Direct Evidence for Intracellular Homeostasis in Mammalian Cells: Insulin-independent Glucose Metabolisms

Abstract

Established Py-3Y1-S2 rat fibroblast cells were used to evaluate whether $NaHCO_3$ or Na_2CO_3 influences glucose metabolism *in vitro*, because factors that contribute to metabolic pathways are much simpler to evaluate in cultured cells than in whole animal bodies. The effects of the carbonates on glucose consumption decreased at high concentrations, >5 mg/ml for Na_2CO_3 and >7 mg/ml for $NaHCO_3$, because of the increased pH of the culture medium. The effects of the carbonates on glucose consumption were additive with those of vanadium and concanavalin A. Streptozotocin, alloxan, and nicotinamide, which induce diabetes in animals, reduced glucose consumption by Py-3Y1-S2 cells, and the inhibitory effects of these reagents were abolished by both Na_2CO_3 and $NaHCO_3$. Finally, the carbonates increased lactate production from glucose in the cells, followed by acceleration of lactate secretion into the culture medium. Glucose was completely converted to lactate, which disappeared gradually from the culture medium. However, the disappearance of lactate from the medium was independent of carbonates. The

present study clarified that $NaHCO_3$ and Na_2CO_3 directly regulate glucose metabolism via an insulin-independent pathway, that is, intracellular homeostasis.

Keywords: Intracellular, Homeostasis, Insulin, Glucose, Metabolism, Diabetes, Anti-diabetic, Carbonates, Lactate, Concanavalin A, Vanadium, Mitochondria.

1. Introduction

The prevalence of diabetes mellitus (DM) is increasing worldwide. The International Diabetes Federation reported that there were 425 million adults with diabetes in the world in 2017 [1]. The symptoms of chronic high blood sugar in DM include frequent urination, increased thirst, and increased hunger. If left untreated, DM can lead to serious complications like cardiovascular disease, stroke, chronic kidney failure, foot ulcers, and eye damage. Insulin via injections is used for treatment of type 1 DM, while insulin and several oral drugs that inhibit glucose production from polysaccharides are used for type 2 DM treatment. However, these drugs only treat the symptoms, and there is no basic remedy for DM. Previous studies have investigated whether insulin plays a role in memory formation *in vivo* [2] and whether insulin contributes to dementia, particularly Alzheimer's disease [3-5].

Blood sugar levels are regulated *in vivo* by insulin and glucagon, which are produced from ß and α cells, respectively, of the pancreatic islets, via homeostatic mechanisms, which maintain *in vivo* vertebrate life. This is a delicate mechanism, which has evolved in vertebrates along with long-term evolution of other biological differentiated functions. The present study aims to clarify the existence of a regulatory mechanism for cellular glucose

metabolism in the absence of insulin before establishment of the endocrine system, in which insulin plays an important role in cells.

Regarding its mode of action as a peptide hormone, insulin binds to its receptor on the plasma membrane, leading to intramolecular phosphorylation within the activated receptor as a tyrosine kinase. The signal transduction proceeds through phosphatidylinositol-3-kinase, protein kinase B, and glucose transporter-4 (GLUT-4), with GLUT-4 plus K^+ accelerating glucose uptake into cells [6]. Vanadium compounds were reported to exhibit insulin-like activity not only *in vitro* [7,8], but also *in vivo* [9-16], and several vanadium compounds have been investigated for their insulin-like activity [17-21]. The insulin-like effects of vanadates are based on inhibition of protein-tyrosine phosphatase [22]. However, to our knowledge, suitable vanadium compounds have not yet been developed as anti-DM drugs because of the serious cytotoxic effects of high vanadium concentrations. It has been suggested that Mt. Fuji subsoil water filtered through basalt can exhibit insulin-like activity, because the water contains vanadium pentoxide (V_2O_5) *in vivo* [23]. Recently, we confirmed that Mt. Fuji subsoil water accelerates glucose consumption *in vitro* using established Py-3Y1-S2 rat fibroblast cells [24] and human primary fibroblasts [25].

Vanadium pentoxide is soluble in the alkaline condition, but its water solubility is quite low (0.7–0.8 g/L). Indeed, the pH value of commercial Mt. Fuji subsoil water (Healthy Vana Water) containing 130 μg/L vanadium was 8.3 [24]. If vanadium-containing water can be prepared by mixing a small amount of Mt. Fuji basalt powder with normal water, the vanadium-containing water could be conveniently used instead of Mt. Fuji subsoil water.

Associations between metabolic acidosis, insulin resistance, and cardiovascular risks have been reported since 1924 [26]. Although correction of metabolic acidosis by nutritional therapy and/or oral administration of sodium bicarbonate is widely applied in chronic kidney disease [27-29], it remains unknown whether metabolic acidosis reduces insulin resistance and/or improves insulin effects on target cells in diabetic subjects. Bellis et al. [30] evaluated whether metabolic acidosis correction by sodium bicarbonate administration can improve peripheral endogen insulin utilization by target organs in diabetic subjects with chronic kidney disease treated with oral antidiabetic drugs. They reported that sodium bicarbonate administration improved insulin resistance in chronic kidney disease at the serum glucose and insulin levels. However, they commented that further investigations were required to evaluate

their results in diabetic and non-diabetic chronic kidney disease patients. Indeed, another group reported that sodium bicarbonate treatment did not improve insulin sensitivity and glucose control in non-diabetic older adults [31]. These contradictory findings seem to arise from the different experimental systems investigated with many different factors. The present study was designed to clarify whether carbonates ($NaHCO_3$ and Na_2CO_3) can directly influence glucose metabolism in simple cultured cells.

2. Materials and Methods

2.1 Cell culture. Py-3Y1-S2 rat fibroblast cells were cultured in Dulbecco's minimum essential medium (DMEM) containing 5% fetal bovine serum (Gibco, Thermo Fisher Scientific K.K., Tokyo, Japan) in the presence of 5% CO_2 at 37°C. The other following cells were used: VERO cells were derived from green monkey kidney [32], and used for virus assay, endotoxin assays, and vaccine production [33]. HepG2 cells were derived from hepatoma [34], and used for investigation of insulin actions [35-37]. Stock cells cultured in 25-cm^2 plastic culture flasks were trypsinized and inoculated into 24-well culture plates. After the cells had almost reached confluent monolayers, they were cultured in the presence or absence of $NaHCO_3$, Na_2CO_3, and other chemical compounds like vanadium, concanavalin A (Con A),

nicotinamide, streptozotocin (STZ), and alloxan monohydrate.

2.2 Glucose assay. Glucose concentrations in culture media were measured by a previously reported method [24] using a Glucose CII-test (Wako Pure Chemical, Osaka, Japan). For this, a 10–50-µl aliquot of culture medium was mixed with the assay solution (0.5 ml) and the absorbance at 505 nm was measured after 15 min. To express the effects of reagents on glucose consumption, the amount of consumed glucose in the sample medium was divided by that in the control medium (Milli-Q water) without a test reagent.

2.3 Lactate assay. Lactate concentrations in culture media were measured with a Lactate Assay Kit-WST (Dojin Chemicals, Tokyo, Japan) according to the manufacturer's protocol. Briefly, a 20-µl aliquot of sample solution was mixed with 80 µl of working solution and incubated at 37°C for 30 min. After the enzyme reaction, the absorbance at 450 nm was measured to calibrate the lactate concentration.

2.4 Protein assay of cultured cells. Cellular protein assays were carried out using a previously reported method [24]. Cells cultured in 24-well plates were washed with phosphate-buffered saline and treated with 0.2 ml of Mammalian

Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA). An aliquot of the solubilized cell solution (4 µl) was added to 156 µl of H_2O, and mixed with Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA, USA). The absorbance at 595 nm was measured. Bovine serum albumin was used as a standard.

