In-vitro study of methylglyoxal and aspirin effects on fibrinolysis parameters

Fahima D. Pouya, Javad Zavar-reza and Beman A. Jalali

Methylglyoxal is a reactive α, β dicarbonyl aldehyde compound that originates from various biochemical pathways. Some studies suggest that increased methylglyoxal in blood leads to changes in fibrinolysis; however, the precise mechanism is not clear. The aim of this study was to compare different concentrations of methylglyoxal and aspirin on fibrinolysis in the plasma of healthy individuals *in vitro*. Different concentrations of methylglyoxal (5, 50, 100, and 500 μmol/l) and aspirin (1, 10, and 100 mg/l) were added to the plasma citrate. They were incubated at 37°C for 24 h. Then, fibrinolysis parameters were analyzed by the turbidimetric procedure at 405 nm. The Independent Samples *t*-test was utilized to compare them ($P<0.05$). Findings revealed that methylglyoxal at 500 μmol/l with aspirin 100 mg/l had significant changes in the maximum lysis velocity (0.163 ± 0.003), half-time lysis (240 ± 10.00), the total lysis time (485 ± 5.00), lag time in lysis (126 ± 5.77), compared with methylglyoxal at 500 μmol/l (0.104 ± 0.005), (276 ± 5.77), (570 ± 10.00), and (186 ± 5.77), respectively ($P<0.05$). Methylglyoxal at 500 μmol/l with aspirin 1 mg/l did not significantly change in either parameter ($P>0.05$). Methylglyoxal at 100 μmol/l with aspirin 1 mg/l did not significantly change in either fibrinolysis parameter ($P>0.05$), compared with methylglyoxal at 100 μmol/l. Methylglyoxal at 5 μmol/l with aspirin (1, 10, 100 mg/l) changed in all fibrinolysis parameters ($P<0.05$), compared with methylglyoxal at 5 μmol/l. The other concentrations were compared in the same way. Aspirin (more than 1 mg/l) had more effect on higher concentrations of methylglyoxal. It increased the velocity of lysis of the clot and shortened clot lysis. *Blood Coagul Fibrinolysis* 24:715–718 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Blood Coagulation and Fibrinolysis 2013, **24**:715–718

Keywords: aspirin, fibrinolysis, methylglyoxal

Shahid Sadoughi University of Medical Sciences and Health Services, Yazd, Iran

Correspondence to Dr Javad Zavar-reza, Shahid Sadoughi University of Medical Sciences and Health Services, Yazd, Iran
Fax: +98 351 8202633; e-mail: jzavar@gmail.com

Received 23 February 2013 **Accepted** 23 March 2013

Introduction

Atherosclerosis (AS) is the major cause of coronary artery disease. It chiefly involves the large and middle muscular arteries, especially the aorta, coronary and cerebral arteries [1]. This takes place during a person's lifetime, but the rapid formation depends on risk factors. When the lipid-rich plaques are separated from vessel walls, the vessels will be damaged and thrombus formation will occur. Clotting disorders are the cause of many deaths. Clot formation is the most important cause of the onset of acute coronary syndromes and sudden death caused by ischemia [2,3].

Modern diets can cause modern diseases. Research has linked a metabolite of sugar, methylglyoxal (MGO), to the development of diabetic complications, but the exact mechanism has not been fully elucidated [4]. MGO, which is a very active compound of –α, β dicarbonyl aldehyde [5–7], is formed during cellular metabolism, glucose oxidation, and peroxidation of lipids or carbohydrates are produced in the food and beverages. Highly reactive dicarbonyl attacks the lysine, arginine and cysteine residues of long-lived proteins, to form irreversible advanced glycation end products (AGEs) [8]. An excess of MGO formation can increase ROS (reactive oxygen species) production and cause oxidative stress. MGO reacts with proteins, DNA and other bimolecular, and is a major precursor of AGEs and AGEs are also associated with the aging and age-related diseases [4].

Under physiological conditions, the glyoxalase system degrades MGO and keeps plasma MGO levels low. Hyperglycemia associated with diabetes drives several damage pathways and raises concentrations of the reactive dicarbonyl, MGO that leads to endothelial damage and atherosclerosis [6,9,10]. By western blotting and mass spectrometry fibrin(ogen), and some other proteins were identified as putative main targets for MGO-derived modification [11].

