METABOLIC, ANTI INFLAMMATORY AND ANTI-OXIDANT EFFECT OF LARGE DAILY DOSES OF METFORMIN IN TYPE 2 DIABETES PATIENTS

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ABSTRACT

Chronic hyperglycemia and an elevation in glycosylated hemoglobin (HbA_{1c}), and approaches to ameliorate oxidative stress have improved IR and decreased HbA_{1c}, leading one to conclude that oxidative stress may be involved in glucose metabolism. Reactive oxygen species can damage cellular DNA, membranes, lipids, proteins and drive inflammatory gene expression that inhibits metabolic pathways induced by insulin, leading to IR. Metformin seems to exert its anti-inflammatory role by reducing pro-inflammatory cytokine secretion in specific cell types. Thirty Iraqi patients aged (35-70) years, newly diagnosed with type 2 diabetes were treated with different doses of oral Metformin and randomized into three groups in a dose of 500mg/day single dose,1000mg/day in 2 divided dose, and 1500mg/day in 3 divided dose for 3 months , 10 patients each group. Serum levels of high sensitivity C-reactive protein (hs-CRP), Serum MDA, glycated hemoglobin HbA_{1c}, total Cholesterol, triglycerides, HDL-C, LDL–C, VLDL-C was measured. There was a significant decrease in HbA_{1c} and serum MDA level ($p<0.05$) and non-significant decrease in hs-CRP level ($p>0.05$) in all patient groups with higher percent of reduction with a dose of (1500mg/day) (27.9%, 49.6%, 22.5%) respectively. There was a significant decrease in total serum cholesterol, TG, Serum (LDL-C), VLDL ($p<0.05$), and a significant increases ($p<0.05$) in the serum HDL-c level after 3 months compared with baseline values. The results of the present study clarified that there are favorable effects of metformin on metabolic, oxidative, and inflammatory profile which may be associated with reduced risk for macrovascular complications in patients with type 2 DM.

KEY WORDS: Diabete mellitus, hyperglycaemia, HbA1c, oxidative stress, metabolic syndrome, metformin, C-reactive protein (CRP), malondialdehyde (MDA), lipid profile.

INTRODUCTION

A combination of nutritional excess and physical inactivity, enhanced by a genetic predisposition, can lead to chronic hyperglycemia, which can increase adiposity in target organs and NADPH oxidase, leading to increases in various reactive oxygen species [1]. Increases in circulating FFA and hyperglycemia, chief characteristics of DM2, can both lead to leakage of reactive oxygen species from the mitochondrial and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [2,3]. Overproduction of (O_2^-) appears to be the first and key event in the activation of other pathways and systems (*e.g.* immune and metabolic) involved in the pathogenesis of vascular dysfunction [4].Within this cascade, ROS from the immune system, adipose tissue and mitochondria mediate/ activate stress-sensitive kinases, such as mitogen-activated protein kinase (p38-MAPK) and inhibitor of kappa B kinase (IKK-)[5]. These kinases activate the expression of pro-inflammatory mediators, such as TNF- , IL-6 which further induces the production of ROS (reactive oxygen species), thus potentiating the positive feedback loop[6]. Reactive oxygen species can damage cellular DNA, membranes, lipids, proteins and drive inflammatory gene expression that inhibits metabolic pathways induced by insulin, leading to IR [7]. This progression leads to chronic hyperglycemia and an elevation in glycosylated hemoglobin (HbA_{1c}), and approaches to ameliorate oxidative stress have improved IR and decreased HbA_{1c}, leading one to conclude that oxidative stress may be involved in glucose metabolism [8]. Elevated levels of malondialdehyde (MDA) indicate an increase in the level of production of oxygen free radicals, suggesting their possible role in atherogenesis, leading to coronary heart disease [9]. In diabetes, MDA formation is increased by glycation products that stimulate breakdown of the lipids[10]. It has been found that AMPK plays an important role in inflammation, and metformin can serve as a potential drug to treat inflammation related disorders [11,12]. Recently, from results obtained in *in vivo* and *in vitro* models, it has been suggested that metformin can be used in other pathophysiological conditions. Moreover, genetic regulation of AMPK activity clearly demonstrated that AMPK has the ability to regulate macrophage functional polarization[13]. And recent clinical studies further suggest that metformin may alter inflammation as determined by decreased inflammatory markers in plasma, in some cases of polycystic ovary syndrome, indicating modulation of inflammation[14]. Metformin may reduce the activity of inflammatory cytokines by increasing the production of IL-1 receptor antagonist (IL1Rn), a protein factor which interferes with pro-inflammatory signaling of IL-1 . It may also promote favorable CRP levels, although not to

