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Full Length Research Paper

Sphenostylis stenocarpa (Hochst. Ex A. Rich) and fermented *Parkia biglobosa* ((Jacq, R.B.R.) supplementation prevents high fat diet induced derangements in kidney of albino rats

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Prevalence of obesity is on the increase globally with high fat diet (HFD) known to be the main contributing factor. This study was carried out to determine the actions of fermented *Parkia biglobosa* (*Iru*) and *Sphenostyles stenocarpa* (*Otili*) on the kidney of obese induced albino rats. The rats were grouped into a control group fed with normal rats chow and three different high fat diet groups (HFD[1], HFD[2], HFD[3]) mixed with different proportions of *P. biglobosa* and *S. stenocarpa*. After feeding *ad-libitum* for six weeks blood samples were collected to determine albumin, total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL) serum low density lipoprotein, enzymes (ALT, ALP, and AST) and Kidney histopathology. Results showed that there was a significant reduced body weight ($p<0.05$) in the treated rats compared with the control animals. Furthermore, the plasma lipid profiles were also improved, with a decrease in total cholesterol (TC), triglycerides (TG), low density lipoprotein (LDL) while boosting the high density lipoprotein (HDL). Similarly, histological examination revealed normal kidney with no negative changes such as dilation in blood vessels, cell infiltration, tubular defects, etc associated with taking high fat diet. In conclusion, supplementing a combination of fermented *P. biglobosa* (*iru*) and *S. stenocarpa* (*otili*) into diets show promise as a natural and safe anti-obesity agent that can ameliorate renal biochemical and histopathological changes associated with obesity.

Key words: *Parkia biglobosa*, *Sphenostyles stenocarpa,* high fat diet, kidney, obesity.

INTRODUCTION

Obesity is one of the leading but avoidable causes of death worldwide (WHO, 2000; Paras et al., 2011).

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Currently, about 1 billion adults are overweight and at least 300 million of them are obese. Obesity is responsible for 2-6% of total health care costs in many developed nations (Puska and Stahl, 2010; Greenway and Smith, 2000).

Obesity is itself a diseased state and is also a risk factor for many other diseases. It decreases life expectancy, increases the risk of heart diseases, stroke and gout (Norris et al., 2005; Slanc et al., 2009; Karallas et al., 2009). It is associated with obstructive sleep apnea and reduces the quality of life; it has been linked with increased risk of some surgical and post-surgical complications (Popkin, 2001; De Ferranti and Monzaffarian, 2008). It is equally estimated that, by the year 2030, about 58% of the world population will be obese (Peltonen et al., 2003; Diament et al., 2003; Finucane et al., 2014). Obese is defined by Body mass index (BMI) greater than 30 and further evaluated in terms of fat distribution via the waist–hip ratio and others (Sweeting, 2007; Flegal et al., 2001; Paras et al., 2011).

Preliminary data evidence revealed that eating beans-based diet such as *Sphenostylis stenocarpa*; known as wild yam beans and Otili in the Yoruba tribe of Nigeria competed favorably with the common edible bean, *Phaseolus vulgaris* in bioactive compound constituents (FAO, 1988; Ejere et al., 2018). So far, we have been able to have an insight into the organic products that underlies the chemo-preventive activities of this underutilized wild bean (Awoyinka et al., 2016). *Parkia biglobosa* (African locust bean) although commonly consumed locally as condiment is overlooked as a gem in disease management (Balunas and Kingworm, 2005; Millogo-Kone et al., 2006; Oguntola, 2019). In past decades there have been records on the use of *P. biglobosa* in traditional medicine-traditional healers in Senegal and in the South Western region of Nigeria use it for the treatment of diabetes mellitus (Dièye et al., 2008). The yellow pulp, containing the seeds is naturally sweet “and can be processed into food as well as seasoning, known as dawadawa among the Hausas in Nigeria and Ashanti tribes of Ghana. The pulp is also used to make beverages (Pieroni, 2005; Olaniyan, 2013).

Recently, many researches have focused on improving health through diet and natural resources, thus, this project would be a contribution to such innovations. The present study aims to rationally investigate the impact of regular intake of *P. biglobosa* and *S. stenocarpa* on the kidney of albino rats fed with a very high fat diet. This is intending to establish scientifically, the benefits (if any) of its intake given the importance and widespread popularity of both beans (Awoyinka et al., 2018).

MATERIALS AND METHODS

Chemicals

All the chemicals/reagents used in the current research were of analytical grade and were purchased from Sigma Chemical Company (St. Louis, Missouri, USA. Diagnostic kits for enzyme analysis were purchased from Randox Laboratories (USA).

Collection and preparation of materials

Dry wild bean, *S. stenocarpa* were sourced from the open bushes within Ado Ekiti metropolis while *P. biglobosa* were bought from a local market and authenticated with a voucher number- (UHAE-1010065). The *P. biglobosa* were collected with the pods. The seeds were identified and authenticated by the Chief Botanist of the Department of Plant Science, Ekiti State University and deposited in the University Herbarium with Voucher Number (UHAE 2020063).

The *S. stenocarpa* beans were further sun dried for some hours and later blended into powdered form using a blender. This was stored in a tight container prior to use. For preparation of *P. biglobosa,* the seeds were selected and rinsed with clean water. Then the seeds were initially boiled for 2 h, followed by de-hulling and washing with cold water. Thereafter, the seeds were boiled again for 45 min and drained using a plastic sieve. For fermentation of the *P. biglobosa* seeds, starter culture that is, *Bacillus subtilis* was introduced after draining, the seeds were then spread into a fermenting can and wrapped with cloth for 48 h. Subsequently, the fermented *P. biglobosa* seeds were dried in an oven at a temperature of 55 to 60°C for 5days and powdered using a blender before storing in a tight container.

