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Pooja Verma
Assistant Professor, School of Medical & Allied Sciences, G.D Goenka University, Gurgaon, Haryana, India

Correspondence
Pooja Verma
Assistant Professor, School of Medical & Allied Sciences, G.D Goenka University, Gurgaon, Haryana, India

Effect of alpha-lipoic acid and its Nano-formulation on streptozotocin induced diabetic neuropathy in Rats

Pooja Verma

Abstract
Diabetic Neuropathy is the most prevalent and costly complication of hyperglycemia that is affecting half of the patient worldwide and its incidence rate has increased upto several folds. It includes impairment of pain signalling pathways. The aim of the investigation was to determine the effect of alpha-lipoic acid in Streptozotocin induced diabetic neuropathic pain in rats by oral route, intraperitoneal route, Preparation of nano-emulsion of alpha lipoic acid and evaluate its effect by oral and intraperitoneal route as well as to determine effect of alpha-lipoic acid on diabetes itself. Alpha-lipoic acid and its Nano-formulation was orally and intraperitoneal administered to STZ induced diabetic rats for 3 weeks. Tail flick latency and tail immersion test were done to determine thermal hyperalgesia, Randall-selitto paw pressure test to determine mechanical hyperalgesia, von-frey hair to mechano-tactile allodynia and Hot plate for mechanical nociception at different time intervals i.e., 2nd and 3rd week.

Keywords: Diabetic neuropathy, Nano-formulation, Allodynia, Hyperalgesia, Neuropathic pain

1. Introduction
Diabetes Mellitus is associated with oxidative stress in both animals and human beings. Long term hyperglycaemia evokes enhanced polyol pathway, increased non-enzymatic glycation of structural proteins, enhanced oxidative stress and in addition PKC activation all interrelated for the cause and development of DN. Streptozotocin (STZ) induced diabetes is the most reliable long term hyperglycaemic model which is considered to be most stable. The mechanism of STZ is still unclear but several times it is reported that it causes pancreatic cell destruction by undergoing methylation through damaging the DNA.

The current mainstream drugs for treatment of diabetic neuropathy includes antiepileptic agents (gabapentin and pregabalin), selective serotonin reuptake inhibitors (SSRIs) and tricyclic antidepressants are Food and Drug Administration (FDA) approved for the treatment of DN. The drugs are largely included within the classifications of antidepressants, anticonvulsants, antipsychotics, anaesthetics/anti-arrythmics, vasodilators, anti-dementia agents, and opioids. For many of these medications, use for neuropathic pain is off-label; they were approved by the Food and Drug Administration for other indications. Many are in the news for questionable side effects (eg, increased blood pressure and oedema from salt retention with fludrocortisones). So more researchers are turning towards the bioactive compounds and herbal remedies to find the better pharmacotherapy for diabetic that will negate the side effects usually associated with the current mainstream drug. Alpha-lipoic acid a well-known antioxidant. It is known for its high chelating property with metal ions with doing so oxidative induced due to iron is prevented. ALA increase the synthesis of GSH by mediating gene expression induced by NrF. Also ALA is have the ability to increase nerve regeneration and regeneration of antioxidants like vitamin c and GSH. Oral formulations of Alpha-lipoic acid present problems due to characteristics of the molecule, Short blood half-life ($t_{1/2}$ ~ 30 minutes). It has poor solubility. Therefore resulting in low- Oral Bioavailability 30%Alpha-lipoic acid (ALA) is an important micronutrient with several pharmacologic as well as antioxidant properties [19]. Oral formulations present problems due to characteristics of the molecule and of the pharmaceutical forms. It is known that ALA is poorly soluble; therefore to increase the solubility was reticulated into a Nano-emulsion formulation. Previously solubility of alpha-lipoic acid is enhanced by techniques such as (microonisation and salification) but these techniques possess several drawbacks. Therefore, in the present research. We are making Nano-formulation particularly Nano-emulsion for bioavailability enhancement of alpha-lipoic acid.

