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Research paper

Potential effects of metformin in DNA BER system based on oxidative status in type 2 diabetes

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ABSTRACT

Metformin is used to reduce hyperglycemia that induces energetic stress and leads to reduction in gluconeogenesis. Also, metformin inhibits complex I in oxidative phosphorylation, thereby decreasing cellular ATP levels. Activation of AMPK by the reduced ATP levels can induce inhibition of reactive oxygen species (ROS) production and activate p53-mediated DNA repair.

DNA polymerase-β and XRCC1 function to repair DNA damages in the BER (base excision repair) system. In type 2 diabetes patients, metformin can enhance AMPK activation therefore suppress oxidative stress. The changes on oxidative stress may alter p53's function and effect many cellular pathways such as; DNA repair. In our project we aim to understand the effects of metformin on p53 and DNA-BER system based on the oxidative status in type 2 diabetes patients.

Oxidative and antioxidative capacity, catalase, SOD, GPx activities and, DNA pol beta, XRCC1 and p53 levels were measured in metformin using or non-using type 2 diabetes patients and controls.

Metformin enhanced SOD and GPx activities in type 2 diabetes patients but the reflection of this increase to the total antioxidant capacity was not significant. Although the increase in DNA pol beta was not significant, XRCC1 and p53 levels were significantly upregulated with metformin treatment in type 2 diabetes patients. Our study reinforces the potential benefit of metformin in antioxidative capacity to protect cells from diabetic oxidative stress and in regulation of DNA BER system.

1. Introduction

Diabetes Mellitus is a multifactorial metabolic condition characterized by impaired insulin action and secretion that leads to disordered levels of serum glucose. The genetic components of this disease still need to be clarified for the prevention and pharmalogical intervention [1]. More than 90% of the diabetes patients are diagnosed with type 2 diabetes and the prevalence for type 2 diabetes is rapidly increasing on a global scale [2]. Metformin is the first-line oral glucose-lowering drug to control hyperglycemia in type 2 diabetes. It is reliable, cost effective, has broad spectrum of pleiotropic effects and good tolerability by patients. Metformin reduces glucose levels by suppression of hepatic glucose production and improves blood glucose control by enhancing insulin-stimulated glucose disposal in the peripheral tissues thus, sensitizing the tissues for insulin [3]. Also, epidemiological studies indicate that the incidence of cancer is reduced in type 2 diabetes treated with metformin [4]. These effects are mostly mediated by the activation of Liver kinase B1 (LKB-1), which directly phosphorylates and activates AMP-activated protein kinase (AMPK), a central metabolic sensor [5,6]. AMPK is a metabolic switch, which is activated when intracellular ATP levels are low and ADP levels are high. It also regulates lipid and glucose metabolism in several tissues by controlling mTOR (mammalian target of rapamycin) pathway, which controls the translation of a number of cell metabolism regulators [7].

AMPK has been shown to directly phosphorylate p53 on serine

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15 and this phosphorylation initiates AMPK-dependent cell-cycle arrest [8]. p53, the guardian of the genome, regulates DNA repair, senescence, apoptosis, differentiation as well as cellular responses to oxidative stresses [9]. Therefore, p53 is a highly important control point for all cellular processes. AMPK activation has been shown to reduce ROS levels and induce antioxidant status of vascular endothelial cells [10]. Metformin has antioxidant properties that reduces reactive oxygen species (ROS) by inhibiting mitochondrial oxidative phosphorylation [11].

