

Glucosamine Sodium Sulfate Can Penetrate Skin and May Affect Glucose Metabolism in Rats

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Abstract

Objectives: To investigate whether glucosamine sodium sulfate (GlcNs) can penetrate the skin and the effects of this dietary supplement on glucose metabolism in rats.

Methods: GlcNs was radiolabeled with ¹⁴C to test its ability to pass through skin. GlcNs was also orally administered to rats at different doses. Changes in body weight and blood glucose levels were subsequently measured. In addition, levels of key enzymes related to glucose metabolism (SLC2A2 (solute carrier family 2, member 2), SLC2A4 (member 4), HK1 (hexokinase 1), NOS2 (nitric oxide synthase 2), and PRKCA (protein kinase C, alpha)) were analyzed in liver and kidney tissues via immunohistochemistry.

Results: Radio labeled GlcNs effectively penetrated rat skin. Rats given a daily dose of 250 mg/kg GlcNs exhibited significantly lower levels of blood glucose ($p < 0.05$) than controls. For the 125-mg/kg group, levels of PRKCA and HK1 in kidney were significantly lower ($p < 0.05$) than controls.

Conclusions: GlcNs can be administered through the skin. Oral administration of GlcNs does not affect body weight, but may influence glucose metabolism in rats. These findings are important for research concerning diabetes and its complications in patients with arthritis.

Keywords: ¹⁴C labeling; Glucose metabolism; Glucosamine sodium sulfate; Rat

Introduction

Glucosamine (GlcN) is an amino monosaccharide (2-amino-2-deoxy-D-glucose) that is naturally present in the human body. It is also found in cartilage of animals and shellfish, and can be prepared from chitin, insect exoskeletons, algae, or mushrooms. Exogenous GlcN is primarily used as a substrate for mucopolysaccharide and biopolymer synthesis in joints and bones [1]. As such, GlcN helps restore damaged cartilage [2]. In cultured human and animal chondrocytes, GlcN is an important component of mesenchymal tissue and synovium [3]. GlcN may also affect gene expression in chondrocytes, as mRNA levels of *aggrecan* are 2-fold higher in treated chondrocytes than in controls [4]. When cartilage is damaged by injury or osteoarthritis, GlcN stimulates cartilage cells to produce proteoglycan, which stabilizes cell membranes. GlcN also appears to act as an anti-inflammatory, but has lower toxicity than many anti-inflammatory drugs. Proponents suggest, therefore, that GlcN could be used to build bones [5]. GlcN also increases the barrier function of skin cells and stimulates intestinal epithelial cells to secrete mucin, which has an anti-ulcerogenic effect [6,7]. Because of these benefits, GlcN represents a potentially useful biologically active compound [8], and it has already been approved as a prescription drug in Europe, a food supplement in the USA, and a health food in China [9,10].

GlcN is metabolized through the hexosamine biosynthetic pathway [11] and is actively imported into cells by glucose transporter proteins (Gluts) [9,12]. Based on metabolism and toxicology studies in both humans and animal model systems [13], GlcN is considered extremely safe. For example, no adverse effects were detected when Sprague Dawley (SD) rats were orally administered 2700 mg/kg GlcN for 12 months [13]. There are concerns, however, that GlcN may adversely affect glucose metabolism, although results from clinical studies

concerning this issue have been controversial. Additional studies are needed to draw a more definite conclusion. In particular, these studies should focus on subjects at high risk for glucose homeostasis impairment [14]. *In vitro*, elevated levels of tissue GlcN impair insulin secretion [15] and induce insulin resistance in isolated rat skeletal muscle [16].

In vivo studies have also revealed GlcN-induced insulin resistance in skeletal muscle and adipose tissues [17-19]. Finally, administration of GlcN to Wistar rats activates the hexosamine pathway and induces insulin resistance in insulin-sensitive tissues (e.g., liver, heart, abdominal muscles, fat, and the submandibular gland). In this context, GlcN completely inhibits glycogen synthesis in the liver.

