

The Mechanism of Hematoxylin on Glucose Metabolism Improvement in Sciatic Nerves from Streptozotocin-induced Diabetic Rats.

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Streptozotocin으로 유도한 당뇨쥐의 대퇴신경에서 Hematoxylin의 당대사 개선 메커니즘

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Abstract Hematoxylin is the main component of *Hematoxylon campechianum* which has been utilized in the southern provinces of Korea as a folk remedy for diabetic complications. In the present study, to investigate the hypoglycemic mechanism of hematoxylin, the 2-deoxyglucose uptake and phospholipid metabolism were examined in sciatic nerves from three groups of rats : normal control, diabetic control, diabetic hematoxylin-treated group. Hematoxylin significantly reduced blood glucose levels in diabetic control rats. On a wet weight basis, the nerves from diabetic rats showed a 20% decrease in total phospholipid from that of controls and a relative decrease in phosphatidylinositide. Hematoxylin treatment increased the incorporation rate of 2-[3H] myo-inositol into total phosphoinositids in diabetic rat. The effectiveness were more potent in higher dose hematoxylin-treated rats than lower dose hematoxylin-treated rats. These results suggest that hematoxylin increases glucose transport and lipid metabolism by partially normalizing concerned with myo-inositol metabolism in diabetic rat. Therefore we propose that hematoxylin can be a promising candidate for diabetes medication.

요약 Hematoxylin은 한국의 남부지방에서 당뇨합병증을 치료하기 위하여 사용해오던 민간약재인 *Hematoxylon campechianum*의 주성분이다. 본 논문에서는 hematoxylin의 혈당저하 메커니즘을 연구하기 위하여 3개 군의 흰쥐-정상군, 당뇨군, hematoxylin 처치군-에서 분리한 대퇴신경을 대상으로 2-deoxyglucose 수송능과 인지질 대사능을 조사하였다. 실험 결과 hematoxylin은 당뇨군에서 혈당치를 현저하게 감소시키는 것으로 나타났다. 또한 무게단위의 기준으로 당뇨군의 신경조직의 경우, 총 인지질의 양은 20% 감소하였으나 상대적으로 phosphatidylinositide의 감소는 작은 것으로 나타났다. 이 경우, hematoxylin을 처치하면 2-[3H] myo-inositol이 총 phosphoinositids로 대사되는 비율이 증가하는 것으로 밝혀졌다. 이러한 효과는 낮은 농도의 hematoxylin 처치군보다 높은 농도의 처치군에서 훨씬 큰 것으로 나타났다. 이상의 실험 결과는 hematoxylin의 당뇨개선 메커니즘이 myo-inositol 대사를 증가시킴으로써 체내 당 수송과 인지질 대사를 정상화시킨다는 점을 제시하고 있다. 따라서 본 연구결과를 토대로 저자들은 hematoxylin은 향후 당뇨 치료제로서 후보 물질이 될 수 있음을 제시하고자 한다.

Key Words : Hematoxylin, Diabetes Mellitus, 2-Deoxyglucose Uptake, Sciatic Nerve, Phosphoinositide, Phospholipid Metabolism.

1. Introduction

Hematoxylin, which is a benzo(b)indeno(2,1-d) pyran

derivative, has been used mainly as a staining material in cytologic research, food coloring agent and an antioxidant [1]. Many physiologic functions of

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hematoxylin have been reported by several scientists [2-5]. It has also been reported that the decoction of *Hematoxylon campechianum* has been used for the treatment of diabetic complications in the folk medicine in the southern provinces of Korea. There have been some positive aspects of the application of the hematoxylin, such as in the improvement of diabetic retinopathy [6-7]. We have *in vitro* evidence that hematoxylin normalizes the glucose utilization in soleus muscle which is one of the active tissues in glucose metabolism. Based on these studies, we set out to elucidate the antidiabetic mechanism of hematoxylin using adipocytes as target cell for their high metabolic activity and hormonal response [8]. For the purpose of the study, we investigated the effects of hematoxylin on glucose transport and phospholipid metabolism in the sciatic nerves from streptozotocin-induced diabetic rats.

