Experimental Research

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Implication of Renal Aquaporin-3 in Fructose-Induced Metabolic Syndrome and Melatonin Protection

Physiology Section

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ABSTRACT

Introduction: Metabolic Syndrome (MetS) can be induced by ingestion of large amounts of fructose as a consequence of oxidative stress and dyslipidemia.

Aim: We investigated the possible protective effects of melatonin administration on MetS induced in fructose-fed rats with special focus on the role of renal aquaporin-3 (AQP-3).

Materials and Methods: Thirty rats were randomly divided into three groups; control, fructose, and fructose plus melatonin. MetS was induced by fructose rich diet and melatonin was injected at a dose of 5 mg/kg dissolved in 1% ethanol in normal saline. After the end of the 6-week experimental period, body weight and fat accretion were assessed. Invasive blood pressure and vascular reactivity were evaluated. Serum lipid profile, glucose, insulin levels, insulin resistance, malondialdehyde (MDA) and uric acid were measured, also underwent renal AQP-3 immunohistochemistry.

Results: Fructose consumption significantly increased fat accretion, systolic blood pressure, serum lipids, insulin levels and insulin resistance, confirming successful establishment of the MetS model. Also serum MDA, uric acid and renal AQP-3 expression increased compared to the control group. Melatonin supplementation significantly decreased the previously measured parameters compared to fructose group.

Conclusion: Increased AQP-3 expression may be implicated in fructose induced MetS. Melatonin protective effect against metabolic consensus and vascular affection may be linked to its antioxidant and lipid lowering effect with reduced renal AQP-3 expression.

Keywords: Aquaglyceroporin-3, Fructose, Insulin resistance, Vascular reactivity

INTRODUCTION

Metabolic Syndrome (MetS) is considered as endocrinopathy associated with a deadly combination of systemic disorders including abdominal obesity, insulin resistance, glucose intolerance or diabetes mellitus, dyslipidemia and hypertension [1]. High fructose intake leads to development of MetS-associated symptoms [2]. Fructose commonly used in food industry and sugar-sweetened drink [3] can provide carbon atoms for both the glycerol and the acyl portions of triglyceride (triacylglycerols) synthesis [4]. The sources of circulating glycerol are fat lipolysis, diet-derived glycerol or glycerol reabsorbed in renal tubules [4]. The regulation of glycerol transport by aquaglyceroporins contributes to the control of fat accumulation, glucose homeostasis, cardiac energy production and pancreatic insulin secretion, among other functions [5].

Aquaporin-3 (AQP-3) is a relatively weak transporter of water but functions as an efficient glycerol transporter [6]. Immunohistochemical studies revealed that AQP-3 is localized along the basolateral membranes of principal cells of the kidney, in cortical distal convoluted tubules and in collecting ducts in the medulla [7]. Aquaglyceporins are key players in shifting the focus of obesity and insulin resistance development, their expression and function in physiological condition and in obesity and type 2 diabetes, suggesting they are potential therapeutic targets for metabolic disorder [8].

Melatonin is a neurohormone produced at night mainly by the pineal gland. The biological action of the hormone is likely to be at the membrane level, either via its interaction with membrane receptors, and/or as a lipoperoxidation radical scavenger [9]. Melatonin, a tryptophan derivative, is a naturally occurring substance with no reported toxicity that may serve as a novel approach for prevention of MetS [10]. Melatonin has been reported to influence blood pressure [11] directly and/or indirectly by affecting cardiovascular risk factors including, amongst others, increased visceral fat accumulation and dyslipidemia [12].

AIM

The present study aims to discuss the metabolic consequences of increased fructose intake, mechanisms leading to fructose-induced insulin resistance, metabolic dyslipidemia and vascular affection. The present study also aims to evaluate the effects of melatonin in modulating renal AQP-3 expressions and dyslipidemia in fructose induced metabolic disorder.

MATERIALS AND METHODS

Animals and Experimental Design

This study was carried out in accordance with the regulations of Animal Experimentation Ethics Committee of faculty of medicine Menoufia University. Thirty adult male Wistar albino rats weighing 120-150 g were used. The animals were housed at 20-24ºC with a 12-h light, 12-h dark cycle and they were provided with standard rat chow and tap water freely available.

