Ameliorative Potential of Spironolactone in Diabetes Induced Hyperalgesia in Mice

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The present study was designed to investigate the ameliorative potential of spironolactone against diabetic hyperalgesia in mice. Tail flick latency, an index of hyperalgesia, was assessed by analgesiometer. Serum nitrite levels, an index of nitric oxide, were analyzed by Griess reaction. Mice were rendered diabetic with streptozotocin (200 mg kg\(^{-1}\) i.p) and kept for 30 days for development of diabetic pain. Thereafter, spleen homogenate supernatant (SHS) was prepared from the mouse spleen and administered in normal mice for 14 days. In both diabetic and SHS-treated mice a significant degree of hyperalgesia was developed, suggesting the key role of spleen-derived factor in induction of diabetic pain. Moreover, the levels of nitric oxide were also elevated in 30th day diabetic mice and SHS-treated mice. Administration of spironolactone (7 and 15 mg kg\(^{-1}\) p.o.) significantly attenuated diabetes-induced decrease of nociceptive threshold and increase of serum nitrite oxide levels. Furthermore, SHS of spironolactone-treated diabetic mice failed to induce hyperalgesia and to increase serum nitrite levels. These results suggest that spironolactone has ameliorative potential in attenuating the hyperalgesia associated with diabetes, which may be possibly mediated through inhibition of release of certain critical factors from spleen.

Key words—diabetes; hyperalgesia; spironolactone; spleen

INTRODUCTION

Diabetic patients frequently exhibit increased responsiveness to noxious stimuli (hyperalgesia) and hyper-responsiveness to normally innocuous stimuli (allodynia) that are often concurrent with a paradoxical loss of stimulus-evoked sensation.1,2 Similar to human diabetic pain, animal models such as streptozotocin (STZ)-induced diabetic mice also demonstrate thermal hyperalgesia and mechanical allodynia.3,4

Spironolactone is an aldosterone receptor antagonist clinically employed as potassium-sparing diuretic. It has also been used clinically to reduce the mortality incidence in congestive heart failure and myocardial infarction.5 Besides, it has also been documented to improve endothelial dysfunction6 and reduce urinary albumin excretion and systolic blood pressure in patients with type 2 diabetes mellitus complicated by diabetic nephropathy.7 Furthermore, spironolactone has also documented to ameliorate the glomerular injury in STZ-induced early diabetic renal injury.8

From our laboratory, it has been reported that splenectomy restores the decrease in antinociceptive effect of morphine in diabetic mice, suggesting the interference of spleen factors with pain alteration during diabetes.9 Very recently it has been reported that spleen-derived factors play a key role in the pathogenesis of diabetic hyperalgesia.10 Therefore the present study was designed to investigate the ameliorative potential of spironolactone against diabetic hyperalgesia and to investigate its modulatory effect on spleen homogenate of diabetic mice.

MATERIALS AND METHODS

Swiss albino mice (20–30 g) of either sex were employed for the present study. STZ (Sigma Aldrich, U.S.A.) was dissolved in 0.1 N citrate buffer (pH 4.5). The suspension of spironolactone (R.P.G. Life Sciences, Ahmedabad) was made in 0.5% CMC immediately before use. The experimental protocol was approved by Institutional Animal Ethics Committee and care of the animals was carried out as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No.-107/1999/CPCSEA).

Induction of Diabetes and Neuropathy in Mice

Single doses of STZ (200 mg kg\(^{-1}\) i.p. 1 ml per 100
g) were administered for induction of diabetes. Blood samples were obtained from retro-orbital sinus for glucose estimation. Mice with fasting blood glucose levels >14 mmol l⁻¹ day 16 after STZ administration were considered diabetic and were included for further study. The diabetic mice were kept for further 14 days for induction of diabetic hyperalgesia.

**Estimation of Blood Glucose** Blood Glucose levels were estimated spectrophotometrically by glucose-oxidase method¹¹ using a commercially available enzymatic kit.

**Measurement of Nociceptive Threshold** Nociceptive threshold was determined by noting withdrawal latency in tail flick test by tail flick analgesiometer.¹² The intensity of the radiant heat was adjusted to obtain a basal or pre-treatment latency of 6–8 s in normal mice. Maximum cutoff latency time was fixed at 10 s. Tail flick latency was expressed in seconds.

