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Original Article

# Effects of Methylglyoxal and Aspirin on *In Vitro* Coagulation and Clot Permeability

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## A B S T R A C T

**Backgrounds and Aims:** Methylglyoxal (MGO) is an –α, β dicarbonyl aldehyde inevitably produced from triose-phosphate intermediates of glycolysis, and amino acid. Increased MGO in blood leads to alterations in coagulation, clot permeability and thus, atherosclerosis in children with diabetes; however, the precise mechanism is not clear. The present study aimed to compare different concentrations of MGO and aspirin on coagulation and clot permeability in the plasma of healthy individuals *in vitro.*

**Materials and Methods**: Different concentrations of MGO (5, 50, 100, 500 µM) and aspirin (1, 10, 100 mg/l) were added to the plasma citrate. They were incubated at 37°C for 24 h. Then, coagulation parameters were analyzed by the turbidimetric procedure and then clot permeability was investigated.

**Results:** MGO at 500 µM with aspirin 100 mg/l made significant changes in the coagulation maximum velocity (0.253±0.006), total coagulation time (803±8.88s) and permeation coefficient ($0.778\times10^{-6}$±0.099) compared to MGO at 500 µM (0.271±0.007), and (500±10.00), ($0.446\times10^{-6}$±0.017), respectively ($P< 0.05$). MGO at 500 µM with aspirin 1 mg/l did not significantly change in either parameter ($p>0.05$). MGO at 100 µM with aspirin 1 mg/l did not significantly change in either parameter ($p> 0.05$), compared to MGO at 100 µM. MGO at 5 µM with aspirin (1, 10, 100 mg/l) changed in all coagulation and clot permeability parameters ($p<0.05$), compared to MGO at 5 µM.

**Conclusions:** Our findings revealed that aspirin (≥1 mg/l) was held to have more effects on higher concentrations of MGO. Moreover, it decreased the velocity of coagulation and increased permeability of clot.

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## Introduction

Atherosclerosis is generally accepted as an inflammatory disease [1]. Inflammatory activity in the atherosclerotic plaque plays a key role in the progress of atherosclerosis and the risk for plaque rupture [2]. When the lipid-rich plaques are separated from vessel walls, vessels will damage and thrombus formation will come about. Clot formation is the most important cause of the onset of coronary heart diseases (CHD) as well as the sudden death caused by ischemia [3].

Methylglyoxal (MGO) can be mentioned as a metabolite of glucose produced from dihydroxyacetone phosphate as a by-product during the formation of glyceraldehyde 3-phosphates in mammalian cells, including vascular smooth muscle cells [4]. MGO is the development of diabetic complications, but its precise mechanism has not in detail been explained [5]. MGO, an active compound of – α, β dicarbonyl aldehyde [6-8], is developed during cellular metabolism, glucose oxidation and peroxidation of lipids or carbohydrates are produced in the food and beverages. Dicarbonyl of MGO attacks the lysine, arginine and cysteine residues of long-lived proteins in order to form irreversible advanced glycation end products [8-10]. As a result, an excess of MGO formation can increase reactive oxygen species production and cause oxidative stress [5, 11]. Some studies determined a positive correlation between serum MGO and Hemoglobin A1c in children with type 1 diabetes [12].

Moreover, plasma levels of MGO are demonstrated to elevate and associate with inflammation in diabetes patients. These indicate that MGO may have an important role in the early phase of the atherosclerotic compliance childhood diabetes [13]. Hyperglycemia associated with diabetes drives several damage pathways and increases concentrations of MGO leading to endothelial damage and atherosclerosis [7, 14, 15]. Some other proteins were categorized as putative major targets for MGO-derived modification via western blotting and mass spectrometry fibrin (ogen) [16]. MGO decreases the number of NH2 side chains in plasminogen, which is associated with overwhelming functional alterations [17]. To thwart this, some compounds have been considered, one of which is aspirin (non-steroidal anti-inflammatory drug), because aspirin inhibits platelet function used in the treatment of atherosclerotic cardiovascular diseases [18]. As a matter of fact, it increases the endothelial nitric oxide synthesis. Nitric oxide is responsible for the maintenance and repair of vascular endothelial, which has a crucial role in preventing cardiovascular syndrome [19, 20]. Another function of aspirin has also reported a decrease in thrombin formation and other coagulation factors [21]. Aspirin also acetylates fibrinogen which is one of coagulation factors [22], shortens the time needed for clot lysis and increases the velocity of clot lysis in the presence of MGO [23, 24].