Thirty rats were randomly divided into three groups (n = 10) as follows; Control: Rats in this group received standard rodent diet. Fructose: Rats in this group fed fructose rich diet (60% fructose (Technogene company, Egypt) mixed with standard rat chow) [13,14]. Fructose plus Melatonin: Rats in this fed fructose rich diet (60% fructose mixed with standard rat chow), and i.p. injected with melatonin (Bio Basic Inc, Canada) (5 mg/kg dissolved in 1% ethanol in normal saline) [13], daily for 6 weeks.

Melatonin solution was prepared freshly every day. Since ethanol was used as a melatonin's vehicle, control and fructose groups received 0.1% ethanol solution proportionately with body weight.

At the end of the experiment (after 6 weeks), body weight was assessed, animals were fasted overnight and blood samples were collected via rat tail vein after rats being anaesthetized. Blood samples were left for clotting for 10 min and centrifuged at 4000 rpm for another 10 min to isolate the serum and kept at -20ºC for further analysis.

Thereafter, rats were subjected for measurement of arterial blood pressure by invasive method and testing the vascular reactivity to vasodilator (acetylcholine and sodium nitroprusside) and vasoconstrictor (noradrenaline and angiotensin-II) agents.

Lastly, rats of all groups were sacrificed and the visceral, epididymal and retroperitoneal fats were estimated. The kidneys were excised, fixed in 10% phosphate-buffered formalin solution, processed through paraffin embedding and prepared for immumohistochemical studies.

Measurement of Invasive Blood Pressure and Vascular Reactivity

Rats were anesthetized by Thiopental sodium 50 mg/kg i.p. The aorta is identified and cannulated using a cannula pre-filled with heparinized normal saline and the other end of the cannula was connected to a three-way stopcock saline filled syringe which was connected to a pressure transducer and the invasive blood pressure was recorded using physiograph system (Narco Bio-Systems, U.K.). Then the responses to various vasoactive drug injections were recorded as following:

- Angiotensin II (AII) (Sigma-Aldrich Chemical Co. Steinheim, Germany): was the first given in doses of 20, 40 and 60 ng/animal.
- Noradrenaline (NE) (Alex. Co. For Egypharma, Egypt): in doses of 100,200 and 400 ng/animal.
- Sodium nitroprusside (El-Gomhoria Company, Egypt): in doses of 1, 2 and 4µg/animal.
- Acetylcholine (Ach) (El-Gomhoria Company, Egypt): in doses of 10^{-9}, 10^{-8} and 10^{-7}.
- Vascular reactivity was judged by the magnitude of change in the mean arterial blood pressure [15].

Serum Biochemical Analysis

Lipid Profile

Total cholesterol and high density lipoprotein (HDL) (mg/dl) levels were estimated after enzymatic hydrolysis and oxidation yielding rose colored quinoneimine derivatives using test reagent kits (Biodiagnostics, Egypt). Triglycerides (TGs) level (mg/dl) was hydrolyzed with lipoprotein lipase to form glycerol, which forms a complex with H_2O_2 giving a derivative that can be colorimetrically estimated using reagent kit (EMAPOL, Poland) [16]. Serum level of Low Density Lipoprotein (LDL) was calculated from the following formula; LDL = Total Cholesterol - (HDL + Triglycerides/5) as described by Friedewald et al., [17].

Blood Glucose

Fasting Blood Glucose Level (FBG) (mg/dl) was determined colorimetrically using a test reagent kit (EMAPOL, Poland) after being oxidized enzymatically to yield a red violet quinoneimine [16].

Insulin Levels

Insulin level (µU/ml) was measured using solid phase enzyme-linked immunosorbent assay (Elisa) based on the sandwich principle (DRG Instruments GmbH, Germany) [18].

Homeostasis Model Assessment index (HOMA-IR)

Insulin resistance was estimated by homeostasis model assessment index (HOMA-IR): Insulin (µU/ml) X glucose (mg/dl) ÷ 405 [19].

