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HIGH FAT / HIGH CARBOHYDRATE LONG TERM DIET ALTERED THE ANTIOXIDANT DEFENSE MECHANISM IN C57BL/6J MICE: EFFECT OF VANILLIC ACID ON LIPID PEROXIDATION MARKERS WITH ANTIOXIDANTS IN LIVER AND KIDNEY TISSUES

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ABSTRACT

Recent studies have revealed that oxidative stress has been suggested as a contributory factor in development and complication of diabetes. The aim of the present study was to determine the protective effect of vanillic acid (VA) on lipid peroxidation and activities of antioxidant defense systems in high fat / high carbohydrate (HF/HC) diet induced C57BL/6J mice. The thirty six male C57BL/6J mice (18-22g) were used. Diabetic mice with hyperlipidemia were induced by oral administration of HF/HC diet on 18 weeks. Mice were divided into six groups of six mice in each cage. Group I served as normal mice intended for control; Group II normal with high dose of VA (40 mg/kg of body weight); Group III-VI receives HF/HC diet for 18 weeks, without VA for Group III and 10, 20 and 40 mg/kg of body weight of VA on last 8 weeks for Group IV, V and VI respectively. End of the study period glucose, insulin, lipid peroxidation markers, antioxidants, lipid profile and histopathological examination were analysed.Diabetic mice exhibited an increase in the levels of plasma glucose, lipid peroxidative markers and lipid profile; whereas, the activities of antioxidant weredecrease inliver and kidneys tissues as compared to control rats. Administration of VA to diabetic mice for last 8 weeks significantly inverted the damage associated with diabetes. These results indicated that VA may act as a safe preventive or therapeutic agent against diabetes mellitus.

Key Words: Vanillic acid, HF/HC, High fat / Highcarbohydrate,Free fatty acids.

INTRODUCTION

Diabetes mellitus, characterized by hyperglycemia is the most common serious metabolic disorder, which is considered to be one of the five leading causes of death in the world [1,2]. Various studies have shown that diabetes mellitus is associated with oxidative stress, leading to an increased production of reactive oxygen species (ROS), including superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$) or reduction of antioxidant defense system [2,3]. Implication of oxidative stress in the pathogenesis of diabetes mellitus is suggested not only by oxygen free radical generation and also due to non-enzymatic protein glycosylation, auto-oxidation of glucose, impaired antioxidant enzyme activites, formation of peroxides[3,4]. Lipid peroxidation (LOOH), a key marker of oxidative stress is a free radical-induced process causing oxidative deterioration of polyunsaturated fatty acids that eventually results in extensive membrane damage and dysfunction. The significant extent of LPO products measured as thiobarbituric acid reactive substances (TBARS) has been reported in diabetes [4, 5]. The formation of ROSwas prevented by an antioxidant system that included non-enzymatic antioxidants (ascorbic acid, glutathione (GSH), tocopherols) and ROS–scavenging antioxidant enzymes such

as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxides (GPx) [6, 7]. Since the synthetic drugs have undesirable side effects or contraindications, the world health organization (WHO) has recommended the evaluation of traditional plant treatments for diabetes [8]. Hypoglycemic effects have been reported in some plants that contain terpenoids, iridoid glycosides, flavonoids, and other phenolic compounds [9]. In addition, a number of secondary metabolites like flavonoids, phenolic acids, phenylpropanoids, and terpenoids have shown significant antioxidant properties [2, 10,11].The cured or fermented beans or pods of the orchid are treated with alcohol and the extract is the natural vanilla. Vanilla (*Vanilla planifolia, Vanilla pompona or Vanilla tahitiensis*) is a tropical, climbing orchid;the main component of vanilla aroma is "vanillin" (4-hydroxy-3-methoxybenzaldehyde), which is accompanied with minor amounts of vanillic acid (VA) along with200 trace components [12]. In addition to flavouring properties, vanillin exhibits several bioactive properties [12] such as antioxidant [13], and antimicrobial activities against yeasts, moulds [14] and bacteria [15].Vanillin has also been reported to possess anticlastogenic, antimutagenic and antitumor properties and therefore it can be considered as a nutraceutical molecule [16]. Moreover, it has found applications as a constituent in perfume and pharmaceutical formulations.

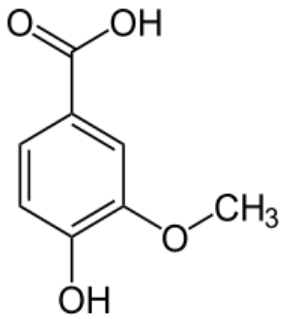

Fig.1. Structure of vanillic acid

MATERIAL AND METHODS

Animals

Healthy adult male C57BL/6J mice, obtained from National Institute of Nutrition (NIN), Hyderabad; and reared in central animal house, Department of experimental medicine, Rajah muthaiah medical college, Annamalai University were used for the experiment. Weight matched animals (18-22g) were selected and housed in polypropylene cages layered with husk; and kept in a semi-natural light/dark condition (12h light/12h dark). The animals were permissible for free access to water and standard pellet diet (Amrut laboratory animal feed, Pranav agro industries ltd., Bangalore, India). Animal handling and experimental procedure were approved by the institutional animal ethics committee, Annamalai University (Register number 166/1999/CPCSEA, Pro.No 778) and animals were cared in accordance with the "Guide for the care and use of laboratory animals" (NIH, 1985) and "Committee for the purpose of control and supervision on experimental animals" (CPCSEA, 2004).