It has been reported previously diabetes patients showed more oxidative damage to DNA, with increased generation of ROS, than controls [12–14]. Oxidative stress, hypoxia and oncogene activation can induce p53 activation. If the intensity of stress is high, p53 would act as a pro-apoptotic molecule and activate cellular death and senescence pathways. However, p53 would act as an antioxidant molecule and reduce ROS accumulation if stress is low [9] (Fig. 1). Thereby it activates DNA repair gene expressions [15]. In general, it is accepted that ROS levels will be high due to the increased intracellular stress in type 2 diabetes; Metformin would decrease ROS levels in which p53 gain antioxidant activity and stimulate cellular survival by inhibiting DNA damage [16]. In addition, it has been shown that metformin decreased p53 protein abundance depending on oxidative stress depletion [17]. Although metformin decreases oxidative stress formation, decreased oxidative stress inhibits p53 activity, which is an unintended consequence for the safety of cellular processes.

Above different DNA repair mechanisms, it is well determined how an array of DNA repair mechanisms appear to function in various DNA damages [18]. Among the DNA repair mechanisms, DNA base excision repair (BER) system is one of the most efficient repair process for correcting oxidation, alkylation, deamination, depurination/depyrimidination lesions and maintain genomic integrity. The short-patch of BER system requires several repair specific proteins and works in a multi stage model. First, DNA glycosidase recognizes the damaged region and hydrolyses the nitrogen carbon linkage between the base and deoxyribose. The resulting abasic site (AP-site) is processed by an AP-endonuclease APE-1. The gap is filled by DNA polymerase beta and XRCC1 proteins correctly. Finally, DNA ligase catalyzes the phosphodiester bond formation between the last unligated nucleotides [19,20]. It has been shown that p53 activates several DNA repair genes such as APE and DNA polymerase beta [21] [22].

In our study, we investigated how metformin effects the oxidant and antioxidant status, and depending on the oxidative stress how p53 and DNA BER system enzymes changed by metformin treatment in type 2 diabetes patients. It can be estimated that metformin treated patients have lesser oxidative stress and higher BER activity than others. The molecular action of metformin may change oxidative and antioxidative capacity. This change can transform the action of p53 between prooxidant and antioxidant status, which directed us to search its effects on BER DNA repair in type 2 diabetes.

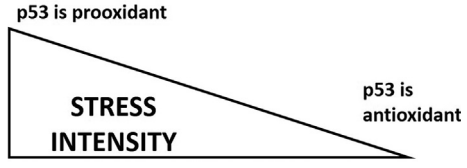

Fig. 1. The responses of p53 to the varying intensity of stress conditions. p53 can have a prooxidant role due to increased stress levels, whereas the decreased or basal stress levels may induce its antioxidant role.

2. Materials and methods

2.1. Study subjects

A total of 57 patients diagnosed with type 2 diabetes and 30 healthy controls matching age and gender proportions were selected from İnternal Medicine Department/Ufuk University. The diagnosis was in line with the diagnostic criteria and classification of diabetes of Turkish Society of Endocrinology and Metabolism. Exclusion criteria were as follows: patients suffering from acute or chronic infection; patients with liver disease; and patients that have concomitant endocrine, metabolic and renal diseases, patients or controls with familial cancer syndrome history, patients using insulin or other oral diabetic agents rather than metformin, female pregnant patients, patients or controls using antioxidant agents. Type 2 diabetes patient group was composed of newly diagnosed patients, who did not get any agent to regulate blood glucose or insulin levels. Metformin using diabetes group was composed of type 2 diabetes patients who receive 1000 mg twice daily metformin for more than 6 months. The control group was composed of individuals with no history of diabetes. The study protocol was approved by the Ethics Committee of Ufuk University School of Medicine, and complied with the guidelines and principles of the Declaration of Helsinki. The written informed consent was taken from all participants.

All the selected subjects were fasted for 8–12 h. About 5 mL of venous blood sample was collected the next morning in an anticoagulant tube at room temperature. The venous blood sample was centrifuged at 3000 rpm for 15 min. The upper supernatant was taken and aliquoted in eppendorf tubes, and frozen at −80 °C for the standby use.