Serum Malondialdehyde (MDA)

Estimation of Malondialdehyde (MDA) was done by assessing thiobarbituric acid reactive products, as oxidative stress markers measuring the peroxidation of fatty acids by using test reagent kits (Biodiagnostics, Egypt) following protocol described in Ohkawa et al., [20].

Serum Uric Acid

Colorimetric method was performed using test reagent kits (Biodiagnostics, Egypt) by using the protocol described in Barham and Trinder [21].

Renal Immunohistochemical Staining with AQP-3

Several sections were cut from the paraffin-embedded blocks with subsequent steps of deparaffinization and rehydration in xylene and graded series of alcohol, respectively. Antigen retrieval was performed by boiling in 10 ml citrate buffer (pH 6.0) for 20 min, followed by cooling at room temperature. The slides were incubated overnight at room temperature with purified rabbit polyclonal to Aquaporin-3 (Abcam (ab125219)). The optimal dilution was 1:500 by using Phosphate Buffered Solution (PBS). All slides were de-paraffinized using xylene and then rehydrated in decreasing concentrations of ethanol. Antigen retrieval using microwave heating (20 minutes; 10 mmol/citrate buffer, pH 6.0) after inhibition of endogenous peroxidase activity (hydrogen peroxidase for 15 min) was used. The primary antibody was applied to the slides, incubated overnight at room temperature in humidity chamber. Sections were then washed by PBS then incubated with secondary antibody for 15 minutes followed by PBS wash. Finally, the detection of bound antibody was accomplished using a modified labeled avidin–biotin (LAB) reagent for 20 minutes then PBS wash. A 0.1% solution of diaminobenzidine (DAB) was used for 5 minutes as a chromogen. Slides were counter-stained with Mayer's haematoxylin for 5–10 minutes. Rat kidney tissue specimens were used as positive controls. Omission of the primary antibody served as a negative control.

Interpretation of immunohistochemical results: A brown cytoplasmic, membranous or membrano-cytoplasmic staining in any number of cells was considered positive in the studied cases and control specimens [22]. Renal tissue in the 3 studied groups (control, fructose metabolic syndrome, fructose metabolic syndrome treated with melatonin) was assessed for:

1. **Intensity of the stain:** Graded as mild (+), moderate (++) or strong (+++).
2. **Staining pattern:** Cytoplasmic, membranous or membrano-cytoplasmic.
3. **Expression Percentage:** Positive cells were counted and given a percentage over 200 cells of the whole section at 200X magnification in renal tissue [23].
4. **Histo-score (H score):** H score was calculated in all positive specimens according to the following equation: H score = 1 X % of mildly stained cells + 2 X % moderately stained cells + 3 X % of strongly stained cells [24].
5. **Distribution of AQP-3:** Patchy (irregular or not uniform distribution) or Diffuse (uniform distribution).

STATISTICAL ANALYSIS

The data were tabulated and analysed by SPSS Statistical Package for Social Science software using statistical package version 20 on IBM compatible computer. Quantitative data were expressed as mean ± standard error of mean (X± SEM). Data from control and test groups were compared using one-way ANOVA, followed by Turkey post-Hoc test, Probability value of less than 0.05 was considered as statistically significant ($p<0.05$).

RESULTS

Body Weight and Fat Accretion

The body weight of fructose and fructose plus melatonin groups were found to be significantly higher ($p<0.05$) than that of the control group.

With regard to the major fat pad accretion, the fructose fed rats showed a significantly higher amount of visceral ($p<0.05$),

retroperitoneal (p<0.05) and epididymal (p<0.001) fat mass compared to their control counterparts indicating abdominal obesity, while fructose plus melatonin treated rats showed a significantly lower amount of visceral (p< 0.05), retroperitoneal (p< 0.001) and epididymal (p<0.001) fat mass compared to fructose-fed rats [Table/Fig-1].

