Original Article

Selective versus non selective cyclooxygenase inhibitors in high fructose-induced metabolic syndrome

Mohamed Abdellah **Ibrahim**[a], Salwa Abdel-tawab **Ibrahim**[a], Entesar Farghaly **Amin**[a], Remon Roshdy **Rofaeil**[a], Aly Mohamed **Abdelrahman**[a]

[a] *Department of Pharmacology, Faculty of medicine, Minia University*

A B S T R A C T

Metabolic syndrome (MS) is a cluster of interrelated abnormalities namely obesity, dyslipidemia, hypertension, and insulin resistance. Chronic inflammation with release of inflammatory mediators including cyclooxygenase (COX) enzymes represents an important pathogenic factor in the development of MS. The present study investigated the effect of selective COX-2 inhibitor, celecoxib, versus non selective, diclofenac, in prevention of high fructose-induced MS in rats. Rats were divided into 6 groups: normal control (received normal diet); high fructose fed (HF) (received 20% fructose plus saline to serve as control MS group; celecoxib-treated (received HF plus either celecoxib 10 or 50 mg/kg/day); diclofenac-treated (received HF plus either diclofenac 6 or 30 mg/kg/day). visceral fat index (visceral fat weight /body weight ratio), insulin resistance , serum levels of triglyceride (TG), high density lipoprotein (HDL), malondialdehyde (MDA), reduced glutathione (GSH), catalase, uric acid, C-reactive protein (CRP), and tumor necrosis factor- α (TNF-α) were measured. The results showed that celecoxib, but not diclofenac, prevented the development of high fructose-induced MS as indicated by significant attenuation in visceral fat index, insulin resistance, and lipid profile. The protective effect of celecoxib was associated with significant improvement in serum levels of oxidative stress markers (MDA, GSH, and catalase), and inflammatory markers (uric acid, CRP, and TNF-α). These results indicate that selective COX-2 inhibitors protect against high fructose-induced MS possibly via antioxidant and anti-inflammatory effects.

Key Words: celecoxib-metabolic syndrome, diclofenac-metabolic syndrome.

Corresponding Author: Mohamed Abdellah Ibrahim   **Email:** mabdellah69@yahoo.com*;* mabdellah69@mu.edu.eg; maim69@yahoo.com

1. INTRODUCTION

Metabolic syndrome (MS) is a worldwide problem that affects 10-25% of adult population. It is a cluster of interrelated abnormalities namely obesity, dyslipidemia, hypertension, and insulin resistance (Grundy et al., 2004). It is associated with several prevalent diseases, such as diabetes, myocardial infarction and inflammatory articular diseases that necessitate the co-administration of multiple drugs (Zambon et al., 2010).

Establishing drug therapies for MS represents a big challenge because it requires understanding the cellular mechanisms that link the metabolic abnormalities with the clinical disease. Currently, it is widely accepted that subclinical chronic inflammation with release of inflammatory mediators including

cyclooxygenase (COX) enzymes represents an important pathogenic factor in the development of MS associated diseases (Shoelson et al., 2006; Van Erk et al., 2010).

Constitutive COX-1 is expressed in most cells including adipocytes, however, COX-2 is induced in response to various stimuli, such as cytokines and oxidative stress (Fujimori and Amano, 2011). Prostaglandins (PGs) are known to play diverse roles in adipocyte differentiation. Precursor adipocyte cells utilize the arachidonate-COX pathway to generate PGs at different stages of the life cycle of adipocytes (Chu et al., 2009). Prostaglandin E2 and prostaglandin F2 are anti-adipogenic factors that suppress the differentiation of adipocytes (Fujimori and Amano, 2011). However, the relative contribution of COX-1

and COX-2 to metabolic syndrome remains to be elucidated.

The present study investigated the effect of selective versus non selective COX-inhibitors in prevention of high fructose-induced MS in rats. The effect of tested drugs on the pathogenesis of MS had been examined

2. MATERIALS AND METHODS

1.1. Animals

Adult male Wistar rats (150-200 g) obtained from the animal house (National Center of Research, El-Giza, Egypt), housed in controlled environmental conditions. They were allowed free access to tap water and normal rats' diet (El-Nile Company, Egypt) for one week, as an adaptation. All experimental protocols were approved by the animal care committee of Minia University and coincide with international guidelines.

