British Journal of Medicine & Medical Research
17(1): 1-9, 2016, Article no.BJMMR.26356
ISSN: 2231-0614, NLM ID: 101570965

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Carbonic Anhydrase Inhibition Affect Antioxidant Enzyme Activity in the Blood of STZ Induced Diabetic Rats

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Authors' contributions

This work was carried out in collaboration between all authors. Author SII was responsible for conception, design and analysis of the study. Authors DAA, SEA and IAU contributed to the supervision and interpretation of the data. Authors SII and FYM were responsible for drafting of the manuscript. Authors DAA, SEA, IAU and FYM contributed to critical revision of the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BJMMR/2016/26356
Editor(s):
(1) Alex Xiucheng Fan, Department of Biochemistry and Molecular Biology, University of Florida, USA.
(2) Masahiro Hasegawa, Department of Orthopaedic Surgery, Mie University Graduate School of Medicine, 2-174 Edobashi, Tsu City, Mie, 514-8507, Japan.
Reviewers:
(1) Boguslaw Lipinski, Harvard Medical School, USA.
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(3) Aaser Mohamed Abdelazim, Egypt.
Complete Peer review History: http://www.sciencedomain.org/review-history/15508

Original Research Article

Received 12th April 2016
Accepted 22nd June 2016
Published 24th July 2016

ABSTRACT

Background: Carbonic anhydrase is found in the blood of all vertebrate and thus playing a fundamental role in the maintenance of acid-base homeostasis. Erythrocytes are intrinsically prone to oxidative stress because of their exposure to high oxygen tension.
Aim: The study aimed to investigate the changes of erythrocytes anti-oxidative enzymes in STZ induced diabetic rats and to determine the antioxidant potential of *Cadaba farinosa* leaves.
Results: The result of the present study showed that inhibition of carbonic anhydrase result in significant decrease in both erythrocyte and plasma catalase activity, whereas erythrocyte and plasma superoxide dismutase activity increased.
Conclusion: Carbonic anhydrase inhibition may alter the activity of anti-oxidative enzymes *in vivo*.

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Keywords: Carbonic anhydrase; catalase; SOD; diabetes.

1. INTRODUCTION

The RBC are intrinsically prone to oxidative stress because they are exposed to high oxygen tension, and have a characteristic structural composition with polyunsaturated fatty acid in the membrane, besides the presence of hemoglobin-bound iron [1,2]. However, membranes and cytoplasmic compartments of RBC have an efficient antioxidant mechanism that maintains their integrity.

In addition to the transport of oxygen from the lungs to tissues, RBCs also transport carbon dioxide (CO_2) from the tissues to the lungs for removal, which is an essential function. Elevated CO_2 content in the body leads to increased plasma proton (H^+) concentrations, which can result in acidosis, malfunction of the central nervous system, coma, and even death [3].

Carbonic anhydrase (CA) is found in the blood of all vertebrate and is involved in the crucial process of CO_2 transport by catalyzing the reversible hydration of CO_2 to carbonic acid, H^+ and HCO_3^-, thus playing a fundamental role in the maintenance of acid-base balance of the body.

Several important physiological and pathological functions are played by the CA isozymes present in organisms, this includes transport of CO_2 and ions (such as H^+, Na^+ and Cl^-) along with pH-regulation in a variety of physiological processes ranging from respiration to intermediary metabolism at the cellular level [4,5].

Carbonic anhydrases are ubiquitous enzymes in vertebrate, carbonic anhydrase inhibition in organs other than the target may result in undesired side effects, the most frequent ones are: numbness and tingling of extremities; metallic taste; depression; fatigue; malaise; weight loss; decreased libido; gastrointestinal irritation; metabolic acidosis; renal calculi and transient myopia [6-9], which are all common among diabetic patients.

