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RESEARCH ARTICLE

Correlation between the Activity of Aldehyde Dehydrogenase and Oxidative Stress Markers in the Saliva of Diabetic Patients

Hina Younus[1],*, Sumbul Ahmad[1] and Md. Fazle Alam[1]

[1]*Enzymology Laboratory, Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, 202002, India*

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Abstract: ***Background:*** Reactive aldehydes are involved in diseases associated with oxidative stress, including diabetes. Human salivary aldehyde dehydrogenase (hsALDH) presumably protects us from many toxic ingredient/contaminant aldehydes present in food.

Objective: This study aimed to probe the activity of hsALDH in patients with diabetes and than to correlate it with various oxidative stress markers in the saliva.

Methods: The saliva samples were collected from total 161 diabetic patients from Rajiv Gandhi Centre for Diabetes, Jawaharlal Nehru Medical College (JNMC), AMU, Aligarh, (India). HsALDH activity and markers of oxidative stress [8-hydroxydeoxyguanosine (8-OHDG), malondialdehyde (MDA) and advanced glycation end products (AGEs)] were measured in the saliva samples.

Results: Patients with early stage of diabetes had higher activity of hsALDH when compared with the control group. As the history of diabetes increases, the activity of the enzyme decreases and also higher oxidative stress markers (8-OHDG, MDA and AGEs) are detected in the saliva samples. Negative significant correlation between hsALDH activity and oxidative stress markers were observed ($p < 0.0001$).

Conclusion: The activity of hsALDH increases in early stages of diabetes most probably to counter the increased oxidative stress associated with diabetes. However, in later stages of diabetes, the activity of the enzyme decreases, possibly due to its inactivation resulting from glycation.

Keywords: Diabetes, saliva, human salivary aldehyde dehydrogenase, oxidative stress markers, glycation, diabetic patients.

1. INTRODUCTION

For both local and systemic diseases, saliva has been extensively studied as a diagnostic biofluid. Researchers have suggested that modification in the salivary chemistry provides insights into disease pathogenesis [1]. Saliva has been considered as a good diagnostic easy to get fluid and its role has been probed in many diseases, such as cystic fibrosis, sarcoidosis, Sjogren's syndrome, hormone dysfunction and neurological disorders [2]. Collecting saliva is a better alternative to collecting blood as it is noninvasive, does not cause any discomfort to the patient, cheaper and safe for both donor and collector. Saliva is good for diagnosis as its molecular profile reflects one's physiological state at the time of collection. Various molecules make entry from serum into the saliva by passive diffusion through capillaries, active transport through secretory cells and ultrafiltration through spaces between ductal and acinar cells [3]. Therefore, serum components can be found in the saliva, while some salivary components produced locally by the salivary glands cannot be detected in the plasma [4]. The saliva as a whole comprises of 99% water, gingival fluid, fluid secreted from the major and minor salivary glands, bacteria and food debris [5].

Oxidative stress which occurs as a result of imbalance between the generation of free oxygen radicals and the inactivation of these reactive species by the antioxidant defense mechanism in the body, is capable of causing damage to various cellular and extracellular constituents [6]. The adverse effects of elevated oxidative stress usually appear after exposure to a relatively high concentration of Reactive Oxygen Species (ROS), and/or impairment in the antioxidant defense system. The antioxidant enzymes like catalase, superoxide dismutases, and glutathione peroxidases are induced to counter ROS generation and to maintain the redox equilibrium [7]. Nevertheless, under oxidative stress conditions, alterations occur in cellular proteins, lipids, and DNA, leading to cell damage or apoptosis. It has been observed that increased oxidative stress is associated with clinical manifestations of coronary artery disease [8], diabetes [9], infertility [10], chronic kidney disease [11], atherosclerotic cardio vascular disease, chronic inflammatory

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*Address correspondence to this author at the Enzymology Laboratory, Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, 202002, India; Tel: +91 571 2720388; Fax: +91 571 2721776;
E-mails: hinayounus@rediffmail.com; hyounus.cb@amu.ac.in

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disease, cancers [12], ageing and neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [13]. Hence, oxidative stress is now being studied as a potential target for therapeutic interventions [14].

