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Critical role of plasma C-peptide on control of ATP/ADP ratio of RBC

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Abstract

Diabetes mellitus is a disorder that contributes whole metabolism of body. The results of this process are a rapid depletion of the intracellular ATP pools, which slows the rate of glycolysis and mitochondrial respiration leading to cellular dysfunction. The ATP/ADP ratio of erythrocytes among four groups of normal individuals (young & old), athletes' subjects and diabetes patients are compared and relationship between ATP/ADP ratio and c-peptide level of plasma are determined. ATP/ADP level and plasma C-peptide in four groups [normal (young & middle age), athletes, diabetes] are measured that show a significant difference between groups (P-value<0.001). A significant positive correlation is found between RBC ATP/ADP content (r=0.716; P<0.001). In this study, a positive relationship between RBC ATP/ADP ratio and C-peptide is found. Based on the obtained results, higher released C-peptide through plasma may control the ratio of ATP/ADP in erythrocytes of different individuals.

Keywords: Diabetes, ATP, ADP, C-peptide, Red Blood Cell.

INTRODUCTION

Diabetes mellitus is a disease involving a deficiency in insulin synthesis and/or a defect in glucose receptors. As a result, glucose levels in the blood and tissues, such as lens, kidney, vascular endothelial cells and erythrocytes are elevated. It has been postulated that prolonged states of hyperglycemia lead to the development of clinical complications, such as retinopathy, neuropathy and nephropathy [1-2]. One may expect that beside the serious disruptions in metabolism, diabetes also affects energetic conversions [3-10]. Decreased lipid oxidation in the basal state of obese and type2 diabetes (T2D) was reported [11]. Moreover, a decreased activity of the electron-transport-chain (ETC) specifically in subsarcolemmal mitochondria in T2D patients compared with obese and lean subjects has also been reported [12]. Numerous studies have shown an altered gene expression (mRNA) of several proteins involved in the oxidation phosphorylation (OXPHOS) [13-15]. Abnormalities in the catalytic subunit of ATP-synthase (a key enzyme in mitochondrial OXPHOS) in skeletal muscle of patients with T2D patients compared to lean subjects have been observed [16]. Other investigation showed poly (ADP ribose) polymerase (PARP) that is a profuse nuclear enzyme of eukaryotic cells that has been implicated in response to DNA injury.

Free-radical- and oxidant-induced cell injury can involve the activation of PARP. Activation of PARP by single-strand DNA (ssDNA) initiates an energy-consuming cycle by transferring ADP ribose units from NAD+ to nuclear proteins. The result of this process is a rapid depletion of the intracellular NAD+ and ATP pools, which slows the rate of glycolysis and mitochondrial respiration leading to cellular dysfunction [17-23]. Note that metabolic changes that make impaired immune function observed in diabetic patients is due to dysfunction in ATP production [24]. Adenine nucleotides play a key role in the energy metabolism of cells. Levels

of ATP, ADP and AMP reflect the rate of energy transformations in mature red blood cells, fueled mainly by glycolysis and the pentose cycle [25]. The adenine nucleotide pool necessary for normal activity of erythrocytes remains under the control of an elaborate set of enzymes [26], which includes three magnesium-dependent regulatory enzymes: hexokinase (EC.2.7.1.1), phosphofructokinase (EC.2.7.1.11) and pyruvate kinase (EC.2.7.1.40). Minor shifts in the level of allosteric activators and inhibitors may grossly affect the rate of enzyme reactions, between them glycolysis, leading indirectly to changes in the level of ATP, ADP and AMP [27] and in the adenylate energetic charge of erythrocytes [28].

ATP can affect thromboregulation and stimulate the immune cells responsible for asthma attacks [29, 30]. ATP (or its metabolite ADP) subsequently binds to endothelial P2Y1 receptors, resulting in vasodilation [31, 32]. It has been proposed that acidemia due to ketoacidosis sometimes seen in severe or untreated diabetics might cause elevation of glucose 6-phosphate levels in the erythrocytes of diabetics, because phosphofructokinase activity is inhibited by the lower pH [33]. In human erythrocytes, the uptake of 3-o-methyl-D-glucose was decreased when the concentration of intracellular ATP was decreased by ca2+ ionophore A23187 [34]. The uptake of 2-deoxy-D-glucose in cultured rat pancreatic β-cells was inhibited by decreased intracellular ATP concentration caused by streptozotocin and also by oligomycin and carbonyl cyanid m-chlorophenylhydrazone (cccp), known as a potent uncoupler [35]. Direct activation of the glucose transporter by physiological concentration of ATP was demonstrated in human Erythrocytes [36-38]. Accordingly, the aim of this study was to study comparison of ATP/ADP level of RBC among three groups of normal, athletes' subjects and diabetes patient and then clarify relationship between ATP/ADP and C-peptide level of plasma.