2. Materias and methods

2.1 Induction of Mild Diabetes Mellitus

Male Sprague-Dawely rats (160-180g) fasted overnight were injected with $40\text{mg}\cdot\text{kg}^{-1}$ streptozotocin (STZ) through a tail vein [9]. STZ was dissolved in citrate buffer (pH 4.0), kept in ice bath, and administered within 10 minutes. These are important precautions in order to prevent the rapid inactivation of STZ at neutral pH and room temperature. After 7 days' stabilization of plasma glucose levels, blood samples were collected from retro plexus orbital in the nonfasting state between 09:00 and 10:00a.m. Plasma glucose was determined by the glucose-oxidase method, plasma insulin was determined by the double antibody radioimmunoassay. Animals with a similar degree of diabetic (blood glucose concentration $335\text{mg}\%$ to $385\text{mg}\%$) were selected for this research. All rats had free access to fresh water and solid laboratory chow (Composition : crude protein 25%, crude fat 3.5%, Ca 20%, P 0.4%, crude cellulose 5.0%, and crude ash 8.0%) throughout the experiment.

2.2 Grouping and Treatment of Animals

Rats were divided into three groups: normal control, diabetic control and diabetic hematoxylin-treated group.

Diabetic hematoxylin-treated rats were administered per oral for two weeks with two different doses ($100\text{mg}\cdot\text{kg}^{-1}$, $10\text{mg}\cdot\text{kg}^{-1}$) of hematoxylin and control rats were administered with the same volume of water. Because of poor solubility of hematoxylin in water and its relative instability in the light, hematoxylin-suspension prepared by about 3 hours' sonication were kept in a brown vacuum bottle. In this way, the preparation of homogeneous suspension of hematoxylin and the prevention of their rapid oxidation to other forms were available.

2.3 Nerve incubations

Nerve incubations were prepared according to the procedure of Bell et al [10]. Immediately after dislocation, about 25mm segment of sciatic nerve was removed from each leg of the normal and diabetic rats by dissection from the sciatic notch to the popliteal fossa. Each nerve was performed in 0.5 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, which contained 5.5mM glucose and $0.5\mu\text{Ci}$ of 2-[3H] myo-inositol. Each tube was flushed with 95% 02:5% CO₂, capped, and incubated for up to 2 h at 37°C. At the end of the incubation period, nerve segments were removed from the medium and immediately washed three times with cold distilled water.

2.4 Measurement of Glucose Transport

Transport studies were performed using the oil flotation as technique described by Olefsky et al [11]. Isolated sciatic nerves (5×10^8 cells.L⁻¹) were preincubated in Krebs-Ringer -Hepes buffer, pH 7.4, containing bovine serum albumin ($10\text{g}\cdot\text{L}^{-1}$) and 2mM pyruvate in the absence or presence of insulin ($25\mu\text{g}\cdot\text{L}^{-1}$). Incubations were carried out in a shaking water bath at 37°C for 30min. At the end of preincubation, glucose transport assay was performed by adding 2-deoxy-[1,2-³H]-D-glucose. This assay measures the total uptake of the radiolabeled 2-deoxyglucose and is based on the principle that while 2-deoxyglucose is transported and phosphorylated by the same process as D-glucose cannot be further metabolized^[12]. The assay was terminated at the end of 3mins by transferring 300μl aliquots from the assay mixture to a plastic microtube containing 100μl dinonylphthalate oil. The tubes were centrifuged for 30

seconds at 10,000rpm in a microcentrifuge and the assay was considered to be terminate when centrifugation began. Dinonylphthalate has a specific gravity intermediate between buffer and nerves, and therefore, after centrifugation, three layers occurred ; nerves on top, oil in middle, and buffer on the bottom. The tubes were cut through the oil layer with a razor blade and the radioactivity in the cell pellet was measured in a liquid scintillation counter. In the case of *in vitro* treatment, various concentrations of hematoxylin ($10^{-6}\text{M}\sim 10^{-3}\text{M}$) was added with or without insulin($25\mu\text{g}\cdot\text{L}^{-1}$) at the beginning of 1 hour preincubation and then glucose uptake was measured as described above.

