

Cholinesterase activity in non-alcoholic-fatty liver disease in diabetic patients taking oral antidiabetic drugs

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) and Alzheimer's disease (AD) are common co-morbidities in patients with diabetes mellitus type 2 (T2DM). Resistance to insulin constitutes a major etiological factor for both conditions as well as in T2DM; thus, hypoglycemic drugs may show some promising prospect in this category of patients. Additionally, cholinesterases (ChEs), mainly butyrylcholinesterase (BChE) are, etiologically, a factor in both AD and DM; also, the effect of antidiabetic drugs on enzyme activity is a reasonable target for response. The present work aimed to investigate the effects of metformin and glibenclamide (MET-SU) with regard to acetylcholinesterase (AChE) and BChE activities and liver biochemical profile in 2 T2DM patient groups: those with a clinical picture of NAFLD and those free from NAFLD. The study is a case-control study, involving 3 groups (30 subjects each). Group 1 comprised of T2DM patients free from clinically evident NAFLD and group 2 comprised of T2DM patients newly diagnosed with NAFLD. The control group consisted of apparently healthy subjects. Groups 1 and 2 were on a metformin-glibenclamide (MET-SU) combination. AChE and BChE activities, as well as levels of transaminases, serum albumin, total bilirubin, alkaline phosphatase and fasting glucose serum levels were tested. AChE and BChE activity in groups 1 and 2 were significantly different compared to control, but much lower in group 2 compared to group 1 and

control. In comparison to controls, plasma levels of transaminases, fasting glucose and alkaline phosphatase in group 2 showed a significant increase, while other parameters were not significantly different. We conclude that diabetic patients with NAFLD who are treated with a metformin-glibenclamide combination showed a better cholinesterase enzyme profile but less favorable liver function panel compared to other groups. These findings suggest a potential protective effect of the drugs in diabetic-NAFLD patients manifested as lowered cholinesterase activities, but they also indicate an apparent worsening of liver condition, in terms of biochemical parameters of liver function.

KEYWORDS: metformin, glibenclamide, cholinesterases, diabetes, fatty liver disease.

INTRODUCTION

Cholinesterases (ChE) are of two types: acetylcholinesterase (AChE), present on the red blood cells (RBCs) and central nervous system (CNS), and butyrylcholinesterase (BChE), or pseudocholinesterase, mainly present in plasma and the liver [1]. Acetylcholine is broken down to its constituent choline and acetic acid components by these enzymes [2]. The AChE activity is traditionally linked to Alzheimer's disease (AD) and AChE inhibitors have long been utilized for AD. While they can improve neurodegenerative changes, more effective drugs that target underlying AD pathophysiology are still lacking [3].

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The risk of developing AD in people with type 2 diabetes mellitus (T2DM) is high [4, 5]. The two diseases share both degenerative pattern and pathology [3]. Insulin resistance (IR) and diabetes contribute to dementia disorders as well [6]. Antidiabetic agents act *via* some mechanisms common for both DM and AD; thus, it is reasonable to consider them eligible candidates to combat cognitive dysfunction and dementia [7]. Very limited number of studies evaluating the impact of antidiabetic drugs on cognitive health exists, and their results show inconsistency [8, 9]. BChE is a common pathological factor in both AD and T2DM. Interestingly, elevated activity of BChE has been reported in diabetic patients [10], but how DM drugs affects its activity is not elucidated in literature [4, 11]. Furthermore, how combined antidiabetic drugs affect both enzymes is not highlighted previously in this category of patients.

Deposition of fat on the liver in excess of 5-10% is termed fatty liver (or hepatosteatosis). When this occurs in a setting where alcohol intake is not involved, the condition is known as non-alcoholic fatty liver disease (NAFLD) [12]. This condition is typically associated with T2DM [13], showing about 55% prevalence in T2DM patients [14]. There is a range of liver injuries that NAFLD is associated with, ranging from mild intrahepatic fat deposition, to the more serious non-alcoholic steatohepatitis (NASH) [15]. Risk of deterioration to NASH and fibrosis is high in NAFLD patients, [16, 17, 18]; also, CVD risk is increased as well in such patients.

Patients with T2DM are highly predisposed to liver damage [19]. Liver disease may be well-established before changes in liver profile are seen [20]. Current recommendations for T2DM patients stress the early screening and intervention for liver damage [15, 19]. Hepatic ultrasonography and biochemical profiling are commonly used to identify NAFLD; but liver biopsy remains the gold standard [18].

For such a critical condition, the ideal treatment for NAFLD is still a matter of debate, and currently drugs for co-morbidities, e.g., DM, hypertension, dyslipidemia, etc. are employed to this end [21]. Drugs like metformin actually ameliorate hepatosteatosis [22] and improve the biochemical and metabolic features of NAFLD [23].