Antioxidative enzymes such as catalase and superoxide dismutase prevent the damage that follows oxidative stress. Catalase (CAT) and Superoxide dismutase (SOD) are the two major anti-oxidative enzymes that remove free radicals *in vivo.* A decrease in the activity of these antioxidants enzymes can lead to an excess availability of superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), which in turn can generate hydroxyl radicals ($^{\bullet}OH$), resulting in initiation and propagation of lipid peroxidation. Since CA is the key enzyme that regulates the acid-base homeostasis in both normal and pathological conditions. It is therefore understandable that hydrogen ion concentration can profoundly influence chemical reactivity as well as anti-oxidative enzyme structure, catalysis, and regulation. Thus, a change in pH may affect the protonation state of enzymes and, therefore, impact the biological activity and conformation of the molecule. Enzymes typically demonstrate maximal catalytic activity at a characteristic pH, referred to as the pH optimum. On either side of the optimum pH, catalytic activity often declines sharply. Therefore, biological control of cellular and extracellular fluid pH is highly important for all aspects of metabolism and cellular activities.

Because enzymes and even their substrates contain acid/base groups, it is not at all surprising that pH is such a dominant factor influencing enzyme action.

Thus, the aim of the present study was to determine the changes of erythrocytes anti-oxidative enzymes in STZ induced diabetic rats under the influence of carbonic anhydrase inhibition by treating the animals with a standard carbonic anhydrase inhibitor (acetazolamide). We hypothesized that CA may be an important protective enzyme against reduction of anti-oxidative enzyme activity in diabetes.

2. MATERIALS AND METHODS

2.1 Experimental Protocol

2.1.1 Chemicals and reagents

Thiobarbituric acid (TBA), (−)-epinephrine and streptozotocin (STZ) were purchased from Sigma–Aldrich, Chemicals (Saint Louis, USA). Other chemicals were of analytical reagent grade.

2.2 Study Animals

Male Wister albino rats of 180–220 grams weight were used for this study. Before initiation, the rats were allowed acclimatization period of 7 days in laboratory condition. Five rats each were housed in polycarbonate cages bedding with husk, 20 to 24℃ temperature and relative humidity between 30 to 70 percent. The dark and light cycle of 12 hours each was

maintained. Standard animal diet (Vitafeeds Nig. Ltd. Nigeria) with pure water in glass bottles *ad libitum* were fed to the animals. The principles of laboratory animal care were followed according to the instructions by the Faculty of veterinary Medicine, Animal Ethics Committee, Ahmadu Bello University, Zaria Nigeria.

2.3 Induction of Diabetes

Diabetes was induced in all the rats except in the normal controls (Group I) by Streptozotocin (STZ) 60 mg per kg body weight, dissolved in ice cold citrate buffer (0.1 M, pH 4.5), through intraperitoneal route. Hyperglycemia was confirmed by the elevated fasting glucose level > 200 mg/dl in plasma, determined at 72 h after STZ injection. Hyperglycemic rats were included for the study along with the normal control animals.

2.4 Study Design

The animals were grouped into five groups of five rats each. Group I rats (Normal control); Group II (Diabetic control); Group III (Diabetic rats treated with Acetazolamide, 250 mg/kg/day for 28 days); Group IV (Diabetic rats treated with Metformin, 1000 mg/kg/day for 28 days) and Group V (Diabetic rats treated with methanol extract of *Cadaba farinosa* leaves 1000 mg/kg/day for 28 day for 28 days). Both animals were fed with standard animal feed mentioned above and distilled water. But Group I (Normal control) and Group II (Diabetic control) rats received only water at the time of treatment of other groups.

2.5 Collection and Preparation of Blood Samples

The blood samples were collected by cardiac puncture in vials with EDTA and centrifuged at 3500 rpm for 10 minutes. The plasma was separated and kept, from the cells and buffy coat removed. The packed red cells were washed three times with normal saline (0.9% NaCl) and were lysed with ice cold water, yielding destroyed plasma membranes.

