

Healthy and Gestational Diabetic Human Placental Fructose 1,6 Bisphosphate Aldolase; Comparative Investigation of Kinetic Properties and Inhibition Effects of DHAP, ATP, and Mg⁺² ionⁱ

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Abstract Fructose-1,6-bisphosphate aldolase plays an effective role in glucose metabolism and gluconeogenic pathway, and reversibly catalyzes the split of fructose 1,6-bisphosphate into the triose phosphates D-glyceraldehyde phosphate and dihydroxyacetone phosphate. Aldolase has 160 kDa molecular weight and three tissue specific isozymes. Gestational diabetes mellitus is defined as glucose intolerance that begins or is first detected during pregnancy. The placenta is a temporary established organ that operates exclusively for the time of pregnancy. It acts as a natural barrier between the maternal and fetal blood circulations and performs a wide range of endocrine and transport functions. In diabetes, the placenta undergoes a variety of structural and functional changes. Healthy and gestational diabetic human placental fructose-1, 6-bisphosphate aldolases were purified and investigated the substrate kinetic properties, in the previous studies. In this comparative study, we wanted to carry out characteristics of inhibition kinetics of aldolase in healthy and diabetic human placenta. The specific activity was defined as the number of activity units per mg of protein. Inhibition kinetics of fructose-bisphosphate aldolase was studied using dihydroxyacetone-phosphate, adenosine triphosphate, and magnesium ion as inhibitors. For healthy placental Aldolase it was detected that, adenosine triphosphate is partial competitive; dihydroxyacetone-phosphate is noncompetitive and magnesium metal is pure-competitive inhibitor. It was found that, dihydroxyacetone-phosphate is competitive; adenosine triphosphate is partial competitive and magnesium is partial competitive inhibitor of gestational diabetic human placental Aldolase.

Keywords Fructose 1,6 Bisphosphate Aldolase, Placenta, Gestational Diabetes Mellitus, Dihydroxyacetone-phosphate, Adenosine Triphosphate, Mg⁺² Inhibition Kinetics

1. Introduction

Fructose 1,6 bisphosphate aldolase (FBPA, EC. 4.1.2.13) is a main glycolytic ubiquitous enzyme that catalyzes the reversible cleavage of one molecule D-fructose 1,6-bisphosphate (FBP), into dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (GA3P) [1-8]. Catalysis proceeds by two distinct chemical pathways in aldolases [1,2]. In class I aldolases, found in plants and higher animals, catalysis depends on “Schiff Base” formation with the substrate, covalently bound to the catalytic lysine [2,4,9–12]. Class I Aldolases are tetramers (160 kDa) of identical polypeptide chains [3]. Class II aldolases are found in bacteria and fungi [1,2] and function as homodimers with a total molecular weight of around 80 kDa [13]. There is an absolute requirement for a divalent metal ion, usually zinc, and in addition the enzymes are activated by monovalent cations such as potassium [8,14].

Aldolases are junction point enzymes which catalyze the production of DHAP and GA3-P in glucose metabolism or the reverse reaction in gluconeogenesis. DHAP is rapidly and reversibly isomerized to GA3-P. In this state, high concentration of DHAP may be able to play a negative role in the activation of enzyme [15,16].

It is clear that aldolase cleavage proceeds through a number of distinct enzyme-substrate intermediates, including carbinolamine, imine (Schiff base), and an enamine/carbanion. A lysine residue in the active site of aldolase attacks the carbonyl carbon of the substrate, leading to the formation of a carbinolamine followed by dehydration to form a Schiff base (Lys229). Protonation of the carbanion and hydrolysis of the resultant Schiff base release the second product DHAP, regenerating the enzyme. The DHAP moiety makes a number of interactions within the active site [8].

Gestational diabetes mellitus (GDM) is defined as glucose intolerance that begins or is first detected during pregnancy.

There are both fetal and maternal complications associated with GDM. [17,18]. In diabetic complications, there are many changes in placental functions, in particular with respect to the uptake, transmission and utilization of glucose, and also in glycolysis and glycolytic enzymes. The placenta plays a critical role in protecting the fetus from adverse effects from the maternal diabetic conditions [17-19]. By the separating maternal and fetal circulation, the placenta is a crucial regulator of fetal nutrition, gas exchange, and maternal immune tolerance. This fetal organ also a target for maternal and fetal metabolic alterations associated with pregnancy pathologies [19-21].

