

## **Elevation of serum methylglyoxal may be used as a screening marker in oral premalignant lesions**

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### **Abstract**

Methylglyoxal (MG) is formed in several metabolic pathways as products, which are highly toxic, and detoxified by glyoxalase enzyme system to lactate. It is a tricky molecule as it has dual role, on one hand its synthesis is increased in oxidative stress and on the other hand it is considered as inhibitor of cell cycle particularly in the rapidly proliferating cells. Considering these aspects of methylglyoxal, the current study was designed to establish the potential role of MG in patients exhibiting oral premalignant lesions and in patients with established malignant disease. The aim of the present study was to determine the serum methylglyoxal concentration in patients with oral premalignant lesions (OPL) as well as in established malignant disease and compare it with the healthy controls. Further to evaluate the oxidative stress and antioxidant status in these OPL patients in comparison with the healthy individuals. Methylglyoxal (MG) was determined in thirty two cases of oral precancerous lesions and thirty two cases malignant cancer patients and compared their findings with thirty-three age/sex-matched controls. Antioxidants and MDA were analyzed to observe the oxidative stress in OPL patients and healthy subjects. The OPL patients were further grouped in two categories based on the extent of oxidative stress and antioxidant status in those patients in order to delineate whether oxidative stress is the sole cause of elevated MG concentration or is there any other factor responsible for elevation in them. The premalignant and malignant cases were confirmed by histopathological examinations. The MG levels were further compared with the patients with established biochemical cancer markers. Methylglyoxal was determined by method of Racker. Superoxide dismutase, Catalase and Glutathione peroxidase was determined by standard methods. The values were expressed as means  $\pm$  standard deviation (SD) and data from patients and control was compared using student's 't'-test. Serum methylglyoxal levels was found to be significantly elevated ( $p < 0.001$ ) in oral premalignant lesions (OPL) and significantly decreased levels ( $p < 0.001$ ) in established malignant disease patients compared to healthy controls. Further analysis among oral precancerous lesion patients was based on the oxidative stress and antioxidant status which were categorized into two groups. Serum malondialdehyde levels were significantly higher and antioxidants were significantly lower in (14/32) 43.75% (Group II) OPL patients where as no significant changes were observed in the remaining patients (18/32) 56.25% (Group I) when compared to controls. Levels of methylglyoxal and specific cancer markers in established cases of malignant disease are shown in Table 1II. Measurement of serum MG levels in suspected OPL- patients will help us to screen the subjects with or without risk of malignant transformation as decreased MG levels increases the risk of malignant change. These high risk subjects could be referred to specialized centers for further evaluation. This could be used as a screening marker which is simple and cost effective.

**Key words:** Methylglyoxal, Superoxide dismutase, Catalase, Glutathione Peroxidase, Malondialdehyde, Oral Precancerous lesions, Malignant disease, India

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### **Introduction:**

Methylglyoxal (MG), also called pyruvaldehyde is formed as a product of several metabolic pathways; however the impor

tant source is glycolysis [1]. It is highly toxic compound which is detoxified by glyoxylase enzyme system to non-toxic form as lactate [2]. Elevated MG levels are observed in diabetes as a precursor of advanced glycation end product

(AGE) [3]. Earlier studies have acclaimed methylglyoxal not only as toxic compound [4], but also as potent anti cancer agent [5]. In 1963, Szent-Gyorgi identified methyl glyoxal and named as 'Retine', which inhibited the rapidly multiplying cells in cancer without hampering the normal cells. It was in 1967, when this molecule was first synthesized in his laboratory as carbonyl compounds which were sensitive to cancer cells than the normal ones, thus exploiting its advantage in controlling the cancer.

Further scientists established that the crux to control the cancer cell was by depleting their energy source which enables them to divide at faster rate, which was only possible when ATP synthesis was inhibited or depleted in those cancer cells [6]. Till late, evidence of methylglyoxal as cancer cell inhibitor were tried in animal models, but it was 2000, when clinical trials were conducted in humans by Indian cancer researchers from Kolkata <sup>(7)</sup>. Their clinical trials showed that methylglyoxal selectively killed the cancer cells without affecting the normal cells, although much more is established as methylglyoxal levels being elevated in oxidative stress, aging process and in diabetes as AGEs <sup>(4, 8, 9, 10, 11)</sup>.

Methylglyoxal is frequently generated under both physiological and pathological conditions. In animal cells the principal route for methylglyoxal catabolism is the glyoxalase pathway, which consists of two enzymes, glyoxalase I (EC 4.4.1.5, lactoylglutathione lyase) and glyoxalase II (EC 3.1.2.6, hydroxyacylglutathione hydrolase). This pathway converts methylglyoxal to d-lactate via S-d-lactoylglutathione <sup>(11)</sup>; reduced glutathione is a required cofactor. Despite advances in understanding the systemic effects of biogenic methylglyoxal, its prominence in metabolic network is still unearthed.