For patients with diabetes and its complications, liver and kidney function are particularly important. The effect of GlcN on glucose metabolism in these two organs, however, has not been well studied. Furthermore, joint tissue contains large amounts of sulfate, which is more favorable for absorption of glucosamine sulfate. Therefore, we investigated the effect of GlcN sodium sulfate (GlcNs) on glucose metabolism in SD rats. We measured circulating blood glucose, body weight, and levels of five enzymes involved in glucose metabolism in the liver and kidneys. These enzymes included: 1) solute carrier family

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2 (facilitated glucose transporter), member 2 (SLC2A2); 2) solute carrier family 2 (facilitated glucose transporter), member 4 (SLC2A4); 3) hexokinase 1 (HK1); 4) nitric oxide synthase 2, inducible (NOS2); and 5) protein kinase C, alpha (PRKCA). Results from this study will potentially indicate whether individuals with diabetes can take advantage of this nutritional supplement.

Experimental

Radiolabeling GlcNs with ^{14}C

GlcNs was radiolabeled with ^{14}C as described [20] with modifications. Twenty milliliters sodium ethoxide (0.25 mol/L) was placed in a conical flask and heated to 40-50°C. We then added 5.39 g glucosamine hydrochloride sodium, of which 1% was labeled with ^{14}C (186333, Sigma-Aldrich, Shanghai, China). The solution was stirred at 750×g for 5 min, and then centrifuged at 3800×g for 10 min. The supernatant was transferred to a three-necked flask, and then 10 ml concentrated sulfuric acid, which contained 20% sulfur trioxide (98%, China National Medicines Corporation, Beijing, China), was added. After stirring the three-necked flask slowly (250×g) for 60 min at 4°C, 500 ml acetone (98%, 10000418, Sinopharm Chemical Reagent Co., Beijing, China) was added. The solution was then stirred for an additional 30 min. Flaxen solid powder was then obtained using vacuum filtration. The solid powder was washed with acetone and ether, and then vacuum dried until it contained <1% water. The product was stored at 4°C under vacuum. The structure of the powder was measured using an infrared method (NEXUS-470 FTIR, Nicolet, Beijing, China). The amount of GlcNs that was labeled with ^{14}C was detected using high-performance liquid chromatography. The chromatographic column was 250 mm×4.16 mm and 4 μm (WAT044355, Waters Carbohydrate, Shanghai, China). The mobile phase was acetonitrile- H_2O (70: 30) with a flow rate of 110 ml/min. The column temperature was 28°C. For evaporative light-scattering detection, the flow rate of the carrier gas was 3100 standard liters per minute, and the temperature of the evaporation tube was 80°C [21].

The ability of GlcNs to permeate rat skin

These experiments were conducted using an auto-diffusion cell [22,23] that contained a supply pool and a receiving tank (TT-6, Keshary-Chien, Tianjin, China). The supply pool contained a GlcNs solution (5% w/w, 3.0 ml). The receiving tank contained saline solution (12 ml). A piece of skin (2.14 cm²) from a SD rat (License No. SCXK-2002000, purchased from the Experimental Animal Center of Peking University, Beijing, China) was affixed between the supply pool and reception tank (the cuticle was towards the supply pool). The flow rate of the GlcNs sodium solution was set at 1 ml/h, and the temperature was maintained at 37°C. Aliquots (0.5 ml) were taken from the receiving tank after 0, 0.5, 1, 2, 4, and 8 h of diffusion. After each aliquot was taken, 0.5 ml saline solution was added to the receiving tank. Receiving-tank aliquots were stored at -20°C until levels of radioactivity (i.e., concentrations of a radioactive substance) were detected using a liquid scintillation counter (Triathler, Hidex Oy, Turku, Finland).

Animals

Forty-eight female SD rats were purchased (Weitong Lihua, Beijing, China). At the start of the experiment, the rats were approximately 7 weeks old. After 5 days of acclimation, the rats were divided into four groups using a stratified-by-weight randomization method. As a result, each group had a similar mean body weight. Each group was then randomly assigned to one of four treatments. Three groups received doses of GlcNs, and one served as the negative control. During the

feeding period, two rats receiving the same treatment were housed in a single stainless steel cage and fed standard laboratory food (AIN-93G diet) [24] and water *ad libitum*. Animal room parameters included a 23 ± 2°C temperature, a relative humidity of 50 ± 10%, 15 air changes per h (fresh air), and a 12-h light/dark cycle. The experimental protocol was approved by the Ethics Committee of The Institute of Medicinal Plant Development (NO. 20090902-1, date 09-02-2009).