In the previous studies, we wanted to examine the presence of aldolase and expose the properties of enzyme in healthy and gestational diabetic human placenta [22,23]. In this study, we mainly have wanted to carry out inhibition effects of DHAP, ATP and Mg^{+2} . The inhibition kinetic properties were performed for both samples these further assays: Inhibition kinetics of fructose-bisphosphate aldolase were studied using DHAP, ATP, and Mg^{+2} as inhibitors.

2. Materials and Methods

2.1. Materials

All reagents used were of analytical grade and were obtained from Sigma Co. (St Louis, MO, USA).

2.2. Purification Procedures

At the previous experiments, purification was carried out on the basis of Penhoet procedure with slightly modifications [25]. All data were presented in previous studies [22,23].

2.2.1. Protein determination

Amount of the protein in the samples was determined with modified Lowry Method [26].

2.2.2. Enzyme Assay

The determinations of the enzyme activity present in the extracts were detected by measuring the cleavage rate of fructose 1,6 bisphosphate spectrophotometrically. A unit of aldolase activity is defined as the amount of enzyme which catalyzes the cleavage of 1 μ mole of substrate per minute at 37°C under conditions of assay [22,23]. The specific activity was defined as the number of activity units per milligram of protein [27].

2.2.3. Kinetic Studies

All experimental conditions were adjust at 37°C, and pH= 7,4 which are optimum enzyme assay conditions [22,23,27]. Inhibition kinetics was performed with DHAP, ATP and Mg^{+2} ion. Effects of organic and metallic inhibitors on Vmax (maximal reaction rate or maximal velocity at saturating substrate levels) and Km (Michaelis-Menten constant) were investigated thereby, inhibitor kinetic properties of FBPA were determined. Substrate affinity constants (Km values)

were determined with the Henri–Michaelis–Menten equation using a nonlinear least squares regression computer program. Vmax and Km constants were calculated with reciprocal Lineweaver-Burke plot of the initial velocity/substrate concentration data by the using 1/(V) vs. 1/(S) values. Enzyme–inhibitor dissociation constant (Ki) were determined with modified Michaelis–Menten equation [28].

For studies of inhibition kinetic properties, different inhibitor concentrations were selected, 0.0, 0.3, 0.5, 0.7, 1.0, 5.0 mM. Inhibition kinetics was determined from the Lineweaver-Burk equation [28].

Non-linear regression analysis module of Systat 11 statistic program was used to make calculation of kinetic parameters. For statistical measurement; the value of alpha (α) coefficient only positive values make sense. The α coefficient is the greatly used objective quantify of reliability.

3. Results

In the previous studies, we wanted to examine the presence of aldolase and expose the properties of enzyme in healthy and gestational diabetic human placenta. Fructose 1,6 bisphosphate aldolase was purified from both healthy and gestational diabetic human placentas by phosphocellulose column chromatography. Purity was controlled by polyacrylamide gel electrophoresis (PAGE). Then, the enzyme substrate interactivity was examined in healthy and gestational diabetic human placenta [22,23].

In this study, we mainly have wanted to carry out inhibition effects of DHAP, ATP and Mg^{+2} . We investigated the kinetic properties of fructose 1,6 bisphosphate aldolase with inhibitors for healthy and gestational diabetic human placenta. The quantitative estimation of aldolase activities present in the enzyme samples were determined by measuring the cleavage rate of the fructose 1,6 bisphosphate spectrophotometrically. A unit of aldolase activity was defined as the amount of enzyme which catalyses the cleavage of 1 μ mole of substrate per minute at 37°C under conditions of assay. The specific activity of the enzyme was defined as the number of activity units per milligram of protein [24].

Ki values of healthy human placental aldolase for:

DHAP = 1,039 \pm 0,107 mM,

ATP = 0,074 \pm 0,026 mM, and

Mg^{+2} = 14,678 \pm 2,702 mM were confirmed.

Ki values of gestational diabetic human placental aldolase for

DHAP = 0,766 \pm 0,035 mM,

ATP = 0,136 \pm 0,049 mM, and

Mg^{+2} = 0,218 \pm 0,113 mM were detected.

3.1. Kinetic Studies

All experimental conditions were arranged at 37°C, and pH 7,4 which were optimum enzyme assay conditions [27].

3.1.1. Inhibition kinetics of healthy human placental aldolase

Studies of inhibition kinetics of healthy human placental aldolase were implemented with DHAP, ATP, and Mg^{+2} . All of inhibition kinetic studies performed in linear zone (3–50mM) which chosen at previously experiments [23].