2.2. Oxidative capacity detection

Reactive oxygen species levels were tested using PerOx (TOS/TOC) commercial kit (Immundiagnostik, Bensheim, Germany) by ChemWell 2910 Automated EIA and Chemistry Analyzer (Awareness Technology, FL, USA). The principle of the kit was to determine the total content of lipid peroxides in a given sample. The measurement of the oxidation of tetramethylbenzidine (TMB) into a colored product was evaluated using photometry at 450 nm.

2.3. Antioxidative capacity detection

Antioxidative capacity was tested using ImAnOx (TAS/TAC) commercial kit (Immundiagnostik, Bensheim, Germany) by ChemWell 2910 Automated EIA and Chemistry Analyzer (Awareness Technology, FL, USA). The principle of the kit to determine the antioxidative capacity of a given sample by adding defined amount of H_2O_2, which allows the elimination of a certain amount of the exogenously provided H_2O_2 by antioxidants in the sample The difference between added and remaining H_2O_2 is determined photometrically by an enzymatic reaction which involves the conversion of tetramethylbenzidine to a colored product. After the elimination reaction, the samples were measured at 450 nm.

2.4. Catalase (CAT) activity assay

Catalase activity was tested using Cayman Chemical Catalase Assay kit (Item no: 707002) (Cayman Chemical, USA) which has peroxidatic function. Catalase detoxifies H2O2 by catalyzing it to molecular oxygen and water. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured spectrophotometrically with 4-amino-3-hydrazino-5- mercapto-

1,2,4-trizazole as the chromogen [23]. The absorbance is read at 540 nm by ChemWell 2910 Automated EIA and Chemistry Analyzer (Awareness Technology, FL, USA) and expressed as μmol/min/mg of protein.

2.5. Superoxide dismutase (SOD) activity assay

The total antioxidant SOD enzyme activity was measured by the Chemical Superoxide Dismutase Assay kit (Item no: 706002) (Cayman Chemical, USA). The principle of the kit is based on detection of O_2 radicals which were generated by xanthine oxidase and hypoxanthine to oxygen and water molecules by tetrazolium salt. One unit of SOD is defined as the amount of enzyme needed to obtain 50% dismutation of O_2 radical. The SOD assay measures all the three types of SOD (Cu/Zn, Mn, and FeSOD). The absorbance is read at 440 nm by ChemWell 2910 Automated EIA and Chemistry Analyzer (Awareness Technology, FL, USA) and expressed as U/ml of protein.

2.6. Glutathione peroxidase (GPx) activity assay

Glutathione peroxidase (GPx) antioxidant enzymes have peroxidase activity, which catalyzes the reduction of hydroperoxides, including hydrogen peroxides, by reduced glutathione, thereby protecting the cell against oxidative stress. The Cayman Chemical Glutathione Peroxidase Assay Kit (Item no: 703102) (Cayman Chemical, USA) measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produced upon reduction of an organic hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in absorbance is directly proportional to the GPx activity in the sample. Glutathione peroxidase activity was expressed as nmol/min/ml.

2.7. DNA polymerase beta (DNA pol beta) protein assay

DNA pol beta level was measured by Elabscience DNA polymerase beta kit (Cat no: E-EL-H3240) (Elabscience Biotechnology, MD, USA). The Sandwich-ELISA method involves attachment of capture antibody to a microplate. Standards or samples containing unknown amount of DNA pol beta are added to microplate wells and bound by the capture antibody. In the next step, a biotinylated detection antibody specific for DNA pol beta is added to each micro plate followed by Avidin-Horseradish Peroxidase (HRP) conjugate, and then they were incubated. Afterwards, free components were washed away. The substrate solution is then added to each well. At this stage, only the wells which contain DNA pol beta, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. By adding a sulphuric acid solution, the enzyme-substrate reaction is terminated and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of DNA pol beta. By comparing the OD of the samples to the standard curve, the concentration of DNA pol beta in the samples calculated and this value is expressed as ng/ml.