MATERIALS AND METHODS

Studies with human subjects:

The subjects were fully informed of any risks and discomforts associated with the experiments before giving their informed written consent to participate. The studies conformed to the code of Ethics of the World Medical Association (Declaration of Helsinki) and were approved by the Ethics Committee of Shariati Hospital, Tehran University, Iran. In the first study 40 diabetes patients (Type2 diabetes) were collected which were treating with insulin and at the time of the study were diagnosed as uncontrolled (HbA1C% 8.79±0.19). Condition comparison studies were carried out, we also collected RBC from 50 healthy volunteer. All control subjects were healthy volunteer living in the community, and none was acutely ill. None exhibited evidence of cardiac or chronic kidney disease and all were euthyroid with normal liver function tests and normal value for plasma urea, creatinine and electrolytes. Furthermore, healthy subjects were drug free and with a negative family history of diabetes mellitus or hypertension. The following parameters were determined in all blood samples which collected at 8 hour after overnight fasting: erythrocyte ATP, ADP content and plasma C-peptide level.

ATP assay method in RBC:

ATP was measured by luciferin-luciferase technique [39, 40, 41]. In which the amount of light generated by the reaction of ATP with recombinant luciferase is dependent on the ATP concentration. Sensitivity was augmented by addition of the D-luciferin to the luciferase. A, 50 µl sample of RBC, lysed with TCA 10%(trichloroaceticacid) and neutralized with KOH 1M and diluted with Hepes buffer 100 mM pH 7.8 (1:64), injected into a cuvette containing 10 µl luciferin (sigma), 10 µl Mgso4, 10 µl luciferase(1 mg/ml). The peak light efflux from cuvette to which either known ATP standards or samples are added was determined using a luminometer (Sirius tube Luminometer, Berthold Detection System, Germany), a ATP standard curve was obtained on the day of each experiment.

RBC ADP assay procedure:

ADP was measured by the coupled assay of pyruvate kinase with luciferin-luciferase technique[42], in which at first we injected 5 µl pyruvate kinase (1 mg/ml) into a cuvettes containing 50 µl RBC(lysed,neutralized and diluted), 5 µl PEP (phospho enol pyruvate 20 mM, sigma), 5 µl KCl and patience for 7 min since the

ADPs existed in the sample converted to ATP, then added 10 µl luciferin(10 mM), 10 µl luciferase (1 mg/ml) and 10 µl Mgso4. the peak light efflux from cuvette to which either known ADP standards or samples are added was determined using a luminometer , a ADP standard curve was obtained on the day of each experiment.

C-peptide assay procedure:

This assay accomplished by automated electrochemiluminescence. C-peptide kit is buied from Cobas (Roche). Inject 500 µl of sample into cup and placed cup into instrument' racks then defined program for instrument [43, 44].

Statistical Method:

Statistical significance among experimental periods and groups was determined with analysis of variance, Tamhane test and Scheffe test for multiple comparisons, Bivariate correlation for relation between variables and regression for prediction. A P-value 0.05 or less was considered statistically significant. Results are reported as the means ± SEM.

RESULTS

Human subject studied. RBCs are taken 40 male Type 2 diabetes who were diagnosed according by American Diabetes Association Guide lie 2009 [45]. Some patient had a family history of diabetes. The average age of the patients was 60.5±1.7 years. The data for average patient with diabetes and control are summarized in table1. In addition, RBC of 50 healthy human volunteers [20 athletes, 30 Normals (20 youngs, 10 middle age)] with equal sex for all was sampled. Average ages for athletic individuals without medication and history of diabetes disease, was 27.7±0.61 and for normal control was 26.7±0.49 and 50 ±0.60 subsequently.

ATP/ADP level between groups

In this study the control individuals divided to three groups, normal and athlete individuals. ATP/ADP level in four groups (Normal(Y) & Normal (M), athletes, Diabetes) was measured and analyzed with ANOVA test, the result showed a significant difference between groups (P value<0.001), the result are summarized in table2 and Fig. 1.

Figure-1: the comparison of ATP/ADP level among 4 groups (diabetes, normal (Y=young, M=middle age) and athletes). The amount of 53, 66, 63, 67 are out of average.

