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Methyl glyoxal elevation is associated with oxidative stress in rheumatoid arthritis

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Abstract

Methyl glyoxal (MG), a metabolic hazard plays a role in pathogenesis of different diseases. We studied the role of MG in cellular oxidative and carbonyl stress in rheumatoid arthritis (RA).

148 RA patients were divided into subgroups according to disease severity, RA factor status and age. They were acute, remission, seropositive, seronegative and JRA group. About 88 normal, young, healthy individuals were taken as control. We estimated serum level of total antioxidant status (TAS), total thiol, GSH, MG, carbonyl compounds and TBARS level of normal control and RA. The synovial fluid (SF) level of above parameters have been also evaluated in RA.

Our observation suggests that MG elevation is associated with increased level of TBARS and decreased level of GSH in all RA subgroups than normal control.

The elevation of MG along with declination of GSH and antioxidant status may be associated with free radical damage in RA.

Keywords: *Methyl glyoxal, free radical, rheumatoid arthritis, total antioxidant status, reduced glutathione*

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory joint disorder and thought to be autoimmune in nature. Here the inflammation is probably induced by the combined action of autoantigen stimulated polymorphonuclear leukocytes, T and B cells and monocytes/macrophages which synthesize and release large amount of reactive oxygen species (ROS). Mediators like prostaglandins, leukotriene, histamine, platelet activating factor and interleukins enhance the inflammatory response in RA [1,2] and promote bone resorption. The pannus of the cartilage in RA joints contains many macrophage like cells which liberate $O_2^{\cdot-}$, H_2O_2, peroxynitrite ($ONOO^-$) which are responsible for tissue injury during chronic inflammation [3].

Methyl glyoxal (MG) is a small ketoaldehyde compound which is electrophilic in nature and an endogenous metabolic hazard, derived from decomposition of glycolytic intermediate *triosephosphate* [4] and biosynthesis by MG *synthetase* [5]. It can be also formed from oxidative decomposition of fatty acids [6] or from catabolism of ketone bodies like acetone [7] and threonine [8]. MG is detoxified to nontoxic D-lactate by reduced glutathione dependent glyoxalase I and II enzyme pathways [9].

MG plays a major role in promoting oxidative stress and carbonyl stress in human body [10,11]. MG reacts with amine group of lysine, arginine, histidine and cysteine residues of proteins [12] and glycates them to form advanced glycation endproduct (AGE) [13]. Thus MG contributes to the aging of proteins and produces *N-ϵ-(carboxyethyl)lysine* (*CEL*), the

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toxic metabolite which generates free radicals by further oxidation. AGE increases the cellular damage by increasing ROS production [14]. Thus, MG plays a definite role in oxidative stress in human body.

AGE accumulation as well as MG elevation have been already reported in diabetes mellitus [15] and although hyperglycemia is not associated with RA but accumulation of AGE in cartilages of RA have been reported [16]. But the report on the level of MG and its correlation with the cellular damage in RA are yet to be published.

In our study, we have subdivided the RA patients according to severity and also according to the status of RA factor. We studied the total antioxidant level and carbonyl stress of different subgroups and tried to draw the correlation between the increased cellular damage and severity of the disease.

We have also studied the correlation between MG level and cellular damage according to the severity of the disease. The possible reason behind the elevation of MG has also been discussed in this study. As far as our knowledge goes, this is the first report of the MG level in synovial fluid (SF) in RA.

Materials and methods

Experimental design and sample collection

A total of 148 RA patients participated in the study who came for their treatment at the in and out patient Department of Rheumatology Unit of NRS Medical College & Hospital, Kolkata, India. Following the revised American Rheumatism Association (ARA) criteria [17], they were divided into five subgroups according to the severity of the disease, status of RA factor and juvenile rheumatoid arthritis (JRA) group. Simultaneously 88 healthy, normal, age and sex matched people were selected randomly for the study as normal control.