Source of chemicals

VA was purchased from sigma chemical co., St. Louis, MO, USA. All other chemicals were of analytical grade obtained from Himedia laboratories pvt. ltd.or S.D Fine chemicals, Mumbai, India.

Experimental design

The mice were randomly divided into six groups of six mice in each cage. Group I - Non diabetic control mice;Group II - Non diabetic mice treated with VA (40mg/kg of body weight); Group III - Mice treated with HF/HC diet for 18 weeks; Group IV, V and VI - mice treated with high fat / high carbohydrate (HF/HC) diet for 18 weeks, and in addition treated with 10, 20 and 40 mg of VA/kg of body weightrespectively for last eight weeks.After 18 weeks, the animals were fasted for overnight (12 h) and anaesthetized between 8:00 - 9:00 am using ketamine (24 mg/kg of body weight, intramuscular injection); and sacrificed by decapitation. Blood was collected in tubes with a mixture of potassium oxalate and sodium fluoride (1:3) for the estimation of plasma glucose and insulin; and also with ethylenediaminetetraacetic acid (EDTA) for the estimation of haemoglobin. Liver and kidney were dissected out, washed withice-cold saline and stored at 4°C for the measurement of various enzyme activities.

Estimation of Blood Glucose

Blood glucose was estimated by the method of Trinder, using reagent kit [17]. 1000µl of enzyme reagent was added and mixed properly in each separate tubes containing 10µl of plasma, standard and distilled water (blank); and kept at 37°C for 15 minutes. The absorbance of colour developed was observed at 505nm against blank and glucose values were expressed in mg/dl of plasma.

Estimation of plasma Insulin

Plasma was analyse by the solid phase system amplified sensitivity immunoassay using reagents kit obtained from Medgenix – INS – ELISA, Biosource, Europe SA Belgium [18]. The assay was based on the oligioclonal system in which several monoclonal antibodies (Mabs) directed against distinct epitopes of insulin were used.

Determination ofthiobarbituric acid reactive substances (TBARS) and lipid peroxidation (LOOH)

The concentration of TBARS in the tissues was estimated by the method of Nichans and Samuelson [19]. In this method, 0.5 mL of sample was diluted with 0.5 mL of double distilledwater and mixed well, and then 2.0 mL of TBA-TCA-HCL reagent was added. The mixture was kept in a boiling water bath for 15 min, after cooling, the tubes were centrifuged at 1000 g for 10 min and the supernatant was estimated. A series of standard solution in the concentrations of 2-10 nmol were treated in a similar manner. The Absorbance of the chromophore was read at 535 nm against a reagent blank. The values were expressed as mmol/100 g of tissues. The lipid hydroperoxide was determined by the method ofJiang et al.[20];0.1 ml of tissue homogenate was treated with 0.9 ml of Fox reagent (88mg of butylated hydroxy toluene (BHT), 7.6mg of xylenol orange and 0.8mg of ammonium iron sulphate were added to 90 ml of methanol and 10 ml of 250mMsulphuric acid) and incubated at 37°C for 30 minutes. Then the absorbance was read at 560 nm. Lipid hydroperoxides were expressed in mM/100g-tissue.

Estimation of reduced glutathione (GSH)

Reduced GSH in the tissues was estimated by the method of Ellman [21]. A known weight of tissue was homogenized in 0.1M phosphate buffer (pH 7.0); 0.5ml of homogenate was taken and precipitated by adding 2.0 ml of 5% TCA.The mixtures were centrifuged; and to 2.0 ml of supernatant, 1.0 ml of Ellaman's reagent and 4.0 ml of 0.3M disodium hydrogen phosphate were added,the absorbance of yellow colour developed was observed in the colorimeter at 412nm. A series of standards with range in between 20 – 100µg were treated in a similar manner along with a blank containing 1.0 ml of phosphate buffer. The amount of GSH was expressed as µg/mg protein for tissues.

Estimation of ascorbic acid (Vitamin C)

Ascorbic acid in the tissues was estimated by the method ofRoe et al. [22]. To 0.5 ml of the sample, 1.5ml of 6% TCA was added and allowed to stand for 5minutes; and then centrifuged. To the supernatant, 0.3g of acid washed norit was added and vigorously shaken;this converts ascorbic acid to dehydroascorbic acid. 0.5ml of filtrate was takenafter filtration and added with 0.5 ml of DNPH, stoppered or placed in a water bath at 37°C for exactly 3 hours;later remove and place in ice-cold water, and add 2.5ml of 85% sulphuric acid drop by drop. The contents of the tubes were mixed well and allowed to stand at room temperature for 30minutes. A set of standards containing 20 – 100µg of ascorbic acid were taken and processed similarly along with a blank containing 2.0 ml of 4% TCA. The colour developed was observed at 540 nm andthe values were expressed in µg/mg protein for tissue.

