

*Full Length Research Paper*

# Nitric oxide level and von Willebrand factor (vWF) secretion are not candidate markers of endothelial cell dysfunction in adenosine triphosphate (ATP) depleted endothelial cells

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Endothelial cells under physiological conditions can alter energy balance by alteration in synthesis, metabolism and transport of adenosine triphosphate (ATP), which failed during endothelial cell dysfunction. ATP depleted endothelial cells are unable to perform their physiological functions as energy dependent protein secretion. Isolated human umbilical vein endothelial cells (HUVEC) from fresh umbilical cords were treated with 10 mM 2-deoxyglucose and 0.1 pg/ml of oligomycin for 6 h to induce ATP depletion. Nitric oxide (NO), von Willebrand factor (vWF), lactate dehydrogenase (LDH) release and trypan-blue exclusion were compared between treated and untreated cells. We observed a slight decrease in nitric oxide levels ( $P = 0.09$ ) and vWF ( $P = 0.395$ ) in the setting of 49.36% ATP depletion. There was no significant change in LDH release and cell viability between treated and untreated cells ( $P > 0.05$ ). Since vWF exocytosis is an energy consuming process, decreased secretion of vWF in the isolated near-half percent of ATP depletion is not seemingly at odds. The application of vWF exocytosis fades as a candidate marker for ATP depletion induced injury, in cultured endothelial cells. Nitric oxide level and vWF secretion are not candidate markers of endothelial cell dysfunction in isolated partial ATP- depleted HUVECs. Measures such as arachidonic acid synthesis may be better alternatives.

**Key words:** Endothelial cell dysfunction, adenosine triphosphate (ATP) depletion, nitric oxide, von Willebrand factor (vWF).

## INTRODUCTION

Endothelial cells under physiological conditions can alter energy balance by alteration in the synthesis, metabolism

and transport of adenosine triphosphate (ATP) (Buxton et al, 2001; Arakaki et al., 2003). These cells release Purinergic nucleotides that are then hydrolyzed to adenosine diphosphate (ADP) and adenosine by the act of ectonucleotidase (Kolossova et al., 2005). The released ATP acts as a protective agent in the maintenance of endothelial cell integrity via its barrier-keeping function (Kolossova et al., 2005). The generated adenosine is a potent autacoid, which prohibits untoward platelet activation and thrombus formation (Bassenge, 1989). In addition, adenine nucleotides modulate the release of endothelial-derive relaxing factors and hence play an important role in flow-mediated arterial vasoregulation (Choi and Barakat, 2004).

In the process of endothelial cell dysfunction, herald

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**Abbreviations:** ATP, Adenosine triphosphate; ox-LDL, oxidized low-density lipoprotein; vWF, von Willebrand factor; HUVEC, human umbilical vein endothelial cells; EDTA, ethylenediaminetetraacetic acid; 2DG, 2-deoxyglucose; OG, oligomycin; PBS, phosphate buffered saline; LDH, lactate dehydrogenase; KATP, adenosine triphosphate dependent potassium channels; RBECs, rat brain capillary endothelial cells.

mechanism toward atherosclerosis and endothelial cells fail to keep their energy balance state. This alteration is due to oxidative mitochondrial dysfunction, oxidized low-density lipoprotein (ox-LDL) and inflammatory or hypoxic injuries (McCully, 2009; Schmitt, 1995; Hultén and Levin, 2009). This energy depletion put cells in energy crisis which mutually results in further mitochondrial damage by accumulation of reactive species. The inactivated ectonucleotidase activity, mainly CD39, results in increased vascular level of ADP and limited bioavailability of adenosine, which is associated with increased platelet aggregation and vasoconstriction (El-Omar et al., 20058; Burnstock, 1987). Energy depletion makes cells susceptible to further injuries, a synergistic factor, which threatens health of the cell and its neighbors; hence they lose energy for defense. Depletion of ATP, major consequence of ischemia disrupts the normal transmembrane ionic gradients and ion channels, which leads to accumulation of sodium and calcium in intracellular and K in extracellular space (Hinshaw et al., 1988; Siesjö, 1992; Köppel et al., 1998). The impaired function of ATP-regulated channels, detrimental in the control of cell membrane polarization, is evident by depolarized state of dysfunctional cells (Figura et al., 2009).