2.5 Chemicals. DMEM powder (Misui, Tokyo, Japan) was dissolved in Milli-Q water, and autoclaved. The medium (1 L) was neutralized by adding 4 ml of 10% $NaHCO_3$, according to the technical protocol recommended by the manufacturer. $NaHCO_3$, Na_2CO_3, vanadium, nicotinamide, STZ, and alloxan monohydrate were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Con A and insulin were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.6 Statistical analysis. Statistical calculations by the *t*-test were performed using Microsoft Excel (version 2010). Values of $p < 0.05$ and $p < 0.01$ were considered significant and highly significant, respectively.

3. Results

3.1 Effects of $NaHCO_3$ and Na_2CO_3. To prepare vanadium-containing water, Mt. Fuji basalt powder (1–2-µm diameter) was treated with 1 mg/ml $NaHCO_3$, which is

safe for the human body, and this water was used to make the cell culture medium. The cell culture medium containing Mt. Fuji basalt-treated water increased glucose consumption in cultured cells (Fig. 1). However, an increase in Mt. Fuji basalt powder for water treatment from 5 mg/10 ml to 200 mg/10 ml did not cause any further increase in glucose consumption by cultured cells. These findings indicate that Mt. Fuji basalt powder treatment may not be involved in acceleration of glucose consumption by cultured cells. Indeed, only $NaHCO_3$ or Na_2CO_3 accelerated glucose consumption by cultured cells (Fig. 1).

Regarding the $NaHCO_3$ concentration, the effect of $NaHCO_3$ was significant at 1 mg/ml and subsequently increased in a dose-dependent manner up to 5 mg/ml. Thereafter, the effect of $NaHCO_3$ gradually decreased in the range of 7–10 mg/ml (Fig. 2A). For Na_2CO_3, a significant effect was observed at 0.5 mg/ml and the maximum effect (~200% of control) was observed at 2 mg/ml (Fig. 2B). The latter concentration is close to the serum bicarbonate concentrations (2.0–2.4 mg/ml) that improved serum glucose and insulin levels in diabetic chronic kidney disease patients with acidosis after oral administration of bicarbonate *in vivo* [30]. The effect of Na_2CO_3 decreased rapidly in the range of 4–5 mg/ml, and completely inhibited glucose consumption by cultured cells at

>7 mg/ml. Both carbonates accelerated glucose consumption in TE-13 cells derived from human esophageal cancers [38].

3.2 Cellular proteins. $NaHCO_3$ and Na_2CO_3 solutions are alkaline, and their pH values in culture media were 8.0–9.5 in the presence of 5% CO_2 in the cell incubator. Of course, culture medium initially contains $NaHCO_3$ to maintain the pH at around 7.5. The cellular protein contents of Py-3Y1-S2 cells were independent of $NaHCO_3$ in the range of 0.5–8 mg/ml, while $NaHCO_3$ at 10 mg/ml reduced the cellular protein to 70% of the control (Fig. 3). The decrease in cellular protein content was linked with the disappearance of glucose consumption acceleration at the high concentration of $NaHCO_3$ (Fig. 2A). Similarly, the Py-3Y1-S2 cellular protein content was markedly reduced by 5 mg/ml Na_2CO_3 to around 30% of the control (Fig. 2B). The cellular protein content was reduced to <10% in the presence of >7 mg/ml Na_2CO_3. This cytotoxic effect of Na_2CO_3 was in accordance with the decrease in glucose consumption (Fig. 3), and was caused by the high pH of 9.5 in the absence of CO_2. The culture medium containing 5 mg/ml Na_2CO_3 had a pH of ~8.5 after incubation in the presence of 5% CO_2. Therefore, conditions involving high pH above 9 led to cell death.

3.3 Additive effects of Con A or vanadium. The lectin Con A was reported to exert insulin-like effects during *in vitro* experiments [39]. We showed that Con A shared insulin receptors with insulin by *in vitro* experiments involving binding assays of labeled Con A and insulin [40,41]. When cultured Py-3Y1-S2 cells were treated with Con A at 0.025 mg/ml, glucose consumption increased in a dose-dependent manner (Fig. 4). Con A at 0.1 mg/ml further increased glucose consumption, and the effect almost reached a plateau. Thus, the effects of Con A at 0.1 mg/ml were examined at different $NaHCO_3$ concentrations. $NaHCO_3$ at 0.5 mg/ml and 1.0 mg/ml further increased glucose consumption with Con A at 0.1 mg/ml, while the effect of $NaHCO_3$ at 5.0 mg/ml was almost null in the presence of 0.1 mg/ml Con A (Fig. 5A). Similarly, Na_2CO_3 at 0.5 mg/ml further increased glucose consumption with Con A at 0.1 mg/ml (Fig. 5B). These results indicate that combinations of $NaHCO_3$ or Na_2CO_3 with Con A show sufficient effects even low concentrations compared with their use alone.

In a previous study [24], vanadium accelerated glucose consumption in a dose-dependent manner in the range of 25–10,000 μg/L, while an inhibitory effect was observed at >1.0 mg/L in Py-3Y1-S2 cells. When cells were cultured with 1.0 mg/L vanadium plus 1.0, 2.5, or 5.0 mg/ml $NaHCO_3$,

glucose consumption was significantly accelerated in the presence of 1.0 and 2.5 mg/ml $NaHCO_3$ (Fig. 5C). However, no additive effect of vanadium was observed at 5.0 mg/ml $NaHCO_3$. Similarly, lower concentrations of Na_2CO_3, 0.5 mg/ml and 1.0 mg/ml, exhibited a significant additive effect with vanadium, while 5.0 mg/ml Na_2CO_3 had no additive effect with vanadium (Fig. 5D).

3.4 Effects of diabetic reagents. STZ, alloxan, and nicotinamide are known chemical compounds that can induce diabetes in animals [42-44]. In animals, alloxan destroys ß-cells in the pancreas at high concentrations, and induces type 2 diabetes at low concentrations. When Py-3Y1-S2 cells were cultured in the presence of the above drugs *in vitro*, the glucose consumption in the culture medium was significantly reduced (Fig. 6A). In the present study, the drugs did not cause significant reductions in cellular protein amounts (Fig. 6B). In contrast, addition of $NaHCO_3$ or Na_2CO_3 to the culture medium abolished the effects of STZ, alloxan, and nicotinamide in Py-3Y1-S2 cells (Fig. 6A). These findings indicate that the reduction in glucose consumption by Py-3Y1-S2 cells was not caused by cell death.

Glucose is metabolized to pyruvic acid during the first stage in the absence of oxygen, and then alcohols or organic acids are formed via the TCA

cycle based on various enzyme reactions in cells. During the second stage in the presence of oxygen, pyruvic acid is finally converted to CO_2 and H_2O. Thus, $NaHCO_3$ or Na_2CO_3 formed from CO_2 could be involved in glucose regulation like insulin. These regulatory mechanisms by carbonates resemble the autocrine or paracrine mechanisms of cytokines. When PY-3Y1-S2 cells were cultured in the presence of $NaHCO_3$ or Na_2CO_3, the concentration of lactate in the culture medium was significantly increased (Fig.6C). Meanwhile, STZ, alloxan, and nicotinamide significantly reduced lactate production, and further addition of $NaHCO_3$ or Na_2CO_3 abolished the effects of these drugs. A parallel relationship between glucose consumption and lactate production was observed in Py-3Y1-S2 cells.