GlcNs was dissolved in distilled water (50 mg/ml) and orally administered by gavage once a day for 28 d. To achieve different dosages, the GlcNs concentration was fixed, but the feeding volume varied. A GlcNs solution volume of 1.0, 0.5, or 0.25 ml was administered to the high-dose (500 mg/kg body weight), mid-dose (250 mg/kg), and low-dose (125 mg/kg) groups, respectively. In contrast, rats in the negative control group were gavaged with 1.0 ml distilled water at the same time and frequency as the treatment groups.

Metabolic enzyme analysis

At the end of the feeding period, all animals were anaesthetized and sacrificed. For each animal, a thorough necropsy was performed *via* visual inspection, with all major organs examined. Four rats from each group were randomly selected for immunohistochemical analysis. Tissue samples from the liver and kidney were excised from these rats and fixed in 4% buffered formaldehyde. Tissues were then embedded in paraffin and sectioned using a microtome. Sections were stained using the PV-9000 general-purpose two-step test kit (Zhongshan Godenbridge Biotechnology Co., Beijing, China). Polyclonal antibodies specific for PRKCA, NOS2, and SLC2A2 were diluted 1:50. Monoclonal antibodies specific for HK1 (1:100) and SLC2A4 (1:70) were also used. All primary antibodies were purchased from Santa Cruz Biotechnology (Zhongshan Godenbridge Biotechnology Co.). Histopathology tests were performed by Sepang Technology Co. (Beijing, China).

Independent pathology technicians used a defined immunohistochemical score [25] to evaluate each slide. Scores were calculated by multiplying a staining intensity score (0=negative, 1=faint, 2=weak, 3=moderate, and 4=strong) by a quantity score, which estimated the percentage of immunoreactive cells (0=no staining, 1=1-30% immunoreactive cells, 2=31-60%, and 3=61-100%). Theoretically, scores could range from 0 to 12.

Blood glucose and body weight

Body weights were measured each week during clinical evaluations. At the end of the study, rats were rendered unconscious by ether inhalation, and blood samples were collected from the abdominal aorta. These samples were stabilized by the addition of heparin (2 USP units per 100 μl blood). To isolate serum, blood samples were centrifuged at 1,500×g for 10 min at 4°C. Glucose levels in sera were measured using a glucose testing kit (YS39-41, BioSino Bio-technology and Science, Beijing, China).

Statistical analysis

Statistical comparisons were designed to detect differences in response variables between groups and to determine whether those differences resulted from GlcNs administration. Data obtained from each treatment group were first compared to the negative control. In addition, data from the high-dose group were compared to the low-dose group. These analyses were performed using a one-way analysis of variance with an adjustment for multiple comparisons. The statistical software was from Statistical Product and Service Solutions (SPSS Inc.,

Chicago, IL, USA). Differences were considered significant at $p < 0.05$. Results are shown as the mean \pm standard deviation.

Investigations and Results

GlcNs permeates rat skin

GlcNs samples labeled with ^{14}C were used to test whether this compound could penetrate rat skin. The structure of GlcNs was not altered by ^{14}C -labeling, as indicated by Fourier transform infrared spectroscopy analysis. The amount of radiolabeled GlcNs in the reception tank was detected using a liquid scintillation counter (Table 1). From 0–1 h, no radioactivity was detected in the reception tank. As such, background levels of radioactivity in the reception tank were very low. After the first hour, however, radioactivity levels in the reception tank increased with time. This indicated that radiolabeled GlcNs was able to pass through the rat skin and enter the reception tank.

