Powder. # $p < 0.05$ vs HSD by one way ANOVA followed by Tukey's post-hoc test. Bars represent mean±SEM. $n \geq 3$ for each group.

3.5. Effect of Jackfruit Seed Powder (JSP) on Lipid Profile Parameters

Blood lipid parameters were measured from serum collected after feeding experiment. HSD-fed mice showed an insignificant increase in serum total cholesterol (TC) and triglyceride (TG) concentrations in comparison to normal diet (ND)-fed group (Figure 5). JSP supplementation significantly attenuated the rise in serum TC and TG concentration that were observed in HSD-fed group (Figure 5). No significant difference was found in serum HDL-cholesterol among the groups. In HSD + JSP group, decrease in LDL-cholesterol concentration (30.27 ± 9.79 g for HSD and 8.15 ± 5.24 g for HSD + JSP) which was statistically insignificant was observed after eight weeks of the experiment (Figure 5).

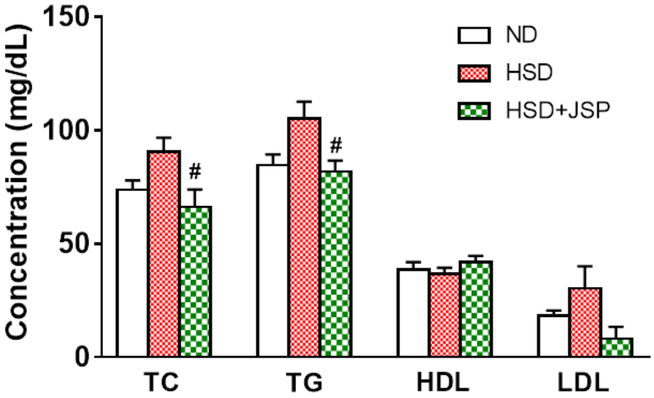

Figure 5. JSP supplementation significantly decreased total cholesterol and triglycerides in HSD-fed mice. ND: Normal Diet; HSD: High Sugar Diet and JSP: Jackfruit Seed Powder. # $p < 0.05$ vs HSD by one way ANOVA followed by Tukey's post-hoc test. Bars represent mean±SEM. $n \geq 3$ for each group.

4. Discussion

Our current findings revealed that the supplementation of jackfruit seed powder (JSP) could effectively preclude the excessive body weight gain caused by high sugar diet (HSD). Importantly, the supplementation of JSP also exerted a remarkable effect to hamper the increase in food intake due to high sugar diet consumption in mice. JSP supplementation in diet also improves glucose tolerance in mice distressed by high sugar consumption for a period of 8 weeks. Moreover, JSP supplementation also significantly reduced liver and WAT weight in mice as compared with HSD supplemented group. Serum TC and TG were significantly attenuated by JSP administration in HSD-fed mice.

Reduction in food intake is associated with complex hormonal and neuronal pathways involving appetite and satiety regulation [30,31]. Reduced food intake simply reduces energy intake that eventually lowers blood glucose and fat mass [31]. The possible mechanism of a substance to prevent the development of diabetes and obesity could be simply due to the reduction of food intake. In support of the above statement, our finding showed that the quantity of food intake was altered by jackfruit seed powder supplementation. Thus, the prevention of diabetes and obesity symptoms by JSP supplementation was likely to be corresponded with reduction of food intake.

Consumption of diets containing high sugar may induce an excessive body weight gain which accelerates the obesity development in rodents [14]. Previous studies reported that consuming high-sugar drinks and fast foods frequently could significantly increase the risk of having obesity and diabetes in humans [32,33]. Our present findings also demonstrated that the HSD-fed mice exhibited a tendency in body weight gain after 8 weeks of treatment, and it was significantly hampered by the 20% JSP supplementation in their diet. However, at the end of treatment, despite the final body weight of HSD-fed mice was higher; it was statistically comparable with control group. In support with our findings, previous study also demonstrated that mice fed with solid sugar diet remained insignificantly different in their body weight as compared with control group [34].

