

Physiologic and Behavioral Assessment of Sciatic Nerve Injury in Wistar Rat Model Treated with Freund's Incomplete Adjuvant

¹Aziez Chettoum, ²Asma Fraia, ²Hacène Frih, ²Bachir Ali Rachedi,
²Moncef Zaafour, ²Selma Guernine, ²Samir Djemli and ³Réda Djenidi

¹Department of Biology, University of Constantine, Algeria

²Department of Biology, Faculty of Science,

University Badji Mokhtar, Annaba, BP 12 El Hadjar, Algeria

³Department of Biology, University of Bordj Bou Arreridj, Algeria

Abstract: Major depression disorders are commonly seen in many medical diseases that share chronic inflammatory pain as a common denominator. Pains whether inflammatory or neuropathic, those caused by damage to the central or peripheral nervous system are the most difficult to treat due to their resistance to conventional analgesic treatments. In our study, we chose a model of neuropathic pain (sciatic nerve ligation) associated with intradermal injection of 0.02 ml of Freund's Incomplete Adjuvant (AIF) in order to achieve an activation of the innate immune system in male Wistar rats subsequently being tested of forced swimming (FST). Our study was completed by an endocrinological study highlighting the importance of sex steroids on the fluctuations of depression by hormonal assays (testosterone and estradiol). Our results showed that the ligation causes a highly significant increase in the depression 10 days postoperative. This is revealed by increased immobility time in the FST. This depression reported among ligated tends to be mitigated over time there has been a significant decrease in twenty days following ligation. Treatment with AIF is in 2 phases: 1st where we see a depressive-like behavior due to the activation of the immune system that causes inflammation and a 2nd phase where there is a less depression antidepressant-like and the inflammation disappears after 20 days. This is also associated with hormonal changes or there is a significant increase in the level of testosterone in subjects ligated and treated (AIF) and a significant decrease of estradiol. These results then takes us to highlight the existence of neuro-immune-endocrine link that acts to benefit the body and restore homeostasis. In case of disruption these systems associated will cause depressive disorders also a major depression.

Key words: AIF • Neuropathic Pain • Inflammation • Testosterone • Estradiol • Depression

INTRODUCTION

The inflammatory response in a normal subject is a key means of protection against bacterial agents and is characterized by a natural balance between cytokines pro- and anti-inflammatory [1]. The four cardinal signs are: redness, heat, swelling and pain demonstrate the mobilization of host defenses [2]. There is a fundamental distinction between acute inflammation and chronic inflammation. Acute inflammation includes the immediate early response to aggression. Chronic inflammation

persists when the trigger or stimulation persists [3]. Peripheral inflammation can directly reach the Central Nervous System (CNS) via mediators of inflammation (interleukins and prostaglandins) that penetrate the brain areas through Blood-Brain Barrier (BBB) [4].

The pain can be considered as contributing to a protective function, an alarm signal, as a result of injury or trauma in peripheral tissue generating an inflammatory defense reaction. Kuner, 2010 reported that pain is one of the most common symptoms encountered in the clinic and inflammatory pain represents an important type of pain.

The latter may extend in time and cause chronic inflammatory pain, but may also involve a nerve causing neuropathic lesion and therefore chronic neuropathic pain. Pain loses its meaning alarm signal to become a disease in itself, a true syndrome.

In addition, more than half of patients with neuropathic pain develop mood disorders such as depression and anxiety as the external stress is widely recognized as a predisposing factor and trigger for depression [6-8]. It's well recognized that depression is accompanied by activation of the immune system, inflammatory and oxidative stress [9]. Especially when this neuronal inflammation is prolonged due to an unknown cause, especially undiagnosed, as an ancient and symptomatic chronic infection, it involves our cellular and humoral defenses, creating a biological disturbance generating oxidative stress. By "oxidative stress" is understood oxidative chemical attack, oxidation of the constituents of our body due to excess particularly harmful molecules called free radicals of oxygen [10, 11]. This oxidation denatures proteins, lipids, sugars and the same DNA and thereby the cell membranes and finally the cells [11]. It was also shown that the depression is one of the primary causes of hyperactivity of the Hypothalamic-Pituitary-Adrenal (HPA) characterized by a negative feedback resistance of the glucocorticoid axis [13]. This would be a major player in the stress response [14].

