

# Flaxseed Lignan Increased Glucose Uptake by Human Red Blood Cells

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**Abstract:** The objective of this study was to determine if flaxseed lignan (secoisolariciresinol diglucoside, SDG) would improve glucose uptake by human red blood cells (RBCs). To measure glucose uptake of RBCs, the RBCs were pretreated, *in vitro*, with SDG (0, 10, 50, or 100  $\mu$ M) and then were incubated with or without insulin (100  $\mu$ U/ml). After incubation, 3-O-(<sup>3</sup>H-Methyl)-D-glucose uptake by the RBCs was measured. RBCs pretreated with 50 or 100  $\mu$ M SDG showed an increase in glucose uptake compared to control (0  $\mu$ M SDG) or RBCs pretreated with 10  $\mu$ M SDG ( $p < 0.0001$ ). In addition, glucose uptake was increased in RBCs with insulin stimulation compared to RBCs without insulin stimulation ( $p < 0.0001$ ). An interaction between SDG and insulin was also observed ( $p < 0.0001$ ). Pretreatment of RBCs with SDG (50 or 100  $\mu$ M) and insulin increased glucose uptake compared to those without insulin stimulation ( $p < 0.0001$ ). The results indicate potential beneficial effects of SDG on glycemic control by increasing glucose uptake of RBCs.

**Key Words:** Glucose uptake, flaxseed lignan, SDG, antioxidant.

## INTRODUCTION

Maintaining glucose homeostasis is important in chronic disease prevention. Many studies have found that hyperglycemia increases reactive oxygen species (ROS), thus leading to increased oxidative stress and oxidative damage as indicated by lipid peroxidation or oxidative DNA damage in humans and animals [1-4]. Increased oxidative damage to the cells may lead to chronic disease development such as diabetes and cardiovascular diseases [5, 6].

Glucose transport into cells is one of the major mechanisms of glucose homeostasis regulation. Human erythrocytes are readily accessible compared to other tissues, so these cells are used in studying glucose homeostasis [7-9]. Erythrocytes are also a good cell model for studying oxidative stress induced damage [4]. Antioxidant pretreatment of erythrocytes decreased oxidative damage by reducing lipid peroxidation of the membrane [10]. In addition, antioxidant pretreatment protected red blood cells (RBCs) from structural and membrane lipid composition changes which caused by oxidative stress. The protection of RBCs from structural and membrane lipid composition changes maintains the integrity of glucose transporter on RBC membrane, thus maintaining normal glucose uptake by RBCs and contributing to glucose homeostasis [7-9].

Antioxidants such as resveratrol, alpha-lipoic acid, or alpha-tocopherol supplementation or pretreatment of cells with an antioxidant significantly decreased blood glucose concentration and increased glucose uptake by erythrocytes, hepatocytes, adipocytes, and skeletal muscles in animals

[11-15]. Secoisolariciresinol diglucoside (SDG), a known antioxidant, is found in flaxseed [16-19]. Many studies have found the beneficial effects of SDG supplementation on glycemic control in animals due to decreased oxidative stress and increased antioxidant status [6, 16, 18]. SDG inhibited *in vitro* DNA scissions and linoleic acid peroxidation which indicate antioxidant activity of SDG [20]. SDG supplementation was associated with decreased serum or pancreatic malondialdehyde (MDA) and ROS in white blood cells, which are used as indicators of oxidative stress in the body [6, 18]. Comparisons of antioxidant activity of SDG to that of vitamin E showed better antioxidant activities in SDG [19].

In our lab, hypoglycemic effect of flaxseed supplementation was found in impaired glucose tolerant people [21]. Twelve weeks of flaxseed supplementation decreased average fasting serum glucose concentrations by 16% while wheat bran supplementation did not change fasting serum glucose concentrations [21]. The current study was conducted as the first step to determine the mechanism of the hypoglycemic effects of flaxseed. Since the glucose lowering effects in the previous study were attributed to the antioxidant in flaxseed, the current study used purified flaxseed SDG. This study was to determine if flaxseed lignan, SDG, would improve *in vitro* glucose uptake by human red blood cells.

## MATERIALS AND METHODOLOGY

The research protocol was approved by the Institutional Review Board at North Dakota State University.

### Reagents

Purified flaxseed SDG was from Chromadex Co. (Santa Ana, CA); Solvable<sup>TM</sup> was from PerkinElmer (Shelton, CT); 30% hydrogen peroxide was from VWR (Batavia, IL); scintillation cocktail was from Research Product International

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Co. (Mount Prospect, IL); and the remainders of the reagents were from Sigma Co. (St. Louis, MO).

