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Quantitative ELISA Analysis of Protein Expression in HepG2 Cells Following Treatment with Insulin, Glucose, and Bis(maltolato)oxovanadium(IV)

(Analisis Kuantitatif ELISA Pengekspresan Protein dalam Sel HepG2 Selepas Rawatan dengan Insulin, Glukosa dan Bis(maltolato)oksovanadium(IV))

ABEER SAMIH AL-QATATI*

Medical Laboratory Sciences Department, Faculty of Science, The University of Jordan, Amman, Jordan

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ABSTRACT

Type 2 diabetes, the most common form of diabetes mellitus, is caused by insulin resistance. Many studies have sought orally active therapeutic compounds to replace insulin injections. This study examines the expression levels of key proteins involved in the insulin receptor (InsR) and AMP-activated protein kinase (AMPK) signaling pathways in HepG2 cells using ELISA, following treatment with insulin, glucose, and/or bis(maltolato)oxovanadium(IV) (BMOV). Treatment with BMOV for 24 h in the presence of glucose significantly increased the levels of glucose transporter type 4 (GLUT4) in the cell lysate from 511 ± 7 ng/L (control) to 882 ± 5 ng/L, and AMPK α1 in the supernatant from 683 ± 9 ng/L to 789 ± 6 ng/L. BMOV treatment for 1 h with glucose increased InsR levels in the lysate from 1.52 ± 0.11 ng/L to 4.01 ± 0.03 ng/L. Additionally, cell-based ELISA showed that AMPK β1 absorbance increased significantly under glucose + BMOV (1 h) treatment (1.32 ± 0.04) compared to the control (0.83 ± 0.03). These findings suggest that BMOV modulates glucose-related signaling pathways and may target the AMPK system - a major therapeutic axis for obesity, diabetes, and metabolic syndrome.

Keywords: AMP-activated protein kinase; BMOV; diabetes; ELISA; insulin receptor

ABSTRAK

Diabetes jenis 2 adalah bentuk diabetes mellitus yang paling biasa yang disebabkan oleh rintangan insulin. Banyak kajian telah mencari sebatian terapeutik aktif secara oral untuk menggantikan suntikan insulin. Penyelidikan ini mengkaji tahap pengekspresan protein utama yang terlibat dalam reseptor insulin (InsR) dan laluan isyarat protein kinase (AMPK) diaktifkan AMP dalam sel HepG2 menggunakan ELISA, selepas rawatan dengan insulin, glukosa dan/atau bis(maltolato)oksovanadium(IV) (BMOV). Rawatan dengan BMOV selama 24 jam dengan kehadiran glukosa dengan ketara meningkatkan tahap pengangkut glukosa jenis 4 (GLUT4) dalam sel lisat daripada 511 ± 7 ng/L (kawalan) kepada 882 ± 5 ng/L dan AMPK α1 dalam supernatan daripada 683 ± 9 ng/L kepada 789 ± 6 ng/L. Rawatan BMOV selama 1 jam dengan glukosa meningkatkan tahap InsR dalam lisat daripada 1.52 ± 0.11 ng/L kepada 4.01 ± 0.03 ng/L. Tambahan pula, ELISA berasaskan sel menunjukkan bahawa penyerapan AMPK β1 meningkat dengan ketara di bawah rawatan glukosa + BMOV (1 jam) (1.32 ± 0.04) berbanding kawalan (0.83 ± 0.03). Penemuan ini mencadangkan bahawa BMOV memodulasi laluan isyarat berkaitan glukosa dan mungkin menyasarkan sistem AMPK-paksi terapeutik utama untuk obesiti, diabetes dan sindrom metabolik.

Kata kunci: BMOV; diabetes; ELISA; kinase protein diaktifkan AMP; reseptor insulin

INTRODUCTION

The prevalence of diabetes mellitus has increased dramatically in recent decades. In 2006, the World Health Organization (WHO) estimated that approximately 180 million people worldwide were affected, with projections suggesting this number could double by 2030 (World Health Organization 2006). Diabetes is classified into two principal types: type 1 and type 2. Type 1 diabetes results from autoimmune destruction of pancreatic β-cells, leading to a complete absence of insulin secretion. In type 2 diabetes mellitus (T2DM), β-cells still produce insulin; however, the hormone's action on target tissues is impaired - a condition known as insulin resistance. T2DM accounts for about 90% of all diabetes cases, while type 1 diabetes comprises the remaining 10%.

Insulin, the main glucoregulatory hormone, is secreted by pancreatic β-cells in response to elevated blood glucose levels. It inhibits hepatic gluconeogenesis and promotes glucose uptake in muscle and adipose tissue (DeFronzo, Bonadonna & Ferrannini 1992). Insulin action begins with

its binding to the insulin receptor (InsR), which consists of two extracellular α-subunits and two transmembrane β-subunits connected by disulfide bonds (Youngren 2007). The β-subunits possess intrinsic protein tyrosine kinase (PTK) activity essential for insulin function. Upon insulin binding to the α-subunit, multi-site tyrosine phosphorylation of the β-subunit activates its PTK activity (Kahn & White 1988; White et al. 1988). The activated β-subunits then phosphorylate insulin receptor substrates (IRSs), which act as scaffolds for recruiting downstream signaling molecules. This leads to the activation of two major signaling pathways, one of which drives the translocation of glucose transporter type 4 (GLUT4) vesicles to the plasma membrane of muscle and adipose cells (Kahn & White 1988; White et al. 1988). The activated β-subunits then phosphorylate insulin receptor substrates (IRSs), which act as scaffolds for binding downstream signaling molecules. This leads to the activation of two major signaling pathways, one of which drives the translocation of glucose transporter type 4 (GLUT4) vesicles to the plasma membrane of muscle and adipose cells.

As diabetes progresses, chronic complications such as retinopathy, neuropathy, and nephropathy may develop. Oral treatment options for T2DM aim to lower hyperglycemia but often have limited effectiveness or are associated with adverse effects, including hypoglycemia, flatulence, weight gain, and gastrointestinal discomfort. Consequently, there is an ongoing search for safer and more effective therapies. A growing body of research indicates that AMP-activated protein kinase (AMPK) may be an important therapeutic target for T2DM.

AMPK, a key energy-sensing enzyme, is activated by cellular stress that depletes ATP. Once activated, it restores cellular energy balance by increasing ATP production and reducing ATP consumption. AMPK is a heterotrimeric complex composed of one catalytic α-subunit and two regulatory subunits (β and γ). Mammals express two isoforms of both the α- and β-subunits, and three isoforms of the γ-subunit. The α2 isoform is primarily expressed in the liver, skeletal muscle, and heart, whereas α1 is expressed more ubiquitously (Stapleton et al. 1996). The AMPK α-subunit contains a catalytic domain (1–312 aa), an autoinhibitory domain (312–392 aa), and a subunit-binding domain (392–548 aa), with Thr172 in the catalytic domain serving as the key phosphorylation site required for activation (Davies et al. 1994). The β-subunit contains a glycogen-binding domain, while each γ-subunit contains four cystathionine β-synthase (CBS) domains, organized into two pairs, each providing a potential AMP-binding site (Stein et al. 2000).