3.1.1.1. Inhibition effects of DHAP on healthy human placental aldolase

Inhibition effects of DHAP have been examined in detail on healthy human placental aldolase (FBPA). It was found that, DHAP is noncompetitive inhibitor of healthy human placental FBPA.

Ki value of DHAP was calculated as $1,039 \pm 0,107$ mM and shown at figure 1:

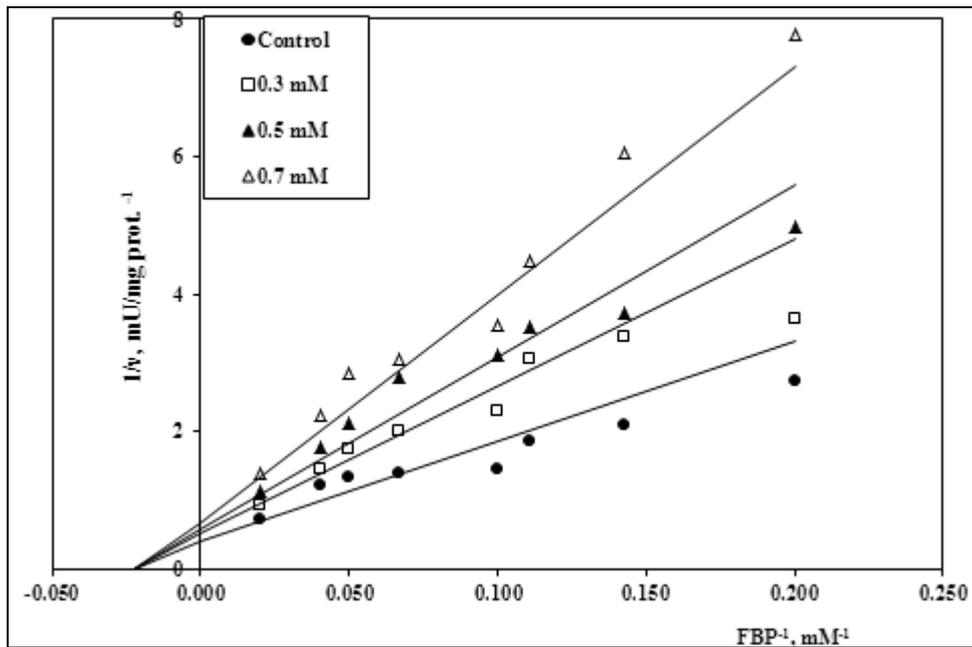


Figure 1. Effects of DHAP on healthy human placental FBPA (0,3, 0,5 and 0,7 mM inhibitor concentrations).