Endothelial cell dysfunction is associated with reduced nitric oxide (NO) release (Florea and Blatter, 2008). Increased plasma levels of von Willebrand factor (vWF) were also detected in endothelial cell dysfunction, but it is not evaluated in endothelial cells exposed to isolated ATP depleted endothelial cells. As a matter of fact, vWF secretion from endothelial cells is an ATP dependent process, which is expected to be inhibited in ATP depleted endothelial cells (Vischer, 2006). Based on these data, it seems unreasonable to use vWF release, a widely used marker of endothelial cell dysfunction, in each setting. Application of vWF as a marker of endothelial cell dysfunction seems questionable in cultured endothelial cells, with the absence of platelets and ischemic tissue, as other sources of vWF.

Therefore, this study was performed to evaluate the role of ATP depletion on the NO levels and vWF secretion in endothelial cells, and their efficacy as candidate markers of endothelial cell dysfunction *in vitro*.

## MATERIALS AND METHODS

### Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated from fresh umbilical cords as described previously. Briefly, human umbilical cords were obtained by written consent; with approval from Institutional review committee of Isfahan University of Medical Sciences. Cells were cultured in M199 culture medium (Gibco-BRL, Scotland) in T25 flasks coated with gelatin (1%). Confluent monolayers were passed using Trypsin/ ethylenediaminetetraacetic acid (EDTA) (Sigma- Aldrich, St. Louis, MO). Morphology of HUVEC monolayer was examined by a phase contrast microscope (Motic digital microscopes equipped with Moticam digital camera/software). Verification of endothelial cell identity was also

performed by the expression of CD31 (PECAM) on their surface which was measured by flow cytometry analysis after staining with phycoerythrin-conjugated murine monoclonal anti-human antibodies (eBiosciences, San Diego, CA, U.S.A.). All experiments were carried out in triplicates on the primary cultures in their third passage.

### Induction of ATP depletion

HUVECs were treated with 10 mM 2-deoxyglucose (2DG), a glycolytic inhibitor, and 0.1 pg/ml of oligomycin (OG), a respiratory chain inhibitor, to induce ATP depletion and incubated at room temperature, in phosphate buffered saline (PBS). The applied dose of these agents was based on the dose-response curve of these compounds (data not shown). According to the previous study carried out by Revtyak et al. (1992), the simultaneous application of these agents were associated with partial ATP-depletion without induction of apoptosis or necrosis. So with the application of this method, the desired ATP-depletion amount is less than 50%. After 6 h, culture media was removed and cells were washed with PBS.

### Assay of cellular ATP/ADP

In order to assess the induced ATP depletion, cellular ATP, ADP and ADP/ATP ratio were measured by the ApoSENSOR™ ADP/ATP Ratio Assay Kit (Biovision, USA), in induced and un-induced cells in 96-well plates. Adenine nucleotides were measured by quantified chemiluminescent signals read out using a CentroLIA LB 961 plate Luminometer (Berthold Technologies, Germany). The quantified signals were expressed in Log<sub>10</sub> relative light units (RLU). Morphological changes after ATP depletion were assessed by phase contrast microscope and viable cells were counted after staining with trypan blue.

### Lactate dehydrogenase (LDH) assay

In order to determine membrane disintegration and increased permeability, LDH levels were measured in test and control cells. The supernatant LDH of treated and untreated HUVECs in 96-well plates were assayed, based on the reduction of NAD<sup>+</sup> at 339 nm, using DGKC Kit (Parsazmun, Iran).