Insulin resistance is central to the pathogenesis of T2DM as well as NAFLD, and hence insulin-sensitizing drugs commonly given to diabetic patients could prove beneficial [24, 15]. Actually, hypoglycemic drugs have been shown to improve histological and functional aspects of the liver, according to earlier systematic reviews, but these studies are of little utility since they were conducted in non-diabetic NAFLD/NASH patients [25]. While they do show good potential for treating both NAFLD and DM, still, available data are conflicting [14]. Only limited number of studies were conducted on patients with T2DM, employing oral antidiabetics in combination, to explore drug impact on liver function [25].

Accordingly, it may be theorized that add-on benefit is achievable by using DM drugs, i.e. while attempting to control DM, these drugs may prove beneficial in terms of the two co-morbidities: AD and NAFLD. To our knowledge, the effect of antidiabetic drug combination in this context has not been evaluated adequately, if any. Thus, this study was carried out to explore the hypothesis that therapy with metformin-glibenclamide (MET-SU) combination in T2DM patients with/without NAFLD is associated with positive impact on both neurologic/cognitive outcome and liver function.

The aim of the present work is to investigate the effects of metformin-glibenclamide combination treatment in both T2DM patients who have NAFLD as well as patients who don't have NAFLD, with regard to AChE and BChE activities and liver profile.

MATERIALS AND METHODS

This study adopted a case-control design, and was performed at a specialized diabetes center (Al-Wafaa). Informed consent was obtained from all subjects enrolled in the study. The subjects included males and females who were not less than 30 years old, and who were diabetic and treated with a combination of metformin (1700 mg/day) and glibenclamide (10 mg/day) for a minimum of at least 6 months prior to the study.

For NAFLD to be present, there should be an imaging or histological evidence of hepatic steatosis; in addition, there should be no secondary liver fatty deposition, e.g. due to excessive intake of

alcohol [26]. In the current study, fatty liver disease was considered present when three or more liver profile tests beyond normal range were obtained [27] in addition to ultrasonographic evidence. Three groups were involved in the study, each consisting of 30 patients:

1. Diabetic patients free of fatty liver disease (Group 1)
2. Diabetic patients diagnosed as having NAFLD (Group 2)
3. Control group: consisted of apparently healthy persons free from diabetes or hepatic disease, who were not exposed to any drugs affecting ChE enzyme.

Exclusion of patients was made when patients: a) were maintained on medications other than for diabetes, b) recent exposure to agents affecting ChE activity, c) had chronic cardiac illness, d) had chronic liver disease, e) renal problems. Blood from fasting subjects was collected in EDTA test tubes. The erythrocyte component and plasma were separated for ChE enzyme assessment. Remaining sample was utilized for the rest of the tests.

The cholinesterase enzyme activity was assessed using a modified version of electrometric method [28, 29] in which a reaction mixture containing distilled water, plasma or erythrocytes and barbital-phosphate buffer of pH 8.1 is prepared; the pH is measured before and after adding the enzyme substrate and the activity is expressed as the change in pH during a period of 20 minutes ($\Delta\text{pH}/20 \text{ min}$), minus change in pH of the blank (mixture containing no sample), i.e. ChE activity ($\Delta\text{pH}/20 \text{ min.}$) = (pH1-pH2)- ΔpH of blank.

Using commercial kits, fasting level of glucose in serum (FSG), serum levels of total bilirubin (TBil), alkaline phosphatase (ALP) activity, serum aspartate

transaminase (AST) activity, alanine transaminase (ALT) activity and serum albumin concentration were determined colorimetrically using standard methods [30, 31, 32, 33, 34]. Student's t-test was utilized to compare means of the study groups [35]. Difference was considered significant at $P < 0.05$. Data were analyzed utilizing SPSS version 15.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Table 1 shows activities of AChE and BChE for control, group 1 and group 2 of study patients. Significant difference in both enzymes activities versus control group are seen, with group 1 showing the highest readings among the 3 groups, and group 2 showing lower readings compared to group 1. Table 2 shows FSG, serum transaminases (AST and ALT), ALP, albumin and total bilirubin of the study groups; fasting glucose was significantly different in both group 1 and 2 versus control; transaminases and ALP were significantly greater, in the second group, versus group 1 and the control group, while TBil and serum albumin did not show a similar difference between the three groups.

DISCUSSION

Abnormality in AChE and BChE in AD as well as in T2DM points to the critical place they hold in the pathogenesis of the two disorders. Both conditions manifest enhanced AChE and BChE plasma activities, indicating systemic inflammation, which *per se* links both disorders [36]. In the current work, both subtypes of ChE were lower in group 2 compared to both the control and group 1. Patients in both groups received MET-SU therapy for diabetes; lower ChE levels may be attributed to therapy with antidiabetic drugs, which is

Table 1. Cholinesterases enzyme activities for control and study groups.

