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# Pyritinol reduces nociception and oxidative stress in diabetic rats

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ABSTRACT

The purpose of this study was to assess the antinociceptive and antiallodynic effect of pyritinol as well as its possible mechanism of action in diabetic rats. Streptozotocin (50 mg/kg) injection caused hyperglycemia within 1 week. Formalin-evoked flinching was increased in diabetic rats as compared to non-diabetic rats. Oral acute administration of pyritinol (50–200 mg/kg) dose-dependently reduced flinching behavior in diabetic rats. Moreover, prolonged administration of pyritinol (12.5–50 mg/kg, every 2 days for 2 weeks) reduced formalin-induced nociception. 1H-[1,2,4]-oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, a guanylyl cyclase inhibitor, 2 mg/kg, i.p.), but not naltrexone (a non-selective opioid receptor antagonist, 1 mg/kg, s.c.) or indomethacin (a non-selective cycloxygenase inhibitor, 5 mg/kg, i.p.), blocked the pyritinol-induced antinociception in diabetic rats. Given alone ODQ, naltrexone or indomethacin did not modify formalin-induced nociception in diabetic rats. Oral acute (200 mg/kg) or prolonged (25 mg/kg, every 2 days for 2 weeks) administration of pyritinol significantly reduced streptozotocin-induced changes in free carbonyls, dityrosine, malondialdehyde and advanced oxidative protein products. Four to 8 weeks after diabetes induction, tactile allodynia was observed in the streptozotocin-injected rats. On this condition, oral administration of pyritinol (50–200 mg/kg) reduced tactile allodynia in diabetic rats. Results indicate that pyritinol is able to reduce formalin-induced nociception and tactile allodynia in streptozotocin-injected rats. In addition, data suggest that activation of guanylyl cyclase and the scavenger properties of pyritinol, but not improvement in glucose levels, play an important role in these effects.



## 1. Introduction

Diabetes is a global health problem and its prevalence is set to increase to more than 360 million worldwide by the year 2025 (Wild et al., 2004). Diabetes leads to several complications including retinopathy, nephropathy and neuropathy. Diabetic neuropathy is the most common complication affecting more than 50% of the diabetic patients. Etiology of diabetic neuropathy is complex and multifactorial. Several pathways contribute to the development of diabetic neuropathy which include: increased activation of polyol pathway, oxidative stress, advanced glycation end product formation, nerve hypoxia/ischemia, protein kinase C and reduction of nerve growth factor support (Van Dam, 2002; Obrosova, 2003; Vincent et al., 2004).

The treatment of pain in diabetic patients is frequently unsatisfactory. Anticonvulsants, tricyclic antidepressants and opioids have become the mainstay in the treatment of chronic neuropathic pain (Sindrup and Jensen, 1999). However, these drugs often have a limited effect or may cause intolerable side effects. Therefore, other options of treatment are needed.

Pyritinol is a nootropic drug (cognition-enhancing agent) used in cognitive disturbances to improve cerebral functions. This drug has been used in patients with Alzheimer's disease (Heiss et al., 1994), senile dementia (Fischhof et al., 1992), cerebral functional disorders (Herrmann et al., 1986), aging (Hartmann et al., 1993), and rheumatoid arthritis (Lemmel, 1993). Moreover, in animals, pyritinol has shown to improve sleep (Wetzel, 1990), energy metabolism (Bielenberg et al., 1986), hypoxic damage (Lun et al., 1989), learning and memory (Jaiswal et al., 1990), cholinergic deficits (Toledano and Bentura, 1994), and epilepsy (Schmidt, 1990). The fact that pyritinol and other nootropic drugs (nefiracetam, levetiracetam) have anticonvulsant effects in rats (Schmidt, 1990, Kitano et al., 2005a,b) lead us to hypothesize that pyritinol could have an effect in streptozotocin-induced pain. Moreover, pyritinol has shown to reduce oxidative stress *in vitro* (Pavlík and Pilar, 1989). It is believed that diabetes-induced hyperglycemia causes neural degeneration *via* the increased oxidative stress, among others (see above). Thus, we have hypothesized that pyritinol could reduce formalin-induced nociception and tactile allodynia in diabetic animals. Therefore, the purpose of this

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work was study the possible antinociceptive and antiallodynic effect of pyritinol as well as its possible mechanism of action in diabetic rats.

## 2. Materials and methods

### 2.1. Animals

Experiments were performed on 204 adult female Wistar rats (body weight range, 220–240 g) of 10–12 weeks of age. The animals were obtained from our own breeding facilities and had free access to drinking water, but food was withdrawn 12 h before experiments. Under this condition, we observed that streptozotocin produced a greater % of diabetic rats (80–90%). Experiments were done in normal light/dark cycle and they were started at the same time (11:00 AM). Efforts were made to minimize animal suffering and to reduce the number of animals used. All experiments followed the Guidelines on Ethical Standards for Investigation of Experimental Pain in Animals (Zimmermann, 1983) and were approved by our local Ethics Committee.

### 2.2. Induction of diabetes

Rats were injected with streptozotocin (50 mg/kg, i.p.) (Research Biochemical International, Natick, MA, USA) to produce experimental diabetes (Courteix et al., 1993). Control animals (age-matched) received distilled water. Diabetes was confirmed 1 week after injection by measurement of tail vein blood glucose levels with the glucose meter Ascensia ELITE (Bayer, Mexico City). Two weeks after streptozotocin injection, glycemia was again determined and only animals with a final blood glucose level ≥300 mg/dl were included in the study. Experiments were started with numbers greater than six considering that only 80–90% of the streptozotocin-treated rats became hyperglycemic or survived at 2 weeks. However, the survival percentage after 4–8 weeks decreases to about 70%. Thus, groups had to be started considering this fact.