### RBC Isolation

Heparinized human blood was centrifuged to remove the plasma, and then the red blood cell (RBC) pellet was washed with phosphate-buffered saline (PBS). Then the RBC pellet was re-suspended at the original blood volume with PBS.

### SDG Pretreated RBC

SDG was dissolved in 0.1% ethanol and diluted into 10  $\mu$ M, 50  $\mu$ M, or 100  $\mu$ M. One ml of 10  $\mu$ M, 50  $\mu$ M, or 100  $\mu$ M SDG or 0.1% ethanol solution (0  $\mu$ M SDG, control) was added to 2 ml of the RBC suspension, and incubated in a dark and sealed system at 37 °C for 1 hour. After incubation, the RBCs were washed with PBS and then re-suspended in PBS at the original blood volume.

### RBC Incubation with Insulin

Porcine insulin was dissolved in 1 M HCl and then diluted into 100  $\mu$ U/ml with PBS. One ml of 100  $\mu$ U/ml insulin or 1M HCl/PBS (control) was added to 1 ml of SDG pretreated RBC suspension, and incubated at 37 °C for 30 minutes. After incubation, the RBCs were washed with PBS, and re-suspended in PBS at the original blood volume. For the glucose uptake assay, 100  $\mu$ l of RBC suspension was transferred into individual tubes.

### Preparation of Radiolabeled Solution

Five ml of 0.5  $\mu$ Ci/ml 3-O-(<sup>3</sup>H-Methyl)-D-glucose was mixed with 5 ml of 0.5 mmol/l 3-O-Methyl-D-glucopyranose (3OMG).

### Stop Solution

Twenty  $\mu$ mol/l mercuric chloride and 10  $\mu$ mol/l phloretin were added into 100 ml of ice-cold PBS immediately before use.

### RBC Glucose Uptake

RBC glucose uptake was measured using a modified method developed by Klepper *et al.* [22]. Glucose uptake

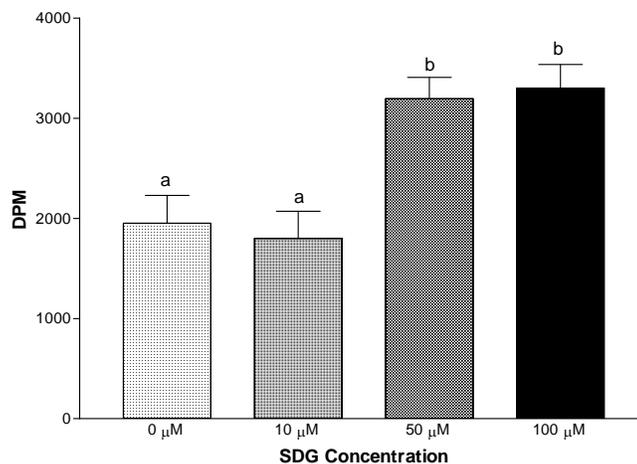
was initiated by adding 200  $\mu$ l of <sup>3</sup>H-labeled 3OMG solution into each 100  $\mu$ l of RBC suspension which prepared at previous steps. Glucose uptake was stopped by adding 1 ml of ice-cold stop solution at 30 sec, 1 min, and 25 min. For the control, 1 ml of stop solution was added to 100  $\mu$ l of RBC suspension, and then 200  $\mu$ l of <sup>3</sup>H-labeled 3OMG solution was added. The RBC suspension was centrifuged at 2,000 g for 5 min and then washed twice with 1 ml stop solution. To digest RBC pellet, 250  $\mu$ l of Solvable™ was added, incubated at 60 °C for 1 hr, and then cooled at room temperature. The RBC pellet was further digested by adding 250  $\mu$ l of 30% hydrogen peroxide and incubated at 60 °C for 30 minutes. Finally, 4 ml of scintillation cocktail was added to each vial and then counted with a scintillation counter (Beckman Coulter, Inc., Fullerton, CA) for 5 min count per sample.

### Data Analysis

RBC glucose uptake data were analyzed with the SAS System (SAS 9.1; SAS Inst., Cary, NC) using an ANOVA model. The original analysis used an effects model specification which included all main effects and two- and three-factor interactions for the primary factors of interest. A Type I error rate (alpha level) of 0.05 was used for all tests. Follow-up comparisons of means were performed using a cell means model approach which allows comparing means from any treatment combinations rather than just main effects means. The Tukey-Kramer method was used to control the experimentwise error rate for these follow-up tests. Data were reported as mean  $\pm$  standard error.

