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ANTI-DIABETIC AND OXIDATIVE STRESS MARKERS IN MEN WITH TYPE 2 DIABETES MELLITUS

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ABSTRACT: Background: The aim of this study is to evaluate metformin and insulin effects on metabolic disorders and oxydatif stress markers, in Algerian men with T2D, in order to recommend the best treatment, which can minimize diabetes complications and recommend/or not a combination between metformin and insulin. Patients and **Methods:** We made this study on 120 subjects men divided into four groups (30 healthy control, 30 T2D without treatment, 30 T2D with metformin, and 30 T2D with insulin). Blood samples are collected for the determination of biochemical parameters (glucose, triglycerides, and cholesterol, high and low-density lipoprotein cholesterol) and oxidative markers (superoxide anion, nitric oxide, malondialdehyde, carbonyl proteins, oxygen radical absorbance capacity, vitamin C, catalase, glutathione). **Results:** Compared with healthy subjects, diabetic patients had altered lipid levels (cholesterol, triglycerides, LDL cholesterol) and high levels of pro-oxidant intra-cellular markers (O2•-, CP and MDA) associated to low concentrations of anti-oxidants (Vitamin C, catalase, and GSH). Our results show that insulin reduces more lipid parameters than metformin, moreover, the oxidant/antioxidant status became normal in patients treated with metformin **Conclusion:** Insulin treatment is more efficient than metformin treatment in improving the lipid profile. In addition, metformin, which reversed redox changes associated with diabetes and insulin, which improve all lipid profiles, should be prescribed in combination, especially in type 2 diabetes patients with hypertriglyceridemia and with severe oxidative stress.

INTRODUCTION: Several lipid disorders have been observed in diabetics and play a key role in the incidence of cardiovascular morbidity and mortality [1-5].

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The main lipid anomalies are quantitative and qualitative. The quantitative abnormalities, are hypertriglyceridemia and low high-density lipoprotein (HDL) cholesterol, and the qualitative abnormalities mainly include large sizes very-low-density lipoprotein (VLDL), enrichment of low-density lipoprotein (LDL) and HDL in triglycerides, LDL oxidation, and glycation of apolipoproteins [6]. Additionally, protein glycation (PG) or non-enzymatic glycosylation is one of the consequences of hyperglycemia [7, 8, 9].

The glycation process affects circulating proteins [10]. PG can be accompanied by an oxidation process, defined as an unfavorable balance, between oxygen free radicals (FR) and antioxidant systems profit first [11, 12, 13, 14]. FR are chemical species atoms derived of oxygen (reactive oxygen species (ROS)). The presence of a single electron in a FR gives them reactivity, which can damage several molecules [15, 16, 17, 18]. However, in T2D, the oxydat if stress (OS) acts as a mediator of IR and its progression to glucose intolerance and the pathology installation [10]. In conditions of severe OS, cell damage occurs with decreased pancreatic ß-cell function, which is due to low expression of antioxidant enzymes [19]. The oxygen radical absorbance capacity (ORAC) has been found to be a good index of oxidative stress in diabetes mellitus [20]. Clinical studies have shown that specific antioxidant concentrations in plasma and erythrocytes of diabetes patients are reduced [21]. Indeed, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) activities scavenging, and ROS, were decreased in patients with T2D [22].