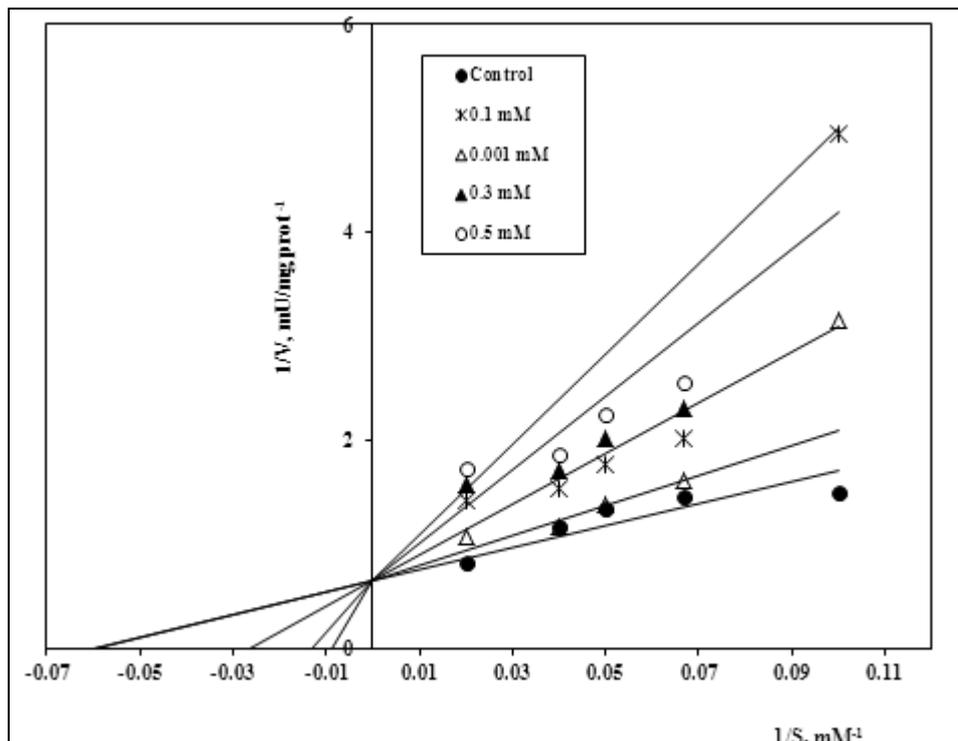


Figure 2. Effects of ATP on healthy human placental FBPA (0.1, 0.001, 0.3 and 0.5 mM inhibitor concentrations).

3.1.1.2. Inhibition kinetic effects of ATP on healthy human placental aldolase

At this part of study, inhibition effects of ATP have been investigated on healthy human placental aldolase. It was detected that ATP is partial competitive inhibitor of healthy human placental FBPA.

K_i value of ATP was calculated as 0.074 ± 0.026 mM and graphed at figure 2. Also, α coefficient was calculated as 3.287 ± 0.434 for healthy human placental aldolase.

3.1.1.3. Inhibitor effects of Mg^{+2} on healthy human placental aldolase

At this part of the study, we have investigated the interaction of healthy human placental aldolase with bivalent Mg^{+2} metal. It was defined that, Mg^{+2} is competitive inhibitor of healthy human placental FBPA. According as the K_i value calculation, Mg^{+2} metal was found that pure-competitive inhibitor of healthy human placental aldolase. K_i value of Mg^{+2} was calculated as $14,678 \pm 2,702$ mM and graphed at figure 3. As is seen in diagram, it was observed that, control

and inhibitor straight lines were intersected at 1/V axis and according to this inhibition type has been approved.

3.1.2. Inhibitor kinetics of gestational diabetic human placental aldolase

Inhibition kinetic studies of gestational diabetic human placental aldolase were performed with DHAP, ATP, and Mg^{+2} . The whole lot of inhibition kinetic studies performed in linear zone (3–50 mM) which elected at previously experiments [23].

3.1.2.1. Inhibition effects of DHAP on gestational diabetic human placental aldolase

Inhibition effects of DHAP have been investigated fully on gestational diabetic human placental aldolase. It was found that, DHAP is competitive inhibitor of gestational diabetic human placental FBPA. It was observed that, control and inhibition straight lines have been intersected at 1/V axis, and K_i value of DHAP was calculated as 0.766 ± 0.035 mM and illustrated at figure 4.

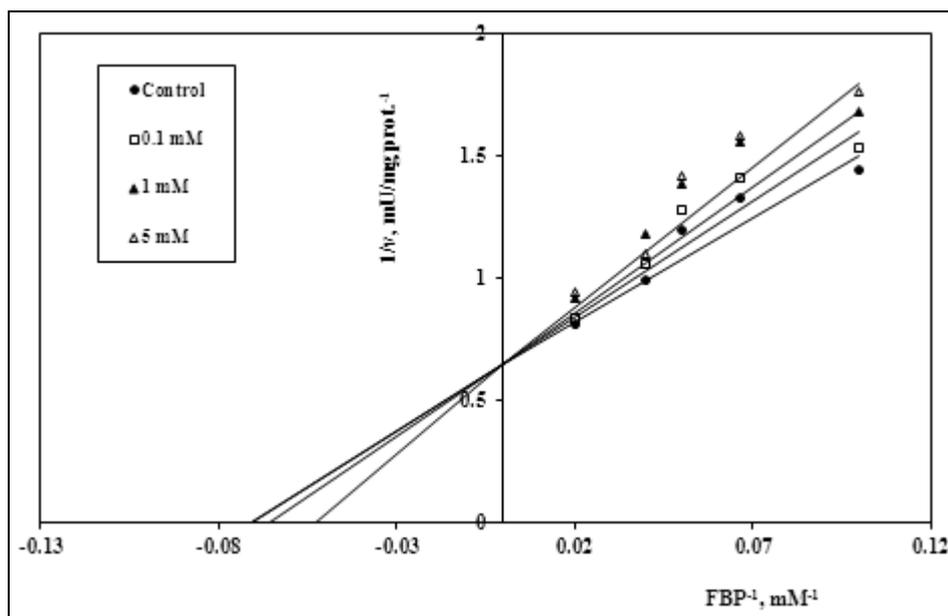


Figure 3. Effects of Mg^{+2} on healthy human placental FBPA (0.1, 1.0, 5.0 mM inhibitor concentrations).