Treatment of plasminogen with methylglyoxal results in the decreased number of NH2 side chains. This structural modification is associated with profound functional alterations. In-vivo fibrinolysis could be impaired under pathological conditions, for example, increased methylglyoxal formation in diabetes mellitus [12]. To prevent this, several compounds have been studied, one of which is aspirin (nonsteroidal anti-inflammatory drug), because aspirin inhibits platelet function used in treating atherosclerotic cardiovascular disease [13]. Aspirin has an anti-inflammatory property that inhibits cyclooxygenase -2(COX-2) which prevents the synthesis of proinflammatory prostaglandins. It increases the endothelial nitric oxide synthesis. Nitric oxide is responsible for

0957-5235 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI:10.1097/MBC.0b013e328361bd68

maintenance and repair of vascular endothelium, and it has a role in preventing cardiovascular syndrome [14,15]. Another function of aspirin is acetylation of fibrinogen that is one of coagulation factors. The structure of acetylated fibrinogen makes clot lysis easier [16].

Thus, because of the importance of cardiovascular disease, the aim of the study was to study the effects of MGO and aspirin on fibrinolysis parameters *in vitro*.

Materials and methods

Isolation of plasma

Blood samples taken from 50 healthy people (aged between 25 and 35 years) who had fasted for 10 h overnight. They were without cardiovascular disorders, allergy and lipid or carbohydrate metabolism disorders, untreated with drugs. Human blood samples were collected into sodium citrate (3.8%) and immediately centrifuged ($4500 \times g$, 5 min) to get plasma. Then, the pooled plasma was frozen at -20°C to be used for fibrinolysis study.

Measuring fibrinolysis parameters

Fibrinolysis was evaluated with a slightly modified method by Williams *et al.* [17]. As 400 μl citrated plasma was diluted at 37°C with 400 μl of a buffer (PBS) (20 mmol/l Na_2HPO_4, 20 mmol/l NaH_2PO_4, 0.15 mol/l NaCl, pH 7.4) containing 25 μl enzyme Streptokinase 750 U/ml (Sigma), 5 μl calcium chloride 2 mol/l (Merck), and 3 μl human thrombin 0.5 U/ml (Sigma). Clot assembly kinetics were monitored spectrophotometrically at 405 nm in duplicate aliquots. Then, by obtaining kinetic curves (OD/time) for aliquots, the maximum lysis velocity (LMV, maximum ΔOD/s), half-lysis time [HLT, (s)], the total lysis time [TLT, (s)] and lag time in lysis [LT, (s)] were determined.

In the next step, a MGO solution with 10 mmol/l was prepared, of which various amounts of MGO (5, 50, 100, and 500 μmol/l) were prepared in 400 μl plasma. They were incubated for 24 h at 37°C with non-MGO plasma. They were analyzed for fibrinolysis parameters.

After that, various amounts of aspirin [acetylsalicylic acid (ASA)] (1, 10, and 100 mg/l) were prepared in 400 μl plasma. They were incubated for 24 h at 37°C with nonaspirin plasma. They were analyzed for fibrinolysis parameters.

Finally, every amount of MGO concentration was collected into different amounts of aspirin in 400 μl plasma consecutively. They were incubated for 24 h at 37°C with different MGO concentrations (5, 50, 100, and 500 μmol/l). They were analyzed for fibrinolysis parameters.

Results

The SPSS software package (SPSS Inc., Chicago, Illinois, USA) was utilized to analyze data by applying Independent Sample *t*-test to compare groups. All the values in this study were expressed as means ± SD ($P < 0.05$).

Measuring methylglyoxal parameters

After collecting MGO into plasma, it was observed that fibrinolysis parameters altered. MGO 500 μmol/l had a significant decrease on LMV (0.104 ± 0.005) compared with the non-MGO group LMV (0.120 ± 0.003) ($P < 0.05$).

It was also observed that HLT (276 ± 5.77), TLT (570 ± 10.00), LT (186 ± 5.77), had significant increase compared with the control group with HLT (195 ± 5.00), TLT (430 ± 4.04), LT (119.00 ± 2.51), respectively ($P < 0.05$) (Table 1).