1.2. Chemicals

Fructose, diclofenac, and celecoxib were obtained as powders from EL-Nasr Pharmaceutical Company; Novartis; and Pfizer companies, respectively. All other chemicals were of high quality commercially available.

1.3. Experimental design

1.3.1. Induction of MS

Metabolic syndrome was induced by dissolving 10 gram fructose in 100 ml drinking water, besides a 10% fructose in diet (i.e. adding 10 gram fructose per 100 gram normal diet). The total ingested fructose was accordingly 20% for 6 weeks. This method was modified from Faure et al. (1999).

1.3.2. Animal grouping

Rats were divided into 6 groups, 8 rats each and treated as follows: group 1, a normal control which received normal diet; group 2, high fructose group (HF), which received 20% fructose (10% with diet (weight/weight) and 10% with water (weight/volume)) and injected with saline to serve as control MS group (Faure et al., 1999).; group 3, celecoxib-treated HF group (HF+ cele.10), received HF plus celecoxib (10 mg/kg/day, P.O) (the dose of celecoxib was chosen according to Khayyal et al.(2009); group 4, (HF+ cele.50), received HF plus celecoxib (50 mg/kg/day, P.O); group 5, diclofenac-treated HF group (HF+ dicl.6), received HF plus diclofenac (6 mg/kg/day i.p.). The dose of diclofenac was chosen according to Nalbant et al.(2006). Group 6 (HF+ dicl.30), received HF plus diclofenac (30 mg/kg/day i.p.). Administration of drugs was started simultaneously at the same day of fructose feeding and continued for 6 weeks, the duration of study.

1.3.3. Sample collection

At the end of the experimental period, the animals were weighted, anesthetized with ether. Blood samples were collected from the abdominal aorta as follows: the rat was fixed on a wooden plate and the abdominal cavity was opened, then the abdominal aorta was explored at its bifurcation after gentle traction of the viscera using soft tissue (Ibrahim et al., 2011). Blood was centrifuged at 5000 rpm for 10 minutes for serum separation, and kept at $-80^{\circ}C$ until further measurements.

1.3.4. Measurements

1.3.4.1. Physical measurements

Visceral fat (adipose tissue surrounding the abdominal and pelvic organs) was dissected and weighed. Visceral fat index was calculated according to the following equation: (visceral fat weight (g) /g body weight) x 100 (Hansen et al., 1997).

1.3.4.2. Biochemical measurements

Fasting blood glucose and serum levels of fasting insulin, triglycerides (TG), high density lipoprotein (HDL), oxidative stress parameters (reduced glutathione (GSH), catalase, malondialadehyde (MDA) and inflammatory parameters (C-reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), and uric acid) were measured.

1.3.4.3. Insulin resistance

Fasting blood glucose was measured using the ACCU-CHEK Active Blood Glucose Meter (Roche, Mannheim, Germany). Serum insulin was measured by enzyme-linked immunosorbent assay (ELISA) Kit (SPI-BIO, France) according to the Kit instruction. Insulin resistance (IR) was calculated using Homeostasis Model Assessment-insulin resistance (HOMA-IR) [fasting glucose (mg/dl) x fasting insulin (μIU/ml)] / 405 (Matthews et al., 1985).

1.3.4.4. Lipid profile

Serum TG and HDL were determined using commercially available kits (human, Germany and Biomed, Egypt, respectively) and expressed as mg/dl according to the kit instructions and quantitated at 500 nm using Beckman-DU-64 spectrophotometer (USA).

1.3.4.5. Oxidative stress markers (Malondialdehyde (MDA), Reduced glutathione (GSH), and catalase)

Serum levels of MDA were measured according to the thiobarbituric acid method as previously described by Buege and Aust (1978). It depends on measuring MDA, the breakdown products of lipid peroxides. The absorbance was read at 535 nm and the corresponding concentration was calculated

from a standard curve using 1,1,3,3-tetraethoxypropane as a standard.