Estimation of α – tocopherol (vitamin E)

α – tocopherol in the tissues was estimated by the method of Baker et al. [23]. 0.5ml of sample was added with 1.5ml of ethanol, mixed properly and centrifuged. The supernatant was evaporated and to the precipitate, 3.0 ml of petroleum ether, 0.2ml of 2, 2' dipyridyl solution and 0.2ml of ferric chloride solution were added, mixed well and kept in dark for 5minutes. An intense red colour was developed. 4.0 ml of n-butanol was added to all the tubes and mixed well. Standard tocopherol in the range of 10 – 100µg was taken and treated similarly along with a blank containing only the reagent. The colour in the n-butanol layer was read at 520nm. The values were expressed as µg/mg protein for tissues.

Assay of superoxide dismutase (SOD)

SOD in the tissues was assayed by the method ofkakkar et al. [24]. Tissue was homogenized by using sodium pyrophosphate buffer (0.025M, pH8.3). 0.5ml of tissue homogenate was diluted to 1.0 ml with water followed by addition of 2.5ml of ethanol and 1.5ml of chloroform (chilled reagents were added). This mixture was shaken for 90 seconds at 4°C and then centrifuged. The enzyme activity in the supernatant was determined as follows. The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1ml of phenazinemethosulphate and 0.3ml of nitroblue tetrazolium and appropriately diluted enzyme preparation in a total volume of 3.0 ml. The reaction was started by the addition of 0.2ml NADH. After incubation at 30°C for 90 seconds, the reaction was stopped by the addition of 1.0 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4.0 ml n-butanol. The mixture was allowed to stand for 10minutes; centrifuged and n-butanol layer was separated. The colour density of the chromogen in n-butanol was measured at 510nm. A system devoid of enzyme served as control. The enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard condition was taken as one unit. The specific activity of the enzyme was expressed as U/min/mg protein for tissues.

Determination of catalase (CAT)

CAT level was determined by Aebi's modified colorimetric method [25]. The reaction mixture (1.5 ml, vol.) contained 1.0 ml of 0.01M phosphate buffer (pH 7.0), 0.1 ml of tissue homogenate and 0.4 ml of 2MH_2O_2. The reaction was stopped by the addition of 2.0 ml of dichromate–acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio). Then the absorbance was read at 620 nm. CAT activity

was expressed as µM of H_2O_2 consumed/min/mg protein.

Estimation of glutathione peroxidise(GPx)

The activity of GP_x in the tissues was measured by the method ofrotruck et al.[26]. The tissue was homogenized using tris buffer. To 0.2ml of tris buffer, 0.2ml of EDTA, 0.01ml of sodium azide, 0.5ml of tissue homogenate or hemolysate were added. To the mixture, 0.2ml of GSH followed by 0.1ml of H_2O_2 was added. The contents were mixed well and incubated at 37°C for 10 minutes; along with a control containing all reagents expect homogenate. After 10minutes, the reaction was arrested by the addition of 0.5 ml of 10% TCA. The tubes were centrifuged and the supernatant was assayed for GSH by the method ellman [21]. The activity was expressed as µmol of GSH consumed/min/mg protein for tissues.

Estimation of total cholesterol

Total cholesterol in the plasma was estimated by the enzymic method described by Allain[27]. To 10 µl of plasma or 10 µl of lipid extract, 1.0 ml of enzyme reagent was added, mixed well and kept at 37°C for 5 minutes. 10 µl of cholesterol standard and distilled water (blank) were also processed similarly. The absorbance was measured at 510 nm. Cholesterol concentration was expressed as mg/dl of plasma.

Estimation of triacylglycerol

Triacylglycerol in the plasma were estimated using the diagnostic kit based on the enzymic method described by McGowan [28]. To 10 µl of plasma or 10 µl of lipid extract, 1.0 ml of enzyme reagent was added, mixed well and incubated at room temperature for 10 minutes. 10 µl of triacylglycerol standard and distilled water (blank) were also processed similarly. The absorbance was measured at 510 nm.The triacylglycerol content was expressed as mg/dl of plasma or mg/g wet tissue.

Estimation of free fatty acids (FFA)

FFAin the plasma and tissues were estimated by the method [29]. 0.5 ml of lipid extract was evaporated to dryness and dissolved in 6.0 ml chloroform-heptane-methanol solvent and 2.5 ml of copper reagent were added. All the tubes were shaken vigorously for 90 seconds and were kept aside for 15 minutes. The tubes were centrifuged and 3.0 ml of the copper layer was transferred to another tube containing 0.5 ml of diphenyl carbazide and mixed carefully. The colour developed was read at 540 nm against a reagent blank containing 3.0 ml solvent and 0.5 ml diphenyl carbazide. The FFA content was expressed inmg/dl of plasma.

