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*Research Paper*

# CHARACTERISTICS OF ANIMAL MODEL OF PAINFUL DIABETIC NEUROPATHY INDUCED BY ALLOXAN IN SUBCUTANEOUSLY IN RATS

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**Abstract**

Animal models of painful diabetic neuropathy were made the subject of several studies. They are mostly induced by chemicals causing intoxication of the pancreas. The ultimate route of administration for these substances is the intraperitoneal route. The deadline by which mechanical allodynia and mechanical hyperalgesia appears varies according to the authors. In our study, we suggest the subcutaneously which is least used to have the characteristics of this model. The objective of this work is to evaluate the animal model of painful diabetic neuropathy by alloxan in subcutaneously. Male Wistar rats were used to induce painful diabetic neuropathy by a single injection of alloxan monohydrate (150mg / kg). Evaluation of mechanical allodynia and mechanical hyperalgesia was carried out respectively by the Von Frey Filaments Test, and the Randall-Sellito Test. Molecular analysis of the expression of c-Fos that is a neuronal activation marker was carried out by Western blot and quantitative RT-PCR. Our results showed that mechanical allodynia appears from 14th day and mechanical hyperalgesia from the 21st day. Molecular analysis of the expression of c-Fos mRNA shows an increase in mRNA levels of this marker. These results therefore showed the simplicity of this administration route and the speed of appearance of the characters of pain.

Key words: Pain; Diabetic Neuropathy; Alloxan monohydrate; subcutaneously; Mechanical Allodynia ; Mechanical hyperalgesia.

## INTRODUCTION

Diabetic neuropathy is one of the major complications of diabetes. It is a common cause of peripheral neuropathy. [1].The prevalence of neuropathy in diabetic patients worldwide is around 30 to 60% according to the authors. This painful neuropathy varies between 8-65% [2]. In 2030, about 472 million people will suffer from diabetes and 236 million neuropathies [1].
Animal models in diabetes research are numerous and most existing models are developed as a conventional model either type 1 or type 2. But very often a classic model of diabetes cannot

demonstrate the specific pathogenesis of complications related to diabetes [3;4]. Therefore, it is necessary to continue seeking a model of specific diabetes complications and reflecting the human disease [5]. Several animal models of diabetic neuropathy have been developed; this is experimental models of spontaneous diabetes or dietary manipulations, or by genetic or chemotherapy-induced pancreatic insufficiency (Streptozotocin, alloxan) [6]. These chemotherapy-induced models of painful diabetic neuropathy in streptozotocin or alloxan mostly use the intraperitoneal route and the Streptozotocin as toxic [7]. However, the intraperitoneal route is more traumatic to the animal and induction of diabetes with Streptozotocin requires several days [8]. Thus, we make an assumption about the characteristics of the animal model of painful diabetic neuropathy induced by alloxan subcutaneously.

The objective of our study is to evaluate the animal model of Painful diabetic neuropathy by alloxan subcutaneously in the Wistar Rat, which could allow us to have ideal choices for product administration and the less traumatic animal model for animal experimentation.

## MATERIALS AND METHODS.

### ANIMALS

Male Wistar rats (125-280 g) from the pet's Laboratory of Physiology and Functional Exploration of Cheikh Anta Diop University of Dakar were used. The animals were given a day / night cycle of 12 hours, with a temperature of 24 ° C ± 3 as well as food and water ad libitum. They were housed in collective cages in groups of 5 or 6. The animals were acclimated for 21 days at the pet before the experiments. All the tests have been carried out during daytime (7h-19h). The ethical guidelines of the International Association for the Study of Pain (AISP) [9] have been respected and the study was approved by the ethics committee of the Cheikh Anta Diop University of Dakar.

### PHARMACOLOGICAL TREATMENT

Experimental diabetes was induced in Wistar rats with a single injection of 150 mg / kg of alloxan monohydrate (Sigma-Aldrich, St Louis, USA) by subcutaneous (sc). Alloxan was prepared by the 0.9% saline solution. The control rats received a dose of 4 ml / kg of 0.9% saline solution.

### MEASUREMENT OF BLOOD GLUCOSE

Blood glucose was determined by a player Accu-Chek Active (Roche Diagnostics GmbH, Germany) for the quantitative determination from capillary blood and test strips. The hyperglycemia threshold value was set at 300 mg / dl [Xu and al. 2011].