Serum Biochemical Analysis

Serum lipid profile (cholesterol, TGs and LDL) in fructose group were significantly higher (p<0.05) compared to control group. In fructose plus melatonin group these parameters were significantly lower (p<0.05) compared to fructose group but still significantly higher compared to control group. While HDL level in fructose and fructose plus melatonin groups was significantly lower (p<0.05) compared to control group, and it was insignificant (p>0.05) in fructose plus melatonin group compared to fructose group [Table/Fig-2].

Blood glucose, serum insulin and HOMA-IR in fructose group were significantly higher (p<0.05) compared to their corresponding levels in control group, while these parameters in fructose plus melatonin group were significantly lower (p<0.05) compared to their corresponding levels in control group [Table/Fig-2].

Serum MDA and uric acid in fructose group were significantly higher (p<0.05) compared to their corresponding levels in control group, while these parameters in fructose plus melatonin group were significantly lower (p<0.05) compared to their corresponding levels in control group [Table/Fig-2].

Invasive Blood Pressure and Vascular Reactivity

Systolic, diastolic and mean arterial blood pressure (measured by invasive technique) in fructose fed rats showed significant increase (p< 0.05) compared to control group of rats while these parameters showed significant (p< 0.05) decrease in fructose plus melatonin treated compared to fructose fed rats [Table/Fig-3a].

The magnitude of increase in blood pressure (mmHg) in response to different doses of AII (20, 40 & 60 ng) and to different doses of NE (100, 200 & 400 ng) were significantly (p<0.05) higher in fructose fed rats compared to corresponding values in control group. While the magnitudes of increase were significantly lower (P<0.05) infructose plus melatonin treated rats, compared to fructose fed rats [Table/Fig-3b&c].

The magnitude of decrease in blood pressure (mmHg) in response to different doses of Ach (10^{-9}, 10^{-8}& 10^{-7} M) were significantly (p<0.05) lower in fructose fed rats compared to corresponding values in control group. While the magnitudes of decrease were significantly higher (p<0.05) in fructose plus melatonin treated rats, compared to fructose fed rats [Table/Fig-3d].

The magnitude of decrease in blood pressure (mmHg) in response to different doses of sodium nitroprusside (1, 2 & 4 µg) were insignificantly (p>0.05) different among groups [Table/Fig-4a-f].

Renal aquaporin-3 (AQP-3) immunohistochemistry

Aquaporin-3 in the control group revealed focal patchy strong cytoplasmic staining in the proximal convoluted tubules and distal convoluted tubules [Table/Fig-4a]. While the collecting ducts, showed diffuse strong membranous staining of aquaporin 3 was noticed [Table/Fig-4d]. The H score ranged from 120 to 200/300.

In fructose group, higher expression of Aquaporin -3 in the form of diffuse strong cytoplasmic and membranous staining in the proximal convoluted tubules, distal convoluted tubules and glomeruli were displayed [Table/Fig-4b]. Strong membranous and moderate cytoplasmic expression of Aquaporin -3 was noticed mainly in the collecting tubules and focally in the loop of Henle [Table/Fig-4e]. The H score ranged from 80 to 240/300.

The fructose plus melatonin group revealed patchy moderate cytoplasmic staining in the proximal convoluted tubules, distal convoluted tubules and glomeruli [Table/Fig-4c]. While a mild to moderate membranous expression of Aquaporin -3 was displayed in the collecting tubules [Table/Fig-4f]. The H score ranged from 100 to 140/300.

	Control	Fructose	Fructose plus Melatonin
Body weight (gm)	211±0.18	270±10.1*	292±15.6*
Visceral fat (gm)	2.7±0.18	3.8±0.24*	2.7±1.86#
Retroperitoneal fat (gm)	4.3±0.15	5.4±0.26*	3.9±0.25#
Epididymal fat (gm)	2.8±0.15	4.7±0.21*	3.2±0.24#

[Table/Fig-1]: Body weight and fat accretion (visceral fat, retroperitoneal fat and epididymal fat weights) (gm) in control, fructose fed and fructose plus melatonin treated groups. Data were expressed as mean ±S.E. (n=10). One way ANOVA: *p<0.05, vs control; #p<0.05, vs fructose group.