AMPK promotes glucose uptake in skeletal muscle by stimulating the translocation of GLUT4-containing vesicles to the plasma membrane. It phosphorylates and inhibits the Rab GTPase-activating protein TBC1D1, thereby enhancing Rab family G-protein activity and facilitating the fusion of GLUT4 vesicles with the membrane (Taylor et al. 2008). Once inside the cell, glucose is metabolized via the pentose phosphate pathway, glycolysis, and glycogen synthesis, with AMPK regulating both glycolysis and glycogen formation (Marsin et al. 2002). Additionally, AMPK suppresses gluconeogenesis by inhibiting transcription factors such as CREB-regulated transcription coactivator 2 (CRTC2) and hepatocyte nuclear factor 4 (HNF4), which stimulate the expression of gluconeogenic enzymes (Hunter et al. 2011).

Vanadium, the 21st most abundant element in the Earth's crust, is a transition metal present in foods such as spinach, mushrooms, parsley, shellfish, black pepper, and dill seeds (0.05-1.8 μg/g), contributing to an estimated daily intake of 10-160 μg (Barceloux 1999). In biological systems, vanadium exists in multiple oxidation states (2+, 3+, 4+, and 5+), with vanadyl (VO^{2+}) predominating under acidic conditions (pH < 3.5) and orthovanadate (VO_4^{3-}) under alkaline conditions. In plasma, metavanadate is the primary species, which enters cells via anion transport and is reduced to VO^{2+} by glutathione. The total body vanadium content in humans is estimated to be 100-200 μg.

Interest in vanadium as an antidiabetic agent arose from the work of Heyliger, Tahiliani and McNeill (1985), who demonstrated that sodium orthovanadate normalized hyperglycemia in diabetic rats - a finding later confirmed by Meyerovitch et al. (1987). Subsequent studies in both humans and animal models of type 1 and type 2 diabetes mellitus have shown that various vanadium compounds - both inorganic salts and organic complexes such as bis(maltolato)oxovanadium (BMOV)—improve glycemic control. For example, BMOV treatment of *fa/fa* Zucker rats reduced neuropeptide Y levels, leading to decreased food intake, reduced body fat, and lower weight gain (Wang, Yuen & McNeill 2001). Other studies have demonstrated that vanadium compounds modulate glucose and lipid utilization in adipose tissue, muscle, and liver, as well as in cultured cell lines (Yale et al. 1995).

In our previous work, we were the first to report that BMOV increases the expression of InsR, GLUT4, and multiple AMPK subunit genes (β1, β2, γ1, γ2) in RBL-2H3 cells (Al-Qatati et al. 2013). The current study aims to investigate whether BMOV exerts similar effects at the protein level in HepG2 cells by examining GLUT4, InsR, and AMPK expression using ELISA techniques, thereby providing further insight into BMOV's role in the regulation of glucose homeostasis.

This study was conducted exclusively *in vitro* using the HepG2 cell line, which - although widely used as a model for human liver metabolism - does not fully replicate the complexity of *in vivo* metabolic regulation. The effects of BMOV observed here may differ in whole-organism systems, where factors such as hormonal feedback, tissue-specific uptake, and pharmacokinetics play significant roles. Additionally, only a limited range of BMOV concentrations and treatment durations were tested; dose–response relationships and longer-term effects remain to be established. Another limitation is that, while

protein expression levels were measured, functional assays (e.g., glucose uptake, glycogen synthesis, AMPK activity assays) were not performed to directly confirm the metabolic impact of BMOV-induced protein changes.

Future studies should examine the effects of BMOV in animal models of type 2 diabetes to evaluate its therapeutic potential *in vivo*, including pharmacokinetics, bioavailability, and safety profiles. It will also be important to assess BMOV's impact on other insulin-sensitive tissues, such as skeletal muscle and adipose tissue, as well as its influence on downstream metabolic pathways. Expanding the scope to include gene expression analysis, phosphorylation status of key signaling proteins, and metabolic flux studies will provide a more comprehensive understanding of BMOV's mechanism of action.

MATERIALS AND METHODS

Adherent HepG2 cells (ATCC® HB-8065™, Manassas, VA, USA) were seeded at a density of 20,000 cells per well in 200 µL of complete culture medium into sterile 96-well plates. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM (Cytiva, USA); high glucose, 4.5 g/L; Gibco, Thermo Fisher Scientific, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco), 1% (v/v) penicillin–streptomycin solution (100 U/mL penicillin and 100 µg/mL streptomycin; Gibco), and 2 mM L-glutamine (Gibco). The medium also contained 1 mM sodium pyruvate (Sigma-Aldrich, USA) to support cellular metabolism.

Cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2, with medium changes every 48-72 h. All culture reagents were handled under sterile conditions in a Class II biosafety cabinet. For suspension or loosely adherent cells, wells were pre-coated with 100 µL of 10 µg/mL Poly-L-Lysine (incubated for 30 min at 37 °C) prior to seeding. Following overnight incubation at 37 °C in a humidified 5% CO_2 atmosphere, cells were treated according to the requirements of each experimental condition. After treatment, the culture medium was aspirated, and cells were washed twice with 200 µL of 1X Tris-buffered saline (TBS). Fixation was carried out by incubating the cells with 100 µL of Fixing Solution for 20 min at room temperature (4% formaldehyde for adherent cells, 8% for suspension/loosely attached cells). Plates were sealed with Parafilm during fixation. Subsequently, the Fixing Solution was then removed, and the cells were washed three times with 200 µL of 1X Wash Buffer for 5 min each on an orbital shaker.

Residual formaldehyde was quenched by adding 100 µL of Quenching Buffer per well and incubating for 20 min at room temperature, followed by three additional washes with 1X Wash Buffer. Non-specific binding sites were blocked by adding 200 µL of Blocking Buffer per well and incubating for 1 h at room temperature. Wells were then washed three times with 200 µL of 1X Wash Buffer. Primary antibody incubation was performed by adding 50 µL of 1X primary antibody solution (e.g., anti-PRKAG1/2/3 or anti-GAPDH) to each well. Plates were sealed with Parafilm and incubated overnight at 4 °C. For targets with high expression, incubation was shortened to 2 h at room temperature with gentle shaking. Following incubation, wells were washed three times with 1X Wash Buffer before proceeding to secondary antibody incubation.Bis(maltolato)oxovanadium(IV) (BMOV) was purchased from Santa Cruz Biotechnology (USA). A 1 mM stock solution was prepared in dimethyl sulfoxide (DMSO; Sigma-Aldrich, USA) and stored at -20 °C in aliquots to avoid repeated freeze–thaw cycles. Working concentrations were freshly prepared in complete culture medium immediately before use, ensuring the final DMSO concentration did not exceed 1% (v/v).