It is already established that oral premalignant lesions are associated with pathological changes in the linings of the mouth and upper aero digestive tract which are not cancerous, but have potency to become cancerous. If these cancerous cells are diagnosed in right time, then the future risk of cancer could be prevented. In India the detection of new lesions of cancer is shown to occur in 0.8/ 1,000,000 persons annually <sup>(12)</sup>, compared to an oral cancer incidence rate of 5/1,000,000 in the same population. The huge economy of the country is utilized in early detection and management of cancer patients. Since no biochemical test is ascertained till date for delineating the malignant potency of cancer cell, however antioxidant therapies are employed as evidence based medicine to patients who has more propensity to have future risk of cancer.

Considering the view points and established facts from all the observational and scientific studies on cancer, the current study was designed to evaluate the potential role of methylglyoxal in oral premalignant lesions as well as malignant disease. The study was conducted to observe the serum concentration of methyl glyoxal in patients and its correlation with other parameters of oxidative stress. The finding of the current study was compared with the serum concentration of methylglyoxal with established cancer markers in patients

with malignant diseases confirmed by histopathological examination.

## Materials and Methods

Ninety-seven subjects were enrolled for the present study with ages ranging from 20-40 years which included thirty-two patients with oral premalignant lesions, thirty-two patients of confirmed malignant disease and the rest thirty-three subjects were healthy controls. The premalignant and malignant cases were confirmed by histopathological examination. The details about the groups are furnished in Table 1.

### Inclusion Criteria

Patients suffering from oral premalignant lesions and established malignant disease were included in the study.

### Exclusion Criteria

Patients with diabetes mellitus, hypertension, dyslipidemia, smokers were excluded. Also patients on lipid lowering drugs and antioxidant vitamin supplements were also excluded.

Serum methylglyoxal, catalase, superoxide dismutase, glutathione and malondialdehyde were determined in all the participants of the study using standard methods.

### Serum Methylglyoxal assay

Methylglyoxals are estimated according to the modified method of Racker <sup>(13)</sup>. Twenty-five  $\mu$ l of serum samples was added to 350  $\mu$ l of DNPH [0.1% DNPH in 2N HCl]. Then to each tube 2.125 ml of distilled water was added. Then it was incubated for 15 minutes at 37<sup>0</sup> C. After the incubation 1.5 ml 10% NaOH was added and absorbance was read at 576 nm using Microlab 200 semi-auto analyser. The results are calculated from the standard curve.

### Catalase assay

The catalase assay was carried out by the method of Aebi (1984) <sup>(14)</sup>. One milliliter of serum was taken with 1.9 ml of phosphate buffer in different test tubes (125 mM, pH 7.4). The reaction was initiated by the addition of 1 ml of hydrogen peroxide (30 mM). Blank without serum was prepared with 2.9 ml of phosphate buffer and 1 ml of hydrogen peroxide. The decrease in optical density due to decomposition of hydrogen peroxide was measured at the end of 1 min against the blank at 240 nm. Units of catalase were expressed as the amount of enzyme that decomposed 1  $\mu$ M H<sub>2</sub>O<sub>2</sub> per minute at 25°C. The specific activity was expressed in terms of units per milligram of protein.

### SOD assay

The assay of SOD was based on the reduction of NBT to water insoluble blue formazan, as described by Fedovich (1976) <sup>(15)</sup>. Serum (0.5 ml) was taken and 1 ml of 125mM sodium carbonate, 0.4 ml of 24 $\mu$ M NBT, and 0.2ml of 0.1mM EDTA were added. The reaction was initiated by adding 0.4ml of 1 mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560 nm followed by recording the absorbance after 5 min at 25°C. The control was simulta-

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neously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required inhibiting the reduction of NBT by 50.0%. The specific activity was expressed in terms of units per milligram of protein.

### Glutathione peroxidase assay (GSH-PX)

GSH-PX was estimated by the method of Paglia and Valentine (1967) <sup>(16)</sup>. The principal of the assay was based on catalyses the oxidation of glutathione (GSH) by cumene hydroperoxide. In the presence of glutathione reductase (GR) and NADPH the oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP<sup>+</sup>. The decrease in absorbance at 340 nm is measured.