Experimental animals

Fifty albino rats with no bias in their sex were obtained from the Animal House, Faculty of Basic Medical Sciences, Ekiti State University Ado Ekiti. They were housed in plastic cages with steel wire lids, at room temperature with adequate access to rat chow and water throughout the experimental period. We got Ethical approval from the Experimental Animal Research Ethics Committee of Ekiti State University, Ado-Ekiti, (ORD/ETHICS/AD/043).The rats were separately grouped into experimental, and control group. They were fed for five weeks with bean and food formation of high fat diet adapted from Monk et al. (2019) while the control group was fed with regular rat chow diet formulated by BioOrganic Feeds (Table 2). Daily food consumption, body weight, behavioral and physiological changes were observed for four weeks as shown in Table 1.

Sacrifice of the animals and collection of tissue

All the animals were anesthetized with chloroform, sacrificed via an aortic cut and immediately dissected. Venous blood was immediately collected from the orbital vein placed in respective tubes before centrifuge to obtain serum. The kidney tissues were collected for biochemical estimation and the remaining portion were fixed using 10% formalin.

Lipid analysis

Preparation of the cholesterol fraction

Blood samples were collected in a test tube with no anticoagulant and allowed to clot at room temperature for half an hour before centrifugation at 2500 × g for 20 min. The serum layer was removed and stored on ice. However, extra care was taken to avoid disturbing the white buffy layer and stored at -80°C prior to performing the assay. Cholesterol level was assessed following Kit

Table 1. The experimental design.

S/N	Experimental group	Feed composition
1	Control	Normal Chow Diet
2	HFD[1]	40 g *Parkia biglobosa* + 40 g *Sphenostylis stenocarpa*+ 20 g fat.
3	HFD[2]	80 g fermented *Parkia biglobosa* + 20 g fat.
4	HFD[3]	80 g Fermented *Sphenostylis stenocarpa*+ 20 g fat.

Table 2. Composition of animal diets.

Ingredient (g)	Normal Chow diet (ND)	High-fat diet (HFD)
Casein,	200	200
L-Cystine	3	3
Corn starch	285	0
Maltodextrin	35	125
Sucrose	325	80
Cellulose	50	50
Soybean oil	25	35
Lard	20	350
Mineral mix,	10	10
Dicalcium phosphate	13	13
Calcium carbonate	5.5	5.5
Potassium citrate,	16.5	16.5
Vitamin mix,	10	10
Choline bitartrate	2	2
Total	**1000**	**896**

manufacturer instruction on the aliquot samples.

Preparation of HDL fractions

Blood was collected in tube containing citrate to avoid hemolyzes and centrifuge at 2000 × g and 4°C for 10 min. The white buffy layer-plasma was gently removed and stored on ice. Dilutions in 1X assay were performed on aliquot samples after storage at -80°C. Later, 200 µl of sample was added to 200 µl of the precipitation Reagent (Randox, USA) and mixed well by vortexing. The mixture was allowed to incubate for 5 - 10 min at room temperature before reading the absorbance at 570 nm.

Preparation of LDL/VLDL fraction

Pellet obtained after removal of the HDL fraction was re-suspended and dissolved in 400 µl of PBS and mixed well to obtain the LDL/VLDL fraction. Assay was carried out immediately by following the instructions of the Kit Manufacturers.

AST, ALT, ALP analysis

Blood samples were maintained at 4°C for 2h then centrifuged at 3000 × g for 20 min at 4°C. The supernatant was stored at -80°C total protein (TP), Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were analyzed assiduously following the method of Reitman and Frankel (1957) as described in the commercial Random Kit. Absorbance was determined using an automatic Biochemical Analyzer - Camspec M106 Spectrophotometer

Histopathological investigation

Following an incision in the anterior abdominal wall, the kidney from each group of rats were removed and fixed in 10% buffered formalin by total immersion for 48hrs as described by Alese et al. (2018). Thereafter, using an automatic tissue processor (Leica TP 1020), the organs were dehydrated using ethanol ranging from 50, 70 and 90%, Absolute 1 and 2 for 1 h each and cleared in xylene. Thereafter, the tissues were sectioned with a rotary microtome (Leica RM 2125 RTS) at a thickness of 4 µm, floated on a water bath and picked using glass slides. Various sections were then stained with H & E for the demonstration of general tissue architecture; photomicrographs were examined and taken at various magnifications under an OMAX 40X-2000X microscope.

Statistical analysis

The results are expressed as mean values ±S.E.M of three replicates. Results were considered significant with $p<0.05$ using statistical Graph Pad- prism software (2003).

RESULTS AND DISCUSSION

Obesity is associated with increased adipose tissue that

Table 3. Albumin and lipid profile.

Group	Parameter (MG/DL)				
	ALB	TG	TC	HDL	LDL
Sphenostyles stenocarpa + *Fat*	35.76±0.2	1.10±0.2	4.35±0.2	0.16±0.2	2.42±0.2
Fermented Parkia biglobosa+ *Sphenostyles stenocarpa*+ Fat	36.34±0.2	0.84±0.2	1.06±0.2	0.30±0.2	1.08±0.2
Fermented Parkia biglobosa + Fat	22.2±0.2	0.35±0.2	1.56±0.2	0.22±0.2	2.69±0.2
Control	41.64±0.2	1.90±0.2	1.60±0.2	0.28±0.2	1.79±0.2

ALB-Albumin, TC-Total cholesterol , TG- Triglyceride, HDL- High Density Lipoproteins, LDL- Low density lipoproteins.

results from both increased fat cell number and size. It is a lipoprotein disorder with derangement in the levels of triglycerides (Karalis, et al., 2009). Once there is an onset of obesity, patients need to use a synthetic drug to control their body weight, body mass index (BMI), and lipid profile levels (Puska and Stahl, 2010). Recently, there is an increasing popularity for the use of natural products in the management of obesity and other ailments. Weight gain is commonly associated with obesity; this occurs when energy uptake surpasses expenditure in an individual such that the store of energy in body fat is enlarged (Greenway and Smith, 2000). Obesity induction using a high fat diet in an animal model was used in this study as the approach has high relevance of mimicking the usual route of obesity occurrence in humans (Flint, 2011; Inukai, 2013).