Materials and Methods

Diabetes was induced in adult wistar rats (Male, 150-250 g). The animals were obtained from animal facility centre, Jamia Hamdard, New Delhi. All experiments were carried out in the duration between 8:00 am and 16:00 pm. This study was approved by the Institutional Animal Ethics Committee (IAEC) [Reg. No & Date of Reg: 173/CPCSEA, 28th JAN-2000] Jamia Hamdard, New Delhi with proposal number 1302. Animals were maintained in clean environment and at a temperature of 24 ◦C±1 ◦C, with relative humidity 50%. The animals were allowed to access food and water. Induction and assessment of diabetes for induction of diabetes, STZ was prepared in citrate buffer at (pH 4.4, 0.1 M) and single dose of STZ (55mg/kg, i.p) was administered to the rats that were fasted overnight for 12 hrs. After STZ administration animals were allowed to access water and food. The diabetes was confirmed after 48h of STZ administration using a glucometer (Dr. Morepen GLUCO One MODEL BG02). The rats with a BSL of >250mg/kg were selected and used further for the study.

Drugs and Chemicals

Streptozotocin was purchased from Sigma Chemicals Co., USA. The drug Alpha lipoic acid was supplied by Kantilal Manilal and Company Pvt limited, Mumbai. Thiobarbituric acid (TBA), Trichloroacetic Acid, Potassium chloride, 5-Dithiobis (2-nitrobenzoicacid) (DTNB), Methanol, Formaldeyhyde solution, EDTA, sod. Salt and Sodium Hydroxide pellets were purchased from S D Fine chemical limited.

Experimental Setup and Design

The duration of the study was 10 weeks, after the basal readings of all the behavioural estimations. The animals were divided into six groups each containing 5 animals.

Groups	Experimental Groups	No of rats	Drugs/dose/route
I	Control	5	Vehicle(1mg/kg,p.o)
II	Diabetic control	5	STZ (55mg/kg) i.p
III	ALA (oral)	5	STZ(55mg/kg)i.p + ALA(100mg/Kg,p.o)
IV	NE-ALA (oral)	5	STZ(55mg/kg) i.p + NE-ALA(100mg/kg)p.o
V	ALA (i.p)	5	STZ(55mg/kg)i.p+ALA(50mg/kg)i.p
VI	NE-ALA (i.p)	5	STZ(55mg/kg)i.p+ NE- ALA(50mg/kg)i.p

ALA- Alpha lipoic acid, NE-ALA- Nano emulsion Alpha-lipoic acid, STZ-streptozotocin

Preparation of α-lipoic acid aqueous solution: Distilled water and 1 N NaOH) were mixed, and Alpha-lipoic acid was added to the solution. After 10 minutes of stirring to completely dissolve the α-lipoic acid.

Behavioural Parameters

1) Thermal Hyperalgesia

a) Tail immersion test: In this parameter, tail of the rat was Spinal thermal sensitivity was assessed by the tail immersion test as described by Necker and Hellon, 1978. In this test, tail of rat was dipped in a water bath maintained at 50◦C until the withdrawal of the tail was noted. (Cut-off time 15-20 s). This reading was taken in three consecutive way. To avoid the error in readings and avoid cooling, the tail was dried with cellulose paper. A less duration of withdrawal of the tail predicts thermal hyperalgesia.

b) Tail flick method: Tail flick latency was assessed by [Dandiya and Menon, 1963] tail flick model, using analgesiometer [Davies *et al*., 1946]. In this test rats were restrained keeping the tail out of the restrainer and tail was placed on the tail flick latency equipment (Tail flick instrument from UGO Basile) on the platform in such a manner that the middle of the tail is in contact with the surface of the equipment from where the infra-red radiation passes and the tail flick latency was noted. A cut off time of 8-10 seconds was planned and similarly three consecutive readings were taken.
Refer figure: 4