### BEHAVIORAL STUDY

The behavioral study was conducted by two experimenters to properly compare values. It was conducted with repeated measurements through the use of different tests for assessing peripheral sensory neuropathy: the Von Frey Filaments test and the Randall-Selitto Test [10;11]. Before these behavioral studies, animals were acclimated to the environment for at least 10 minutes.

### Mechanical Allodynia test (Von Frey Filaments).

Mechanical allodynia is a technique based on the progressive application of von Frey filaments weighing from 0.16 to 26 g on on the plantar surface of the hind legs of the animals. The rats were placed in a Plexiglas cage and were acclimated for 20 minutes before testing. Five separate tests for 3 minutes was required to validate or not the motor response of the animal. Painful diabetic neuropathy in rats was materialized by a withdrawal movement or described in terms of the tab. A positive response was considered when the animal withdraws the paw abruptly to mechanical stimulus Von Frey filaments to more than 50% [12].

### Mechanical Hyperalgesia Test (Randall-Selitto test).

Prior to testing, each animal received 5 minutes to accommodate so get used to handling; then it was placed in a soft cotton cloth and carefully locked with the same hand used to hold the tested leg. The test involves using the stylus of a mechanical pacemaker activated by the experimenter (algesimeter Ugo Basile, 92370 Chaville) to exert increasing pressure on the dorsum of the right hind paw of the rat. The pressure threshold is determined during the gradual increase of the

applied pressure that corresponds to the pressure at which the rat trying to withdraw its paw (withdrawal threshold) which is a reflex movement involving spinal neural network. The application point has been marked with ink in order to maintain the location during the repeated testing. The maximum applied force was limited to 250 g to prevent damage to the skin of the animal [13].

**SAMPLING OF SPINAL CORD.**

The animals were anesthetized with 3% isoflurane in oxygen delivered via a mask mounted on the nose and mouth of the animal. Rectal temperature was monitored and kept constant (37.5 ± 0.2 ° C) rats by placing in a temperature controlled key. The animal is placed in the prone position on absorbent paper, then the skin is incised and the vertebral axis cleared its entire Neck thoracolumbar length. Laminectomy performed using a rat to retrieve the medullary axis without tissue damage. Using a scalpel, the marrow is cleansed of spinal nerves in a 0.9% saline solution and immediately frozen at -80 ° C in liquid nitrogen. L5 and L6 section was used [14].

**C-FOS EXPRESSION BY WESTERN BLOT.**

For Western blotting, tissue samples were homogenized by sonication in a protein extraction buffer containing 50 mmol / L Tris, pH 7.5, 150 mmol / L NaCl, 1 mmol / L EDTA Na2 dihydrate 2% sodium dodecyl sulfate, 1 mmol / L dithiothreitol, 1 mmol / L phenylmethylsulfonyl fluoride, containing protease inhibitors and phosphatase (Santa Cruz Biotechnology, Heidelberg, Germany). 20µg of lysate were loaded and separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins incorporated in the gel were then transferred onto polyvinylidene difluoride membranes (PVDF). The membranes were washed with TBS-Tween 0.1% buffer, and then incubated with 5% nonfat dry milk for 30 minutes at room temperature. Then, they were incubated with anti-c-Fos (1: 1000; Santa Cruz Biotechnology, Heidelberg, Germany) overnight on a shaker at 4 ° C. After three washes with 0.1% TBS-Tween , the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase goat anti-rat IgG (Santa Cruz Biotechnology, Heidelberg, Germany) diluted 1: 1500 for 45 minutes and the membrane was washed 3 times in TBS-Tween and the proteins were revealed using the Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology, Heidelberg, Germany). Images were scanned with a Densitometer Scanner (GS800, Bio-Rad), and optical density (OD) values were analyzed using Quantity One software (Bio-Rad). To normalize for protein loading, antibodies directed against β-actin were used, and the proteins expression level was expressed as a relative value to that of β-actin.

**GENE EXPRESSION BY QUANTITATIVE RT-PCR.**

The RNA of the cells was obtained by phenol-chloroform extraction methods standards using a cDNA synthesis kit, iScript cDNA (Bio-Rad, USA). Total RNA solution of 1µl was used for the reaction. It was performed by denaturation of RNA for 5 minutes at 65 ° C followed by reverse transcription at 37 ° C for 1 hour and finally by the inactivation of the enzyme at 95 ° C for 10 minutes.