Blood glucose and body weight

Body weight and the blood glucose level were used to evaluate the effect of GlcNs on glucose metabolism in SD rats. Compared to negative controls, rats in the mid-dose group (250 mg/kg) had significantly lower levels of blood glucose ($p < 0.05$, Figure 1A). The high- (500 mg/kg) and low-dose groups (125 mg/kg), however, exhibited no significant differences in blood glucose levels when compared with the negative control ($p > 0.05$). These results suggest that oral administration of GlcNs may, at least at some doses, decrease blood glucose levels in SD rats.

The effect of GlcNs on the body weight of rats was also evaluated (Figure 1B). No statistically significant differences in final mean body weight were detected among the groups. Thus, at the doses tested, GlcNs did not affect body weight.

Effect of GlcNs on metabolic enzymes in the liver

To evaluate the effect of GlcNs on glucose metabolism in the liver, we examined the levels of five metabolic enzymes *via*

immunohistochemistry (Figure 2, Table 2). These five enzymes were SLC2A2, SLC2A4, HK1, NOS2, and PRKCA. For these five enzymes, no significant differences in protein levels in the liver were detected when comparing the negative control and each of the three treatment groups ($p > 0.05$). Thus, the tested doses of GlcNs (125–500 mg/kg) did not affect expression of these five enzymes in the liver.

Effect of GlcNs on metabolic enzymes in the kidney

We also evaluated the effect of GlcNs on SLC2A2, SLC2A4, HK1, NOS2, and PRKCA levels in the kidney (Figure 3, Table 2). Compared with the negative control group, oral administration of GlcNs (125–500 mg/kg for 28 d) did not affect expression levels of SLC2A2, SLC2A4, and NOS2 in rat kidney ($p > 0.05$). In contrast, PRKCA was down-regulated ($p < 0.05$) by oral administration of GlcNs at doses of 500 mg/kg and 125 mg/kg. However, no effect on PRKCA was detected with the intermediate dose of 250 mg/kg. Finally, HK1 was down-regulated ($p < 0.05$) only when a low dose of GlcNs was administered (125 mg/kg).

Discussion

GlcN is frequently used to treat or prevent osteoarthritis in humans. Results from a number of experiments indicate that the bioavailability of orally administered GlcN is extremely low (i.e., articular cartilage cannot obtain enough GlcN sulfates *via* this method to maintain normal metabolism) [26]. Therefore, we sought to identify an alternative GlcN administration approach based on GlcN diffusion through skin. We used ^{14}C -labeled GlcN hydrochloride sodium to generate radiolabeled GlcN sodium sulfate. This radio labeled product was clearly able to penetrate rat skin. The bioavailability of GlcN seems to be improved by applying GlcNs to the skin and allows it to diffuse into subcutaneous tissue. So we supposed that the sulfate seems to play a critical role to the improved bioavailability of GlcN.

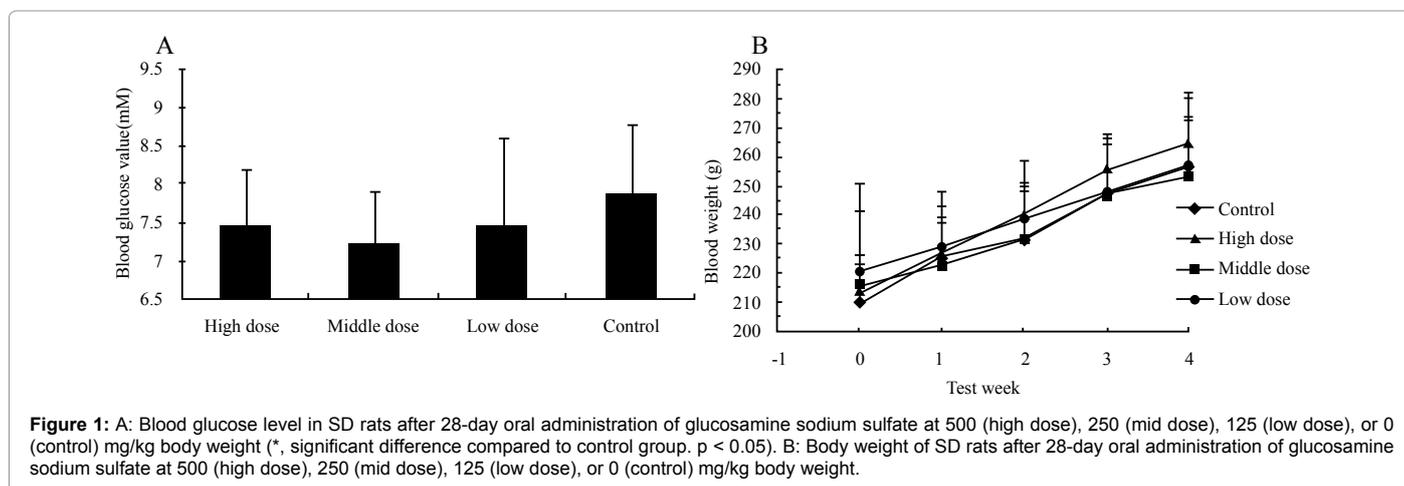
We then investigated the effect of orally administered GlcNs on glucose metabolism according to above hypothesis. We administered different doses of GlcNs and measured effects on blood glucose, body weight, and expression of five metabolic enzymes (SLC2A2,