Estimation of phospholipids

Phospholipids were estimated by the method of zilversmith et al. [30]. 0.5 ml of lipid extract was evaporated to dryness. 1.0 ml of 5.0 N sulphuric acid was added and digested till light brown. Then 2 to 3 drops of concentrated nitric acid was added and the digestion was continued till it became colourless. After cooling, 1 ml of water was added and heated in a boiling water bath for about 5 minutes. Then, 1.0 mlof ammonium molybdate and 0.1 ml of ANSA were added. The volume was then made up to 10.0 ml with distilled water and the absorbance was measured at 680 nm within 10 minutes. Standards in the concentration range of 2-8 mg were treated in the similar manner. The values obtained were multiplied with a factor 25 to convert inorganic phosphorus to its phospholipids equivalents. The amount of phospholipids was expressed as mg/dl of plasma or mg/g wet tissue.

Histopathological study

For histopathological study, the organs were removed and perfuse with cold physiological saline, followed by formalin (10% formaldehyde). The liver, kidneys were excised immediately and fixed in 10% formalin. Then dehydrated on treatment with a series of different concentration of ethanol and embedded in paraffin wax. 3-5 µm thick sections were cut using microtome and stained with hematoxylin and eosin. The specimens were evaluated under light microscope. All histopathological changes were examined by a pathologist.

Data presentation and statistical analysis

Statistical test was performed using SPSS Version 11. Data is reported as the mean ±SD Differences between means were evaluated by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT). The level of significance was set at $p < 0.05$.

RESULTS

Effect of VA on plasma glucose and insulin

Table 1 represented the changes of plasma glucose and insulin in control, HF/HC induced diabetic mice and VA treated mice. The level of plasma glucose was significantly increased, whereas the level of plasma insulin wasconsiderably decreased in HF/HC induced diabetic mice. Oral administration of VA (10,20 and 40mg/kg of body weight) daily for a period of 8 weeks to diabetic mice significantly decrease the levels of plasma glucose, and appreciable increase in levels of plasma insulin were observed at the end of experimental period. VA at a dose of 40 mg/kg of body weight showed a highly significant effect than 10 and 20 mg/kg of body weight. Based on the data, the effective dose of VA was fixed at 40 mg/kg of body weight and applied for further studies.

Table.1.Effect of vanillic acid on plasma insulin and glucose, in control and HF/HC induced diabetic C57BL/6J mice

Groups	Insulin (µU/ml)	Glucose (mg/dl)
Normal	41.78 ± 0.17a	83.12 ± 7.97a
Normal + Vanillic acid (40mg)	35.36 ± 0.15a	86.35 ± 7.76a
HF/HC	18.32 ± 3.33b	269.54 ±26.15b
HF/HC + Vanillic acid (10 mg)	20.01 ± 1.11a	234.14 ± 22.37c
HF/HC + Vanillic acid (20 mg)	29.32 ± 2.55d	183.67 ± 17.96d
HF/HC + Vanillic acid (40 mg)	37.41 ± 2.68c	133.95 ± 12.44a

Values are means ± SD from 6 mice in each group. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT), HF/HC, high fat/high carbohydrate.

Effect of VA on TBARS and hydroperoxides (HP)

Table 2 shows significant elevation in the levels of TBARS and LOOH in the tissues of control, HF/HC induced diabetic mice and VA treated mice. The levels of TBARS and HP in tissues were increased in HF/HC mice, and administration of VA showed significant decline in the levels of TBARS and HP in their experimental groups.

Table.2.Effect of vanillic acid on thiobarbituric acid reactive substances (TBARS) and lipid peroxidation (LOOH) activity, in liver and kidney of control and HF/HC induced diabetic C57BL/6J mice

Groups	TBARS (mmol/mg tissue)		LOOH (mmol/mg tissue)	
	Liver	***Kidney***	***Liver***	***Kidney***
Normal	1.03 ± 0.09a	1.37 ± 0.07a	91.45 ± 7.96a,e	82.73±1.40a,b
Normal + Vanillic acid (40 mg)	0.98 ± 0.07a	0.94 ± 0.08b	87.33 ± 8.34a	81.07 ± 2.03[a]
HF/HC	4.53 ± 0.37b	4.79 ± 0.39c	153.09 ± 14.72b	120.83± 2.30e
HF/HC + Vanillic acid (10 mg)	4.02 ± 0.35c	3.89 ± 0.28d	135.42 ± 13.52c	104.73 ± 4.43,d
HF/HC + Vanillic acid (20 mg)	3.98 ± 0.26c	3.25 ± 0.25e	127.64 ± 11.65d	91.52 ±1.91c
HF/HC+Vanillic acid (40 mg)	2.80 ± 0.14d	2.88 ± 0.16f	102.35 ± 8.98e	84.48 ±3.05b

Values are means ± SD from 6 mice in each group. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT). HF/HC, high fat/high carbohydrate

Effect of VA on non enzymic antioxidants

Table 3 shows the effect of VA on vitamin C, vitamin E and GSH in liver and kidney of control, HF/HC induced diabetic mice and VA treated mice.