In this study, the initial weights of the rats were between 120-125 g but after a week of obesity induction, there was an increase to133-142 g. This increase in body weight may be attributed to a disproportionate increase in organs like kidney and liver as reported by Christiansen et al. (1981) and Thomson et al. (2001) respectively. The consumption of high fat diets led to obesity because it facilitates the development of a positive energy balance, leading to an increase in visceral fat deposition, and thus abdominal obesity (Mercer and Archer, 2005). However, it was observed that feeding the rats on high fat diet with the powder of fermented *P. biglobosa* and *S. stenocarpa* might have had a hypolipidemic effect on the rats by significantly reducing their weight from an average of 133 to 115 g.

In the biochemical analysis of the lipid profile (Table 3), the results showed a significant decrease in albumin, triglycerides (TG), total cholesterol (TC) and Low density lipoproteins-cholesterol; (LDL), as well as increase in High density lipoprotein-cholesterol (HDL) for the groups fed with fermented locust beans+otili+fat (Figure 1). Albumin is the protein with the highest concentration with the highest concentration in plasma and it transports many small molecules in the blood (for example, bilirubin, calcium, drugs etc (Duncan et al., 1994). The significant albumin may suggest that the fermented *P. biglobosa* and *S. stenocarpa* have ability to inhibit in vivo protein biosynthesis.

Cholesterol is transported via blood by lipoproteins. HDL (good cholesterol) transports it from tissues to liver and LDL (bad cholesterol) does it in the opposite direction. Therefore decrease in serum LDL cholesterol is an indication of low rate of transportation of cholesterol from liver to tissues and subsequent transformation of triglycerides and cholesterol into bile acid by liver enzymes. Meanwhile, increase in albumin, TG, TC, LDL and decrease in HDL was observed in high fat diet only (HFD). This is because dietary cholesterol raises TC, TG and LDL levels. The intake of cholesterol rich food has been positively related to hypercholestolemia and risk of cardiovascular diseases (Zulet et al., 1999). Thus it can be suggested that the extract of the two plants could have an effect on dietary cholesterol which could result in the level of cholesterol in the blood. This is similar to the work of Jorge et al. (1998) who carried out work on the effect of eggplant juice on plasma lipid levels.

The kidney is an important organ that is responsible for the metabolism, detoxification, storage, and excretion of xenobiotics and their metabolites. It can be damaged by external substances. Intracellular enzymes including AST, ALT and ALP appear in the plasma and are indicative of cellular damage (Evans et al., 2014; Debelo et al., 2015). Hence, their serum levels could be used to assess any situation of organ damage, particularly with the presence of established normal ranges for the detection of organ damage (Oh and Hustead, 2011). As seen in Figure 2, results from this work show that feeding the HFD rats with fermented *P. biglobosa* and *S. stenocarpa* significantly reduces the activities of these enzymes in the kidney when compared with the group fed the HFD alone. However, in Figure 3 biochemical analysis of the serum revealed that the activities of the enzymes were increased in the treatment animals when compared with the animals fed with HFD only. Injac et al. (2008) explained that the increase in the serum enzyme levels helps in contributing to increased leakage from damaged and necrotic cells preventing the kidney and other organs from effects of atherosclerosis.

As seen in Figure 4, the photomicrographs show the architecture of the representative kidney sections from the experimental groups. In Figure 4A, the HFD group fed with fermented *P. biglobosa* shows an essentially normal kidney tissue but with splitting of the glomerulus in few areas. Similarly, as seen in Figure 4B, the HFD group fed