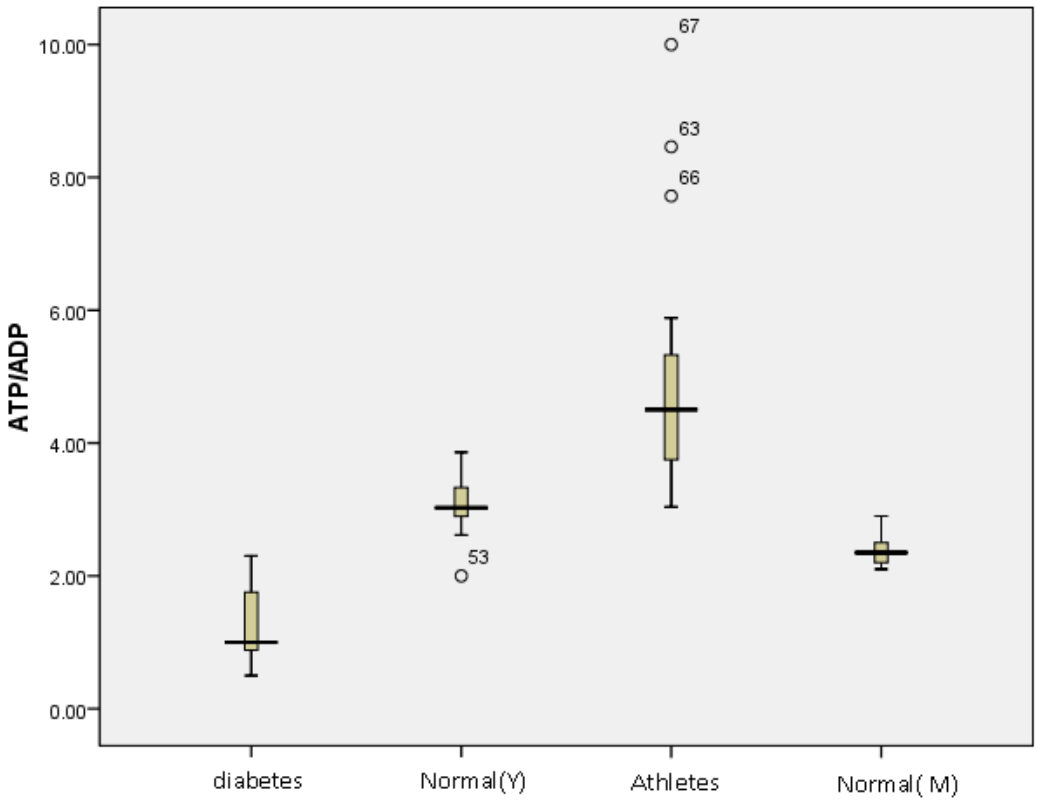


Table1- Age and sex characteristics of studied subjects: Erythrocyte ATP/ADP and plasma C-peptide content.

	Normal (Y)*	Normal (M)**	Athletes	Diabetes
N	20	10	20	20
Age (year)	26.7±0.49	50±0.60	27.7±0.61	60±1.7
Sex	Male	Male	Male	Male
BMI[a]	25	28	20	33
ATP/ADP[b]	3.08±0.09	2.4±0.07	4.96±0.411	1.26±0.08
C-peptide(ng/ml)[c]	7.59±0.32	5.4±0.3	8.4±0.36	2.39±0.16
HbA1C%	4.86±0.13	5.31±0.11	4.09±0.08	8.79±0.19
FBS[d]	84.05±2.5	102.5±2.7	76±0.94	172±5.9

a=Body Mass Index [weight/(height)$^{2]}$ =kg/m^2, b = P value<0.001 c= P value<0.001 , d=fasting blood sugar
* Y= young, ** M= middle age.

C-peptide level between groups:

In this study we measured C-peptide by Electrochemiluminescence technique, and result was analyzed with ANOVA test. As the difference variance between groups was not significant, data were analyzed with fisher and Scheffe test. The results are summarized in table3 and Fig. 2. Relationship between ATP/ADP and C-peptide was analyzed with Pearson correlation that shows a 0.716 unit increase in C-peptide is accompanied with 1 unit increase in ATP/ADP level (table 4).

Correlation between RBC ATP/ADP content and C-peptide:

A significant positive correlation was found between RBC ATP/ADP content (r=0.716; P<0.001). In this study, a positive relationship between ATP/ADP and C-peptide is found, accordingly; a 0.716 unit increase in C-peptide is accompanied by 1 unit increase of ATP/ADP ratio (Fig. 3). Therefore, based on experimental measurements reported here, using B Coefficient a formula for prediction of ATP/ADP ratio could be obtained : ATP/ADP= 0.429 + 0.420 [C- peptide] (Table 5).

Figure-2: The comparison of C-peptide level among 4 groups (diabetes, Normal (Y=young, M=middle age) and athletes)