MGO 50 μmol/l had a significant increase on HLT (244 ± 6.02), TLT (488 ± 12.05), LT (136 ± 5.77), compared with the control group with HLT (195 ± 5.00), TLT (430 ± 4.04), LT (119.00 ± 2.51), respectively ($P < 0.05$). They are reported in Table 3, and results for other concentrations are incorporated in Tables 2 and 4.

Measuring aspirin parameters

In the study, aspirin had significant effects on parameters ($P < 0.05$), that is, when 100 mg/l was applied, LT (70.00 ± 5.00), TLT (290 ± 6.00) and HLT (140 ± 6.02) decreased significantly, but LMV (0.192 ± 0.004) increased significantly compared with the nonaspirin group (Table 5).

Concentrations of 1 and 10 mg/l underwent significant alterations compared with the nonaspirin group as shown in Table 5 ($P < 0.05$).

Measuring methylglyoxal and aspirin parameters

When various concentrations of MGO and aspirin were mixed together, the following results were obtained:

Table 1 Comparison of different concentrations of methylglyoxal (500 μmol/l) with aspirin on fibrinolysis parameters (24 h, 37°C)

Variables	LMV	*P*	HLT	*P*	TLT	*P*	LT	*P*
MGO 500 μmol/l	0.104 ± 0.005	–	276 ± 5.77	–	570 ± 10.00	–	186 ± 5.77	–
MGO 500 μmol/l + ASA 100 mg/l	0.163 ± 0.003	0.000*	240 ± 10.00	0.005*	485 ± 5.00	0.000*	126 ± 5.77	0.000*
MGO 500 μmol/l + ASA 10mg/l	0.113 ± 0.003	0.062	265 ± 5.00	0.057	532 ± 7.21	0.006*	176 ± 5.29	0.078
MGO 500 μmol/l + ASA 1mg/l	0.109 ± 0.005	0.139	270 ± 5.00	0.205	559 ± 9.53	0.240	182 ± 5.29	0.360

ASA, acetylsalicylic acid or aspirin; HLT (s), half-lysis time; LMV (maximum ΔOD/s), maximum lysis velocity; LT (s), lag time of lysis; MGO, methylglyoxal; TLT (s), total lysis time; Variables are given as (mean ± SD). *statistically significant ($P < 0.05$).

Table 2 Comparison of different concentrations of methylglyoxal (100 μmol/l) with aspirin on fibrinolysis parameters (24h, 37°C)

Variables	LMV	*P*	HLT	*P*	TLT	*P*	LT	*P*
MGO 100 μmol/l	0.111 ± 0.003	–	255 ± 5.00	–	526 ± 11.54	–	155 ± 5.00	–
MGO 100 μmol/l + ASA 100 mg/l	0.174 ± 0.005	0.000*	226 ± 5.77	0.003*	471 ± 13.52	0.006*	110 ± 10.00	0.002*
MGO 100 μmol/l + ASA 10 mg/l	0.131 ± 0.010	0.034*	238 ± 8.08	0.041*	485 ± 13.22	0.015*	146 ± 2.88	0.067
MGO 100 μmol/l + ASA 1 mg/l	0.115 ± 0.005	0.398	251 ± 12.58	0.692	520 ± 20.00	0.643	150 ± 10.00	0.482

ASA, acetylsalicylic acid or aspirin; HLT (s), half-lysis time; LMV (maximum ΔOD/s), maximum lysis velocity; LT (s), lag time of lysis; MGO, methylglyoxal; P, P-value; TLT (s), total lysis time; Variables are given as (mean ± SD). *statistically significant ($P < 0.05$).

MGO 500 μmol/l with ASA 100 mg/l significantly decreased HLT (240 ± 10.00), TLT (485 ± 5.00), and LT (126 ± 5.77) but increased LMV (0.163 ± 0.003) compared with the MGO group with 500 μmol/l, as were HLT (276 ± 5.77), TLT (570 ± 10.00), LT (186 ± 5.77), and LMV (0.104 ± 0.005), respectively ($P < 0.05$) (Table 1).

In the group MGO 500 μmol/l with ASA 1 mg/l, there were no significant changes in fibrinolysis parameters compared with the MGO 500 μmol/l group. The comparison of the other concentration is shown in Table 1.

