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Research Article

The Effect of Pure Andrographolide Compound on Insulin Resistance Inbred Strain of Male Mice with High Fat Diet Condition

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ABSTRACT
The potential anti-diabetic effects of andrographolide have been reported in earlier work by other investigators. Therefore, the focus of the current work is on cellular and ultra-structural changes. associated with andrographolide intervention in insulin resistant mice. Most therapeutic agents for diabetes are able to reduce blood glucose level. Few therapeutic agents are capable of protecting and preserving the pancreatic cells mass, insulin sensitivity and hepatocellular, and adipocyte functions. Insulin resistant was induced in male mice using high fat diet (HFD) (10 % butter; 45% dietary energy from fat) where mice were fed for 24 weeks. The insulin resistance mice were divided into one control negative group (CN) with normal chow and three high fat diet groups, which were; control positive group HFD (CP), high fat diet group with andrographolide 25 mg/kg (HFA25), and high fat diet with andrographolide 50 mg/kg (HFA50) Following the induction of insulin resistance, andrographolide was administrated to HFA24, HFA50 groups respectively for 15 days by oral gavages. At the end of the treatment, mice were sacrificed; pancreas and liver were sectioned for histological study. The results showed that andrographolide exerted evident ameliorative effects on insulin sensitivity. There were also evidences pointing to the regeneration of beta cells and acini, reduced inflammation of pancreatic cells and liver, all of which nearly reverted to their normal structures. Therefore, it is concluded that the anti-insulin effects seen in andrographolide treatment is associated with restoration of insulin sensitivity

Keywords: Andrographolide, insulin resistance, inflammation, cell–regeneration

INTRODUCTION

Insulin resistance is the salient feature of type 2 diabetes mellitus (T2DM). It occurs when normal circulating concentration of insulin fail to regulate body glucose homeostasis[1,2]. Insulin resistance has been common sequelae of obesity; insulin resistance syndrome implies a series of abnormalities that happen frequently in insulin resistant individuals[3-5]. These include dyslipidaemia, endothelial dysfunction changes, glucose intolerance, elevated inflammatory markers[7,8]. Even though the key to understanding the nature and significance of insulin resistance has been the assessment of functional and morphological alterations of islets and their causes, data on this aspect of diabetes are still scarce[9-11]. Hundreds of herbal extract have been used to reduce blood glucose, but only a small number of these herbal extracts have received scientific and medical evaluation to assess their efficiency especially as anti-insulin resistance agent and anti-inflammation effects in mice[12,13]. Andrographolide is the primary bioactive compound of *Andrographis paniculata*, a type of herb well known throughout South East Asia, India, and China[14-17]. The reported biological activities are antibacterial, antifungal, antiviral, anthelmintic, anticancer, anti-diabetic, anti-inflammatory, anti-venomic, antiasthemic, hepatoprotective and used to treat cold and fever[18-21]. Oral administration of *Andrographis paniculata* considerably reduces streptozotocin-induced diabetes mellitus in rats[22,23]. Ethanolic leaf extract of this plant reduced oxidative stress and have been reported to demonstrated antihyperglycaemic properties in rats[22]. Anti-diabetic property of aqueous extract of andrographliode was further confirmed in a recent study[24,25]. Insulin resistance and T2DM are associated with a range of pathologies in the pancreas and liver. Characteristic pathological lesions associated with insulin resistance includes; multiple hyperplastic and hypertrophic islets, mononuclear cell infiltration, vascular congestion and hemorrhage, presence of fibrous tissue, increased fibrous tissue separating beta cells, sparse islet inter beta cell perivascular tissue and in extra-islet periductal and vascular locations[26-28]. In the liver, lesions associated with HFD include fatty degeneration and steatosis, vascular congestion and infiltration of inflammatory cells[29,30]. Indeed, it has been suggested these damages, particularly

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in pancreas is associated with oxidative damage, because pancreatic islet cells were reported to have anti-oxidative enzymes activity[31,32]. The current study was postulated that andrographolide ameliorate the pathological changes associated with insulin resistance and improved insulin sensitivity. This could have occurred not only via the enhanced antioxidant protection, which reduces oxidative damages of the tissues, but also through effects that directly stimulated cellular repair and growth. This eventually will lead to functional restoration of the affected cells. Understanding these pathophysiological changes would greatly enhance our understanding of how functional deficiencies of these organs eventually lead to insulin resistance and obesity. This study therefore aimed at investigating the ameliorative effects of andrographolide on insulin sensitivity, and histopathological changes associated with insulin resistance in high fat diet induced insulin resistance in ICR male mice.

MATERIALS AND METHODS

Animals

Forty-eight, apparently healthy ICR male mice of six weeks old, weighing 22±2g were used in this study. The mice were housed in a temperature-controlled (21±2˚C) room on a 12:12 h dark-light cycle (light on at 8:00 hrs) and a constant relative humidity (60±10%). They were fed with a standard laboratory rat chow and water was provided *ad-libitum*. The experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia, with the approval number of UPM/IACUC.

Induction of insulin resistance

The male mice were placed in animal house for 2 weeks for acclimatization, after which they were divided into four groups (n=12 for each group), and fed different diets during 24 weeks' period. The treatment group: high fat diet (HFD 45% of calories from fat, 20% protein and 35% carbohydrate), or standard chow. Fresh chow was provided daily and any chow remaining from the previous day was discarded. The body weight was measured weekly. After the acclimatization period the mice were randomly divided into four groups (n=12 for each group) as follows

CN: mice receiving standard mice diet (CN)

CP: mice receiving high fat diet (CP) (10% butter)

HFA25: mice receiving high fat diet plus andrographolide 25 mg/kg /daily

HFA50: mice receiving high fat diet plus andrographolide 50 mg/kg/daily

Measurement of insulin sensitivity

Intraperitoneal glucose tolerance test (IPGTT)

IPGTT procedure was carried out on 6 randomly selected mice after 4 weeks of feeding high fat diet and 2 weeks after andrographolide treatments. A drop of blood was used for the measurement of glucose levels using a glucose reagent strip and glucometer (Roche Diagnostics Aust. Pty. Ltd, Castle Hill, Australia.). Blood glucose was measured at time 0 (before glucose injection) and at 5, 15, 45, 90 and 120 min post Intraperitoneal glucose (1 g/kg, Sigma-Aldrich, St. Louis, MO, USA) injection according to the method of Abdollahi et al. (2011).

Intra peritoneal insulin tolerance test (IPITT)

IPITT was carried out on the next day after IPGTT. Fasted mice were injected with human recombinant insulin (Humulin, Eli Lilly and Company Indianapolis, USA) intraperitoneally at a dose rate of 0.7IU/kg of BW. Blood glucose was analyzed at time 0 min (before insulin injection) and 5, 15, 45, 90 and 120 min after insulin injection. The onset of insulin resistance was verified according the methods described by[34,35].