### NO measurements

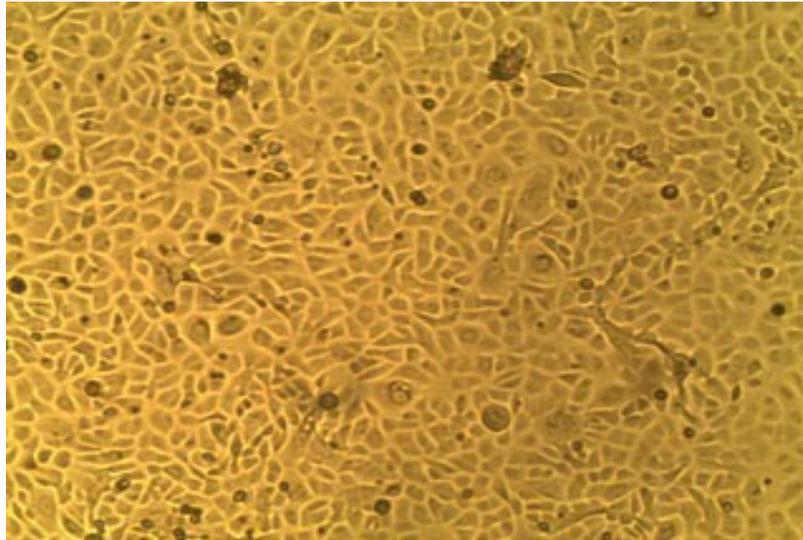
NO in cell-conditioned medium was measured indirectly by quantification of nitrites, using the total nitric oxide and nitrate/nitrite parameter assay kit according to manufacturer's instructions (R&D). NO measurement was performed in both treated and untreated cells.

### vWF measurements

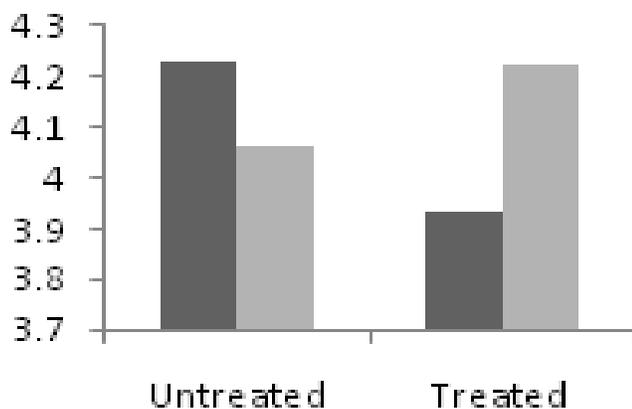
Extracellular vWF was measured in HUVECs in 96-well plates before and after ATP depletion using Asserachrom vWF Kit according to manufacturer's instructions (Diagnostica Stago, France).

### ATP-dependent ion transport

In order to evaluate the induced ionic changes after ATP depletion in HUVECs, the extracellular Na and K were measured by emission flame photometry before and after ATP depletion.



**Figure 1.** Round morphology of human umbilical vein endothelial cells depicted by phase-contrast microscope.



**Figure 2.** Influence of 2-deoxyglucose and oligomycin treatment on cellular ATP and ADP levels in HUVECs. The amounts of ATP (black bars) and ADP (grey bars) were assessed in HUVEC cells before and after 6 h of treatment with 2-deoxyglucose and oligomycin. The results are expressed as Log<sub>10</sub> relative light units (RLU) (mean for three repeats).

#### Statistical analysis

Data representations and statistical tests were made with paired samples Student's t-test using the Statistical Package for the Social Sciences (SPSS) software Ver. 15. P-value less than 0.05 was considered to be significant.

## RESULTS

### Induction of ATP depletion by simultaneous application of 2DG and OG

The study was based on the HUVEC cells, well known

cells in terms of endothelial cell injury and dysfunction. Cells were verified as endothelial cells by their polygonal cobblestone morphology. Phase contrast microscopic photographs of the cultured endothelial cells are shown in Figure 1. Flow cytometry results demonstrated that cells were stained positively with anti-CD31 antibody. By simultaneous application of 2DG and OG, 49.36% ATP depletion was induced in the treated cells and cellular ADP demonstrated a 45% increase in treated when compared with untreated cells. ADP/ATP ratio was 0.51 in untreated cells which increased to 3.33 after treatment with 2DG and OM. These data were mostly in favor of growth arrest (Figure 2). There was no difference in the cell number between treated and untreated cells, counted by trypan blue exclusion within 15 min ( $92.66 \pm 1.11$  vs.  $96.36 \pm 0.53\%$  per 40 mm dish,  $P = 0.071$ ,  $n = 3$ ). After 6 h of ATP depletion, some endothelial cells appeared round and retracted. Only a few numbers of ATP-depleted endothelial cells were detached from the culture media and large gaps were seen between endothelial cells. There was no difference in LDH release between treated and untreated cells ( $7.40 \pm 0.56$  vs.  $7.55 \pm 0.07$  IU/L,  $P = 0.75$ ,  $n = 3$ ), which is in favor of intact membrane and absence of the necrotic events in ATP depleted endothelial cells (Table 1).