### 2.3. Assessment of nociception

Nociception in non-diabetic and diabetic (2 weeks) rats was assessed using the 0.5% formalin test (Juárez-Rojop et al., 2006). The rats were placed in open plexiglas observation chambers for 30 min to allow them to acclimate to their surroundings; then they were removed for formalin administration. Fifty μl of diluted formalin (0.5%) were injected subcutaneously into the dorsal surface of the right hind paw with a 30-gauge needle. The animals were returned to the chambers and nociceptive behavior was observed immediately after formalin injection. Mirrors were placed in each chamber to enable unhindered observation. Nociceptive behavior was quantified as the number of flinches of the injected paw during 1-min periods every 5 min, up to 60 min after injection (Wheeler-Aceto and Cowan, 1991). Flinching was readily discriminated and was characterized as a rapid and brief withdrawal, or as a flexing of the injected paw. Formalin-induced flinching behavior was biphasic (Wheeler-Aceto and Cowan, 1991). The initial acute phase (0–10 min) was followed by a relatively short quiescent period, which was then followed by a prolonged tonic response (15–60 min). Animals were used only once and at the end of the experiment they were sacrificed in a $CO_2$ chamber.

### 2.4. Assessment of allodynia

Tactile allodynia was tested in diabetic rats 4 to 8 weeks after streptozotocin injection as previously described (Chaplan et al., 1994). Rats were transferred to a clear plastic, wire mesh-bottomed cage and allowed to acclimatize for 30 min. von Frey filaments (Stoelting, Wood Dale, IL) were used to determine the 50% paw withdrawal threshold using the up-down method of Dixon (1980). A series of filaments, starting with one that had a buckling weight of 2 g, was applied in consecutive sequence to the plantar surface of the right hind paw with a pressure causing the filament to buckle. Lifting of the paw indicated a positive response and prompted the use of the next weaker filament whereas that absence of a paw withdrawal after 5 s indicated a negative response and prompted the use of the next filament of increasing weight. This paradigm continued until four more measurements had been made after the initial change of the behavioral response or until 5 consecutive negative (assigned a score of 15 g) or four consecutive positive (assigned a score of 0.25 g) responses had occurred. The resulting scores were used to calculate the 50% response threshold by using the formula: 50% g threshold = $10^{(Xf+\kappa\partial)}/10000$, where $Xf$ = the value (in log units) of the final von Frey filament used, $\kappa$ = the value (from Table in Chaplan et al., 1994) for the pattern of positive and/or negative responses, and $\partial$ = the mean difference (in log units) between stimulus strengths. Behavioral tests (threshold assessment) were performed immediately before and every 30 min until 5 h after drug administration. Allodynia was considered to be present when paw withdrawal thresholds were <4 g. Diabetic rats not demonstrating allodynia were not further studied.

### 2.5. Determination of free carbonyls

Quantification of free carbonyls was done using a method proposed elsewhere (Dalle-Done et al., 2003). One-hundred μl of plasma were mixed with 1 ml of 2,4-dinitrofenilhidrazine 10 mM in HCl 2.5 M. Samples were incubated at room temperature, in darkness, and stirred up every 15 min during 1 h. Then, they were precipitated with a 20% solution of trichloacetic acid, centrifuged for 10 min at 3500 rpm, and then washed again with 10% solution of trichloacetic acid for the collection of precipitated protein. The samples were centrifuged for 10 min at 3500 rpm, and washed again with a 10% solution of trichloacetic acid, for the collection of precipitated protein. Finally, the precipitated was washed with a 3 ml solvent mixture (1:1) of ethanol plus ethyl acetate in order to eliminate the excedent 2,4-dinitrofenilhidrazine. The product was centrifuged again, and the new precipitate was dissolved in 1 ml of guanidine 6 M in a potassium phosphate solution 20 nM, and incubated for 10 min at 37 °C. Samples were analyzed spectrophotometrically, in a wavelength of 370 nm. The coefficient of molar extinction of 2,4-dinitrofenilhidrazine is $\varepsilon = 22{,}000/M^{-1}\,cm^{-1} = 22{,}000/10^6$ nmol/ml, and it was used to calculate the concentration of free carbonyls, expressed in osazone/ml plasma, corrected for mg of protein, quantified according with Lowry's method (Lowry et al., 1951).

### 2.6. Determination of malondialdehyde

Malondialdehide was measured according to the method of Yagi (1998) based in the quantification of reactive compounds to thiobarbituric acid, which are markers of lipid peroxidation. The procedure was done mixing 400 μl of buffer Tris-preset 7.2 mM at a pH of 8.0 with 100 μl of plasma and 1 ml of acid thiobarbituric 0.375% in HCl 0.2 N. The mixture was warmed at 90 °C during 15 min. Later on 0.5 ml de HCl 0.2 N were added and the solution was analyzed spectrophotometrically at 532 nm wavelength in a Perkin Elmer UV/VIS spectrophotometer model B050-9914 at 25 °C, using 1,1,3,3-tetramethoxipropane as standard.

### 2.7. Determination of dityrosines

Quantification of dityrosines was done according with the method proposed by Lehrer and Pasman (1967) using a plasma sample (10 μl) re-suspended in a 6 M urea solution in $NaHCO_3$ 0.1 M, pH 9.8, incubated at 23 °C during 30 min. The excitation spectrum for fluorescence of dityrosines was 280 to 370 nm wavelength. A spectrofluorometer PTI (Photon Technology International), registering the emission at 405 nm was used.