Due to the importance of CHD, the present study aimed to investigate the effects of MGO and aspirin on coagulation and clot permeability parameters *in vitro*.

## Materials and Methods

### Isolation of plasma

Blood samples were taken from 50 healthy people, who were reported not to suffer from any 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×g, 5 min) to get plasma. Then, the pooled plasma was frozen at -20°C to be used for the coagulation and clot permeability.

### Measuring coagulation pparameters

Due to the time limit of the study, plasma samples of patients suffering from cardiovascular diseases were not used. The study was conducted on fibrinolysis parameters in vitro in the presence of MGO and aspirin as a previous study [23, 24]. Coagulation was evaluated with a slightly modified method by Williams et al. [25]. A total of 400 µl citrated plasma was diluted at 37°C with 400 µl of Phosphate-buffered saline containing 5 µl calcium chloride 2M (Merck), and 3 µl human thrombin 0.5U/ml (Sigma) to activate coagulation factors. Clot assembly kinetics was monitored spectrophotometrically at 405 nm in duplicate aliquots. Then, the coagulation maximum velocity (CMV, maximum ΔOD/second), and the total coagulation time [TCT, (second)] were determined by obtaining kinetic curves (OD/Time) for aliquots.

In the next step, of a 10 mM MGO solution, various amounts of MGO (5, 50, 100, 500 µM) were prepared in 400 µl plasma. They were incubated for up to 24 hours at 37°C along with non-MGO plasma, and then were analyzed in regard with coagulation parameters. Subsequently, various amounts of aspirin (1, 10, 100 mg/l) were prepared in 400 µl plasma. They were incubated for up to 24 hours at 37°C along with non-aspirin plasma, and analyzed for coagulation parameters. Ultimately, every amount of MGO concentration was collected into different amounts of aspirin in 400 µl plasma consecutively. They were incubated for up to 24 hours at 37°C along with different MGO concentrations (5, 50, 100, 500 µM), and analyzed for coagulation parameters.

### Measuring clot permeation coefficient

As shown before fibrin clot permeation was determined [26]. A total of 5 µl calcium chloride 2M (Merck) and 3 µl human thrombin 0.5U/ml (Sigma) were added to 400 µl citrated plasma. The mixture was placed in plastic tubes, which were allowed to clot. After incubation for 120 minutes in a wet chamber, tubes were connected with a reservoir of a buffer (0.05 mol/L Tris HCl, 0.15 mol/L NaCl, pH 7.5), and its flow rate through the gel was measured. The permeation coefficient (Darcy constant [Ks]), which represents the surface of the clot allowing flow through a fibrin network, was calculated from the equation $Ks= (Q. L. \eta)/ (t. A. \Delta p)$, where Q is the volume of the buffer flowing through the gel in time t, L is the length of the fibrin gel (1.6 cm), η is the viscosity of buffer ($10^{-2}$poise), A is the area of the gel perpendicular to the flow (4.17 $cm^2$), and Δp is the differential pressure (in dyne/$cm^2$) [27]. In the next step, of a 10

mM MGO solution, various amounts of MGO (5, 50, 100, 500 µM) were prepared in 400 µl plasma. They were incubated for 24 hours at 37°C along with non-MGO plasma, that were analyzed for clot permeation coefficient.

Afterwards, various amounts of aspirin (1, 10, 100 mg/l) were prepared in 400 µl plasma. They were incubated for 24 hours at 37°C along with non-aspirin plasma, which were analyzed for clot permeation coefficient. Finally, every amount of MGO concentration was collected into different amounts of aspirin in 400 µl plasma consecutively. They were incubated for 24 hours at 37°C along with different MGO concentrations (5, 50, 100, 500 µM), that were analyzed for clot permeation coefficient the study was approved by the Ethics Committee of Shahid Sadoughi University of Medical Sciences.

### Statistical analysis

The SPSS software package was utilized to analyze the study data applying independent-samples t-test to compare the groups. All the values were expressed as means ± SD. $P<0.05$ was considered statistically significant.

## Results

### Measuring methylglyoxal parameters

After collecting MGO into plasma, it was observed that coagulation and clot permeation coefficient parameters altered. MGO with 500 µM revealed a significant increase on CMV (0.271±0.007) compared to non- MGO group CMV (0.220±0.017) ($p<0.05$). Moreover, TCT (500±10.00), and Ks ($0.446\times10^{-6}$±0.017) were held to have a significant decrease compared to the control group with TCT (735±5.50), and Ks ($1.058\times10^{-6}$±0.103),respectively ($p<0.05$). MGO 50 µM revealed a significant decrease on TCT (660±10.00) compared to the control group with TCT (735±5.50), ($p<0.05$). The results are shown in table 1.