### ATP depletion decreases NO levels and vWF secretion in HUVECs

There was a slight decrease (16.32%) in NO levels between induced and un-induced endothelial cells ( $7.23 \pm 1.13$  vs.  $8.64 \pm 0.85$   $\mu\text{m/L}$ ,  $P = 0.09$ ,  $n = 3$ ). In addition, our results demonstrated a marginal decrease (15.73%) in secreted vWF between treated and untreated cells

**Table 1.** Effect of ATP depletion on NO level, vWF secretion, LDH release and extracellular Na/K of human umbilical vein endothelial cells.

Parameter	Mean $\pm$ SD	
	Before	After
NO	8.64 $\pm$ 0.85	7.23 $\pm$ 1.13
vWF	0.087 $\pm$ 0.008	0.105 $\pm$ 0.023
LDH	7.55 $\pm$ 0.07	7.40 $\pm$ 0.56
Na	158.3 $\pm$ 1.52	138.3 $\pm$ 1.52*
K	4.40 $\pm$ 0.1	3.83 $\pm$ 0.05*

Primary cultures of HUVECs in 96-well plates were incubated for 6 h in the absence and presence of 10 mM 2-deoxyglucose (2DG) and 0.1  $\mu$ g/ml oligomycin (OG) in PBS buffer, pH 7.4 at 37°C. NO level, vWF secretion, LDH release and extracellular Na/K were assessed before and after ATP depletion. Data are expressed as the mean  $\pm$  S.D for three repeats. \* denotes  $P < 0.05$ .

(0.0876  $\pm$  0.0087 vs. 0.1056  $\pm$  0.023885  $\mu$ m/L,  $P = 0.395$ ,  $n = 3$ ) (Table 1).

### Altered ionic gradient in ATP depleted HUVECs

A significant decrease in extracellular Na was observed between induced and un-induced endothelial cells (138.33  $\pm$  1.52 vs. 158.33  $\pm$  1.52 mEq/L,  $P = 0.006$ ,  $n = 3$ ). This decrease (12.63%) is in parallel with inhibition of Na/K/ATPase pump. Interestingly, a significant decrease (12.95%) in extracellular K level was also seen in ATP depleted cells when compared to normal ATP level cells (3.8333  $\pm$  0.057 vs. 4.4000  $\pm$  0.100 mEq/L,  $P = 0.023$ ,  $n = 3$ ) (Table 1).

### DISCUSSION

In our experiment, vWF factor was slightly decreased (15.73%) in ATP-depleted endothelial cells. Hence, it is considered as a marker of endothelial cell dysfunction and we expected its secretion to be increased. vWF functions (Holmsen, 1994) and its exocytosis from endothelial cells are shown to be an ATP-dose dependent process (Hegeman et al., 1998) and consequently, after ATP depletion, vWF exocytosis will be impaired and decreased vWF secretion in this setting, is explainable by its energy dependent protein exocytosis.

Enhanced vWF exocytosis is seen in the HUVECs exposed to isolated acute hypoxia, in contrast to human retinal endothelial cells which showed decreased vWF secretion under oxygen-glucose deprivation (Kim et al., 2007). It seems that the secretion of vWF in response to endothelial cell stimulants is different by endothelial cells with various origins, but the importance of this difference needs to be determined. High levels of cytokines *in vitro* were not able to raise the secretion of vWF in treated endothelial cells, which hints the diverse effects of diffe-

rent stimuli on vWF exocytosis (Zavoico et al., 1989). Considering energy depletion as a cellular insult with effects on vWF secretion, the effects of isolated metabolic inhibition on the vWF exocytosis pattern in HUVECs needs to be determined. ATP and its metabolites exert an agonist-induced exocytosis of vWF (Parker and Gralnick, 1997). Contrarily, ATP depletion acts as an antagonist-inhibited exocytosis of vWF (Dorner et al., 1990). So, enhanced vWF exocytosis from dysfunctional endothelial cells is not attributable to energy crisis and we suggest that other stimuli such as membrane shedding may provoke it. On the other hand, activated platelets and reperfused ischemic tissues may be source of secreted vWF which are absent in studies on HUVECs. Also, for endothelial cells, it has been demonstrated that vWF secretion in platelets is an ATP-dependent process which is decreased in the ATP-depleted settings (Parker and Gralnick, 1997). However, it seems that vWF is not a candidate marker of endothelial cell dysfunction in the setting of ATP depletion induced endothelial cell injury.