Measurement of the area under curve (AUC)

The area under the curve was determined at 24, 26 weeks of the trial based on the total plasma insulin levels during IPITT test and total glucose concentration during IPGTT test according to[36]. AUC of glucose and insulin concentration was measured using the trapezoid rule. The formula was as follows:

$$AUC_{0\text{-}n} = \frac{Cp1+Cp2}{2}(t2-t1)$$

AUC: (area under curve)

CP1: (first value)

T1 :(first time)

T2: (second time)

0-n: numbers of treated mice

The AUC was used to measure the insulin sensitivity because it quantifies changes of glucose or insulin concentration by the measurement of the area under the curve rather than selecting a single time point. To further determine the state of insulin resistance in each treatment group, the insulin resistance index was employed. The index was measured by multiplying the total glucose concentration during IPGTT with the total insulin concentration during IPGTT[37,38].

Sampling

Mice were sacrificed by cervical dislocation after anaesthetize with (ketamine 0.1 ml and xylazine 0.5) ml intraperitoneal injection of this combination at 26 weeks of experiment. The 26 weeks are inclusive of 24 weeks of feeding with high fat diet and 2 weeks for treatment with andrographliode at 25, 50 mg/kg for the HFA25, HFA50 groups only, respectively. The pancreas and liver were carefully removed and weighed. The tissues (3 sections per mice) were then fixed in freshly prepared fixative solution (10% buffered formalin). The tissues were routinely processed and stained with Hematoxylin and Eosin for histological evaluation, masson trichrome stain (fibers stain). The degree of injury of pancreas under the light microscopy was scored by two pathologists[39-41].

Structural and quantitative approaches

To study the morphological aspects and islet mass of endocrine pancreas, five pancreases from each group were excised, cleared of fat and lymph nodes, weighed, immersion-fixed for 12 h in Bouin's fixative solution, dehydrated and embedded in paraffin. At 250 µm intervals, four serial sections (5 µm) were cut on a rotary microtome and adhered to individual normal or salinized glass. The first section from each series was stained with Masson trichrome to perform the morphological and stereological analysis. The second, third and fourth sections were

Table 1: Lesion scoring for the pancreatic tissues on H & E stain for all experimental groups at the end of trial. Mean±SE (n=12 mice per group)

	islet size	pyknotic nuclei	inflammation	degeneration	fibrosis	acini structure	P-Value
CN	0.66±0.14[d]	0.58±0.14[b]	0.25±0.13[c]	0.33±0.14[b]	_	0.41±0.14[c]	0.0001
CP	3.16±0.25[a]	3.08±0.25[a]	3.66±0.14[a]	3.58±0.19[a]	0.50±0.19[a]	2.91±0.22[a]	0.0001
HFA25	2.36±0.34[b]	2.81±0.28[a]	2.81±0.28[b]	3.27±0.26[a]	1.54±0.23[b]	2.00±0.25[b]	0.0001
HFA50	1.69±0.22[c]	0.92±0.20[b]	0.84±0.17[c]	0.84±0.22[b]	2.15±0.28[c]	0.29±0.22[c]	0.0001

[a,b,c,d] Values with the different superscripts/within column differ significantly at $P<0.05$. [a,b,c,d] Values with the different superscripts/within column differ significantly at $P<0.05$. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/kg

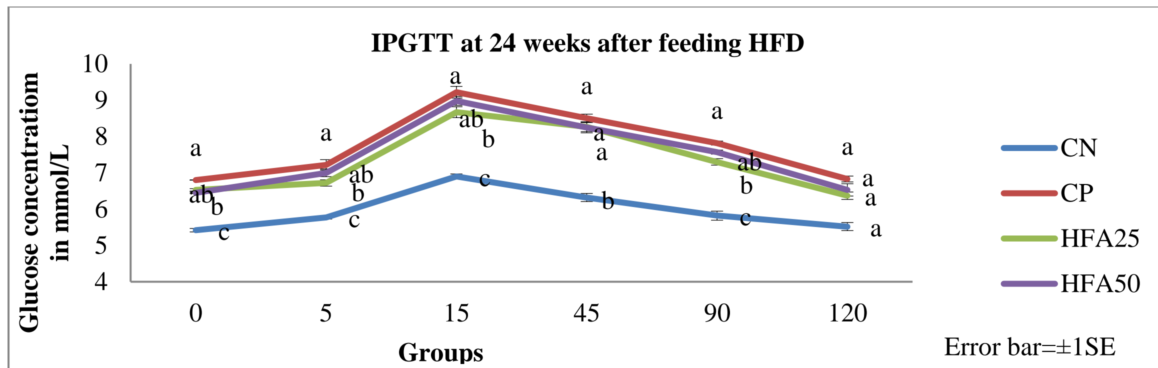

Figure 1: Intraperitoneal glucose tolerance test at the 24 weeks after feeding high fat diet. Glucose values for all the groups at week 24 before onset of treatment, [(a, b. c. d)] showing significant difference(P<0.05) among blood glucose levels at 0, 5, 15,45, 90, 120 min between the high fat fed diet groups (CP, HFA25, HFA50) at 5,15, 45, 90, 120 min compare to (CN) which had lower glucose level. Data is expressed as means ± 1 standard error. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/kg

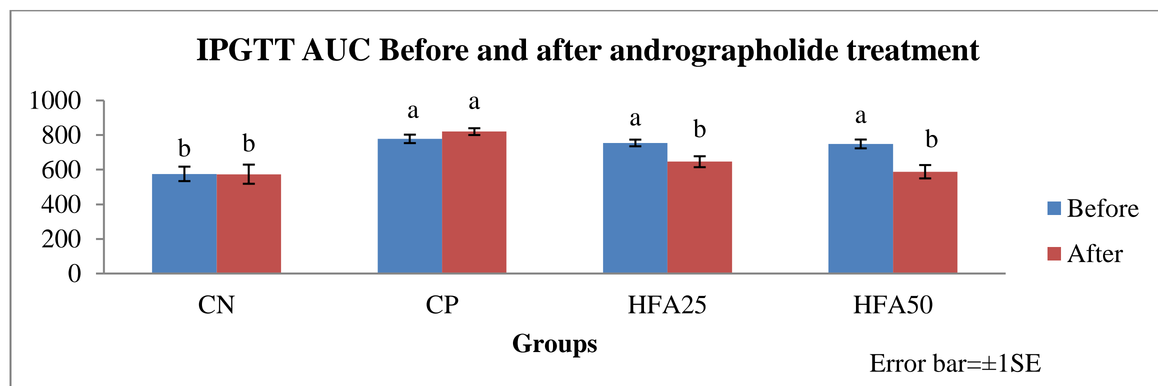

Figure 2: Glucose AUC of IPGTT at 24 week after feeding high fat diet and Glucose AUC of IPGTT after 2 week andrographolide treatment.[a,b] different superscripts showing significant differences P<0.05 in HFA25,HFA50 after treatment 2 week with andrographolide compare the same treatment (HFA25,HFA50) at 24 weeks feeding HFD indicated to reduce effect of andrographolide compouned on IPGTTAUC values. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/kg

immunoperoxidase stained for insulin to qualify the distribution of pancreatic beta and alpha cells and the presence of proliferation in beta cells[42,43].