Sampling time (h)	0	0.5	1	2	4	8
Levels of radioactivity	–	–	+	+	+++	++++

(+): A radioactive substance (^{14}C -labeled GlcNs) was detected in the reception-tank sample. The number of “+” symbols is proportional to the amount of radioactivity detected.

(–): A radioactive substance was not detected in the reception tank sample.

Table 1: The amount of radiolabeled GlcNs that penetrated rat skin.



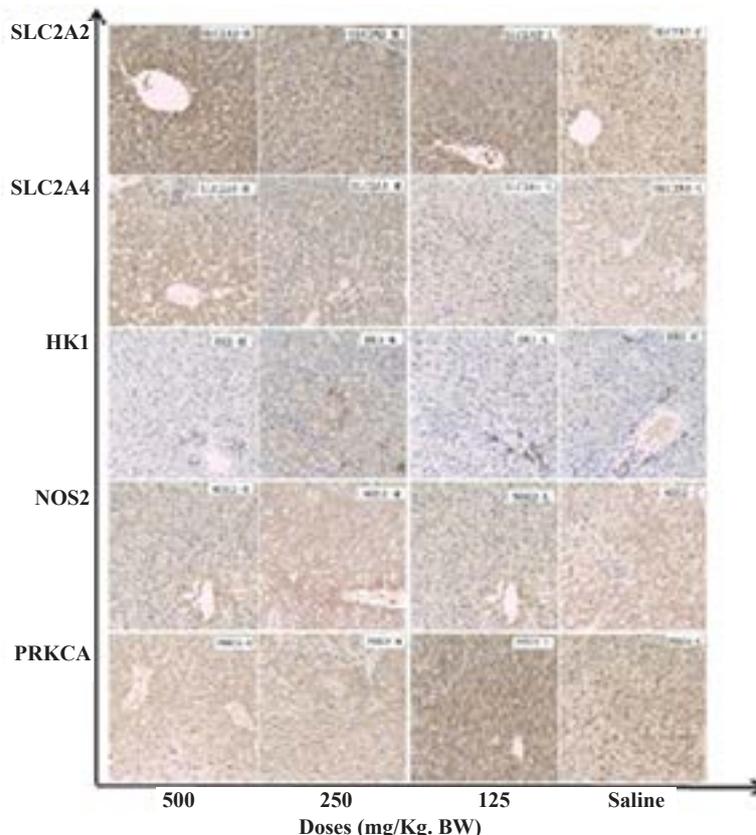


Figure 2: Immunohistochemical staining for SLC2A2, SLC2A4, HK1, NOS2, and PRKCA in rat liver ($\times 33$). The violet coloring is positive immunolabeling.