**Estimation of Serum Nitrite as Index of Nitric Oxide Production** Nitric oxide (NO) is rapidly oxidized to nitrite/nitrate and its half-life in biological systems is very short.¹³ Therefore the measurement of nitrite concentration is routinely used as an index of NO production.¹⁴¹⁶ Serum nitrite levels were estimated by Griess reaction;¹⁷ values are expressed in µmol l⁻¹.

**Preparation of Spleen Homogenate Supernatant**

After sacrificing the mice by cervical dislocation, the spleen was removed and immersed in 1% Minimal Essential Media (MEM, pH = 7.8). The spleen was mashed, homogenized, and centrifuged at 3000 rpm for 10 min. The supernatant of spleen homogenate (SHS) was used for the study, in place of mononuclear spleen cells, to avoid any implication of immunogenic response.

**EXPERIMENTAL DESIGN**

Nine groups were included in the present study, each group comprised six animals.

1. **Group I (Non-diabetic control group)** Mice were administered citrate buffer to serve as non-diabetic control animals. Tail flick latency was noted before and after administration of citrate buffer on days 1, 4, 10, 14, 20, 24 and 30. Serum nitrite levels were noted before and after day 30 of buffer administration. Fasting glucose levels were monitored before and after administration of citrate buffer on days 16 and 30.

2. **Group II (Streptozotocin-induced diabetic group)** Mice were administered a single dose of STZ (200 mg kg⁻¹ i.p.) to serve as diabetic control animals. Tail flick latency, serum nitrite levels, and fasting glucose levels were noted on the days as described in group I.

3. **Group III (Spironolactone-treated diabetic group; 7 mg kg⁻¹)** Mice were administered spironolactone (7 mg kg⁻¹ i.p.), daily for 14 days, starting on day 16 after STZ administration. Tail flick latency, serum nitrite levels, and fasting glucose levels were noted on the days as described in group I.

4. **Group IV (Spironolactone treated diabetic group; 15 mg kg⁻¹)** Mice were administered spironolactone (15 mg kg⁻¹ i.p.), daily for 14 days, starting on 16th day after STZ administration. Tail flick latency, serum nitrite levels, and fasting glucose

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**Table 1. Effect of Streptozotocin, Spironolactone, Spleen Homogenate of Diabetic and Non-diabetic Mice on Blood Glucose Levels (mg/dl)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>0th day</th>
<th>8th day</th>
<th>16th day</th>
<th>30th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>87.8±10.3</td>
<td>82.4±18.4</td>
<td>92.4±16.9</td>
<td>94.8±9.4</td>
</tr>
<tr>
<td>II</td>
<td>88.9±7.4</td>
<td>235.3±21.5</td>
<td>252.7±28.4</td>
<td>248.2±25.4</td>
</tr>
<tr>
<td>III</td>
<td>82.5±4.1</td>
<td>257.1±21.6</td>
<td>249.3±18.4</td>
<td>236.5±16.6</td>
</tr>
<tr>
<td>IV</td>
<td>80.1±7.4</td>
<td>236.3±18.4</td>
<td>261.7±17.7</td>
<td>248.0±14.1</td>
</tr>
<tr>
<td>V</td>
<td>80.9±4.9</td>
<td>236.8±10.5</td>
<td>239.8±12.5</td>
<td>256.0±21.8</td>
</tr>
<tr>
<td>VI</td>
<td>76.8±7.9</td>
<td>258.3±16.8</td>
<td>251.9±18.3</td>
<td>265.8±24.9</td>
</tr>
<tr>
<td>VII</td>
<td>93.1±10.1</td>
<td>250.7±18.2</td>
<td>251.0±12.8</td>
<td>262.9±17.9</td>
</tr>
<tr>
<td>VIII</td>
<td>876±14.9</td>
<td>260.8±13.7</td>
<td>248.9±13.5</td>
<td>265.0±23.9</td>
</tr>
</tbody>
</table>

Each value is mean±S.E.M. for six animals. a”<p<0.05 vs 0th day of each corresponding group.
levels were noted on different days as described in group I.

5) Groups treated with spleen homogenate supernatant (SHS)

(a) Group V: Non-diabetic mice were administered SHS (1 ml kg\(^{-1}\) daily) of non-diabetic mice (Group I mice) for 14 days. Tail flick latency was noted before and after administration of SHS on days 1, 2, 4, 8, 10 and 14. Serum nitrite levels and fasting glucose levels were noted before and on day 14 after SHS administration.