Today the pains that they either inflammatory or neuropathic, those caused by damage to the Central or Peripheral Nervous System are the most difficult to treat due to their resistance to standard analgesic treatments. The treatments currently used involve not specifically targeted therapeutic classes on pain, especially antidepressants. Their limited effectiveness is in fact based on empirical observations. A better understanding of the pathophysiological processes underlying neuropathic pain, is a prerequisite for any therapeutic innovation [15].

In this case we applied to develop neuropathic pain model [7, 8] in rats by performing a ligation of the sciatic nerve at the level to study the behavioral characteristics through the forced swimming test parameters (time swimming, immobility and climbing) and hormonal assays (testosterone and estradiol). So we developed this experimental protocol in order to highlight the activation of the immune system by intradermal injection of Incomplete Freund's Adjuvant (IFA) to demonstrate the links between the nervous immune and endocrine system.

MATERIALS AND METHODS

Biological Material: Our biological material is male Wistar rat aged about six (06) months and average weight of 250 ± 10 g purchased from the Pasteur Institute of Algiers. The animals were raised in polyethylene cages. They underwent an adjustment period of four (04) weeks in the environmental conditions of the experiment room (natural photoperiod, humidity, temperature, etc ...). Their food consisted of corn, barley, milk and vitamin supplements. Drinking water is served *ad libitum*. The rats were subjected to natural photoperiodic regime. Because of the extreme susceptibility to stress response systems to all kinds of aggression, we attached great importance to the conditions of daily manipulation and the environment where the noise is reduced. The animals were handled by the same experimenters.

The animals were divided into three (03) experimental groups (n=08): Control (Have not suffered anything), Ligation (Sciatic nerve ligation under general anesthesia) and IFA (Intradermal tail injection of 0.02 ml Freund's incomplete adjuvant).

Sciatic Nerve Ligation «Sciatic Nerve Injury»: The rat model *Ratus ratus* Wistar strain underwent ligation of the sciatic nerve [7, 8] while the sural nerve left intact. The results lesions of hypersensitivity is marked in the lateral region of the leg that is innervated by the sural nerve was spared. The non-operated side of the rat was used as control.

The rats suffered surgery "sciatic nerve ligation" (*sciatic nerve injury*). Following this operation, the subjects received a treatment of antibiotics for five (05) days by intraperitoneal injection, after a period of complete rest for ten (10) days (including days of treatment antibiotic) before restarting a second battery of tests similar to the previous one. At the end of the second battery, the rats were injected with 0.02 ml of IFA and experimental protocol resulted with a third and final set of tests.

Anesthesia:

- Rats anesthetized by intraperitoneal injection of a ketamine 0.3ml / 100g + a drop orally of Largactil (chlorpromazin).
- Animals placed in a calm and quiet place until fully anesthetized.

- Verification of rats reflexes by pinching the tip of the tail and legs with a pair of tweezers to ensure the immobility of animals before any surgery.
- Shaving drapes (using an electric razor) slightly below the knee area to the hip area.
- Application of an ophthalmic ointment to the eyes of animals using a cotton swab.
- The animal was placed on its right side (or left) and placement of the left hind limb (or right) on a small platform in order to keep it high. Leg fixation with tape.
- Disinfection of the operative field with alternating scrubs of ethanol and betadine outside the surgical site.

Surgery:

- Location knee with the thumb of the left hand and use a scalpel to make an application. A few cm incision in the proximal longitudinal direction of the knee.
- Open the skin by blunt dissection using the tip of a pair of sterilized scissors.
- Separation of the muscular layer by dissection just next to the clearly visible blood vessel, close to the femur (thigh bone). The muscle layers are easily separated without bleeding, revealing the sciatic nerve right or left below the muscle.