Reduced glutathione (GSH) was measured using colorimetric kit (Biodiagnostic, Egypt) according to kit instructions. The method based on the reduction of 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) with glutathione to produce a yellow compound. The reduced chromogen is directly proportional to GSH concentration and its absorbance was measured at 405 nm using Beckman-DU-64 spectrophotometer (USA).

Catalase activity in the serum was determined using colorimetric methods (Biodiagnostic, Egypt). Catalase reacts with a known quantity of H_2O_2. In the presence of peroxidase, the remaining H_2O_2 reacts to form a chromophore with color intensity inversely proportional to the amount of catalase in sample.

1.3.4.6. Determination of inflammatory markers (C-reactive protein, Tumor necrosis factor-α, and Uric acid)

Serum C-reactive protein (CRP) was measured by enzymatic colorimetric kits (Agappe Diagnostic LTD, India). Determination and semi-quantitation of CRP depends on rapid agglutination procedure.

Tumor necrosis factor-α (TNF- α) was measured by ELISA Kit (ID Labs Inc., Canada) according to manufacture instruction. It depends up on using wells coated with a polyclonal antibody specific for rat TNF-α. After incubation with the rat TNF-α antigen and a biotinylated polyclonal antibody and washing to remove the unbound enzyme, a substrate solution was added to induce a colored reaction product. The intensity of this colored product was directly proportional to the concentration of rat TNF-α present in the samples. The values were read at 450 nm in an ELISA reader.

Serum uric acid was determined using an enzymatic colorimetric kit (Biomed diagnostic, Egypt). The method was based on oxidation of uric acid by uricase to allantoin and hydrogen peroxide. The hydrogen peroxide causes oxidative reaction in the presence of peroxidase, forming a red colored quinoneimine dye. The intensity of the color produced is directly proportional to the concentration o f uric acid in the sample, with maximum absorbance at 520 nm.

1.4. Statistical analysis of the data:

Results were expressed as means ± standard error of mean (SEM). One-way analysis of variance (ANOVA) followed by the Tukey-Kramar post analysis test was used to analyze the results for statistically significant difference. p values less than 0.05 were considered significant. Graph Pad Prism was used for statistical calculations (version 5.03 for Windows, Graphpad Software, San Diego California, USA, www.graphpad.com).

3. RESULTS

3.1. Effect of celecoxib and diclofenac on body weight and visceral fat index

There was no significant difference in the body weight between groups. High fructose feeding increased significantly visceral index compared with normal control. Celecoxib (50 mg/kg) significantly decreased visceral fat index to almost normal values. Neither diclofenac nor celecoxib (10 mg/kg) produced any significant changes in visceral index (Table 1).

3.2. Effect of celecoxib and diclofenac on insulin resistance, and lipid profile

High-fructose intake significantly caused insulin resistance indicated by increased fasting glucose and insulin levels as well as by increased HOMA-IR compared to control value. Celecoxib (50 mg/kg) treatment reduced significantly insulin resistance parameters to near control values (Table 1). Moreover, high-fructose intake produced dyslipidemia manifested by significant increase in serum TG and decrease serum HDL as compared to control group. Celecoxib (50 mg) treatment almost normalized the fructose-induced dyslipidemia (Table 1). Celecoxib 10 mg/kg as well as diclofenac in all doses did not produce any significant change in all measured parameters (Table 1).

3.3. Effect of celecoxib and diclofenac on serum levels of oxidative stress markers (MDA, GSH, and catalase)

In high fructose-fed rats, there was significant increase in MDA and decrease in GSH and catalase activity compared to normal control. Treatment with celecoxib significantly attenuated the effect of high fructose intake on the measured oxidative stress parameters. Diclofenac in all doses did not produce any significant change in oxidative stress markers compared with high fructose-fed group (Table 2).

3.4. Effect of celecoxib and diclofenac on serum level of inflammatory markers (CRP, uric acid, and TNF-α)

Serum CRP level significantly increased in high fructose-fed rats compared to normal control. Treatment with celecoxib significantly decreased the effect of high fructose intake on CRP serum level (Table 2). Diclofenac in all doses did not produce any significant changes compared with high fructose-fed group (Table 2).