the same extent as weight loss [15]. Increased levels of serum CRP has been found virtually in all diseases associated with active inflammation or tissue destruction, a significant decrease in serum CRP concentrations was monitored after metformin therapy, the improvements of Body Mass Index during metformin treatment were also marked as significant determinants of serum CRP level [16]. Metformin has suppressed IL-8 release from human adipose tissue in *vitro* [17]. Thus, metformin seems to exert its anti-inflammatory role by reducing pro-inflammatory cytokine secretion in specific cell types [18].

This study was primarily designed to evaluate the anti-inflammatory effect and oxidative stress status of different doses of metformin in newly diagnosed patients with type 2 diabetes, also to determine the comparable effect of metformin doses on Lipid profile.

PATIENTS & METHODS

The study was conducted between October 2012 up to March 2013; during this period thirty Iraqi patients newly diagnosed with type 2 diabetes were attend the National Diabetes Center during their periodic visit seeking for medical advice concerning their diet modification, weight reduction and drug prescription. Patient's age range was (35-70) years with mean $\pm$SD (51.5$\pm$10.248) years, table 1.

TABLE 1: Demographic parameters distribution among newly diagnosed T2DM patients on different doses of metformin therapy

parameter	Metformin 500 mg/day	Metformin 1000 mg/day	Metformin 1500 mg/day	*P* value
Age (years)	55.6$\pm$11.4	47.4$\pm$8.8	51.4$\pm$9.5	0.83
Sex				
Female	4(40%)	3(30%)	5(50%)	0.66
Male	6(60%)	7(70%)	5(50%)	
BMI (kg/m2)				0.50
Normal (18.5-24.9) (kg/m2)	(30%)	2(20%)	1(10%)	
Over weight (25-29.9)(kg/m2)	3 (30%)	6(60%)	4(40%)	
Obese (=>30) (kg/m2)	4 (40%)	2(20%)	5(50%)	

Values are presented as mean $\pm$SD and percentage; n= 10 patients in each group $ Significant using Pearson Chi-square test at 0.05 level

Eighteen patients were male and twelve were female, most of patients are either overweight having a BMI ranging from (25-29.9) kg/m² or obese having a BMI (30) kg/m²and all of them are newly diagnosed with no history of other associated illnesses or infection at time of study also those with known diseases, which are associated with disordered glucose metabolism; such as Cushing's disease, acromegaly, chronic pancreatitis and pancreatactomy, as well as, those with chronic kidney disease and pregnant women. Patients did not take oral hypoglycemic agents or any other medication before the time of enrollment and they had no history of smoking or alcohol drinking. The diagnosis of T2DM was made on the basis of the recommended criteria by WHO (1999)[19]

The selected diabetic patients were treated with different doses of oral anti-diabetic agents [Metformin 500mg (Glucophage ®) tablets -Merck, France-500, 1000, 1500 mg/day].Patients agreement for treatment have been taken according to their condition and physician opinion are randomized into three groups:

1. First group: includes 10 diabetic patients were treated with oral hypoglycemic agent metformin in a dose (500mg/day) in single dose for 3 months.
2. Second group: includes 10 diabetic patients were treated with metformin in a dose (1000mg/day) in 2 divided doses for 3 months.
3. Third group: included 10 diabetic patients were treated with metformin in a dose (1500mg/day) in 3 divided dose for 3 months.

All patients were fasting (12-14) hr calories free diet.
Glycated hemoglobin was measured by using the Variant HbA$_{1c}$program developed by BIO-Rad[20]. American diabetes association (2001) recommends that the goal of therapy should be an HbA$_{1c}$of <7% [20].

Serum levels of high sensitivity C-Reactive Protein (hs-CRP) was quantitatively determined in patients by means of sandwich ELISA test using commercially available kits (DRG International Inc., USA), and MDA was quantitatively tested by sandwich ELISA test, according to the literatures (Talaro, 2005)[21] using commercially available kits (CUSABIO Biological Laboratories Company, China).