Five micrograms of cDNA obtained by RT reaction was incubated with 1 U of Taq polymerase (Qiagen, USA), 1 µM of each primer, 0.5 mM each dNTP, 1.5 mM MgCl2, in a buffer containing 10 mM Tris-HCl pH 8.3 and 50 mM KCl. The primers were synthesized in order to be compatible with the sequence of the rat gene. The primer sequence for GAPDH were: 5'-AATGGGAGTTGCTGTTGAAG-3 '(sense), 5'-CTGGAGAAACCTGCCAAGTA-3' (antisense) ; the primer sequence for c-Fos were: 5'-CCCGTAGACCTAGGGAGGAC-3 '(sense), 5'-CAATACACTCCATGCGGTTG-3' (antisense). PCR conditions are as follows: initial denaturation at 94 ° C for 1 minute; for the PCR cycles: denaturation at 94 ° C for 30 s, annealing at 55 ° C for 30 s and elongation at 72 ° C for 30 sec. The last cycle is followed by a final extension at 72 ° C for 7 min. All reactions were performed at 35 cycles in a thermal cycler C1000 Touch (Biorad, USA). The variation in the copy number of the target gene was determined by the formula $2^{-\Delta\Delta CT}$ where ΔΔCT = [CT of the target gene - CT control gene] treated group - [CT of the target gene - CT control gene] control group untreated. The control gene GAPDH was used [15].

**STATISTICAL ANALYSIS.**

All data were presented as mean ± SEM and P <0.05 was considered statistically significant in all cases. Data analysis was performed by Student's t-test and the GraphPad Prism 5 Demo software.

**RESULTS.**

**Time evolution of body weight and blood glucose.**

To determine the health status of the animals in this study, we conducted the weight measurement (Fig 1-A.) and as well as blood glucose (Fig.1-B) of animals for the following dates: D0 before injection and D3, D7, D14 and D21 after injection of alloxan monohydrate (150 mg / kg). All drugs were administered subcutaneously.(figure 1)

The body weights of rats treated with alloxan monohydrate (150 mg / kg) showed significantly lower levels (91 g to 110 g; n = 25) compared to rats treated with saline solution (145 g to 192 g, n = 15) at 14th day after the injection of alloxan monohydrate (Figure 1-A). Rats treated with alloxan monohydrate (150 mg / kg) showed significantly higher blood glucose levels (217-543 mg / dl; n = 25) compared to the rats treated with 0.9% saline solution (89-96 mg / dl; n = 15) after the third day of injection of alloxan monohydrate (Figure 1-B). Analysis of these data showed a statistically significant difference between the two groups, ** P <0.002 for body weight (from 14th day). *** P <0.0001 for glucose (from third day). These observations indicate that there is a body weight drop from 14th day and diabetes appears from third day [16].

**Behavioral study.**

To determine the presence and the date of onset of mechanical allodynia and hyperalgesia induced by alloxan monohydrate (150 mg / kg), we used the Von Frey filaments test and the Randall-Selitto test in the control group (CG) and group under alloxan monohydrate (GE) at different times.(figure 2).

Statistical analysis between control group of 0.9% saline solution and group of alloxan monohydrate (150 mg / kg) showed a statistically significant difference, ** p <0.005, n = 15 , from 14$^{th}$ day for mechanical allodynia (Fig .2-A) and ** p <0.007, n = 15, for mechanical hyperalgesia from 21$^{st}$ day (Fig.2 B). These results confirm the presence of mechanical allodynia and mechanical hyperalgesia. In addition, these data also demonstrate that the mechanical allodynia appears first with respect to the mechanical hyperalgesia.

**Hyperglycemia causes the neuron activation in the dorsal horn of the spinal cord.**

We therefore analyzed neuronal responsiveness of the spinal cord in rats allodynic and hyperalgesic using molecular biological methods. Since nociceptive responses evaluated above mainly reflect the behavioral aspect of the nerve damage. Therefore we explored whether the injection of alloxan monohydrate (150 m / kg) by subcutaneous route may have molecular effects on neurons in particular centrally to the dorsal horn of the spinal cord. With Western blot and quantitative RT-PCR, we have expressed the c-Fos, which is a marker of neuronal activation [17]. (figure 3).