2.6 Biochemical Analysis

2.6.1 Assay of carbonic anhydrase activity

Carbonic anhydrase activity was determined as mentioned by [10], with the modification described by [11], using spectrophotometer. In this assay, the esterase activity of carbonic anhydrase was determined from the hydrolytic rate of 3 mM p-nitrophenyl acetate to p-nitrophenol. The assay system contained 100 µL hemolysate placed in 1 cm spectrometric cell containing 1.4 ml 0.05 M Tris- HCl buffer, pH: 7.4 and 1.5 ml p-nitrophenyl acetate. The change in absorbance at 348 nm was measured over the period of 3 min before and after adding the sample. The absorbance was measured by a UV-Vis spectrophotometer (Shimadzu UV-2600 Spectrophotometer). One unit of enzyme activity was expressed as µmol of p-nitrophenol relased/min/µL from hemolysate at room temperature (25℃) [11,12].

2.6.2 TBARS content

The method of [13], with some modification was used to estimate the rate of lipid peroxidation LPO. 0.25 ml homogenate was pipetted into 15 mm ×100 mm test tubes and incubated at 37℃ in a metabolic shaker for 1 h. After 1 h of incubation, 0.5 ml of 5% (w/v) chilled trichloroacetic acid (TCA), followed by 0.5 ml of 0.67% TBA (w/v) was added to each test tube and centrifuge tube, and centrifuged at 1000 × g for 15 min. Thereafter, the supernatant was transferred to other test tubes and was placed in a boiling water bath for 10 min. The absorbance of pink color produced was measured at 535 nmin a spectrophotometer (Shimadzu-1601, Japan). The TBARS content was calculated by using a molar extinction coefficient of 1.56×105 M^{-1} cm^{-1} and expressed as nmol of TBARS formed min^{-1} mg^{-1} of protein.

2.6.3 Assay for CAT

CAT activity was assayed by the method of [14]. Briefly, the assay mixture consisted of 0.05 M phosphate-buffer (pH7.0), 0.019 Mhydrogen peroxide (H_2O_2), and 0.05 ml PMS in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. CAT activity was expressed as nMol H_2O_2 consumed min^{-1} mg^{-1} protein.

2.6.4 Assay for SOD

SOD activity was measured as described by [15], with some modification the reaction mixture contained 0.8 ml of 50 mmol/l glycine buffer (pH 10.4), and 0.2 ml supernatant. The reaction was initiated by the addition of 0.02 ml of a 20 mg/ml solution of (−)-epinephrine. Absorbance was recorded at 480 nm in a spectrophotometer (Shimadzu-1601, Japan). SOD activity was

expressed as nmol of (−)-epinephrine protected from oxidation by the sample compared with the corresponding readings in the blank cuvette. The molar extinction coefficient of 4.02×103M−1 cm−1 was used for calculations.

2.7 Statistical Analysis

Results were presented as mean ± standard Deviation (SD). Within and between groups, comparisons were performed by the analysis of variance (ANOVA) (using SPSS 20.0 for windows Computer Software Package). Significant differences were compared by Duncan"s new Multiple Range test was considered significant [16].

3. RESULTS

3.1 Carbonic Anhydrase Inhibition Decreased Erythrocyte and Plasma Catalase Activity in STZ-induced Diabetic Rats

Fig. 1: Shows the diabetic group treated with Acetazolamide (Standard carbonic anhydrase inhibitor) to significantly decrease ($P < 0.05$) erythrocyte catalase activity when compared with normal control group. Methanol leaf extract of *Cadaba farinosa* was found to significantly ($P < 0.05$) increased the activity of Erythrocyte catalase when compared with diabetic control group.

Fig. 2: Shows a significant reduction of plasma catalase activity in diabetic groups treated with Acetazolamide when compared with both normal and diabetic control group. Methanol leaf extract of *Cadaba farinosa* was found to significantly ($P < 0.05$) decreased the activity of plasma catalase when compared with diabetic control group.

3.2 Carbonic Anhydrase Inhibition Increased Erythrocyte SOD Activity in STZ-induced Diabetic Rat

Fig. 3: Inhibition of erythrocyte carbonic anhydrase increases SOD activity, though the increase was not significant when compared with diabetic control group. Diabetes results in significant decrease ($P < 0.05$) in erythrocyte SOD activity when compared with normal control.

