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Banana (*Musa balbisiana* Colla) Peels Flour Modulates HTR2B Receptor Expression in the Liver Diabetic Rats

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ABSTRACT

Uncontrolled diabetes mellitus (DM) has been linked to depression and lipid metabolism impairment. Serotonin (5-HT) has been known to improve lipid metabolism and affect mood. The 5-Hydroxytryptophan (5-HTP) in banana peel flour (BPF) is a precursor to synthesize 5-HT in the body, which is linked to initiating liver regeneration via HTR2B receptors. The impact of 5-HTP in the diet on HTR2B receptor expression has yet to be thoroughly investigated. We aimed to elucidate the correlation between serum lipid profile and immobility time in Tail Suspension Test (TST) as depressive-like behavior and compare the expression of HTR2B receptor in healthy and diabetic rats. Male Wistar (*Rattus norvegicus*) rats were assigned to the control and treatment groups in a DM model with streptozotocin (60 mg/kg) injected intraperitoneally, then either fed standard diet or BPF 10% supplemented standard diet for 21 days. Immunohistochemical staining was used to examine the expression of 5-HT, HTR2B receptors. We determined that diabetic rats fed the standard diet supplemented 10% BPF group showed significantly lower concentrations of total cholesterol and triglycerides compared to diabetic rats fed a standard diet and it was positively correlated between total cholesterol and LDL with the duration of immobility time in TST. The average immunoreactivity score in diabetic rats fed 10% BPF was the highest among the other groups, indicating that the available BPF dose is sufficient for HTR2B activation, which will support the liver cell regeneration process, and should be investigated further.

1. Introduction

Diabetes is a chronic disease that frequently leads to a variety of complications, including lipid metabolism disorders, also known as dyslipidemia. (Schofield *et al.* 2016). Dyslipidemia is a condition of growth in plasma cholesterol concentrations, triglycerides, or both and can arise when low high-density lipoprotein (HDL) cholesterol concentrations. In patients with DM, hypercholesterolemia is a common concern, which demands secondary attention to the aggregation of triacylglycerol-rich lipoproteins due to the impaired enzymatic ability of lipoprotein lipase (Kingman 1991).

Dyslipidemia can lead to a variety of psychological issues, including depression (Ancelin *et al.* 2010). Previous research has suggested that the prevalence of depression is almost twice as high in people with DM and that depression may also increase the risk of developing type 2 DM (Ali *et al.* 2006; Roy and Lloyd 2012). High cholesterol levels place the DM patient at increased risk for depression and that abnormal blood lipid levels can increase the risk of depression and controlling HDL and LDL levels is important in reducing risk factors for depression (Nakao *et al.* 2004). The relationship between high cholesterol and depression may be complicated (Kim *et al.* 2019).

Effective care and treatment are needed to overcome depression in DM patients, one of the easiest is to adopt a healthy lifestyle by choosing healthy food (Mohd Akhter Ali *et al.* 2017). Distress and various mental disorders happened in DM patients are closely related to serotonergic malfunctions frequently associated with the

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increased conversion of tryptophan to kynurenine and decrease 5-HT synthesis, and it will exacerbate the complications (Mackay *et al.* 2009). Consumption of foods containing 5-HTP plays a role in supporting the availability of 5-HT formation precursors. Several components of food play a role in influencing the availability of 5-HTP in the body for later use in the synthesis of 5-HT, one of which is complex carbohydrate compounds (Khalid *et al.* 2016). One of the fruits that are rich in complex carbohydrates and known to have anti-anxiety and improve the lipid profile is bananas. Not only the pulp, but banana peels also have the potential to be processed further (the portion of banana peels is about 40% of the total banana weight), because it contains phytoserotonin as an antidepressant content of around 170,000 ng/g and the pulp contains around 35,000 ng/g (Rayne 2010). Several studies have demonstrated the effect of banana peel consumption on improving serum lipid profiles, in rats with acute liver failure, intervention of dried banana peel with doses of 5% and 10% increased HDL-c levels significantly compared to a positive control group. (Mosa *et al.* 2015), and the banana peel has hypolipidemic effects by a reduction in total cholesterol serum and triglycerides (Edenta *et al.* 2014).