Lines of evidences demonstrated that NO level decreases in dysfunctional endothelial cells, a favored index of endothelial cell dysfunction (Kawai et al., 1996). But interestingly, NO level has never been measured as a marker of endothelial cell dysfunction in isolated ATP depleted HUVECs. In our experiment, 16.32% decrease in NO level was observed in ATP depleted when compared to untreated cells. This statistically non-significant change in NO level may be due to the fact that constellation of insults are needed for impairment of endothelial cell function, hence endothelial cells are considered as resistant cells to imposed stresses. Isolated ATP depletion may lead to aberrant endothelial cell function, synergistically, with other stressors especially by predisposing cells to further injuries. It seems that isolated partial energy deprivation may be tolerable or even considered as a sub-lethal injury by cells. The threshold of endothelial cell injury needed to perturb NO activity is not well determined, but it seems that depletion of near fifty percent of ATP stores is less pronounced to induce dramatic fall in NO production. This level of ATP depletion for cells in conditioned medium will be certainly different from *in vivo* conditions with complex interplay microenvironment. Determination of the threshold of ATP depletion requisite for induction of lethal cellular injury, measurable by impaired NO production may be helpful for better understanding of ischemia and reperfusion injuries.

Consistent with previous data, isolated partial ATP depletion has not been associated with induction of apoptosis or necrosis of endothelial cells, as demonstrated by the percentage of viable cells and LDH release from treated cells (Revtyak and Campbell, 1992). Six hours of ATP deprivation, induced by simultaneous application of 2-DG and OG, is shown to be associated with endothelial cell dysfunction, determined by impaired synthesis of arachidonic acid (Revtyak and Campbell, 1992). We aimed to induce purinergic changes less than

50% ATP depletion to prevent cell death. Statistically, non-significant changes of NO and vWF secretion in our study demonstrated that candidate markers of endothelial cell dysfunction should be sought *in vitro* based on design of the experiment, not universally.

ATP depletion is considered as a key factor in the disruption of normal cellular transmembrane ionic gradient (Pepe, 2000). Decreased extracellular concentration of Na, an indirect measure of increased intracellular retention, is in accordance with Na/K/ATPase pump failure under hypoxic conditions (Palpant et al., 2008; Kawai et al., 1996). Intracellular alteration of Na and calcium concentrations following ATP depletion is associated with activation of inwardly rectifier K channels (KIR) of high conductance, to facilitate cell membrane re-polarization (Lien et al, 2003; Mintert et al., 2007; Harrell et al., 2007; Pouget, 2008). During hypoxia, ischemia or metabolic inhibition, ATP-dependent K channels ( $K_{ATP}$ ) open, which facilitate K influx and shortening of action potential duration (Harrell, 2007; Pouget, 2008; Lebuffe et al., 2003; Cameron et al., 2003; Lascano et al., 2002). Therefore, reduced extracellular K concentration, following ATP depletion seems to be due to the activation of ATP-dependent KIR channels, located in the membrane of endothelial cells (Cameron et al., 2003; Hu et al., 2003). The other explanation for reduced extracellular K concentration might be increased K uptake through Na-K-Cl cotransport, as seen in rat brain capillary endothelial cells (RBECs) under hypoxic conditions (Kawai et al., 1996). Membrane alteration may precede alterations in cytoplasm and nucleus. Pump failure and membrane dysfunction may be the earlier events in the process of endothelial cell dysfunction, proceeding NO impairment and vWF exocytosis.

The cascade of events involved in the process of ATP-depletion induced endothelial cell dysfunction, are not clearly identified. Further studies are needed to clarify the role of vWF exocytosis in the stressful conditions provided by isolated energy crisis for endothelial cells. Regulated secretion of vWF in graded ATP-depleted endothelial cells may give us a comprehensive insight in this regard. The lethal dose of ATP depletion for endothelial cells is more than 50%. But the exact threshold needs to be determined by the grade of ATP depletion associated with impaired NO production. Besides, earlier markers of endothelial cell dysfunction should be sought, which proceed impaired NO production. So, measures such as ionic transport pump failure and arachidonic acid synthesis, as earlier markers of endothelial cell dysfunction, may be better alternatives in this setting.

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