In diabetic mice the level of vitamin E, vitamin C and GSH levels were observed to be decreased in the tissues. The oral administration of VA altered the non enzymic antioxidant like vitamin E, vitamin C and GSH levels to almost normal.

Table.3.Effect of vanillic acid on vitamin C, vitamin E and reduced glutathione, in the liver and kidney of control and HF/HC induced diabetic C57BL/6J mice

Groups	Vitamin C (µg/mg protein)		Vitamin E (µg/mg protein)		Reduced Glutathione (µg/mg protein)	
	Liver	*Kidney*	*Liver*	*Kidney*	*Liver*	*Kidney*
Normal	0.84 ± 0.06a	0.78 ± 0.05a	5.96 ± 0.50a	4.02 ± 0.02a	14.74 ± 0.14a	13.79 ± 0.10a
Normal+Vanillic acid (40 mg)	0.80 ± 0.07a	0.75 ± 0.06a	5.80 ± 0.48a	3.90 ± 0.30a	14.69 ± 1.02a	13.73 ± 0.91b
HF/HC	0.40 ± 0.02b	0.44 ± 0.02b	2.96 ± 0.19b	1.78 ± 0.12b	7.06 ± 0.69b	5.98 ± 0.46c
HF/HC+Vanillic acid (10 mg)	0.57 ± 0.04c	0.51 ± 0.01c	3.99 ± 0.30c	2.08 ± 0.10c	9.60 ± 0.83c	7.74 ± 0.61d
HF/HC+Vanillic acid (20 mg)	0.68 ± 0.05d	0.60 ± 0.04d	4.27 ± 0.33c	2.77 ± 0.16d	11.45 ± 0.91d	9.22 ± 0.78e
HF/HC+Vanillic acid (40 mg)	0.79 ± 0.06a	0.63 ± 0.05d	5.33 ± 0.45d	3.86 ± 0.27a	13.78 ± 0.91a	12.07 ± 0.90a

Values are means ± SD from 6 mice in each group. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT). HF/HC, high fat /high carbohydrate.

Effect of VA on enzymic antioxidants

Tables 4 show the activities of enzymatic antioxidant in liver and kidney of control, HF/HC induced diabetic mice and VA treated mice. The activities of SOD, CAT and GPx significantly decreased in the tissues of diabetic mice. VA administration resulted in a significant increase in the activities of enzymatic antioxidants in the tissues of diabetic mice.

Table.4.Effect of vanillic acid on SOD, CAT and GPx, in the liver and kidney of control and HF/HC induced diabetic C57BL/6J mice

Groups	SOD (U^{α}/mg protein)		CAT (U^{β}/mg protein)		Glutathione peroxidase (Gpx) (U^{γ}/mg protein)	
	Liver	*Kidney*	*Liver*	*Kidney*	*Liver*	*Kidney*
Normal	10.87 ± 0.94a	12.63 ± 0.10a	67.31 ± 5.22a	31.43 ± 2.27a,e	11.58 ± 0.90a	9.73 ± 0.84a
Normal+Vanillic acid (40 mg)	10.90 ± 0.88a	12.69 ± 0.12a	70.22 ± 6.26a	33.19 ± 2.32a	11.64 ± 0.10a	9.79 ± 0.83a
HF/HC	5.77 ± 0.50b	5.07 ± 0.45b	30.54 ± 2.24b	14.63 ± 0.25b	4.37 ± 0.34b	4.51 ± 0.33b
HF/HC+Vanillic acid (10 mg)	7.13 ± 0.67c	7.72 ± 0.71c	43.42 ± 3.56c	20.51 ± 1.05c	6.09 ± 0.55c	5.95 ± 0.41c
HF/HC+Vanillic acid (20 mg)	8.08 ± 0.77c	9.47 ± 0.80d	55.28 ± 4.66d	27.03 ± 1.66d	8.72 ± 0.84d	7.43 ± 0.58d
HF/HC+Vanillic acid (40 mg)	10.11 ± 1.00a	11.24 ± 1.09e	60.05 ± 5.54d	30.02 ± 2.22e	10.17 ± 1.02e	8.98 ± 0.75a

Values are means ± SD from 6 mice in each group. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT). HF/HC, high fat / high carbohydrate

$^{\alpha}$ enzyme concentration required to inhibit the NBT to 50% reduction in one minute

$^{\beta}$ µmole of H_2O_2 consumed / minute

$^{\gamma}$ µg of GSH utilized / minute

Effect of VA on lipid profiles

Table 5 shows the levels of total cholesterol, TGL, FFA and PLP in the plasma of control, HF/HC induced diabetic mice and VA treated mice. These levels have been found to be increased in the plasma of the HF/HC treated mice. The oral administration of VA drastically decreased the levels total cholesterol, TGL, FFA and PLPclose to normal.