	Control	Fructose	Fructose plus Melatonin
Cholesterol (mg/dl)	100.9±2.3	126±4.5*	108±2.3*#
Triglycerides (mg/dl)	62.1±1.5	79.3±2.6*	69.7±1.0*#
HDL (mg/dl)	40.9±1.5	31.8±1.3*	32.4±2.4*
LDL (mg/dl)	47.5±2.8	79.3±3.6*	62.4±2.5*#
Glucose level (mg/dl)	90.4±2.1	114.6±4.2*	102.2± 2.8*#
Insulin level(µU/ml)	16.6±1.2	33.2±1.8*	20.2±1.8#
HOMA-IR	3.6±0.29	9.3± 0.51*	5.4± 0.41*#
MDA (nmol/ml)	3.3±0.26	8.0± 1.4*	5.3± 0.49#
Uric acid (mg/dl)	1.6±0.08	3.4±0.13*	1.9±0,06#

[Table/Fig-2]: Serum lipid profile, glucose, insulin, HOMA-IR, MDA and uric acid levels in control, fructose and fructose plus melatonin treated groups. Data were expressed as mean ±S.E. (n=10). One way ANOVA: *p<0.05, vs control; #p<0.05, vs fructose fed group.

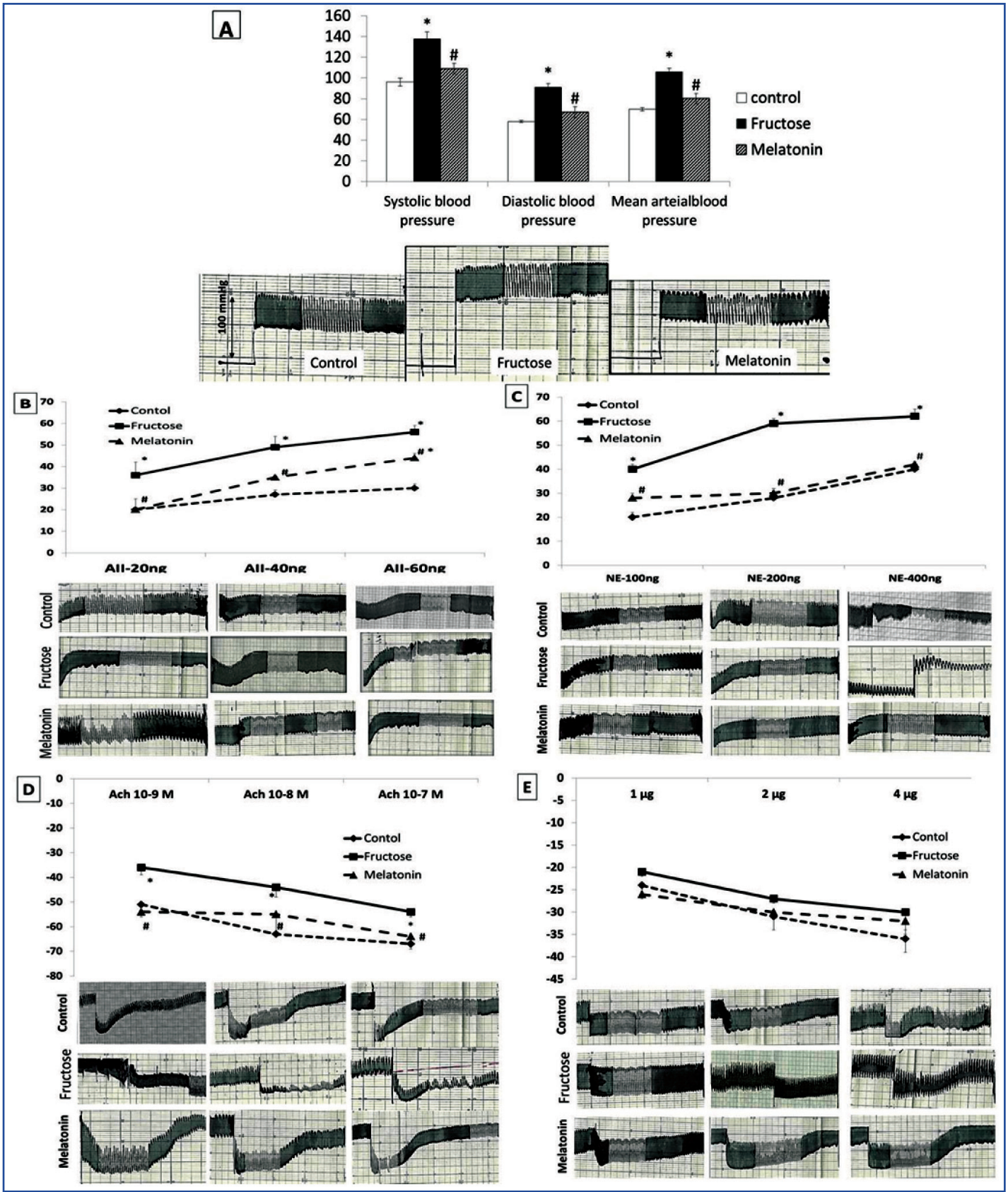
DISCUSSION

We are currently in the midst of an epidemic of metabolic disorders, which may, in part, be explained by excess fructose intake [25]. Metabolic syndrome components improved after Melatonin supplementation [26].

In the present study metabolic syndrome was successfully established in fructose fed rat model as evidenced by the significant increase in body weight and fat accretion, dyslipidemia, hyperglycaemia, hyperinsulinemia, insulin resistance, hypertension and disturbed vascular reactivity, which were associated by hyperuricaemia increased lipid peroxidation parameter; Malondialdeyde (MDA), mild renal degenerative changes and increased expression of renal aquaporin-3 (AQP-3).

The hypotheses concerning the mechanisms by which fructose promotes metabolic syndrome, focused on the lipogenic nature of the sugar with deposition of triglycerides in adipose tissue and ectopic tissues, eventually resulting in impaired insulin signaling and dyslipidemia [27]. There was evidence that fructose rich diet increased plasma triglycerides, through several mechanisms, among which was a stimulation of hepatic de novo lipogenesis [28], and a decreased VLDL triacylglycerol clearance [29]. It seems also that, the overexpression of renal AQP-3 presented in this study may have a role in these dyslipedemic complications, considering that they are channels in the basement membrane of distal convoluted tubules and collecting ducts of the kidney, that are responsible for glycerol reabsorption [7]. Glycerol is the backbone of triglycerides [4,5].