For the experiments, HepG2 cells were treated with BMOV at a final concentration of 10 µM for either 1 h or 24 h, in the presence or absence of 16 mM D-glucose (Sigma-Aldrich, USA), according to the experimental design. Cells were assigned to one of eight treatment groups:

(1) Complete medium with vehicle only (1% DMSO, v/v) for the designated treatment period.
(2) 200 nM human recombinant insulin (Sigma-Aldrich, USA) for 1 h.
(3) 10 µM BMOV for 1 h.
(4) 10 µM BMOV for 24 h.
(5) 16 mM D-glucose for 1 h.
(6) 16 mM D-glucose for 1 h in combination with 200 nM insulin for the final 1 h.
(7) 16 mM D-glucose for 1 h in combination with 10 µM BMOV for the final 1 h.
(8) 16 mM D-glucose with 10 µM BMOV concurrently for 24 h concurrently.

PROTEIN EXTRACTION

Protein extraction was performed using a Radioimmunoprecipitation Assay (RIPA) kit (Chem Cruz, sc-24948). Approximately one million HepG2 cells were lysed in RIPA buffer supplemented with a protease inhibitor cocktail. Protein concentration was determined using a Bicinchoninic Acid (BCA) kit (iNtRON Biotechnology, 21071). All samples were normalized to a uniform concentration of 2 mg/mL.

ELISA (ENZYME-LINKED IMMUNOSORBENT ASSAY)

Protein expression levels were quantified using the CytoGlow™ AMPK β1 Colorimetric Cell-Based ELISA Kit (Catalog No MBS9500029; (MyBioSource Inc., San Diego, CA, USA) according to the manufacturer's instructions. A kit was used for each target. The kit includes rabbit polyclonal antibody and mouse monoclonal anti-GAPDH antibodies, as well as HRP-conjugated

secondary antibodies for detection. Colorimetric detection was performed at 450 nm, and normalization was achieved using GAPDH absorbance and/or Crystal Violet staining at 595 nm. Absorbance readings were taken by Multiskan Go Spectrophotometer, Model 1510, Thermo Fisher, UK. Data was analyzed using Microsoft Excel (Microsoft Office 2019). The expression levels of glucose transporter type 4 (GLUT4), AMP-activated protein kinase α1 (AMPKα1), AMP-activated protein kinase α2 (AMPKα2; PRKAA2), and insulin receptor (InsR) were quantified in HepG2 cell lysates and supernatants using commercially available ELISA kits (MyBioSource Inc., San Diego, CA, USA) according to the manufacturer's instructions. Additionally, cell-based ELISA assays were performed to measure AMPK β1 and AMPK γ (PRKAG) subunit protein levels using specific rabbit polyclonal antibodies (anti-AMPKβ1; anti-PRKAG1/2/3) with GAPDH as a loading control.

STATISTICAL ANALYSIS METHODS

Concentrations and normalized absorbance values were summarized as mean ± standard deviation (SD). Differences in concentrations were assessed using either a one-way ANOVA or Welch's ANOVA, A significant test was followed up with specific multiple comparisons applying Sidak correction for multiple testing. All analyses and figures were produced with GraphPad Prism 8.

RESULTS

Significant differences were observed in the concentrations of the four ELISA antigens across the eight treatment groups, in both cell lysates and supernatants (Table 1).

The mean concentration of GLUT4 in the supernatant of HepG2 cells treated with BMOV for 1 h was lower than that observed with insulin treatment for 1 h (p-value = 0.018). Similarly, the mean GLUT4 concentration in the supernatant of HepG2 cells treated with glucose + BMOV for 1 h was lower than that in the cells treated with glucose + insulin for 1 h ($p < 0.0001$). Conversely, treatment with BMOV for 24 h or glucose + BMOV for 24 h was associated with higher mean GLUT4 concentrations in the supernatant (insulin 1 h vs. BMOV 24 h, p-value = 0.095; glucose + insulin 1 h vs. glucose + BMOV 24 h, $p = 0.004$) (Table 1 & Figure 1(A)). All treatments were significantly different from the control (p-value < 0.001) except for glucose + insulin for 1 h (p-value = 0.050).

BMOV treatment of HepG2 cells for 1 h resulted in a higher mean GLUT4 concentration in the cell lysate compared to both insulin treatment for 1 h and BMOV treatment for 24 h (p-value < 0.0001 in both cases). In the presence of glucose, BMOV treatment for 24 h yielded the highest GLUT4 concentration in the cell lysate (p-value < 0.0001 for all pairwise comparisons) (Table 1 & Figure 1(B)). All treatments differed significantly from the untreated control (p-value < 0.01) except for glucose + insulin for 1 h (p-value = 0.609).

Small differences in AMPKα1 concentration in HepG2 supernatant were observed in response to insulin for 1 h and BMOV treatment for 1 or 24 h. However, in the presence of glucose, BMOV induced a substantial increase in AMPKα1 in the supernatant after both 1 and 24 h compared to insulin for 1 h (p-value < 0.0001 for both) (Table 1 & Figure 2(A)). The untreated control differed significantly from glucose, glucose + BMOV (1 h), and glucose + BMOV (24 h) groups (p-value < 0.0001).

Treatment of HepG2 cells for 1 h resulted in higher AMPKα1 concentration in the cell lysate compared to insulin for 1 h or BMOV for 24 h (p-value < 0.0001 and p-value = 0.0007, respectively). In the presence of glucose, BMOV increased AMPKα1 concentration in the cell lysate in a time-dependent manner (1 h vs. 24 h), both significantly higher than insulin treatment (p-value < 0.0001 for both) (Table 1 & Figure 2(B)). The untreated control differed significantly from glucose, BMOV (1 h), BMOV (24 h), glucose + BMOV (1 h), and glucose + BMOV (24 h) groups (p-value < 0.01).

Treatment with BMOV for 24 h was associated with significantly higher PRKAA2 concentrations in the HepG2 cell supernatant compared to both BMOV treatment for 1 h or insulin treatment for 1 h (p-value < 0.0001 and p-value = 0.0002, respectively). Similar results were observed in the presence of glucose (p-value < 0.0001 for both comparisons) (Table 1 & Figure 3(A)). The untreated control differed significantly from BMOV for 24 h, glucose + insulin for 1 h, glucose + BMOV for 1 h, and glucose + BMOV for 24 h (p-value < 0.05). Conversely, BMOV treatment for 24 h was associated with significantly lower PRKAA2 concentrations in the HepG2 cell lysate compared to BMOV treatment for 1 h (p-value = 0.031) (Table 1 & Figure 3(B)).