### Lipid peroxidation assay

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532 nm (Buege and Aust, 1978) <sup>(17)</sup>. Liver homogenate (0.5ml) and 1ml of 0.15M KCl were taken. Peroxidation was initiated by adding 250  $\mu$ L of 0.2 mM ferric chloride. The reaction was run at 37 °C for 30 min. The reaction was stopped by adding 2ml of an ice-cold mixture of 0.25 N HCl containing 15% trichloroacetic acid, 0.30% TBA and 0.05% BHT and was heated at 80°C for 60min. The samples were cooled and results were expressed as MDA an equivalent, which was calculated by using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ . One unit of lipid peroxidation activity was defined as the amount of TBA that converts to thiobarbituric acid reac-

tive substances (TBARS). The specific activity was expressed in terms of units per milligram of protein.

### Determination of specific cancer markers

The prostate specific antigens-Total (TPSA) and Carcinoembryonic antigen (CA-125) levels in established cancer patients were determined by standard ELISA kit obtained from Ranbaxy Diagnostics.

The study was conducted for a period of six months from December 2009 till May 2010. The design of this study was pre-approved by the institutional ethical committee board of the Institution and informed consent was obtained from the participants.

## Results

Serum methylglyoxal levels were significantly higher ( $p < 0.001$ ) in patients with oral premalignant lesions where as significantly lower ( $p < 0.001$ ) in patients with established malignant diseases compared to healthy controls (Table 1).

Serum malondialdehyde levels were significantly higher in 43.75% (group II) patients with oral precancerous lesions associated with significantly lower ( $p < 0.001$ ) levels of antioxidants in them compared to healthy controls where as no significant changes in oxidative stress parameters were observed in 56.25% (group I) patients (Table II).

Levels of methylglyoxal and specific cancer markers in established cases of malignant disease are shown in Table III.

**Table 1.** Comparison of Serum Methylglyoxal levels in cancer patients compared to healthy controls

Serum Methyl Glyoxal levels ( $\mu\text{mol/l}$ )			
Healthy Controls (n=33)	Patients with oral premalignant lesion (n=32)	Patients with malignant disease (n=32)	P Value
102 $\pm$ 12	133.5 $\pm$ 13*	66.6 $\pm$ 12*	< 0.001

\* indicates the level of significance ( $p < 0.001$ )

**Table 2.** Oxidative stress markers in oral premalignant lesion patients compared to healthy controls

Parameters	Healthy Controls (n=33)	Group I patients (n=18)	Group II patients (n=14)	P Value
MDA ( $\mu\text{mol/l}$ )	1.4 $\pm$ 0.7	1.5 $\pm$ 0.8	3.1 $\pm$ 0.6*	< 0.001
GSH ( $\mu\text{mol/l}$ )	97.4 $\pm$ 6.4	103 $\pm$ 14.2	28 $\pm$ 6*	< 0.001
SOD ( $\mu\text{mol/l}$ )	56 $\pm$ 3.8	52 $\pm$ 5	26 $\pm$ 7.2*	< 0.001
Catalase (U/L)	104 $\pm$ 9.2	110 $\pm$ 12.3	40.6 $\pm$ 8.1*	< 0.001

\* indicates the level of significance between group I and II patients

Group I and Group II patients are oral premalignant lesions patients

**Table 3.** Levels of Methylglyoxal and Cancer Markers in some malignant patients

Serial number:	Age	Serum MG levels( $\mu\text{mol/l}$ )	Marker levels
<i>Ca Prostate</i>			
1	65	51.2	76.9 †
2	74	55.1	96.1 †
3	77	55.1	47.2 †
4	57	59.1	40.7 †
5	58	59.1	42.4 †
6	52	51.2	66.8 †
7	70	63.0	29.9 †
8	54	47.3	61.2 †
9	55	35.5	107.1 †
<i>Breast Cancer</i>			
1	38	70.9	99.7 ‡
2	48	70.9	96.8 ‡
3	38	82.7	62.6 ‡
4	63	27.6	183.5 ‡

† *TPSA levels (ng/ml)*; ‡ *CA-125 levels (U/ml)*

## Discussion

The current study was conducted to determine the serum methylglyoxal (MG) levels in patients with oral premalignant lesions (OPL) and malignant diseases which were compared with healthy controls. Our study observed significantly higher levels of MG in the entire OPL patients when compared to controls. Report from the previous studies based on antioxidant and lipid peroxidation status in oral precancerous lesion (OPL) patients observed elevated peroxides and depleted antioxidants with concomitant increase in serum methylglyoxal in all OPL patients<sup>(4, 8, 9, 10, 11)</sup>. Our study did not conform with their findings as we observed only 43.75% of OPL patients had increased oxidative stress and depletion of antioxidants which is justified by significantly higher levels of malondialdehyde (MDA) and lower levels of superoxide dismutase, catalase and glutathione peroxidase compared to healthy controls.