It should be noted that in case of bleeding due to damage of a blood vessel near the knee, they must be sterilized using a cotton bud body to absorb the blood by pressing until the bleeding stops.

- Rat under a stereo microscope to gently separate the muscles with a pair of tweezers sterilized to clearly visualize the sciatic nerve.
- Identification of the area and the collateral saphenous branches of the sciatic nerve knowing that the sural nerve is the smallest of the three branches.
- Creation of a surgical tight knot around the sciatic nerve. If the first node is a member of the tight contractions will be observed.
- Cut the suture ends with a pair of micro scissors and gently closing the muscle layer. Adding a drop of lidocaine on the wound and suture with surgical knots.

Post-Surgery Period:

- Verification of the sufficiency of eye ointment.
- Rat placed in a clean cage under a paper towel in a comfortable posture to room temperature.
- Water and food are easily accessible for the animal operated.
- Intra-peritoneal injection of the antibiotic in a 0.3ml dose for five (05) days after surgery.
- Conduct a battery of behavioral tests (neuropathic pain and anxiety and depression measures) after ten (10) days of the surgery.

Treatment by the Incomplete Freund's Adjuvant (IFA):

Incomplete Freund's Adjuvant (IFA) is composed of mineral oil and mannide monooleate emulsifier. This adjuvant, causes enhanced immunogenicity by increasing local inflammation stimulates the proliferation of non-specific lymphocytes and prolonging the persistence of the antigen [16]. In this context, the adjuvant plays an important role because it improves the recruitment of various types of immune system cells. Induces an increased expression of endogenous inflammatory cytokines [17].

The IFA used comes from the Pasteur Institute of Algiers. In our study, it was injected intradermally at the tail at a dose of 50 µl / rat was reduced to 2 µl / rat for the needs of the protocol, namely a stimulation of the immune response [18].

Blood Sample Collection and Hormonal Assays (Testosterone and Oestradiol):

Retro-orbital blood samples were collected. Blood samples were centrifuged at 5000 r/min to be used for hormone assays of testosterone and oestradiol, which measured these levels by the BIOTECH conventional ELISA test kit, with a TECAN ELISA reader equipped with Magellan computer software that automatically calculates the standard range and provides the value of the hormone to the desired unit directly.

Forced Swimming Test: The forced swim test [19, 20] is a behavioral test of inducing despair in rats by placing the animal 15min in a glass aquarium 54 cm height (34 × 60cm). This dimension ensures that the rat can't escape by climbing to the edges of the device. The aquarium is filled with water (26°C) to a height of 40 cm, in order to ensure that the rat will not use his legs to keep the surface and

thus force him to swim. The procedure of forced swimming (FST) in rats occurs in two phases: the pre-test and test, separated by an interval of 24 hours. During the pre-test, the rat was placed for 15 min. At the end of session, the animal is immobile. The next day, the animal plunged into the aquarium for 5 min. The swimming session on each day was videotaped for behavioral analysis. The time of immobility, swimming and climbing are calculated.

Statistical Analysis of Results: Data are presented as mean \pm SEM. Data were analyzed by one-way ANOVA and Newman and Keuls or Benferroni as the post hoc test. Results were considered significant at $p < 0.05$. Graph Pad Prism 5 for windows version 5.01 was used to do the analysis.

RESULTS

Effect of Ligation and IFA on Depressive-Like Behavior in the Swimming Test: The ANOVA1 for swimming time (A) reported a significant treatment effect ($F = 6.327$, $R \text{ square} = 0.5686$, $p < 0.0007$). The Newman-Keuls test indicated a significant increase in swimming time in lots: Lig vs Lig10 ($q = 5.096$, $p < 0.05$), Lig vs Lig 20 ($q = 7.007$, $p < 0.05$), Lig vs IFA 20 ($q = 4.282$, $p < 0.05$). By cons, we reported a significant decrease in groups: IFA 10 vs Lig20 ($q = 4.848$, $p < 0.05$), Lig vs control ($q = 5.388$, $p < 0.05$).