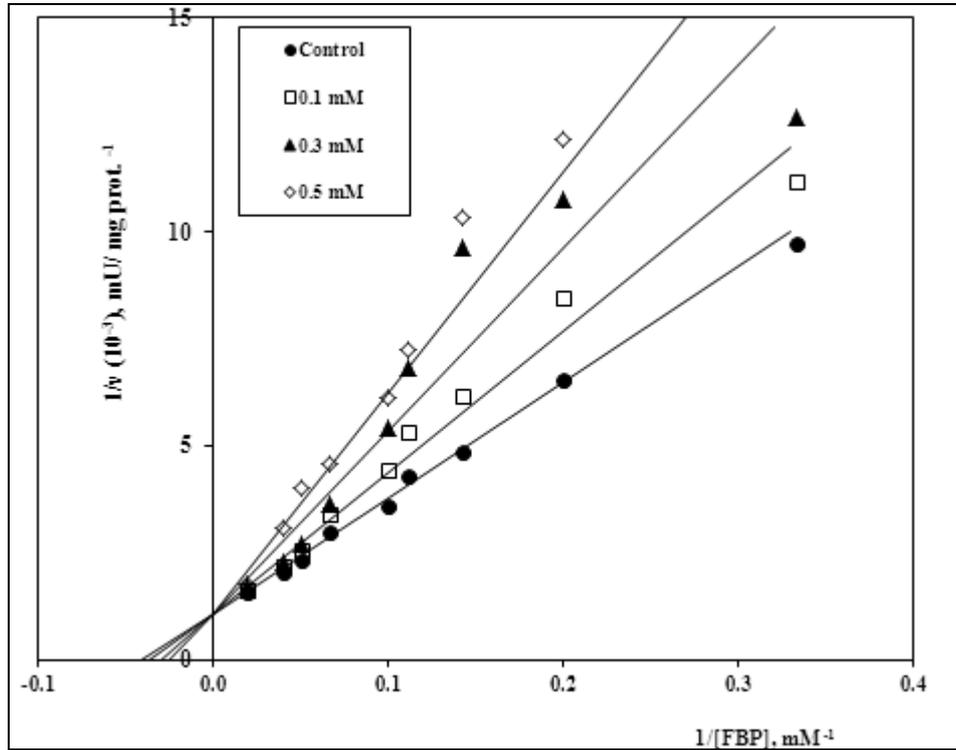


Figure 4. Effects of DHAP on gestational diabetic human placental FBPA (0.1, 0.3 and 0.5 mM inhibitor concentrations).

3.1.2.2. Inhibition effects of ATP on gestational diabetic human placental aldolase

After investigation of inhibitor effects of ATP on GDM FBPA, ATP has been found as partial competitive inhibitor of gestational diabetic human placental FBPA. K_i value of ATP was calculated as 0.136 ± 0.049 mM and α coefficient was determined as 4.089 ± 0.539 . These data were illustrated at figure 5.