3.3 Carbonic Anhydrase Inhibition Increased Plasma SOD Activity in STZ-induced Diabetic Rats

Fig. 4: Inhibition of erythrocyte carbonic anhydrase increases SOD activity significantly ($P < 0.05$) when compared with both normal and diabetic control group.

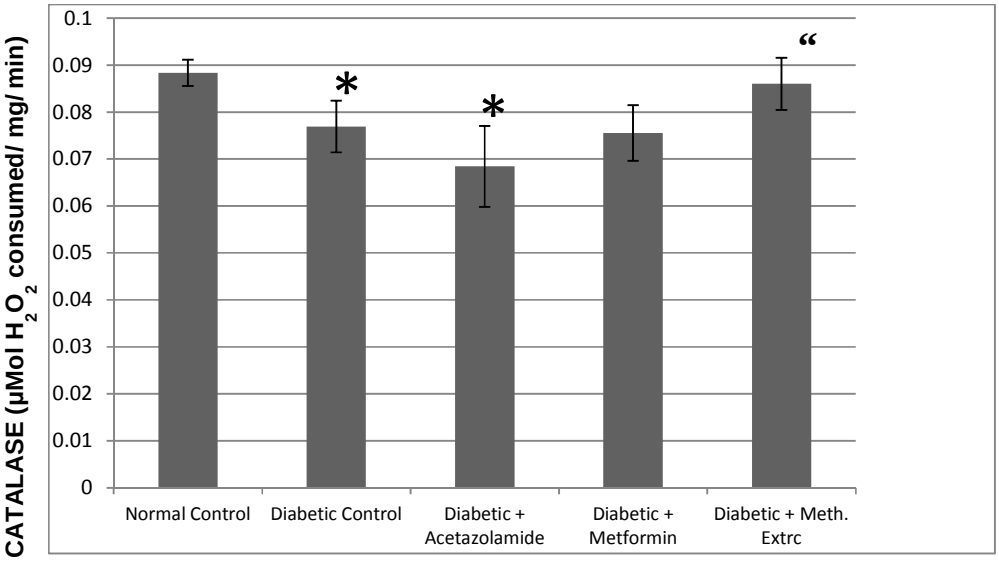

Fig. 1. Effect of acetazolamide, metformin and methanol extract of *Cadaba farinosa* on red blood cell catalase activity levels in STZ induced diabetic rats treated at 250 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day doses for 28 days