5-HT is a biogenic amine that aids in the maintenance of energy homeostasis. When 5-HT enters the bloodstream, it interacts with various organs, preparing the body for energy storage by promoting insulin secretion in the pancreas and initiating the process of liver cell regeneration (Lesurtel *et al.* 2006). The presence of a 5-HT receptor subtype, HTR2B in hepatocytes will form a parenchymal mass in the liver, after 5-HT depletion and after inhibition of 5-HT antagonist activity (Bonhaus *et al.* 1995). During the process of liver regeneration, hepatocytes are thought to have replicated once or twice, and after reaching the previous liver size and volume, the hepatocytes return to their original state (Rmilah *et al.* 2019).

It is hypothesized that BPF's lipid-lowering effect is due to its 5-HTP content, involved in the synthesis of 5-HT, which initiates cell regeneration via the HTR2B receptor in the liver, thereby improving lipid metabolism. As a result, DM complications can be managed and the risk of depression reduced (Gilgenkrantz *et al.* 2018). However, the effect of tryptophan in the diet on HTR2B receptor expression has yet to be thoroughly investigated. Based on the previous reviews, this study aimed to prove the effect of BPF intervention on serum lipid profiles, to study the correlation between serum lipid profile to immobility time as a marker of depressive-like behavior in rats, and compare the expression of 5-HT2B receptors of the liver in healthy and diabetic rats.

2. Materials and Methods

2.1. BPF Preparation

The samples, classified as *Musa balbisiana* Colla species, were collected in the city of Sleman, Yogyakarta, Indonesia. The process of making BPF consists of the following stages: washing the banana peel (*Musa balbisiana* Colla) with running water, slicing the size smaller, soaking in a 0.5% citric acid solution, steaming for 5 minutes, crushing the ingredients using a blender with the ratio of ingredients: water = 1: 2 for 3 minutes, drying using a drum dryer with a pressure of 3-4 bar, at a temperature of 140°C, then refining the flakes using a disk mill to produce flour particles that pass 80 mesh sieve.

2.2. Chemical Analysis

Moisture content, the substance of protein, crude fat, ash, crude fiber, and dietary fiber were based on the description of BPF by the Association of Official Analytical Chemist (AOAC) methods. (AOAC 2010).

2.3. Estimation of 5-HT and 5-Hydroxytryptophan Compounds by HPLC

Detection of 5-HT and 5-hydroxytryptophan present in the banana peels used the HPLC (Shimadzu, Kyoto, Japan) system coupled to a Shimadzu Fluorescence Detector (FD) and controlled by Chromatography Data Software (Shimadzu, Japan) (Oktarina Tisadewi 2016).

2.4. Animals Experimental Protocol

Healthy adult male Wistar rats (*Rattus norvegicus*) weighing 150-220 g were used in this study after approval and ethical clearance from the Medical and Health Research Ethics Committee, Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia (Ref: KE/FK/0313/EC/2020). The animals were handled individually in polypropylene cages under a 12 h light-dark cycle (light on at 6:00 h) with room temperature (22±2°C) with free access to standard diet food and tap water during the experiment. Before starting the experimental work, test animals underwent a one-week acclimation period.

Rats were assigned to control non-diabetic and experimental diabetic groups. Diabetic conditions were induced by intraperitoneal injection of streptozotocin (60 mg/kg body wt; Sigma-Aldrich, St Louis, MO) diluted in citrate-buffered saline (0.1

mol/l, pH 4.5; Sigma-Aldrich). The control non-diabetic group (n = 10) was fed standard dieting (AIN-93) and the experimental diabetic group (n = 10) was fed a standard diet supplemented with 10% BPF. Both groups had free access to 5% dextrose for 72 h. Twenty rats with blood glucose concentrations of more than 250 mg/dl were included in this study.

The dosage for using a standard diet supplemented with 10% BPF was determined by the results of a preliminary study, which revealed that the tryptophan content in BPF at a dose of 20% had no significant impact on HTR2B expression and lipid profile improvement. Furthermore, we chose to focus on the 10% BPF dose; this finding is consistent with the fact that tryptophan metabolism involved a rate-limiting enzyme, implying that higher tryptophan supplementation will not increase the antidepressant effect any further.