(b) Group VI: Non-diabetic mice were administered SHS (1 ml kg\(^{-1}\) daily) of 30\(^{th}\) day diabetic mice (Group II mice) for 14 days. Tail flick latency, serum nitrite levels, and fasting glucose levels were noted on the days as described in group V.

(c) Group VII: Non-diabetic mice were administered SHS (1 ml kg\(^{-1}\) daily) of 30\(^{th}\) day diabetic mice, which were treated with spironolactone (7 mg kg\(^{-1}\) i.p.) (Group III mice) for 14 days. Tail flick latency, serum nitrite levels, and fasting glucose levels were noted on the days as described in group V.

(d) Group VIII (n=6): Non-diabetic mice were administered SHS (1 ml kg\(^{-1}\) daily) of 30\(^{th}\) day diabetic mice, which was treated with spironolactone (15 mg kg\(^{-1}\) i.p.) (Group IV mice) for 14 days. Tail flick latency, serum nitrite levels, and fasting glucose levels were noted on different days as described in group V.

(e) Group IX (n=6): Non-diabetic mice were administered SHS (1 ml kg\(^{-1}\) daily) of 30\(^{th}\) day diabetic mice, which were treated with 0.5% CMC (Group V mice) for 14 days. Tail flick latency, serum nitrite levels, and fasting glucose levels were noted on the days as described in group V.

Data Analysis

All the results are expressed as mean±S.E.M. The data were analyzed by two-way analysis of variance (ANOVA) followed by Tukey’s multiple range test. The level of significance was fixed at \(p<0.05\).

RESULTS

Effect of Pharmacological Agents on Blood Glucose Levels

A significant rise in glucose levels was noted in STZ-treated mice as compared with normal non-diabetic mice. Administration of spironolactone did not modulate STZ-induced rise in glucose levels. Administration of SHS of diabetic mice did not affect basal glucose levels. Moreover, spironolactone also did not modulate glucose levels in SHS-treated mice (Table 1).

Effect of Spironolactone on Tail Flick Latency

Time in Diabetic Mice

As shown in Fig. 1, tail flick latency time was gradually decreased in STZ-induced diabetic mice compared with non-diabetic mice. Significant hyperalgesia was noted on and after day 10 following STZ administration, and peak hyperalgesia was noted on day 30.

Administration of spironolactone (7 or 15 mg kg\(^{-1}\) p.o.) for 14 days, starting on day 16 after STZ ad-

![Fig. 1.](image-url)

Fig. 1. Effect of Spironolactone on Diabetic Hyperalgesia in Terms of Tail Flick Latency, Measured on Different Days, before and after STZ Administration

Each value is mean±S.E.M. for six animals; a=\(p<0.05\) vs non diabetic mice; b=\(p<0.05\) vs diabetic mice; c=\(p<0.05\) vs spironolactone (7 mg kg\(^{-1}\) treated.
ministration, significantly suppressed the further progression of diabetic hyperalgesia.

**Effect of Spironolactone on Serum Nitrite Level in Diabetic Mice**

Serum nitrite levels were significantly increased in diabetic mice measured on day 30 after STZ administration as compared with basal levels (Fig. 2). Administration of spironolactone (7 mg kg\(^{-1}\) or 15 mg kg\(^{-1}\) p.o.) for 14 days, starting on day 16 after STZ administration, significantly attenuated STZ induced increase in serum nitrite levels (Fig. 3).

**Effect of Spleen Homogenate Supernatant (SHS) on Tail Flick Latency Time and Serum Nitrite Levels**

Administration of SHS of non-diabetic mice, daily for 14 days, in normal mice did not produce significant effect on tail flick latency time measured on days 1, 2, 4, 8, 10 and 14 and on serum nitrite levels measured on day 14 as compared with basal levels (Figs. 3 and 4). However, administration of SHS of STZ-induced diabetic mice, daily for 14 days, significantly decreased tail flick latency time and increased serum nitrite levels in normal mice (Figs. 3 and 4).

**Effect of Spleen Homogenate Supernatant (SHS) of Spironolactone-treated Diabetic Mice on Tail Flick Latency Time and Serum Nitrite Levels**

Administration of SHS of diabetic mice treated with spironolactone (7 mg kg\(^{-1}\) and 15 mg kg\(^{-1}\) p.o.) did not alter tail flick latency time and serum nitrite levels in normal mice (Figs. 3 and 4).