Cholesterol was determined by enzymatic hydrolysis and oxidation according to method of Richmond, 1973,[22] using a ready-made kit (SPRINEACT Company, SPAIN), HDL fraction which is determined by using cholesterol kit (Randox Company, U.K.) according to the method of Burstein et al., 1970 [23]. The triglycerides are determined by enzymatic hydrolysis according to method of Young and Pestaner, (1975); Fossati and principle, (1982)[24,25] by using a ready- made kit (SPRINEACT Company, SPAIN). LDL– cholesterol can be calculated mathematically from the total cholesterol, the triglycerides and the HDL – cholesterol concentrationusing Friedwald's method, 1972 [26]. VLDL-C concentration is calculated as one – fifth of the serum TG according to method of Fiancis and David, 2001[27].

Statistical Analysis

Statistical analyses were carried out using the computer program SPSS version 21(Statistical Package for Social Sciences-version 21).The results were expressed as mean $\pm$SD. Data were statistically evaluated using paired *t*-test to compare between pre and post treatment results. The way analysis of variance (ANOVA) test was utilized to

compare between the results of studied parameters among different patients groups. Values with *P*<0.05 were considered significantly differen[28].

RESULTS

There was a significant decrease in HbA_{1c} level (p<0.05) in all groups of type 2 DM patients treated with different doses of metformin after 3 months, compared with pre-treatment values, table 2.

TABLE 2: Metabolic, anti-oxidant, and anti inflammatory changes pre- and post treatment among newly diagnosed T2DM patients on different doses of metformin therapy

Variables	Period	Metformin 500 mg/day	Metformin 1000 mg/day	Metformin 1500 mg/day
$HbA_{1c}\%$	Pretreatment(Baseline)	9.54 ± 1.95^a	9.16 ± 1.55^a	9.80 ± 1.94^a
	Post treatment(After 3 months)	$8.55\pm1.89^{a*}$	$7.47\pm1.08^{a*}$	$7.06\pm1.23^{a*}$
MDA (µg/dl)	Pretreatment(Baseline)	6.07 ± 1.70^a	5.61 ± 1.69^a	5.46 ± 1.97^a
	Post treatment(After 3 months)	$5.09\pm1.9^{a*}$	$3.59\pm0.89^{b*}$	$2.75\pm0.86^{c*}$
hs-CRP (mg/l)	Pretreatment(Baseline)	5.005 ± 3.18^a	5.40 ± 3.02^a	5.82 ± 3.37^a
	Post treatment(After 3 months)	4.82 ± 3.22^a	4.47 ± 3.39^a	4.51 ± 2.84^a
	Pretreatment(Baseline)	201.70 ± 42.14^a	200.20 ± 28.86^a	200.40 ± 13.14^a
TC(mg/dl)	Post treatment(After 3 months)	$182.50\pm40.57^{a*}$	$170.90\pm30.20^{a*}$	$161.70\pm26.39^{a*}$
TG(mg/dl)	Pretreatment(Baseline)	164.00 ± 53.38^a	164.20 ± 43.69^a	164.60 ± 21.77^a
	Post treatment(After 3 months)	$137.10\pm41.68^{a*}$	$107.80\pm12.65^{b*}$	$95.40\pm5.04^{c*}$
HDL-c(mg/dl)	Pretreatment(Baseline)	42.20 ± 4.85^a	42.40 ± 5.68^a	42.40 ± 5.68^a
	Post treatment(After 3 months)	$47.10\pm5.82^{a*}$	$49.70\pm5.64^{a*}$	$50.20\pm7.91^{a*}$
LDL-c (mg/dl)	Pretreatment(Baseline)	126.7 ± 38.15^a	125.1 ± 28.87^a	131.9 ± 20.15^a
	Post treatment(After 3 months)	$107\pm36.83^{a*}$	$99.9\pm31.21^{a*}$	$91.5\pm28.61^{a*}$
VLDL (mg/dl)	Pretreatment(Baseline)	32.80 ± 11.06^a	32.70 ± 8.68^a	32.80 ± 4.32^a
	Post treatment(After 3 months)	$27.50\pm8.42^{a*}$	$21.40\pm2.50^{b*}$	$19.30\pm1.34^{c*}$

Data were presented as Mean±SD.
Data with identical superscript letter (a) among treatment groups considered non-significantly differ, (*p*>0.05) during the same period.
Data with non-identical superscript letters (a,b,c) among treatment group considered significantly differ, (*p*<0.05)during the same period.
* represent significant difference, (*p*<0.05) comparing pre and post treatment, within the same group.