Blood (5 ml) from both patients and normal people was collected aseptically and serum was separated by centrifuging the blood at 5000 rpm for 5 min and then processed for further assays. With patients' consents, SF was collected from the knee joints of RA patients with effusion. SF could be collected from 12 patients.

Estimation of different biochemical parameters

Total antioxidant status (TAS). TAS was estimated according to Re et al. [18], based on the inhibition of radical cation $ABTS^{+}$, which has a characteristic long wavelength absorbance maxima at 734 nm. ABTS radical cations are formed by interaction with potassium persulphate.

Total thiol. Thiol level was estimated spectrophotometrically, based on DTNB method [19].

GSH. Reduced glutathione is measured according to the method of Miao-Lin-Hu [19]. This is a fluorimetric method based on a fluorescence dye *O*-phthalaldehyde and fluorescence intensity is measured at 420 nm, exciting at 350 nm.

Carbonyl compounds. Carbonyl compounds were measured spectrophotometrically using 2,4-dinitro phenyl hydrazine (DNPH) reagent in alkaline condition [20].

MG. MG was estimated spectrophotometrically, based on 1,2 di-amino benzene following Ghosh et al. method [21] which is a modified method of Cordeiro et al. [22].

TBARS. The cellular damage can be measured by estimating MDA like compounds which is called *thiobarbituric* acid reacting substances (TBARS). This is a spectrophotometric assay based on *thiobarbituric* acid (TBA) reaction, read at a wavelength of 532 nm following Okhawa method [23].

Statistical analysis

The results of different assays of RA patients and normal control were subjected to statistical evaluation. The evaluation was not only done in between the normal control and each subgroup of RA but also in between the RA subgroups. The mean values and standard errors were computed for the different biochemical parameters of both patients and normal control. Correlations and significances of the serum variables were determined by Pearson correlation. Differences in mean values of the variables between RA patients and normal control and also between the RA subgroups were analyzed with the two sample *t*-test. Significance value was considered as $p < 0.05$.

Results

148 RA patients, coming from the eastern part of India and neighboring country Bangladesh were included for the study and divided into five subgroups. The name of the groups, number of patients in each group, mean ($\pm$ SD) age of each group and distribution of sex are given in Table I. None of them had any other co-morbid illness and they did not receive any antioxidant drug immediately before the study.

Clinical observations

According to severity, 117 of total RA patients (JRA not included), have been divided into two following groups:

Table I. Shows the different groups of RA patients and normal control with their total number, age (mean ± standard deviation) and sex distribution.

Groups	Total	Male	Female	Age (year) (mean ± SD)
Normal control	88	51	37	32 ± 10.3
RA	148	39	109	39.1 ± 12.1
Acute	58	10	48	35.42 ± 12.9
Remission	59	14	45	41.6 ± 10.6
Seropositive	56	13	43	38.31 ± 12.9
Seronegative	49	10	39	32.73 ± 13.5
JRA	31	15	16	13.1 ± 2.8

1. *Acute group*: at the time of presentation, the patients who were in acute or severe disease condition and under no medication, have been included in this group. These patients belonged to less than 2 years duration. Most of them had swelling of the small joints of hand with spindling as the only deformity. They had mostly metacarpophalangeal (MCP) and proximal interphalangeal (PI) joint involvement (85–90%), followed by knee, ankle, wrist and subtalar joint involvement in 78, 72, 76 and 69%, respectively. Cervical spine and hip joint were involved in 35 and 40% of the patients. Temporo-mandibular in 23% and shoulder joint was involved in 12% patients. Overall 18% patients had monoarticular disease. About 30–35% of the RA patients had knee joint or ankle joint effusion that could be aspirated.
2. *Remission group*: Patients who were in remission stage and when presented, had late disease with mean disease duration of 5.6 years, belonged to this group. Most of these patients were receiving non steroidal anti-inflammatory drugs (NSAIDs), some with intermittent mono or combination of Disease Modifying Anti Rheumatoid Drugs (DMARD's, i.e. methotrexate, sulfasalazine, hydroxychloroquine, etc.) with poor compliance and poor affordability. About 75% of these patients had some forms of deformities with radiographic evidence of joint destruction.