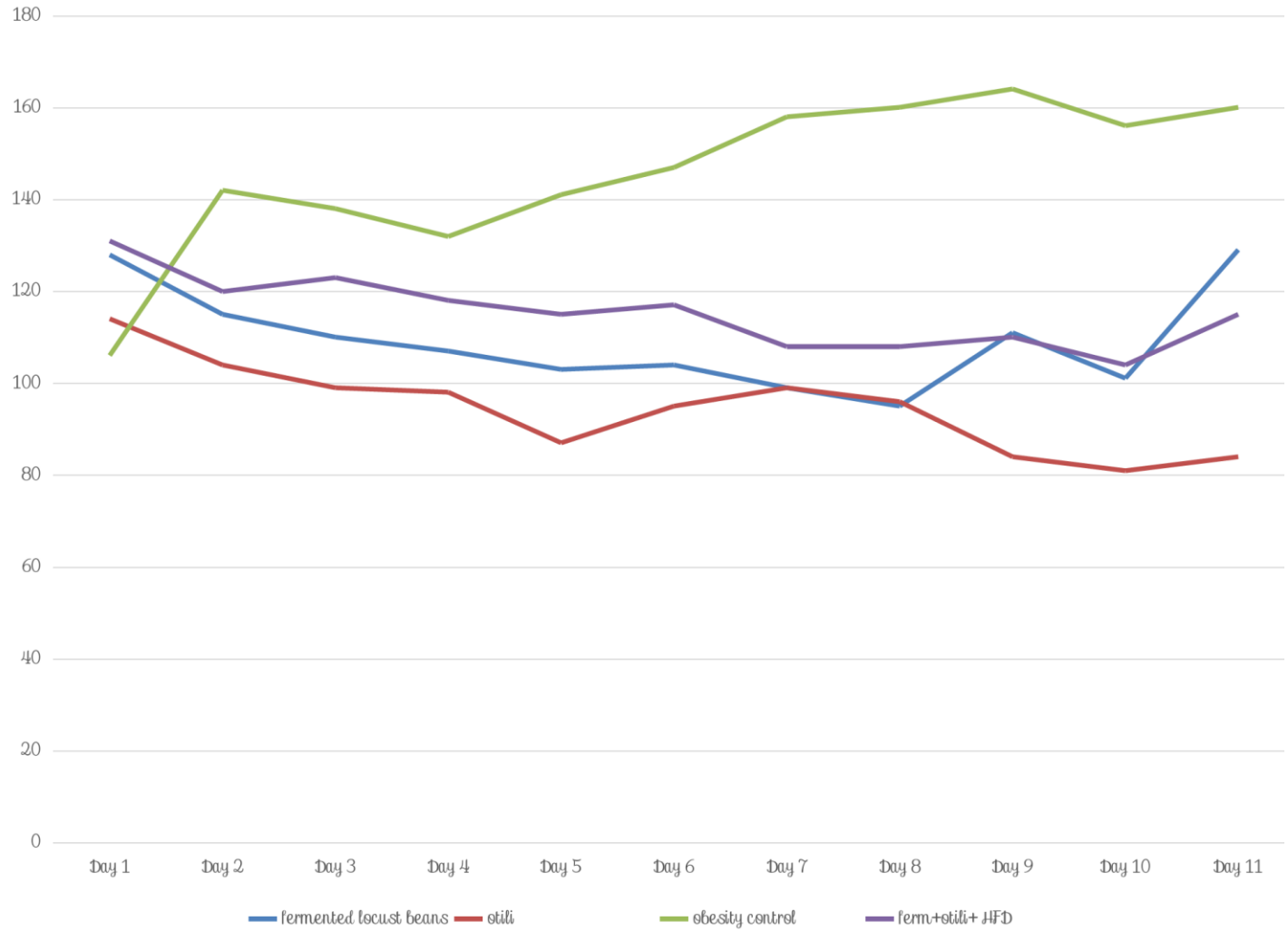

Figure 1. Mean Weights of rats taken every 3 days through the duration of the feeding.

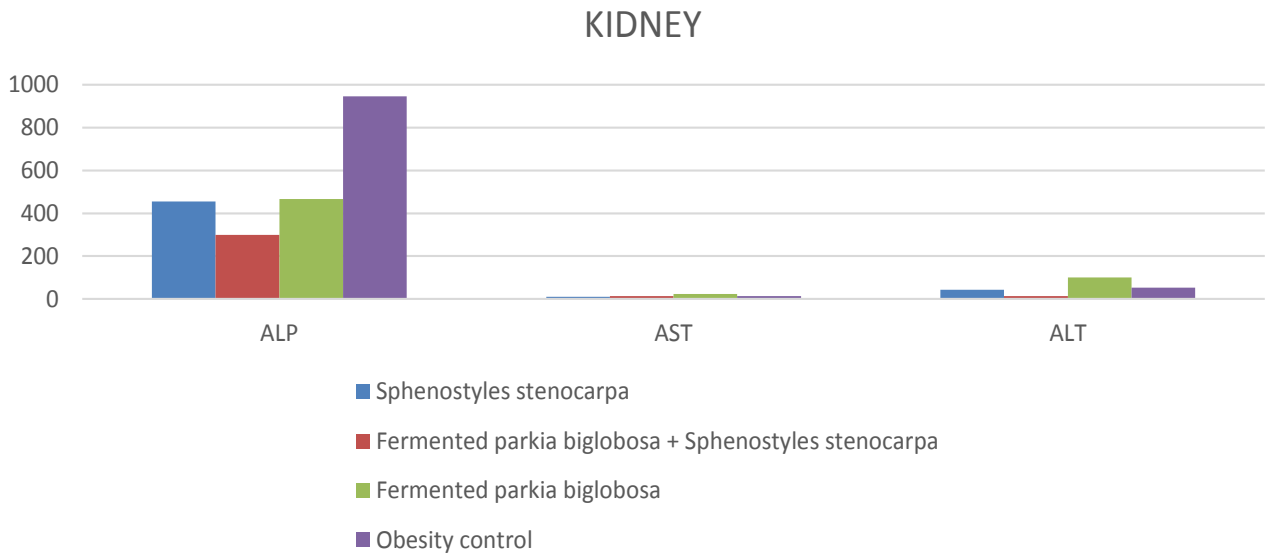

Figure 2. Level of ALP, AST, ALT in Kidney tissue after treatment.

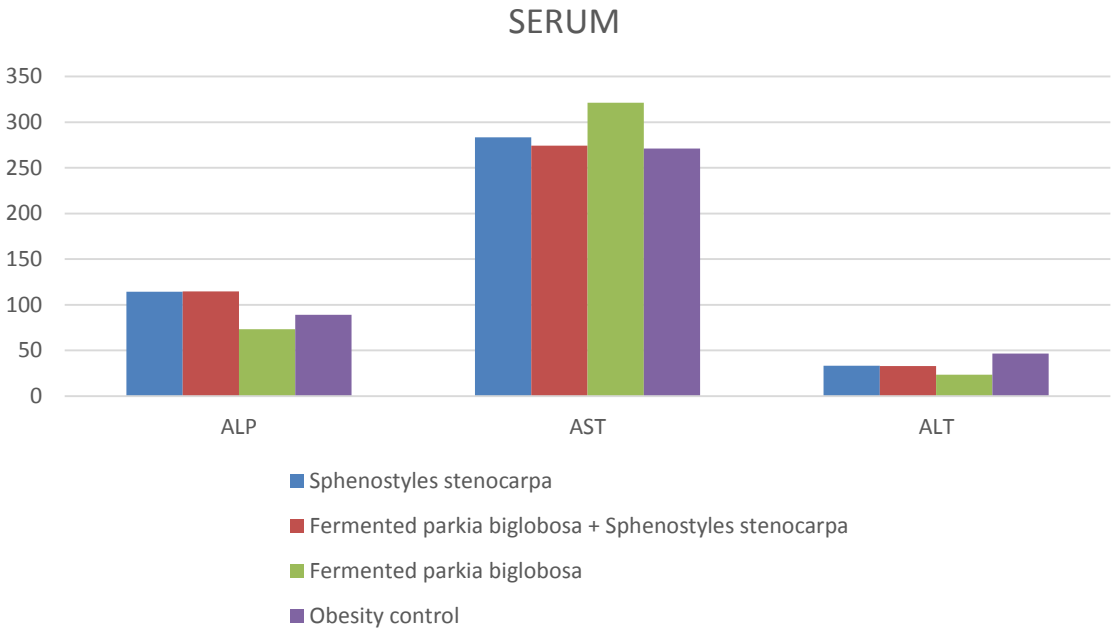

Figure 3. Level of ALP, AST, ALT in Serum tissue after treatment.