In high fructose-intake rats, there was significant increase serum uric acid compared to normal control. Treatment with celecoxib significantly attenuated the effect of high fructose intake on serum uric acid (Table 2). Both doses of diclofenac did not

produce any significant changes compared with high fructose-fed group (Table 2).

In high fructose-fed rats, there was significant increase in serum TNF-α compared to control group.

Celecoxib treatment almost restored the control values (Table 2). Diclofenac did not produce any significant change compared with high fructose-fed group (Table 2).

Table (1): Effect of celecoxib and diclofenac on metabolic disorders in fructose-induced metabolic syndrome

	Control	**FF**	**Cele.10**	**Cele. 50**	**Dicl. 6**	**Dicl. 30**
BW (g)	225 ± 7.32	245 ± 6.43	231 ± 6.97	225 ± 6.84	226 ± 5.72	231 ± 6.51
VW/BW ratio	0.23 ± 0.17	2.12 ± 0.16[a]	1.64 ± 0.12[a]	1.25± 0.10 [a b c d]	2.14 ± 0.26 [a]	2.02 ± 0.30 [a]
Fasting blood Glucose (mg/dl)	94.6 ± 5.98	137 ± 5.25[a]	136± 3.37[a]	111 ± 4.35[b c d]	136 ± 2.33 [a]	134 ± 4.06 [a]
Fasting serum insulin (μIU/ml)	12.0 ± 1.63	43.9 ± 1.87[a]	31.0± 5.66 [a]	13.94 ± 0.93[b c d]	54.7 ± 2.09 [a]	45.87 ± 1.74 [a]
HOMA-IR	2.85 ± 0.30	14.8 ± 1.06[a]	10.6 ± 1.95 [a]	3.91 ± 0.39 [b c d]	15.4 ± 4.09 [a]	15.1 ± 0.16 [a]
Serum TG (mg/dl)	74.6 ± 5.69	175 ± 5.48[a]	146 ± 9.91 [a]	114 ± 4.19[a b c d]	170 ± 6.29[a]	161 ± 7.36[a]
Serum HDL (mg/dl)	41.1 ± 4.47	19.3 ± 1.58[a]	23.2 ± 5.61 [a]	37.1 ± 3.00 [b c d]	19.2 ± 2.20[a]	18.6 ± 1.59 [a]

Values represent the mean ± SEM. (n= 4-7). [a, b, c, d] significantly different from control group, FF, celecoxib (10), and diclofenac-treated groups, respectively, at $p < 0.05$. FF = fructose fed; cele.= celecoxib; Dic. = diclofenac. HOMA-IR = Homeostasis Model Assessment-insulin resistance [fasting glucose (mg/dl) x fasting insulin (μIU/ml)] / 405. BW = body weight. VW = visceral fat weight.

One-way analysis of variance (ANOVA) followed by the Tukey-Kramar post analysis test was used to analyze the results for statistically significant difference.

Table (2): Effect of celecoxib and diclofenac on serum levels of oxidative stress and inflammatory parameters in fructose-induced metabolic syndrome

	Control	FF	Cele.10	Cele. 50	Dicl. 6	Dicl. 30
MDA (nmol/dl)	212 ± 17.4	338 ± 19.0[a]	242 ± 14.3[b]	227 ± 17.9 [b c d]	288 ± 26.0	315 ± 19.5[a]
GSH (mg/dl)	50.1 ± 5.41	23.1 ± 2.34[a]	23.3 ± 2.78 [a]	31.7 ± 3.17[a b c d]	23.4 ± 4.02 [a]	24.7 ± 2.63 [a]
Catalase (U/dl)	42.4 ± 5.22	19.5 ± 1.36[a]	38.9 ± 3.99 [b c]	46.1 ± 4.69 [b c d]	23.9 ± 2.91 [a]	23.2 ± 2.85 [a]
CRP (mg/dl)	1.48 ± 0.30	20.4 ± 2.67[a]	9.02 ± 1.34 [b d]	6.67 ± 1.61 [a b c d]	17.8 ± 1.26 [a]	19.8 ± 2.02 [a]
Uric acid (mg/dl)	3.03 ± 0.28	7.29 ± 0.42[a]	3.36 ± 0.42 [b d]	3.29 ± 0.31 [b d]	5.92 ± 0.29 [a]	7.39 ± 0.54 [a]
TNF-α (pg/ml)	12.8 ± 2.17	32.9 ± 3.70[a]	12.9 ± 2.51 [b d]	13.7 ± 2.19 [b c d]	26.7 ± 4.45 [a]	24.2 ± 3.86 [a]

Values represent the mean ± SEM. (n= 4-7). [a, b, c, d] significantly different from control group, FF, celecoxib (10), and diclofenac-treated groups, respectively, at $p < 0.05$. FF = fructose fed; cele.= celecoxib; Dicl. = diclofenac. CRP = C-reactive protein.