2.5. Tail Suspension Test (TST)

TST is a validated test for depression in rats based on the duration of immobility time when rats are exposed to the inescapable situation (Castagné *et al.* 2010) and TST detects the anti-immobility effects of a wide array of antidepressants (Chahardehi *et al.* 2012). Concisely, mice were suspended separately 50 cm over the tabletop by a paper adhesive tape placed around 1 cm from the tip of the tail. Mice were suspended for 6 min and the Cumulative immobility time during 6 minutes recording was considered as a depressive score. The mice were considered motionless when they remained passive and motionless for more than 5 s (Liu *et al.* 2015).

2.6. Serum Lipid Measurement

Blood specimens were collected from their eyes and set into liver-coated tubes. Blood specimens were centrifuged at 6,000 rpm for 10 minutes at 4°C and serum was collected to determine serum total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglyceride concentrations. HDL-C and LDL-C were based on an HDL and LDL cholesterol Quantification Assay Kit (Abcam, Cambridge, MA). The triglyceride concentration was based on a colorimetric measurement using a Triglyceride Quantification Assay Kit (Abcam, Cambridge, MA).

2.7. Procedure of Immunohistochemistry

Immunohistochemistry (IHC) analysis was performed to visualize and compare the distributions of 5-HT receptors in healthy and diabetic rats. Anti-5-HT HTR2B Receptor antibody produced in rabbit (Sigma Chemicals Co, St Louis, MO, USA) antibodies and Fine Test rabbit-DAB (Poly-HRP) were used to detect the target proteins in paraffin sections of the liver. The careful selection for biopsies, including the most representative areas of histological alterations of the paraffin-embedded liver, aimed at minimizing the effects of heterogeneity of liver lesions (Fedchenko and Reifenrath 2014).

Slides next underwent deparaffinization in xylene, after rehydrating tissues utilizing graded ethanol, washed with purified water for 2 min, then washed with tris buffered saline (TBS) 3 times for 3 min each. Antigen recovery was activated in citrate support pH 6 for 20 min, and the slides were put into TBS and afterward washed by TBS multiple times for 3 min each. Finally, impeding serum was added to the tissue segment to obstruct vague immunostaining and incubated for 1 h at 37°C. An abundance of any hindering serum was blotched from areas.

A solution of hematoxylin Meyer's was diluted with purified water (1:4) for 1-3 min. The slides were plunged into a hematoxylin answer for staining. At that point, slides were washed in running water and dried out by plunging in ethanol. Finally, the slides were washed in xylol and mounted in Frontier Duo EFH (Canada) and examined with an Olympus CX21/ Optilens Optilab Standard light magnifying lens (Carl Zeiss, Oberkochen, Germany) and captured with a computerized camera.

The principle of IHC examination is the detection of antigens by specific antibodies. In IHC staining, positive control and negative control are always included, based on the method used by (Fedchenko and Reifenrath 2014). The immunoreactivity of the 5-HT receptors in the liver was determined by a semiquantitative method by looking at the absorption of color. In the histological preparations that had been painted, five fields of view were selected randomly and continuously. Then, pictures were taken using Optilab (Miconos). For each image, the number of cell nuclei was counted that showed as brown cytoplasm in non-necrotic areas. The calculation was done manually using cell encounters with the Image-J software. Each field of view counts the number of cells according to their intensity. Intensity is expressed in numbers, where 3 for strong intensity, 2 for medium intensity, 1 for weak intensity, and 0 for negative intensity.

2.8. Allred Score Determination

This technique has been depicted in past research, where the numerical value for overall intensity used the formula: [intensity score (IS)] depended on a 4-point framework: 0, 1, 2, and 3 (for none, light,

medium, or dull staining). The numerical value for percent stained [proportion score (PS)] was dictated by a mathematical = rather than direct division, no stain = 0, ≤1/100 cells stained = 1, ≤1/10 cells stained = 2, ≤1/3 cells stained = 3, ≤2/3 cells stained = 4, all cells stained = 5. The expansion of the two values gave the all-out Allred score, with the Allred score in the range of 0 and 8 (Chand *et al.* 2018).

2.7. Statistical Analysis

Data are presented as the mean ± SD. The chemical composition of banana peel, level of profile lipid, immobility time, and expression of HTR2B in the liver. The results were compared statistically by the One Way Anova and followed by the least significant difference (LSD) test. The differences significant was indicated as p-value <0.05. Pearson's correlation was used to determine the relationship between serum lipid profile concentrations and immobility time with the tail suspension test as a parameter for depressive-like behavior.