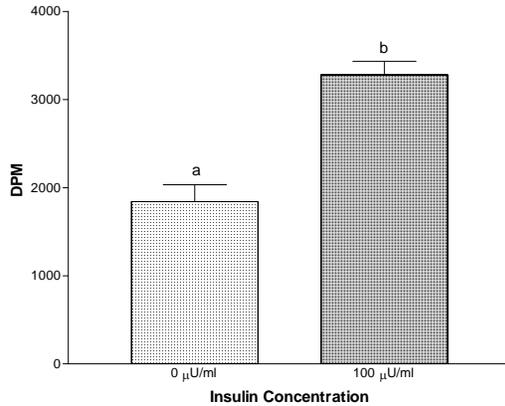
### RESULTS

Hemocytometer counting of the RBC suspension showed an average number of  $7.88 \times 10^6$  RBC/ $\mu$ l, and also showed a >95% of cell viability. There was no time effect on glucose uptake of RBCs. However, the pretreatment of RBCs with either 50  $\mu$ M or 100  $\mu$ M SDG increased glucose uptake compared to RBCs without or with 10  $\mu$ M SDG pretreatment ( $p < 0.0001$ , Fig. 1). No difference was observed in glucose uptake between the 50  $\mu$ M and 100  $\mu$ M SDG groups.



**Fig. (1). Effects of SDG on RBC glucose uptake.** The data shown are representative of three independent experiments, with the mean and standard error of the mean shown for each treatment group. Since there were no time effects on RBC glucose uptake, the mean of all different times for each treatment group was used for data presentation. Values with different lower case letters are significantly different ( $p < 0.0001$ ).

RBCs incubated with 100  $\mu\text{U/ml}$  insulin increased glucose uptake compared to RBCs without insulin ( $p < 0.0001$ , Fig. 2). Increased glucose uptake followed by SDG (50  $\mu\text{M}$  or 100  $\mu\text{M}$ ) treatment was also observed in groups without insulin stimulation ( $p < 0.0001$ , Fig. 3). An interaction between SDG and insulin was observed. Pretreatment of RBCs with SDG (50  $\mu\text{M}$  or 100  $\mu\text{M}$ ) and insulin increased glucose uptake compared to those without insulin stimulation ( $p < 0.0001$ , Fig. 3). When comparing an increase of glucose uptake followed by 50  $\mu\text{M}$  SDG or 100



**Fig. (2). Effects of insulin on RBC glucose uptake.** The data shown are representative of three independent experiments, with the mean and standard error of the mean shown for each treatment group. Since there were no time effects on RBC glucose uptake, the mean of all different times for each treatment group was used for data presentation. Values with different lower case letters are significantly different ( $p < 0.0001$ ).

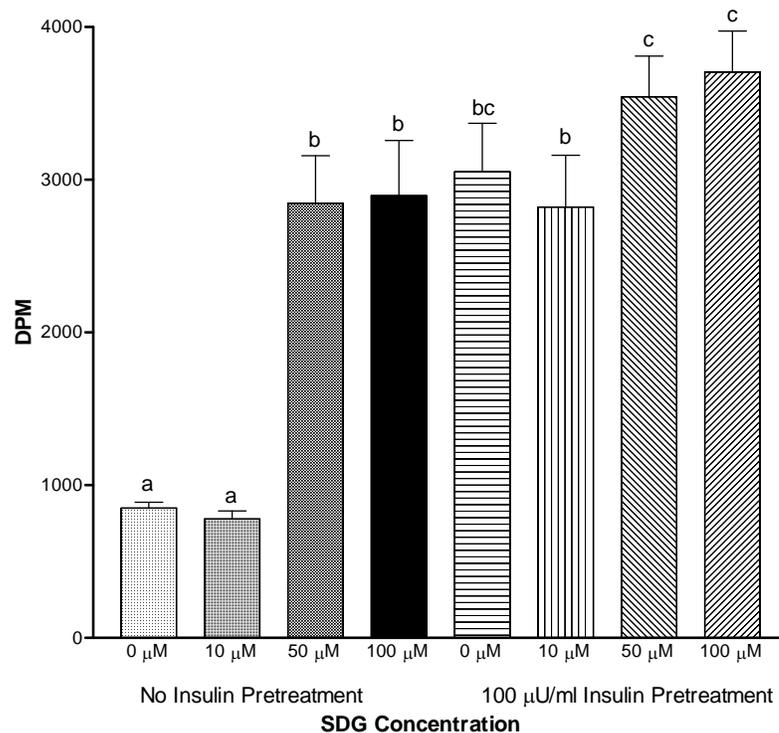
$\mu\text{M}$  SDG without insulin pretreatment to that of cells with 100  $\mu\text{U/ml}$  insulin and SDG (0  $\mu\text{M}$  or 10  $\mu\text{M}$ ) pretreatment, a similar increase of glucose uptake was observed ( $p < 0.0001$ , Fig. 3). However, no interaction between time and SDG or time and insulin was observed.