According to the status of RA factor, 105 of total RA patients (JRA not included) have been divided into two following groups and 12 patients had no available data of RA factor status.

1. *Seropositive group*: The patients who had positive RA factor in their blood, belonged to this group.
2. *Seronegative group*: The patients with negative RA factor were included in this group.

According to age and other criteria, the patients were grouped as JRA group and the number of JRA patients out of 148 was 31.

JRA group: The RA patients who fulfilled the criteria for JRA, belonged to this group. The mean age and sex distribution of this group have been mentioned in Table I. About 25% of the JRA patients were seropositive and rests were seronegative. Thirty five per cent of the JRA patients had polyarticular disease. Twenty seven per cent patients had systemic features like lymphadenopathy, spleenomegaly and rash. JRA patients had early and aggressive disease course with deformities particularly in those who did not get any treatment earlier. Multiple large joint deformities were more common in JRA patients, present in 45% cases. Most of the JRA patients were under no treatment and rest received very irregular and symptomatic treatment schedule. JRA patients had overall more extra-articular features compared to other RA patients. Extra-articular features like iritis, rheumatoid nodules, ulcers, rash etc were present in 45% of JRA patients compared to only 12% of other RA patients.

Normal controls were mainly healthy individuals without any illness and other confounding factors and they also did not receive any antioxidant drug before presentation.

Analysis of different biochemical parameters

Different biochemical parameters like TAS, thiol content, reduced-glutathione, carbonyl compounds, MG and TBARS level have been estimated in normal and RA serum and SF of RA.

TAS expresses the overall picture of antioxidant status of serum. Actually TAS is the sum of all the various antioxidants found in the serum regardless of their origin.

In RA, the mean TAS is 0.58 ± 0.02 mM which has been dropped down by 56% ($p < 0.005$) than the normal control. Possibly it indicates the increased ROS level in RA patients cannot be scavenged due to the poor TAS of serum which facilitates every kind of oxidative damage in RA, resulting in the severe pathogenesis.

The increased ROS attack the lipoproteins, deoxyribose, etc. and produces MDA like compounds which is measured by estimating TBARS. The mean concentration of normal TBARS level is 1.96 ± 0.05 nmol/ml (mean ± SEM) which has been significantly ($p < 0.005$) increased by 118% in RA where the mean (± SEM) concentration of TBARS is 4.28 ± 0.40 nmol/ml. Poor TAS probably supports the evidence of increased TBARS level in RA.

Mean thiol concentration in normal serum is 568 ± 26.7 μM which has been decreased by 37% in RA, being 360 ± 18.6 μM ($p < 0.005$).

Reduced glutathione level has been very significantly ($p < 0.005$) declined in RA. Mean concentration (± SEM) of GSH is 5.88 ± 0.28 μM in normal serum and 3.64 ± 0.1 μM in RA. Low GSH level not

Table II. Shows mean (± SEM) concentration of different oxidant and antioxidant parameters in SF of RA patients.

Biochemical parameters	Concentration (mean ± SEM)
TAS (mM)	0.42 ± 0.17
Total thiol (μM)	91.1 ± 23
GSH (μM)	1.15 ± 0.29
Carbonyl compounds (nmol/ml)	196 ± 50
MG (nmol/ml)	14.6 ± 3.13
TBARS (nmol/ml)	2.6 ± 0.45

only indicates the poor antioxidant level of RA patients but also plays an important role to maintain the level of MG in serum, described in the discussion part.

Carbonyl compounds are found to be increased by 47% in RA compared to normal control. It is 72.5 ± 2.3 nmol/ml (mean ± SEM) in normal control and 107 ± 6.2 nmol/ml (mean ± SEM) in RA.