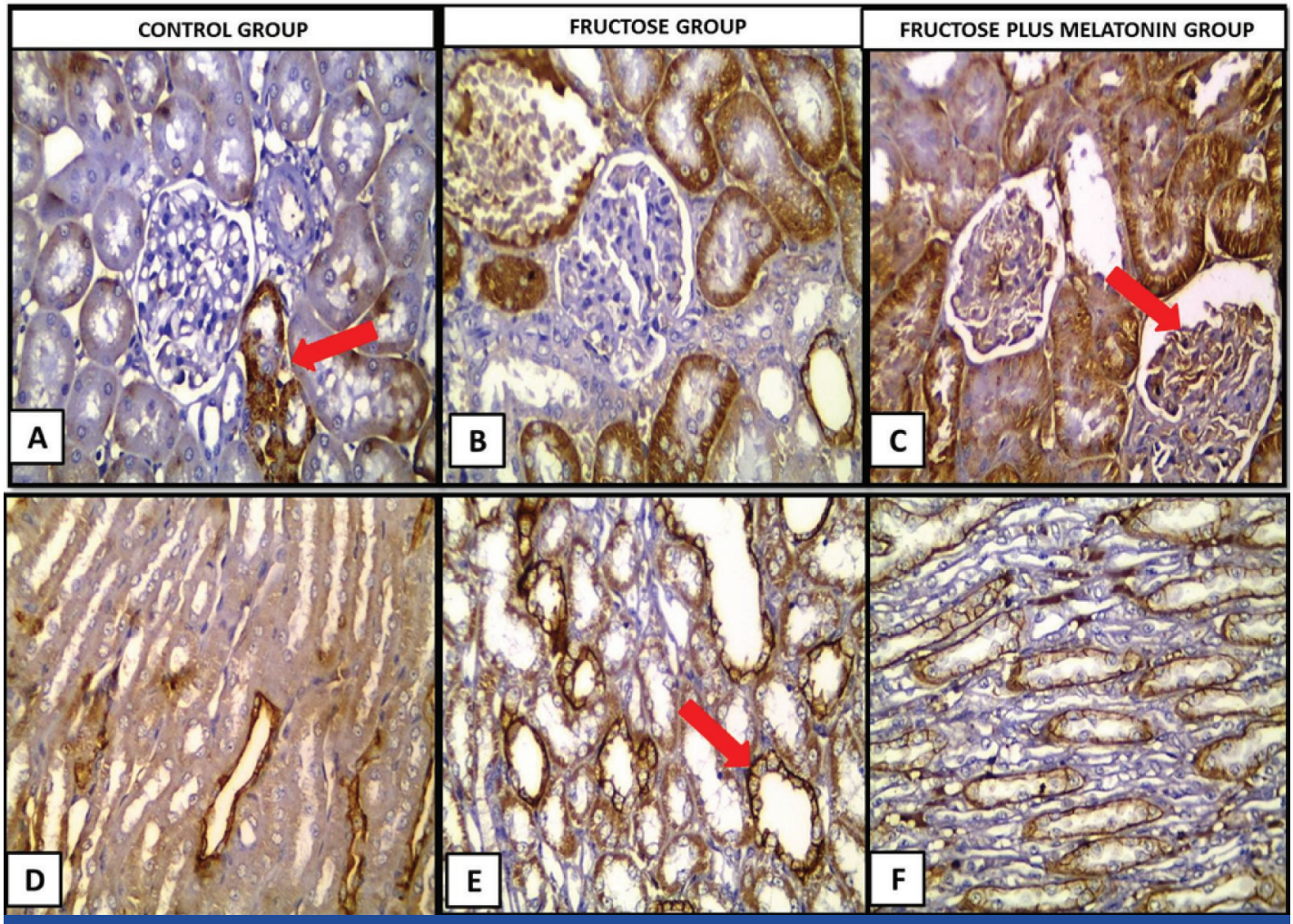
Fructose is known to induce oxidative stress by different mechanism one of them may be implicated to the increased expression of AQP-3 presented in this study. AQP-3 enhanced hydrogen peroxide (H_2O_2) permeability according to Galán-Cobo and colleagues [30]. To our knowledge this is the first study to examine the expression of renal AQP-3 in fructose induced metabolic syndrome rat model.



[Table/Fig-3]: Invasive blood pressure and vascular reactivity among control, fructose and fructose plus melatonin groups A. Level of systolic, diastolic and mean arterial blood pressure (mmHg) among groups B. Magnitude of increase in blood pressure (mmHg) in response to different doses of angiotensin II (AII) C. Magnitude of increase in blood pressure (mmHg) in response to different doses of noradrenaline (NE) D. Magnitude of decrease in blood pressure (mmHg) in response to different doses of acetylcholine (Ach) E. Magnitude of decrease in blood pressure (mmHg) in response to different doses of sodium nitroprusside. Data were expressed as mean ±S.E. (n=6-8). One way ANOVA: *p<0.05, vs control; #p<0.05, vs fructose-fed group.

Melatonin treatment was efficient in preventing fructose induced metabolic syndrome as it significantly reduced fat accretion, serum lipid profile, glucose level, insulin level and HOMA-IR. Nduhirabandi and colleagues [12] stated that melatonin treatment should be capitalized in the future for effective management of obesity-induced complications, including cardiovascular disease, gaining benefit from its actions on obesity-related metabolic disorders (increased body fat accumulation, glucose intolerance, atherogenic dyslipidemia, and raised blood pressure).