Tissue	Group	SLC2A2	SLC2A4	HK1	NOS2	PRKCA
Liver	High dose	6.25 \pm 5.06	3.00 \pm 1.41	0.38 \pm 0.25	5.25 \pm 4.11	2.13 \pm 2.72
	Intermediate dose	7.75 \pm 4.92	4.00 \pm 4.24	2.00 \pm 1.78	5.25 \pm 5.74	3.75 \pm 3.86
	Low dose	7.75 \pm 5.31	1.75 \pm 1.50	3.12 \pm 3.71	2.00 \pm 2.83	5.25 \pm 5.74
	Negative cont.	1.50 \pm 1.00	5.75 \pm 2.87	0.25 \pm 0.29	6.00 \pm 5.48	5.50 \pm 4.80
Kidney	High dose	8.50 \pm 1.91	3.87 \pm 1.25	5.62 \pm 2.84	7.00 \pm 3.32	3.87 \pm 1.93 ^a
	Intermediate dose	4.75 \pm 4.25	3.12 \pm 3.20	3.87 \pm 3.22	4.12 \pm 4.77	5.87 \pm 3.40
	Low dose	3.37 \pm 2.95 ^b	4.75 \pm 2.96	1.00 \pm 1.35 ^a	4.37 \pm 4.53	3.50 \pm 1.47 ^a
	Negative cont.	4.12 \pm 2.39	4.25 \pm 3.40	7.00 \pm 1.35	5.00 \pm 5.08	8.37 \pm 3.09

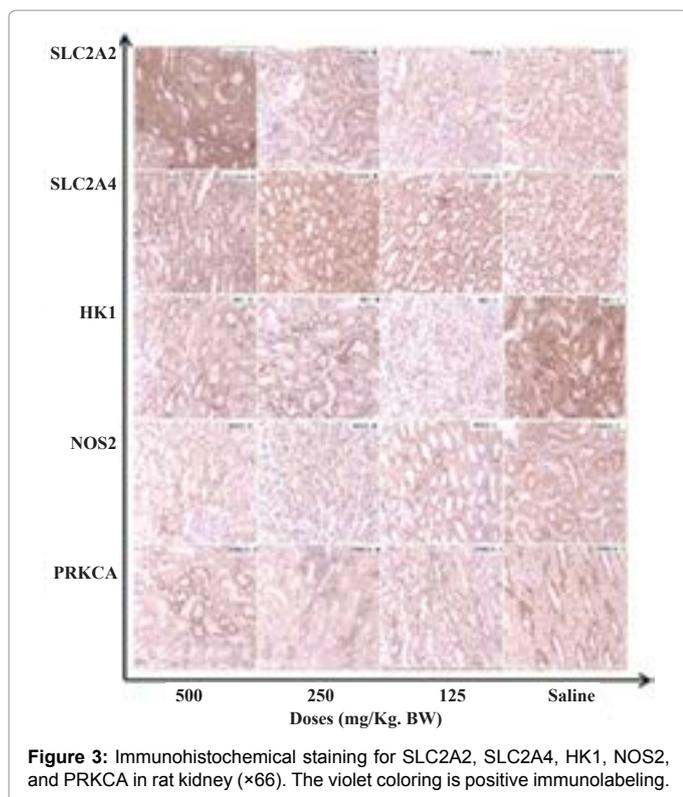
^ap < 0.05 compared to the negative control group.

^bp < 0.05 compared to the high-dose group.

Table 2. The effect of GlcNs on immunohistochemistry scores (see Methods) in liver and kidney tissue samples (mean \pm standard deviation, n = 4).

SLC2A4, HK1, NOS2, and PRKCA) in liver and kidney tissue. A number of studies have shown that oral administration of GlcN at very high doses does not affect blood glucose levels in rats [1]. To treat osteoarthritis in humans, a dose of 1.5 g/d GlcN sulfate (~25 mg/kg) is typically prescribed [27], resulting in serum GlcN levels of ~0.02 mM [28]. In contrast, the bioavailability of GlcN is very low when orally administered to rats, which likely results from low absorption in the gastrointestinal tract [11]. Therefore, we used GlcN sulfate to treat rats in this study. We orally administered 500, 250, and 125 mg/kg GlcNs to female SD rats. Compared to recommended doses in humans, these doses are 20, 10, and 5 times higher, respectively. SD rats were chosen because they are sensitive to insulin and other factors that affect glucose metabolism. We found that an intermediate dose of GlcNs (250 mg/kg) decreased blood glucose levels (compared to negative controls). This finding differs from a previous report [1].