2) Mechanical hyperalgesia

Randall selitto paw pressure test Mechanical nociceptive threshold, an index of mechano hyperalgesia, was assessed by method described by Randall and Selitto, 1957 [51]. The mechanical hyperalgesia was checked using the Randall-Selitto paw pressure device (IITC Life sciences), it applies mechanical force in (g) to the rat's hind paw, and an increasing force was applied until the rat vocalise. The withdrawl of the paw was used to assess mechanical hyperalgesia. Refer figure: 3

3) Mechano-tactile allodynia

Vonfrey-hair test: Mechanotactile allodynia (non-noxious mechanical stimuli) was assessed as described by Chaplan *et al*., 1994 [52]. Rats were brought from the animal house and acclimatize by keeping them in the elevated mesh of the von-frey equipment i.e (IITC, Woodland Hills, USA). This activity was performed for 4 days for 20 minutes before the actual performance of the experiment. After Acclimatization Von-Frey hairs were calibrated with bending forces to produce stimuli of different forces. After placing the rat on the elevated mesh the vonfrey hair were applied from below the mesh to the hind paw for 1 s. A response was considered positive only if the paw was immediately withdrawn. Refer figure: 2.

4) Thermal nociception

Hot plate method: It was proposed by Eddy and Leimbach in 1953. Nociception was assessed using the hot plate test (IITC Life sciences). In this test the rat was placed on plate at 55°C, and the time taken to lick a hind paw was noted. The rat was removed from the plate if no response was noted till 1 min.

Results

Effect of Alpha-lipoic acid and its Nano-formulation on diabetes-induced mechanical hyperalgesia (Randal-selittos). There was no significant difference in the mean paw withdrawal threshold in diabetic control rats on day 0 before induction of diabetic neuropathy as compared to normal non-diabetic rats. (As shown in table 4) significant decrease ($P < 0.001$) in mean paw withdrawal threshold was produced in the diabetic (STZ) control rats after 4 weeks of STZ injection as compared to normal non-diabetic rats. Chronic treatment with Nano-emulsion of Alpha-lipoic acid orally for 4 weeks

significantly ameliorated this decrease in mean paw withdrawal threshold (P <0.05) compared to diabetic control rats Moreover, attenuation of decreased mean paw withdrawal threshold by Intraperitoneal ALA and NE-ALA was more significant (P < 0.01) and (p<0.001) respectively. Also, NE-ALA intraperitoneally showed more significant increased (P<0.05) in amelioration as compared to one another.

Effect of Alpha-lipoic acid and its Nano-formulation on diabetes-induced mechano-tactile allodynia (Von-frey hair)

The mean paw withdrawal threshold in diabetic control rats before the induction of diabetic neuropathy was not significantly different than that of normal non-diabetic rats. (As shown in table 5) Four weeks after intraperitoneal injection of STZ, in response to von-Frey hair stimulation, a significant decrease (P < 0.001) in mean paw withdrawal threshold was produced in diabetic control rats as compared to normal nondiabetic rats. Rats treated with ALA and NE-ALA orally showed significant amelioration in the decreased in mean paw withdrawal threshold(P < 0.05)as compared to STZ control rats However, treatment with ALA and NE-ALA by intraperitoneal route significant increased (P < 0.01, p<0.001)respectively.

Effect of Alpha-lipoic acid and its Nano-emulsion on diabetes-induced thermal hyperalgesia (tail immersion test)

Before the induction of diabetic neuropathy by intraperitoneal injection of STZ there was no significant difference in mean tail withdrawal latency normal non diabetic rats and diabetic (STZ) control rats. (As shown in table 6) Four weeks after intraperitoneal injection of STZ resulted in significant decrease (P < 0.001) mean tail withdrawal latency of diabetic (STZ) control as compared to normal non-diabetic rats. Chronic treatment with NE-ALA orally for 4 weeks significantly ameliorated (P < 0.05). These decreased mean tail withdrawal latency as compared to diabetic control rats. Also chronic treatment with ALA and NE-ALA intraperitoneally for 4 weeks significantly increased mean tail withdrawal latency(p< 0.05,p< 0.01) respectively compared to diabetic control rats.