High sugar diet consumption is associated with the development of metabolic dysregulations including diabetes and obesity [35–37]. In this study, as expected, mice fed with HSD exhibited a slight increase in fasting blood glucose as observed at 0 min of glucose tolerance test (GTT). This finding might indicate an impaired blood glucose homeostasis toward diabetic development [38]. As observed in ipGTT, HSD-fed mice showed an impaired resistance to glucose (2 g/kg BW) challenge. However, supplementation of JSP with HSD remarkably improved glucose tolerance in mice. Previous study showed that the inclusion of soluble fiber in food of diabetic mice significantly reduced glucose basal levels in relation to mice that were fed without fiber [39]. The area under the curve at 8 weeks was significantly lower in the control group compared to that in the HSD group. Again, JSP supplementation significantly lowered the area under the curve in the HSD + JSP group, which was similar to the control group. In contrast to our findings, methanolic extract of jackfruit seed has been reported to increase the blood glucose levels compared to control animals in glucose-loaded mice [40]. Previous and current findings may differ due to the way of administration as we incorporated JSP into the diet instead of methanolic extract of seeds.

These results showed that the actual liver wet weight of mice in the HSD group was insignificantly higher than that of the control group. However, actual liver wet weight of the HSD + JSP group was significantly lower than that of the HSD group, indicating that JSP prevented liver enlargement. We did not find any significant difference in heart and kidney weight of the mice. The weight of WAT in the HSD group was relatively higher than that in the control group. However, JSP supplementation significantly decreased the weight of WAT in HSD + JSP mice. Mice in the HSD group showed a tendency to increase the weight of BAT than those in the control group. However, JSP slightly reduced the weight of BAT in HSD diet-fed mice. Jackfruit seed powder supplementation resulted in lowering of fat deposit and improvement of insulin sensitivity in high sugar diet fed mice.

Maintaining healthy levels of lipids circulating in blood stream is important to prevent cardiovascular diseases. Consumption of high sugar diet induces fatty liver or hepatic steatosis in mice. In this study, HSD consumption increased plasma total cholesterol (TC) and triglycerides (TG) levels in blood but it was insignificant. However, JSP supplementation significantly prevented the rise of plasma TC and TG in HSD-fed mice. Although there was a decreasing tendency in plasma LDL-C level in HSD+JSP group, plasma HDL-C levels were comparable among the group. The hypocholesterolemic action of jackfruit seed powder may be attributed due to the presence of flavonoids and phenolic compounds that enhanced lipid metabolism [41]. Besides this, jackfruit seed powder also contains appreciable quantity of non-digestible carbohydrates that has been reported to be associated with lowering the plasma cholesterol [42]. Indifference in HDL-C level may be due to absorption of intestinal cholesterol and enhanced cholesterol turnover to bile acids by bioactive compounds present in jackfruit seeds [43]. Further investigation is needed to completely understand the beneficial effects of jackfruit seed powder consumption in maintaining metabolic homeostasis.

5. Conclusions

The jackfruit seed powder could effectively sustain a normoglycemic state as well as body weight and food intake against the development of diabetes and obesity caused by HSD in mice. Addition of jackfruit seeds in diets may help to improve blood lipid profile that may attenuate the deleterious effect of high sugar diets consumption. As the seeds are usually discarded after consumption of the pulps, seed powder can be an alternative or complementary for wheat flour to prepare ready-made food. This powder could be potentially used as a supplemental diet to overcome the metabolic dysregulation in addition to achieve food security.

Author Contributions: Conceptualization, C.G. and R.C.; methodology, C.G. and R.C.; software, O.A.; validation, K.K., R.J. and M.H.; formal analysis, M.K.H.K.; investigation, M.K.H.K., O.A. and C.G.; resources, C.G.; data curation, C.G. and R.C.; writing—original draft preparation, C.G.; writing—review and editing, R.C.; visualization, O.A. and C.G.; supervision, C.G.; project administration, C.G.; funding acquisition, C.G. and R.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Council for International Organizations of Medical Sciences international guiding principles of biomedical research involving animals, and approved by the institutional Animal Welfare and Experimentation Ethics Committee of Bangladesh Agricultural University, Bangladesh (AWEEC/BAU/2020_30).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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