2.5 Measurement and separation of phospholipids.

Phospholipids were separated by one-dimensional high performance thin layer chromatography on 10 cm x 10 cm plates coated with silica gel 60 (E. Merck, Scientific Products, Houston, TX) in chloroform-methanol-20% methylamine (60:36:10, vol/vol). The solvent was poured into small chromatography tanks lined with filter paper and allowed to equilibrate for at least 1h before use. Duplicate aliquots(30-60 μl) of each lipid extract were spotted approximately 1.5 cm from the bottom edge of the plates, which were placed in the tanks on small glass stands which prevented the bottom edges of the plates from touching the solvent. After 15-20min, the plates were lowered into the running solvent and developed for approximately 45min until the solvent was within 0.5cm of the top of the plates. The positions of the radioactive compounds were located by standard graphy method, and the appropriate areas of silica gel were scraped and placed in glass scintillation vials containing 10 ml of toluene scintillation fluid. The sampels were counted in a Beckman liquid scintillation counter.

2.6 Statistical Analysis

To determine if the means of data were significantly different from each other or controls, the data were subjected to analysis followed by Duncan's Multiple Range test. In all case, $p < 0.05$ was used to determine significance.

3. Results

3.1 General Characteristics of Experimental Animals

Some characteristics of the experimental rats used in this study are summarized in Table 1.

[Table 1] General characteristics of experimental animals.

	Check Time	Group			
		NC(5)	DC(8)	DT1(8)	DT2(9)
Body Weight (g)	Initial	182 \pm 14	170 \pm 10	168 \pm 12	173 \pm 12
	Final	275 \pm 20	220 \pm 15	245 \pm 13	250 \pm 15
Plasma Insulin ($\mu\text{U/L}$)	Initial	30 \pm 2	19 \pm 2	21 \pm 2	19 \pm 2
	Final	33 \pm 3	20 \pm 3	21 \pm 2	21 \pm 2
Plasma Glucose (mg%)	Initial	110 \pm 20	370 \pm 15 [#]	345 \pm 18	350 \pm 15
	Final	120 \pm 15	380 \pm 20 [#]	195 \pm 20 [@]	180 \pm 25 [@]

Each value represents mean \pm SE. The number of experimental animals are given in parenthesis. Hematoxylin was administered per oral for two weeks. Control group was administered the same volume of water. Initial and Final represent the values obtained before and after treatment. NC; normal control group, DC; diabetic control group, DT1; hematoxylin-treated diabetic group ($10\text{mg}\cdot\text{kg}^{-1}$), DT2; hematoxylin-treated diabetic group ($100\text{mg}\cdot\text{kg}^{-1}$). # $p < 0.05$, NC vs DC. @ $p < 0.05$, DC vs DT group.

The STZ-induced diabetic rats consumed more food and water, but gained weight less rapidly than normal control rats. The plasma glucose levels were markedly increased but the plasma insulin levels were decreased in diabetic rats. From these results, the model of STZ-induced diabetic rat might be explained by reduction in plasma insulin level. The plasma glucose levels in hematoxylin-treated rats were significantly lower than those in diabetic control rats, while the difference of plasma insulin levels between the treated group and non-treated group was not found. Also, the body weights of diabetic hematoxylin-treated rats were somewhat higher than those of diabetic control rats, but the difference between the two groups was not statistically significant.

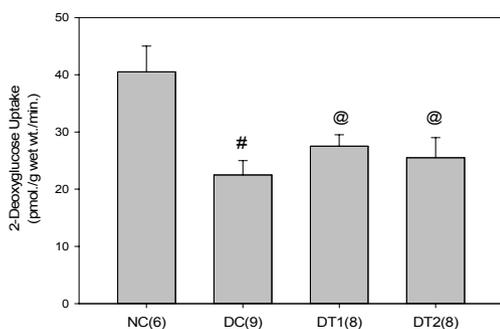
These results suggest that hematoxylin may affect plasma glucose levels without changing insulin secretion/level in diabetic status and it means hematoxylin could act in a different mode from the action of insulin in improving diabetic status. Thus to elucidate the

hypoglycemic mechanism of hematoxylin, we have assessed the rate of glucose uptake which is not only the first step but the rate limiting step in lipid metabolism using sciatic nerve.