**P < 0.05 vs Normal control, "P < 0.05 vs Diabetic control (n=5)*

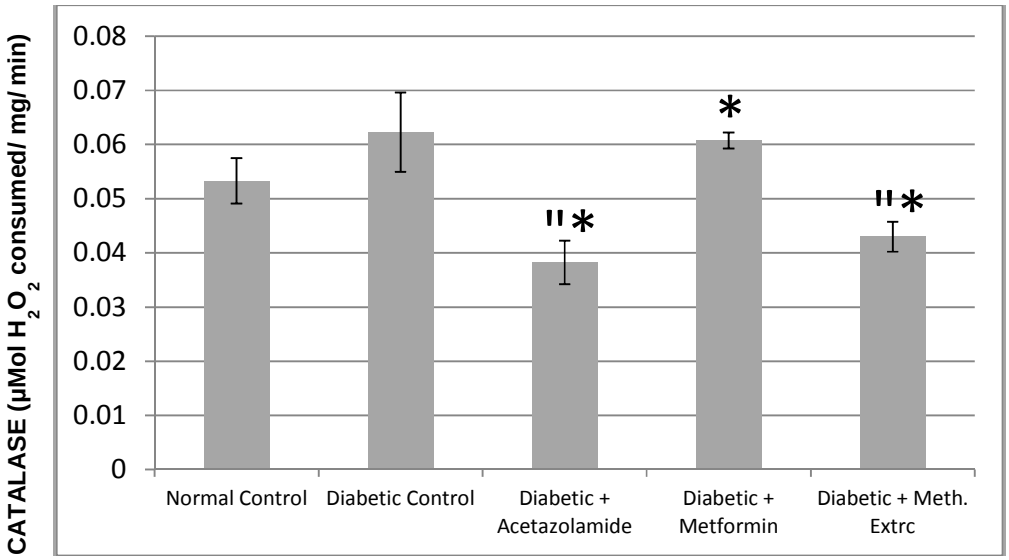

Fig. 2. Effect of acetazolamide, metformin and methanol extract of *Cadaba farinosa* on plasma catalase activity levels in STZ induced diabetic rats treated at 250 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day doses for 28 days

**P < 0.05 vs Normal control, "P < 0.05 vs Diabetic control (n=5)*

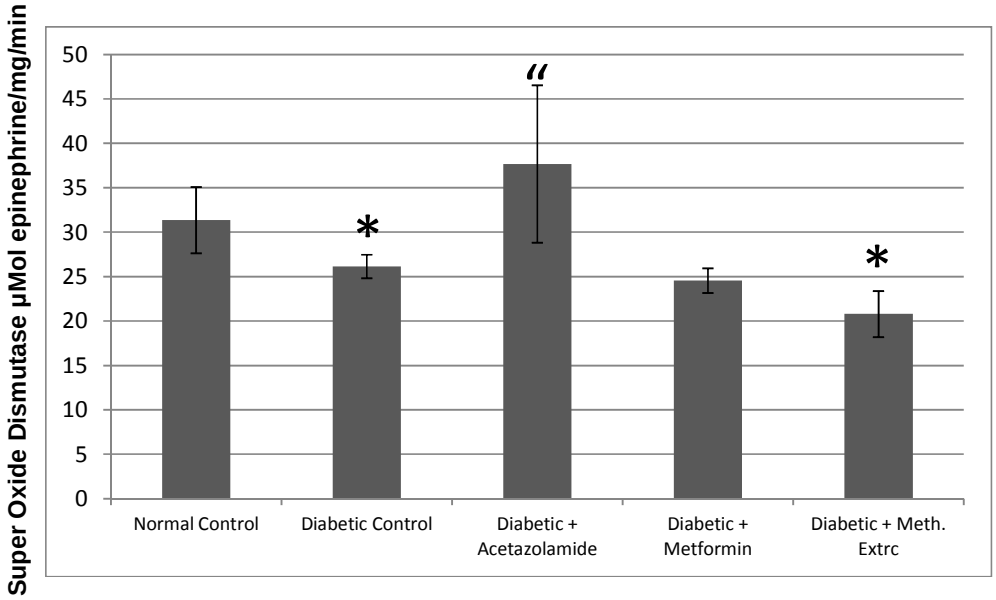

Fig. 3. Effect of acetazolamide, metformin and methanol extract of *Cadaba farinosa* on erythrocyte superoxide dismutase activity levels in STZ induced diabetic rats treated at 250 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day doses for 28 days

**P < 0.05 vs Normal control; "P < 0.05 vs Diabetic control (n=5)*

3.4 Carbonic Anhydrase Inhibition Decreased TBARS Level in STZ-induced Diabetic Rats

Fig. 5: Inhibition of erythrocyte carbonic anhydrase with Acetazolamide significantly (P < 0.05) decreased TBARS level when compared with diabetic control group. Methanol leaf extract of *Cadaba farinosa* also significantly decreased TBARS level when compared with diabetic control.