BMOV treatment for either 1 or 24 h was associated with significantly lower insulin receptor (InsR) concentrations in HepG2 cell lysates compared to insulin treatment for 1 h (p-value < 0.0001 in both cases). However, in the presence of glucose, BMOV treatment for 1 h led to higher InsR concentrations in the lysate than insulin treatment for 1 h or BMOV treatment for 24 h (p-value < 0.0001 and p-value = 0.0003, respectively) (Table 1 & Figure 4). No significant differences were observed between the untreated control and any of the treatment groups.

CELL-BASED ELISA

Significant differences were observed in absorbance values for the four ELISA antigens among the eight treatment groups (Table 2 & Figure 2).

Post-hoc comparisons for PRKAG1/2/3 showed significant differences between insulin treatment for 1 h and BMOV treatment for 24 h (p-value = 0.0004). Additionally, BMOV treatment for 1 h differed significantly from BMOV treatment for 24 h (p-value = 0.0003). There was no significant difference between insulin for 1 h and BMOV for 1 h, with both treatments showing similar absorbance

values (1.21 ± 0.06 vs. 1.26 ± 0.02). Conversely, BMOV treatment for 24 h was associated with a markedly lower absorbance (0.34 ± 0.30). Furthermore, BMOV treatment for 1 h in the presence of glucose yielded significantly higher absorbance compared to BMOV treatment for 24 h with glucose (p-value = 0.015) (Table 2 & Figure 2(A)). The untreated control differed significantly from BMOV for 24 h, glucose + insulin for 1 h, glucose + BMOV for 1 h, and glucose + BMOV for 24 h (p-value < 0.05). Significant differences were also observed between the untreated control and both glucose treatment alone and glucose + BMOV for 24 h (p-value < 0.05). For AMPKβ1, a significant difference was observed between insulin treatment for 1 h in the presence of glucose and BMOV treatment for 24 h in the presence of glucose (p-value = 0.036) (Table 2 & Figure 2(B)). No significant differences were found between the untreated control and any other treatments.

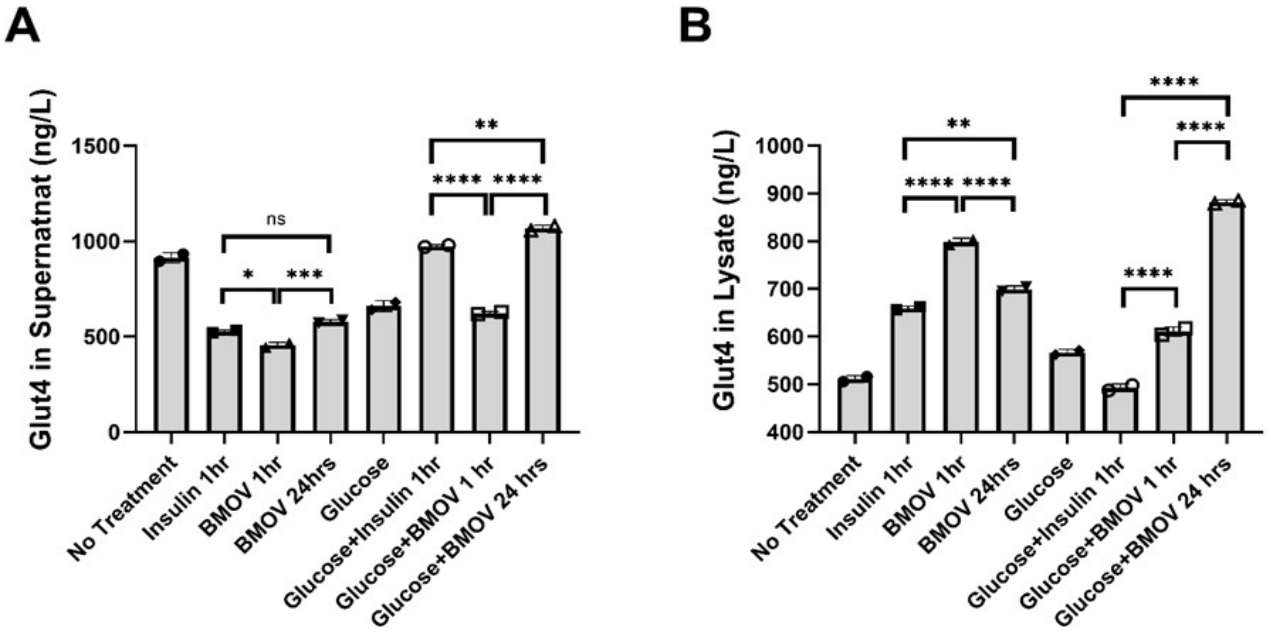
DISCUSSION

Diabetes mellitus remains a major global health challenge despite the availability of insulin therapy and oral hypoglycemic agents. By 2030, it is projected