3.2 *in vivo* Effects of Hematoxylin on 2-Deoxyglucose Uptake

First of all, to check the effectiveness of hematoxylin on glucose transport system in diabetic status, we examined the *in vivo* effects of hematoxylin using epididymal sciatic nerve from diabetic rats. Fig.1 and Fig.2 showed the effects of hematoxylin on 2-deoxyglucose uptake of sciatic nerve at the basal and insulin-stimulated status.

This means the effectiveness of hematoxylin on diabetic status might have relation to the action of insulin. But it is not clear whether these results are due to direct effects on glucose transport system *per se* or simply due to secondary effects of amelioration of hyperglycemia. To clarify this problem, we examined the *in vitro* effects of hematoxylin on sciatic nerve cultures.



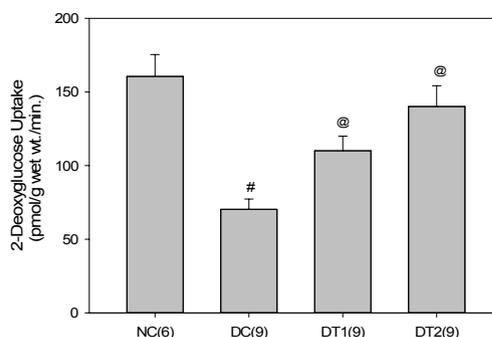
[Fig.1] *in vivo* Effects of hematoxylin on the basal 2-deoxyglucose uptake in sciatic nerve from streptozotocin -induced diabetic rat.

NC; normal control group, DC; diabetic control group, DT1; diabetic hematoxylin-treated group (10mg.kg⁻¹), DT2; diabetic hematoxylin-treated group (100mg.kg⁻¹). # p<0.05, NC vs DC. @ p<0.05, DC vs DT group.

Diabetic status led to a decrease in both basal and insulin-stimulated rates of 2-deoxyglucose uptake, while hematoxylin increased only the insulin-stimulated rate of 2-deoxyglucose without affecting basal status.

3.3 *in vitro* Effects of Hematoxylin on 2-Deoxyglucose Uptake

Isolated sciatic nerve from diabetic rats were incubated with various concentrations of hematoxylin (10⁻⁶M~10⁻³M) in the basal and insulin stimulated status. After 20 hours of incubation, 2-deoxyglucose uptake was measured and the results are shown in Fig. 3 and Fig.4. As the same results reported in *in vivo* tests, only the rate of insulin-stimulated 2-deoxyglucose uptake was significantly increased by the treatment of various concentrations(from 10⁻⁶M to 10⁻⁴M) of hematoxylin and the increase pattern in 2-deoxyglucose uptake was dose dependent manner (when the values of treated groups were compare to that of diabetic control group : values were 171%, 157% and 142% respectively).



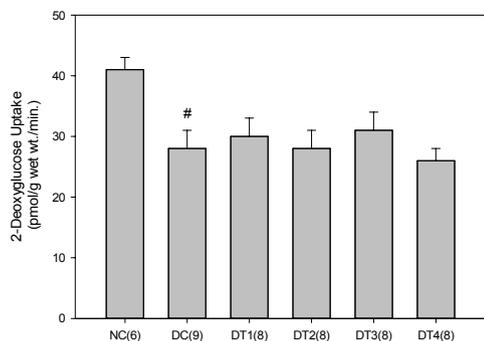
[Fig.2] *in vivo* Effects of hematoxylin on the insulin-stimulated 2-deoxyglucose uptake in sciatic nerve from streptozotocin-induced diabetic rat.

NC; normal control group, DC; diabetic control group, DT1; diabetic hematoxylin-treated group (10mg.kg⁻¹), DT2; diabetic hematoxylin-treated group (100mg.kg⁻¹). # p<0.05, NC vs DC. @ p<0.05, DC vs DT group.