2.8. XRCC1 (X-ray repair cross-complementing Gene1) protein assay

XRCC1 level was measured by Elabscience XRCC1 kit (Cat no: E-EL-H3326) (Elabscience Biotechnology, MD, USA). This ELISA kit uses Sandwich-ELISA as the method as described above.

2.9. p53 protein assay

p53 level was measured by Elabscience p53 kit (Cat no: E-EL-H0910) (Elabscience Biotechnology, MD, USA). This ELISA kit uses Sandwich-ELISA as the method as described above.

2.10. Statistical analysis

SPSS 20.0 software (SPSS Inc, Chiago, IL, USA) was used for statistical analysis. Comparisons between normally distributed variables were conducted using One Way Anova and non-normally distributed variables using the Mann-Whitney *U* test. $P < 0.05$ was considered statistically significant.

3. Results

In our study, we collected blood samples from 30 type 2 diabetes patients using metformin, 27 type 2 diabetes patients not using metformin and 30 healthy controls. We determined the oxidative damage on those blood samples and we examined the changes in some of the defense mechanisms like SOD, CAT and GPx; additionally we examined the total antioxidant capacity. At the same time, by ELISA method, we determined the p53 activities of the patients and controls using or not using metformin and we checked if the DNA-BER system is suppressed or not, by evaluating XRCC1 and DNA pol beta protein levels.

According to our results, oxidative stress levels were increased both in the patients using or not using metformin considering the control group; but this increase is not statistically significant (Fig. 2a). On the other hand, total antioxidant capacity was

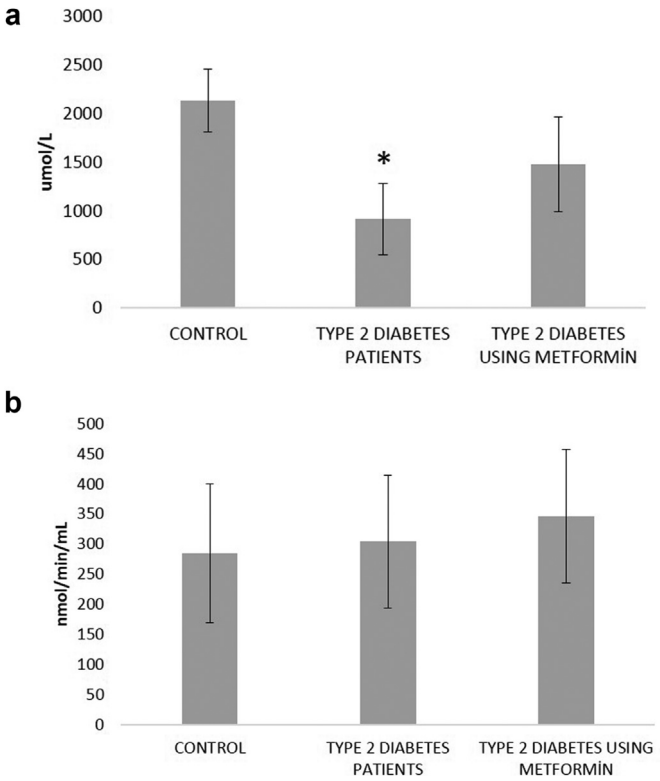

Fig. 2. (a) The changes of ImAnOx capacity in controls, type 2 diabates patients and type 2 diabetes patients using metformin. * = significant difference when compared with control group ($p < 0.05$) (b) The changes of PerOx capacity in controls, type 2 diabates patients and type 2 diabetes patients using metformin. * = significant difference when compared with control group ($p < 0.05$).

significantly decreased in the type 2 diabetes patients, compared with the control group (Fig. 2b).