3. Results

3.1. The Chemical Composition of Banana Peel

The proximate compositions of banana peel at three different conditions are shown in Table 1. The fresh banana peel has more moisture content compared to the others. The results of decreasing moisture content, due to the drying process, ranged from 9.92 to 83.47%, with BPF showing the lowest moisture content. This is likely due to the effect of heat on the sample during drying. The dietary fiber of banana peel fresh and flour was 7.88% and 10.50%, respectively. Table 2 shows the quantities of 5-HT and 5-HTP present in the fresh banana peel, BPF, and the standard diet containing 10% BPF. The HPLC profiles of 5-HT and 5-HTP are shown in Figure 1.

3.2. Effect of the BPF on Serum Lipid Concentration

The results of total cholesterol, triglyceride, cholesterol-HDL, and cholesterol-LDL are presented in Table 3. The table reveals that the administration of 10% BPF supplementation on standard diets reduced the total triglyceride and increased HDL-cholesterol with a significant difference compared with the non-diabetic and diabetic rats group fed the standard diet.

3.3. Effect of the BPF Diet on Depressive-like Behaviors

The tail suspension test was used to determine whether the diabetic condition produced a depressive condition, where the immobility time represents helplessness found in depression in the rats. Immobility time was significantly higher in the diabetic group. Group analyses revealed that immobility time was significantly lower in rats fed with BPF in both the diabetic group and non-diabetic group, indicating lower depressive-like behavior in rats fed with BPF (Table 4).

3.4. Correlation between Immobility Times and Serum Lipid Concentrations

The correlations between the duration of immobility time in the TST and serum lipid profile of non-diabetic and diabetic rats are shown in Table 5, there was no significant correlation between the duration of immobility time and the serum lipid profile of non-diabetic rats. However, in the diabetic

Table 1. Chemical analysis (% dry weight basis) of banana peel fresh, BPF, and the standard diet containing 10% BPF

Sample	Banana peel fresh	BPF	The standard diet containing 10% BPF
Proximate (% wb)			
Moisture	83.47±0.25[a]	9.92±0.01[b]	11.50±0.16[c]
Ash	1.91±0.08[a]	13.71±0.03[b]	3.97±0.18[c]
Protein	1.00±0.00[a]	6.77±0.07[b]	9.97±0.03[c]
Crude Fat	0.29±0.14[a]	7.05±0.01[b]	3.67±0.37[c]
Crude fiber	10.25±0.16[a]	21.00±0.78[b]	9.11±0.09[c]
Dietary fiber	7.88±0.11[a]	10.50±0.04[b]	10.12±0.04[c]

Data are expressed by mean ± SD. Different letters in the same line indicate statistical significance, p<0.05

Table 2. Quantitative analysis of 5-HT and 5-hydroxytryptophan in BPF

Sample	Banana peel fresh	Level (ppm) BPF	The standard diet containing 10% BPF
5-HT and its precursor			
5-HT	5.49±0.15[a]	5.90±0.34[a]	0.99±1.11[b]
5-Hydroxytryptophan	0.41±0.29[a]	0.22±0.02[b]	0.06±0.05[c]

Data are expressed by mean ± standard deviation (SD). Different letters in the same line indicate statistical significance, p<0.05

rat's group, the immobility time was positively correlated with total cholesterol and LDL-cholesterol, which means the increase in total cholesterol and LDL-cholesterol make immobility time with the TST significantly increased.

3.5. Effect of BPF Diet on 5-HT Receptor Expression in Liver

The average result of the observation of the immunoreactivity score in each group had a normal data distribution, then the One Way Anova test was done followed by the LSD test. The results showed there were differences in the diabetic rat groups with standard feed and those fed 10% BPF (Table 6 and Figure 2).