These results suggest that hyperglycemia induced by alloxan monohydrate causes nerve damage materialized at the molecular level by the activation of neurons in the dorsal horn of the spinal cord.

**DISCUSSION.**

In this study, we evaluated the animal model of neuropathic pain induced by alloxan subcutaneously. We noted a significant reduction in the weight of the animals from 21$^{st}$ day. Our data showed hyperglycemia from the 3rd day after the injection of alloxan monohydrate by subcutaneously. We also determined in rats, mechanical allodynia from the 14th day, and mechanical hyperalgesia from 21$^{st}$ day. Finally, we demonstrated that mechanical allodynia and mechanical hyperalgesia causes molecular changes in the expression of c-Fos mRNA which is a neuronal activation marker.

Our data showed a fall in body weight in Wistar rats which made them diabetic by a single injection of alloxan by subcutaneously. These results confirm those of the literature that reports

that the injection of streptozotocin intra peritoneal causes a drop weight [18]. This weight regression may be due to lipolytic actions exerted by glucocorticoids in adipose tissue [19].
We also observed that the drop of body weight is associated with hyperglycemia from the third day after injection of alloxan monohydrate by subcutaneously [Auberval and al. 2009]. This hyperglycemia is related to destruction of islets of Langerherans. Our data showed that hyperglycemia appears more quickly compared with the results of some authors [20;21]. Although subcutaneously appears slow compared to intra-peritoneal route, however, the substance used diffuses more easily in adipose tissue and most easily destroyed pancreatic [22].
It must be remembered in this study, the problem of the interpretation of the pain and the issue of pain assessment is thus asked. During surgery, it is convenient, first assessment compared with similar situations in humans. This could not really be realistic if the pain was felt in the same way in every individual, human or animal. It is known that the neurological mechanisms of transmission of nociceptive stimuli are similar in humans and other mammals. It is also known that the necessary stimuli to trigger these mechanisms are very similar. However, it is unable to understand the perception of pain in animals: one cannot determine whether an animal feels a noxious stimulus in the same way and with the same intensity as human. However, we cannot do without the tests based on the paw withdrawal to determine mechanical allodynia and mechanical hyperalgesia [23].
In our study, we demonstrated that mechanical allodynia appears at 14th and mechanical hyperalgesia at 21st day after the injection of alloxan monohydrate. Indeed, several studies have reported that mechanical Allodynia and mechanical hyperalgesia appears slower in induction methods in Streptozotocin diabetes by intraperitoneal route and genetic method [24]. Indeed, mechanical allodynia and mechanical hyperalgesia appear 4-5 weeks after injection of Streptozotocin according to the authors [25;26]. Our results contrast with previous studies that these substances were injected by the intraperitoneal route. In addition, the mechanism of allodynia in diabetes is associated with hyperglycemia [27]. The mechanism underlying the action of glucose on the neuron is known. It uses two channels, one of the non-enzymatic glycation and the glucose oxidation [28]. These arguments show that injection subcutaneously could have a faster action on adipose tissue.
We also demonstrated that hyperglycemia causes neuronal damage. Indeed, hyperglycemia would cause oxidative stress in animals, causing nerve damage [29]. In addition, hyperglycemia itself could change the homeostasis of cells of the central nervous system [30]. These studies confirm previous studies by the significant expression of c-Fos. However, none of these hypotheses may explain first the appearance of mechanical allodynia compared with mechanical hyperalgesia as in most studies of neuropathic pain induced pat toxic substances or genetically modified.