The antioxidant enzyme activities such as SOD, GPx and CAT vary between groups. SOD was decreased significantly in type 2 diabetes patients. On the other hand, it was increased significantly in the patients using metformin compared to diabetes patients not using metformin (Fig. 3a). GPx was decreased significantly in the patients with diabetes compared to the control group in our experiment. When patients with diabetes not using metformin were compared to patients with diabetes using metformin, it was observed that the GPx activity was increased significantly in metformin using group (Fig. 3b). Although it was not as obvious as controls, the GPx activity was increased with metformin treatment. This indicates that metformin usage increased GPx levels almost up to the control levels. CAT was decreased in the patients with diabetes whether they use metformin or not; but this decrease is not statistically significant. Interestingly, the CAT activity of the patients with diabetes using metformin is less than that of the other groups (Fig. 3c).

DNA-BER mechanism is a DNA repair mechanism which is responsible for the repair of critical damages like oxidation, alkylation, deamination, and depurination/deprimidination; all of which can cause DNA damage. Two of the major proteins in DNA-BER mechanism are XRCC1 and DNA pol beta. The effect of metformin on XRCC1 and DNA pol beta levels which have important roles in DNA-BER system in type 2 diabetes is not known. In our study XRCC1 level was significantly reduced in patients with diabetes compared with the control group, whereas it significantly increased in the metformin using diabetes patients (Fig. 4a). DNA pol beta level is increased in the diabetes patients using or not

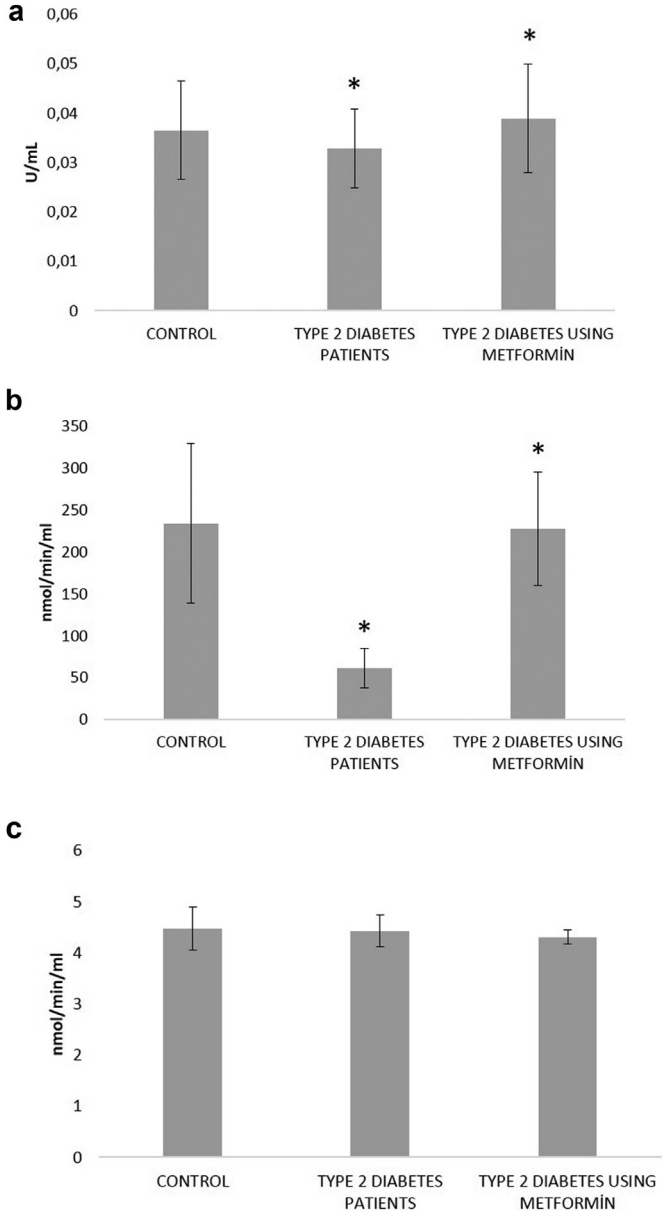

Fig. 3. Comparison between the activity of antioxidant enzymes SOD (a), GPx (b) and CAT (c) in controls, type 2 diabates patients and type 2 diabetes patients using metformin. * = significant difference ($p < 0.05$) between control and type 2 diabetes patients or type 2 diabetes patients using metformin.