Diabetes is a metabolic disease resulting from multiple causes, which progresses slowly in steps [15]. It initially begins with insulin resistance, which progresses slowly with time until the body fails to maintain glucose homeostasis which leads to glucose intolerance. These physiological alterations are accompanied with changes in a number of biochemical processes like obesity, an abnormal lipid profile and lipid peroxidation [16]. The resulting lipid peroxidation resulting from free radicals causes significant changes in the cell membrane. Oxidative stress increases in diabetes, due to elevated ROS and an impaired antioxidant defense system [17, 18]. ROS induce membrane lipid peroxidation, and the resulting fatty acids peroxides are toxic and cause cell malfunction [14]. The measurement of malondialdehyde (MDA) is the most widely used assay for lipid peroxidation. Therefore, the presence of lipid peroxides in blood is important information for the prognosis of diabetes where the secondary complications are usually fatal [19].

Reactive aldehydes are known to be involved in diseases associated with oxidative stress, including diabetes [20]. Aldehyde dehydrogenases (ALDHs) are important detoxifying enzymes which oxidize a wide range of aliphatic and aromatic aldehydes formed from various exogenous/ endogenous precursors to their corresponding carboxylic acids [21, 22]. Human salivary ALDH (hsALDH) enzyme presumably protects individuals from various toxic aldehydes present in food items either as natural ingredients or as contaminants [23, 24]. This enzyme is activated by suforaphane (SF) which is a bio-active compound found in cruciferous vegetables [25]. The objective of the present study was to examine the activity of hsALDH in patients with diabetes of various categories, and to correlate it with various oxidative stress markers in the saliva. Herein, we have also given explanation for the up/down regulation of the activity in these patients.

2. MATERIALS AND METHODS

2.1. Materials

6-Methoxy-2-naphthaldehyde, 6-methoxy-2-naphthoic acid, bovine serum albumin (BSA), bicinchoninic acid solution (BCA) and NAD^+ were procured from Sigma Chemicals Co., USA. Dithiothreitol (DTT) was obtained from Sisco Research Laboratories, India. Thiobarbituric acid, Di-sodium hydrogen phosphate, sodium phosphate monobasic anhydrous, EDTA and Tris were the products of Himedia chemicals (India). Tricholoroacetic acid was from Qualigens, India. NaOH and HCl (35%) were obtained from Merck Specialities Pvt. Ltd. The other reagents and chemicals used were all of analytical grade.

2.2. HsALDH Activity Measurements

HsALDH activity was measured using the substrate 6-methoxy-2-naphthaldehyde (5 μM) and the coenzyme NAD^+ (100 μM) in 50 mM sodium phosphate buffer (pH 7.5) at 25°C, in the presence of 0.5 mM EDTA and 0.5 mM DTT [26, 27]. The reaction was initiated by the addition of the enzyme in the reaction mixture at 25°C and monitoring continuously for 5 min. If any fluorescence background drift was there, it was measured before adding the enzyme and was subtracted from the final slope. Fluorescence assays were performed on a Shimadzu RF-5301PC Spectrofluorometer with excitation and emission wavelengths as 315 nm and 360 nm, respectively. The inner filter effect was nullified by using the following equation (1):

$$F_{cor} = F_{obs}10^{(Aex+Aem)/2} \tag{1}$$

Where, F_{cor} and F_{obs} are the corrected and observed fluorescence intensity, respectively. Aex and Aem are the absorbance at excitation and emission wavelength, respectively. The reaction velocity was converted in terms of product formation using a standard curve of 6-methoxy-2-naphthoic acid. One unit (U) of enzyme activity was defined in terms of number of micromoles of product produced per min per microgram of the enzyme [28].

2.3. Activity of hsALDH in Diabetic Patients

2.3.1. Study Design and Sampling of Diabetic Patients

A cross-sectional case control study was conducted in our laboratory at Interdisciplinary Biotechnology Unit, Aligarh Muslim University. Saliva samples were collected from diabetic patients, total 161 individuals (97 males and 64 females), who were recruited for the study from the Out Patient Department of Rajiv Gandhi Centre for Diabetes, Jawaharlal Nehru Medical College (JNMC), AMU, Aligarh, India. The diabetic patients were categorized into the following four groups on the basis of duration of diabetic history and addiction:

Group 1: Diabetes Mellitus (DM) patients having less than 5 years of diabetic history and were non-smokers