Otherwise, the principal markers of lipid peroxidation (LP) are substances, which react with malondialdehyde / thiobarbituric acid, conjugated dienes, lipid hydroperoxides, and isoprostanes [23, 24]. However, the two main biological markers of protein oxidation are the formation of protein carbonyls and nitrotyrosines groups [25]. Several studies have demonstrated increased values of LP products [26], and high levels of carbonyls and AGEs in T2D plasma [27]. In T2D, metformin is often the first glucose-lowering treatment by its effect on IR [28]. Metformin decreases hepatic glucose output, lowers fasting glycemia, increases glucose uptake in peripheral tissues, reduces insulin resistance (IR) 29, 30, and decreases high blood sugar, by suppressing liver glucose production [31]. Moreover, a number of studies have established the favorable effect of metformin on body mass index (BMI) and body mass composition through the reduction of fat mass. In addition, it reduces hyperinsulinemia, lipid parameters, arterial hypertension, and endothelial dysfunction [32]. Metformin has been demonstrated to have a role in preventing the conversion of impaired glucose tolerance (IGT) to type 2 diabetes mellitus (T2DM) and has a beneficial effect on the blood lipid profile [33. 34]. In addition, intensified treatment with insulin eventually becomes necessary to maintain acceptable glycemic control in most patients with T2D [35], although this intervention has not been proven to reduce the risk of cardiovascular disease [36, 37, 38]. Other studies showed a reduction in cardiovascular risk during the first 18 months of treatment with insulin alone, despite a significant increase in total and LDL cholesterol levels and body weight [39]. In Patients treated with insulin, fasting plasma glucose and glycated hemoglobin (HbA1c) levels were reduced but weight increased [39]. The aim of this study is to compare metformin to insulin effects on metabolic disorders and OS markers in Algerian men with T2D, in order to select the best treatment which can minimize diabetes complications and recommend/or not a combination between metformin and insulin.

MATERIALS AND METHODS:

Participants: Between January 2018 and June 2019, 120 men were recruited from the diabetic clinic of Tlemcen (Algeria), with primary criteria including non-obese (BMI< 30 kg/m^2), age range between 50 and 60 years, not taking any medication, and not having a chronic disease. The subjects were divided into four groups: group I consisted of control health, group II consisted of control diabetic men without treatment, group III consisted of type II diabetic men treated with metformin, group IV consisted of type II diabetic treated with insulin. All men were non-smokers. None of subject's history of HTA, liver or renal diseases, or a history of cardiovascular diseases. Information concerning age, BMI, duration of treatment, and blood pressure were collected by questionnaire. Participation in this study was voluntary, and all subjects gave their written, informed consent. The ethical committee of the Tlemcen-University Hospital (number 01 MDU 531) approved the study.

Blood Collection: Fasting venous blood samples were collected in two tubes; EDTA tubes and dry tubes were centrifuged. Serum was separated for glucose, lipid parameters. Plasma was separated for oxidant/ antioxidant determinations. Superoxide anion and vitamin C were measured in fresh plasma samples**.** The remaining erythrocytes were washed, hemolyzed by the addition of cold distilled water (1/4), and the cell debris was removed by

centrifugation (2000g for 15 min). The hemolysates were assayed for antioxidant enzyme activities and GSH contents.

Biochemical Analysis: Serum glucose, triglycerides, and cholesterol contents were determined by enzymatic methods (Kits Sigma Chemical Company, St Louis, MO, USA).

Lipoprotein Isolation: Total lipoproteins were isolated from plasma by precipitation according to the method of Burstein *et al.* [40]. Lipoprotein (LDL and HDL) triglyceride and total cholesterol contents were determined by enzymatic methods (Kits from Sigma).

Oxidant/antioxidant Marker Determination: Scavenging capacity of plasma: The oxygen radical absorbance capacity of plasma (ORAC) employs the oxidative loss of the intrinsic fluorescence of allophycocyanin (APC) [20].

Determination of Plasma Level Vitamin C: Plasma vitamin C levels were determined in plasma by using the method of Roe and Kuether [41].

Determinations of Erythrocyte Antioxidant Enzyme Activity: Determination of Erythrocyte catalase (EC 1.11.1.6) activity was measured by spectrophotometric analysis of the rate of hydrogen peroxide decomposition, at 240 nm (Sigma Aldrich kit). Erythrocyte-reduced glutathione (GSH) levels were assayed by a colorimetric method, according to a Sigma Aldrich kit (Saint Louis, USA).

Determination of Superoxide Anion: The spectrophotometric determination of the O_2•- was based on the reduction of nitrobluetetrazolium (NBT) in the presence of superoxide anion (O_2•-), a chromophor that absorbs at 550 nm [42].

Determination of Nitric Oxide NO: Plasma and erythrocyte NO was determined by the method of Guevara *et al.,* [43] after deproteinization, using the colorimetric method of Griess.

Determination of Malondialdehyde: Plasma-malondialdehyde (MDA) levels were determined by the reaction of MDA with thiobarbituric acid (Sigma Aldrich kit; St. Louis, MO, USA).