Islet mass

This was determined by point counting stereology[44] on Masson trichrome-stained sections. Each section was counted systematically with a grid of 100 points (final magnification × 320). The numbers of points over endocrine, exocrine and non-exocrine pancreatic tissue were counted. The relative islet volume was calculated by dividing the number of points over the endocrine tissue by the number of points over the total tissue. Islet mass was determined by multiplying the relative volume by the total weight of the pancreas. A minimum of 500 fields per mice was counted. The islet images for documentation were

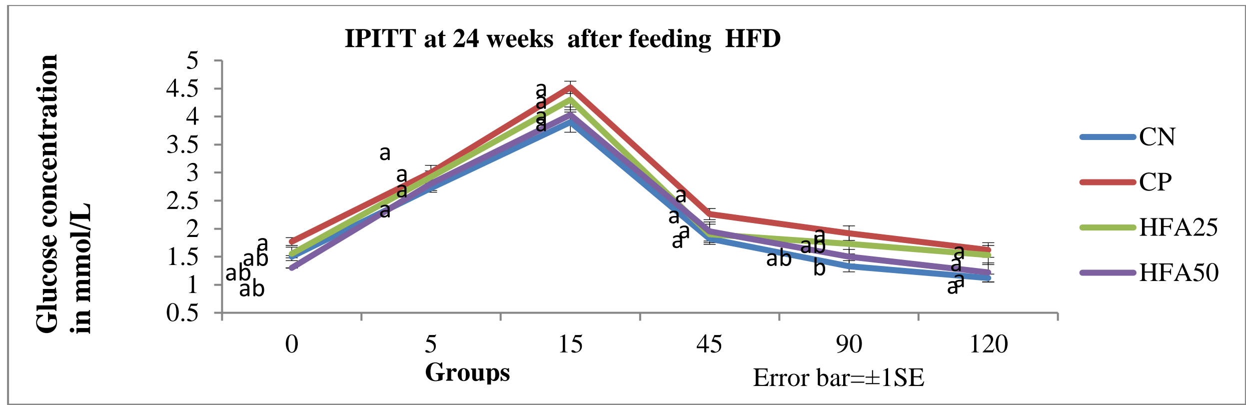

Figure 3: Intraperitoneal insulin tolerance test values at week 24 after feeding high fat diet (n=12 mice each group). Different superscripts ([a,b]) Showing significant difference (P<0.05) among blood insulin levels at 0, 5, 15,45, 90,120 min for high fat diet groups (CP, HFA25, HFA50) compare to (CN). Data is expressed as means ± 1 standard error. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/kg

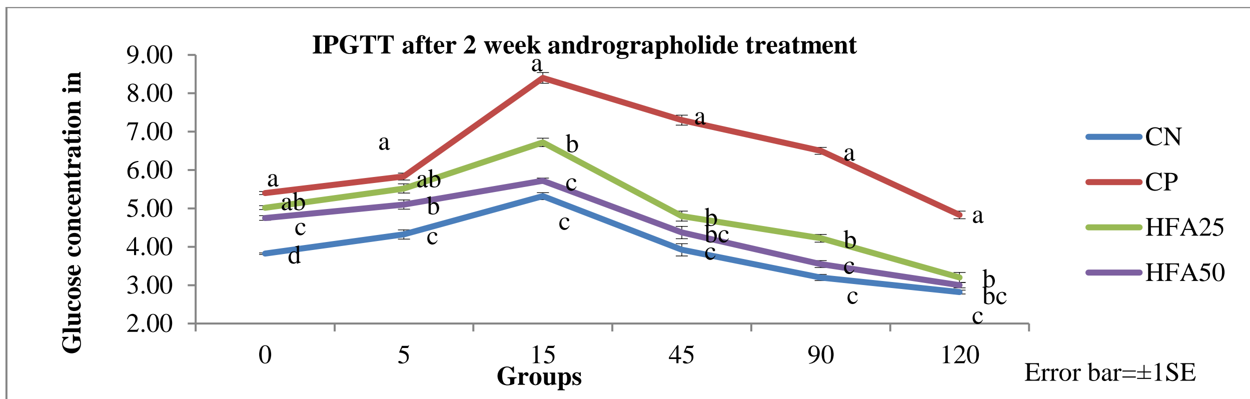

Figure 4**:** Intraperitoneal glucose tolerance test values 2 week after treatment with andrographolide. Glucose values. Different superscripts ([a,b,c,d]) showing significant difference (P<0.05) between (CP, HFA25, HFA50) (n=12 mice per group) at 0, 5, 15, 45, 90,120 minutes compare to (CN) which had lower glucose level. Data is expressed as means ± 1 standard error. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/k

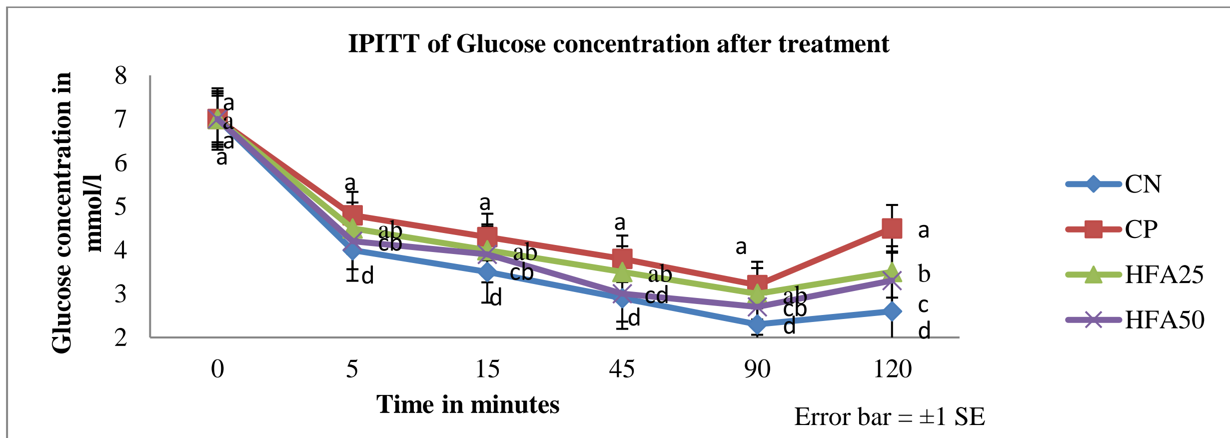

Figure 5: Intraperitoneal insulin tolerance test values after 2 weeks andrographolide treatment (n=12 mice each group). Different superscripts ([a,b,c,d]) Showing significant difference (P<0.05) among blood insulin levels at 0, 5, 15,45, 90,120 min for high fat diet groups (CP, HFA25, HFA50) compare to (CN) at 5,15,45,90,120 min and(HFA50) compare to CP group at5,15,45,90,120 min . Data is expressed as means ± 1 standard error. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/kg