Group 2: DM patients having less than 5 years of diabetic history and are smokers

Group 3: DM patients having more than 5 years of diabetic history and are non-smokers

Group 4: DM patient having more than 5 years of diabetic history and are smokers

Control: Non-diabetic and non-smokers

2.3.2. Collection and Processing of Saliva Samples

All saliva samples were obtained in the morning (8 - 10 a.m.), before the first meal and after thorough washing of the mouth. Whole saliva samples (unstimulated) were collected by participants who were made to in spit in disposable collection tubes containing cold 50 mM Tris-HCl buffer (pH 8.0) with 0.5 mM DTT and 0.5 mM EDTA. The final dilution of the saliva with buffer was 1:1. Crude saliva samples were centrifuged at 9723 x g for 10-12 min at 4°C, and the supernatant was collected and kept in ice [29]. After 1-3 h, the activity of hsALDH in the supernatant was determined in the presence of the 6-methoxy-2-naphthaldehyde and NAD^+. The protein concentration was routinely estimated by the BCA method as described by Smith *et al.* (1985) [30], taking the standard protein BSA.

2.4. Assay of MDA Levels

The levels of MDA were determined in the clinical saliva samples by the procedure of Jain *et al.* (1989) [31]. This procedure is based on the reaction of MDA with thiobarbituric acid, which produces a colored complex that can be measured spectrophotometrically. The sample (0.2 ml) was mixed thoroughly with 0.8 ml of sodium phosphate buffer saline (pH 7.4) and 25 µl of 0.88% butylated hydroxytoluene solution. After the addition of 0.5 ml of trichloroacetic acid (30%), the samples were kept over ice for 2 h and then centrifuged at 2000 x g at 25°C for 15 min. One ml of the supernatant was added to 75 µl of 0.1 M EDTA and 0.25 ml of 1% thiobarbituric acid in 0.05 N NaOH. The samples were placed in boiling water for 15 min, cooled to room temperature, and then the absorbance was determined at 532 nm.

2.5. Assay of 8-Hydroxydeoxyguanosine (8-OHDG) Levels

The saliva samples were centrifuged at 10,000 rpm for 10 min, and 8-OHDG levels in the supernatant were determined by using a competitive ELISA kit (Highly Sensitive 8-OHDG Check, Japan Institute for the control of Aging, Shizuoka, Japan). The determination range was 0.125 - 200 ng/ml.

2.6. Determination of Advanced Glycation End Products (AGEs)

Salivary AGEs were assessed by the spectrofluorometric method of Münch *et al.* (1997) [32]. The wavelength used for excitation was 370 nm, and the emission was recorded at 440 nm. The saliva samples were diluted 10-fold with sodium phosphate buffer saline (pH 7.2), and the specific fluorescence of the AGEs was expressed in arbitrary units.

2.7. Statistical Analysis

Differences in demographic and clinical parameters between healthy controls and diabetic patients were analyzed by an unpaired t test. Differences in hsALDH activity, MDA level, 8-OHDG level and AGEs formation between groups were analyzed by the Mann-Whitney's U-test. The correlations between variables were determined by Spearman's rank test. A value of $P < 0.05$ was considered to be significant. All values are expressed as mean ± standard deviation. Graph pad Prism 6.0 for Windows was utilized for these analyses.

3. RESULTS AND DISCUSSION

3.1. Characteristics of the Study Participants

Table **1** shows the demographic variation and diabetic parameters of the study groups. One hundred sixty one diabetic patients participated in this study with the ratio of 1.5 Male : 1 Female. No statistically significant differences were observed in terms of weight, height and BMI ($p > 0.05$). However, there were statistically significant differences in terms of ages and blood glucose levels ($p < 0.05$). The average age of the participants was 50 years and the patients with DM were found to be older than the participants of the control group. All the four diabetic groups had obviously much elevated levels of blood glucose as compared to the control, with Group 3 and 4 (patients having more than 5 years of diabetic history) having the highest levels. Smoker with diabetes (Group 2 and 4) had slightly higher blood glucose level as compared to their respective diabetic alone group (Group 1 and 3).