One-way analysis of variance (ANOVA) followed by the Tukey-Kramar post analysis test was used to analyze the results for statistically significant difference.

4. DISCUSSION

High fructose feeding animal models are frequently used to understand the pathogenesis and therapeutic interventions of MS (Tran et al., 2009). The results of the present study showed that high fructose feeding caused insulin resistance indicated by increased fasting glucose and insulin levels and HOMA-IR. It also produced dyslipidemia manifested by increased serum TG and decreased HDL. Additionally, it significantly increased the visceral fat index. These results are in consistence with previous studies (Faure et al., 1999).

Several explanations for the metabolic abnormalities in high fructose feeding have been put forward. Impaired activity of the carbohydrate metabolizing enzymes (Van Den Berghe, 1986) and increased oxidative stress (Fuare et al., 1999) have been reported. In the liver, fructose is metabolized into glyceraldehyde and dihydroxyacetone phosphate. These particular fructose end products converge with the glycolytic pathway. Of key importance is the ability of fructose to by-pass the main regulatory step of glycolysis, the conversion of glucose-6-phosphate to fructose 1,6-bisphosphate, controlled by phosphofructokinase. Thus, while glucose metabolism is negatively regulated by phosphofructokinase, fructose continuously enters the glycolytic pathway and promotes the overproduction of TG (Park et al., 1992). Excess TG with subsequent excess free fatty acids may cause insulin resistance by stimulating gluconeogenesis and activating protein kinase C (PKC) and Jun N-terminal kinase (JNK), which may interfere with tyrosine phosphorylation of insulin receptor substrates (IRS) (Dey et al., 2005).

In the present study, celecoxib was used in 2 doses; 10, and 50 mg/kg/day. Only the dose 50 mg/kg improved fructose-induced metabolic disorders. On the other hand, the present results showed that diclofenac, at low and high doses, did not change the fructose-induced metabolic abnormalities. In consistence with the present results, it has been reported that celecoxib, but not piroxicam attenuated insulin resistance in fructose-fed rats (Hsieh et al., 2008).

In a trial to understand the role of oxidative stress and inflammatory mediators in the mechanisms of actions of the investigated drugs, we measured serum levels of MDA, GSH, catalase, CRP, uric acid and TNF-α. The results showed that high fructose feeding was accompanied with increase in MDA and decrease in GSH and catalase enzymes as well as by increase in CRP, uric acid and TNF-α. These mediators are potential contributors to low grade inflammation associated with MS (Rayssiguier, et al., 2006).

Several evidences have supported the link between inflammation, oxidative stress and MS. The females, which are protected against the pro-oxidant effects of a high fructose diet, do not develop insulin resistance when compared with males (Busserolles et al., 2002a). The lipooxygenase/cyclooxygenase inhibitors, which decrease the inflammatory response, attenuate the hypertriglyceridemic effect of high fructose diet (Kelley and Azhar, 2005).

In obesity, macrophages infiltrate adipose tissue and induce chronic inflammation by secreting pro-inflammatory cytokines, including COX-2 and iNOS. Enhanced expression of COX-2 in adipocytes was reported in the early phase of adipogenesis (Fujimori and Amano, 2011). Suppression of COX-2 and iNOS ameliorates the effect of MS (Jungbauer and Medjakovic 2012).