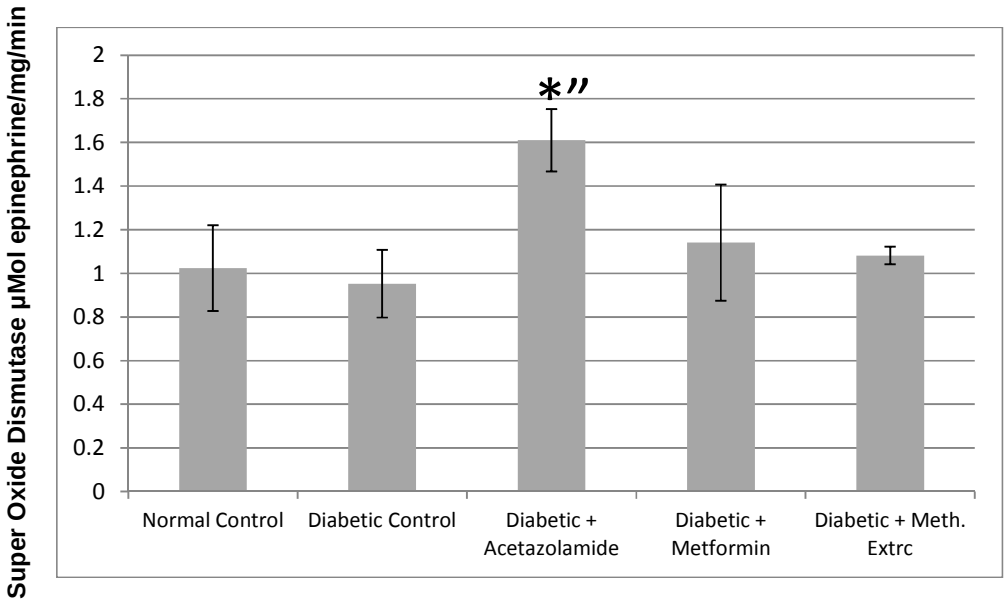

Fig. 4. Effect of acetazolamide, metformin and methanol extract of *Cadaba farinosa* on plasma superoxide dismutase activity levels in STZ induced diabetic rats treated at 250 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day doses for 28 days

**P < 0.05 vs Normal control, "P < 0.05 vs Diabetic control (n=5)*

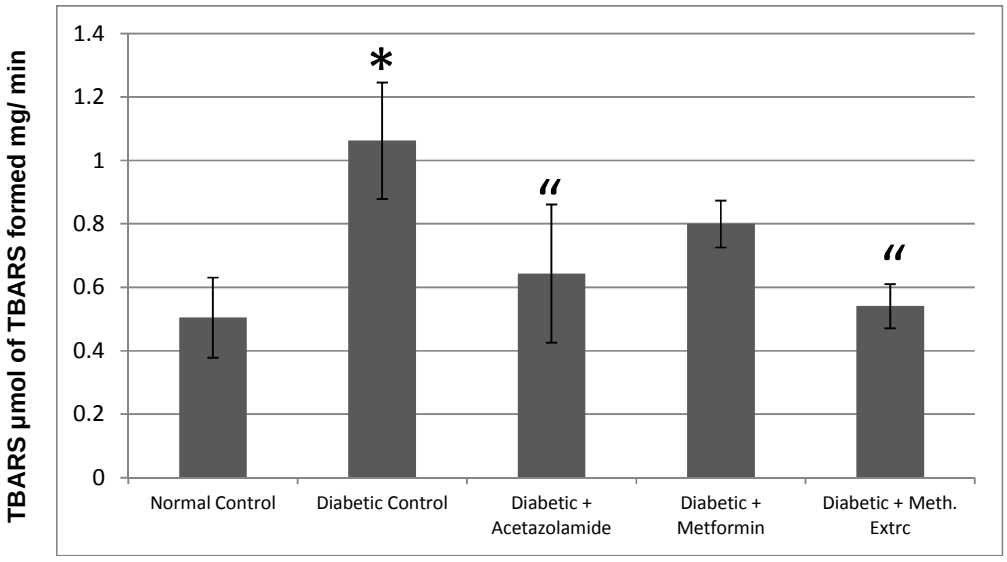

Fig. 5. Effect of acetazolamide, metformin and methanol extract of *Cadaba farinosa* on red blood cell TBARS in STZ induced diabetic rats treated at 250 mg/kg/day, 500 mg/kg/day and 1000 mg/kg/day doses for 28 days