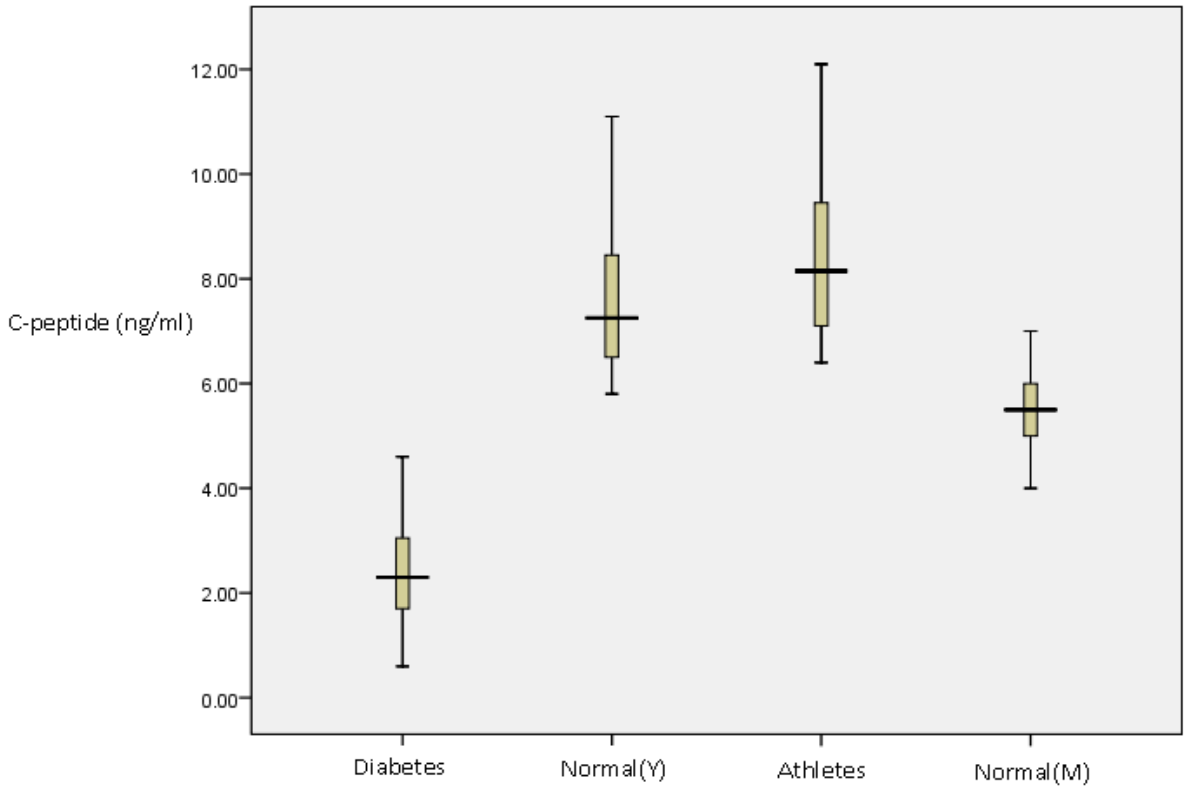


Table-2. Tamhan test for ATP/ADP group comparison, as shown in this table the difference between groups are significant (P-value <0.001).

Factor(I)		Factor(J)	Sig.*
Tamhan (ATP/ADP)	Diabetes	Normal(Y)[1]	0.000
		Normal(M)[2]	0.000
		Athletes	0.000
	Normal(Y)	Diabetes	0.000
		Normal(old)	0.000
		Athletes	0.001

*. The mean difference is significant at the 0.05 level. 1=young, 2=middle age.

Table-3. Scheffe test for comparison between groups shows that c-peptide differences between Normal and athletes individuals are not significant.

Factor(I)		Factor(J)	Sig.*
Scheffe (C-peptide)	Diabetes	Normal(Y)[1]	0.000
		Normal(M)[2]	0.000
		Athletes	0.000
	Normal(Y)	Diabetes	0.000
		Normal(M)	0.001
		Athletes	0.272

*. The mean difference is significant at the 0.05 level. 1=young, 2=middle age.

Table-4. Pearson correlation between ATP/ADP and C-peptide, the correlation between C-peptide and ATP/ADP is 0.716.

	ATP/ADP & C-peptide	Sig.
Pearson correlation	0.705*	0.000

*. Correlation is significant at the 0.01 level (2-tailed).

DISCUSSION

Impairment in glucose metabolism in diabetic patients when it goes into cell is a main problem in treatment of diabetic patients [46]. It has been suggested that decrease in ATP/ADP ratio in pancreatic- β cells makes decrease in insulin and C-peptide secretion into plasma and led to type 2 diabetes [47]. The present study investigates the simultaneous effects of exercise and C-peptide level on the RBC metabolism rate, ATP/ADP content. In healthy control (normal and athletes) and type 2 diabetes (Fig.1, Table 2) , a positive significant correlation (Fig.3, Table 4) between plasma C-peptide and ATP/ADP content of RBC among four groups has been observed. This observation

Figure-3. Correlation between RBC ATP/ADP content and C-peptide level between four groups.