The most interesting finding in the present work is that MG increases in RA patients by 76% than normal control which is statistically highly significant ($p < 0.005$). The mean (± SEM) concentration of MG level in normal control is 4.77 ± 0.34 and 8.41 ± 0.77 nmol/ml in RA.

The variables of SF of RA patients have been given in the Table II, but as there is no control of SF (because normal SF is not available and osteoarthritic SF shows more oxidative damage than the SF of RA; (unpublished data)), no comparison or no statistical analysis could have been drawn against the data of SF in RA. But the antioxidant parameters of SF have been found to be depleted and oxidative stress factor like TBARS level, have been found increased than the control serum. Surprisingly carbonyl compounds and MG level have been observed to be elevated very high in SF which are not reported earlier.

Comparison of different variables of serum among subgroups of RA

Acute and remission group. Table III shows the mean concentration (± SEM) of different parameters of acute and remission group of RA and their significance levels have been mentioned in Table IV. It is clear from the data of Tables III and IV, that the TAS and thiol content are strikingly lower in acute patients than the remission ones whereas carbonyl compounds and TBARS levels are higher.

Not only that, here the GSH level is less (1.6-fold), simultaneously MG level has been found higher by 1.76-fold in acute group than the remission group of RA.

Seropositive and seronegative group. Table III shows the comparison between the mean concentration (± SEM) of serum antioxidants and oxidative damage indicating factors among normal, seropositive and seronegative group. The significance levels of parameters between normal and each subgroup and also between the subgroups themselves have been mentioned in Table IV.

From Table III it is evident that the antioxidant markers like thiol content and TAS are in better status in seronegative group than the seropositive one and accordingly carbonyl compound level is also significantly higher in seropositive group. Though MG and TBARS levels are higher in seropositive patients but not statistically significant.

From the clinical history of patients we have observed that the seropositive patients who are in acute disease condition remain in lower profile of antioxidant status than the acute but seronegative patients.

JRA group. The antioxidant levels and oxidative stress factors are compared between the JRA group and normal controls and the significance levels of different parameters between normal control and JRA group have been shown in Table V.

In JRA patients, increase of carbonyl compounds, MG and TBARS levels are highly significant simultaneously TAS, thiol content and GSH levels have been found significantly low, compared to normal control.

Discussion

There are growing evidences to indicate that elevated oxidative stress plays significant role in tissue damaging and inflammation perpetuating processes in RA [24,25]. It is clear from our extensive clinical study that the oxidative and carbonyl stress are directly correlated with the severity and prognosis of the disease RA. When the disease is in control position (remission group), the antioxidant status is in better level, compared to onset situation (acute group). Although the significant difference is not found regarding the cellular damage (as shown by TBARS level) still the seronegative group of patients remain in higher degree of TAS than seropositive ones. Whether RA factor plays any role in oxidative stress is not clearly understood from our study.

In JRA, the antioxidant markers are depressed and cellular damage markers are highly elevated yet TBARS level is not as much high as acute or seropositive group (though the mean value is statistically significant than normal control). Probably JRA patients are getting advantage of anabolic repair for their young age to cope up with the increased ROS and compensate the cellular damage.

The elevation of MG and its related clinical significance in RA seems to be of paramount interest. The accumulation of MG in RA is surprising. It has

Table III. Shows comparative study of mean concentration (± SEM) of different biochemical parameters of normal control and acute, remission, seropositive and seronegative subgroups of RA.