Determination of Carbonyl Proteins: Plasma carbonyl proteins were determined by the derivatization of protein carbonyl groups, with 2, 4-dinitrophenylhydrazine, leading to the formation of stable dinitrophenylhydrazone adducts (Sigma Aldrich kit).

Statistical Analysis: The results are presented as means and Standard deviations, a priori power analysis, was performed to determine the sample size, using power and sample size calculator (Statistical solutions, Sigma). The results were tested for normal distribution using the Shapiro-Wilk test. The comparison of means between the four groups is performed by ANOVA one factor. This analysis is completed by the Tukey's test to locate the source of significant difference. All tests were performed using STATISTICA 4.1 program (StatSoft, Tulsa, OK).

RESULTS:

Clinical and Biochemical Parameters: Table 1 shows that BMI was significantly higher in T2D without treatment and T2D treated with insulin and metformin compared with control subjects. No significant difference was found between T2D with metformin and T2D with insulin for BMI ($P>0.05$). Blood pressure (systolic, SBP and diastolic, DBP) did not differ significantly among the four groups. Additionaly, significant differences were found between T2D with metformin, T2D with insulin compared to control subjects and T2D without treatment for plasma glucose levels **Table 2**. The highest glucose concentrations were apparent in T2D without treatment (ANOVA, $P<0.001$).

Lipid and Lipoprotein Levels: T2D Without treatment and T2D with metformin patients demonstrated significantly higher plasma levels of total cholesterol, compared with their control and T2D with the insulin treatment group. The highest values were observed in T2D without treatment **Table 2** ($P<0.001$, $P>0.05$, $P<0.01$, respectively). Triglycerides levels were higher in subjects with untreated T2D compared to the control and treated T2D. Metformin and insulin reduce triglyceride concentration in T2D men. A greater reduction was observed with insulin treatment ($P<0.05$).

Lipoprotein concentrations were markedly different among the four groups studied (Table 2) LDL-C amounts were significantly higher in T2D without treatment compared to T2D treated with metformin or insulin compared to control. LDL-C concen-

trations were significantly higher in T2D with metformin compared to T2D with insulin. HDL-C concentrations were significantly higher in T2D with metformin and T2D with insulin compared to control subjects and T2D without treatment. No significant difference between T2D with metformin and T2D with insulin for HDL-C. The report LDL-C/HDL-C levels were significantly lower in T2D with metformin and T2D with insulin than controls group and T2D without treatment (P<0.001), but the differences did not reach statistical significance between T2D with metformin and T2D with insulin (P > 0.05).

TABLE 1: CHARACTERISTICS OF THE STUDY POPULATION

Characteristic	Control	T2D without treatment	T2D with metformin	T2D with insulin	ANOVA
Number (n)	30	30	30	30	
Age (years)	53 ± 3	52 ± 2	54 ± 3	56 ± 4	
BMI (Kg/m²)	22,49±1,23^{c}	27,06±1,89^{a}	25,83±1,85^{b}	25,94±1,60^{b}	0.0001
Duration of treatment	-	-	7±3	5±1	
SBP (mm Hg)	124±5.25	130±8.25	127±7.11	120.43±5.26	0.020
DBP (mm Hg)	76.53±4.55	84.35±5.79	80.43±5.38	86.32±5.12	0.020

Values are means ± SD. BMI, body mass index; DBP, diastolic blood pressure; SBP, systolic blood pressure. Statistical comparison between the four groups (Control, T2D without treatment, T2D treated with metformin and T2D treated with insulin) was performed by one-way ANOVA test, followed by Tukey post hoc test. Values for each parameter with different superscripts (a,b,c,d) are significantly different for P < 0.05, as determined by one-way ANOVA and the least significance test. P values for ANOVA test.