4. Discussion

Uncontrolled diabetes can lead to a variety of complications, one of which is depression. The effect of BPF intervention containing 5-HTP as a precursor for the formation of 5-HT in the liver via the HTR2B receptor was investigated in this study. 5-HT in the liver aids in the regeneration of liver cells, which

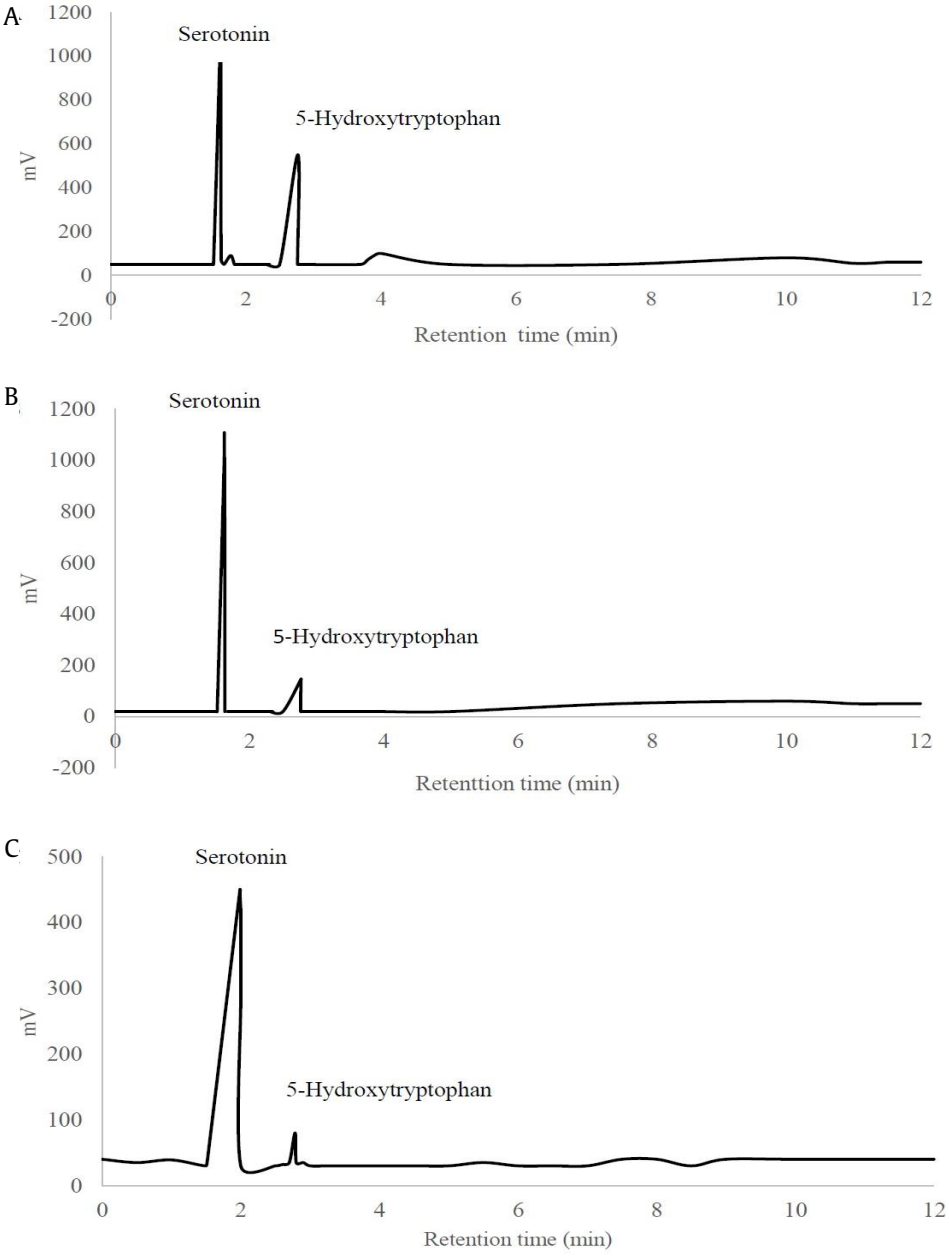

Figure 1. HPLC chromatogram showing serotonin and 5-Hydroxytryptophan in banana peel fresh (A), BPF (B), and the standard diet containing 10% BPF (C)

Table 3. Effect of BPF on serum total cholesterol, triglycerides, HDL and LDL in streptozotocin-diabetic male albino rats