TABLE 1. ELISA antigen concentrations

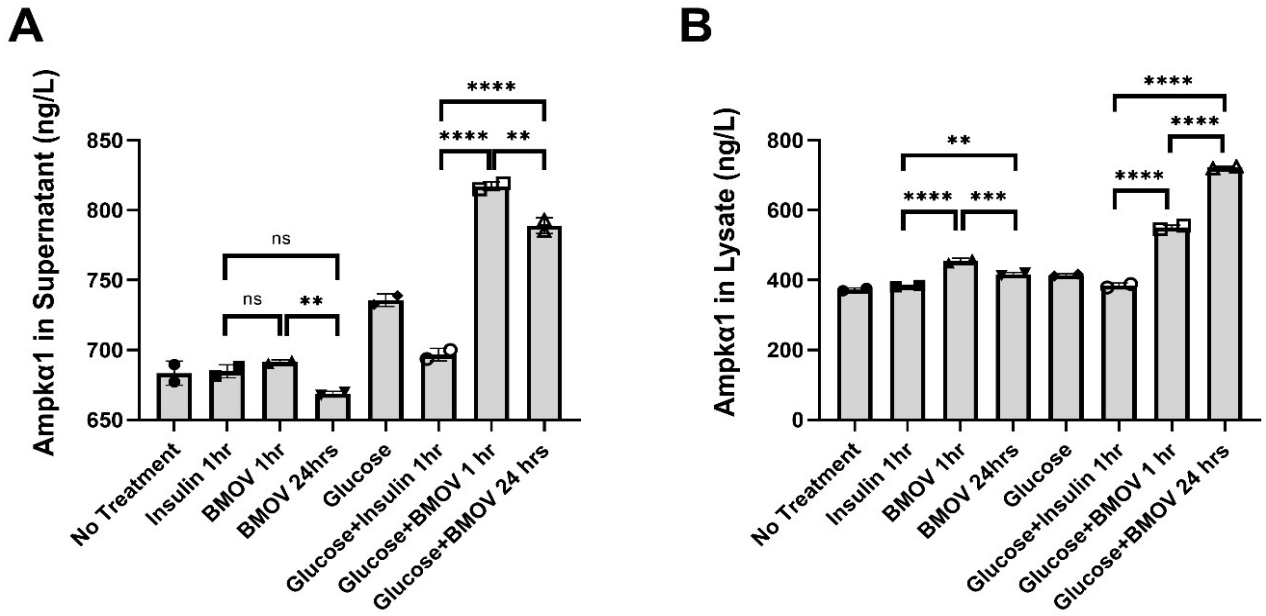
Antibody Treatment	Glut4 Sup (ng/L)	Glut4 Lys (ng/L)	AMPKa1 Sup (ng/L)	AMPKa1 Lys (ng/L)	InsR Lys (ng/L)	PRKAA2 Sup (ng/L)	PRKAA2 Lys (ng/L)
Control	914±27	511±7	683±9	373±5	1.52±0.11	0.46±0.01	75±1
Insulin 1 h	527±9	659±5	685±5	383±2	3.29±0.23	0.45±0	76±5
BMOV 1 h	456±16	798±8	692±2	455±8	2.31±0.04	0.42±0.01	82±0.3
BMOV 24 h	578±11	699±7	669±2	417±5	2.26±0.05	0.54±0.01	69±4
Glucose	660±28	567±6	736±5	414±4	3.10±0.04	0.43±0.01	65±6
Glucose + Insulin 1 h	975±7	493±8	697±5	384±7	2.84±0.01	0.39±0.01	71±4
Glucose + BMOV 1 h	624±8	611±10	817±3	551±6	4.01±0.03	0.41±0.01	71±3
Glucose + BMOV 24 h	1068±16	882±5	789±6	722±4	2.10±0.03	0.67±0.01	66±2
ANOVA p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.050

Concentrations were summarized as mean ± SD for two replicates each. Sup: Supernatant of HepG2, Lys: Lysate of HepG2



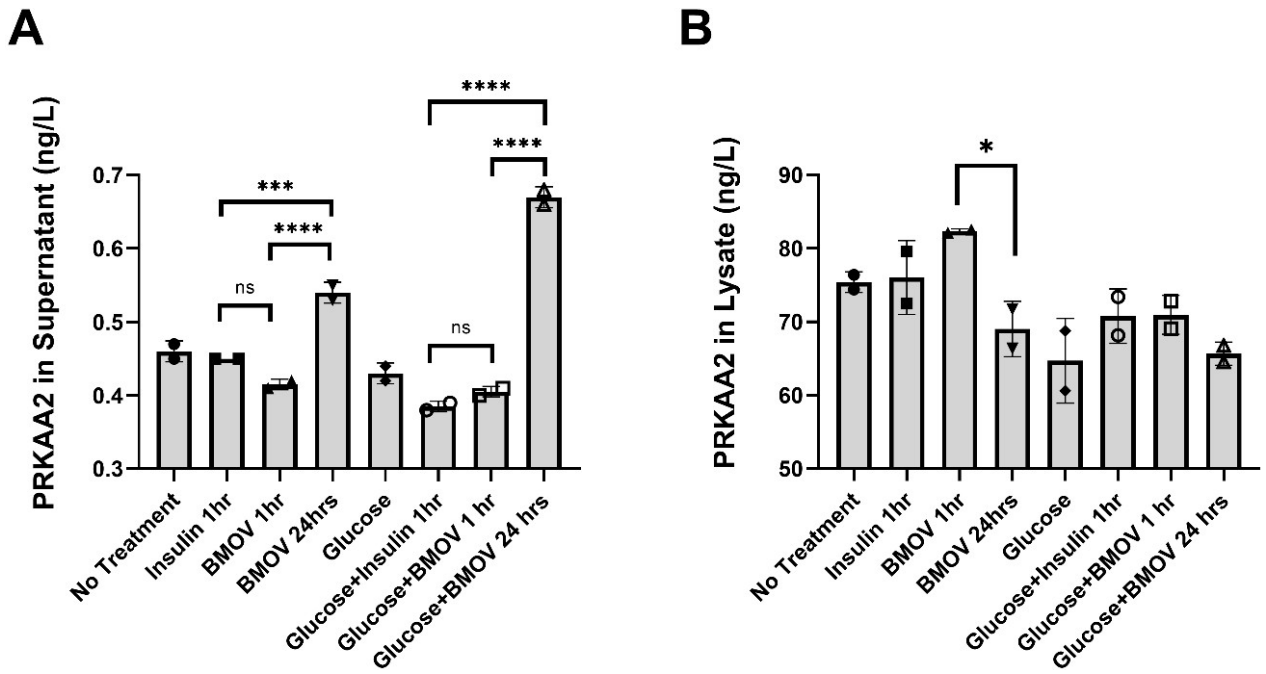
Bar heights represent mean concentrations from two replicates; vertical bars indicate standard deviations. Comparisons represent Sidak-corrected post hoc tests following significant ANOVA results. ns: not significant; *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001; ****: p-value < 0.0001

FIGURE 1. GLUT4 ELISA results and ANOVA tests. A) Concentration of GLUT4 in HepG2 supernatant across eight treatment groups, and B) Concentration of GLUT4 in HepG2 lysate across eight treatment groups



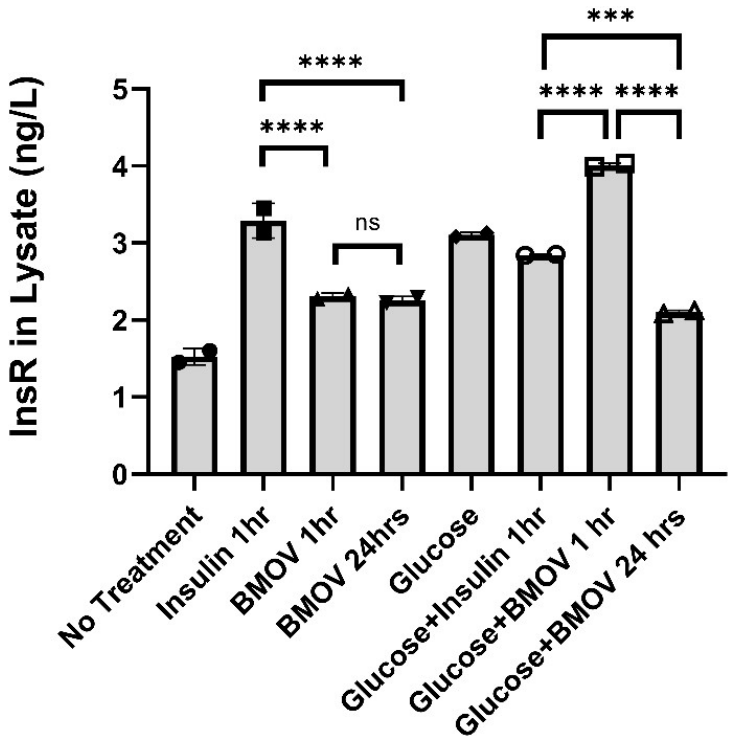
Bar heights represent mean concentrations from two replicates; vertical bars indicate standard deviations. Comparisons show Sidak-corrected post hoc tests following significant ANOVA results. ns: not significant; *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001; ****: p-value < 0.0001