**DISCUSSION**

STZ is a well reported chemical agent to induce insulin-dependent diabetes mellitus (IDDM) in mice. It is reported from our\(^9,10,14\) as well as from other laboratories\(^3\) that a significant degree of hyperalgesia develops in mice after 4 weeks of STZ administration. Therefore in the present study mice were kept for 4 weeks after STZ administration to provide sufficient time for hyperglycemia to affect pain perception.

We observed that nociceptive threshold was significantly decreased in STZ-induced diabetic mice as assessed by decrease in tail flick latency on different days and decrease in nociceptive threshold was directly related to duration of hyperglycemia, with peak hyperalgesia observed on day 30 after STZ administration. However, administration of spironolactone significantly attenuated decrease in nociceptive threshold in STZ-induced diabetic mice without affecting blood glucose levels. Clinically, spironolactone has been employed to reduce mortality incidence in patients with congestive heart failure and myocardial infarction.\(^5,6\) Spironolactone has been reported to ameliorate glomerular injury in STZ-induced diabetic renal injury.\(^8\) However, this is the first report suggesting usefulness of spironolactone in attenuating hyperalgesia in diabetic mice.

In the present investigation, serum nitrite levels

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Fig. 2. Effect of Spironolactone on Diabetic Changes in Serum Nitrite Levels (\(\mu M\)) Measured on Different Days, before and after STZ Administration

Each value is mean ± S.E.M. for six animals; a = \(p < 0.05\) vs non diabetic mice; b = \(p < 0.05\) vs diabetic mice; c = \(p < 0.05\) vs spironolactone (7 mg/kg) treated.
Fig. 3. Effect of Spleen Homogenate Supernatant (SHS) of Diabetic and Non-diabetic Mice on Tail Flick Latency in Normal Mice and Its Modulation by Treatment with Spironolactone

Each value is mean ± S.E.M. for six animals; a = p < 0.05 vs SHS of non diabetic mice; b = p < 0.05 vs SHS of diabetic mice; c = p < 0.05 vs spironolactone (7 mg/kg) treated.

Spironolactone is a mineralo-corticoid (aldosterone) receptor antagonist, and is a mild diuretic. There have been several reports demonstrating that spironolactone administration decreases expression of pro-inflammatory mediators and cytokines such as TNF-α, IL-6, interferon (IFN)-γ, MCP-1, and NF-κB. Furthermore, the role of cytokines has been well documented in the pathogenesis of diabetic neuropathy and associated hyperalgesia. Therefore it may be tentatively proposed that spironolactone-
mediated decrease in cytokine expression is responsible for its anti-hyperalgesic effect noted in the present study. Indeed, cytokines are well documented to increase the production of NO. Therefore spironolactone-mediated attenuation of STZ-induced increase in NO in diabetic mice may be possibly linked to decrease in cytokine production. However, our study does not directly support the hypothesis that anti-hyperalgesic effects of spironolactone in diabetic mice are due to suppression of cytokine generation.

In the present investigation, administration of SHS of 30th day diabetic mice resulted in induction of hyperglycemia and increase in serum nitrite levels in normal non-diabetic mice. These results suggest that certain factors are released from spleen during diabetes, which contribute significantly to induction of hyperglycemia. These results are in consonance with our earlier report. However, administration of SHS of spironolactone-treated diabetic mice failed to induce hyperglycemia and to increase serum nitrite levels. It is possible to suggest that spironolactone may have inhibited the production of spleen-derived factors involved in the induction of hyperglycemia in diabetic mice.

Spleen is a principal organ of the immune system and a rich source of cytokines. Long-standing hyperglycemia is reported to induce cytokine production from mononuclear cells. There have been several reports suggesting the presence of mineralo-corticoid and glucocorticoid receptors on mononuclear cells. Therefore spironolactone is documented to attenuate cytokine production from mononuclear cells. Therefore in the present investigation, administration of SHS of 30th day diabetic mice failed to increase factors involved in the pathogenesis of diabetic hyperglycemia, which contribute significantly to induction of diabetic hyperglycemia, which may be possibly linked to interference with release of spleen-derived factors involved in the pathogenesis of diabetic hyperglycemia.

**CONCLUSION**

Spironolactone has ameliorative potential in attenuating diabetic hyperalgesia, which may be possibly linked to interference with release of spleen-derived factors involved in the pathogenesis of diabetic hyperalgesia.

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**REFERENCES**