3.5 Different cell lines. To confirm that carbonates, i.e., $NaHCO_3$ and Na_2CO_3, contribute to glucose consumption in cellular metabolisms as a general rule, several different established cell lines were examined. TE-13 cells were derived from human esophageal cancer [38]. These cells were used in our previous study with Mt. Fuji subsoil water, which contains vanadium pentoxide (V_2O), and vanadium ions accelerated glucose consumption [24,25]. In addition, VERO and HepG2 were examined. When the cells were cultured in a medium to which

1.0 mg/ml $NaHCO_3$ or Na_2CO_3 had ben added, glucose consumption was significantly accelerated (Fig. 7A). The effects of carbonates on glucose consumption varied slightly among different cell lines. After culturing for a sufficient time period, the glucose in the medium in the 24-well culture plates was almost completely consumed, whereas lactate production reached a plateau. There was no significant difference between lactate production by the control cells and that by the carbonate-treated cells, and among different cell lines (Fig. 7B). The plateau concentration of lactate was 10–12 mmol/L. This value is twice the initial glucose concentration (5.6 mM). This suggests that glucose in the medium was completely converted to lactate in these cell lines.

3.6 Lactate metabolism. When Py-3Y1-S2 cells were continuously cultured, the lactate concentration decreased gradually with incubation time (Fig. 8). After culturing for 2 days, about 50% reduction was observed, and after 3 days more than 60% of the lactate had disappeared. No significant effect of carbonates on lactate reduction was observed (Fig. 8). Eventually, the carbonates apparently accelerated only glucose consumption. Similar lactate reduction occurred in the other cell lines, i.e., TE-13, VERO, and HepG2, although the reduction rates differed. The fact that

the secreted lactate from cells was further metabolized by cells seems to have a certain physiological functional significance. The rate difference between glucose consumption and lactate reduction may contribute to maintenance of a cellular steady state, i.e., intracellular homeostasis. Lactate was largely considered a dead waste product of glycolysis due to hypoxia, the primary cause of O_2 debt following exercise, a major cause of muscle fatigue. However, its physiological significance has been reevaluated [44].

3.7 Non-insulin effect. To determine whether insulin receptors are involved in glucose consumption by carbonates in cells, Py-3Y1-S2 cells were cultured in the presence of insulin. No significant acceleration of glucose consumption was observed in the range 0.1–50 μg/ml insulin (Fig. 9).

4. Discussion

Plants and some bacteria are autotrophs and are able to grow by using photosynthetic energy, CO_2, and H_2O. Other autotrophs are the chemolithotrophs, which use an inorganic substrates such as hydrogen or thiosulfate as a reductant and carbon dioxide as a carbon source. However, animals and many bacteria, except for the above-mentioned autotrophic bacteria, require organic carbon for growth via catabolism and

anabolism, which involve biochemical reactions using chemical energy. These biochemical reactions occur in living cells as well as in cell growth. In general, to produce chemical energy, glucose or hydrolyzed carbohydrates are used as nutrients. Differentiation of vertebrates has led to blood sugar levels in whole bodies being maintained *in vivo* by the actions of insulin and glucagon. However, not only single-cell organisms such as bacteria and protozoa, but also multicellular organisms such as invertebrates, have certain primitive glucose regulatory mechanisms which enable them to survive without an endocrine system. It would not be surprising if these less-developed organisms have characteristic carbohydrate metabolisms which differ from the endocrine system established in vertebrates. In addition, these primitive glucose regulatory mechanisms could still be preserved in vertebrate cells under dedifferentiated conditions.

Cell cultures provide a useful tool for investigating cellular metabolisms *in vitro* because such a system is much simpler than a whole body, which consists of various different cells and metabolic pathways *in vivo*. In 1955–1959, Eagle and his coworkers developed a method for culturing isolated cells *in vitro* [45]. His developed medium for *in vitro* cell culture, i.e., Eagle's minimum essential medium (MEM), consists of amino acids, glucose,

vitamins, and salts. In certain cases, 5%–10% bovine serum is added. Some amino acids such as alanine, asparagine, aspartic acids, glycine, hydroxyproline, proline, and serine have been removed from the medium because these amino acids are biosynthesized in cells. It is possible to remove serum from certain cell cultures, and glutamine and tyrosine have been deleted from the medium for rat hepatoma cells, Ry121B [47]. Sato and coworkers added hormones to a chemically defined culture medium instead of serum [48,49]. (To our knowledge, however, a culture medium which does not contain glucose has not yet been developed.) Glucose is essential for organisms, except autotrophs, for energy production and to provide a carbon source. Various culture media such as BME [50], MEM [51], Fisher [52], F12 [53], RPMI [54], DM-160 [56], and DME [57] contain 900–2,000 mg/L glucose. My former supervisor, Emeritus Professor Yasumura, who established famous cell lines such as VERO [32], Y1 [57] and GH [58] tried to establish cells which can grow in a glucose-free chemically defined medium, by using green monkey kidney cell VERO. However, he was unable to complete this work during his research life at the Dokkyo Medical University.

Glucose metabolism has been completely clarified based on biochemical reactions, not only in prokaryotes but also in eukaryotes, and glucose metabolisms

are almost the same among various organisms. However, some biochemical reactions occur at different places in prokaryotes than in eukaryotes. One major difference between the cellular structures is the presence of mitochondria in eukaryotes, and this organelle contributes to the respiratory function, which metabolizes carbohydrates, i.e., glucose. In general, one molecule is converted to two pyruvate molecules, and finally converted to CO_2 and H_2O via the tricarboxylic acid (TCA) cycle in the presence of oxygen. In contrast, in the absence of oxygen, pyruvate produced from glucose is converted to lactate via the Embden–Meyerhof pathway or the alternative Entner–Doudoroff pathway. Eventually, one glucose molecule is converted to two lactate molecules in the absence of oxygen. In the present study, the glucose contained in the culture medium was almost all converted to lactate, although the cells were cultured in the presence of oxygen (Fig. 7). This indicates that the TCA cycle is not involved in glucose metabolism in the cultured cells, i.e., glucose was metabolized via an aerobic pathway to lactate in the cells.

In prokaryotes, the complete genome of *Mycoplasma plumonis* consists of 782 protein genes and 963,879 nucleotides [59], and that of *Ureaplasma urealyticum* consists of 646 protein genes and 874,478 nucleotides [60]. In

eukaryotes, the *Homo sapiens* (human) genome consists of 20,109 protein genes and 2,851,330 mb nucleotides [61,62]. These facts indicate that biological evolution diverged along with increases in the number of protein genes and nucleotide numbers in chromosomal DNA. Glucose metabolism maintains life not only in prokaryotes but also in eukaryotes, which have mitochondria. The *Reclinomonas americana* (Protist) (~70 kb), consisting of 97 genes, is thought to be an ancestral mitochondrial DNA, whereas vertebrate mitochondrial DNA (~16 kb), consisting of 13 respiratory genes, seems to be constructed with only essential genes for respiration reactions. These decreases in protein and total nucleotide numbers along with mitochondrial evolution are the reverse of the phenomena observed in chromosomal DNA. It has been suggested that mitochondria developed from the protobacterium *Rickettsia* or its relatives, on the basis of gene similarities between these two cellular organelle DNAs [63,64]. We showed that normalization of the nucleotide contents of a complete genome indicates the characteristics of an organism [65]. For example, this procedure was used to classify prokaryotes into two groups, namely *Escherichia coli* and *Staphylococcus aureus* types [66], and for construction of phylogenetic trees [67]. The use of normalized nucleotide values enables