**Table 1.** Comparison of different concentrations of methylglyoxal (5, 50, 100, 500 µM) with controls on coagulation and clot permeation coefficient parameters (24 h, 37 ˚C).

| Variables | TCT | P-value | CMV | P-value | Ks | P-value |
|---|---|---|---|---|---|---|
| Control | 735±5.50 | - | 0.220±0.017 | - | $1.058\times10^{-6}$±0.103 | - |
| MGO 500 µM | 500 ± 10.00 | 0.000 | 0.007± 0.271 | 0.009 | $0.446\times10^{-6}$±0.017 | 0.001 |
| MGO 100 µM | 610± 10.00 | 0.000 | 0.250 ±0.002 | 0.040 | $0.553\times10^{-6}$±0.025 | 0.001 |
| MGO 50 µM | 660±10.00 | 0.000 | 0.246±0.007 | 0.074 | $0.858\times10^{-6}$±0.184 | 0.176 |
| MGO 5 µM | 720±10.00 | 0.081 | 0.255±0.005 | 0.656 | $0.939\times10^{-6}$±0.075 | 0.182 |

MGO = methylglyoxal; TCT (second)= total coagulation time; CMV (maximum ΔOD/sec)= maximum coagulation velocity; Ks ($cm^2$)= clot permeation coefficient
Variables are presented as mean ± SD.

### Measuring aspirin parameters

Aspirin had significant effects on parameters ($p<0.05$), that is to say, when 100 mg/l was applied, CMV (0.163±0.015) decreased significantly, though TCT (897±5.19) and Ks ($2.169\times10^{-6}$±0.363) increased significantly compared to the non-aspirin group. Concentrations of 1 and 10 mg/l underwent significant alterations

compared to the non-aspirin group, as it is shown in table 2 ($p<0.05$).

**Table 2.** Comparison of different concentrations of aspirin on coagulation and clot permeation coefficient parameters. (24 h, 37 ˚C).

| Variables | TCT | P-value | CMV | P-value | Ks | P-value |
|---|---|---|---|---|---|---|
| Control | 735±5.50 | - | 0.220±0.017 | - | $1.058\times10^{-6}$±0.103 | - |
| ASA 100 mg/l | 897±5.19 | 0.000 | 0.163±0.015 | 0.034 | $2.169\times10^{-6}$±0.363 | 0.007 |
| ASA 10 mg/l | 774±5.50 | 0.001 | 0.170±0.010 | 0.040 | $1.615\times10^{-6}$±0.203 | 0.013 |
| ASA 1 mg/l | 753±5.77 | 0.017 | 0.186±0.010 | 0.046 | $1.320\times10^{-6}$±0.086 | 0.029 |

ASA= acetylsalicylic acid or aspirin; TCT (second)= total coagulation time; CMV (maximum ΔOD/sec)= maximum coagulation velocity; Ks ($cm^2$)= clot permeation coefficient
Variables are presented as mean ± SD.

**Measuring methylglyoxal and aspirin parameters**

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

MGO 500 µM with aspirin 100mg/l significantly decreased CMV (0.253±0.006), but increased TCT (803±8.88) and Ks ($0.778\times10^{-6}$) ±0.099 compared to the MGO group with 500 µM, as were CMV (0.271±0.007), TCT (500±10.00) and Ks ($0.446\times10^{-6}$±0.017), respectively ($p<0.05$). In this group, MGO 500 µM with aspirin 1mg/l, demonstrated no significant changes in coagulation as well as Ks parameters compared to the MGO 500 µM group. The comparison of other concentrations is displayed in table 3.