MGO 100 μmol/l with ASA 100 mg/l had significant changes compared with the MGO 100 μmol/l in all fibrinolysis parameters ($P < 0.05$) (Table 2). The comparison of the other concentrations in the group is shown in Table 2.

MGO 50 μmol/l in the three concentrations of ASA significantly changed in all parameters of clot lysis compared with the MGO 50 μmol/l, but LT was not significant as MGO 100 μmol/l with ASA 100 mg/l (Table 3).

MGO 5 μmol/l in the three concentrations of ASA significantly changed in all parameters of clot lysis compared with MGO 5 μmol/l (Table 4).

Discussion

Oxidative stress is one of the pathogenic factors in the development of endothelial dysfunction in experimental models of diabetes [18]. MGO has been shown to increase production of reactive oxygen species (ROS) in other animal models of disease [19]. Apart from directly increasing ROS production, MGO can also increase oxidative stress by inducing AGE formation [20].

In the in-vitro study it was observed that MGO increased the time for clot lysis. Various concentrations of MGO (100 and 500 μmol/l) decreased significantly the maximum velocity of lysis compared with the non-MGO group. Lerant *et al.* [12] showed treatment of plasminogen with methylglyoxal results in the decreased number of NH2 side chains. This structural modification is related with profound functional alterations: the rate of activation by streptokinase, tissue-type plasminogen activator, urokinase-type plasminogen activator and trypsin decreased. In the present work, the other parameters, HLT, TLT, LT significantly increased in the concentrations compared with the non-MGO group. Therefore, increased HLT, TLT, LT are reasons for formation of fibrin clots resistant to lysis in the presence of MGO, therefore, leading to thrombus in patients with high MGO. Lund *et al.* [11] noted that MGO modification may also influence fibrinolysis, as a cleavage site for plasmin between R491 and H492 of the α-chain involved an arginine that was modified *in vitro* by 5 μmol/l MGO, but in the present work, MGO 5 μmol/l showed no significant change in fibrinolysis parameters. It did not mean that there was no change in the concentration, but it was not significant (Table 4).

The study revealed that aspirin properties affected clot lysis. It raised clot lysis, that is, it shortened the time needed for clot lysis and increased the velocity of clot lysis in the presence of MGO. Ajjan *et al.* [13] demonstrated by scanning electron microscopy thicker fibers with looser clot structure when clots were made from aspirin-treated fibrinogen. The lower density of fibrin fibers and larger pores of clots made from aspirin-treated fibrinogen may be important for perfusion of plasminogen and plasminogen activators into the clot, so it makes clot lysis easier. More studies are warranted to fully evaluate the *in vivo* best dose of aspirin in using a monoclonal antibody and acetylation of lysine residues on the α-chain of aspirin-treated fibrinogen [13]. This posttranslational modification in the fibrinogen molecule may lead to changes in charge distribution and possibly conformation [21]. Alternatively, acetylated fibrinogen may affect the rate of conversion of plasminogen to plasmin by tissue plasminogen activator, or it may increase affinity to t-PA or plasmin, resulting in increased

Table 3 Comparison of different concentrations of methylglyoxal (50 μmol/l) with aspirin on fibrinolysis parameters (24 h, 37°C)

Variables	LMV	P	HLT	P	TLT	P	LT	P
MGO 50 μmol/l	0.115 ± 0.005	–	244 ± 6.02	–	488 ± 12.05	–	136 ± 5.77	–
MGO 50 μmol/l + ASA 100 mg/l	0.181 ± 0.007	0.002*	198 ± 18.92	0.016*	403 ± 15.27	0.002*	90 ± 10.00	0.002*
MGO 50 μmol/l + ASA 10 mg/l	0.138 ± 0.003	0.005*	221 ± 7.63	0.016*	448 ± 2.30	0.005*	118 ± 2.30	0.007*
MGO 50 μmol/l + ASA 1 mg/l	0.130 ± 0.005	0.023*	223 ± 5.77	0.012*	463 ± 5.77	0.030*	128 ± 2.88	0.089

ASA, acetylsalicylic acid or aspirin; HLT (s), half-lysis time; LMV (maximum ΔOD/s), maximum lysis velocity; LT (s), lag time of lysis; MGO, methylglyoxal; TLT (s), total lysis time; Variables are given as (mean ± SD). *statistically significant ($P < 0.05$).