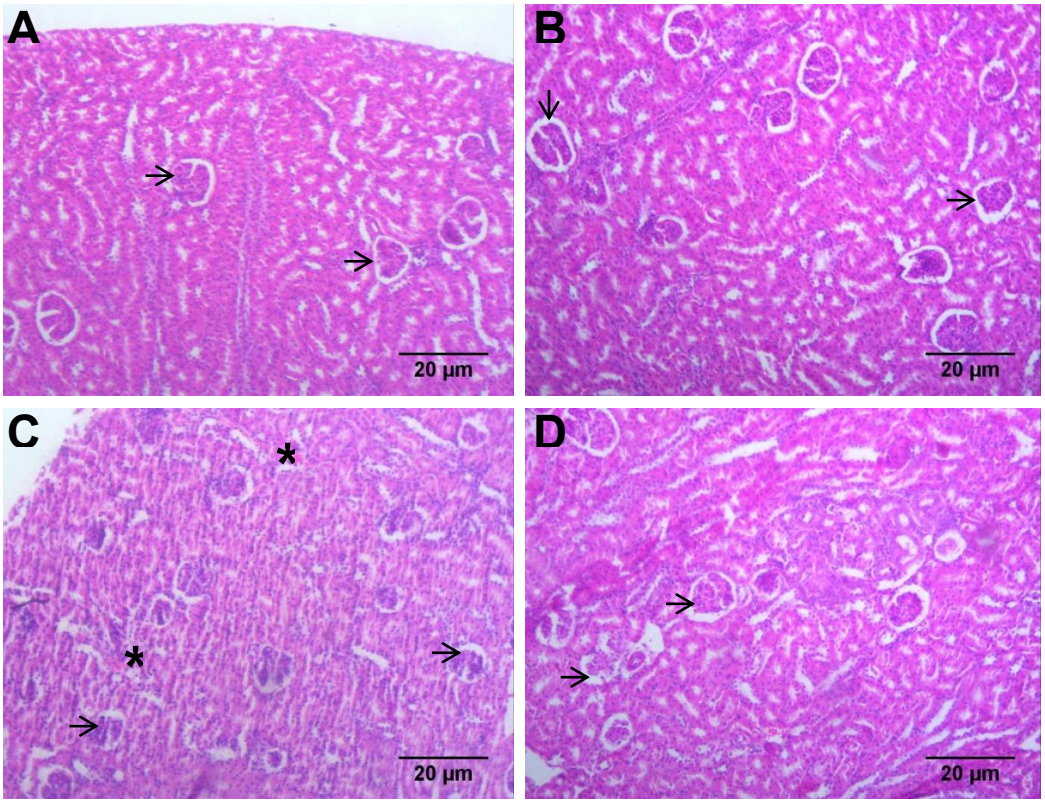

Figure 4. Photomicrographs of the representative kidney sections in experimental rats (H & E x100). (A) shows well defined glomeruli (arrows) and tubules in most areas of the group fed with fermented *P. biglobosa.* (B) shows normal looking kidney tissues with few areas of increased Bowman's space in the glomerus (arrows) of the group fed with fermented *P. biglobosa* + *S. stenocarpa.* (C) shows degenerative changes in the glomeruli (arrows) with dilatation of blood vessels (asterisk) in the group fed with HFD. (D) shows evidence of restorative changes with normal looking glomeruli in most areas (arrows) of the group fed with *S. stenocarpa.*

with fermented *P. biglobosa* + *S. stenocarpa* shows normal kidney tissue with evidence of restorative changes as there is increased size of the Bowman's space in few areas. The representative kidney of the obesity control group fed with HFD alone revealed dilatation in a few blood vessels with enlargement of Bowman's space (Figure 4C). Also, evidence of mononuclear cell infiltration is present in the renal cortices. Glomerulosclerosis and necrosis which are features of degenerative changes in the nephrons are also seen. Figure 4D shows essentially normal kidney tissue with restorative changes in the glomerulus in few areas. The evidence of degenerative changes observed in the HFD group in this study is similar to that of Altunkaynak et al. (2008) in a study of the structure of the kidneys of adult Sprague-Dawley rats fed a HFD for 3 months. They concluded that a fatty diet is responsible for the observed obesity and renal abnormalities as a result of histopathological changes such as dilation, tubular defects, inflammation and connective tissue hypertrophy of the kidney. However, the treatments in our study were able to produce ameliorate effects when compared with the HFD group alone. Adeyeye (2013) confirmed the presence of high levels of lecithin and phytosterols in both fermented and unfermented samples of *P. biglobosa.* Lecithin and phytosterols are bioactive substances that are proven to be effective for the prevetion of obesity as well as lowering cholesterol (Spilburg et al., 2003; Furlan et al., 2013). Ndidi et al., (2014) suggested the potentials of *S. stenocarpa* seeds in reducing cholesterol levels and preventing disorders.

Conclusion

This study is novel for demonstrating the effectiveness of the combination of fermented *P. biglobosa* and *S. stenocarpa* in reducing the risk of obesity. This could be a cost effective approach in the treatment of obesity especially in low income countries such as Nigeria.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Influence of the genetic polymorphism of haptoglobin in the occurrence of retinopathy and nephropathy in diabetics subjects

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The objective of this study was to determine the frequency of haptoglobin (Hp) genotypes and their association with nephropathy and retinopathy in diabetic subjects. This is a case-control study conducted in diabetic subjects developing nephropathy and/or retinopathy. Each patient was matched to a control of the same sex and the same age ± 2 years. Hp genotyping was performed by conventional PCR without enzymatic digestion on the Proflex® System PCR (Biosystems, Spain) and the biochemical parameters were determined using enzymatic techniques with the Cobas c311 system (Roche Diagnostics, Switzerland). The study population consisted of 60 diabetic subjects with an average age of 56 years and a sex ratio of 0.43. The Hp2-2 genotype was more frequent in diabetic subjects (40%) compared to control subjects (28.3%) (p= 0.18). The distribution of Hp1-1 and Hp2-1 genotypes in diabetic subjects showed rates of 46.7 and 13.3% respectively. Multivariate analysis showed that the Hp 2-2 genotype was more associated with nephropathy (OR=1.77; p=0.8). These results showed an association between Hp polymorphism and diabetic microangiopathy. Thus, Hp2-2 genotype could be associated with a risk factor predisposing to the onset of diabetic kidney disease.