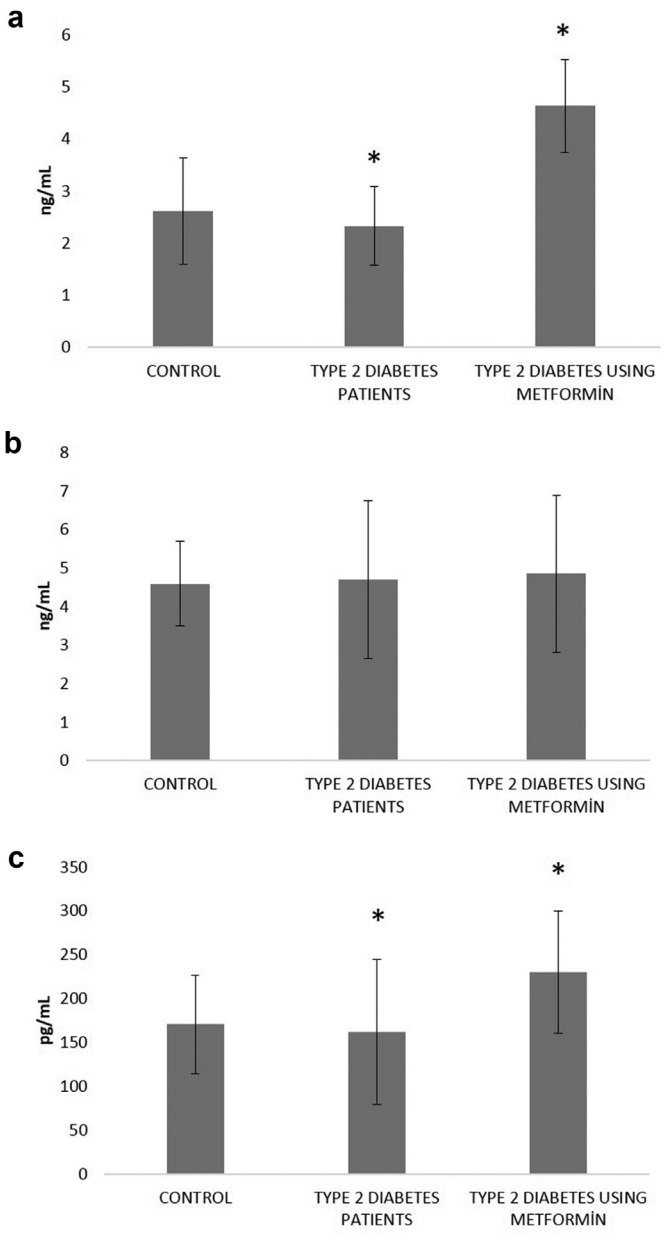

Fig. 4. Comparison between the levels of XRCC1 (a), DNA pol beta (b) and p53 (c) in controls, type 2 diabates patients and type 2 diabetes patients using metformin. * = significant difference when compared with control group ($p < 0.05$).

using metformin, compared with the control group. However, this increase is not statistically significant (Fig. 4b). p53, the multifunctional protein was also investigated in diabetic patients using or not using metformin. While p53 level was decreased significantly in type 2 diabetes patients, it was increased significantly in metformin using cases (Fig. 4c). This may be a consequence of increased antioxidant activity in metformin using diabetic cases.

Our results suggest that although XRCC1 and p53 levels are downregulated in diabetic patients, their expression were upregulated with metformin treatment. Based on these findings, metformin induces DNA-BER system and activates cellular survival as specified with p53's antioxidant role. Furthermore, p53 concentration is decreased with increased oxidative conditions but metformin reverses this reduction and enhances p53 levels in metformin using diabetes patients.