**Table 3.** Comparison of different concentrations of methylglyoxal (500 µM) with aspirin on coagulation and clot permeation coefficient parameters (24 h, 37 ˚C).

| Variables | TCT | P-value | CMV | P-value | Ks | P-value |
|---|---|---|---|---|---|---|
| MGO 500 µM | 500 ± 10.00 | - | 0.007± 0.271 | - | $0.446\times10^{-6}$±0.017 | - |
| MGO 500 µM+ASA 100 mg/l | 803±8.88 | 0.000 | 0.006±0.253 | 0.035 | $0.778\times10^{-6}$±0.099 | 0.005 |
| MGO 500 µM+ ASA 10 mg/l | 7.63 ±691 | 0.000 | 0.002 ±0.262 | 0.125 | $0.527\times10^{-6}$±0.034 | 0.022 |
| MGO 500 µM+ ASA 1mg/l | 5.00 ±510 | 0.196 | 0.005±0.269 | 0.683 | $0.461\times10^{-6}$±0.026 | 0.445 |

MGO= methylglyoxal; ASA= acetylsalicylic acid or aspirin; TCT (second)= total coagulation time; CMV (maximum ΔOD/sec)= maximum coagulation velocity; Ks ($cm^2$)= clot permeation coefficient
Variables are presented as mean ± SD.

MGO 100 µM with aspirin 100 mg/l had significant changes in all coagulations and Ks parameters compared to the MGO 100 µM ($p<0.05$). The comparison of other concentrations in the group is shown in table 4.

**Table 4.** Comparison of different concentrations of methylglyoxal (100 µM) with aspirin on coagulation and clot permeation coefficient parameters (24 h, 37 ˚C).

| Variables | TCT | P-value | CMV | P-value | Ks | P-value |
|---|---|---|---|---|---|---|
| MGO 100 µM | 610± 10.00 | - | 0.250 ±0.002 | - | $0.553\times10^{-6}\pm0.025$ | - |
| MGO 100 µM+ASA 100 mg/l | 844 ±9.29 | 0.000 | 0.224±0.007 | 0.005 | $1.060\times10^{-6}\pm0.213$ | 0.014 |
| MGO 100 µM+ ASA 10 mg/l | 758 ±10.26 | 0.000 | 0.235±0.005 | 0.010 | $0.801\times10^{-6}\pm0.050$ | 0.002 |
| MGO 100 µM+ ASA 1mg/l | 625± 6.42 | 0.089 | 0.244±0.006 | 0.212 | $0.643\times10^{-6}\pm0.064$ | 0.870 |

MGO= methylglyoxal; ASA= acetylsalicylic acid or aspirin; TCT (second)= total coagulation time; CMV (maximum ΔOD/sec)= maximum coagulation velocity; Ks ($cm^2$)= clot permeation coefficient
Variables are presented as mean ± SD.

MGO 50 µM in the three concentrations of aspirin significantly changed in all parameters of coagulation and Ks compared to the MGO 50 µM, but Ks was not significant as MGO 50 µM with ASA 1 mg/l (Table 5).

**Table 5.** Comparison of different concentrations of methylglyoxal (50 µM) with aspirin on coagulation and clot permeation coefficient parameters (24 h, 37 ˚C).

| Variables | TCT | P-value | CMV | P-value | Ks | P-value |
|---|---|---|---|---|---|---|
| MGO 50 µM | 660±10.00 | - | 0.246±0.007 | - | $0.858\times10^{-6}\pm0.184$ | - |
| MGO 50 µM+ASA 100 mg/l | 871±11.06 | 0.000 | 0.215±0.014 | 0.029 | $1.555\times10^{-6}\pm0.235$ | 0.016 |
| MGO 50 µM+ ASA 10 mg/l | 780±10.01 | 0.000 | 0.224±0.004 | 0.014 | $1.309\times10^{-6}\pm0.099$ | 0.020 |
| MGO 50 µM+ ASA 1 mg/l | 696±11.54 | 0.014 | 0.230±0.005 | 0.043 | $1.134\times10^{-6}\pm0.130$ | 0.102 |

MGO= methylglyoxal; ASA= acetylsalicylic acid or aspirin; TCT (second)= total coagulation time; CMV (maximum ΔOD/sec)= maximum coagulation velocity; Ks ($cm^2$)= clot permeation coefficient
Variables are presented as mean ± SD.

MGO 5 µM in the three concentrations of aspirin significantly changed in all parameters of coagulation and Ks compared to MGO 5µM (Table 6).