Parameters	Control	Group 1	Group 2
BChE $\Delta \text{pH}/20 \text{ min}$	1.32 \pm 0.07	1.45 \pm 0.12*	0.64 \pm 0.09*
AChE $\Delta \text{pH}/20 \text{ min}$	0.97 \pm 0.12	1.02 \pm 0.08*	0.74 \pm 0.13*

The values are expressed as means \pm SE,

*Significantly different control, $P < 0.05$

BChE: Butyrylcholinesterase; AChE: acetylcholinesterase

Table 2. Fasting glucose/liver biochemical profile in control and study groups.

Parameters	Control	Group 1	Group 2
FSG (mmol/L)	5.65 ± 1.02	9.10 ± 1.32 *	9.3 ± 0.90 *
S. TBil (mmol/L)	9.73 ± 2.24	9.32 ± 4.12	9.5 ± 2.61
S. ALT (U/L)	4.62 ± 3.14	3.92 ± 1.32	26.41 ± 5.73 *
S. AST (U/L)	6.89 ± 3.74	7.96 ± 3.2	21.84 ± 4.12 *
S. ALP (U/L)	97.00 ± 29	113.20 ± 10.21	250.6 ± 15.27 *
S. Alb (g/dl)	4.1 ± 1.16	4.2 ± 0.23	4.0 ± 1.26

The values are expressed as means ± SE,

*Significantly different from control, P<0.05

FSG: fasting serum glucose; TBil: Total bilirubin; AST: aspartate transaminase;

ALT: alanine transaminase; ALP: alkaline phosphatase; S.Alb: serum albumin.

consistent with previous works conducted on rats with streptozocine-induced diabetes which were treated with metformin [37, 38]. Metformin treatment also reduced brain AChE activity in rats with D-galactose-induced aging [39]; similar findings were reported in glibenclamide-treated patients [40].

Conversely, metformin did not improve the AChE activity excess in scopolamine-elicited memory deficit rat model [41] and diabetic rat model [42]; additionally, Bradamante *et al.* [43] reported that BChE activity in non-diabetic rats increases after glibenclamide treatment. Thus, literature shows conflicting findings in this respect.

The enzymes AChE and BChE are well known to play a pivotal role in the etiology of AD [36]; in addition to its role in pathogenesis of AD, by reducing insulin sensitivity, BChE may contribute to the etiological picture of T2DM; thus, insulin sensitivity links both morbidities [44]. The antidiabetic drugs used in the current study, *via* normalizing ChE status, may have contributed therefore to improving neurological outcome. The current study findings showed that the NAFLD group is benefited in this respect. Current study patients were maintained on MET-SU combination, and no similar study could be located for comparison; thus, current results should be cautiously weighed against existing evidence.

The current study reported fasting glucose level significantly in excess in study groups (1, 2) versus their values in control group. Previous work have shown MET-SU combination to effectively reduce FSG [45], while a related work found combined

MET-SU to lag in producing beneficial effect in this respect [46], in line with current findings, which probably suggest poor glycemic control in diabetic patients, or non-compliance of patients with general recommended guidelines for managing DM.

As can be seen in Table 2, transaminases were non-significantly different between group 1 and control but significantly in excess in group 2 versus control. Elevated transaminases points to hepatic necrotic-inflammatory changes and their level is a hepatic function outcome index. Although designated as tests for liver function, they actually assess degree of hepatocytic damage [47]. They can also be altered in conditions other than liver dysfunction, and they maybe an inaccurate marker of NAFLD in otherwise histologically evident disease [20]; thus, in this regard, they show minor diagnostic utility and relevance. Additionally, increased transaminase levels that fluctuate within and beyond normal values are typically seen in those with fatty liver [25]. Accordingly, current findings do suggest greater hepatic injury in group 2, compared to the other groups.

Evidence points toward an improvement in liver function in terms of these enzymes associated with antidiabetic use. In previous studies, metformin decreased ALT and AST, and improved liver histology in NAFLD patients with T2DM [48-53]. ALT and AST improved significantly in the metformin-treated group in comparison to controls in another study, with benefit linked to improved insulin resistance (IR) [54]. Prolonged therapy with metformin was associated with

significant decline in transaminases in NASH as per studies in humans [55]. Several studies demonstrated a favorable influence of metformin on transaminase levels in NAFLD [53, 56, 22].

Conversely, lack of substantial benefit was reported in the literature as well. Rebound increase in ALT was reported after initial decline in one study [48]. Metformin was found in a meta-analysis to improve weight and IR, but not liver histological picture compared to placebo, in NAFLD patients [57]. Similarly, ultrasonographic and histologic features of the liver, and ALT and AST levels were not improved by metformin in NAFLD in another study [58]; ALT was slightly improved in metformin-treated patients [25]. These findings coincide with current study findings in group 2. The controversy in literature could stem from different population background characteristics and heterogenous study design schemes. Biochemical liver profile, in terms of ALT and AST levels, was not improved in the present work, probably due to improvement of IR running parallel to liver function status; additionally, case control study design may preclude any clear beneficial effects of MET-SU compared to earlier studies adopting different designs.