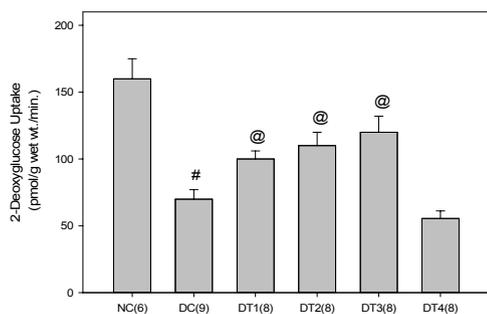
But, the concentration of 10⁻³M group showed decrease in 2-deoxyglucose uptake. This might be toxic effect of hematoxylin on sciatic nerve.

These results indicated that hematoxylin might indirectly affect the glucose transport system or its related factors without any influence on the basal rate of 2-deoxyglucose uptake. Based on this observation, we went on to investigate the effect of hematoxylin on phospholipid metabolism (next step in glucose metabolism) in sciatic nerves from diabetic rats. Nerves from diabetic rats showed a significant decrease in the labeling of

phosphatidylinositol and phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-biphosphate.



[Fig.3] *in vitro* Effects of hematoxylin on the basal 2-deoxyglucose uptake in sciatic nerve from streptozotocin-induced diabetic rat. NC; normal control group, DC; diabetic control group, DT1; hematoxylin-treated group(10^{-6} M), DT2; hematoxylin-treated group(10^{-5} M), DT3; hematoxylin-treated group(10^{-4} M), DT4; hematoxylin-treated group(10^{-3} M). # $p < 0.05$, NC vs DC.



[Fig.4] *in vitro* Effects of hematoxylin on the insulin-stimulated 2-deoxy -glucose uptake in sciatic nerves from streptozotocin-induced diabetic rat. DT1; hematoxylin-treated group(10^{-6} M), DT2; hematoxylin-treated group(10^{-5} M), DT3; hematoxylin-treated group(10^{-4} M), DT4; hematoxylin-treated group(10^{-3} M). . # $p < 0.05$, NC vs DC. @ $p < 0.05$, DC vs DT group.

3.4 Effects of Hematoxylin on Phosphatidyl -inositol metabolism.

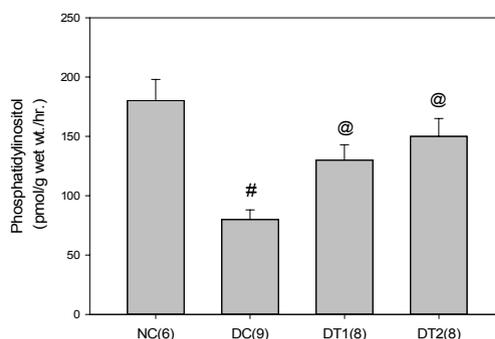
Fig. 5 gives the results obtained when sciatic nerves

from normal and diabetic rats treated with two different concentrations of hematoxylin(10mg/kg b.w and 100mg/kg b.w) were incubated in the presence of 0.5uCi 2-[3 H]myo-inositol. hematoxylin treatment significantly increased incorporation of myo-inositol into phosphatidylinositol(PI) in both groups. There were no changes myo-inositol incorporation into phosphatidyl-4-phosphate(PIP) and phosphatidylinositol-4,5-diphosphate(PIP2) in normal treated rats(Fig.6-7).

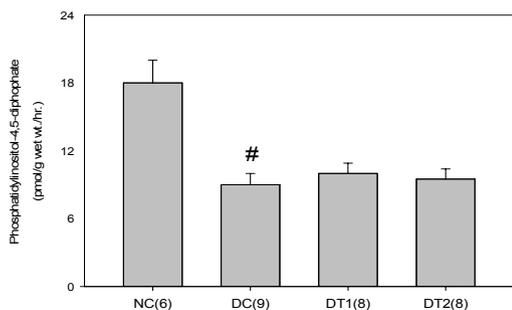
However, myo-inositol incorporation into phosphatidy -linositol-4,5-diphosphate was significantly increased but phosphatidylphosphte decreased by the two different doses of hematoxylin administration in diabetic rat.

But there was no significant change of incorporation rate between high and low concentrations of hematoxylin. Data presented in Fig.6 show that only incorporation into phophatidyl-4-phosphate was significantly decreased in nerves from diabetic rat.