FIGURE 2. AMPKα1 ELISA results and ANOVA tests. A) Concentration of AMPKα1 in HepG2 supernatant across eight treatment groups, and B) Concentration of AMPKα1 in HepG2 lysate across eight treatment groups



Bar heights represent mean concentrations from two replicates; vertical bars indicate standard deviations. Comparisons represent Sidak-corrected post hoc tests following significant ANOVA results. ns: not significant; *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001; ****: p-value < 0.0001

FIGURE 3. PRKAA2 ELISA results and ANOVA tests. A) Concentration of PRKAA2 in HepG2 supernatant across eight treatment groups and B) Concentration of PRKAA2 in HepG2 lysate across eight treatment groups



Bar heights represent mean InsR concentrations in HepG2 lysates across eight treatment groups, based on two replicates. Vertical bars indicate standard deviations. Comparisons show Sidak-corrected post hoc tests following significant ANOVA results. ns: not significant; *: p-value < 0.05; **: p-value < 0.01; ***: p-value < 0.001; ****: p-value < 0.0001

FIGURE 4. InsR ELISA results and ANOVA tests

TABLE 2. Cell-based ELISA absorbances for anti-PRKAG1/2/3 and anti-AMPKβ1 antibodies in HepG2 cells

Antibody Treatment	PRKAG123	AMPKB1
No treatment	0.82±0.02	1.41±0.95
Insulin 1 h	1.21±0.06	2.89±0.48
BMOV 1 h	1.26±0.02	2.49±1.21
BMOV 24 h	0.34±0.30	0.67±0.08
Glucose	0.21±0.06	1.45±0.65
Glucose + Insulin 1 h	0.38±0.08	1.41±0.12
Glucose + BMOV 1 h	0.64±0.06	1.00±0.31
Glucose + BMOV 24 h	0.14±0.001	0.24±0.14
ANOVA test p-value	<0.0001	0.043

Absorbance values are presented as mean ± SD of two replicates each

that approximately 366 million people will be affected worldwide (Nathan et al. 2009). Limitations of current therapies, along with their side effects, underscore the urgent need for alternative treatments. Among promising candidates, vanadium-containing compounds have demonstrated significant insulin-mimetic and antidiabetic effects in both *in vitro* and *in vivo* models (Lyonnet, Martz & Martin 1899; Tolman et al. 1979).

The earliest evidence of vanadium's insulin-like effects dates back to 1899, when sodium orthovanadate was shown to reduce glucosuria in diabetic patients (Lyonnet, Martz & Martin 1899). Later, Tolman et al. (1979) demonstrated that vanadium salts suppress gluconeogenesis, enhance glycogen synthesis, and increase glucose transport and oxidation in various cell types. While the precise molecular mechanisms remain to be fully elucidated, the accumulated evidence supports a key role for vanadium compounds in regulating glucose metabolism.

In the present study, BMOV treatment for 24 h in the presence of glucose significantly increased GLUT4

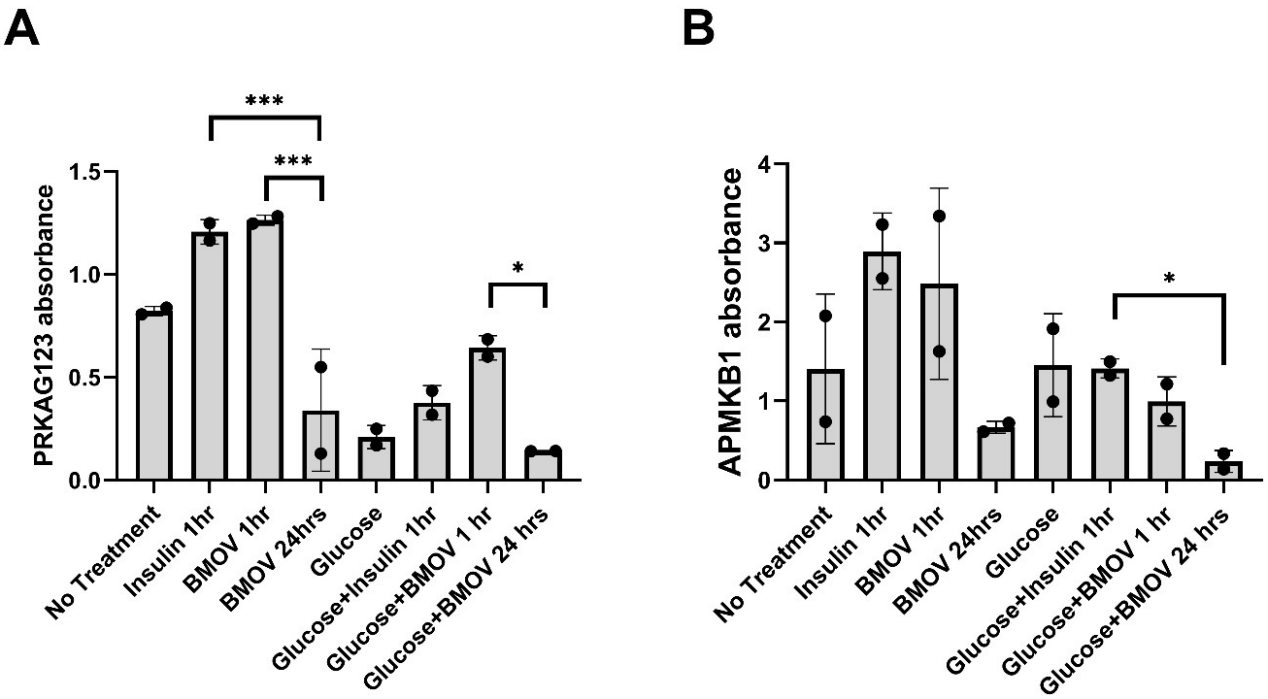


FIGURE 2. Absorbance values for anti-PRKAG1/2/3 and anti-AMPKβ1 in cell-based ELISA using HepG2 cells under eight different treatment conditions

concentrations in both HepG2 cell lysates and supernatants. These findings align with previous reports showing that vanadium compounds restore GLUT4 expression and promote its translocation to the plasma membrane in insulin-resistant tissues (Mohammad, Sharma & McNeill 2002; Shafrir et al. 2001; Zorzano et al. 2009). Similarly, studies with sodium orthovanadate and arylalkylamine vanadium derivatives have demonstrated increased GLUT4 mobilization and glucose uptake in adipocytes and myotubes (Molero et al. 1998; Tsiani et al. 1998).