Effect of Alpha-lipoic acid and its Nano-emulsion on the thermal hyperalgesia (tail flick latency)

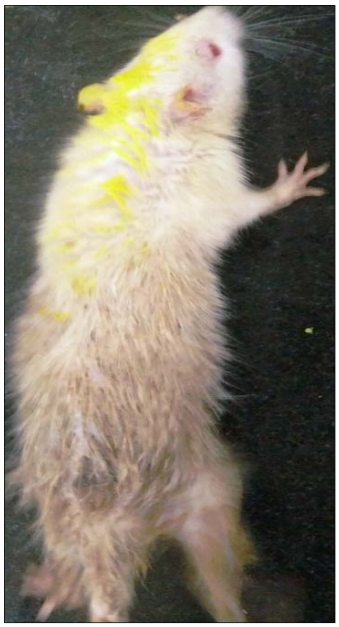
(As shown in table 7) There was significant increase (p < 0.001) in the tail flick latency of the diabetic control rats as compared to normal non diabetic control rats. Treatment with oral dose of ALA and NE-ALA significantly decreased (p < 0.05) the tail flick latency as compared to STZ-induced diabetic control rats. In addition, intraperitoneal injection of ALA and NE-ALA significantly decreased (p< 0.05, p<0.01) respectively as compared to STZ-induced diabetic control rats. However, intraperitoneal injection of NE-ALA showed remarkable decrease (p < 0.05) in tail flick latency when compared to other groups.

Discussion

To discover the potential of drugs used in diabetic neuropathic pain tail flick latency, hot plate latency, tail immersion test, von-frey hair and Randall selitto are the widely used and already well-established methods. There is a significant decrease in the pain sensitivity in the STZ induced wistar rats as compared to the non-treated control rats. Therefore, it can be concluded that there was development of Hyperalgesia and allodynia.

The metabolism of glucose through glucolytic pathway and tricaboxylic acid (citric acid) pathway produces reducing intermediate equivalents to produce perfect energy "currency" of cells called adenosine triphosphate (ATPS) via mitochondrial oxidative phosphorylatiom. The process in which ATP is produced, called oxidative phosphorylation. By products of oxidative phosphorylation include free radicle most commonly superoxide anion and the amount of the free radicles. So SOD production due to oxidative stress can be the reason for painful neuropathy. The investigation done on tail immersion test, tail flick latency, von-frey hair and randall selitto's equipment indicates that there was a significant increase in the nociceptive parameters by treatment with alpha-lipoic acid and its nano-formulation.

1. Diabetic rats



2. von-frey hair containing net mesh, pricking probe and threshold measuring device.

3. Randall selitto paw withdraw apparatus containing probe

4. Tail flick apparatus

Conclusion

In conclusion, the present investigation showed that hyperglycemia leads to generation of free radicals responsible for elevated levels of oxidative stress that caused neuronal damage via activation of various pathways in peripheral neuropathic pain. The individual treatment with antioxidants like Alpha-lipoic acid and Nano-emulsion Alpha-lipoic acid oral and Alpha-lipoic acid intraperitoneal treatment showed the inhibition of the elevated levels of oxidative stress but partially reversed the neuropathic pain. However, the intraperitoneal treatment with NE-ALA not only attenuated the hyperglycemia and effects in body weight. But also showed significant decrease in neuropathic pain via inhibition of elevated levels of oxidative–nitrosative stress as well as elevation in the membrane bound inorganic phosphate enzyme that leads to diminution in the allodynia and hyperalgesia.

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