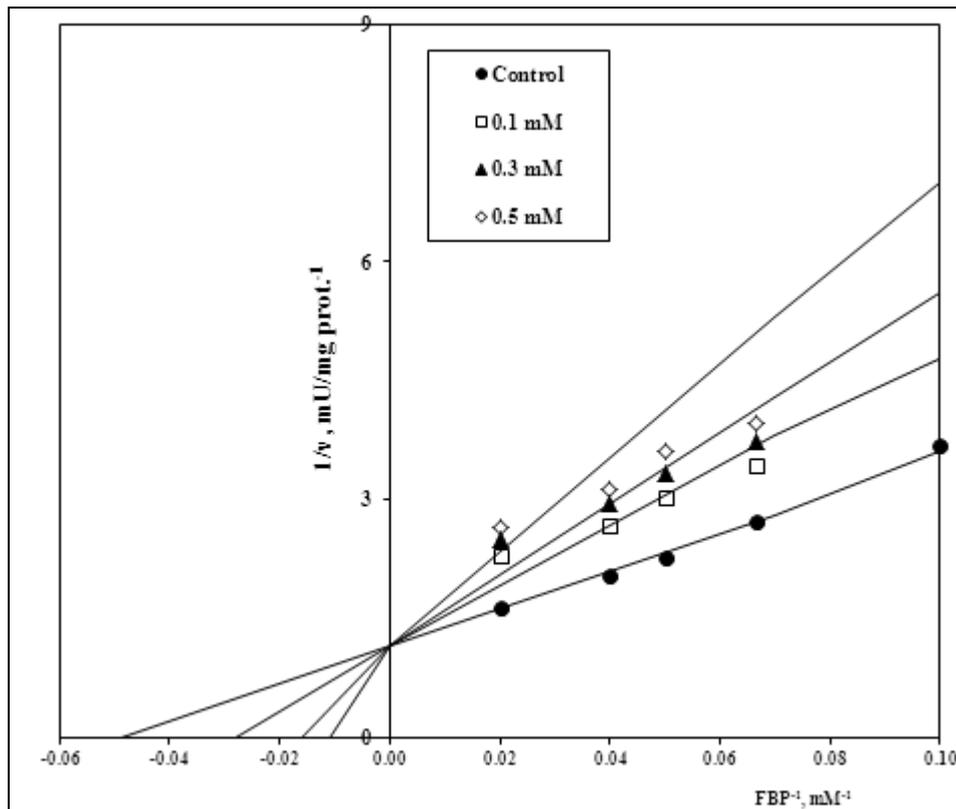


Figure 5. Effects of ATP on gestational diabetic human placental FBPA (0.1, 0.3 and 0.5 mM inhibitor concentrations).

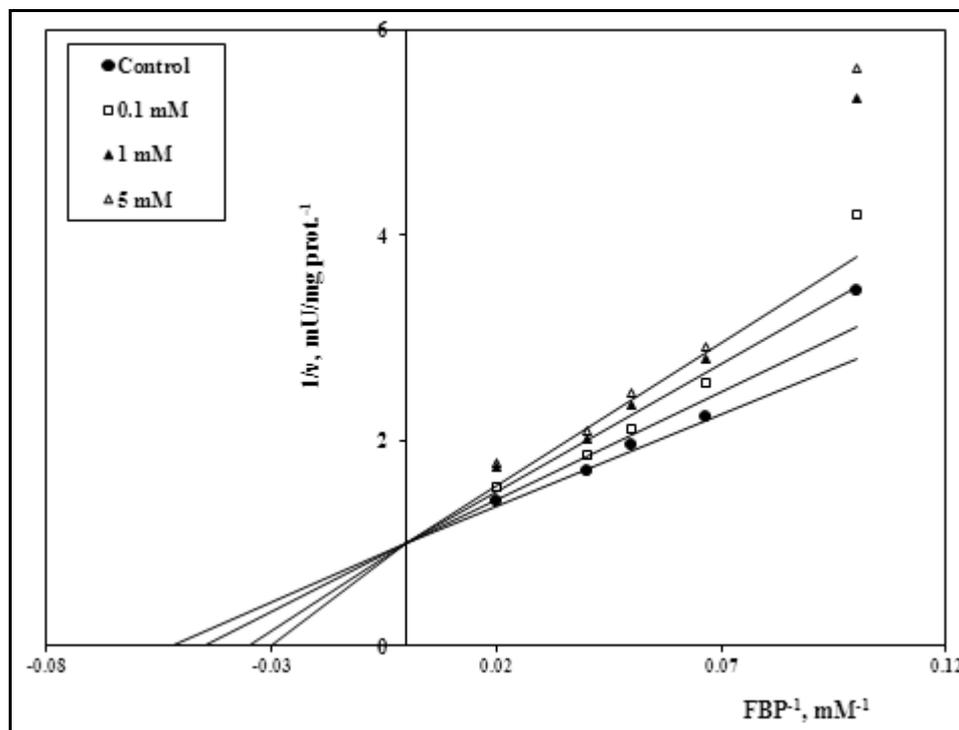


Figure 6. Effects of Mg^{+2} on gestational diabetic human placental FBPA (0.1, 1.0 and 5.0 mM inhibitor concentrations).

Table 1. Kinetic parameters of FBPA from healthy and gestational diabetic human placenta

Parameter	Healthy Placental		Gestational Diabetic Placental	
Km (for 3-50 mM FBP cons.)	23.063 ± 6.845 mM		26.686±1.913 mM	
Vmax (for 3-50 mM FBP cons.)	1885.457 ± 292.48		981.093±38.483	
Ki DHAP	1.039±0.107 mM	noncompetitive	0.766±0.035 mM	competitive
Ki ATP	0.074±0.026 mM	partial competitive	0.136 ± 0.049 mM	partial competitive
Ki Mg^{+2}	14.678±2.702 mM	competitive	0.218±0.113 mM	partial competitive

3.1.2.3. Kinetic effects of Mg^{+2} on gestational diabetic human placental aldolase

At this stage of study, we have investigated the interaction of gestational diabetic human placental aldolase with bivalent Mg^{+2} metal. It was pointed out that, Mg^{+2} is partial competitive inhibitor of GDM human placental FBPA. K_i value of Mg^{+2} was calculated as 0.218±0.113 mM and graphed at figure 6. According to this diagram, α coefficient was detected as 1.676±0.118.