Our results also showed that 1-h BMOV treatment with glucose significantly increased InsR levels in HepG2 cell lysates. To the best of our knowledge, this is the first report demonstrating such an effect in this cell line. Previous studies have indicated that BMOV can alter insulin receptor compartmentalization and enhance its localization within membrane microdomains (Winter et al. 2012), potentially facilitating insulin signaling. Furthermore, BMOV has been shown to increase InsR and IRS-1 phosphorylation, supporting its insulin-mimetic potential (Mehdi & Srivastava 2005).

Regarding AMPK regulation, we observed that 1-h BMOV treatment increased AMPKα1 levels in cell lysates, whereas 24-h treatment elevated AMPKα2 in supernatants. This time-dependent modulation may reflect distinct roles of AMPK isoforms in glucose metabolism. AMPK, a heterotrimeric enzyme composed of α, β, and γ subunits, is a key regulator of cellular energy homeostasis, promoting glucose uptake, inhibiting gluconeogenesis, and regulating lipid metabolism (Hunter et al. 2011; Shamshoum et al. 2021).

Several natural compounds that activate AMPK have shown beneficial effects on glucose metabolism. For example, rosemary extract, asprosin, and *Ganoderma lucidum* enhance AMPK phosphorylation and GLUT4 expression (Lee et al. 2020; Zhang et al. 2021). Differentiation-inducing factor-1 (DIF-1), sesquiterpene glycosides, and chrysin improve glucose uptake in insulin-resistant cell models by activating AMPK (Kubohara et al. 2021; Li et al. 2020; Zhou et al. 2021).

Our cell-based ELISA data indicate that 1-h BMOV treatment in the presence of glucose produced higher AMPKβ1 and PRKAG absorbance compared to 24-h treatment, suggesting early activation of the AMPK complex followed by potential feedback regulation. These patterns underscore the importance of treatment duration in modulating AMPK activity.

Collectively, our findings suggest that BMOV exerts antidiabetic effects by upregulating GLUT4, InsR, and AMPK subunits, with both time- and glucose-dependent influences. This supports BMOV's potential as a therapeutic agent for improving insulin sensitivity and metabolic control in T2DM. However, further studies - including animal models and clinical trials - are necessary to confirm these findings and to assess the long-term safety and efficacy of BMOV. It is essential to note that, despite a small sample size (n), ANOVA still has sufficient power to detect large differences between treatments. Indeed, our analysis was able to detect such significant differences between treatments. However, we acknowledge that small replicate size is a limitation of our study, and we recommend more replicates in future studies to confirm the non-significant differences.

CONCLUSION

Diabetes mellitus is increasing in prevalence worldwide, driven largely by lifestyle changes. Type 2 diabetes mellitus (T2DM) is characterized by elevated blood glucose levels and impaired glucose metabolism. AMPK signaling plays a central role in glucose regulation by promoting the translocation of glucose transporters GLUT1 and GLUT4, thereby enhancing glucose uptake in muscle and other tissues and lowering serum glucose concentrations. Vanadium-containing compounds have demonstrated insulin-like effects in both *in vitro* and *in vivo* models, improving glucose homeostasis and reducing insulin resistance in animal models of type 1 and type 2 diabetes mellitus (Heyliger, Tahiliani & McNeill 1985; Meyerovitch et al. 1987; Yale et al. 1995). BMOV, an organic vanadium complex with improved bioavailability and tolerability, shows promise as an antidiabetic agent. Our study demonstrated that BMOV treatment increases the protein levels of AMPK subunits, GLUT4, and insulin receptor (InsR). This ability to upregulate key proteins involved in glucose metabolism likely underlies BMOV's insulin-mimetic and glucose-regulatory effects. Further investigations are warranted to elucidate the precise molecular mechanisms of BMOV, evaluate its therapeutic efficacy *in vivo*, and explore its potential integration into treatment regimens for T2DM.

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REFERENCES

Al-Qatati, A., Wolf-Ringwall, A.L., Bouma, G.J., Crans, D.C., Barisas, B.G. & Roess, D. 2013. Using real time RT-PCR analysis to determine gene expression patterns in RBL-2H3 cells in response to insulin, glucose, and the anti-diabetic bis(maltolato) oxovanadium (IV). *Journal of Al Azhar University-Gaza (Natural Sciences)* 15: 129-152.

Barceloux, D.G. 1999. Vanadium. *Journal of Toxicology: Clinical Toxicology* 37: 265-278.

Davies, S.P., Hawley, S.A., Woods, A., Carling, D., Haystead, T.A. & Hardie, D.G. 1994. Purification of the AMP-activated protein kinase on ATP-gamma-sepharose and analysis of its subunit structure. *European Journal of Biochemistry* 223: 351-357.

DeFronzo, R.A., Bonadonna, R.C. & Ferrannini, E. 1992. Pathogenesis of NIDDM: A balanced overview. *Diabetes Care* 15: 318-368.

Heyliger, C.E., Tahiliani, A.G. & McNeill, J.H. 1985. Effect of vanadate on elevated blood glucose and depressed cardiac performance of diabetic rats. *Science* 227: 1474-1477.

Hunter, R.W., Treebak, J.T., Wojtaszewski, J.F.P. & Sakamoto, K. 2011. Molecular mechanism by which AMP-activated protein kinase activation promotes glycogen accumulation in muscle. *Diabetes* 60: 766-774.

Kahn, C.R. & White, M.F. 1988. The insulin receptor and the molecular mechanism of insulin action. *Journal of Clinical Investigation* 82: 1151-1156.

Kubohara, Y., Homma, Y., Shibata, H., Oshima, Y. & Kikuchi, H. 2021. Dictyostelium differentiation-inducing factor-1 promotes glucose uptake, at least in part, via an AMPK-dependent pathway in mouse 3T3-L1 cells. *International Journal of Molecular Sciences* 22(5): 2293.

Lee, H.A., Cho, J.H., Afinanisa, Q., An, G.H., Han, J.G., Kang, H., Choi, S.H. & Seong, H.A. 2020. *Ganoderma lucidum* extract reduces insulin resistance by enhancing AMPK activation in high-fat diet-induced obese mice. *Nutrients* 12(11): 3338.