Total ALP values were higher in group 2 than control, in the current study. While ALP is derived from bone and liver mainly (minor contribution from intestine), only hepatic form increases in the serum when there is underlying liver problem [59]. Consistent with this, a previous study [60] found significantly greater liver ALP but not bone ALP in diabetic subjects against control group; thus, it can be presumed that the rise in ALP values in the current study may be attributed to the liver isozyme. Group 1 showed insignificant difference vs. control in the present study. Metformin is presumed to have hepatoprotective capacity, and significantly lower levels of ALP were reported in metformin-treated fatty liver mice model in one study [61]. Altered ALP levels occur in patients with renal/bone disorders, which commonly occur in DM patients [62]. When combining SUs with metformin, stronger impact on reducing ALP was reported, as they in effect show a hepato-supportive effect on liver especially in those with liver disease [25]; current study findings did not reflect improved ALP in group 2, thus suggesting

co-existing bone/liver morbidity which may contribute to the clinical picture and diminish any positive impact the antidiabetic drugs (MET-SU) may have.

No significant differences between the 3 groups in terms of total bilirubin (TBil) were found in the present study. Total bilirubin and T2DM appear to be causally linked [63]. Additionally, bilirubin presumably has cytoprotective effects, as its levels correlate with diminished risk for developing NAFLD [64], and thus is proposed as a protective biomarker for NAFLD [65], probably due to antioxidant potential of bilirubin. Previous work had shown bilirubin effects on NAFLD to be repressive [66]. Low levels of bilirubin are often seen in diabetic patients and they usually predispose to many complications of DM; thus, efforts to artificially boost its levels were studied extensively [67, 68]. However, the present study did not report improved TBil; this underlies the risk these patients may be exposed to, probably reflecting poor DM control as indicated by high FSG levels, requiring further up-titration of drug doses.

Glibenclamide, a second-generation sulphonylurea (SU), was administered in the current study combined with metformin (MET-SU). This combination is one of the most commonly used in clinical practice [69]. Sulphonylureas are commonly employed in the management of T2DM and they currently hold second-line status, being used when metformin fails [70]. The combination is efficient as it tackles both deficiency and resistance of insulin [71].

The SU class appears to have negative impact. Retrospective studies indicate that fibrosis in diabetic patients with NAFLD is more prevalent in those treated with SUs; this, however, was attributed to insulin [72]. Risk of progressive NAFLD is higher with insulin secretagogues, and patients treated with sulphonylureas showed poor glycemic control and longer disease interval [73]. Moderate decline in liver function tests (LFTs) when gliclazide alone or in combination was administered was reported in another study [74], in line with present study group 2 findings, where SU is taken by patients. In the present work, group 2 exhibited deteriorated LFT as compared to both control and group 1; as DM itself is a risk factor for NAFLD, it may be concluded that this group may have had

a poorer glycemic control, compared to group 1, which may contribute to the findings, in addition to the role of SU in the combination taken by patients.

Actually, previous data are in favor of using metformin in T2DM-NAFLD patients, while some reported SUs are associated with hepatic damage preceding NAFLD [73]. No prospective studies on SU use in NAFLD diabetic patients, combined with metformin, are available [72], and hence the current report sheds some light in this respect.

The present study had some limitations. Due to the case-control design of the present study, no causal relationships can be inferred. Additionally, the sample size was relatively small. In view of absence of data on duration of T2DM, and dose and duration of the drugs used in the study, it will be difficult to draw firm conclusions on actual disease/drug impact on studied parameters. Furthermore, information on baseline IR, which is linked to T2DM and NAFLD, was not available.

CONCLUSION

In conclusion, the current study supports the hypothesis that therapy with MET-SU combination in diabetic patients with NAFLD is associated with positive impact on AChE and BChE activities. Diabetic patients with NAFLD who are treated with a MET-SU combination showed a better cholinesterase enzyme profile but less favorable liver function panel, compared to other groups. These findings suggest a potential neuroprotective effect of the drugs in diabetic-NAFLD patients as indicated by lowered cholinesterase activities, which in light of possible risk on cognitive function in diabetic patients may prove beneficial in the long run. Conversely, an apparent worsening of liver status was also noted, in terms of biochemical parameters of liver function, which could be attributed to the secretagogue in the combination. Further studies, set prospectively, to explore causal effects regarding combined metformin and SUs are warranted in the future.

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CONFLICT OF INTEREST STATEMENT

The authors declare that no conflicts of interest exist.

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