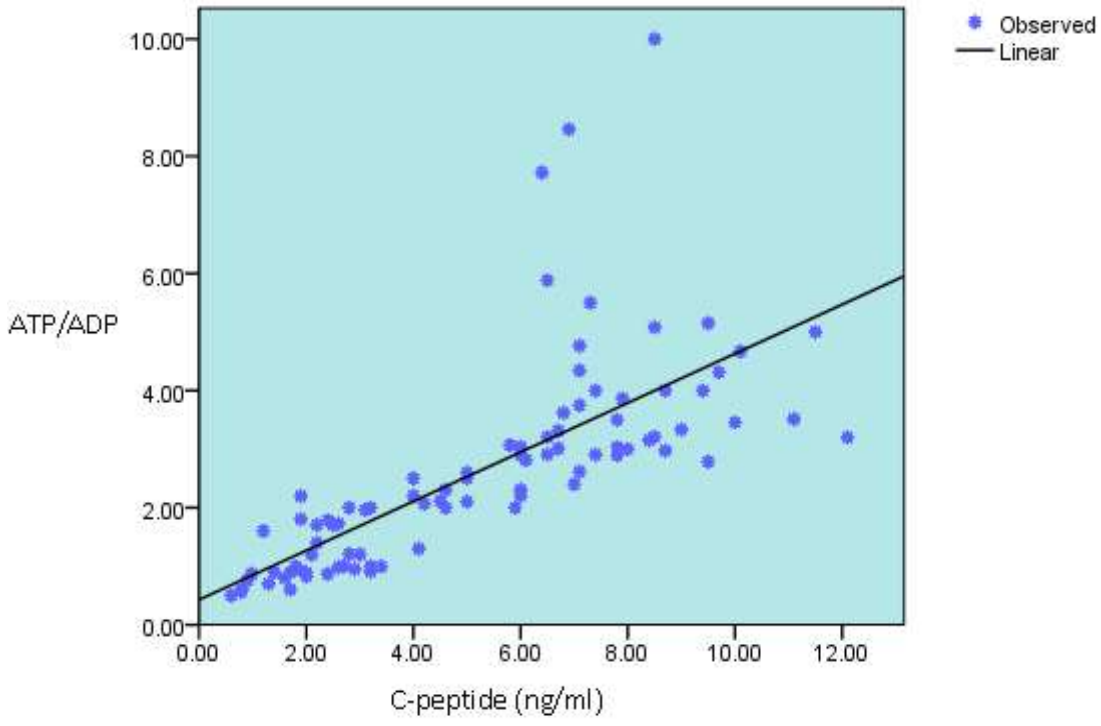
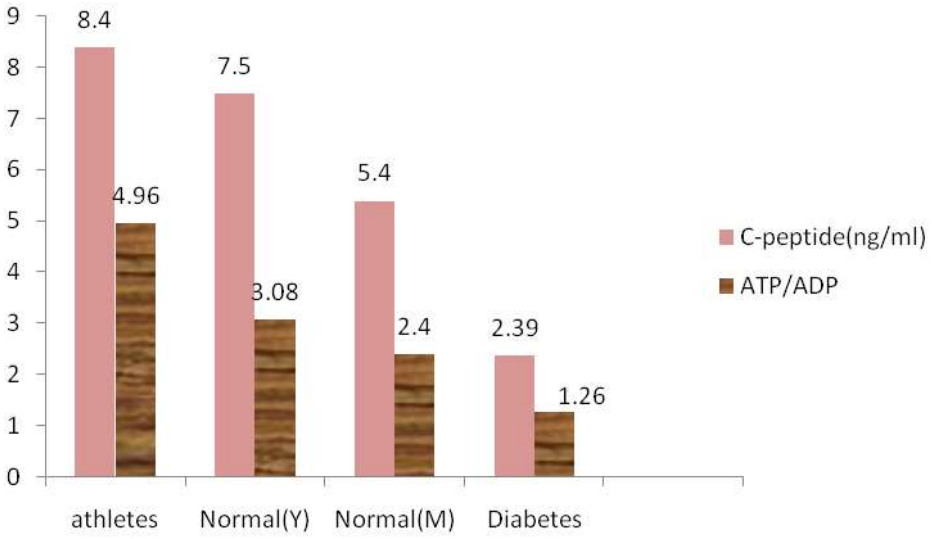


Figure-4. Correlation between c-peptide and RBC ATP/ADP with bar curve.



suggests higher metabolic rate (ATP/ADP ratio) in athletes in comparison with normal and type2 diabetes. Other study reported that athletes have high ATP/ADP content in comparison with normal subject [48]. Also a similar positive correlation between exercise and erythrocyte Na+, K+-ATPase has been reported [49]. Indeed, Rabini et al. [50] and Petruzzi et al. [51] showed an increase in ATP concentration in erythrocytes in patients with the Type 1 diabetes while Na+, K+ ATPase activity that determines the intracellular ATP concentration was decreased. Moreover, other evidences reported that dysfunction of Na+-K+ ATPase and Ca2+-ATPase activity in the erythrocyte membrane is not directly connected with the degree of diabetic control because there is no correlation between enzymatic activity and fructose amine or glycemia in diabetic rats [52]. This enzymatic dysfunction is probably connected with the decreased ATP concentration, showed by the positive correlation between ATP concentration and the activity of Na+-K+ ATPase [49]. Many evidences suggested that Na+, K+ ATPase increases K+ concentration of cytoplasm and export Na+ into plasma which is accompanied by increase of cytoplasmic calcium [53]. Calcium itself activates calmodulin and calmodulin-dependent proteins such as CAMK, calcineurine [54, 55].

Through a cascade of signals these proteins activate metabolism and increase in ATP/ADP in cells [56, 57]; there are reciprocal relationship between ATP/ADP and Na+, K+-ATPase [58]. On the other hand, other evidences showed that dysfunction in Na+, K+-ATPase increases cytoplasmic concentration of Na+ and this ion led to calcium to be exited from mitochondria. It is known that some of enzymes in mitochondria such as dehydrogenases need calcium ion and this deficiency can led to impairment in mitochondria activity [59]. In this study, we report that released C-peptide from pancreatic cells activates metabolism in RBC and increases ATP/ADP ratio (Fig.3-4, table 4). In fact, measurements of plasma C-peptide by electrochemiluminescence and concomitant measurements of ATP/ADP level of the same individuals RBC by bioluminescence method [60-61] confirmed a direct relationship. C-peptide affects on a G-protein receptor and thereby resulted in an increase in Na+, K+-ATPase activity [62]. Different evidences implicate on increase of intracellular calcium by C-peptide [62, 63]. Interestingly, C-peptide stimulated specifically the classical PKC-α, calcium-dependent mediator of Na+, K+-ATPase phosphorylation in tubular cells [64]. These observations interpret strong reasons for increase in ATP/ADP content upon increase in plasma C-peptide. It is well known that the properties of diabetic erythrocytes are abnormal [65, 66]. These abnormalities include decreased deformability [67, 68], increased membrane viscosity [69] and increased erythrocyte aggregation [45, 70]. The decreased Na+, K+-ATPase activity observed in the diabetic erythrocyte membrane

leads to an intracellular accumulation of sodium with subsequent accumulation of free calcium ions due to competition. A well-known reason for ATP/ADP low level in diabetic subject is due to anaerobic pathway of glucose metabolism in RBC which as mentioned earlier, dysfunction in mitochondria and pH decreasing can led to prevent the activity of some of glycolytic enzymes such as phosphofructokinase, hexokinase, pyruvate kinase [63,71] .