Li, J., Ding, X., Jian, T., Lü, H., Zhao, L., Li, J., Liu, Y., Ren, B. & Chen, J. 2020. Four sesquiterpene glycosides from loquat (*Eriobotrya japonica*) leaf ameliorate palmitic acid-induced insulin resistance and lipid accumulation in HepG2 cells via AMPK signaling pathway. *PeerJ.* 8: e10413.

Lyonnet, B., Martz, M. & Martin, E. 1899. L'Emploi Thérapeutique des Dérivés du Vanadium. *La Presse Médicale* 32: 191-192.

Marsin, A.S., Bouzin, C., Bertrand, L. & Hue, L. 2002. The stimulation of glycolysis by hypoxia in activated monocytes is mediated by AMP-activated protein kinase and inducible 6-phosphofructo-2-kinase. *Journal of Biological Chemistry* 277: 30778-30783.

Mehdi, M.Z. & Srivastava, A.K. 2005. Organo-vanadium compounds are potent activators of the protein kinase B signaling pathway and protein tyrosine phosphorylation: Mechanism of insulinomimesis. *Archives of Biochemistry and Biophysics* 440: 158-164.

Meyerovitch, J., Farfel, Z., Sack, J. & Shechter, Y. 1987. Oral administration of vanadate normalizes blood glucose levels in streptozotocin-treated rats: Characterization and mode of action. *Journal of Biological Chemistry* 262: 6658-6662.

Mohammad, A., Sharma, V. & McNeill, J.H. 2002. Vanadium increases GLUT4 in diabetic rat skeletal muscle. *Molecular and Cellular Biochemistry* 233: 139-143.

Molero, J.C., Martinez, C., Andres, A., Satrustegui, J. & Carrascosa, J.M. 1998. Vanadate fully stimulates insulin receptor substrate-1 associated phosphatidylinositol 3-kinase activity in adipocytes from young and old rats. *FEBS Letters* 425: 298-304.

Nathan, D.M., Buse, J.B., Davidson, M.B., Ferrannini, E., Holman, R.R., Sherwin, R. & Zinman, B. 2009. Medical management of hyperglycemia in type 2 diabetes: A consensus algorithm for the initiation and adjustment of therapy. *Diabetes Care* 32: 193-203.

Shafrir, E., Spielman, S., Nachliel, I., Khamaisi, M., Bar-On, H. & Ziv, E. 2001. Treatment of diabetes with vanadium salts: General overview and amelioration of nutritionally induced diabetes in the *Psammomys obesus* gerbil. *Diabetes/Metabolism Research and Reviews* 17(1): 55-66.

Shamshoum, H., Vlavcheski, F., MacPherson, R.E.K. & Tsiani, E. 2021. Rosemary extract activates AMPK, inhibits mTOR, and attenuates the high glucose- and high insulin-induced muscle cell insulin resistance. *Applied Physiology, Nutrition, and Metabolism* 46(7): 819-827.

Stapleton, D., Mitchelhill, K.I., Gao, G., Widmer, J., Michell, B.J., Teh, T., House, C.M., Fernandez, C.S., Cox, T., Witters, L.A. & Kemp, B.E. 1996. Mammalian AMP-activated protein kinase subfamily. *Journal of Biological Chemistry* 271(2): 611-614.

Stein, S.C., Woods, A., Jones, N.A., Davison, M.D. & Carling, D. 2000. The regulation of AMP-activated protein kinase by phosphorylation. *Biochemical Journal* 345 Pt 3(Pt 3): 437-443.

Taylor, E.B., An, D., Kramer, H.F., Yu, H., Fujii, N.L., Roeckl, K.S., Bowles, N., Hirshman, M.F., Xie, J., Feener, E.P. & Goodyear, L.J. 2008. Discovery of TBC1D1 as an insulin-, AICAR-, and contraction-stimulated signaling nexus in mouse skeletal muscle. *Journal of Biological Chemistry* 283(15): 9787-9796.

Tolman, E.L., Barris, E., Burns, M., Pansini, A. & Partridge, R. 1979. Effects of vanadium on glucose metabolism *in vitro*. *Life Sciences* 25: 1159-1164.

Tsiani, E., Bogdanovic, E., Sorisky, A., Nagy, L. & Fantus, I.G. 1998. Tyrosine phosphatase inhibitors, vanadate, and pervanadate stimulate glucose transport and GLUT translocation in muscle cells by a mechanism independent of phosphatidylinositol 3-kinase and protein kinase C. *Diabetes* 47: 1676-1686.

Wang, J., Yuen, V.G. & McNeill, J.H. 2001. Effect of vanadium on insulin sensitivity and appetite. *Metabolism* 50: 667-673.

White, M.F., Shoelson, S.E., Keutmann, H. & Kahn, C.R. 1988. A cascade of tyrosine autophosphorylation in the β-subunit activates the phosphotransferase of the insulin receptor. *Journal of Biological Chemistry* 263(6): 2969-2980.

Winter, P., Al-Qatati, A., Wolf-Ringwall, A.L., Schoeberl, S., Chatterjee, B., Barisas, G., Roess, D. & Crans, D. 2012. The anti-diabetic bis(maltolato) oxovanadium(iv) decreases lipid order while increasing insulin receptor localization in membrane microdomains. *Dalton Transactions* 41: 6419-6430.

World Health Organization (WHO). 2006. *Diabetes*. Fact Sheet No. 312.

Yale, J.F., Lachance, D., Bevan, A.P., Vigeant, C., Shaver, A. & Posner, B.I. 1995. Hypoglycemic effects of peroxovanadium compounds in sprague-dawley and diabetic BB rats. *Diabetes* 44(11): 1274-1279.

Youngren, J. 2007. Regulation of insulin receptor function. *Cellular and Molecular Life Sciences* 64: 873-891.

Zhang, Y., Zhu, Z., Zhai, W., Bi, B.Y., Yin, Y. & Zhang, W. 2021. Expression and purification of asprosin in *Pichia pastoris* and investigation of its increase glucose uptake activity in skeletal muscle through activation of AMPK. *Enzyme and Microbial Technology* 144: 109737.

Zhou, Y.J., Xu, N., Zhang, X-C., Zhu, Y.Y., Liu, S-W. & Chang, Y-N. 2021. Chrysin improves glucose and lipid metabolism disorders by regulating the AMPK/PI3K/AKT signaling pathway in insulin-resistant HepG2 cells and HFD/STZ-induced C57BL/6J mice. *Journal of Agricultural and Food Chemistry* 69(20): 5618-5627.

Zorzano, A., Palacín, M., Marti, L. & García-Vicente, S. 2009. Arylalkylamine vanadium salts as new anti-diabetic compounds. *Journal of Inorganic Biochemistry* 103: 559-566.

*Corresponding author; email: a.alqatati@ju.edu.jo