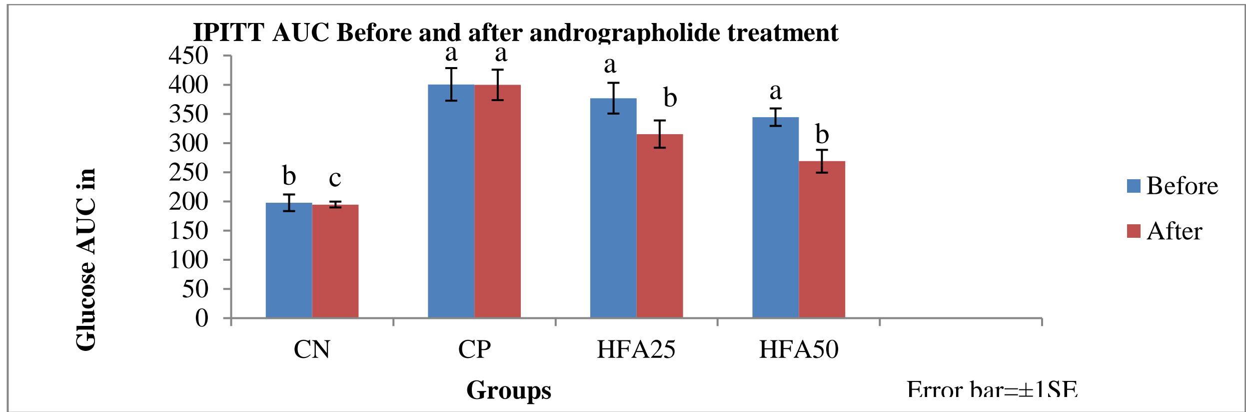

Figure 6: Glucose AUC of IPITT at 24 week after feeding high fat diet and Glucose AUC of IPITT after 2 week andrographolide treatment .Different superscripts[(a,b)]. showing significant differences P<0.05 in HFA25,HFA50 after treatment 2 week with andrographolide compare the same treatment (HFA25,HFA50) at 24 weeks feeding HFD indicated to reduce effect of andrographolide compouned on IPITT AUC Values. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/kg

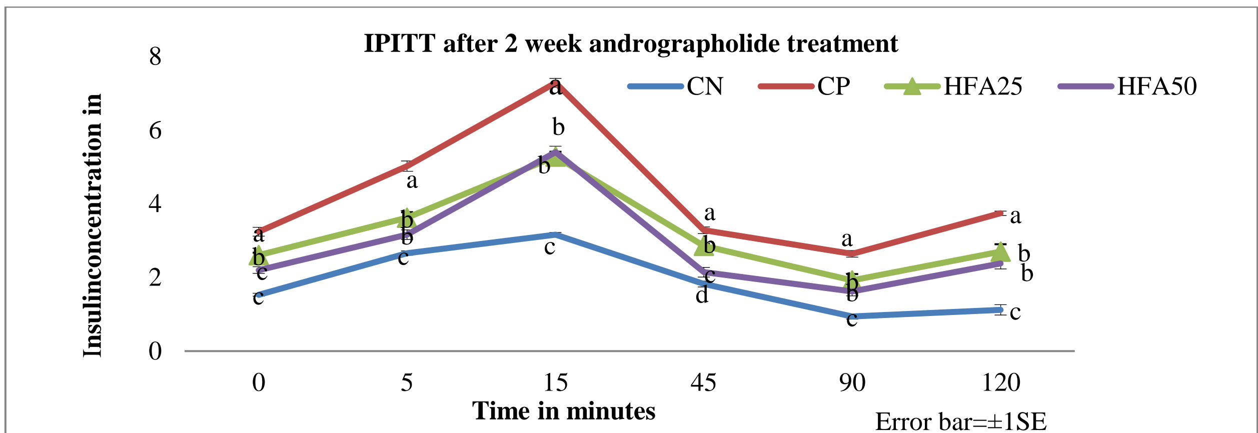

Figure 7: Intraperitoneal insulin tolerance test values 2 weeks after andrographolide treatment, showing different levels of IPITT in all the groups (n =12 mice per group) [(a,b,c,d)] showing significant difference (P<0.05) between the high fat fed diet (CP) at 15,45, 90,120 min which is take longer time to normalize compare to. Data are expressed as means ± standard error. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/kg

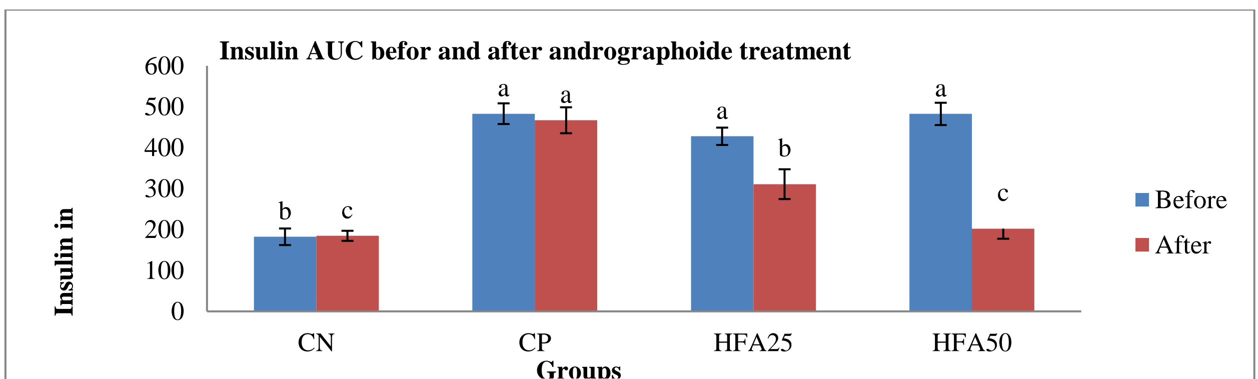

Figure 8: Insulin AUC of IPGTT at 24 week feeding HFD and after 2 weeks andrographolide treatment. [a,b,c,d] different superscripts showing significant differences p<0.05 between (HFA25,CP) (HFA50,CP), (CN,CP),and (CP,HFA25,HFA50) compare to CN group that indicated to effect of andrographolide to reduce insulin levels after glucose injection. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/kg

Table 2: Lesion scoring for the liver tissues on H & E stain for all experimental groups at the end of trial. Mean±SE, (n=12 mice per group)

	steatosis	ballooning	inflammation	activity	cell infiltrations	degeneration	P-Value
CN	0.33±0.14^{c}	0.41±0.14^{d}	0.41±0.14^{b}	0.16±0.14^{b}	0.58±0.19^{c}	0.16±0.11^{b}	0.0001
CP	3.33±0.22^{a}	3.50±0.23^{a}	3.83±0.11^{a}	3.25±0.30^{a}	3.75±0.13^{a}	3.75±0.13^{a}	0.0001
HFA25	2.91±0.19^{a}	2.91±0.19^{b}	3.58±0.25^{a}	2.91±0.25^{a}	2.83±0.29^{b}	3.50±0.15^{a}	0.0001
HFA50	1.58±0.22^{b}	1.41±0.22^{c}	0.91±0.22^{b}	1.16±0.16^{b}	1.08±0.28^{c}	0.58±0.19^{b}	0.0001

^{a,b,c,d}. Values with the same superscripts/within column do not differ significantly at P<0.05. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/kg

registered by a CCD camera, coupled to an Olympus BX-60 photomicroscope (Olympus, Tokyo, Japan).

Histopathology scoring of pancreas

An average of 6 fields per slide was examined at 200x and the lesions were scored and the averages were recorded[45,46]. The criteria that were selected for scoring the pancreas were; islets size, inflammation, fibrosis, and degeneration where Scoring 0-4, 0 No signifying changes, 1 signifying mild changes, 2 signifying moderate changes. 3 signifying sever changes, and 4 signifying so sever changes as described by[45].