certain nucleotide contents to be expressed by a linear regression line [68]; for example, the cytosine content can be expressed by $C = aG + b$, where C and G are nucleotide contents and a and b are constants, based on Chargaff's second parity rule [69], as shown in Fig. 10. The regression lines obtained from complete chromosomal, plant mitochondrial, and chloroplast DNA overlap, whereas those obtained from complete animal mitochondrial DNA deviate from these regression lines. Although it was reported that mitochondrial DNA deviated from Chargaff's second parity rule [69], two regression lines can be obtained, dividing animal mitochondria into two groups, namely groups with high and low C/G contents [68.70]. As *Monosiga brevicollis* mitochondria have the lowest C/G contents among all cellular organelles, as shown in Fig. 10, we concluded that *Monosiga brevicollis* mitochondria may be the most primitive extant ancestor of the species examined [70]. In addition, the fact that all the regression lines crossed at a single point indicates that all organisms might diverge from a single origin of life [68.70], as speculated in Darwin's theory. Our previous study indicated that more highly evolved organisms have greater normalized cytosine contents in their complete genomes, and the highest cytosine content was observed in primate and avian complete mitochondrial genomes [68,70].

This is consistent with results based on complete genome analysis, which were reported by another group [71]. In contrast, the normalized cytosine contents of plant mitochondrial genomes which obey Chargaff's second parity rule [69] showed lower evolutionary divergence than in the case of vertebrate mitochondrial genomes [68,70]. Vertebrate mitochondrial evolution therefore seems to be linked with expansion of animal active behaviors, which consume a lot of energy. The numbers of mitochondria in the liver, kidney, muscle, and brain are larger than those in other organs. Cells cultured *in vitro* might not need a large number of mitochondria, which produce energy, because their mobility is limited. In the presence of oxygen, the TCA cycle produces CO_2 and H_2O as the final products of glucose metabolism. This means that a carbon source is lost from the system, whereas lactate, which is produced from glucose in the absence of oxygen, can be reused later in the system. In the present study, many cell lines produced lactate as the final product from glucose (Fig. 7). These results indicate that the Embden–Meyerhof and Entner–Doudoroff pathways are active in cultured cells, even if oxygen is present. Lactate metabolism by cells was slower than glucose consumption (Fig.8). The lactate production pathway therefore seems to assist a rapid decrease in the

blood glucose level *in vivo*. In addition, the rapid secretion of lactate into the culture medium is necessary for cells to maintain a neutral pH inside the cells, and the metabolism of lactate secreted from cells may contribute clinically to recovery from lactate acidosis. The results of the present study indicate that mammalian cells basically have to metabolize extracellular lactate.

It is well known that tissue cultures lose their differentiated cellular functions *in vitro*. It is therefore impossible to establish cell lines which reserve full organ specific functions, and only certain differentiated functions are randomly maintained. To our knowledge, there is no cell line in which gluconeogenesis takes place *in vitro*. In addition, the control of blood sugar levels is based on homeostasis by the endocrine system, which is established in highly evolved organisms such as vertebrates. This endocrine system is also a differentiated function. In the present study, glucose metabolism of Py-3Y1-S2 cells was independent of insulin (Fig. 9), although insulin receptors are present on the plasma membranes of various established cell lines [72]. However, addition of carbonates, namely $NaHCO_3$ and Na_2CO_3, to the culture medium accelerated glucose consumption (Figs. 1, 2, 6, and 7). It is therefore clear that there is an insulin-independent glucose metabolic pathway in Py-3Y1-S2 cells.

The addition of $NaHCO_3$ to the basic culture medium maintains a neutral pH in a 5% CO_2 incubator, rather than cell nutrient or glucose metabolism regulation.

Con A [39] and vanadium compounds [7-22] showed insulin-like activity not only *in vivo* but also *in vitro*. Our present study and previous [23,24] studies confirmed these results for Py-3Y1-S2 cells. Con A is a lectin and protein, and vanadium is a metal and its salts are metal compounds. Carbonates such as $NaHCO_3$ and Na_2CO_3 are inorganic compounds. Their molecular structures clearly differ not only from that of insulin but also from those of Con A or vanadium. Carbonates seem not to bind to insulin receptors on the plasma membrane to induce signal transductions, followed by activation of a glucose transporter (GLUT 4). In addition, the dissociation constants (K_d) of insulin with its receptors on cultured rat hepatoma cells (Ry121B) were ~4 $x10^{-9}$ M and ~3 $x10^{-8}$ M, at the high and low insulin-binding sites, respectively [73]. These values are much lower than ~1 mg/ml (17 mM) of $NaHCO_3$ (Fig. 2). The acceleration of glucose consumption by these small molecules therefore takes place via an insulin receptor-independent pathway.

Addition of nicotinamide, which is normally present in the basic culture medium, reduced glucose consumption, but this inhibitory effect was abolished by carbonates (Fig. 6). Nicotinamide, alloxan,

and STZ induce diabetes [42-44]; this is consistent with the present results. Insulin acts on target tissues to reduce blood glucose levels. However, the present study indicates that insulin-independent glucose metabolisms occurs in cells. It is necessary to consider this newly discovered pathway to achieve a more precise understanding of glucose metabolisms, not only *in vitro* but also *in vivo*.

The basic structure of both $NaHCO_3$ and Na_2CO_3, which accelerate glucose consumption, is a carbonyl group, =C=O, while the structures of STZ, alloxan, and nicotinamide, which induce type 1 diabetes and reduce glucose consumption in cultured cells, also contain a carbonyl group. The carbonyl group of diabetes-inducing reagents like STZ, alloxan, and nicotinamide is a part of an amide structure, -CONHR. Ceramides consisting of an amino group-bound carbonyl group were shown to induce insulin resistance [44]. However, amino acids containing a carboxyl group did not affect glucose uptake in the present study, although their structures contained a carbonyl group that was not bound to an amino group. These findings indicate that glucose consumption by $NaHCO_3$ or Na_2CO_3 is controlled by side groups that bind to the carbonyl group, such that the $–O^-$ accelerates glucose consumption and the –N- reduces glucose consumption.

$NaHCO_3$ is used as baking soda

in daily life and as a medicine for alkaloids. However, because $NaHCO_3$ and Na_2CO_3 are alkaline chemical compounds, their concentrations for usage should be carefully controlled. The present results may contribute to the development of alternative medicines as well as new medicines for DM and dementia.

5. Conclusions

Two carbonates, $NaHCO_3$ and Na_2CO_3, directly accelerated glucose consumption in established Py-3Y1-S2 rat fibroblast cells. Combinations of the carbonates with vanadium or Con A further increased glucose consumption, while combinations of the carbonates with STZ, alloxan, or nicotinamide abolished the effects of the drugs in Py-3Y1-S2 cells. Both carbonates increased lactate production by Py-3Y1-S2 cells. Taken together, the present study indicates that $NaHCO_3$ and Na_2CO_3 directly regulate glucose metabolism in Py-3Y1-S2 cells via insulin-independent cellular glucose metabolisms based on intracellular homeostasis.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing

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Figure Legends

Figure 1. Effects of basalt powder on glucose consumption by cultured Py-3Y1-S2 cells in the presence of $NaHCO_3$ or Na_2CO_3. Mt. Fuji basalt powder with ~2-μm diameter was kindly supplied by Mt. Fuji Yogan Institute (Yamanashi, Japan), and 5 or 200 mg of powder was treated with 1 mg/ml $NaHCO_3$ for 30 min at room temperature. After treatment with $NaHCO_3$, the solution was centrifuged at 3,000 rpm for 10 min, and the supernatants were used for preparation of culture media. Data represent means ± SD of 3 or 13 independent experiments. *p < 0.05; **p < 0.01. This original figure was published in Int. J. Pharm. Phytopham. Res., 2019; 9 (3): 1-5. [74].