4. Discussion

Mitochondria produce significant amounts of cellular ROS during normal aerobic metabolism [24]. Hyperglycemia in type 2 diabetes stimulates oxidative stress which can be mediated through; NAD(P)H oxidase, xanthine oxidase, monoamine oxidase and electron transport chain. Most of the reactive oxygen types are produced in mitochondria by NADPH oxidase. The inhibition of complex I can minimize the oxidative damage in the cell. Metformin, the most commonly used drug for diabetes, decreases ROS production by inhibiting NADPH oxidase and class 1 respiratory chain enzymes [25] that can be considered as an antioxidant agent.

Oxidative stress is defined as an imbalance between the prooxidants and antioxidants which is characterized as increased reactive oxygen species and/or impaired antioxidant support [26]. Also, oxidative stress plays a key role in type 2 diabetes progression and the pathogenesis for its complications [27].

According to our results, although it is not statistically significant, oxidative stress was tend to increase in diabetic patients. The increase in oxidative stress in metformin using patients may be explained with individual constitutional differences within groups. Also, the type 2 diabetes patients who participated in our study was newly diagnosed diabetes patients. On the other hand, metformin using patients were diagnosed formerly thus they are under oxidative stress conditions for a long while. Also, total antioxidant capacity was significantly decreased in the type 2 diabetes patients, compared with the control group. This result is in line with the results from the similar studies in the literature [28–30]. Total oxidant and antioxidant capacity of the patients using metformin was increased compared to that of the cases not using metformin. However, this increase is not statistically significant. This situation also is in line with the existing studies in the literature [31,32].

Several reactions in biological systems contribute to the steady state concentrations of free radicals. SOD which is one of the cellular antioxidant enzymes, eliminates damaging effects of super oxide under normal physiologic conditions [33]. In our study, SOD was significantly reduced in type 2 diabetes patients compared to the control group. Also, it was significantly increased in type 2 diabetes patients using metformin compared to type 2 diabetes patients. Many studies observed in the literature show similar findings [34–37]. However, Akkuş et al. didn't find a significant difference between the SOD values of the control group and diabetes cases [28]. Diniz-Vilelea et al. showed the increased SOD levels in diabetes and metformin treated diabetes group compared with non-diabetic rats [38]. Also, Ciechanowski pointed out the fact that the decrease of extracellular superoxide dismutase activity in diabetes develops due to excessive glycation [39]. As suggested by Chen et al. it is possible that changes in SOD activity may occur in early stages of diabetes [40]. Based on these findings, the hyperglycemia can promote an increase in the generation of ROS leading to different activities of SOD.

GPx which is another enzyme showing antioxidant activity, reduces hydroperoxides to water. We found significantly decreased GPx activity in diabetics whereas it was significantly upregulated in metformin using group. Many previous studies reported the metformin's effects and decreased GPx levels in diabetic organisms compared with normal controls [36,41]. Our results are in agreement with these studies. Thus, we propose that metformin may induce antioxidant capacity by inducing the activity of GPx. However, GPx activity was increased in diabetic rat muscles compared to control rats. Also, metformin treatment did not enhance GPx activity in those diabetic rats [38].

Activity of the antioxidant enzyme CAT which catalyzes the transformation of hydrogen peroxide to water and oxygen, was higher in controls compared to type 2 diabetes patients. However, metformin use could not reverse CAT decrease in diabetes cases. Similar to our results, Erejuwa et al. showed SOD and GPx were upregulated, while CAT activity was significantly reduced in diabetic rats [41]. However, Dai et al. observed metformin increased the catalase activity in mice with oxidative liver injury [42]. In another study it was shown that metformin again induces the catalase activity in metformin treated macrophages [34]. According to these results, our findings contradict with some of the studies in the literature. However, we suggest the blocking of mitochondrial complex I enzymes by metformin may decrease the free radical production which may result in the decrease in CAT activity. Also, it may be possible, that the decreased CAT activity in diabetes patients using metformin in our study may rely on the diabetes diagnosis time, life style variations, calorie restriction, metformin dose and time of treatment, or other unknown factors.