(Normal) 0-5% of the cells showed enlargement of the islet size, inflammation, fibrosis and degeneration

(Mild) 5-10% of the cells showed enlargement of the islet size, inflammation, fibrosis and degeneration,

(Moderate) 10-25 % showed enlargement of the islet size, inflammation, fibrosis and degeneration,

(Very severe) more than 50% enlargement of the islet size, inflammation, fibrosis and degeneration,

Organ damage was assessed by evaluating changes in the pancreatic acini, the ducts, the arteries and islets of Langerhans. A subjective rating for each slide ranging from 0 (None) to 4 (So severe damage) was assigned to each components of the organ[47,48]. Damage of the islets of Langerhans was evaluated based on the presence or absence of nuclei, nuclear pyknosis and inflammation. Acinar damage was evaluated according to the appearance of the acinar pyramidal structure, the regular patency of the acinar lumen, the number of vacuolation, pyknotic nuclei and the amount of intraluminal secretion of zymogenic granules, intima hypertrophy and presence of inflammatory cells in the adventitia, and the presence of intraluminal haemorrhages. The ductal changes reflected a more irregular disposition of the epithelial cells and the presence both of oedema in the wall and of inflammation[48].

Histopathological scoring of the liver

Lesions were scored based on semi quantitative evaluating of steatosis, hepatocellular ballooning, and lobular inflammation, presence of hemorrhage, and number of liver lesions for all slides according to SAF score was created including Steatosis (S), Activity (A) and Fibrosis (F) as described by[49].

(Normal) 0-5% showed steatosis, inflammation, and fibrosis from all lesions

(Mild) 5-10% showed steatosis, inflammation, fibrosis lesions from all lesions

(Moderate) 10-25 % showed steatosis, inflammation, and fibrosis from all lesions

(Sever) 25-50 % showed steatosis, inflammation, and fibrosis from all lesions

(Very severe) more than 50% showed steatosis, inflammation, and fibrosis from all lesions

Data analysis

All Data presented as mean ± SE. One-way analysis of variance (ANOVA) least significant difference (LSD). Were used for statistical analysis to compute possible differences between treated the groups. *P*-values of less than 0.05 were considered Insulin sensitivity were analysed using the general linear model procedure in SPSS (version 20.0 for windows, SPSS Inc., Chicago) repeated measurement test

RESULTS

Intraperitoneal glucose tolerance test and intraperitoneal insulin tolerance test (IPGTT, IPITT)

In the IPGTT, the serum glucose increased to the maximum after 15 min after intraperitoneal injection of glucose in treated groups, but this maximum was higher in the (CP) mice than in (CN) and (HFA25 and HFA50) groups (P<0.05). Period of glucose clearance in (CP) mice was delayed compared to control negative group, remaining higher for 15 min after glucose administration, qualifying for characterization as glucose intolerance (Figures.1, 2, 3, 9). After oral gavage with andrographolide for 15 days, animal from the treated groups HFA25, HFA50 had lower glucose levels. The IPITT demonstrated a quick decline in serum glucose after 45 min of insulin administration in CN, CP, HFA25, and HFA50 groups which mean no significant differ between treatment groups at 24 weeks after feeding high fat diet (Figures.3, 4, 8) but the serum glucose remained higher in (CP) mice than in (CN, HFA25, HFD50) (P<0.05) at all-time points up to 120 min (Figure 5) characterizing the CP mice as insulin resistant. After treatment with andrographolide oral gavage for 15 days, insulin levels had significant different (P<0.05) CP had the highest value at all-time point up to 120 than CN, HFA 50, HFA25 groups. (Figure. 7).

Serum levels of HbA1c

The mean haemoglobin A_{1c} (HbA_{1c}) at week 26 is shown in (Figure 10). High fat diet (CP group) was observed to induce increase in HbA_{1c} level following 24 weeks feeding with the HFD, suggesting HFD is associated with increase in HbA_{1c}. However, 15 days' treatment with andrographolide at the doses of 25 mg/kg and 50 mg/kg body weight (HFA25 and HFA50 respectively) significantly reduced the level of HbA_{1c}, implying that

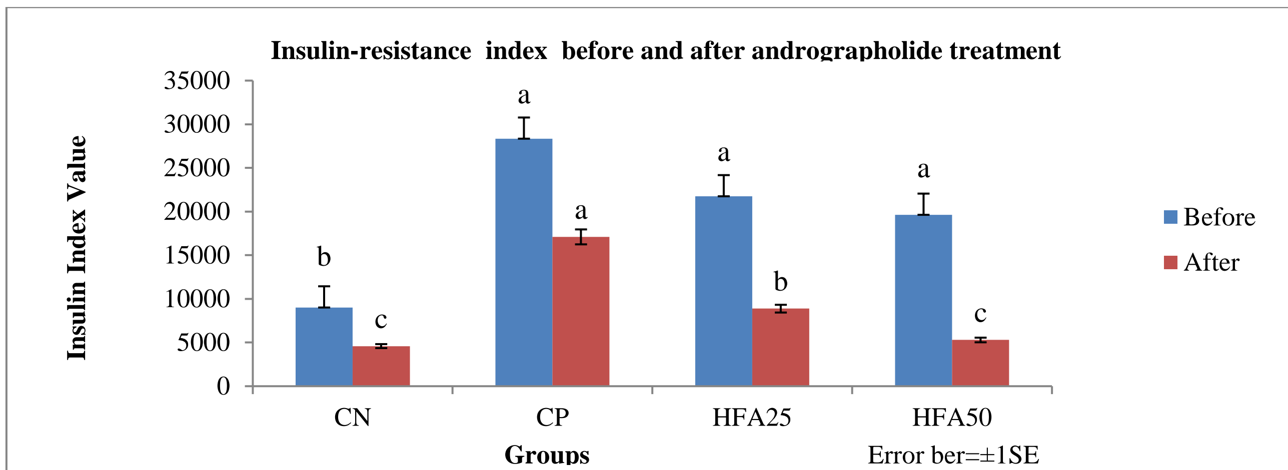

Figure 9: Insulin –Resistance index before and after 2 weeks andrographolide treatment[a,b,c,d]. different superscripts showing significant differences P<0.05 between groups indicated reduce insulin resistance in HFA25,HFA50 compare to CP group. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/kg