Figure 2. Effect of $NaHCO_3$ (A) or Na_2CO_3 (B) on glucose consumption by Py-3Y1-S2 cells. Data represent means ± SD of 3–7 independent experiments. *p < 0.05; **p < 0.01. This original figure was published in Int. J. Pharm. Phytopham. Res., 2019; 9 (3): 1-5. [74].

Figure 3. Effect of $NaHCO_3$ (A) or Na_2CO_3 (B) on cellular protein. Data represent means ± SD of 5 independent experiments. *p < 0.05; **p < 0.01. This original figure was published in Int. J. Pharm. Phytopham. Res., 2019; 9 (3): 1-5. [74].

Figure 4. Effect of Con A on glucose consumption by Py-3Y1-S2 cells. Data represent means ± SD of 5 independent experiments. *p < 0.05; **p < 0.01. This original figure was published in Int. J. Pharm. Phytopham. Res., 2019; 9 (3): 1-5. [74].

Figure 5. Effect of vanadium or Con A on glucose consumption by Py-3Y1-S2 cells in the presence of $NaHCO_3$ (A and B) or Na_2CO_3 (C and D). Data represent means ± SD of 5 independent experiments. *p < 0.05; **p < 0.01. This original figure was published in Int. J. Pharm. Phytopham. Res., 2019; 9 (3): 1-5. [74].

Figure 6. Effect of $NaHCO_3$ or Na_2CO_3 on glucose consumption by Py-3Y1-S2 cells in the presence of STZ, alloxan, or nicotinamide. The concentration of each reagent was 1 mg/ml. Data represent means ± SD of 3 independent experiments. *p < 0.05; **p < 0.01. This original figure was published in Int. J. Pharm. Phytopham. Res., 2019; 9 (3): 1-5. [74].

Figure 7. Effect of $NaHCO_3$ or Na_2CO_3 on glucose consumption (A) and lactate production (B) by different cell lines. Data represent means ± SD of 6–8 independent experiments. *p < 0.05; **; p < 0.01.

Figure 8. Gradual decrease in lactate concentration in culture medium treated with

Py-3Y1-S2 cells. A 10 μl sample of culture medium was removed after incubation for 1, 2, 3, and 4 days for lactate assays.

Figure 9. Insulin effect on glucose consumption. Concentrations of insulin were 0.1, 0.5, 1.0, 5.0, 10, and 50 μg/ml. Data represent means ± SDs of six independent experiments.

Figure 10. Regression lines based on plotting of C content against G content. Nucleotide contents of complete genomes of various cellular organelles were normalized. This is a modified version of a figure in our previous article: Natural Science, 2018; 10 (9): 338-369 [70]. Large red and blue closed circles represent *Monosiga brevicollis* and *Homo sapiens* mitochondria, respectively. Vertebrate mitochondria; (asterisk), high C/G invertebrate mitochondria; (triangle), low C/G invertebrate mitochondria, (cross), bacteria; (circle), non-animal mitochondria and chloroplasts; (diamond), and chromosomes; (square).

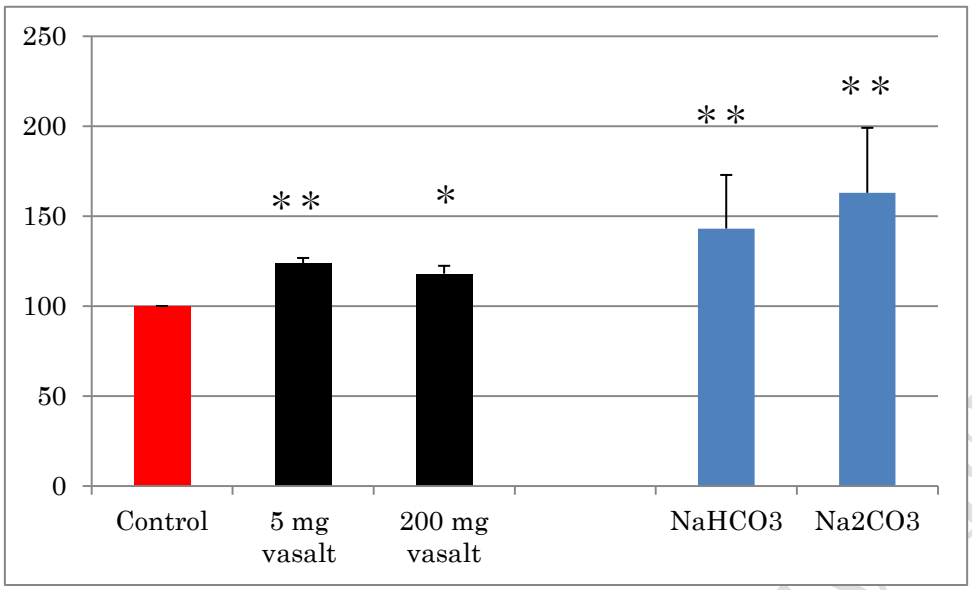
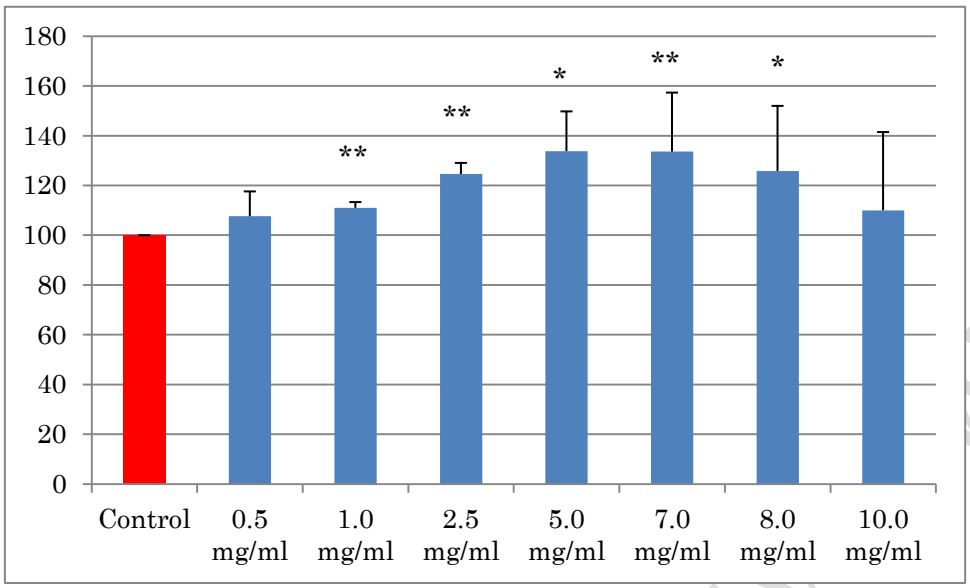