Table.5.Effect of vanillic acid on triglycerides, total cholesterol, free fatty acids and phospholipids, in the plasma of control and HF/HC induced diabetic C57BL/6J mice

Groups	Triglycerides (mg/dl)	Total cholesterol (mg/dl)	Free fatty acids (mg/dl)	Phospholipids (mg/dl)
Normal	67.74 ± 5.01a	75.86 ± 7.13a	52.75 ± 4.58a	77.62 ± 7.00a,e
Normal + VA(40 mg)	62.04 ± 5.64a	71.28 ± 6.65a	49.83 ± 4.02a	72.36 ± 6.78a
HF/HC	170.22 ± 16.61b	176.07 ± 17.04b	139.32 ± 12.74b	166.27 ± 15.84b
HF/HC+VA (10mg)	152.12 ± 14.51c	143.72 ± 14.54c	108.14 ± 10.41c	131.09 ± 13.39c
HF/HC+VA(20mg)	124.52 ± 11.42d	115.32 ± 10.42d	83.79 ± 7.89d	111.47 ± 10.09d
HF/HC+VA (40mg)	87.24 ± 8.31e	93.15 ± 9.08e	64.38 ± 5.76e	89.32 ± 8.21e

VA - Vanillic acid
Values are means ± SD from 6 mice in each group. Values not sharing a common superscript letter differ significantly at $p < 0.05$ (DMRT). HF/HC, high fat/high carbohydrate.

Effect of VA on histopathology of liver, kidney

Fig.2 (A) depicts the photomicrographs of hematoxylineeosin staining of hepatic tissues of control and experimental mice. Fig.2(A) shows the section of liver tissue of control mice demonstrating the normal architecture of hepatocytes. Similarly the normal group treated with VA also revealed an sinusoids congested in normal hepatocytes (Fig.2(B)). Fig.2(C) shows the hepatic tissues of HF/HC induced diabetic mice exhibiting fatty acid changes in all zone. Fig. 2(D) shows the diabetic mice treated with VA presenting sinusoidal dilation to a lesser degree and exhibited limited fatty changes.

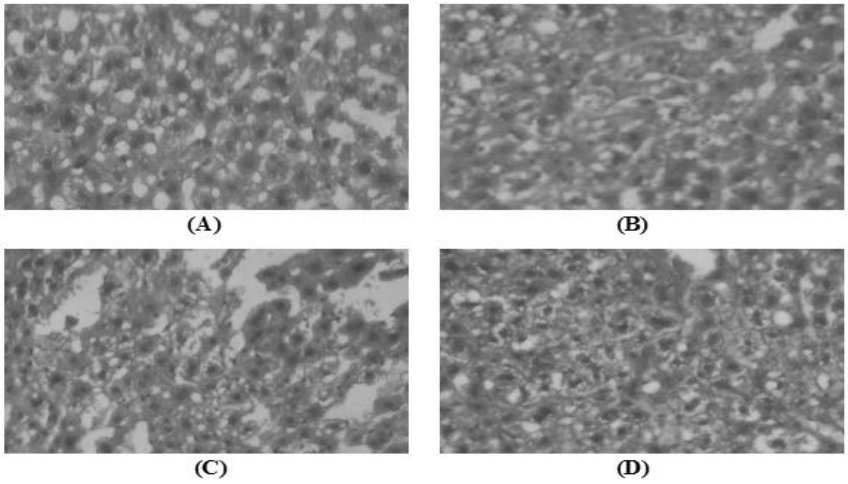

Fig.2. Photomicrographs of hematoxylin-eosin staining of hepatic tissues of control and experimental mice

Histological photograph of (A) control, (B) Control + VA (40 mg/kg of body weight), (C) HF/HC induced diabetic mice and (D) HF/HC induced diabetic mice +VA (40 mg/kg of body weight) treated mice in hepatic tissue sections were showed at 20X magnification.

Fig.3(A) represents the photomicrographs of hematoxyline eosin staining of renal tissues of control and experimental mice. Fig.3(A) shows the section of kidney tissue of control mice showing normal kidney architecture with glomeruli and tubules. Similarly the normal group treated with VA shows normal tubular cells (Fig.3(B)). Fig.3(C) shows the renal tissues of HF/HC induced diabetic mice exhibiting a fatty infiltration in large area of hemorrhage in tubules. Fig.3(D) shows the diabetic mice treated with VA presenting mild infiltration in tubules.