Key words: Polymorphism, gene, haptoglobin, diabetes, nephropathy, retinopathy.

INTRODUCTION

Diabetes is not only a major public health problem because of its progression, but also because of the significant medical, social and human costs it generates. This progression is such that it is considered by the World Health Organization (WHO) to be a rampant epidemic (Beaudeux and Durand, 2011). About 425

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million people, or 8.8% of adults, are living with diabetes worldwide in 2017, 79% of whom live in low- and middle-income countries. According to the International Diabetes Federation (IDF), by 2045, 693 million people will have diabetes (Diabetes Atlas, 2017).

Diabetes is all the more serious in that it causes many debilitating complications, particularly affecting the cardiovascular system but also other organs such as the eyes, the kidneys and the nervous system. The onset of complications is associated with several factors which may be environmental in nature, but the role played by genetic factors is increasingly being recognized. Epidemiological studies looking for candidate genes have indicated differences in genetic susceptibility to the development of vascular complications in diabetic patients of different haptoglobin (Hp) genotypes (Szafranek et al., 2002; Nakhoul et al., 2001). The influence of genetic polymorphism of Hp on the evolution of diabetic disease has been reported by several authors. Indeed, Hp is a plasma protein synthesized by the liver and coded by two alleles rated 1 and 2 that give rise to three large genotypes Hp 1-1, Hp 2-1 and Hp 2-2; it has an antioxidant power that is genotype dependent (Brown, et al., 2013; Szafranek et al., 2002; Dalan et al., 2016). Considerable evidence has been reported regarding the involvement of oxidative stress in the development of vascular complications of diabetes (Giugliano et al., 1996; Gurler et al., 2000). The protein product of the Hp 2 allele is a lower antioxidant compared to the Hp 1 allele (Dalan et al., 2016). The genotype Hp 1-1 would protect better against oxidative stress compared to the genotype Hp 2-2 which has low antioxidant activity; the genotype Hp 2-1 has intermediate activity (Brown et al., 2013). However, in our regions, little data exists on the role played by genetic factors in the onset of diabetes complications. Thus, the objective of this study was to determine the frequency of Hp genotypes in order to search for a possible association with nephropathy and retinopathy in diabetic subjects.

METHODOLOGY

Place and design of study

This is a case-control analytical study carried out at the Biochemistry Laboratory of the Fann National University Hospital (FNUH) between June 2018 and September 2019.

Study population

The patients were recruited from the MARC SANKALE Center of AbassNdao Hospital in Dakar (Senegal) specializing in the national management of diabetes. Diabetic patients developing nephropathy with microalbuminuria ≥ 30 mg / 24 h and / or diabetic retinopathy were included in our study. The controls were recruited at the Biochemistry Laboratory of the Fann National University Hospital (FNUH) and at the National Blood Transfusion Center (NBTC) among blood donors. For each patient, a control of the same sex and the same age ± 2 years was recruited. Pregnant women and non-consenting patients were not included.

Sampling

Blood samples were taken from subjects fasting, at rest and by venipuncture at the elbow crease with tourniquet using a tube with EDTA for haptoglobin genotyping.

Genotyping of the haptoglobin gene

The DNA was extracted from whole blood using the QIAmp® genomic DNA and RNA kits (QIAGEN, Paris) using the semi-automatic microcentrifuge technique following the instruction of manufacturer.

The different genotypes were obtained using conventional PCR without enzymatic digestion with the Proflex System PCR (Biosystems, Spain). Primers A (5'GAGGGGAGCTTGCCTTTCCATTG3 ') and B (5'GAGATTTTTGAGCCCTGGCTGGT3') were used for the amplification of a sequence of 1757pb specific for the Hp1 allele and of a sequence of 3481pb specific for the Hp2 allele respectively. Primers C (5'CCTGCCTCGTATTAACTGCACCAT3') and D (5'CCGAGTGCTCCACATAGCCATGT3') were used to amplify a 349 bp sequence specific for the Hp2 allele (Koch et al., 2002). The reaction medium consisted of 1 µl of each of the 4 primers mentioned above, 12.5 µl of a synthetic Taq polymerase, 6.5 µl of sterile water and the whole added with 2 µl of DNA extract. The PCR was then performed following a programming of 35 amplification cycles (denaturation at 95°C for 1 min; hybridization at 69°C for 2 min and elongation at 72°C for 7 min for each cycle) preceded by initial denaturation at 95°C for 5 min. The electrophoretic migration of the PCR product was performed with Mupid-One (Labpro Scientific Laval, Canada) on 0.7% agarose gel supplemented with ethidium bromide (BET) and in the presence of a molecular weight marker. The DNA bands were then visualized by reading with the Doc Rx Gel.

Statistical analysis

The data was processed and analyzed with Epi info 7.2.2.6 software. The Student's t-test was used to compare the averages and the comparison of the proportions was carried out with the Chi² test. The odds ratio has been used to measure associations between Hp genotypes and diabetic microangiopathy. A p-value less than 0.05 was considered statistically significant.

RESULTS

The study population consisted of 60 diabetic subjects whose average age was 56 years. A female predominance was found with a sex ratio of 0.43. The average BMI value was 26.06 and that of the diabetes duration was 8.1 years. Among the microangiopathic complications sought, diabetic nephropathy was more common with a frequency of 88.33% followed by retinopathy with a rate of 11.67% (Table 1).

Statistical analysis of the data also showed higher average blood sugar level in diabetic patients (1.71g/L) than in controls (0.95 g/L) (p = <0.0001). The same variations were observed for HbA1c with a rate of 8.43% in the patients versus 5.48% in the controls (p =<0.0001).

Table 1. General characteristics of the population.