The five metabolic enzymes analyzed in this study play important roles in signal transduction pathways involved in glucose metabolism. In certain tissues, Gluts have a higher affinity for GlcN than for glucose [15]. SLC2A2 is a high-affinity GlcN transporter that is localized in the basolateral membrane of cells in the liver, kidney, and small intestine [29,30]. SLC2A4 is expressed almost exclusively by insulin-sensitive tissues such as muscle and adipose tissue and is an insulin-sensitive Glut that is responsible for rapid glucose transport following insulin stimulation.^[30] Previously, GlcN was shown to induce insulin resistance in Wistar rats, particularly in insulin-sensitive tissues such as liver, heart, abdomen muscles, and fat [19]. However, in that study, the authors infused rats with GlcN for a total of 6 h, rather than administering 28 daily doses by gavage (as was done in the current study). Another study also reported that GlcN can lead to insulin resistance and reduce the transport capacity of SLC2A4 [31]. High



levels of glucose can elevate expression levels of *secreted phosphoprotein 1*, potentially by activating PRKCA and the hexosamine pathway [32]. This can lead to atherosclerosis [33]. GlcN also inhibits the production of nitric oxide by NOS2 [34]. Finally, most GlcN that is imported into cells is phosphorylated by HK1 into GlcN-6-phosphate, which effectively bypasses glutamine: fructose-6-phosphate amidotransferase to enter the hexosamine pathway [15,35]. As such, HK1 is an important regulator of GlcN metabolism.

Here we show that administration of 125 mg/kg GlcNs significantly decreased the expression of PRKCA and HK1 in rat kidney. This dose did not affect expression levels of other enzymes included in the analysis. In addition, 125 mg/kg GlcNs did not affect levels of these five enzymes in liver tissue. We postulate that in the kidney, decreased levels of blood glucose may down-regulate PRKCA expression. PRKCA is an important component of many signal transduction pathways and can activate HK1. Thus, expression of HK1 is down-regulated following PRKCA down-regulation [36]. We also demonstrated that SLC2A2 expression was significantly elevated in the high-dose group compared with the low-dose group. Thus, we conclude that SLC2A2 has a higher affinity for GlcN than for glucose and that this effect is dose dependent. This is consistent with a previous study [15].

Besides the above explanation, there is another speculation for the decreased HK1 and PRKCA. Since the HK can phosphorylate the glucose and promote the glycogen synthesis, its decrease would make the glucose to another alternative in the existing condition of sulfate, such as incorporate into an extracellular matrix glycoprotein as N-acetyl glucosamine or glucosamine sulfate. The sulfate moiety in the GlcNs supplement may take part in and play a critical role this process. In diabetes, a key factor that lacked is the availability of GlcNs, which is the molecule we are providing in this research. So, the sulfate contained in the GlcNs maybe a critically important component of the health

benefit to diabetes patients. And this can explain the contradictions in glucose level observed between our research and the other research using glucosamine. The PRKCA is also inhibited in kidney in this research. Its activation requires the entry of calcium, angiotensin and adrenergic agonists, which implicated in hypertension, diabetes and heart disease. This suggests the sulfate supplied in GlcNs maybe play a beneficial effect in kidney [37,38].

In conclusion, these results suggest that GlcNs can penetrate skin, which may represent an alternative method for GlcN administration. In addition, oral administration of GlcNs may influence glucose metabolism through a variety of routes. And the sulfate contained in the GlcNs seems to be more critical to the glucose metabolism in diabetes than glucosamine. Although results concerning a rat model do not always correlate with human outcomes, these results are interesting and should be considered when dealing with humans. Additional studies are required to understand the mechanism by which GlcN decreases blood glucose and down-regulates PRKCA and HK1 expression. Results from these additional studies will help clarify how GlcNs influences glucose metabolism specifically. Finally, it is important to repeat these types of experiments using a mouse model of diabetes. This will provide information on whether it is safe for humans with diabetes (or diabetes complications) to use this nutritional supplement and benefit to health.

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