After all experimental studies, we proved that both human placental aldolase activity was competitively inhibited in the presence of DHAP, ATP and Mg^{+2} (Summarized at table 1).

4. Discussion

In our preceding studies, we purified and demonstrated the presence of fructose 1,6 bisphosphate aldolase in healthy and gestational diabetic human placenta. In order to determine of optimal assay conditions of placental aldolase, enzyme

activity method was optimized for both enzyme samples. Also, we demonstrated that molecular weight of placental aldolase is 40 kDa, and homotetramer [22,23]. We also carried out the substrate kinetics on healthy and gestational diabetic human placental aldolases [23].

The beginning points of this research were investigating the activity differences and causes of both aldolases and how were these inhibitors influence on both enzymes? And also the main question is if the DHAP, ATP and Mg^{+2} are an activator or inhibitor for both enzymes?

In this study we searched thoroughly the inhibition kinetic properties of both placental aldolases. Therefore, we carried out the inhibition kinetic characteristics of both purified enzymes. It was investigated that inhibition effects of DHAP, ATP and Mg^{+2} on healthy and gestational diabetic human placental aldolase. Activity values for calculation of kinetic parameters of fructose 1,6 bisphosphate aldolase, which studied at all substrate concentrations for both enzymes, were calculated via non-linear regression analysis module of Systat 11 statistical program.

Lineweaver Burk plots were graphed by predicated on $1/V$ vs $1/S$ values. It was observed that, DHAP is a noncompetitive inhibitor of healthy human placental FBPA. K_i value of DHAP was calculated as 1.039 ± 0.107 mM (Fig. 1).

A non-competitive inhibitor reacts with the enzyme-substrate complex, and slows the rate of reaction to form the enzyme-product complex. In non-competitive inhibition, inhibitor has no directly effect on substrate binding region. Substrate and inhibitor bind to different regions as irreversible, randomly and independently. This means that increasing the concentration of substrate will not relieve the inhibition, since the inhibitor reacts with the enzyme-substrate complex which named as ESI complex. Inhibition effect occurs with conformational variation. ESI complex is inactive and can't constitute product [28].

It was found that, DHAP has competitive inhibition effect on gestational diabetic human placental FBPA. K_i value of DHAP was calculated as 0.766 ± 0.035 mM (Fig. 4). It was determined that, control and inhibition straight lines have been intercepted at $1/V$ axis. A competitive inhibitor competes with the substrate for the active site of the enzyme. So, increasing the concentration of substrate will decrease the chance of inhibitor binding to the enzyme [28].

The 1-phosphate binding side of FBPA is consist by Arg 148 and Lys 146 residues. The 6-phosphate binding side is made up by Lys 41, Arg 42. C-terminal Tyr residue is appeared to be within hydrogen binding distance of the Schiff base forming Lys residue. Fructose 1,6 bisphosphate which substrate of FBPA, holds two phosphate groups and adhere Arg and Lys residues that locate in active side of enzyme [1,3,29-32]. DHAP performed the product inhibition by its phosphate group thereby, binding Arg and Lys residues, locate in substrate binding side of FBPA. In the study, inhibition effect of DHAP on healthy placental FBPA was observed non-competitive, and on diabetic placental FBPA, inhibition effect of DHAP was detected competitive. It's thought that, this difference occurs because of some conformational defects on enzyme due to diabetes. It's known that the enzyme activity of the aldolase decreases in lots of metabolic disorders [30-32]. By all means, it can be said that also diabetes causes some damages on placenta tissue which effects negatively the enzyme activity [33,34].

If the inhibitor binds at the active side completely, competitive inhibition can be total or pure. If the inhibitor interferes with substrate binding without completely, inhibition occurs as partial. Inhibitor, unlike pure competitive inhibition, bounds to both free enzyme and ES complex at partial competitive inhibition. Substrate and inhibitor constitutes the ES, EI and ESI complex by binding to different sides of enzyme. Inhibitor competes partially with its substrate [28].