However, the percent of reduction in $HbA_{1c}$was in a dose dependent manner (10.3%, 18.3% and 27.9% respectively) compared with baseline values. There was non- significant difference (p>0.05) among the three groups treated with metformin doses after 3 months. Serum MDA level show a significant decrease in (*p*<0.05) in all groups of type 2 DM patients treated with different doses of metformin after 3 months compared with baseline values, according to paired t-test. Moreover, treatment with (1500mg/day) of metformin produced a higher percent of reduction in this parameter after 3 months (49.6%) compared with baseline values, while treatment with (1000 and 500mg) produced (36% and 16.14%) respectively, compared with baseline values. There was a significant difference (*p*<0.05) among the three groups treated with metformin doses after 3 months, table 1. There was non-significant decrease in hs-CRP level (*p*>0.05) in all groups of type 2 DM patients treated with different doses of metformin after 3 months compared with baseline values. Moreover, treatment with (1500mg/day) of metformin produced a higher percent of reduction in this parameter after 3 months (22.5%) compared with baseline values, while treatment with (1000mg and 500mg) produced (17.22% and 3.6%) respectively, compared with baseline values. There is a non-significant difference (*p*>0.05) among the three groups treated with metformin doses after 3 months. The lipid profile show that there was a significant decrease in T.C. level (*p*<0.05) in all groups of type 2 DM patients treated with different doses of metformin after 3 months compared with baseline values. And the treatment with (1500mg/day) metformin produced a higher percent of

reduction (19.31%) in this parameter after 3months compared with baseline values, while metformin treated with (1000 and 500mg) produced (14.63%and 9.51%) respectively, when compared with baseline values. There was non-significant difference among the three groups treated with metformin doses after 3 months. Total Serum T.G. show that there was a significant decrease (*p*<0.05) in level in all groups of type 2 DM patients treated with different doses of metformin after 3 months compared with baseline values. And the treatment with (1500mg/day) of metformin produced a higher percent of reduction in this parameter after 3 months (42.04%) compared with baseline values, while treatment with (1000 and 500mg) of metformin produced (34.3% and16.4%), respectively compared with baseline values. There was a significant difference (p<0.05) among the three groups treated with metformin doses after 3 months. Effect of different doses of metformin on High Density Lipoproteins (HDL-c) levels of newly diagnosed patients with type 2 DM after 3 months of treatment show that there was a significant increases (*p*<0.05) in the serum HDL-c level compared with baseline values according to paired t-test. Moreover higher dose (1500mg/day) of metformin produced a higher percent of increase in this parameter after 3 months (19.5 %) compared with baseline values, while treatment with (1000 and 500mg) produced (17.21% and 11.61%) respectively, compared with baseline values. There was non-significant difference among the three groups treated with metformin doses after 3 months. Effect of different doses of metformin on serum Low Density Lipoproteins(LDL-C) level of newly

diagnosed patients with type 2 DM after 3 months of treatment show that there was a significant decrease ($p<0.05$) in Serum LDL-C level in all groups compared with baseline values, according to paired t-test. Moreover, the treatment with (1500mg/day) of metformin produced a higher percent of reduction in this parameter after 3 months (30.62%) compared with base line values, while treatment with (1000 and 500mg) produced (20.14% and 15.54%) respectively, compared with baseline values . There was non-significant difference among the three groups treated with metformin doses after 3 months.

There was a significant decrease ($p<0.05$) in Serum VLDL in all groups of type 2 DM patients treated with different doses of metformin after 3 months compared with baseline values, according to paired t-test. Moreover, the treatment with (1500mg/day) of metformin produced a higher percent of reduction in this parameter after 3 months (41.15%) compared with base line values, while treatment with (1000 and 500mg) produced (34.55% and 16.15%) respectively, compared with baseline values. There wasa significant difference among the three groups treated with metformin doses after 3 months.