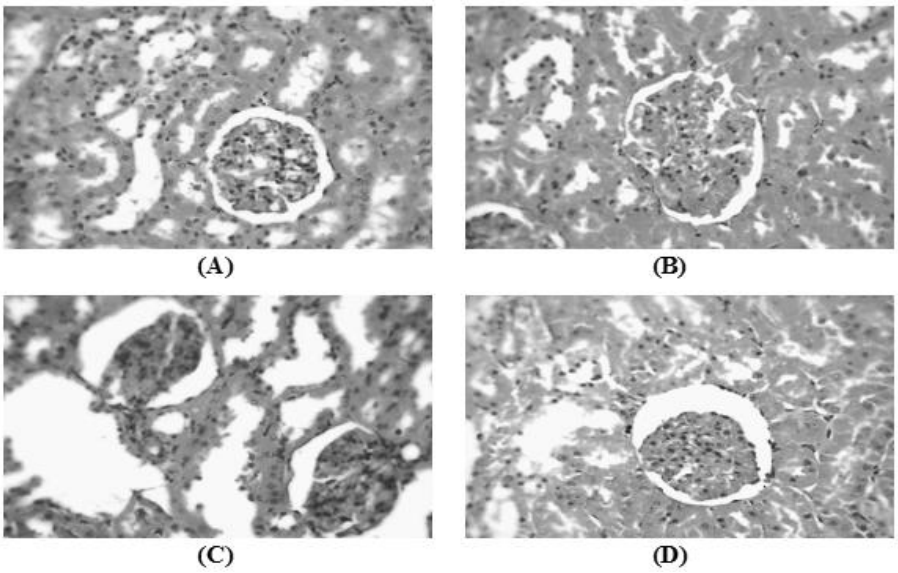

Fig.3. Photomicrographs of hematoxylin-eosin staining of renal tissues of control and experimental mice

Histological photograph of (A) control, (B) Control + VA (40 mg/kg of body weight), (C) HF/HC induced diabetic mice and (D) HF/HC induced diabetic mice +VA (40 mg/kg of body weight) treated mice in hepatic tissue sections were showed at 20X magnification.

DISCUSSION

The inability to maintain blood glucose homeostasis is the hallmark of diabetes, yet frank T_2DM ensues only after years of a slowly developing pre-diabetic state involving obesity, progressive insulin resistance and loss of glucose tolerance [31, 32]. The induction of T_2DM by a HF/HC diet in C57BL/6J mice [33] is a highly relevant animal model to study the relationship between diet and the development of T_2DM. In mice, as in humans; a HF/HC diet leads to obesity, accompanied by insulin resistance and impaired glucose tolerance pose substantial risks for development of hyperglycemia. Eventual loss of pancreatic β-cell mass leads to frank diabetes, characterized by fasting hyperglycemia [33,34]. Although fasting blood glucose levels are used clinically to diagnose diabetes, non-fasting blood glucose levels are also important, and even transient hyperglycemia can produce oxidative tissue damage, such as neuronal cell death[35]. In the present study, oral administration of VA produces a significant improvement in glucose metabolism which might be due to improved glycemic control. During diabetic state, increased generation of ROS occur and cause an imbalance between the oxidant and antioxidant status [36]. Increased amount of free radicals observed in diabetic mice is attributed to chronic hyperglycemia that damage antioxidant defense system [37]. Free radicals may also be formed via the auto-oxidation of unsaturated lipids in plasma and membrane lipids. The free radical produced may react with polyunsaturated fatty acids in cell membrane leading to LOOH. LOOH will in turn result in elevated production of free radicals [38]. Increased LOOH impairs membrane functions by decreasing membrane fluidity and changing the activity of membrane-bound enzymes and receptors [39]. Its products are harmful to most of the cells in the body and are associated with a variety of diseases [40]. Our present study showed a significant increase of tissue TBARS and LOOH level in diabetic rats. The increased TBARS content of diabetic rats suggests that peroxidative injury may be involved in the development of diabetic complications. TBARS and LOOH levels in liver and kidneys were significantly lower in the VA treated group compared to the diabetic control mice. Vitamin E is a well-known physiological antioxidant and membrane stabilizer. The decreased level of vitamin E observed in the HF/HC treated diabetic mice is compatible with the hypothesis that in tissues vitamin E excess plays a protective role against increased peroxidation in diabetes. Vitamin C is a hydrophilic antioxidant in tissues, because it disappears faster than other antioxidants when the tissues are exposed to ROS. The observed significant decrease in the level of tissues vitamin C could be caused by increased utilization of vitamin C as an antioxidant defense against ROS or by a decrease in GSH, which is required for the recycling of vitamin C. Treatment with VA brought vitamin E to near