Parameters		Non-diabetic rats		Diabetic rats	
		Standard diet	BPF	Standard diet	BPF
Total cholesterol (mg/dl)	Pre	73.40±4.24	66.80±2.40	80.60±16.54	79.30±3.68
	Post	75.30±4.67	67.00±0.57	92.40±24.75	75.80±1.56
	Δ	1.90±0.42	0.20±1.83	11.80±8.20	-3.50±2.12#
Triglycerides (mg/dl)	Pre	73.20±10.04	68.10±6.90	86.47±4.47	182.80±125.32
	Post	101.53±35.64	69.17±4.96	56.30±17.91	78.43±6.10*,#
	Δ	28.33±25.80	1.07±8.83	-22.53±26.46	-104.38±128.91*#
HDL-C (mg/dl)	Pre	42.05±1.06	41.60±0.14	42.05±0.63	40.90±0.57
	Post	42.35±5.73	53.30±0.00	43.50±7.78	60.45±10.82
	Δ	-1.90±0.99	11.70±0.14	1.45±7.14	19.55±10.25*,#
LDL-C (mg/dl)	Pre	25.15±1.48	21.52±4.68	23.55±4.98	25.95±11.24
	Post	19.20±3.96	18.24±2.52	23.43±4.65	28.10±2.838*#
	Δ	-5.95±5.44	-3.28±4.97	-0.13±6.93	2.15±14.07

Values are expressed as mean ± standard deviation (SD). The symbols represent statistical significance: *, # (p<0.05) *Significant difference vs. non-diabetic rats fed the standard diet, # Significant difference vs. diabetic rats fed the standard diet. HDL-C: high-density lipoprotein cholesterol, LDL-C: low-density lipoprotein cholesterol

Table 4. Depressive-like behaviors in non-diabetic and diabetic rats

Groups and diet	Immobility time (s) Immobility time (s)
Non-diabetic group	
Standard	70.50±23.33
BPF	53.00±9.89*#
Diabetic group	
Standard	164.00±16.97*
BPF	108.00±1.41*#

Values are expressed as mean ± standard deviation (SD)
The symbols represent statistical significance: *, #, (p<0.05).
*Significant difference vs. non-diabetic control, # significant difference vs. diabetic control

Table 5. Analysis of the correlation between duration of immobility time in tail suspension test and serum lipid profile of non-diabetic and diabetic rats.

Index	r- value	p- value
Non-diabetic group		
Total cholesterol	0.881	0.119
Triglyceride	-0.416	0.584
HDL-C	0.216	0.861
LDL-C	0.396	0.741
Diabetic group		
Total cholesterol	0.976	0.024*
Triglyceride	-0.548	0.260
HDL-C	-0.966	0.034*
LDL-C	0.955	0.045*

Tested by pearson correlation
*Significantly different, p<0.05

Table 6. Immunoreactivity of rat liver cell score by immunohistochemical staining with 5-HT HTR2B anti-receptor

Groups and diet	Immunoreactivity score
Non-diabetic group	
Standard	7.00±0.72
Diabetic group	
Standard	6.26±0.31
BPF	8.53±0.80*

Values are expressed as mean ± standard deviation (SD)
The symbols represent statistical significance: *, #, (p<0.05).
*Significant difference vs. non-diabetic control, # significant difference vs. diabetic control

can support the improvement of lipid metabolism (Lesurtel *et al.* 2006). The improvement in lipid metabolism will also reduce the level of depression in rats with diabetes, which is indicated by a significantly lower immobility time value (p<0.05) compared to diabetes control without BPF intervention. 5-HT is a neurotransmitter that regulates mood in the human brain. The high concentration of 5-HTP, a 5-HT precursor, indicates the possible antidepressant properties of banana, particularly present in its peel. The depressive condition was strongly correlated with serum total cholesterol, HDL-cholesterol, and LDL-cholesterol concentrations. The pathophysiological