DISCUSSION

Numerous studies suggest positive antihyperglycemic and metabolic effects of metformin, with a wide safety profile. Therapeutic profile of metformin has been evaluated for more than five decades[29]. High HbA_{1c} regarded as predicator for the development of microangiopathy, which can be partially reversed by additional intensive therapy for hyperglycemia[30]. Iftikhar et al. (2011) documented that each 1% increase inHbA_{1c} poses 15-18% relative risk of cardiovascular disease in T1DM and T2DM respectively is regarded as risk factor for development of microangiopathy [31].The present study show that treatment with maximum dose of metformin (1500mg) produced the highest percent reduction, similar to other result[32]. However, the percent changes in HbA_{1c} produced following the use of different doses of metformin, appeared significantly different after 3 months, and this finding disagrees with other previous studies [33, 34]. As was noticed metformin treatment reduces oxidative stress as well as cholesterol level in the blood, this may be the basis for Na^+-K^+ ATPase activity to be restored almost to the control level in DM subjects during metformin therapy [35, 36, 37]. Hyperglycemia may induce oxidative stress and the possible mechanisms for inducing such stress are well documented [38, 39]. Our results support the idea that oxidative stress may be an early event in the natural history of diabetes [40]. In this regard, it has been reported that oxidative stress can activate multiple serine kinase cascades including IKKB/ NF-kB that can impair insulin action. Thus, hyperglycemia and diabetic complications may induce cycle of oxidative stress which further exacerbates an existing condition of increased oxidative stress[41]. The present study, shows that there is a significant decrease in MDA level in all groups of patients with maximum dose of metformin (1500mg) produced a higher percent reduction in this parameter after 3 months compared with baseline values, and other metformin treated group (500 and 1000) mg, these results were in agreement with the study conducted by Pavlovic et

al.,(2000) [42] who reported significant reduction in MDA levels in both erythrocytes and plasma in newly-diagnosed patients with Type 2 DM who had been on metformin therapy for a period of 4 weeks [43]. These results were also in line with the study conducted by Abdulkadir AA.,(2012)who reported that Metformin positively affected the oxidant/antioxidant balance in newly diagnosed Type 2 DM patients with no significant effects on acute phase reaction protein [44]. Conversely, Skrha et al.,(2007)observed that 3-months therapy with metformin was accompanied by significantly increased plasma MDA levels in Type 2 DM patients and they concluded that initiation of metformin treatment in such patients was associated with activation of oxidative stress [45]. Gupta et al. (2010) observed no change in MDA levels at the end of the 12 weeks treatment with metformin, in newly diagnosed Type 2 DM patients [46]. The present study has shown that metformin therapy for 3 months in newly diagnosed Type 2 DM patients decrease MDA levels significantly. Metformin has antioxidant properties which are not fully characterized, it reduces reactive oxygen species (ROS) by inhibiting mitochondrial respiration and decreases AGE indirectly through reduction of hyperglycemia and directly through an insulin dependent mechanism,[47] metformin inhibition of insulin receptor signaling pathways is a central mechanism through which inflammatory and stress responses mediate insulin resistance[48]. The current study demonstrated a high improvement and significant decline in MDA levels in a dose dependent manner. A recent prospective study found that serum CRP was determinant of risk for development of type 2 diabetes mellitus in apparently health middle-aged women [49].Also such association between inflammation, as measured by CRP levels, with the development of diabetes over a 3 to 4 year period has been reported in elderly subjects 65 years[50]. Similarly, Freeman et al.,(2002)showed in a retrospective study that there was a strong and graded association of hs-CRP level with the incidence of diabetes independent of established risk factors[51]. Meanwhile, in the present study, as shown in table and figure (3-10) shows that there was a non-significant decrease in hs-CRP in all groups of patients treated with different doses of metformin (500, 1000 and 1500mg). Moreover, treatment with the highest dose of metformin (1500mg) produced a highest percentage of reduction in this parameter after 3 months compared other metformin treated group (1000 and 500) mg, our finding were in agreement with the results of previous studies [44, 52, 53]. Also, Dragan Micicetal (2010) study was found that metformin has an effect on CRP and insulin sensitivity in type 2 diabetes patients after 12 weeks of therapy, this effect was associated with significant reductions in HbA_{1c} levels and insulin sensitivity independent of changes in hs-CRP [54]. Accumulating evidence shows that metformin reduces CRP concentrations in patients with type 2 DM. However, this effect is probably dependent on improving glycemic control. These findings support the hypothesis that DM has inflammatory aspects, but the causal pathway between DM and inflammation was not clear[55]. However, the present study revealed that no significant difference observed among these treated groups in hs-CRP after 3 months, Prior studies on the effect of metformin on CRP