TABLE 2: BIOCHEMICAL PARAMETERS OF THE STUDY POPULATION

Parameter	Control	T2D without treatment	T2D with metformin	T2D with insulin	ANOVA
Glucose (mmol/L)	4,98±0,27^{d}	7,27±0.49^{a}	6,52±0,54^{b}	5,96±0,24^{c}	0.0001
TG (mmol/L)	1,45±0,09^{c}	1,85±0,22^{a}	1,66±0,15^{b}	1,56±0,17^{b}	0.0400
TC (mmol/L)	4,67±0,31^{b}	5,53±0,22^{a}	5,45±0,50^{a}	4,72±0,46^{b}	0.0001
LDL-C (mmol/L)	2,72±0,44^{b}	3,72±0,20^{a}	2,28±0,34^{c}	1,85±0,45^{d}	0.0001
HDL-C (mmol/L)	1,58±0,25^{a}	1,12±0,10^{c}	2,32±0,26^{b}	2,44±0,24^{b}	0.0001
LDL-C/HDL-C	1,81±0,48^{b}	3.37±0,39^{a}	0,99±0,23^{c}	0,81±0,12^{c}	0.0001

Values are means ± SD. TG, triglycerides; TC, total cholesterol; LDL-C, LDL cholesterol; HDL-C: HDL cholesterol. Statistical comparison between the four groups (Control, T2D without treatment, T2D treated with metformin and T2D treated with insulin) was performed by one-way ANOVA test followed by Tukey post hoc test. Values for each parameter with different superscripts (a, b, c, d) are significantly different for P < 0.05, as determined by one-way ANOVA and the least significance test. P values for ANOVA test.

Oxidative Stress Biomarkers:

Oxidant Status: For erythrocyte O_2•-, statistical study demonstrated a significant difference among the four groups. The levels of O_2•- were significantly higher in T2D with metformin and T2D with insulin compared to T2D without treatment and controls (P<0.001) **Table 3**. The highest level of O_2•- was observed in T2D with insulin. A significant difference between T2D with metformin and T2D with insulin was found for Erythrocyte NO. The highest NO levels were observed in T2D treated with insulin compared to T2D without treatment and controls. However, no significant difference of NO was observed between T2D with Metformin and T2D without treatment **Table 3**. Erythrocyte MDA levels were higher in all diabetic patients, treated with metformin or insulin and not treated compared with control men, but T2D without treatment men's values were the highest (ANOVA P<0.001 and P <0.001, respectively) **Table 3**. Similarly, erythrocyte carbonyls protein level was enhanced in T2D without treatment and T2D treated with metformin or with insulin compared to control cases (P<0.0006) and significantly decreased with the two treatments compared to T2D without treatment.

TABLE 3: OXIDANT INTRACELLULAR MARKERS IN THE STUDY POPULATION

Parameter	Control	T2D without treatment	T2D with metformin	T2D with insulin	ANOVA
O2 (µmol/L)	50,91±2,01^{d}	56,17±2,16^{c}	76,27±5,53^{b}	84,10±3,62^{a}	0.0001
NO (µmolL)	38,16±3,06^{b}	34,82±4.41^{c}	35,42±2,92^{c}	48,93±6,05^{a}	0.0001
MDA (µmol/L Lyat)	2,11±0,27^{c}	6.17±0,26 ^{a}	3,35±0,45^{b}	4,02±0.33^{b}	0.0001
CP (nmol/mg protein)	2,7±0,33^{c}	6,15±0,29^{a}	3,98±0,40^{b}	3,82±0,38^{b}	0.0006

Values are means ± SD. $O2^{-}$, superoxide anion; NO, nitricoxide; MDA, malondialdehyde; CP, carbonyl proteins. Statistical comparison between the four groups (Control, T2D without treatment, T2D treated with metformin and T2D treated with insulin) was performed by one-way ANOVA test followed by Tukey post hoc test. Values for each parameter with different superscripts (a, b, c, d) are significantly different for P < 0.05, as determined by one-way ANOVA and the least significance test. P values for ANOVA test.

Antioxidant Status: Oxidative stress biomarkers Plasma total antioxidant status (ORAC) was significantly lower in T2D without treatment compared to controls **Table 4**. Values tended to increase among the two-treated group, but there is no significant difference between the group treated with metformin and insulin. Vit C levels were significantly lower in T2D without treatment compared to the control and treated groups, but its levels were significantly higher in T2D treated with metformin compared to T2D treated with insulin (P<0.001) **Table 4**. Erythrocyte catalase activities were markedly different among the group studied, it was significantly lower in T2D without treatment and T2D treated with metformin and insulin compared to control subject **Table 4**. Diabetes medication induced a rise in catalase activities but not reaching control values after treatment with insulin and metformin. Erythrocyte GSH values were significantly lower in T2D without treatment compared to control subjects and T2D treated with metformin and T2D with insulin (P<0.001) Table **4**. However, no significant difference in erythrocyte GSH activities was observed between controls cases and in both T2D treated patients.