**Table 6.** Comparison of different concentrations of methylglyoxal (5µM) with aspirin on coagulation and clot permeation coefficient parameters (24 h, 37 ˚C).

| Variables | TCT | P-value | CMV | P-value | Ks | P-value |
|---|---|---|---|---|---|---|
| MGO 5µM | 720±10.00 | - | 0.225±0.005 | - | $0.939\times10^{-6}\pm0.075$ | - |
| MGO 5 µM+ASA 100 mg/l | 880±5.50 | 0.000 | 0.173±0.007 | 0.001 | $2.169\times10^{-6}\pm0.363$ | 0.005 |
| MGO 5 µM+ ASA 10 mg/l | 853±7.63 | 0.000 | 0.186±0.008 | 0.002 | $1.615\times10^{-6}\pm0.203$ | 0.006 |
| MGO 5 µM+ ASA 1 mg/l | 759±8.14 | 0.006 | 0.210±0.004 | 0.020 | $1.333\times10^{-6}\pm0.076$ | 0.003 |

MGO= methylglyoxal; ASA= acetylsalicylic acid or aspirin; TCT (second)= total coagulation time; CMV (maximum ΔOD/sec)= maximum coagulation velocity; Ks ($cm^2$)= clot permeation coefficient
Variables are presented as mean ± SD.

## Discussion

Methylglyoxal, a metabolic by-product, reacts with certain proteins to yield irreversible advanced glycation end products (AGEs) and increases oxidative stress that causes the

pathophysiological changes in diabetes, hypertension, and aging [10, 28, 29]. Oxidative stress is one of the pathogenic factors in the development of endothelial dysfunction in experimental models of diabetes [30].

In the previous study, aspirin 100 mg/l was observed to affect all MGO concentrations (5, 50, 100, 500 µM), that is to say, fibrinolysis proceeded 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 [23]. In this in vitro study, MGO was observed to decrease the time for clot formation. Various concentrations of MGO (50, 100, 500 µM) significantly decreased and increased TCT as well as CMV, respectively compared to the non-MGO group. Therefore, methylglyoxal may speed up coagulation and decrease the time for clot formation.

Lurd et al. [16] demonstrated that fibrin (ogen) was identified by western blotting and LC-MS/MS as a putative main target for MGO modification. Fibrin (ogen) α-chain was identified in three different spots that corresponded to spots in western blotting by MGO AGEs antibody, ensuring the identity of the modified protein. The α-chain seems to be extensively modified compared with other proteins, since it was scarcely visible by Coomassie staining in contrast to high MGO AGEs immunoreactivity. Gugliucci et al. [31] demonstrated both aminoguanidine and carnosine were able to prevent MGO-induced loss of antithrombin III and plasminogen activities in a dose dependent manner. As a result, methylglyoxal may intervene in coagulation by reducing the AT III activity, hence increasing coagulation. In the current study, CMV significantly increased and TCT significantly decreased in the presence of MGO. In addition, another parameter, Ks, was examined. It was indicated that clot structure has changed. In the presence of various concentrations of MGO (100, 500 µM), the clot permeation coefficient significantly decreased compared to the non-MGO group. Pieters et al. [32] demonstrated that a purified fibrinogen model enables us to determine the effects of fibrinogen glycation on fibrin network structure independent of other plasma components. A significant increase was reported in permeability with achievement of glycaemic control and consequent decrease was demonstrated in the level of fibrinogen glycation in the diabetic subjects. Therefore, it can be possibly concluded that fibrinogen glycation decreases clot permeability. As MGO is regarded as a factor to form AGEs, it may affect fibrinogen and modify its structure, end up to thick clot, and decrease clot permeability at the aforementioned concentrations.

The study results revealed that aspirin properties affected clot formation and permeability. It increased TCT, and Ks parameters and decreased CMV in the presence of MGO. Undas et al. [21] showed that aspirin can reduce thrombin generation with the subsequent attenuation of thrombin-mediated coagulant reactions such as factor XIII activation. Aspirin also acetylates lysine residues

in fibrinogen resulting in increased fibrin clot permeability and enhanced clot lysis as well as direct fibrinolysis promoting with high-dose aspirin. Hence, aspirin delays coagulation and enhances clot permeability.

In the present study, it was observed that aspirin 100mg/l affected all MGO concentrations (5, 50, 100, 500µM), that is, coagulation is more slowly formed compared to that of MGO. The coagulation maximum velocity decreased, whereas TCT and Ks increased. Therefore, aspirin could reduce the effects of MGO on clot formation and clot permeation coefficient parameters.

## Conclusion

It may be concluded that aspirin 100mg/l affects all MGO concentrations (5, 50, 100, 500µM), delays coagulation, and increases clot permeability. Although aspirin in lower amounts highly affected MGO 5, 50 µM, no change was observed in higher concentrations.

## Conflict of Interest

The authors declare that there is no conflict of interest.

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