**P < 0.05 vs Normal control; "P < 0.05 vs Diabetic control (n=5)*

4. DISCUSSION

The essential finding from this study revealed that inhibition of carbonic anhydrase may be responsible for the altered level of catalase and superoxide dismutase activity in both the erythrocyte and plasma of STZ induced diabetic rats. To our knowledge, this is the first study demonstrating an association between decreased catalase activity; elevated SOD activity and carbonic anhydrase inhibition in both the erythrocyte and plasma of STZ induced

diabetic rats. Previous studies had demonstrated that in diabetics, activity of erythrocyte SOD has been shown to be decreased [17], increased [18] and unchanged [19,20]. Red blood cell SOD activity is frequently measured in humans as an index of defence against superoxide radical in the blood.

It has been reported that inhibition of Carbonic anhydrase decreased the rate of acidification of urine, producing alkaline urine and eventually metabolic acidosis [21].

Hyperglycemia enhances intracellular acidosis and accumulation of intracellular calcium [22,23]. In addition, decrease in blood pH is often observed in metabolic acidosis. A pH-dependent reduction in the affinity of hemoglobin for O_2 (Bohr effect) has been reported in metabolic acidosis [24]. The final effect on the affinity of hemoglobin for O_2 will depend on the duration of the acidosis. Complications associated with diabetes are augmented by increased O_2 consumption, ROS production, lipid peroxidation and decreased mitochondrial antioxidant substrates (GSH, NADH, CoQ, etc) [25] has been reported that diabetic rats display higher O_2 consumption and reduced mitochondrial antioxidant GSH and coenzyme Q pools than non-diabetic rats [25]. We may therefore suggest that, the enhanced SOD activity and decreased TBARS level in diabetic rats treated with Acetazolamide (carbonic anhydrase inhibitor) might be due to decreased O_2 delivery and consumption brought about by inhibition of carbonic anhydrase.

Any increase in SOD catalytic activity produces an excess of H_2O_2 that must be efficiently neutralized by catalase or glutathione peroxidase [26]. In tissues lacking significant catalase activity, detoxification of hydrogen peroxidase becomes critically dependent on Glutathione peroxidase [27].

In the present study catalase activity was found to significantly decrease when carbonic anhydrase was inhibited, suggesting CA and CAT might influence each other. [28] demonstrated that CA III overexpressing cells grew faster and were more resistant to cytotoxic concentrations of H_2O_2 than control cells. Their results suggest that CA III functions as an oxyradical scavenger and thus protects cells from oxidative damage.

We may suggest that catalase and carbonic anhydrase together can be more effective in mediating defence against free radical damage. [29] suggest that CA would mediate detoxification of peroxide.

$$H_2O_2 + CO_2 \longleftrightarrow H_2CO_4$$

Recently glutaraldehyde-crosslinked Poly-SFHb-SOD-CAT-CA was prepared by [30]. They suggested that the incorporation of CA resulted in a soluble nanodimension complex of PolySFHb- SOD-CAT-CA that has enhanced antioxidant properties in addition to acting as carrier for both O_2 and CO_2 [30].

Lack of convincing evidence on the decreased catalase activity due to inhibition of carbonic anhydrase does not mean that there is no link or interaction. However, assessing oxidative stress induced by inhibition of carbonic anhydrase is not easy. Carbonic anhydrase (CA) is the enzyme in RBC responsible for the transport of CO_2. CA inhibitor can block RBC–CA activity, resulting in marked decrease in CO_2 transport [3]. The result is a decrease in blood pH due increased CO_2 concentration. Decreased levels of CA in STZ induced diabetic rats may provide a convenient media for reduced catalase activity by creating an acidic media.

5. CONCLUSION

Acetazolamide and leaf extract of *Cadaba farinosa* may enhance the activity of anti-oxidative enzymes and thus may serve a protective role against erythrocyte membrane lipid peroxidation in diabetes.

CONSENT

It is not applicable.

CONFLICT OF INTEREST

Authors declare no conflict of interests.

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Peer-review history:
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