Parameter	Diabetics	Control
Number	60	60
Average age (years)	56	56
Sex-ratio	0.43	0.43
BMI	26.06	-
Average follow-up time for diabetic subjects (years)	8	-
Nephropathy (%)	88.33	-
Retinopathy (%)	11.67	-

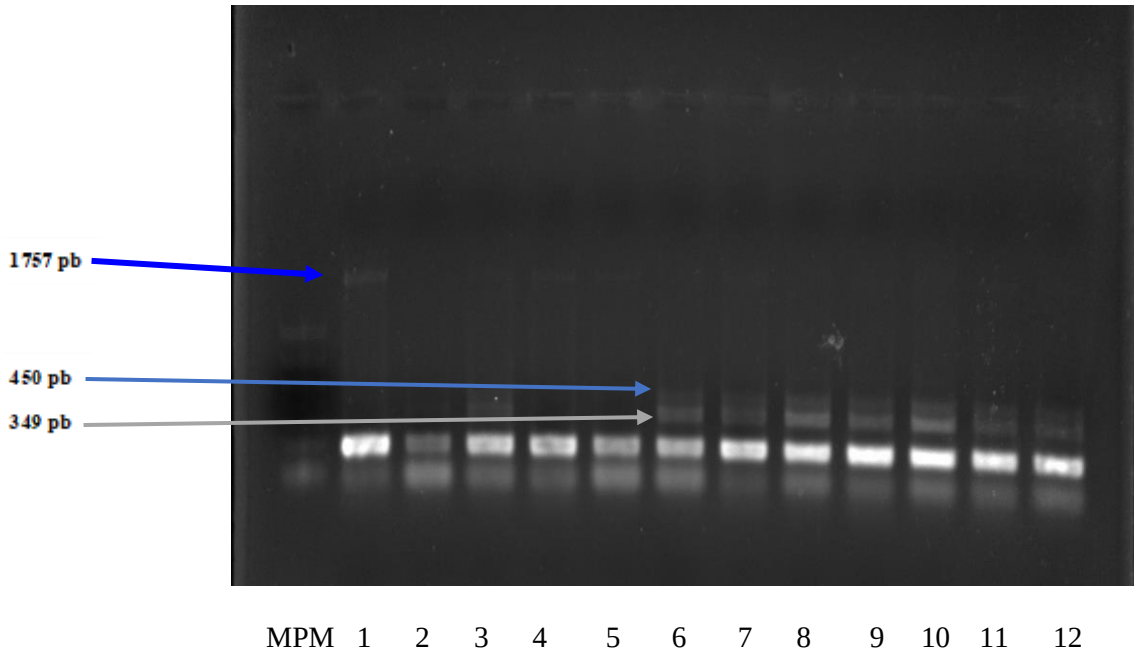

Figure 1. Visualization of DNA bands on agarose gel. 1= Hp1-1; 2= Hp2-2; 3= Hp2-2; 4= Hp1-1; 5= Hp1-1; 6= Hp2-1; 7= Hp2-2; 8= Hp2-2; 9= Hp2-2; 10= Hp2-2; 11= Hp2-2; 12= Hp2-2; MPM= Molecular weight marker.

The DNA amplification using the four primers A, B, C and D generated different PCR products: one Hp1-specific product of 1757 bp and two Hp2-specific products of 349 and 450 bp. The Hp2-specific product of 3481 bp was not generated (Figure 1).

The distribution of Hp alleles and genotypes showed variability in frequencies between diabetic subjects and control subjects. Thus, the results showed a higher frequency of the Hp2 allele with a rate of 46.7% in diabetics against 45% in control subjects (p = 0.157). In contrast, the Hp2-2 genotype was significantly more frequent in diabetic subjects (40%) compared to control subjects (28.3%) (p = 0.18). In addition, for the Hp1-1 and Hp2-1 genotypes, we found frequencies of 46.7 and 13.3% respectively in diabetic subjects; in the control subjects they were 38.4 and 33.3% respectively. The bivariate analysis showed a significant decrease in the Hp2-1 genotype in diabetic subjects compared to control subjects (p = 0.009). No significant difference was found for the Hp1-1 and Hp2-2 genotypes (Figure 2).

In multivariate analysis, the Hp2-2 genotype was the determinant most associated with nephropathy (OR = 1.77) with a statistically insignificant link (p = 0.8). Retinopathy was more associated with the Hp2-1 genotype (OR = 3.13; p = 0.5) (Table 2).

DISCUSSION

Diabetes is a chronic metabolic disease associated over time with macroangiopathic complications but also very often with diabetic microangiopathy. The occurrence of the latter is today linked to the expression of certain genes but also to a genetic polymorphism. Thus, the

Table 2. Association of the different Hp genotypes with nephropathy and retinopathy in multivariate analysis.

Hp genotypes	Nephropathy		Retinopathy	
	OR (IC 95%)	P	OR (IC 95%)	P
Hp 1-1	1.19 (0.24-5.84)	1.0	0.84 (0.17-4.12)	1.0
Hp 2-1	0.32 (0.05-2.02)	0.5	3.13 (0.49-19.86)	0.5
Hp 2-2	1.77 (0.31-9.99)	0.8	0.56 (0.10-3.17)	0.8