Fig. 2

A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Control
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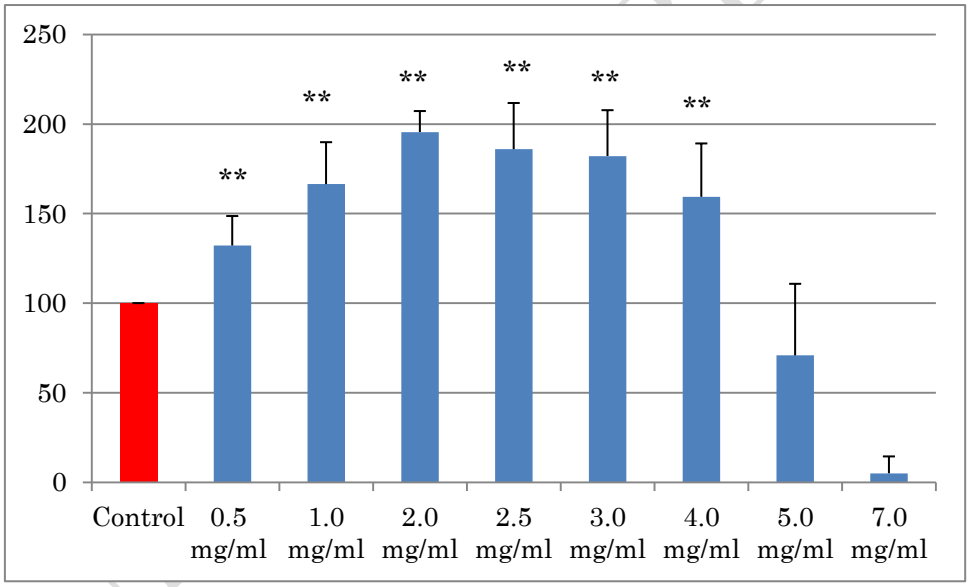
2.5 mg/ml
5.0 mg/ml
7.0 mg/ml
8.0 mg/ml
10.0 mg/ml

B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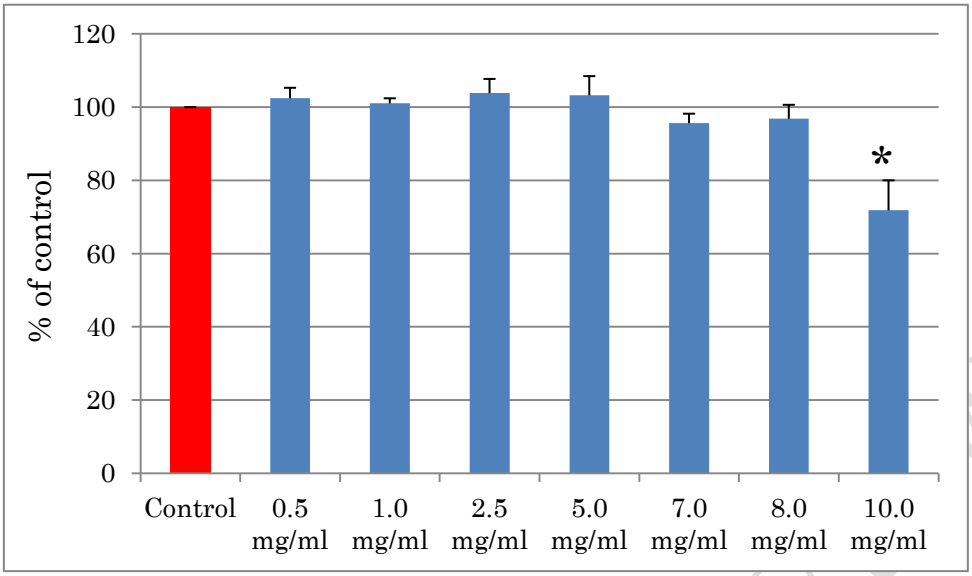
3.0 mg/ml
4.0 mg/ml
5.0 mg/ml
7.0 mg/ml

Fig. 3

A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10.0 mg/ml

B

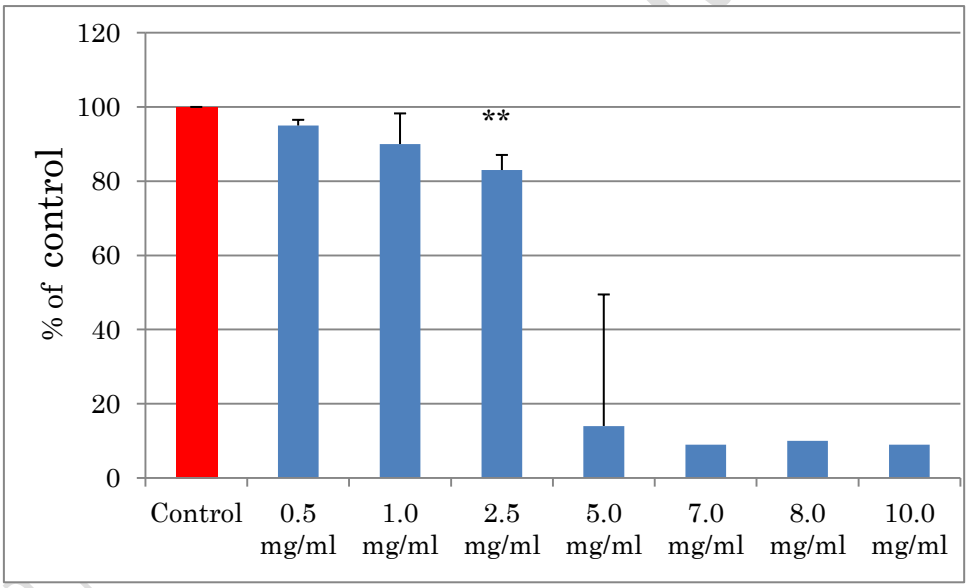
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Fig. 4

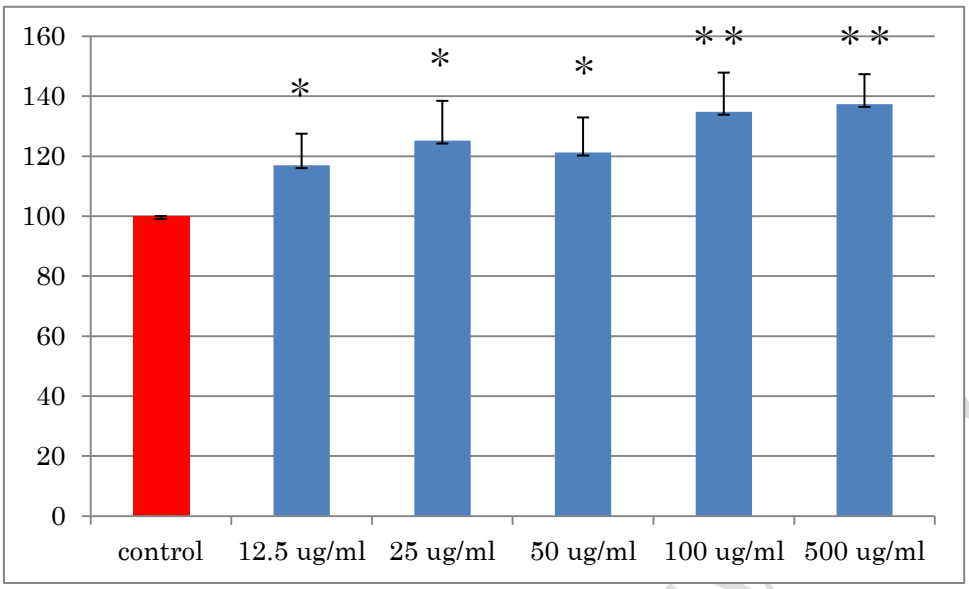
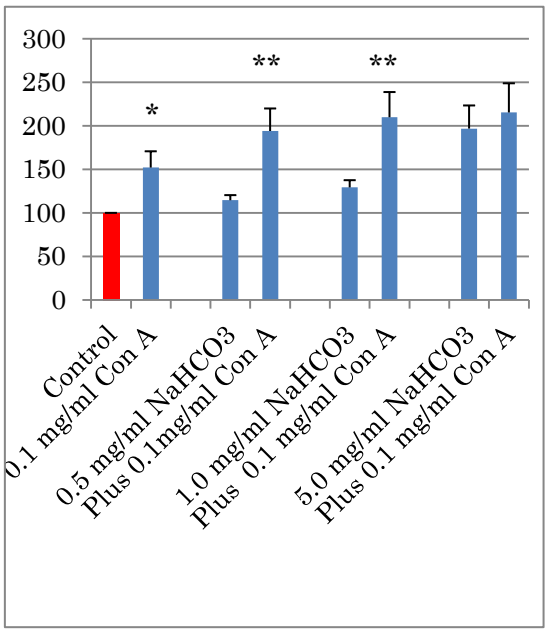