Metformin's effects on DNA damage under oxidative conditions is inconsistent. This contradiction may depend on the type and duration of oxidative stress as well as tissue or organism difference. Ohnisi et al. reported metformin dramatically enhanced oxidative DNA damage under oxidative condition in isolated DNA [43]. Whereas Lee et al. showed that metformin inhibited UVC-induced upregulation of p53 and DNA repair and downregulated DNA damage markers in A549 lung cancer cells [44]. Also, Na et al. demonstrated that metformin has inhibitory effects on DNA damage accumulation due to low AKT activity which was generated by age and oxidative stress in Drosophila cells [45].

DNA base modifications are the most common defect of oxidation, deamination and alkylation reactions. There are more than 100 types of oxidative base damage to DNA as the result of normal mitochondrial respiration activity which produces ROS [46]. To protect DNA against these harmful modifications, BER system has recruited several enzymes and proteins such as XRCC1 and DNA pol beta. In diabetes, the repair of oxidative damage is critical to maintain the genomic integrity. We found XRCC1 level was significantly lower than controls. On the other hand, metformin use significantly upregulated its levels and induce BER system in diabetic patients. Although it was not significant, other BER enzyme, DNA pol beta, level was increased in diabetic patients. In line with our results, Blasiak et al. found elevated level of oxidative DNA damage, increased susceptibility to mutagens and decreased efficacy of DNA repair in type 2 diabetes patients [47]. On the other hand, Grindel et al. found no significant difference in oxidative stress parameters, antioxidant enzyme activities, damage to DNA and base excision repair capacity between diabetic patients and controls [48]. Also, metformin attenuates ROS production both in AMPK wild type and −/− mouse embryonic fibroblasts (MEFs) and prevents paraquat-induced DNA damage in MEFs in an AMPK independent manner [16].

Although dosage and time of treatment matters, metformin

reduces cellular stress and decreases ROS levels. Thus, this situation brings antioxidant activity to p53 and stimulates cellular survival. As expected, due to decreased antioxidative capacity, p53 level was decreased significantly in type 2 diabetes patients. Whereas it was increased significantly in metformin using cases in line with the increased antioxidative capacity. Othman et al. showed, metformin treated diabetic animals showed lower induction of oxidative stress and DNA damage represented by increased p53 levels [49]. The p53 induction in metformin using patients may be due to the activation of AMPK. AMPK activation induces p53 phosphorylation and this promotes cellular survival in response to glucose deprivation [8]. The p53's antioxidant activity hereby lowers stress conditions and induces cellular survival which is a desired condition in diabetes.

Several studies showed that metformin has antioxidative activity and some reported that metformin can act as prooxidant due to p53 activity, which represents an interesting point that could be considered in different diseases and conditions. Overall, our study is powered for the outcomes and reinforces the potential benefit of metformin in antioxidative capacity to protect cells from diabetic oxidative stress and in regulation of DNA BER system. Through these findings, it is expected to contribute not only to elucidation of molecular events underlying the conditions of type 2 diabetes but also reflects how metformin works in terms of antioxidative activity, enhance longevity and cancer prevention as a multifunctional agent. Hereafter, it would be better if metformin's effects on another DNA repair systems and signaling pathways based on oxidative status are detailed in the further studies.

5. Conclusion

In our study, metformin increased SOD and GPx activities in type 2 diabetes patients but the reflection of this increase to the total antioxidant capacity was not significant. Although DNA pol beta levels were not enhanced significantly, XRCC1 and p53 levels were significantly upregulated with metformin treatment in type 2 diabetes patients. This findings reinforce the potential benefit of metformin in antioxidative capacity to protect cells from diabetic oxidative stress and in regulation of DNA BER system.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Declarations of interest

None.

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