3.2. Activity of hsALDH in Control and Diabetic Patient Samples

ALDHs are very useful enzymes that contribute very significantly to the management of oxidative/electrophilic stress within the living systems [20]. HsALDH acts on a variety of toxic aldehydes including 4-hydroxy-2-nonenal which is the most toxic [33, 34], formed during lipid auto-oxidation. During diabetic pathogenesis, bursts of free radical generation occurs which leads to lipid peroxidation. Since reactive aldehydes have been shown to be implicated 0

Table 1. Clinical and demographic characteristics of the different study groups.

-	Non-Diabetic	Diabetic				-
Variable	(Control)	(Group 1)	(Group 2)	(Group 3)	(Group 4)	p-value
Number of subjects	62	42	37	51	31	-
Gender (male/female)	39/23	16/26	25/12	28/23	28/3	-
Ages (years)	43.8 ± 8.3	45.7 ± 9.8	44.6 ± 4.7	55.9 ± 8.3	53.9 ± 8.1	< .001
Weight (kg)	61.9 ± 10.8	58.3 ± 12.3	63.9 ± 10.6	62.6 ± 13.2	61.5 ± 12.0	0.6520
Height (cm)	169.6 ± 5.4	165.2 ± 8.2	169.5 ± 8.2	169.3 ± 9.0	165.1 ± 9.8	0.178
BMI (kg/m^2)	21.5 ± 2.9	21.5 ± 4.9	22.4 ± 4.4	21.8 ± 4.3	22.7 ± 4.6	0.691
Blood glucose (mmol/l)	5.5 ± 0.5	10.8 ± 1.7	10.9 ± 2.1	12.4 ± 2.7	13.9 ± 3.2	< .001
Addiction (smoker)	-	No	Yes	No	Yes	-

BMI = Body Mass Index.

in diseases associated with oxidative stress including diabetes, hsALDH activity in diabetic patients was examined. The activity profile of hsALDH in saliva samples of different diabetic groups and the control group is shown in Figure **1**. It was found that Group 2 had the highest activity, and Group 4 had the least. Group 2 patients were smokers with early stage of diabetes and therefore, the high activity is likely to be due to the combined effect of diabetic and smoking status which leads to enhanced free radical formation, and therefore due to this reason hsALDH is induced to manage the oxidative stress. The activity decreased at the later stage of diabetes (Group 3), which might be due to the glycation induced inactivation of hsALDH. The least activity in Group 4 is implicated to be due to excessive damage/inactivation of hsALDH because of high oxidative stress resulting from both prolonged diabetes and smoking.

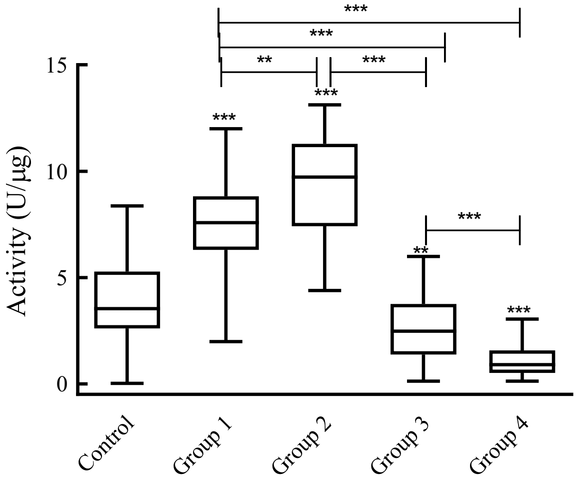

Figure 1. HsALDH activity in different groups of diabetic patients and control group. ** Denotes p <0.001 and *** denotes p <0.0001.

3.3. Oxidative Stress Parameters of Diabetic Patients and Correlation with hsALDH Activity

Oxidative stress has a key role to play in the pathogenesis of DM [35, 36]. It is believed that salivary markers are potential better alternatives to serum for diagnostic purposes [37]. The products of oxidative damage (*e.g.* 4-hydroxyalkenals, 8-OHDG, protein carbonyls, MDA) and antioxidant enzyme activities (*e.g.*, catalase, glutathione peroxidase and superoxide dismutase) have been shown to be present in the human saliva [38]. The changes in redox homeostasis of the saliva as manifested by deviations in absolute levels or expression patterns of these indices may reflect the occurrence and severity of various oral (*e.g.*, periodontitis) and systemic (*e.g.*, scleroderma and inflammatory bowel disease) defects [39, 40].