TABLE 4: ANTIOXIDANT MARKERS IN THE STUDY POPULATION

Parameter	Control	T2D without treatment	T2D with metformin	T2D with insulin	ANOVA
ORAC (UI)	4,54±0,26[a]	1.58±0,25[c]	2,55±0,27[b]	2,35±0,38[b]	0.0001
Vit C (µmol/L)	47,48±2,25[a]	27,92±1.28[c]	45,88±5,87[a]	35,36±3,12[b]	0.0001
Catalase (UI/min/ml)	88,73±2,57[a]	49,21±1,62[c]	69,60±5,92[b]	67,58±4,86[b]	0.0001
GSH (mmol/L)	1,55±0,06[a]	0,59±0,14[b]	1,51±0,08[a]	1,54±0,06[a]	0.0010

Values are means ± SD. ORAC, oxygen radical absorbance capacity which represents total plasma antioxidant capacity; Vit C, plasma vitamin C, erythrocyte catalase levels; GSH, erythrocyte reduced glutathione levels. Statistical comparison between the four groups (Control, T2D without treatment, T2D treated with metformin and T2D treated with insulin) was performed by one-way ANOVA test followed by Tukey post hoc test. Values for each parameter with different superscripts (a, b, c, d) are significantly different for P < 0.05, as determined by one-way ANOVA and the least significance test. P values for ANOVA test.

DISCUSSION: Diabetes may induce multiple changes in lipids, lipoproteins, and oxidant / antioxidant status. The results obtained from the present study are found to be consistent with previous findings [44, 45, 46]. Indeed, our results indicate that metformin and insulin are efficacious substances in treating diabetes because there is a significant decrease in plasma glucose levels in T2D patients treated with metformin and insulin as compared to untreated diabetics. The effect of metformin on blood glucose is attributed to decreased hepatic glucose production and increased glucose transport in muscle cells [47].

The lipid levels are altered, and the content in lipoproteins is significantly modified in diabetic patients compared to control subjects. The diabetic patients presented high levels of plasma triglycerides, cholesterol, and LDL-C but low levels of HDL-C. These findings are consistent with those reported in previous studies [48-51]. In addition, an elevated atherogenicity ratio (LDL-C/HDL-C) was observed in T2D patients. These abnormalities could be attributed to hyperglycemia, insulin resistance, lipase, and CETP activities [51-53]. Our findings showed that T2D patients presented high levels of pro-oxidant intra-cellular markers (O2•-, CP and MDA) and low concentrations of anti-oxidants (Vitamin C, catalase, and GSH). These results are consistent with those reported in previous studies [54]. The ORAC values were significantly lower in untreated T2D patients compared with nondiabetic control subjects. In diabetes, several mechanisms seem to be involved in the genesis of oxidative stress. Indeed, glucose auto-oxidation in the presence of iron causes the generation of reactive oxygen species (ROS) [55, 56], and leads to protein glycation, overproduction of superoxide radicals at the mitochondrial and NADPH oxidase levels [57-62]. Reduced (NO) levels were observed in (T2D) patients, which is in agreement with the results reported in a previous study [57, 63]. Overproduction of (NO) is associated with various inflammatory conditions, including diabetes [64-68]. The results of this study show a decrease in plasma triglyceride and LDL-C levels and an increase in HDL-C levels. Metformin and insulin therapies allow normalizing the lipid and lipoprotein levels in (T2D). However, the total cholesterol concen-trations can be normalized by insulin only. In another study, it was indicated that metformin could play a major role in lowering blood cholesterol [69, 70].

In this study, it is shown that patients with T2D who were prescribed metformin presented a reduction in LDL cholesterol, unlike untreated T2D patients, which is in agreement with findings reported in previous studies [71, 72]. Moreover, metformin and insulin also help to lower the atherogenicity ratio (LDL-C/HDL-C) in (T2D) patients, unlike untreated patients. The greatest reduction in this ratio was observed in patients treated with insulin. This helps to reduce major cardiovascular risk factors. Several studies have shown that elevated triglyceride and blood sugar levels contribute to higher (OS) [73]. The findings from the present investigation confirm a strong association between glucose plasma concentrations and (OS) parameters. Oxidative stress in diabetes is generally caused by a diminution in the antioxidant defense system and an elevation in ROS production due to hyperglycemia [74]. In addition, chronic hyper-glycemia induces an increase in protein oxidation in T2D patients [75].