The ANOVA1 for the immobility time (B) reported a significant treatment effect ($F = 14.74$, $R \text{ square} = 0.7544$, $p < 0.0001$). The Newman-Keuls test indicated a significant increase in immobility time in groups: control vs Lig ($q = 10.88$, $p < 0.05$), control vs IFA 10 ($q = 6.322$, $p < 0.05$),

control vs Lig 10 ($q = 5.213$, $p < 0.05$) et Lig20 vs IFA 10 ($q = 3.814$, $p < 0.05$) and significantly decreased in lots: IFA 2 vs Lig ($q = 8.619$, $p < 0.05$), IFA 20 vs IFA 10 ($q = 4.065$, $p < 0.05$), Lig 10 vs Lig ($q = 8.368$, $p < 0.05$), IFA 10 vs Lig ($q = 4.553$, $p < 0.05$) and Lig 10 vs Lig ($q = 5.662$, $p < 0.05$).

The ANOVA1 for time climbing (C) did not report a significant treatment effect ($F = 1.378$, $R \text{ square} = 0.2230$, $p < 0.2677$).

Effects of Ligation and IFA on Hormonal Parameters

Variation in Plasma Testosterone Level (ng / ml): The ANOVA1 revealed a significant treatment effect on the testosterone level ($F = 9.183$, $R \text{ square} = 0.4440$). IFA has significantly increased the level of testosterone (Control vs Lig IFA: $q = 5.117$, $p < 0.05$), (Lig vs Lig IFA: $q = 5.733$, $p < 0.05$).

Variation in Plasma Estradiol Level (ng / ml): The ANOVA1 revealed a significant treatment effect on estradiol level ($F = 27.50$, $R \text{ square} = 0.7857$). Ligation significantly decreased the level of oestradiol (Lig vs Lig IFA: $q = 5.733$, $p < 0.05$), Control vs Lig IFA: $q = 5.117$, $p < 0.05$).

DISCUSSION

After our study the results obtained regarding the degree of depression show that: ligation causes a highly significant increase in the depression. This is revealed by increased immobility time in the FST (Fig. 1). We believe that this depression is induced by inflammation due to sciatic nerve ligation.

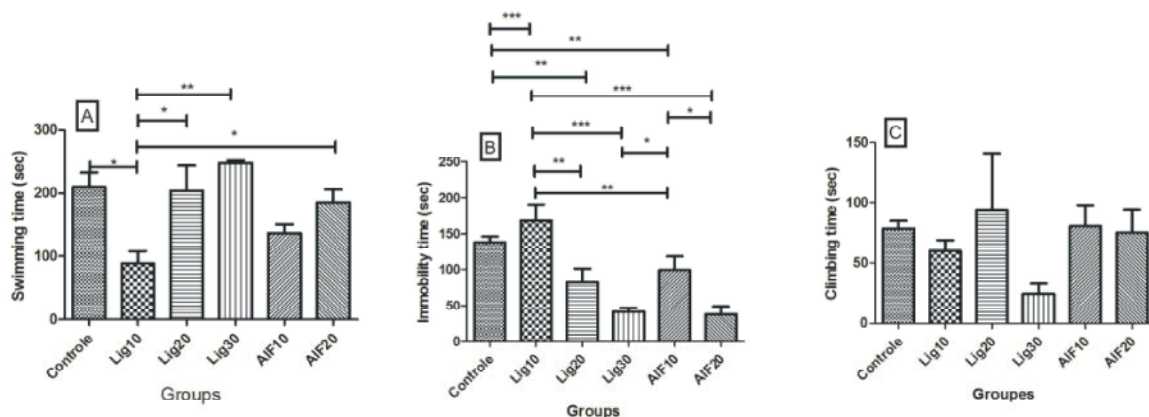


Fig. 1: Effect of ligation and IFA injection on forced swimming test parameters in the *Wistar* rats. One-way ANOVA and Newman-Keuls test multiple comparison test post hoc test were used to compare groups to another (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

The FST is a widely used model in which behavioral change is induced by acute stress [21]. If the immobility time decreases or increases this is interpreted as depressive or antidepressive action [22]. In addition to immobility, animals exhibit behaviors that reflect active interest in actively try to avoid this aversive situation. Indeed, it has been reported that a large proportion of the individuals nervous system injury (sciatic nerve ligation) can lead to a debilitating chronic neuropathic pain, which is currently considered a neuro-immune disorder, since data recent indicate a critical involvement of the innate and adaptive immune responses in response to nerve injury [22].