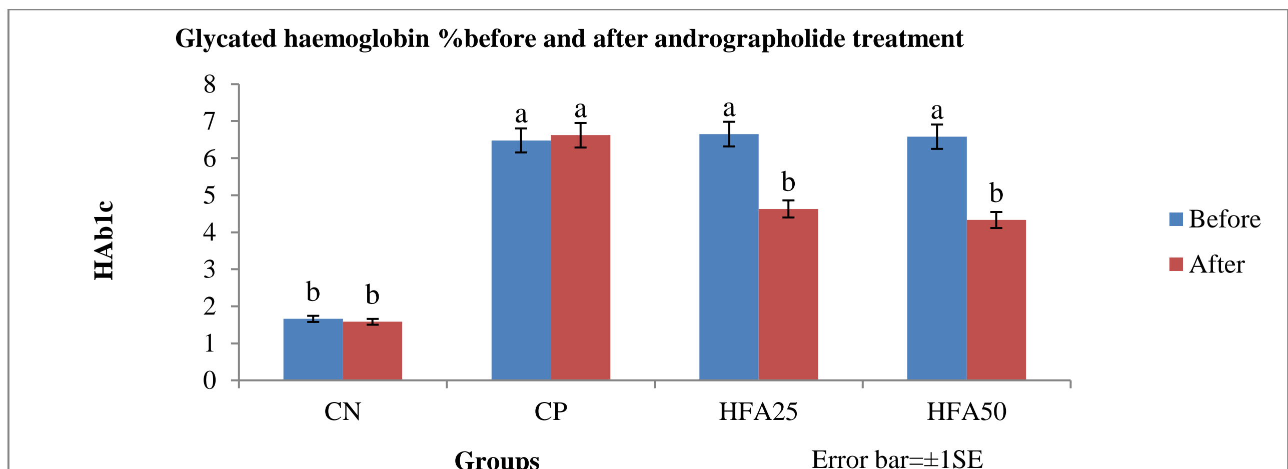

Figure 10: presenting glycated haemoglobin (%) at 24 week feeding HFD and after 2 weeks andrographolide treatment. [a,b,] different superscripts showing significant differences P<0.05 between HFA25,HFA50 before and after andrographolide treatment indicted to reduce % of HbA1c after treatment compare to CP , CN groups. CN=control negative group, CP=control positive group, HFA25=high fat diet +andrographolide 25mg/kg, HFA50=high fat diet+ andrographolide 50 mg/kg

treatment with andrographolide has a beneficial influence on HbA_{1c}.

Lesion score of pancreas tissues

The lesion scoring for the tissues as observed in this study is presented in (Table 1 and 2). There was significant (p<0.05) difference between the CP and HFA25, HFA50 groups. The result obtained has shown that even though animals treated with andrographolide showed reduced lesion scores following the 15 days' treatment with andrographolide, HFA25, HFA50 groups' demonstrated better overall cellular improvement (HFA50). The pathological scores for six cellular lesions analyzed in sections of the pancreas of the four groups are presented (Table 1). It was observed that the mice treated with andrographolide for 15 days' post high fat diet feeding had significantly (P<0.05) reduced inflammation, degeneration and improved islet size in HFA25, HFA 50 compared to the control positive group. The negative control group shows no significant lesions. Table 2. Shows liver scores across treatment groups, statistically significant differences between the CP and HFA50 in liver tissues lesions (steatosis, hepatocellular ballooning and lobular inflammation and eosinophlic stain action, different degree of degeneration like fatty degeneration and infiltration). CP group had no significantly different in liver lesion scores (steatosis, inflammation, activity, degeneration) compare with HFA25.

DISCUSSION

The mechanisms underlying the development of insulin resistance are complex; however, excessive levels of circulating free fatty acids (FFAs) appear to play an important role, particularly in the liver, skeletal muscle,

Histology of panceratic tissue of treated groups (Hematoxylin and Eosine stain)