The results from the present study indicate that T2D patients treated with insulin present higher levels of some pro-oxidant markers (NO, O_2•-) as compared to T2D patients treated with metformin. Moreover, significant differences were found between metformin and insulin-treated groups with regard to erythrocytes and NO. Furthermore, low levels of MDA and CP were observed in T2D patients treated with both insulin and metformin, unlike untreated diabetic patients, these findings are in good agreement with those reported in previous studies [76].

The improved control of glycemia, observed in T2D patients treated with insulin and metformin, can explain the diminution in the production of radicals and the decrease in lipid peroxidation. In our study, markers of protein oxidation, such as carbonyls, showed a significant decrease in protein oxidation in diabetic patients treated with insulin and metformin, unlike untreated patients. This decrease is attributed to low blood glucose levels in diabetic individuals treated with insulin and metformin. This is consistent with the findings of a study, which indicated that appropriate glycemic control lowers plasma levels of carbonyls in T2D patients [75]. Moreover, the results of the present study indicate a significant decrease in erythrocytes, superoxide anions, and nitric oxides (NO) in metformin-treated T2D patients as compared to those treated with insulin. Therefore, the decrease in (NO) production in diabetic patients, is at the origin of the alteration of the endothelial vasodilatation [77]. On the other hand, (NO) levels are higher in insulin-treated T2D patients as compared to those treated with metformin and those not treated. This is probably due to improved glycemic control, which induces the reduction in the (OS). The present study is an attempt to measure some antioxidant defense markers, vitamin C level, catalase activity, (OS) biomarkers, plasma total antioxidant (ORAC), as well as glutathione concentration. The levels of antioxidants, vitamin C, catalase, and GSH were found higher after treatment. The (ORAC) was significantly lower in untreated T2D patients as compared to control patients, but it was found unchanged in patients treated with metformin and insulin.

The results from this study indicated higher plasma levels of vitamin C in T2D patients treated with metformin as compared to those treated with insulin. The increase in vitamin C levels can be explained by the rise in the concentration of glutathione, which is an essential factor for the enzymatic regeneration of ascorbic acid from dehydroascorbate [78].

The results obtained revealed a significant increase in erythrocyte catalase activity in treated diabetes patients as compared to untreated subjects. These results are consistent with those of several other authors who reported a decrease in the erythrocyte catalase activity of diabetic patients [20].

Glutathione is the main soluble antioxidant in cells [79, 80]. The results of this study indicated a significant difference in the erythrocyte GSH levels between treated diabetic patients and untreated ones, in favor of improved antioxidant defense. Our results highlight the beneficial effects of metformin and insulin on lipid and lipoprotein profiles in T2D patients. Favorable changes in lipid and lipoprotein parameters were observed in T2D patients treated with insulin, moreover, it was noted a decrease in plasmatotal LDL cholesterol and triglyceride levels and an increase in HDL-C levels. These treatments normalize lipid and lipoprotein levels in diabetic patients.

The present work found that after therapy with metformin and insulin, plasma MDA and carbonyl protein levels significantly decreased, whereas vitamin C, GSH levels, and catalase activities remarkably increased. However, the results obtained suggest that insulin reduces more total and LDL cholesterol than metformin does; it also increases the production of NO and O-2.

In conclusion, the present study suggests that glycemia reduction in T2D subjects leads to a definite reduction in ROS generation. Metformin and insulin present beneficial effects on plasma lipids or oxidative stress markers in diabetic patients, with preexisting plasma lipid and redox abnormalities. In addition, insulin improves the lipid profile, and metformin reverses the redox changes associated with diabetes. It may be concluded that the combination of metformin and insulin may help to correct the oxidative status and lipid disorders observed in diabetic patients.

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CONFLICTS OF INTEREST: The authors have no relevant conflict of interest to enclose.

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