Table 4 Comparison of different concentrations of methylglyoxal (5 μmol/l) with aspirin on fibrinolysis parameters (24 h, 37°C)

Variables	LMV	*P*	HLT	*P*	TLT	*P*	LT	*P*
MGO 5 μmol/l	0.121 ± 0.003	–	206 ± 5.77	–	438 ± 7.63	–	125 ± 6.02	
MGO 5 μmol/l + ASA 100 mg/l	0.185 ± 0.005	0.000*	137 ± 7.50	0.000*	388 ± 12.58	0.004*	80 ± 5.00	0.001*
MGO 5 μmol/l + ASA 10 mg/l	0.144 ± 0.006	0.004*	181 ± 10.40	0.022*	401 ± 19.42	0.037*	91 ± 7.63	0.004*
MGO 5 μmol/l + ASA 1 mg/l	0.137 ± 0.007	0.030*	182 ± 6.80	0.010*	412 ± 13.11	0.040*	108 ± 7.63	0.037*

ASA, acetylsalicylic acid or aspirin; HLT (s), half-lysis time; TLT (s), total lysis time; LMV (maximum ΔOD/s), maximum lysis velocity; LT (s), lag time of lysis; MGO, methylglyoxal; Variables are given as (mean ± SD). *statistically significant ($P < 0.05$).

Table 5 Comparison of different concentrations of aspirin on fibrinolysis parameters (24h, 37°C)

Variables	LMV	*P*	HLT	*P*	TLT	*P*	LT	*P*
Control	0.120 ± 0.003	–	195 ± 5.00	–	430 ± 4.04	–	119 ± 2.51	–
ASA 100 mg/l	0.192 ± 0.004	0.000*	140 ± 6.02	0.000*	290 ± 6.00	0.000*	70 ± 5.00	0.000*
ASA 10 mg/l	0.147 ± 0.003	0.001	171 ± 3.51	0.003	356 ± 7.63	0.000*	106 ± 5.29	0.016
ASA 1 mg/l	0.135 ± 0.005	0.012	181 ± 5.77	0.039	390 ± 10.00	0.003*	111 ± 3.60	0.027

ASA, acetylsalicylic acid or aspirin; HLT (s), half-lysis time; LMV (ΔOD/s), maximum lysis velocity; LT (s), lag time of lysis; TLT (s), total lysis time; Variables are given as (mean ± SD). *statistically significant ($P < 0.05$).

rate of lysis [22]. In the present study, it was observed that aspirin 100 mg/l affects all MGO concentrations (5, 50, 100, and 500 μmol/l), that is, fibrinolysis went faster than it was just with MGO. The maximum velocity of lysis increased and decreased the half-lysis, the total lysis time and lag time in lysis. Therefore, aspirin could reduce the effects of MGO on clot lysis and shorten the lysis time.

Conclusion

It may be concluded that aspirin 100 mg/l affects all MGO concentrations (5, 50, 100, and 500 μmol/l), and speeds up fibrinolysis, but aspirin in lower amounts affected MGO 5, 50 μmol/l, however, no change was observed in higher concentrations.

Acknowledgements

No other individuals or institutions contributed to the project financially or by any means.

Conflicts of interest

Any support for the work in the form of grants, equipment, materials, or any combination and financial support of these were provided by the Shahid Sadoughi University of Medical Sciences and Health Services, Yazd, Iran.