ROS interact with the nitrogenous bases (purine and pyrimidine) of DNA and oxidize them, generating multiple oxy-products. One of the major products of nucleotide oxidation in DNA is 8-OHDG [41, 42]. It was observed that the level of 8-OHDG rises as the duration of diabetes increases, and the results are statically significant (Figure **2**). Smoker with diabetes (Group 2 and 4) had still higher levels of 8-OHDG as compared to their respective diabetic alone groups (Group 1 and 3). The level of 8-OHDG is negatively correlated with the activity of hsALDH (Table **2**). During the early stage of diabetes (Group 1 and 2), the concentration of 8-OHDG is slightly greater as compared with that in the control Group (Figure **2**). This might be due to higher activity of hsALDH in patients's saliva of these groups. Therefore, hsALDH is contributing to lowering 8-OHDG level in early stages of diabetes. However, in later stage of diabetes (Group 3 and 4), the level of 8-OHDG is much higher as compared to the control (Figure **2**) due to perhaps partly highly reduced activity of hsALDH (Figure **1**).

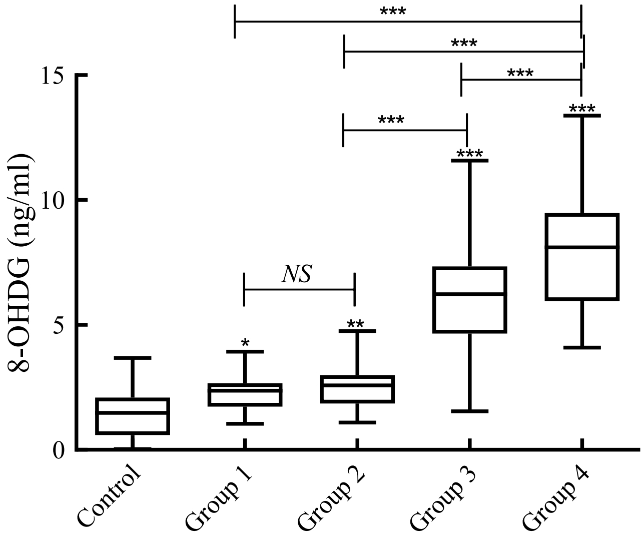

Figure 2. DNA oxidative profile of different diabetic groups as compared with the control group. NS denotes not significant, * denotes p <0.005, ** denotes p <0.001 and *** denotes p <0.0001.

Table 2. Correlation among the different parameters in diabetic group.

Parameter	HsALDH	MDA	8-OHDG	AGEs
HsALDH	1	-	-	-
MDA	-0.769**	1	-	-
8-OHDG	-0.709**	0.790**	1	-
AGEs	-0.577**	0.634**	0.725**	1

MDA is a stable end product of free radicals resulting from lipid peroxidation [43], and hence it is used as a reliable marker for assessing free radical induced damage to tissues [44]. Hyperglycemia is the main factor responsible for increased free radical production in diabetes, through the auto-oxidation of glucose. The levels of MDA are also elevated in the saliva of diabetic patients, since salivary MDA levels are directly affected by systemic oxidative stress [45]. Figure **3** shows the levels of MDA in the different groups of diabetic patients. As compared to the control group, Group 3 and 4 had much higher MDA levels, while Group 1 and 2 had slightly higher MDA levels. MDA is also a substrate of hsALDH, therefore the level of MDA is not much enhanced in Group 1 and 2, because in these groups the activity of hsALDH also increases. From the correlation (Table **2**), it can also be observed that the activity of hsALDH and the level of MDA is highly negatively correlated ($r = -0.769$, $p < 0.0001$). The higher levels of MDA in Group 3 and 4 (Figure **3**) are perhaps partly due to highly reduced activity of hsALDH (Figure **1**).