Parameter	Group				
	Normal control	Acute	Remission	Seropositive	Seronegative
TAS (mM)	1.33 ± 0.07 ($n = 52$)	0.59 ± 0.03 ($n = 41$)	0.67 ± 0.04 ($n = 35$)	0.62 ± 0.03 ($n = 38$)	0.65 ± 0.05 ($n = 32$)
Total thiol (μM)	568 ± 27 ($n = 52$)	301 ± 15 ($n = 37$)	419 ± 34 ($n = 32$)	321 ± 16 ($n = 36$)	400 ± 32.7 ($n = 35$)
GSH (μM)	5.88 ± 0.28 ($n = 35$)	3.7 ± 0.25 ($n = 37$)	4.37 ± 0.25 ($n = 27$)	3.95 ± 0.2 ($n = 36$)	4.26 ± 0.26 ($n = 31$)
Carbonyl compounds (nmol/ml)	72.5 ± 2.3 ($n = 51$)	114 ± 9.1 ($n = 42$)	99.5 ± 8.7 ($n = 37$)	109 ± 5.4 ($n = 42$)	87.9 ± 5.8 ($n = 33$)
MG (nmol/ml)	4.77 ± 0.34 ($n = 34$)	10.78 ± 1.48 ($n = 14$)	6.54 ± 0.78 ($n = 20$)	8.79 ± 1.0 ($n = 18$)	7.71 ± 1.38 ($n = 16$)
TBARS (nmol/ml)	1.96 ± 0.1 ($n = 53$)	4.81 ± 0.56 ($n = 43$)	3.21 ± 0.36 ($n = 29$)	4.63 ± 0.54 ($n = 43$)	3.96 ± 0.42 ($n = 32$)

Table IV. Shows the statistical significance levels of different variables of RA patients. Here the mean concentration of each variable of each subgroup has been statistically correlated with the normal control and significance level has been also evaluated between acute vs. remission and seropositive vs. seronegative subgroups.

Parameter	Group					
	Normal vs. acute	Normal vs. remission	Acute vs. remission	Normal vs. seropositive	Normal vs. seronegative	Seropositive vs. seronegative
TAS (mM)	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.01$
Total thiol (μM)	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.01$
GSH (μM)	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$	n.s.
Carbonyl compounds (nmol/ml)	$p < 0.005$	$p < 0.005$	n.s.	$p < 0.005$	$p < 0.005$	$p < 0.01$
MG (nmol/ml)	$p < 0.005$	$p < 0.025$	$p < 0.01$	$p < 0.005$	$p < 0.01$	n.s.
TBARS (nmol/ml)	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$	$p < 0.005$	n.s.

n.s: not significant.

Table V. Comparative analysis of the mean concentration (Mean ± SEM) of antioxidant and oxidant parameters between JRA group and normal control and also their significance level.

Parameter	Group		
	Normal control	JRA	Normal Vs. JRA
TAS (mM)	1.33 ± 0.07 ($n = 52$)	0.59 ± 0.08 ($n = 18$)	$p < 0.005$
Total thiol (μM)	568 ± 27 ($n = 52$)	311 ± 19.2 ($n = 19$)	$p < 0.005$
GSH (μM)	5.88 ± 0.28 ($n = 35$)	3.73 ± 0.25 ($n = 19$)	$p < 0.005$
Carbonyl compounds (nmol/ml)	72.5 ± 2.3 ($n = 51$)	98.81 ± 11.2 ($n = 20$)	$p < 0.005$
MG (nmol/ml)	4.77 ± 0.34 ($n = 34$)	9.26 ± 1.6 ($n = 9$)	$p < 0.005$
TBARS (nmol/ml)	1.96 ± 0.1 ($n = 53$)	2.99 ± 0.25 ($n = 16$)	$p < 0.005$

n.s: not significant.

been reported that its level rises significantly in diabetes mellitus and very recently we have also reported the higher level of MG is associated with poor level of glutathione in diabetes mellitus [26]. But in normoglycemic disease like RA, the mechanism of its formation is possibly quite different.

Level of MG in serum and SF has been increased in all RA subgroups when compared to normal control. It is also found that GSH level has been decreased significantly in all groups accordingly and Figure 1 shows it is inversely related to MG concentration in RA.