normal levels which could be as a result of decreased membrane damage as evidenced by decreased LOOH. GSH is a major endogenous antioxidant which counterbalance free radical mediated damage. Studies have shown that the tissue GSH concentrations of HF/HC induced diabetic mice are significantly lower when compared with the control mice [41]. It is well known that GSH is involved in the protection of normal cell structure and function by maintaining the redox homeostasis, quenching of free radicals and participating in detoxification reactions. It is a direct scavenger of free radicals as well as a co-substrate for peroxide detoxification by GPx [42]. Increased oxidative stress, resulting from a significant increase in aldehyde products of LOOH has probably decreased the tissue GSH content [42]. In the present study, the elevation of GSH levels in liver and kidneys was observed in the VA treated diabetic mice. This indicates that the VA can either increase the biosynthesis of GSH or reduce the oxidative stress leading to less degradation of GSH. SOD is an important defense enzyme which catalyses the dismutation of superoxide radicals to produce H_2O_2 and molecular oxygen [43], hence diminishing the toxic effects caused by their radical. Wohaieb et al. [44] had suggested that in diabetic mice, the reactive oxygen free radicals could inactivate and reduce the tissue SOD activities. The observed decrease in SOD activity could result from inactivation by H_2O_2 or by glycation of enzymes [45]. Oral treatment of VA caused a significant increase in SOD activities in diabetic mice. This means that the VA can reduce reactive oxygen free radicals and improve the activities of the tissue antioxidant enzymes.CAT is a haemeprotein which catalyses the reduction of hydrogen peroxides and protects the tissues from highly reactive hydroxyl radicals [46]. CAT reduces hydrogen peroxide produced by dismutation reaction. It also prevents the generation of hydroxyl radicals thereby protecting the cellular constituents from oxidative damage in peroxisomes. The reduced activity of CAT in HF/HC treated mice results in the accumulation of H_2O_2, which produces deleterious effects. These findings suggest that the VA caused a significant increase in the activity of CAT in diabetic mice. This action is predominantly due to the antioxidant nature of VA and could involve a mechanism related to scavenging activity. GPx a selenium containing enzyme present in significant concentrations; detoxifies H_2O_2 to H_2O through the oxidation of reduced GSH [42]. GPx works together with GSH in the decomposition of H_2O_2 or other organic HP to non-toxic products at the expense of reduced GSH[43]. Reduced activities of GPx may result from radical-induced inactivation and glycation of the enzyme [42]. The low activity of GPx could be directly explained by the low content of GSH found in diabetic state, since GSH is a substrate and cofactor of GPx. Reduced activities of GPx in the liver and kidney have been observed during diabetes and this may result in a number of deleterious effects due to the accumulation of toxic products. In this context, other studies also reported a decrease in the activity of GPx in the liver and kidneys of diabetic mice [47]. The observed level shows that, the administration of VA increased the activities of GPx in HF/HC diet induced diabetic mice. HF/HC diet induced mice showed increase in the plasma cholesterol and triglyceride concentrations [48]. The levels of plasma lipids are usually elevated in diabetes mellitus and such an elevation represents a risk factor for coronary heart disease [49]. Further, the abnormal high concentration of plasma lipids in the diabetic subjects is mainly due to increase in the mobilization of FFA from fat depots.The marked hyperlipidemia that characterizes the diabetic state may be regarded as a consequence of uninhibited actions of lipolytic hormones on fat depots [50]. Insulin is required for the inhibition of hormone sensitive lipase and on the other hand, glucagons and other hormones enhance lipolysis. Under normal circumstances, insulin activates the enzyme lipoprotein lipase, which hydrolyses triglycerides [51]. However, in diabetic state lipoprotein lipase is not activated due to insulin deficiency resulting in hypertriglyceridemia. Diabetic mice treated with VA significantly decreased TC and TG towards normal level. A significant increase in the level of FFA was observed in the plasma of HF/HC diet induced mice. This increase in FFA could be due to increased transport of FA as a result of excessive mobilization of FFA from depots [52], the increased FFA level may lead to accumulation of FFA metabolites namely acetyl-CoA and acyl carnitine, both of which have been demonstrated to interfere with Na^+-K^+ ATPase activity [53]. A significant reduction of FFA was observed in the plasma of diabetic mice treated with VA when compared to diabetic control rats. The decreased level of FFA is also associated with decreased actions of lipolytic hormones, which, in turn, decrease the activity of hormone sensitive lipases on fat depots. Phospholipids are vital component of biomembranes and play an important role in the transport of triglyceride [54]. According to Venkateswaran [55] phospolipids levels were found to be elevated in diabetic mice. The abnormally high concentration of plasma lipids in diabetes is mainly a result of the increase in mobilization of FFA from peripheral depots, because insulin inhibits the hormone-sensitive lipase. Enhanced LOOH in diabetes causes membrane damage, which may be responsible for the increased phospholipids. In the diabetic mice fed with VA, a significant

reduction of phospholipids was observed in the plasma.

CONCLUSION

In conclusion increasing evidence in both experimental and clinical studies suggests that oxidation stress plays a major role in the pathogenesis of diabetes mellitus. The present study revealed the ability of VA on antioxidant stress and lipid profiles in liver and kidney tissues of HF/HC induced diabetic mice which provide the formulation of specific therapies for an early intervention and better management of the disease. VA at a dose of 40 mg/kg of body weight showed a more pronounced effect than the other doses 10, 20 mg/ kg of body weight. These findings suggest that VAsupplement, functioning as an excellent antioxidant, and having projective action against hyperglycemia, lipid-lowering actions in the liver and kidney injury associated with diabetic conditions.

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Abbreviations:
ANOVA, analysis of variance;
CAT, catalase;
DMRT, Duncan's multiple range test;
EDTA, ethylene diamine tetra acetic acid;
FFA, free fatty acids;
GPx, glutathione peroxides;
GSH, glutathione;
HF/HC, high fat / high carbohydrate;
HP, hydroperoxides;
LOOH, Lipid peroxidation;
Mabs, monoclonal antibodies;
NIN, national institute of nutrition;
ROS, reactive oxygen species;
SOD, superoxide dismutase;
TBARS,thiobarbituric acid reactive substances;
VA, vanillic acid;
WHO, world health organization.

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