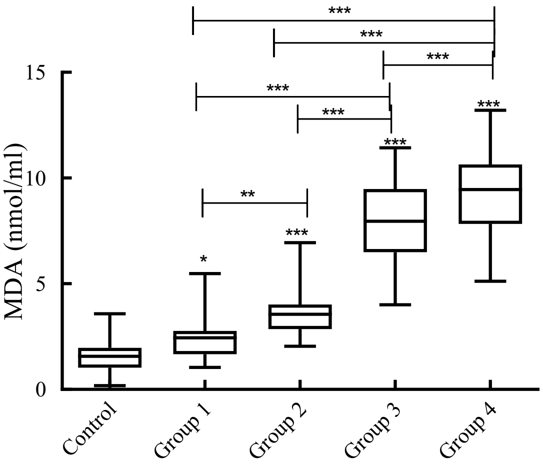

Figure 3. Lipid peroxidation profile of different diabetic groups as compared with the control group. * denotes $p < 0.005$, ** denotes $p < 0.001$ and *** denotes $p < 0.0001$.

AGEs are another important consequence of diabetes and other ROS related diseases [46]. Non-enzymatic reaction of a ketone/aldehyde group of a sugar with the free amino group of proteins and other biomolecules leads to the formation of AGEs. The formation of AGEs is a multi-step process, in which firstly a Schiff base is formed which is labile and hence subsequently it rearranges into the Amadori products, which undergo further rearrangements, cyclization, dehydrations, *etc* to form AGEs [47]. The AGEs *in vivo* contribute to numerous dysfunctions associated with normal ageing. Enhanced formation of AGEs occurs due to chronic hyperglycemia. It was observed that the amount of AGEs in the saliva of patients with diabetes increases as the history of diabetes increases (Figure **4**). The diabetic smoker groups (2 and 4) had more AGEs in the saliva as compared to the diabetic groups which were non-smoker (1 and 3), and this might be due to the external AGEs entering in the body through smoking.

From the correlation (Table **2**), it was observed that all markers of oxidative stress studied (8-OHDG, MDA and AGEs) are positively correlated with each other, and each of them is negatively correlated with the activity of hsALDH. Therefore, in early stages of diabetes as oxidative stress increases, hsALDH activity is up regulated and hence contributes to lowering the oxidative stress generated in the body. However, in later stages of diabetes it is expected that proteins including hsALDH get increasingly glycated and hence the activity reduces, leading to much greater oxidative stress and hence damage. Patients with diabetes also show higher activity of salivary lactate dehydrogenase, aspartate aminotransferase and alanine aminotransferase due to autoimmunological damage to the salivary glands in diabetes mellitus [48].

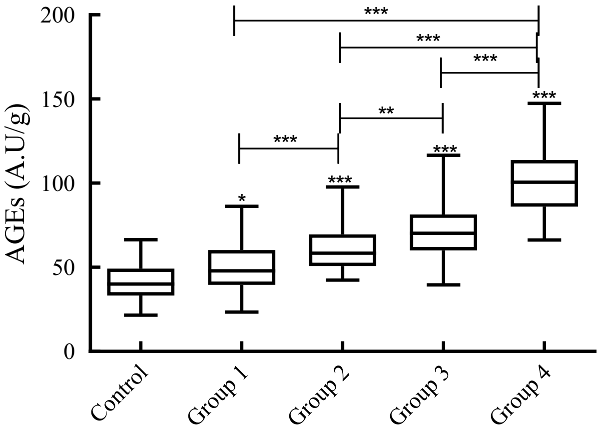

Figure 4. Concentration of AGEs in different diabetic sample groups compared with those in the control group. Where * denotes $p < 0.005$, ** denotes $p < 0.001$ and *** denotes $p < 0.0001$.

CONCLUSION

The activity of hsALDH increases in early stages of diabetes to counter the increased oxidative stress associated with diabetes. However, in later stages of diabetes, the activity of the enzyme decreases, expectedly due to its inactivation resulting from glycation. The hsALDH enzyme appears to be the first line of defense in the body against toxic aldehydes of exogenous origin, however much work has not been performed on this important member of the ALDH family. This study is expected to encourage researchers to further probe this important enzyme, its mechanism of detoxification, importance and involvement in oral disorders.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study is approved by the institutional ethics committee of Aligarh Muslim University, Aligarh, India.

HUMAN AND ANIMAL RIGHTS

No animals were used in this study. The reported experiments on humans were followed in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2013 (http://ethics.iit.edu/ecodes/node/3931).

CONSENT FOR PUBLICATION

Prior to initiating the study, each human participant was briefed about the purpose and design of the research study and a written consent was obtained.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

FUNDING

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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