## DISCUSSION AND CONCLUSION

Generally, the contribution of glucose uptake by erythrocyte to glucose homeostasis is rather small and may not be a major mechanism of the blood glucose lowering effect. However, due to easy accessibility, erythrocytes were used in this study to measure basal glucose uptake as the first step in the identification of the mechanism of glucose lowering effects of flaxseed. Since this study was to measure glucose uptake by RBCs, the antioxidant activity of SDG was not measured in the current study. However, twelve weeks of flaxseed supplementation decreased plasma MDA concentration in glucose intolerant people in our previous study (Unpublished result). This result indicates that SDG in flaxseed functioned as an antioxidant.

Significant improvement of RBC glucose uptake by SDG treatment is in agreement of findings of other studies of antioxidants [11, 12]. These studies suggested that the protective function of an antioxidant against oxidative stress improved *in vitro* glucose uptake and led to a decreased insulin resistance in animals [11, 13, 23, 24]. Although SDG effects on oxidative stress in RBCs were not determined in the current study, SDG may have acted similarly as other antioxidants used in other studies [23, 24].

High blood glucose levels generate ROS in individuals who have diabetes or impaired glucose tolerance [25-27]. It



**Fig. (3). Effects of SDG and insulin on RBC glucose uptake.** The data shown are representative of three independent experiments, with the mean and standard error of the mean shown for each treatment group. Since there were no time effects on RBC glucose uptake, the mean of all different times for each treatment group was used for data presentation. Values with different lower case letters are significantly different ( $p < 0.0001$ ).

is theorized that glucose in the red blood cell cultures had similar effects on ROS generation. As a result, this generated ROS may have caused a lower glucose uptake by RBCs without SDG treatment. Moreover, increased ROS may have increased membrane lipid peroxidation which disturbed membrane integrity and decreased glucose transporter activation on red blood cells.

Improved glucose uptake by RBCs with SDG pretreatment may be explained by decreased oxidative stress, since SDG functions as an antioxidant. As seen in other studies of antioxidants [9], SDG is an antioxidant which may have decreased lipid peroxidation and oxidative damage on RBCs, thus maintaining the integrity of the cell membranes and cellular structures including glucose transporters. Therefore, decreased lipid peroxidation may have led to increased glucose uptake by RBCs.

Insulin regulates glycemic control by stimulating cellular glucose uptake. RBCs with SDG treatment increased glucose uptake to a similar level as that of insulin stimulated cells. This result indicates that SDG increased basal glucose uptake by RBCs and also SDG has the same or similar hypoglycemic effect as insulin.

Insulin pretreatment of RBCs should not have affected glucose uptake directly, since RBCs transport glucose *via* non-insulin mediated glucose transporter. However, the interactions between insulin and SDG suggest that SDG potentiates insulin action leading to increased glucose uptake by RBCs.

The significant increase in glucose uptake by RBCs with SDG treatment may explain the blood glucose lowering effects of flaxseed in humans as seen in our previous clinical study [21]. Although there is a report on availability of SDG metabolites, enterodiol and enterolactone, in blood after SDG consumption [28], no studies measured the blood concentration of SDG after normal flaxseed lignan consumption. Therefore, it would be difficult to extrapolate the amount of SDG in the RBC suspension into an amount of SDG needed for a whole human body to exert similar improvement of glucose uptake. However, individuals with an insulin/oral medication regimen for diabetes control would benefit by consuming flaxseed in addition to insulin/oral medication. SDG in flaxseed would reduce the required amount of insulin or oral medication.

These results demonstrate the beneficial effects of SDG on *in vitro* glucose uptake by human RBCs. However, further investigation is needed to determine the effects of SDG on the mechanism of cellular glucose uptake in humans. For example, the *in vitro* cellular glucose uptake following SDG metabolite, enterodiol and/or enterolactone stimulation and *in vivo* cellular glucose uptake following SDG supplementation need to be measured to identify the mechanism of hypoglycemic effects of flaxseed in humans.

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