In conclusion, according to the result obtained in this investigation; ATP/ADP ratio is high in athletes and normal individuals in comparison with diabetic patients. It seems that ATP/ADP content may be considered as a suitable parameter for diabetes control and also for athlete's individuals as the higher ATP/ADP ratio can be suggested as a doping test similar to erythropoietin (EPO) effect because ATP/ADP ratio determines metabolic rate in individuals. However, it may be suggested that complementary C-peptide-insulin (digested proinsulin) for drug treatment to diabetes subject, as C-peptide increases ATP/ADP content in cells.

References

[1] Haring HU. (1991)The insulin receptor: signaling mechanism and contribution to the pathogenesis of insulin resistance. Diabetologia. 34, 848-61.
[2] Moller DE, Flier JS. (1991) Insulin resistance-mechanism, syndromes, and implications. N Engl J Med. 325, 938-48.
[3] Kelley DE, Mandarino LJ. (2000) Fuel selection in human skeletal muscle in insulin resistance: a reexamination. Diabete, 49, 677-683.
[4] Ritov VB, Menshikova EV, He J, Ferrell RE. (2000) Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. Diabete. 54, 8-14.
[5] Shulman GI. (2004) Unraveling the cellular mechanism of insulin resistance in humans: new insights from magnetic resonance spectroscopy. Physiology (Bethesda). 19, 183-190.
[6] Petersen KF, Befroy D, Dufour S, Dziura. (2003) Mitochondrial dysfunction in the elderly: possible role in insulin resistance. Science. 300, 1140-1142.
[7] Mootha VK, Lindgren CM, Eriksson KF. (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet. 34, 267-273.
[8] Patti ME, Butte AJ, Crunkhorn S. (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci U S A. 100, 8466-8471.
[9] Sreekumar R, Halvatsiotis P, Schimke JC. (2002) Gene expression profile in skeletal muscle of type 2 diabetes and the effect of insulin treatment. Diabetes. 51, 1913-1920.
[10] Hojlund K, Wrzesinski K, Larsen PM. (2003) Proteome analysis reveals phosphorylation of ATP synthase beta -subunit in human skeletal muscle and proteins with potential roles in type 2 diabetes. J Biol Chem. 278, 10436-10442.
[11] Kelley DE, Mandarino LJ. (2000) Fuel selection in human skeletal muscle in insulin resistance: a reexamination. Diabetes. 49, 677-683.
[12] Ritov VB, Menshikova EV, He J. (2005) Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. Diabetes. 54, 8-14.
[13] Mootha VK, Lindgren CM, Eriksson KF. (2003) PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. Nat Genet. 34, 267-273.
[14] Patti ME, Butte AJ, Crunkhorn S. (2003) Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: Potential role of PGC1 and NRF1. Proc Natl Acad Sci U S A. 100, 8466-8471.
[15] Sreekumar R, Halvatsiotis P, Schimke JC. (2002) Gene expression profile in skeletal muscle of type 2 diabetes and the effect of insulin treatment. Diabetes. 51, 1913-1920.
[16] Hojlund K, Wrzesinski K, Larsen PM. (2003) Proteome analysis reveals phosphorylation of ATP synthase beta -subunit in human skeletal muscle and proteins with potential roles in type 2 diabetes. J Biol Chem. 278, 10436-10442.
[17] Pieper, A.A., Verma, A., Zhang, J. & Snyder, S.H. (1999) Poly (ADP-ribose) polymerase, NO and cell death. Trends Pharmacol. Sci. 20, 171–181.
[18] FRANCISCO GARCIA SORIANO, LÁSZLÓ VIRÁG, PRAKASH JAGTAP. (2001) Diabetic endothelial dysfunction. the role of poly(ADP-ribose) polymerase activation, volume7, number1
[19] Eliasson, M.J. (1997) Poly(ADP-ribose) polymerase gene disruption renders mice resistant to cerebral ischemia. Nature Med. 3, 1089–1095.
[20] Zingarelli, B., Salzman, A.L. & Szabó, C. (1998) Genetic disruption of poly (ADP-ribose) synthetase inhibits the expression of P-selectin and intercellular adhesion molecule-1 in myocardial ischemia/reperfusion injury. Circ. Res. 83, 85–94.
[21] Burkart, V. (1999) Mice lacking the poly(ADP-ribose) polymerase gene are resistant to pancreatic β-cell destruction and diabetes development induced by streptozocin. Nature Med. 5, 314–319.
[22] Newsholme P, Gordon S & Newsholme EA. (1987) Rates of utilisation and fates of glucose, glutamine, pyruvate, fatty acids and ketone bodies by mouse macrophages. Biochemical Journal. 242, 631–636.
[23] Szabó, C., Cuzzocrea, S., Zingarelli, B. (1997) Endothelial dysfunction in a rat model of endotoxic