Study conducted by Subapriya et al on oral precancer patients<sup>(18)</sup> observed enhanced lipid peroxidation and depletion of antioxidants levels in preoperative compared to post operative oral precancerous lesions patients. They observed decreased lipid peroxides and increased antioxidants levels twelve weeks after surgery. So it is evident that cancer is linked with derangements of oxidants and antioxidants. Another study conducted by Khanna et al on twenty new cases of oral squamous cell carcinoma observed similar facts on oxidants/antioxidants misbalance. Both the above mentioned study does not conform with the current study as 56.25 % of OPL patients had increased serum MG levels without oxidative stress. Thus our study confirms the multifactorial nature and etiopathogenesis of OPL. There might be several unrevealed factors other than oxidant/antioxidant imbalance which could elevate MG levels in OPL patients<sup>(19)</sup>.

Earlier studies on cancer patients observed significantly higher levels of methylglyoxal (MG) in established cancer patients compared to controls<sup>(20)</sup> which is not in accordance with the current study since significantly decreased

( $p < 0.001$ ) MG levels was observed in malignant disease. As per published findings, methylglyoxal is toxic to cells which are involved in numerous pathologic processes *in-vivo*, causing carcinogenesis. Earlier studies conducted (Takahashi et al., 1989) on an experimental animal models proved methylglyoxal as a key molecule in carcinogenesis which has tumor promoting activity in adenocarcinomas of stomach in rat. Evidence based on their experiment observed sudden increase in glyoxalase I and II enzymes, a protective mechanism against methylglyoxal, when animal models were fed on diet containing 3'-methyl-4-dimethylaminoazobenzene to induce carcinogenesis. Apparently they observed drastic reduction of glutathione levels in animal models, which justifies the fact when cells are exposed to carcinogens; it causes induction of methylglyoxal metabolizing enzyme to prevent the cells proceeding to cancerous state from precancerous state<sup>(21)</sup>.

In another study the toxicity of methylglyoxal (MG) causing neuroblastoma was protected by phenolic acid which further proves the role of MG causing cancer<sup>(22)</sup>. Contrary to the above study, researchers suggested the growth inhibitory, anticancer and tumoricidal properties of methyl glyoxal<sup>(23)</sup>.

Study conducted elsewhere reported glyoxylase being as a pro-survival factor of the p53 family and plays a critical role in the normal development and in the pathogenesis of various diseases including cancer by controlling the levels of methylglyoxal in cells<sup>(24)</sup>.

Based on the findings of the above studies it can be presumed that alteration of MG level occurs at the time of oxidative stress which is involved in the pathogenesis of cancer but at times it acts as a protective mechanism as we observed in our study. The MG level alters at the stage of cell transformation from precancerous state to cancerous state indicating the link in early detection of cancer in its premalignant stage.

Our study is an eye-opener since it points the alarming nature of cancerous cells much before they exhibit clinical

signs and symptoms just by determining methylglyoxal levels by simple colorimetric measurement. This has merits of being adopted even in rural health set up due to its cost effectiveness and affordability to lower socio economic strata of population. Though previous studies suggested increased levels of methylglyoxal is a phenomenon observed in oxidative stress, but our study presumes it as cell cycle checker in controlling the cell proliferation.

Its levels become optimized to safe guard the cellular proliferation but no sooner it fails the level of methylglyoxal drops back to normal. However, this is a preliminary report of our study. We are looking forward to perform an *in vitro* study in future in cell culture model of oral cancer cell line (HSC2 or HSC3 etc ) and stimulate with MG in varying doses and perform a simple flow cytometry analysis to see if cell cycle is perturbed. That would enable us with better understanding of real effect of MG on these cells. The drawback of our study is small sample size but our findings are justified by comparing the levels of MG in some established cases of prostate and breast cancer diagnosed with the specific cancer markers (Table III).

## Conclusion

Measurement of serum MG levels in suspected OPL- patients will help us to screen the subjects with or without risk of malignant transformation as decreased MG levels increases the risk of malignant change. These high risk subjects could be referred to specialized centers for further evaluation. This could be used as a screening marker which is simple and cost effective.

## Limitations of the study

The results obtained from the current study is a tip of the iceberg though the study has limitations of involving less number of participants due to financial constraints and duration of the study.

## Future directions of the study

In future a multicentre study should be carried out involving large sample size to support the findings of the current study.

## What this study contributes

Measurement of methylglyoxal levels in cancer prone patients at the right time will avoid them from future cancer risk. As the measurement of methylglyoxal involves simple colorimetric assay, so it is cost effective and can be done in small laboratories. Also it can be used for screening of large scale population.

## Conflict interest

The authors do not have any conflict interest from this study.

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