At the molecular level, the link between oxidative stress, inflammation, and MS abnormalities is not fully explored; however several mechanisms have been suggested. First, high fructose feeding activates NADPH oxidase enzyme with subsequent overproduction of reactive oxygen species (Busserolles et al., 2002a). This oxidative stress response was evidenced in the present study by elevation in MDA, reduction in GSH, and increase in catalase activity. Increased oxidative stress stimulates nuclear receptors including liver X receptor and peroxisome proliferator activators receptors that release inflammatory mediators such as induced nitric oxide synthase (iNOS), COX-2, TNF-α, interleukine-1 (IL-1) and IL-6 (Armutcu et al., 2005; Busserolles et al., 2002b). These mediators induce dramatic changes in lipid metabolism, particularly in serum TGs via increasing hepatic secretion and/or delayed clearance of VLDL (Sparks et al., 2012). Evidence suggests that TNF-α induces insulin resistance by the inhibition of the insulin receptor substrate 1 signaling pathway (Shoelson, et al., 2006). Second, visceral fat plays an important role in metabolic syndrome. It is a rich source of cytokines and peptides including TNF-α, angiotensinogen, plasminogen activator inhibitor-1, leptin, and complement components. Theses mediators participate in development on insulin resistance through modulation of the insulin receptor/IRS-1 axis. (Shoelson et al., 2006). Third, uric acid has been shown to be involved in metabolic syndrome via several mechanisms. It leads to oxidative stress, endothelial dysfunction, and to a vascular and systemic inflammatory response. It reduces nitric oxide biovailability with subsequent reduction in glucose uptake in the skeletal muscle leading to hyperinsulinemia and insulin resistance (Stellato et al., 2012). High fructose increases uric acid production via activation of hypoxanthine-guanine-phosphoribosil transferase enzyme (Nakagawa et al., 2006).The data

of the present study support the above mentioned concepts.

The results of the present experiment are in agreement with studies reported that celecoxib antagonized low-grade inflammation (Hsieh et al., 2010) and oxidative stress (Kirkova, et al., 2007) which is linked to insulin resistance. On contrary to celecoxib, the present work showed that diclofenac did not cause any changes in serum levels of oxidative stress, TNF-α, and uric acid. The presence of variability between non selective and selective COX-inhibitors had been previously reported. Hsieh et. al. (2008) reported that a therapeutic dose of celecoxib, but not piroxicam, could significantly attenuate fructose-induced insulin resistance in rats. Similarly, in human study, it has been reported that celecoxib, but not acetylsalicylic acid, increased insulin sensitivity in human subjects (Gonzaliz-Ortiz, et al., 2001). In conflict with the current observation, a human study by Van Erk et al (2010) reported a possible role of diclofenac in modulation of inflammatory mediators of obesity. Species and model differences are possible causes for such conflict.

The protective effect of celecoxib compared with diclofenac might be attributed to several factors including; selectivity of celecoxib for COX-2 (Hsieh et. al. 2008), antioxidant (Kirkova, et al., 2007) and anti-TNF-α (Hsieh et al., 2010) effects. However, other mechanisms could not be ruled out. For example, it has been reported that non cyclooxygenase-derived prostanoid (8-isoprostan) increased in metabolic syndrome and that celecoxib treatment, but not piroxicam, attenuated 8-isoprostane levels (Hsieh et. al. 2008). Both celecoxib and piroxicam significantly diminished fructose-induced elevation in plasma thromboxane B2 and 6-keto PGF$_1$-α; but only celecoxib treatment significantly attenuated a fructose-induced increase in 8-isoprostane levels. Accordingly, celecoxib, but not piroxicam, was reported to attenuate fructose-induced whole body and muscular insulin resistance in rats (Hseih et al., 2008). A recent study reported overexpression of COX-2 as well as increased oxidative stress in MS induced by high fat diet. Selective COX-2 inhibitors, celecoxib and nimsulide attenuated high fat induced insulin resistance and suprresed COX-2 overexpression. Both selective COX-2 inhibitors increased glucose uptake mediated by insulin and GLUT4 (Glucose transporter-4) translocation in skeletal muscles (Tian et al., 2011).

In conclusion, the results of the current work clearly demonstrate that the selective COX-2 inhibitor, celecoxib, but not the non selective one, diclofenac, has a protective effect against high fructose-induced metabolic disorders. Such effect might be due to its antioxidant effect as well inhibition of uric acid and TNF-α.

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