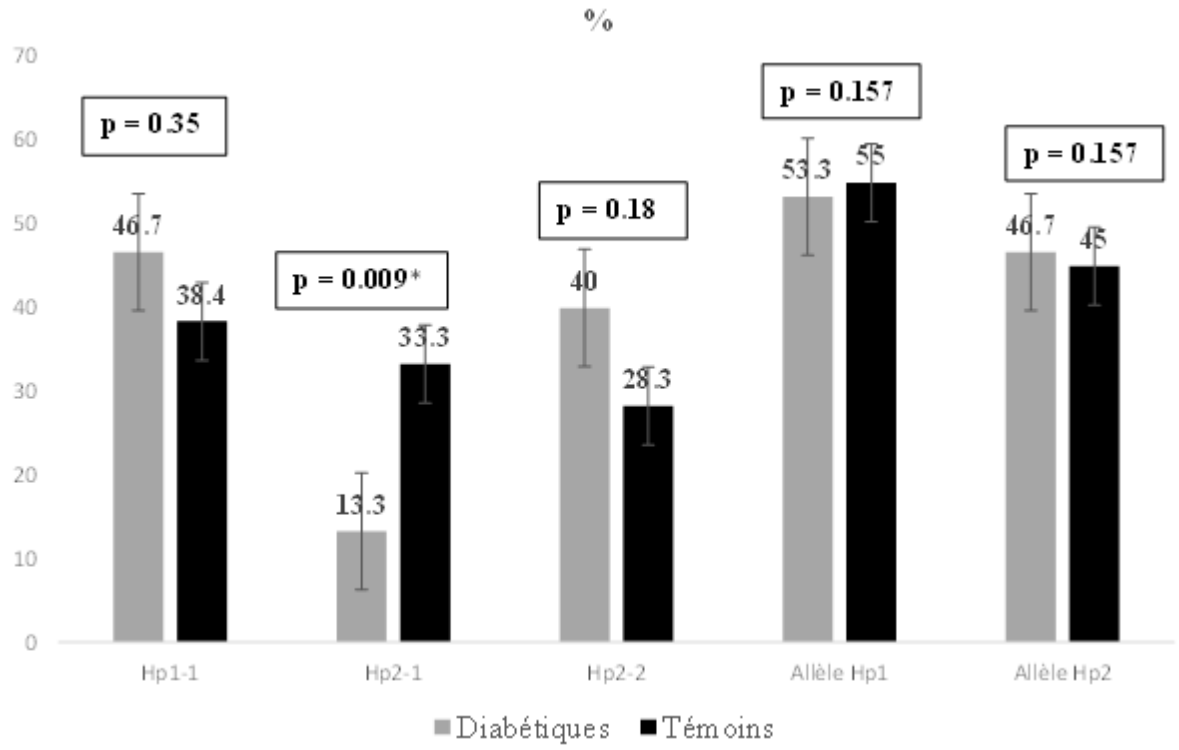

Figure 2. Frequencies of alleles and genotypes of haptoglobin in diabetic and control subjects.

objective of the study was to search for genetic biomarkers predisposing to the occurrence of vascular complications in diabetes.

The population was characterized by an average age of 56 years and a sex ratio of 0.43. A similar trend was also found in the studies of Ndour-Mbaye et al. (2011) in Senegal (75.1% women) and Djrolo et al. (2012) in Benin (62.1% women) thus confirming our results. However, in the literature studies have also reported a male predominance (Drabo et al., 1996; Millogo et al., 2015).

The evaluation of data related to the type of microvascular complications showed a high prevalence of nephropathy with a frequency of 88.33% compared to retinopathy (11.67%). This result is supported by several studies including that of Demnati et al. (2010) in Tunisia. In this study, the frequency of retinopathy is probably underestimated due to the limited accessibility to diagnosis and the high cost of these investigations, which are mostly borne by the patient.

The study of the genetic polymorphism of Hp showed in the controls a higher frequency of the Hp1-1 genotype in controls with a rate of 38.4%. Similar results were found by Harris et al. (1959) who found in a study carried out in The Gambia a predominance of the Hp1-1 genotype with a rate of 30.57% followed by the Hp2-1 (21.66%) and Hp2-2 genotypes (7.01%). However, a predominance of the Hp2-2 genotype with a frequency of 48.2% was found by Koch et al. (2002) in a study carried out in Germany. There is indeed certain variability in frequencies across the world. Authors have shown a predominance of the Hp1-1 genotype in black populations; while in Caucasian populations the predominant form is Hp2-1 followed by Hp2-2 (Moullec et al., 1961).

Genotyping performed in diabetic subjects showed a higher frequency of the Hp2-2 genotype (40%) compared to controls (28.3%) (p = 0.18). These results are similar to those of Brown et al. (2013) who found a majority frequency around 38% for the Hp2-2 genotype in a

Ghanaian study of 50 diabetic black patients. In addition, this increase in the frequency of the Hp2-2 genotype has been found in the literature in diabetes but also in the context of other pathologies such as cardiovascular diseases (Guèye, 2007; Holme et al., 2009).

In multivariate analysis, the Hp2-2 genotype increases by 1.77, the risk of developing nephropathy (OR = 1.77) although this link is not statistically significant (p = 0.8). Retinopathy is more associated with the Hp2-1 genotype (OR = 3.13 and p = 0.5). This result could be explained by the fact that retinopathy was very infrequent (11.67%) in the study population. In the literature, the Hp2-2 genotype is found to be more associated with the occurrence of diabetic microangiopathy (Nakhoul et al., 2001; Levy et al., 2000; Nakhoul et al., 2000). Indeed, HP is considered an antioxidant because of its ability to recover free Hb from extra and intravascular hemolysis and prevent the initiation of reactions leading to the production of free radicals. However, this antioxidant activity varies greatly among different Hp genotypes (Melamed-Frank et al., 2001). Some authors have shown that the protein Hp1-1 eliminates Hb faster than the Hp2-2 protein. Consequently, there are more Hp-Hb molecules in the plasma of individuals with the Hp2-2 genotype. This mechanism would be of even greater importance in diabetics, already exposed to significant oxidative stress (Szafranek et al., 2002; Dahan et al., 2015). However, this study presents certain limitations relating to very narrow selection criteria and to the limited sample size of cases, especially for those developing retinopathy. In addition, we did not take into account certain confounding factors that could influence the development of microangiopathy, such as smoking.

Conclusion

These results suggested an association between haptoglobin polymorphism and diabetic microangiopathy. The Hp2-2 genotype was associated with nephropathy while retinopathy was more associated with the Hp2-1 genotype. The Hp2-2 genotype could thus be associated with a risk factor predisposing to the onset of diabetic nephropathy. However, these results should be confirmed by larger-scale studies associating the other risk factors involved in the pathogenesis of diabetic microangiopathy.

CONSENT

Written and informed consent was obtained from each participant.

ETHICAL APPROVAL

This study was approved by the Research Ethics Committee (CER) of the Cheikh Anta Diop University (UCAD) in accordance with the rules issued by the National Ethics Committee for Health Research (CNERS) of Senegal under number: 0227/2017/CER/UCAD

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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