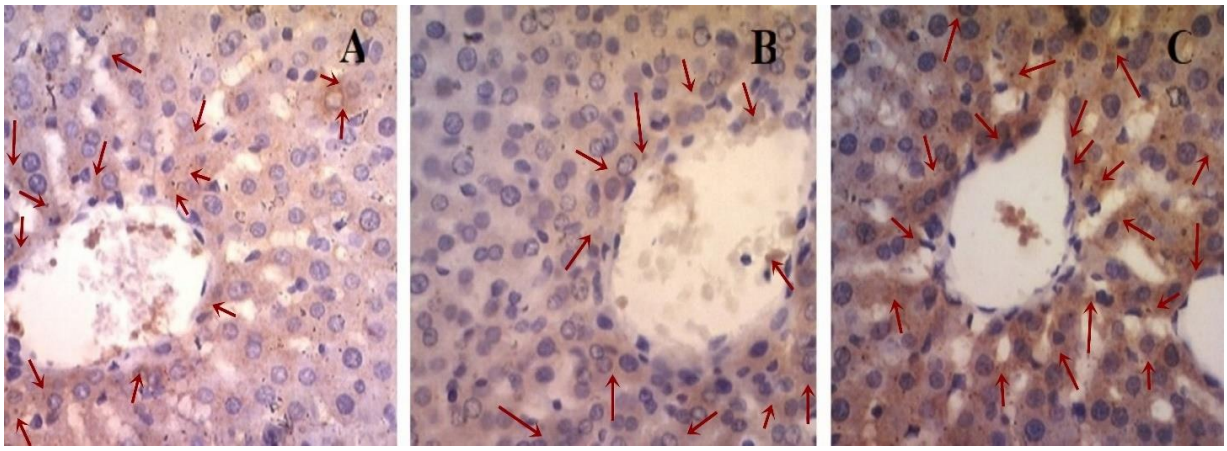

Figure 2. Microscopic photographs of liver cells stained with IHC, showing weak (A), intermediate (B), and strong positive (C) HTR2B 5-HT receptor expressions in the liver. Redarrows indicate HTR2B expression

mechanism associated with the relationship between cholesterol levels and depression is still being debated. Low serum cholesterol levels are associated with decreased serotonergic function (Papakostas *et al.* 2004). The exact mechanisms are still unclear by which serum cholesterol, HDL, and LDL affect depressive and depression in DM. Nevertheless, a previous study has reported similar results (Remage-Healey and Romero 2001), they found a stress-responsive increase in glucose and cortisol concentrations as well as a decrease in triglyceride concentrations.

5-HTP contained in BPF will be used for 5-HT formation in the digestive tract, especially in enterochromaffin cells which will synthesize, store and release most of the body's 5-HT. 5-HTP can also cross the blood-brain barrier so that it can play a role in the formation of 5-HT in the central nervous system, wherein in stressful conditions, there is a reduction in 5-HT levels in the brain. Peripheral 5HT in the liver has an effect on glucose and lipid metabolism in a variety of cell types, including white and brown adipocytes, -cells, myocytes, and hepatocytes (Yang *et al.* 2017; Cataldo *et al.* 2019). Furthermore, serotonin functions are mediated by various serotonin receptor subtypes, these findings suggest that peripheral serotonin speeds up lipid metabolism by increasing the concentration of bile acids in circulation (Watanabe *et al.* 2010).

The liver is the first organ that is directly related to the increased production of Gut Derived 5-HT (GDS) that circulates through the portal vein. Excess availability of 5-HT will activate the 5-HT system by activating 5-HT receptors, where the activated receptor is HTR2B in the liver. The expression of HTR2B receptors is closely related to the presence of HSC activation in the normal liver, but in a diseased liver, HTR2B expression is very abundant (Ebrahimkhani *et al.* 2011). Research on HTR2B activation has been conducted, and it was found that using selective 5-HT2B antagonists will increase hepatocyte growth, reduce fibrogenesis and improve hepatic function in an already diseased liver. Accordingly, using drugs that target the action of 5-HT2B is an option and is promising in patients with a diseased liver (Oh *et al.* 2016). It was found that the HTR2B expression was significantly different between groups, where the mean score of immunoreactivity in diabetic rats fed BPF 10% had the highest expression among other groups because DM-modeled mice fed with 10% BPF had a 5-HT dose sufficient for the activation of HTR2B which would be related to the regeneration process of hepatic cells or perhaps prevention of hepatocyte fibrogenesis which should be investigated further.

This study supports the hypothesis that diabetic rats given the 10% BPF group diet supplement had significantly lower concentrations of total cholesterol and triglycerides than diabetic rats fed the standard diet and that total and LDL cholesterol were positively correlated with duration of immobility time in TST. The average immunoreactivity score in diabetic rats given 10% BPF was the highest compared to the other groups, indicating that the available BPF dose was sufficient for HTR2B activation, which would aid in the regeneration of liver cells. The presence of a serum lipid profile will have an impact on controlling depression as a manifestation of diabetes, as evidenced by improvement of serum lipid profile.

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