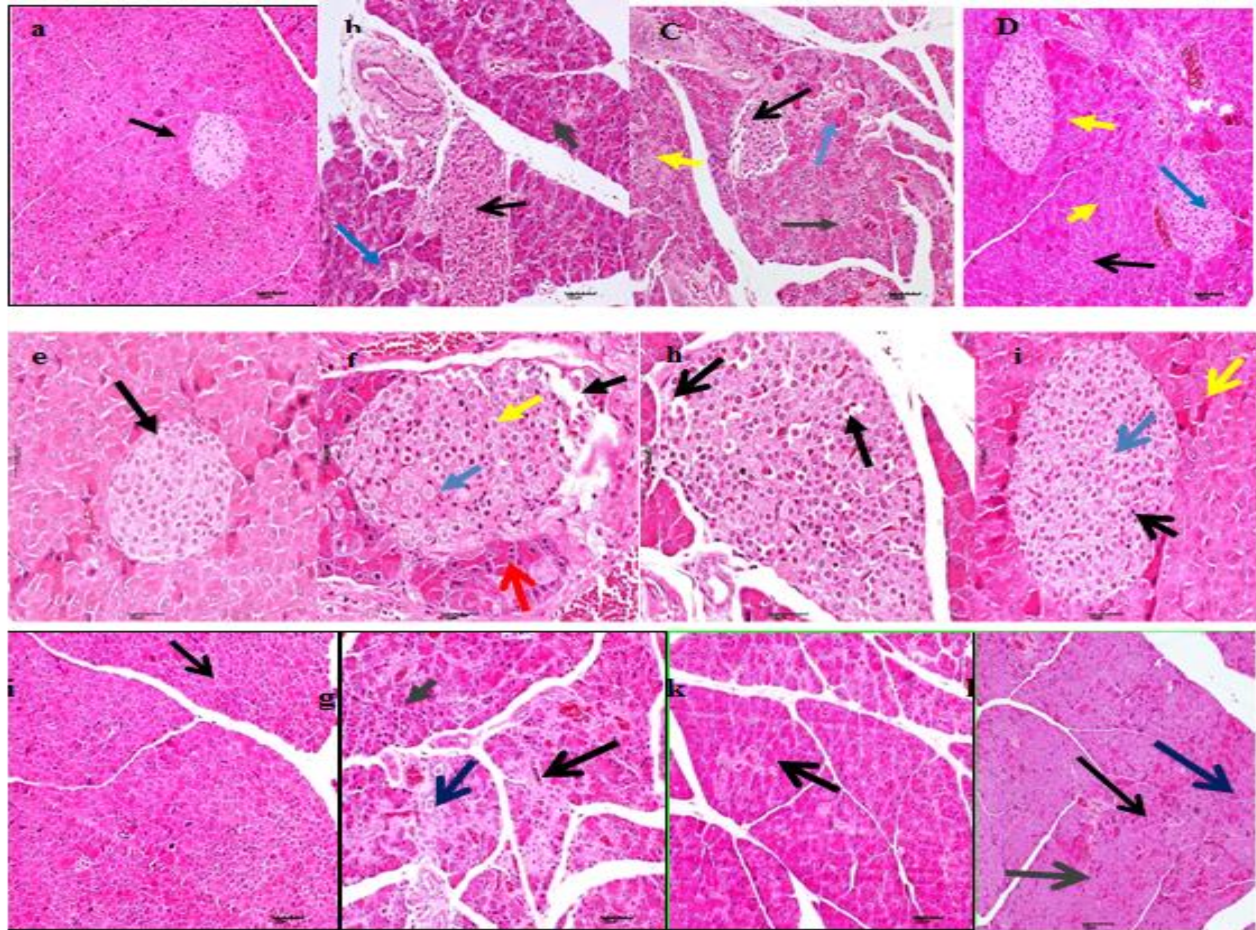

Figure 11: Photomicrographs of pancreas tissues of mice from different experimental groups. (a) Normal control mice pancreatic (CN) endocrine and exocrine showing islets of Langerhans and acini (200x). (b) Insulin resistance mice pancreas (CP). shows degeneration of acini (gray arrow) and hypertrophy of Langerhans (black arrow), necrotic cells with pyknotic nuclei and acidophilic cytoplasm (blue arrow), and disruption of normal endocrine architecture (200x). (c) Pancreas of andrographolide treated insulin resistance mice (HFA25) shows degeneration and acidophilic cytoplasm (irregular space in the islets of Langerhans—black arrow), swelling of the pancreatic acinar and irregular arrangements (luminal disappearing—blue arrow). Pancreatic cells necrosis (yellow arrow) (200x). (d) Pancreas andrographolide treated insulin resistance (HFA50) shows significantly reduced necrosis (black arrow), reduced cellular degeneration (blue arrows), and normal endocrine and exocrine normal islets of Langerhans acinar, and no vacuolar degeneration (yellow arrow) (200x). (e) Pancreas control negative (CN) endocrine and exocrine showing islets of Langerhans and acini (black arrow) (400x).(f) Insulin resistance mice pancreas (CP) shows destruction of islet of Langerhans structure (black arrow) ,severe inflammation and degenerative changes like fatty degeneration (blue arrow), nuclei pyknotic (yellow arrow) and acidophilic cytoplasm(red arrow) (400x).(g) HFA25 shows Pancreas of andrographolide treated insulin resistance mice reduced slight cellular degeneration and necrosis (black arrows) (400x).(j) HFA50 treated mice shows significant cellular and architectural restoration, reduced necrosis (black arrow), reduced degeneration (blue arrow), and normal endocrine and exocrine slightly acidophilic cytoplasm (yellow arrow).(i) CN shows normal pancreatic acini structure (black arrow).(h) CP shows degeneration pancreatic acini (black arrow) , cell infiltrations(gray arrow), multi necrosis area (blue arrow)(400x).(i) HFA25 shows reduce degeneration of pancreatic acini (black arrow).(l) HFA50 shows significant reduce degeneration and necrosis (black arrow),slight acidophilic reaction in pancreatic acini (blue arrow).restoration pancreatic acini architecture(gray arrow).

and pancreatic islet cells[50,51]. It is evident that the mice in this study were insulin resistant and obese after being fed with the high fat diet for 24 weeks. The induced insulin resistance developed in mice using high fat diet as observed in this study would mimic the natural sequence of disease initiation and progression and it demonstrated the characteristic metabolic features of both type 2 diabetes and insulin resistance as similarly reported in a previous study[53-56]. Although the exact mechanism through which high fat diet induce insulin resistance was not investigated in this study, previous studies have linked it to defects in the insulin signal cascade via Serine phosphorylation of insulin receptor substrate 1 (IRS-1), leading to impaired glucose usage[57,58]. Postulated that the accumulation of

Histology of panceratic tissue of treated groups (Masson trichrom stain)

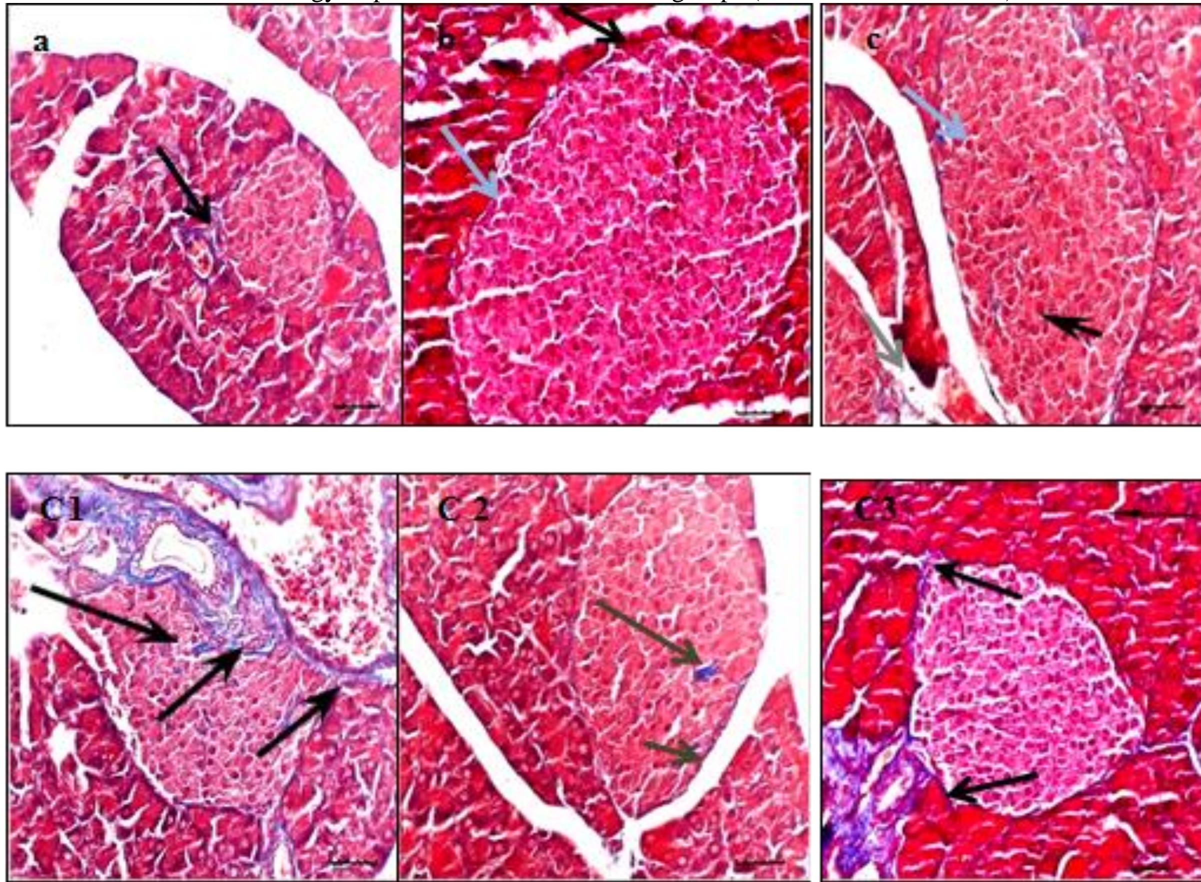

Figure 12: Photomicrograph sections of pancreas tissue for treated mice using Masson trichrome stains (a) CN mice pancreas shows collagen fibers around Langerhans islets (black arrow)(400x).(b) CP shows hypertrophy in the islets of Langerhans cells (black arrow) ,no collagen fibers deposition (blue arrow).(c) HFA25 shows mild collagen fibers around(blue arrow) inside of (black arrow) the Langerhans islets and pancreatic acini (gray arrow)showed beginning stages of pancreatic recovery (collagen fiber aggregation), (c1) ,(c2) ,(c3) HFA50 shows heavy collagen fibers accumulation in pancreatic acini, a round blood vessels(black arrow), and islet of Langerhans (gray arrow)due to cell healing processes compare to treated group.