Melatonin has potent antioxidant activity; it effectively reduced MDA level in the current study, which plays a pivotal role in the patho-physiology of many diseases including metabolic syndrome and its vascular complications [31]. However, a particular attention must be paid to the fact that the overall role of melatonin in metabolic syndrome



[Table/Fig-4]: Immunohistochemistry staining of renal AQP-3. *In control group* AQP-3 showed focal patchy strong brown cytoplasmic staining in the proximal convoluted tubules (red arrow) and distal convoluted tubules (A) and focal moderate to strong membranous brown staining in the collecting tubules (D). *The fructose group* revealed higher expression of AQP-3 in the form of diffuse strong brown cytoplasmic and membranous staining in the proximal convoluted tubules, distal convoluted tubules and glomeruli (B) and strong brown membranous (red arrow) and moderate cytoplasmic expression of in the collecting tubules mainly and few of the loop of Henle (E). *Fructose plus melatonin group* shows patchy moderate brown cytoplasmic staining in the proximal convoluted tubules, distal convoluted tubules and glomeruli (red arrow) (C) and mild to moderate brown membranous expression of AQP-3 in the collecting tubules (F) (A,B,C,D,E and F immunoperoxidase 400XHPF).

is not only due to its antioxidant activities alone, but more likely to its combined different regulatory effects [12]. In this study beside its MDA reducing effect, melatonin significantly reduced uric acid level which in a key player in induction of metabolic syndrome [27].

Melatonin also decreased the membranous expression of renal AQP-3. This mechanism seems to be implicated in preventing the increase in circulating triglycerides and decreased fat accretion. According to Nduhirabandi et al., increased triglycerides are responsible for body fat accumulation and body weight gain [12].

Melatonin administration inhibits insulin secretion in rat pancreatic islets [32] and could explain why melatonin reduced the fasting insulin levels. Hyperinsulinemia and insulin resistance are the common links between obesity and its vascular complications [33].

According to Lee and colleagues [34], the expression of AQP-3 channels was increased in the kidney of spontaneously hypertensive rats. Our trial similarly demonstrated higher membranous expression of renal AQP-3 in association with hypertension in fructose induced metabolic syndrome rat model. Lower AQP-3 membranous expression after melatonin treatment which was associated with reduced blood pressure level compared to control group. This may reflect some sort of correlation between over expression of AQP-3 and elevated blood pressure but it needs further investigation for confirmation of this possible relationship. Fructose feeding induced hyper responsiveness to both angiotensin-II and norepinephrine and hypo responsiveness to acetylcholine (Ach) but not to sodium nitroprusside-induced relaxation, denoting abnormal vascular reactivity and endothelial dysfunction. Melatonin successfully corrected these altered vascular reactivity responses, the underlying mechanism of improved vascular reactivity in melatonin group may be linked to beneficial effects of melatonin in protecting against endothelial dysfunction [35], and its antioxidant effect, because increased vascular reactivity to vasoconstrictors is related to increased oxidant stress [31]. Triglycerides and LDL lowering effect of melatonin have a share in restoring endothelial function and vascular response to vasoactive agents, Liu et al., have shown that these particles can accelerate senescence and interfere with the function of endothelial progenitor cells and elicit proinflammatory responses [36].

LIMITATION

Limitations in this study included; death of few rats before completion of the vascular reactivity test, so the sample size for this parameter were only 6-8 rats per group instead of 10 rats.

CONCLUSION

Fructose induced metabolic syndrome characterized by increased fat accretion, insulin resistance, metabolic dyslipidemia, increased oxidant, uric acid levels and complicated with impaired vascular reactivity, which were associated with increased expression of renal AQP-3. The over expression of renal AQP-3 presented in this study may have a role in these dyslipedemic complications. Melatonin was protective against fructose vascular and insulin resistance consensus through reducing free radicals, renal AQP-3 expressions and improving dyslipidemia in fructose induced metabolic disorder.

LIST OF ABBREVIATIONS

Ach: Acetylcholine; AII: Angiotensin II; AQP-3: aquaporin-3; FBG: Fasting blood glucose level; HDL: high density lipoprotein; HOMA-IR: Homeostasis Model Assessment index; H2O2: hydrogen peroxide;LDL: low density lipoprotein; MDA: malondialdehyde; MetS: Metabolic syndrome; NE: Noradrenaline; PBS: phosphate buffered solution; TGs: Triglycerides.

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