shock. Importance of the activation of poly(ADP-ribose) synthetase by peroxynitrite. J. Clin. Invest. 100, 723–735
[24] Sacks DB. In Burtis CA,Ashwood ER(eds). (1999) Tietz Textbook of clinical chemistry, WB Saunders,Philadelphia,3rd edition 750-808, Chapter8 metabolism
[25] Ataullakhonow, Fj, Vitvitsky, Vm, Zhabotinsky. (1981) The regulation of glycolysis in human erythrocytes. Eur J Biochem. 115, 359-365
[26] Dabrowska. (1997) A Red cell pyruvate kinase maybe control of oxygen delivery from erythrocyte. Post Hig Med Dosw. 51, 305-318.
[27] Atkinson, DE. (1968) The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. Biochemistry. 7, 4030-4034.
[28] Pelleg A, Schulman ES. (2002) Adenosine 5-triphosphate axis in obstructive airway diseases [Review]. Am J Ther. 9, 454–64.
[29] Vial C, Pitt SJ, Roberts J. (2003) Lack of evidence for functional ADP-activated human P2X1 receptors supports a role for ATP during hemostasis and thrombosis. Blood. 102, 3646–51.
[30] Bergfeld GR, Forrester T. (1992) Release of ATP from human erythrocytes in response to a brief period of hypoxia and hypercapnia. Cardiovasc Res. 26, 40–7.
[31] Gorman MW, Ogimoto K. (2003) Savage MV Nucleotide coronary vasodilation in guinea pig hearts. Am J Physiol Heart Circ Physiol. 285:H1040–7.
[32] N Kono, M Kuwajima, and S Tarui. (1981) Alteration of glycolytic intermediary metabolism in erythrocytes during diabetic ketoacidosis and its recovery phase. Diabetes April. 30, 346-353.
[33] Mitsuhiro SOFUE, Yoshiyuki YOSHIMURA, Mikio NISHIDA. (1993) ADP modifies the function reconstituted liposomes, Biochem. J. 292, 877-881.
[34] Sofue,M.,Youshimura,Y.,Nishida,M.,and Kawada,d . (1991) human erythrocytes and uptake of 3-o-methyl-D-glucose decreased by intracellular ATP concentration, J.Endocrinol. 131, 135-138
[35] Jaguez.J.A. (1983) The uptake of 2-deoxy-D-glucose in cultured rat pancreatic β-cells Biochem.Biophys.Acta. 727, 367-378
[36] Hebret,D.N.and carruthers,A. (1986) Transport processes in eukaryotic and prokaryotic organisms, J.Biol.chem. 261, 10093-10099.
[37] Carruthers, A. (1986a)Proceedings of the National Academy of Sciences of the United States of America, Biochemistry. 25, 3592-3602.
[38] Sprague RS, Ellsworth ML, Stephenson AH. (1996) ATP: The red blood cell link to NO and local control of the pulmonary circulation. Am J Physiol. 271, H2717–H2722.
[39] Sprague RS, Ellsworth ML, Stephenson AH. (1998) Deformation-induced ATP release from red blood cells requires cystic fibrosis transmembrane conductance regulator activity. Am J Physiol .275, H1726–H1732.
[40] Strehler BL, McElroy WC. (1957) Assay of adenosine triphosphate. In: Colowick SP, Kaplan NO, Eds. Methods in Enzymology. New York: Academic Press. pp, 871–873.
[41] Kimmich,GA.,Randles,J.,Brand,JS. (1975) Assay of picomole amounts of ATP,ADP, and AMP using the luciferase enzyme system. Anal Biochem. 69(1), 187-206.
[42] Slotte, H. Gustafson, O. Nylund'L, Pousette. (1990) A ATP and ADP in human pre-embryos. Human Reproduction. 5, 319-322.
[43] Houssa P,Dinesen B ,Deberg M. (1998) First direct assay for intact human proinsulin.clin chem. 44(7), 1514-1519.
[44] Zilkens TM,Eberle AM,Schmidt-Gayt H. (1996) Immunoluminometric assay (ILMA) for intact human proinsulin and its conversion intermediates.clin chim Acta. 247, 23-37.
[45] Amal A. Hassan, Esam H. Mansour, Abo El-Fath A. El Bedawey and Mohamed S. Zaki. Effect of α-amylase enzyme on rheological properties and quality of betifore-type cookies. *Biolife* 2015; 3(1); 31-39.
[46] Angie McAdams American Diabetes Association Guideline; (2009). http://EzineArticles.com/?expert=Angie_McAdams.
[47] Attie AD,Kendziorski CM. (2003 Jul) PGC-1alpha at the crossroads of type 2 diabetes. Nat Genet. 34(3), 244-5.
[48] Larsen PR,et al. (2003)Williams Textbook of Endocrinology,9th ed.saunders.
[49] Clark PM. (1999) Assays for insulin,proinsulin(s) and C-peptide. Ann Clin Biochem. 36(5), 541-564.
[50] D.R.Wgner,J.Felber,U.Gresser. (1991) Muscle metabolism and Red cell ATP/ADP concentration during Ergometer in patients with AMPD-Deficiency.klin Wochenscher. 69, 251-255.
[51] W Dudzinska, AJ Hlynczak. (2004) Purine nucleotides and their metabolites in erythrocytes of streptozotocin diabetic rats, Diabetes Metab. 30, 557-567 • www.e2med.com/dm
[52] Rabini RA, Fumelli P, Staffolani R. (1993) Effects of diabetes mellitus on structural and functional properties of erythocyte membranes. Membr Biochem. 10, 71-9.
[53] Michael I. Lindinger and Simon P. Grudzien. (2003) Exercise-induced changes in plasma composition increase erythrocyte Na+,K+-ATPase, but not Na+–K+–2Cl_ cotransporter, activity to stimulate net and unidirectional K+ transport in humans, DOI: 10.1113/jphysiol;052860,
[54] Ganong's. (2010) Review of Medical physiology Endocrine functions of the pancreas & Regulation of Carbohydrate Metabolism. chapter 21, twenty-third editions
[55] Chin, E. R., E. N. Olson, J. A. Richardson. (1998) A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. Genes Dev.12, 2499–2509.
[56] Delling, U., J. Tureckova, H. W. Lim. (2000) A calcineurin-NFATc3-dependent pathway regulates skeletal muscle differentiation and slow myosin heavy-chain expression. Mol. Cell. Biol. 20, 6600–6611.
[57] Wu, H., S. B. Kanatous, F. A. Thurmond. (2002) Regulation of mitochondrial biogenesis in skeletal muscle by CaMK. Science. 296, 349–352.
[58] Potthoff, M. J., H. Wu, M. A. Arnold. (2007) Histone deacetylase degradation and MEF2 activation promote