It was investigated that the inhibition effects of ATP on both placental aldolases. And it was proved that, ATP is partial competitive inhibitor of both healthy and gestational diabetic human placental FBPA. For healthy human placental aldolase K_i value of ATP was calculated as

0.074 ± 0.026 mM and α coefficient was found as 3.287 ± 0.434 (Fig. 2). K_i value of ATP was calculated as 0.136 ± 0.049 mM and α coefficient were determined as 4.089 ± 0.539 for gestational diabetic human placental FBPA (Fig. 5).

In 1965, Spolter and coworkers showed that adenine nucleotides are competitive inhibitors of aldolase isozymes. ATP was found to be the most effective inhibitor for muscle [35]. With another research, interaction of adenine mononucleotides and phosphates with rabbit muscle aldolase has been studied. It is shown that each sub-unit of aldolase has one strong and one weak binding site. This study also demonstrated that affinity of the tight site is much greater. They also gave point to the interaction of ATP with aldolase in the presence of saturating concentration of FBPA might argue against the possibility that the weak site is situated at the active center and reported that ATP is a pure competitive inhibitor for the fructose-1,6-P2 cleavage reaction [36].

It's supposed that, ATP inhibits the enzyme through phosphate groups by binding to Arg and Lys residues, which situated in catalytic active side of enzyme. In our study, for both enzymes, low K_i values of ATP indicate the power of inhibition.

Aldolase is one of the most important enzymes in the pathway known as glycolysis which the main aim is produce ATP. In cellular conditions, the presence of high concentrations of ATP, will lead to lower yield. It's considered that, also fructose 1,6 bisphosphate aldolase (FBPA) is being controlled by ATP through "negative feedback mechanism", like fructose 1,6 bisphosphate.

In our study, it's also performed that the interaction of healthy and gestational diabetic human placental aldolase with bivalent Mg^{+2} ion. It was defined that, Mg^{+2} is pure-competitive inhibitor of healthy human placental FBPA (K_i : 14.678 ± 2.702 (Fig. 3), and partial competitive inhibitor of GDM human placental FBPA (K_i 0.218 ± 0.113 mM and α coefficient: 1.676 ± 0.118 (Fig. 6).

When compared K_i values of both placental enzymes, it's proved that Mg^{+2} ion is even more effective at inhibiting the gestational diabetic placental FBPA. It's thought, this difference is grow out of the deformation of enzyme which generated by diabetes. We predict that, this sensibility of diabetic placenta to inhibition effect of bivalent Mg^{+2} ion, is correlate with the diabetes and enzyme affected by the conditions.

According to Kasprzak's study, Mg-ATP complex binds to aldolase with the affinity of at least two orders of magnitude lower than free nucleotide. Their kinetic measurements revealed pure competitive inhibition of aldolase by ATP. They reported that, although electrostatic forces are essential for binding of nucleotides to aldolase, this interaction cannot be considered as simple ionic association in aqueous solution [36].

In present study, as compared with all inhibitors, it's clear that, ATP is potent inhibitor for healthy human placental aldolase. And for same enzyme, it's seemed the Mg^{+2} is less effective inhibitor. This situation is suggests, there is no

structural and biochemical deformation on healthy human placental enzyme.

As compared with all inhibitors, it's seen that, ATP is potent inhibitor for gestational diabetic human placental aldolase, too. When compared Ki values, it's understood that, diabetic placental aldolase is more sensitive to all inhibitors, than healthy placental aldolase.

It's conceived that, the different kinetic behaviors of both enzyme forms, is proceed from diabetic deformational complications on tissue.

Although, there was no difference between the electrophoretic analysis of both enzyme, it's deemed that, activity may have affected by missing or difference of any amino acid which locate in active side of enzyme.

Hence, we estimate that, general effect of gestational diabetes mellitus may be occur in this direction on enzyme.

Because of the aldolases occupy a critical position in the glycolytic pathway, our research particularly focused on inhibition of this glycolytic enzyme. We especially interested in kinetic properties and, activation and inhibition of gestational diabetic placental aldolase.

Thus, our further study should be investigated the activation of the enzyme at the molecular level, and the affection and deformations of diabetes on aldolases.

The possibly mutations on amino acids, three dimensional structure deformations, and advance inhibition structures, glyucose metabolism anomalies depend on the diabetic complications of placental aldolase will be mostly important key points.

At further investigations of placental FBPA may be more helpful for clinical situations.

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