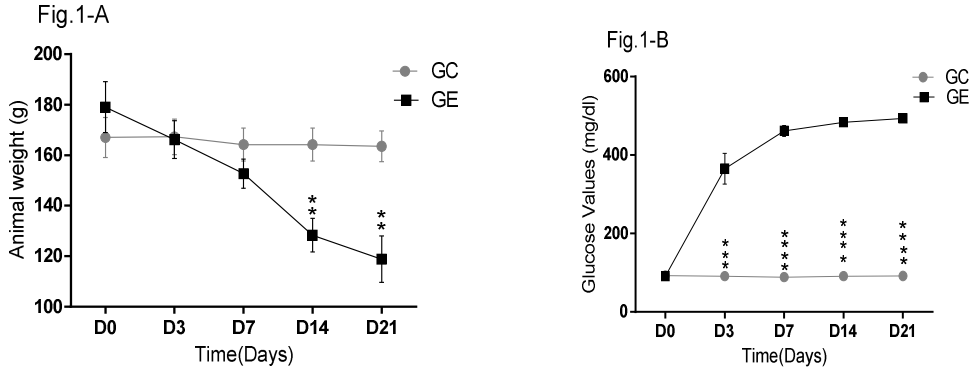


**Fig.1: Time evolution of body weight and blood glucose in Wistar rats.**
GC: control group (N = 15) and GE: group under alloxan monohydrate (150 mg / kg) (N = 25). The analysis of these data showed a drop in weight between the control group rats under 0.9% saline solution (GC) and group under alloxan monohydrate (GE) (150 mg / kg), ** p <0.002 , n =

40 (Fig.1-A). It was also noted hyperglycemia between two aforementioned groups, *** p <0.0001, n = 40 (Fig.1 B).

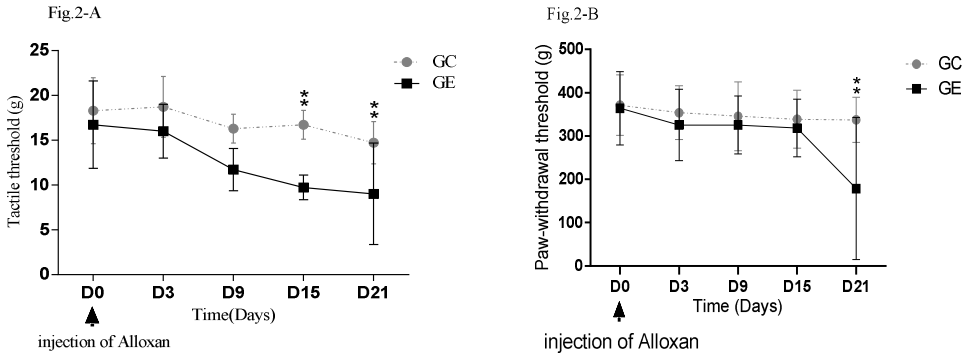


**Fig.2: Determination of allodynia and mechanical hyperalgesia.**
GC: control group (N = 15) and GE: group under alloxan monohydrate (150 mg / kg) (N = 25). The data of this figure showed mechanical allodynia from 14th day (** p <0.005) (Fig.2-A) and mechanical hyperalgesia from 21st day (** p <0.007) (Fig. 2-B).

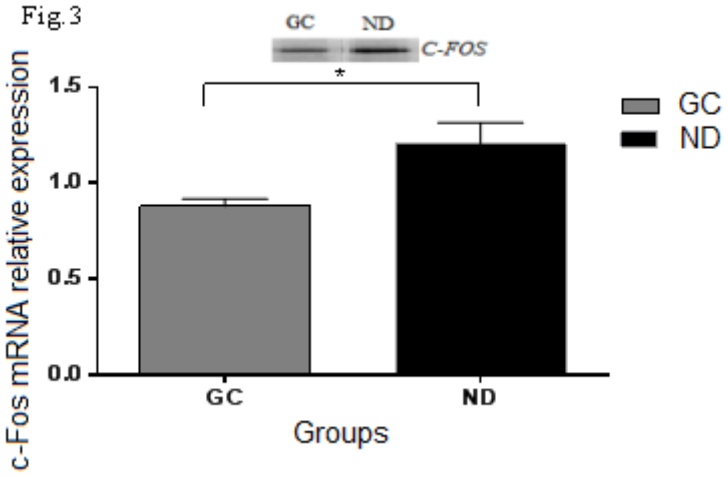


**Fig. 3: c-Fos expression in rats.**
Analysis of mRNA expression in the rats allodynic and hyperalgesic showed a significant difference between control group (GC) under 0.9% saline solution and the group under alloxan monohydrate (150 mg / kg) * P <0.02, n = 5.

## CONCLUSION

Definitely, this study allowed us to determine the presence of mechanical allodynia and mechanical hyperalgesia within a reasonable time by the injection of alloxan monohydrate by subcutaneously. The emergence of these parameters is correlated with an increase of hyperglycemia in Wistar rats.

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## CONFLICTS OF INTEREST.

There was no conflict of interest in this work.

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