Thiols by virtue of their ability to reversibly oxidized are recognized as key components involved in the maintenance of redox balance in cellular system. GSH, an important thiol containing compound functions in diverse role such as regulating anti oxidant defences, xenobiotics and in the redox regulation of signal transduction [27].

GSH is not only the potent antioxidant, it also plays a role in MG catabolism. MG reacts spontaneously with GSH and forms the intermediate hemimercaptal derivative which is the substrate of glyoxalase I. So GSH prevents MG accumulation in the body, thus minimizes the toxicity of MG and may reduce the cellular damage.

From our study it can be thought that in due course of oxidative stress, GSH is declined and facilitates MG accumulation. But accumulated MG may further uses GSH for its degradation which in turn further suppresses the GSH pool in RA.

So it is evident from the discussion that inclined MG level not only promotes the oxidative stress and cellular damage, it causes the imbalance in cellular redox system also. As a result, overall redox balance may be altered in RA and it is reported that altered redox balance can act as a signal for altering cellular fate [27].

MG, being a potent glycating agent, produces AGE like CEL, etc. Verzijl et al. [28] reported the decrease in instantaneous deformation as a measure of the stiffness of collagen network after incubation in MG. So it is clear from this investigation [28] that AGE cross linking, produced by MG, results in increased stiffness of the cartilage collagen network and make it more brittle. Increased stiffness and brittleness may in turn contribute to the failure of cartilage to resist mechanical damage and helps to develop RA pathogenesis.

Recent reports have shown that oxidative stress response pathways are triggered by MG [29] and MG

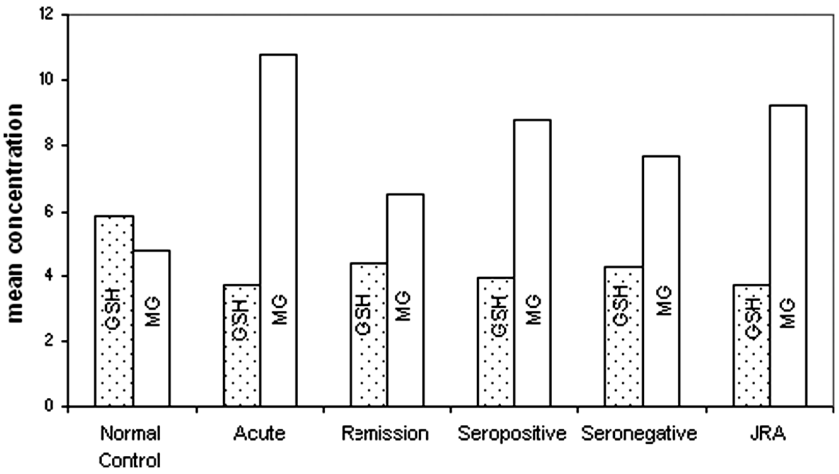

Figure 1. The correlation study between the MG level and GSH level in the RA subgroups. Here is an inverse relation between the serum MG level and GSH level found in RA subgroups in respect to the normal control.

regulated gene expression causes the pathobiology of various situations like cancer and diabetic vascular diseases [30].

Although the precise biological function of this metabolite is uncertain but this potentially important molecule has also significant growth inhibitory [31] and antitumour activity [32].

Thus this paradoxical nature of the molecule arises the question, whether its formation is an undesirable side metabolite or its accumulation in cellular system of certain free radical mediated pathological condition may be a tricky defence mechanism for cell homeostasis and/or signaling for apoptosis.

Thus, we can conclude that level of MG in RA may be a good biomarker to understand the pathophysiological damage and complications. Supplementation of glutathione precursor like N-acetyl-cysteine (NAC) with other antioxidants may be beneficial to prevent cellular damage in RA. Though the short term antioxidant intake cannot influence the overall situation in advanced life forms because it is completely under genetic control [33]. So further work has to be done to know the exact role of MG in RA and the other free radical-mediated disease pathogenesis.

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