References

1 Xu H, Shi D, Chen K. Atherosclerosis: an integrative East-west medicine perspective. *Evid Based Complement Alternat Med* 2012; **2012**:148413.
2 Vilahur G, Padro T, Badimon L. Atherosclerosis and thrombosis: insights from large animal models. *J Biomed Biotechnol* 2011; **2011**:907575.
3 Badimon L, Vilahur G, Padro T. Lipoproteins, platelets and atherothrombosis. *Rev Esp Cardiol* 2009; **62**:1161–1178.
4 Sena CM, Matafome P, Crisostomo J, Rodrigues L, Fernandes R, Pereira P, *et al.* Methylglyoxal promotes oxidative stress and endothelial dysfunction. *Pharmacol Res* 2012; **65**:497–506.
5 Thornalley PJ. Pharmacology of methylglyoxal: formation, modification of proteins and nucleic acids, and enzymatic detoxification–a role in pathogenesis and antiproliferative chemotherapy. *Gen Pharmacol* 1996; **27**:565–573.
6 Desai K, Wu L. Methylglyoxal and advanced glycation endproducts: new therapeutic horizons? *Recent Pat Cardiovasc Drug Discov* 2007; **2**:89–99.
7 Kalapos MP. The tandem of free radicals and methylglyoxal. *Chem Biol Interact* 2008; **171**:251–271.
8 Sassi-Gaha S, Loughlin DT, Kappler F, Schwartz ML, Su B, Tobia AM, *et al.* Two dicarbonyl compounds, 3-deoxyglucosone and methylglyoxal, differentially modulate dermal fibroblasts. *Matrix Biol* 2010; **29**:127–134.
9 McLellan AC, Thornalley PJ, Benn J, Sonksen PH. Glyoxalase system in clinical diabetes mellitus and correlation with diabetic complications. *Clin Sci (Lond)* 1994; **87**:21–29.
10 Wang H, Meng QH, Gordon JR, Khandwala H, Wu L. Proinflammatory and proapoptotic effects of methylglyoxal on neutrophils from patients with type 2 diabetes mellitus. *Clin Biochem* 2007; **40**:1232–1239.
11 Lund T, Svindland A, Pepaj M, Jensen A-B, Berg JP, Kilhovd B, *et al.* Fibrin (ogen) may be an important target for methylglyoxal-derived AGE modification in elastic arteries of humans. *Diab Vasc Dis Res* 2011; **8**:284–294.
12 Lerant I, Kolev K, Gombas J, Machovich R. Modulation of plasminogen activation and plasmin activity by methylglyoxal modification of the zymogen. *Biochim Biophys Acta* 2000; **1480**:311–320.
13 Ajjan RA, Standeven KF, Khanbhai M, Phoenix F, Gersh KC, Weisel JW, *et al.* Effects of aspirin on clot structure and fibrinolysis using a novel in vitro cellular system. *Arterioscler Thromb Vasc Biol* 2009; **29**:712–717.
14 Doutremepuich C, Aguejouf O, Desplat V, Eizayaga FX. Paradoxical effect of aspirin. *Thrombosis* 2012; **2012**:676237.
15 Taubert D, Berkels R, Grosser N, Schroder H, Grundemann D, Schomig E. Aspirin induces nitric oxide release from vascular endothelium: a novel mechanism of action. *Br J Pharmacol* 2009; **143**:159–165.
16 Undas A, Sydor WJ, Brummel K, Musial J, Mann KG, Szczeklik A. Aspirin alters the cardioprotective effects of the factor XIII Val34Leu polymorphism. *Circulation* 2003; **107**:17–20.
17 Undas A, Brozek J, Jankowski M, Siudak Z, Szczeklik A, Jakubowski H. Plasma homocysteine affects fibrin clot permeability and resistance to lysis in human subjects. *Arterioscler Thromb Vasc Biol* 2006; **26**:1397–1404.
18 Ding H, Triggle CR. Endothelial dysfunction in diabetes: multiple targets for treatment. *Pflügers Arch* 2010; **459**:977–994.
19 Guo Q, Mori T, Jiang Y, Hu C, Osaki Y, Yoneki Y, *et al.* Methylglyoxal contributes to the development of insulin resistance and salt sensitivity in Sprague-Dawley rats. *J Hypertens* 2009; **27**:1664–1671.
20 Forbes JM, Cooper ME, Thallas V, Burns WC, Thomas MC, Brammar GC, *et al.* Reduction of the accumulation of advanced glycation end products by ACE inhibition in experimental diabetic nephropathy. *Diabetes* 2002; **51**:3274–3282.
21 Henschen-Edman AH. Fibrinogen noninherited heterogeneity and its relationship to function in health and disease. *Ann N Y Acad Sci* 2001; **936**:580–593.
22 Gabriel DA, Muga K, Boothroyd EM. The effect of fibrin structure on fibrinolysis. *J Biol Chem* 1992; **267**:24259–24263.