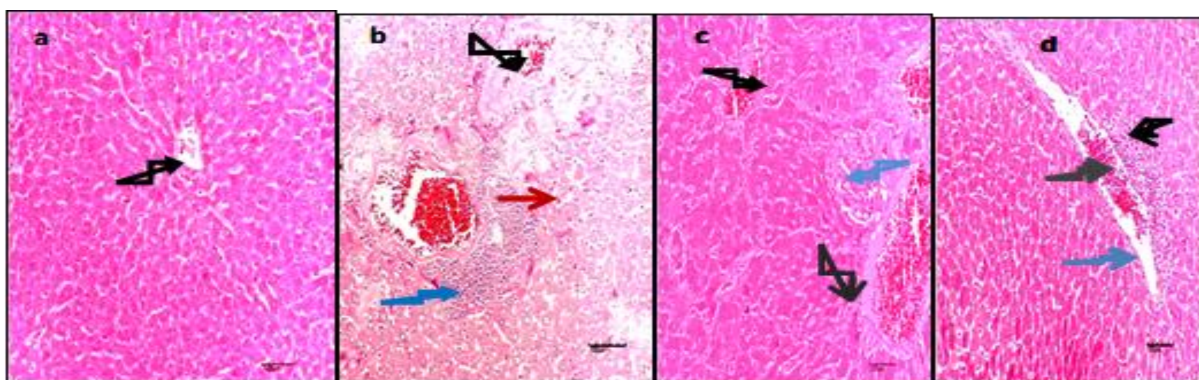

Figure 13: Photomicrographs of liver tissues of mice from different experimental groups. (a) CN hepatocytes of normal control mice shows normal liver cell structure (200x). (b) CP liver show severe degeneration, loss of hepatic architecture (black arrow), cell infiltrations (inflammation) (blue arrow), and sever steatosis (fat accumulation) (red arrow) (200x). (c) HFA25 liver of andrographolide treated insulin resistance shows mild granular degeneration (gray arrow) and mild swelling of liver cell (blue arrow), and sinusoidal congestion (black arrow) (200x). (d) HFA50 liver of andrographolide treated insulin resistance mice shows significantly reduced fatty change, normal morphological architecture and significant degenerative cells reduction (blue arrow), reduced multifocal infiltration of inflammatory cells (black arrows), reduced sinusoidal Congestion (gray arrow) (200x).

excess fats due to high fat diet as lipid in pancreas, liver, and adipose tissues is responsible for reduced insulin sensitivity and this precedes the initiation of insulin resistance, obesity and mitochondrial dysfunction. Findings from other studies have suggested that high levels of free fatty acids are associated with decreased expression of insulin receptor substrate-1 (IRS-1) in peripheral tissues and impaired glucose utilization, resulting in subsequent hyperglycaemia and hyperinsulineamia[59,60]. This ultimately leads to insulin resistance and its subsequent histopathological changes seen for CP groups (Figures 5, 6, 7). The histopathological changes in pancreas such as islet hypertrophy, disrupted cellular architecture of the islets, inflammatory cell infiltration and the fatty degeneration, steatosis, vascular congestion and hepatocellular degenerations observed in the CP (Figure.10) in this study is consistent with findings in other previous studies[33,34,12] to be a consistent feature of obesity-induced insulin resistance[4]. In chronic stage, the pancreas fails in its compensatory responses and most beta cells and acini of the pancreas begin to experience degenerative changes, which lead to the significant histopathological changes seen in the islets. In the liver, excess systemic free fatty acids as a result of high fat diet modulates increased intracellular diacylglycerol, which in turn results in lower insulin stimulated liver glycogen synthesis and subsequently, decreasing the suppression of hepatic gluconeogenesis as similarly reported in a previous study[17,31]. This could ultimately be the genesis of the significant difference in the hepatocellular changes seen in CP mice against the CN, HFA25 and HFA50 mice. Glycated hemoglobin which is also known as hemoglobin A1c, A1C, HbA1c, or Hb1c is one of the forms of hemoglobin that is usually measured principally to identify the average plasma glucose level over protracted periods of time[18]. Measuring the levels of glycated hemoglobin gives assess to the effectiveness of treatment by checking protracted serum glucose regulation. Level of HbA1c has been reported to be directly proportional to the blood glucose level over the last four weeks to about 3 months[31,10]. In this study, 24 weeks feeding with HFD was observed to induce increase in HbA1c level. However, 15 days' treatment with andrographolide was observed to reverse the increase in HbA1c level. This finding is in agreement with the report of a previous study[11] who observed a fall ion HbA1c following treatment of the subjects with andrographolide. The decline in HbA1c level was opined to be due to increased blood glucose utilization which was proposed to be one of the mechanisms of andrographolide in combatting diabetes and insulin resistance. On the other hand, the groups treated with andrographolide demonstrated significant amelioration in the histopathological changes induced by the high fat diet as shown in (Fig. 5, 6, 7 and 8). These were also reflected in (Table 1) scoring for the pancreatic tissues. Treatment with andrographolide was seen to significantly induce amelioration and regeneration of the acini and islet cells. It has been shown that oxidative stress plays a key role in the pathogenesis of insulin resistance and beta cell dysfunction via their ability to activate stress-sensitive signaling pathways[5,8]. Although this study did not investigate the exact mechanisms involved in the damage amelioration induced by treatment with andrographolide, previous studies have found[13,9] that andrographliode have potent antioxidant activity. Antioxidants are known to be one of the body's first lines of defense against cellular damage due to oxidative stress[60].

The damage amelioration and regeneration seen (Fig. 8) in the islets of Langerhans and pancreatic acini following treatment with andrographliode for 15 days is suggestive of pancreas protective effects exerted by andrographolide. The ameliorative effects observed in this study could be due to reduction in the generation of reactive oxygen species in the liver and pancreas increased antioxidant activity, concomitantly reducing the pancreas and hepatocellular damage. This finding is in agreement with a previous study[58] where hepatoprotective properties of andrographliode were investigated in both normal and diabetic rats. Previous investigations suggest that increased oxidative stress is potential mechanisms of action for hyperglycemic toxicity on pancreatic beta cells[44]. Disturbances in the metabolism of lipids in insulin resistance are important determinant factor in the course and status of the disease[56]. In the adipocytes, the larger size and volume of adipocytes and abnormal cellular architecture seen (Fig. 13) could be attributed to inhibition of cholesterol catabolism, leading to accumulation of free fatty acids. The accumulation of free fatty acids in the adipose tissues could consequently reduce insulin sensitivity and eventually insulin resistance. The normal size, structure and volume of adipocytes seen in the treated group could be associated with damage amelioration induced by andrographolide through enhanced lipid catabolism. This finding is in accordance with earlier findings[25] where extracts of andrographliode were investigated in diabetes-induced rats and mice respectively[17,46,58]. studies has established that the anti-inflammatory effect of andrographolide on adipose tissue could be explained by its ability to inhibit neutrophil adhesion/transmigration through suppression of Mac-1 up regulation, more importantly; this study showed that andrographolide is invaluable in inducing cellular repair and regeneration. Thus, andrographolide could be useful for the improvement of pancreas beta cell mass and insulin functions.

CONCLUSION

The findings of this study concluded that treatment with pure extract of andrographliode could significantly ameliorate insulin sensitivity, and the pathological changes associated with insulin resistance as observed in this study. Andrographolide has a credibly high potential anti-insulin resistance effects. Future studies should attempt to investigate the exact mechanisms associated with the ameliorative effect of the pure extract of andrographolide at the molecular and cellular levels.

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