Fig. 5

A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**
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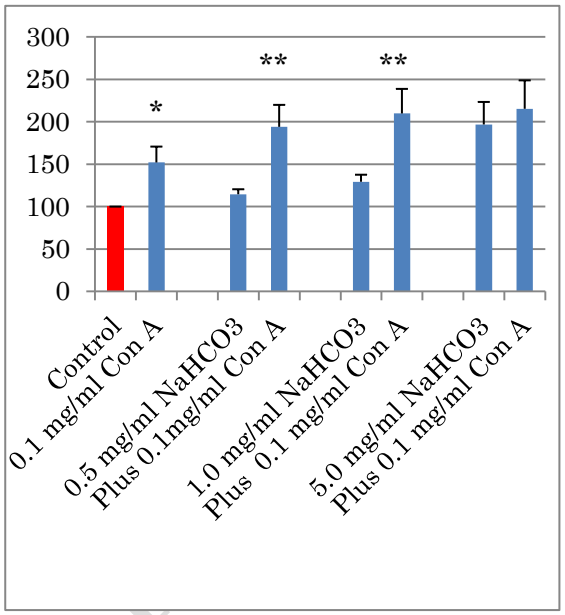
0.5 mg/ml NaHCO3
Plus 0.1mg/ml Con A
1.0 mg/ml NaHCO3
Plus 0.1 mg/ml Con A
5.0 mg/ml NaHCO3
Plus 0.1 mg/ml Con A

B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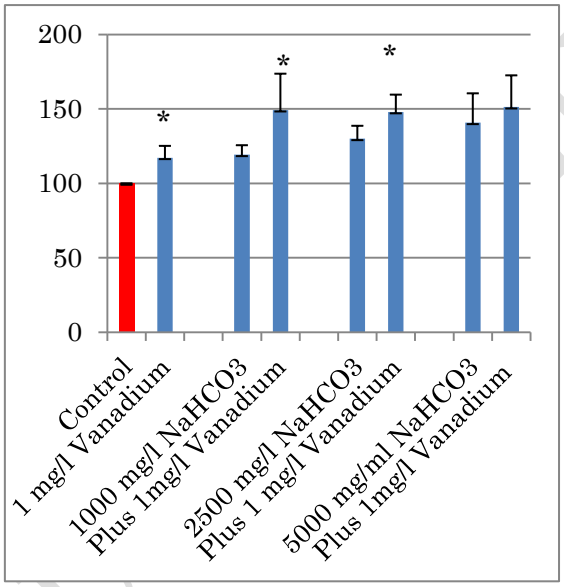
Plus 0.1 mg/ml Con A
5.0 mg/ml NaHCO3
Plus 0.1 mg/ml Con A

C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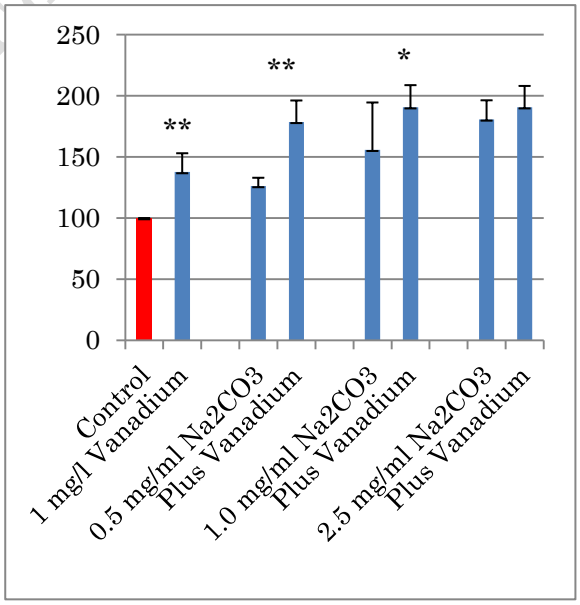
Plus 1 mg/l Vanadium
5000 mg/ml NaHCO3
Plus 1mg/l Vanadium

D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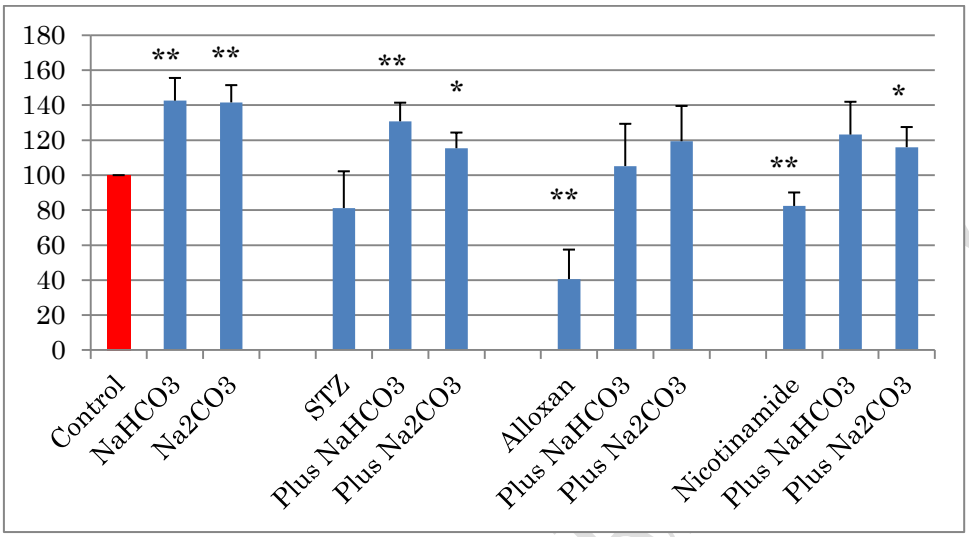
2.5 mg/ml Na2CO3
Plus Vanadium

Fig. 6

A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NaHCO3
Na2CO3
STZ
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Plus Na2CO3
Alloxan
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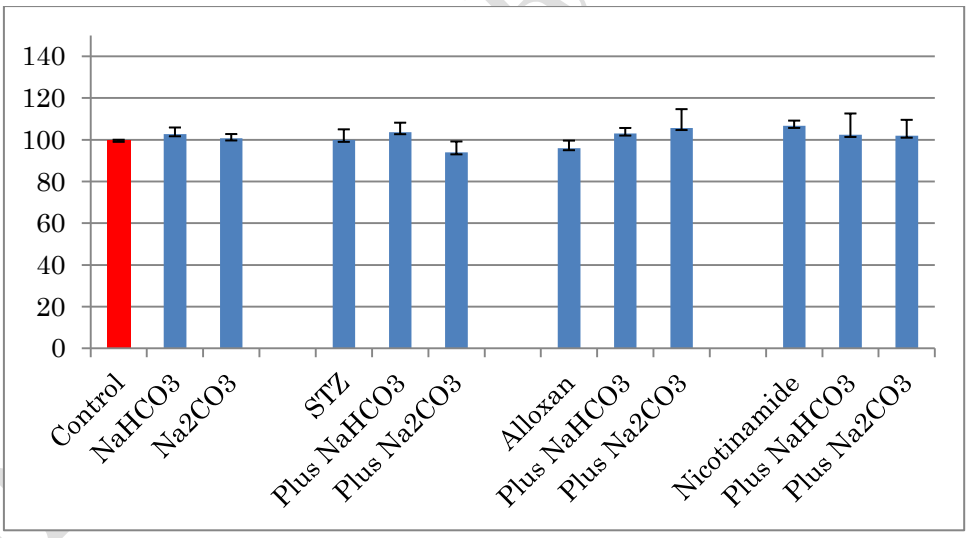
Plus Na2CO3
Nicotinamide
Plus NaHCO3
Plus Na2CO3