the formation of slow-twitch myofibers. J. Clin. Invest. 117, 2459–2467.
[59] Clausen T, van Hardeveld C, Everts ME. (1991) Significance of cation transport in control of energy metabolism and thermogenesis. Physiol Rev. 71, 733–774.
[60] DiLisa F, Fan C, Gambassi G, Hogue B, Kudryashova I, Hansford R. (1993) Altered pyruvate dehydrogenase and mitochondrial free Ca2+ in hearts of cardiomyopathic hamsters. Am J Physiol. 264(6 Pt. 2), H2188–H2197.
[61] Bagher Said Alipour, Saman Hosseinkhani. (2009) The effective role of positive charge saturation in bioluminescence color and thermostability of firefly luciferase, Photochemical & Photobiological Sciences. 8, 847–855
[62] Moosavi SM, Ashtiyani SC, Hosseinkhani S. (2010) Comparison of the effects of L: -carnitine and alpha-tocopherol on acute ureteral obstruction-induced renal oxidative imbalance and altered energy metabolism in rats. Urol. Res. 38(3), 187-94.
[63] Ohtomo, Y., Aperia, A., Sahlgren, B. (1996) C-peptide stimulates rat renal tubular Na+, K(+)-ATPase activity in synergism with neuropeptide Y. Diabetologia. 39, 199–205.
[64] Tarakalakshmi, Y and Viveka Vardhani V. Protein, DNA, RNA and amino acids contents from stomach of mice infected with Ancylostoma caninumlarvae Biolife 2014;2(2); 486-492.
[65] Shafqat, J., Juntti-Berggren, L., Zhong, Z. (2002) Proinsulin C-peptide and its analogues induce intracellular Ca2+ increases in human renal tubular cells. Cell. Mol. Life Sci. 59, 1185–1189.
[66] Tsimaratos, M., Roger, F., Mordasini, D. (2003) C-peptide stimulates Na,K-ATPase activity via PKC alpha in rat medullary thick ascending limb. Diabetologia. 46, 124–131.
[67] Finotti, P., and Verbaro, R. (1987) Identification and partial purification of a Na,K-ATPase stimulating serine protease from plasma of insulin-dependent diabetics. Clin. Chim. Acta. 170, 121–134.
[68] McMillan, D. E. (1975) Deterioration of the microcirculation in diabetes. Diabetes. 24, 944–957.
[69] Vague, P., and Juhan, I. (1983) Red cell deformability, platelet aggregation, and insulin action. Diabetes. 32, 88–91.
[70] Baba, Y., Kai, M., Kamada, T. (1979) Higher levels of erythrocyte membrane microviscosity in diabetes. Diabetes. 28, 1138–1140.
[71] Schmid-Schonbein, H., and Volger, E. (1976) Red-cell aggregation and red-cell deformability in diabetes. Diabetes. 25, 897–902.
[72] Gardner, K., and Bennett, V. (1986) A new erythrocyte membraneassociated protein with calmodulin binding activity. Identification and purification. J. Biol. Chem. 261, 1339–1348.
[73] Pamela C.Champe Richard A.Harvey Lippincott's. (2008) Illustrated Reviews Biochemistry. ,metabolism of monosaccarides and disaccharides, chapter 12

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