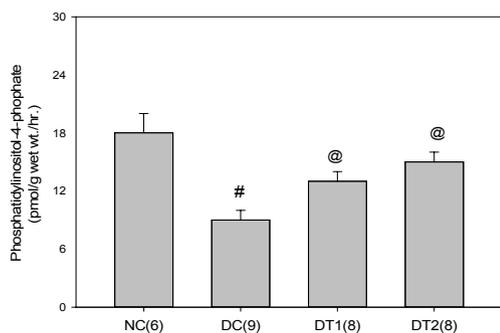
These results suggest that the condiderable alteration in incorporation of these lipid precursors in diabetic nerves may be related to the impaired inositol uptake and these deficits were ameliorated by hematoxylin treatment and this effectiveness was more potent in diabetic state than in normal state.



[Fig.5] Effects of hematoxylin on incorporation of 2-[3 H]myo-inositol into phosphatidylinositol in rat sciatic nerves. NC; normal control group, DT1; hematoxylin-treated group (10ppm), DT2; hematoxylin-treated group (100ppm). Values are mean \pm SE. The number of experimental rats are given in parenthesis. # $p < 0.05$, NC vs DC. @ $p < 0.05$, DC vs DT group.



[Fig.6] Effects of hematoxylin on incorporation of 2-[³H]myo-inositol into phosphatidylinositol-4-phosphate in rat sciatic nerves. NC; normal control group, DT1; hematoxylin-treated group (10ppm), DT2; hematoxylin-treated group (100ppm). Values are mean±SE. The number of experimental rats are given in parenthesis. # p<0.05, NC vs DC.



[Fig.7] Effects of hematoxylin on incorporation of 2-[³H]myo-inositol into phosphatidylinositol-1,4-diphosphate in rat sciatic nerves. NC; normal control group, DT1; hematoxylin-treated group (10ppm), DT2; hematoxylin-treated group (100ppm). Values are mean±SE. The number of experimental rats are given in parenthesis. # p<0.05, NC vs DC. @ p<0.05, DC vs DT group.

4. Discussion

This investigation has studied the effects of treatment of diabetic rats with hematoxylin on two common defects expressed with impaired glucose transport system and increased phospholipid metabolism in sciatic nerves

[12-14]. Also in this study, sciatic nerves tissue was used as target organ for determining glucose transport, since it is active hormonally and metabolically in glucose metabolism.

In the study, the treatment of hematoxylin had a significant effect on the glucose level not insulin level in the diabetic rats. This phenomenon could be explained that hypoglycemic mechanism of hematoxylin was not at least due to normalization of insulin secretion in diabetic rat. There have been several reports concerning agents which improved diabetic status not affecting insulin level [15-16]. We considered that hematoxylin might have a similar action mode like these reagents. From the observations in *in vivo* and *in vitro* studies, it was more cleared that hematoxylin normalized in part glucose level by increasing glucose transport and this increased uptake by hematoxylin was resulted from change of insulin sensitivity in diabetic status. The normalization degree by hematoxylin was higher in the presence of insulin than that of in the absence of insulin and its pattern was dose dependent manner (Fig. 2 and Fig. 4). But it is noticeable that both basal and insulin-stimulated rate of the 2-deoxyglucose uptake was reduced at the highest concentration of hematoxylin (10⁻³M). This could be explained as toxic effects on sciatic nerves due to the unusually high concentration of hematoxylin.

But in the sciatic nerves from hematoxylin-treated rats, even though glucose level taken into account at diabetic status, the decrease rate of glucose uptake was much lower than that of untreated-rats. In conclusion, we have found that hematoxylin decreased blood glucose level by increase utilization of glucose metabolism in diabetic status regardless of insulin level and its mechanism could be explained by an increase V_{max} of 2-deoxyglucose in sciatic nerves, indicating that it could be used as an antidiabetic therapeutic agent. Despite this finding, to get reach the accurate hypoglycemic mechanism of hematoxylin, many problems including insulin signaling system, glucose metabolism pathway such as glucose oxidation and lipogenesis should be further investigated. Especially peripheral nerves sodium-potassium ATPase is fundamental to both energy and substrate, metabolism, myo-inositol related sodium-potassium ATPase perturbations might have widespread consequences in diabetic nerves. Therefore we need to investigate several

hypoglycemic mechanisms of hematoxylin.

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