B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STZ
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Alloxan
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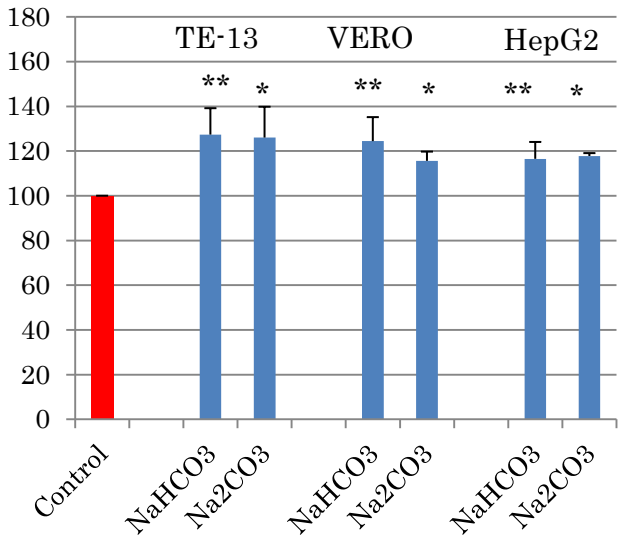
Plus Na2CO3
Nicotinamide
Plus NaHCO3
Plus Na2CO3

Fig. 7

A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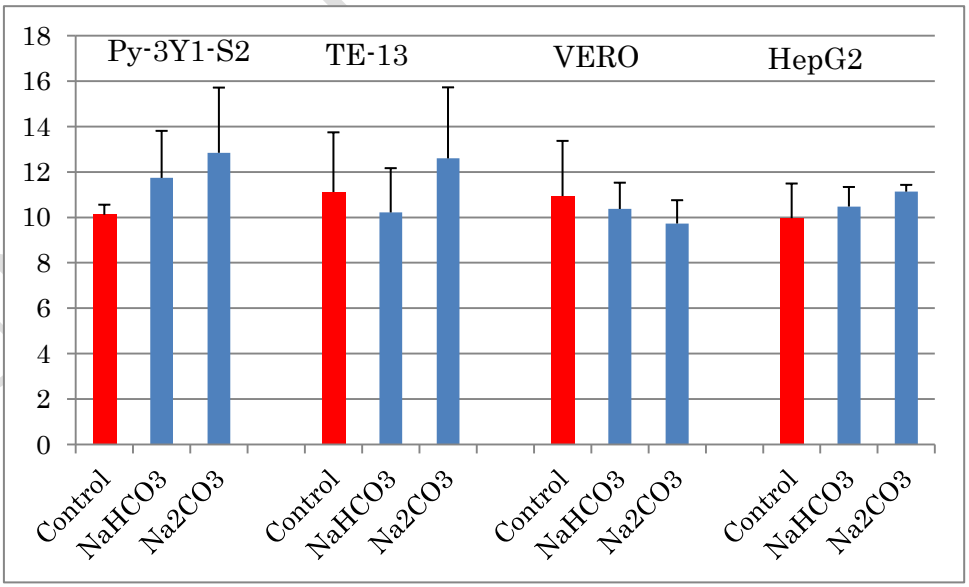
NaHCO3
Na2CO3
NaHCO3
Na2CO3

B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Na2CO3
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Control
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Na2CO3

Fig. 8

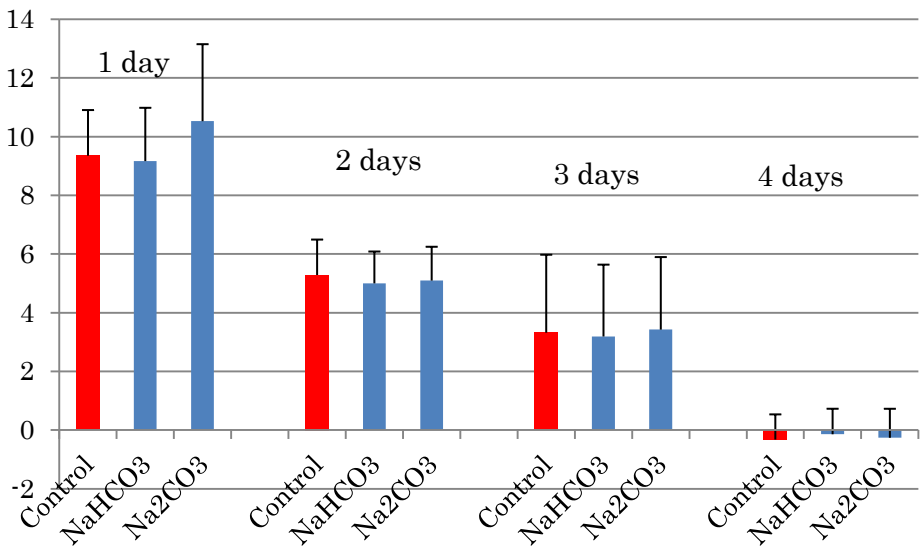

Fig. 9

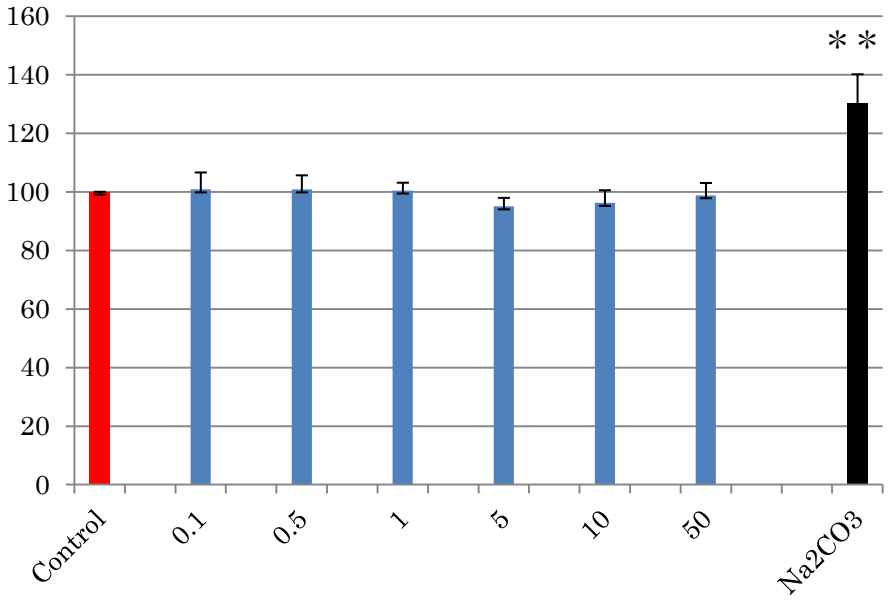

Fig. 10