have led to different conclusions, in a recent placebo-controlled study, metformin decreased CRP after 24 weeks, although other investigators have found more modest effects or no effect on CRP, Our data are consistent with the absence of a meaningful effect of metformin on subclinical inflammation reflected by CRP [56].

Our patients are obese and overweight, the role of adipose tissues in metabolic dysfunctions has long been considered but their potential role in inflammatory processes is a new expanding concept, because this tissue is an important metabolically active endocrine organ that secretes various hormones and cytokines (like IL-6), known as adipokines [57]. Interleukin-6 is a multifunctional cytokine that targets several tissues and cell types, and one of its major actions is to control hepatic production of inflammatory proteins such as CRP, which is an important cardiovascular risk factor [58]. Accordingly, all these findings can explain the non-significant differences between groups and a different percent of reduction in this parameter after 3 months compared with baseline values after administration of increased doses of metformin. According to prospective population based studies, TG is a powerful risk factor for coronary heart disease (CHD) in type 2 diabetes mellitus patients as in non diabetic subjects, in contrast, high TG and low HDL-C maybe even stronger risk factors for CHD in type 2 diabetes mellitus patients than in non diabetic individuals, but more prospective studies are needed to substantiate this view[59, 60]. In present study, all the utilized approaches of treatment with different doses of metformin in newly diagnosed patient, T2DM &high total triglycerides and low HDL-C produced significant decreases in serum TC, TG and LDL-C levels, with an increase in the level of HDL-C level compared to baseline values. Also, a highest value achieved following the use of (1500) mg metformin dose compared to other doses (500 and 1000) mg. This effect is compatible with the studies of Wulffele MG et al. (2004) [61] and Mourao-Junior CA et al.,(2006) [62]. Moreover, metformin treatment results in a moderate to significant reduction in circulating TG levels, particularly in patients with marked hypertriglyceridemia and hyperglycemia; this has been attributed to a reduction in hepatic very low density lipoprotein (VLDL-C) synthesis [63]. Emral et al. (2005)[64] investigated the effect of short term glycemic regulation with either metformin or gliclazide or both on postprandial lipemia in patients with type 2 DM; fasting TC was reduced, while no change was observed in fasting HDL-C and LDL-C levels and both fasting and postprandial TG decreased after the intervention in comparison with the baseline values, the main finding of this study was that the improvement of glycemia reduces both fasting and postprandial TG [64]. Metformin has been proposed to cause a mild and transient inhibition of mitochondrial complex I which decreases ATP levels and activates AMPK-dependent catabolic pathways, increasing lipolysis and ß-oxidation in white adipose tissue and reducing gluconeogenesis [65, 66]. The resultant reduction in triglycerides and glucose levels could decrease metylglyoxal (MG)[MG is a potent protein-glycating agent and an important precursor of advanced glycation end products (AGEs)]production through lipoxidation and glycoxidation, respectively, accordingly, metformin improves glycemic control and

lipoprotein metabolism in patients with type 2 DM, it lowers postprandial glucose, insulin and FFAs levels [67, 68]. Moreover, metformin reduces postprandial total triglycerides and may increase HDL cholesterol; these favorable effects of metformin on lipids may be associated with reduced risk for macrovascular complications in patients with type 2 DM [59, 60].

CONCLUSION
The results of the present study clarified that metformin, in a dose- dependent pattern produce significantly improvement in HbA$_{1C,}$ Lipid profile and serum MDA levels in all diabetes patient groups after 3 months of treatment, and the serum levels hs-CRP as an inflammatory marker was decreased. These favorable effects of metformin on metabolic, oxidative, and inflammatory profile may be associated with reduced risk for macro vascular complications in patients with type 2 DM.

ACKNOWLEDGMENT

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