The activation of immune cells (glia) in the injured nerve causes the release of two cytokines pro- and anti-inflammatory [23]. Smith (1991) was hypothesizes that cytokines proinflammatory IL-1 (interleukins) and IL-6, IFN γ (interferon) and Tumor Necrosis Factor (TNF α) in serum were treated as main symptoms of depression [25].

This depression reported in ligated, tends to be attenuated with time as a significant decrease is observed there of twenty (20) days after ligation. We justify this by the fact that the subjects were ligated suitable for inflammation with the intervention of the Hypothalamic-Pituitary-Adrenal (HPA) axis *via* glucocorticoids such repair has been completed.

The activation of the HPA axis is triggered by an increased secretion of Corticotrophin Releasing Hormone (CRH) is attributable to proinflammatory cytokines [26, 27]. CRH stimulates the pituitary gland to secrete Adrenocorticotropin hormone (ACTH) in the bloodstream. The latter promotes the synthesis of glucocorticoids (cortisol in humans and corticosterone in rodents). Increased glucocorticoid regulates, in a negative way, the activity of the HPA axis and thus its own production through a negative feedback mechanism. They inhibit the release of ACTH, synthesis and release of CRH [28, 29].

Despite this adaptation reported previously, we see that after thirty (30) days some degree of residual depression persists. This explains the absence in the body of endogenous resources sufficient to overcome depression.

As such, it has been reported that the inflammation may extend over time and lead to chronic inflammatory pain, but can also affect nerve tissue causing neuropathic injury and therefore chronic neuropathic pain: pain loses its meaning alarm signal to become a disease in itself, a true syndrome [30]. We see then that the ligated subjects

seem to be permanently installed in a chronic depression process. In order to find ways to combat chronic neuroinflammation and therefore depression, we tried in subjects ligated stimulation of the immune system by Incomplete Freund's Adjuvant (IFA). It appears from this test that the IFA is trying causes in a first stage inflammation which disappears after twenty (20) days.

IFA causes enhanced immunogenicity by increasing inflammation, by stimulating the proliferation of non-specific lymphocytes and the extension of the persistence of the antigen [31]. The administration of the IFA is to be considered in current and long-term. In the short term, the inoculation of the AIF causes a non-specific immune response that favors production of TNF, interferon the IFN γ , from IL-6, IL-17, IL -4, IL-10, IL-1 β and IL-2 [32]. Among the roles of IFN γ is activation of IDO (indoleamine 2,3-dioxygenase) this enzyme is highly expressed in humans and rodents, by dendritic cells and macrophages and by brain endothelial cells, astrocytes, microglia and neurons [33, 34]. IDO then inhibits the conversion of tryptophan which is a precursor amino acid biosynthesis of 5-HT (5-hydroxytryptamine). Tryptophan decreased rates resulting in a reduction of the synthesis of 5-HT and that decline may be involved in the development of mood disorders and also cause depression [35]. Thus, the immune reaction to long-term changes shape and is no longer expressed IFN γ which induces inhibition of IDO and thus recovery of the conversion of tryptophan where the biosynthesis of 5-HT in namely increased metabolism thereof and follows a decrease in depressive disorders.

Our results regarding the immobility time show a significant reduction thereof to the detriment of the swimming time. This depression is linked to a reduction in monoamine transmission, especially serotonin (5-HT) and noradrenaline (NA) [36], as well as by an increase in the dopaminergic transmission in the Central Nervous System (CNS) [37].

Our study was completed by an endocrinological study highlighting the importance of sex steroids on the fluctuations in the state of depression. Our results (Fig. 2) show a significant increase in the testosterone level in subjects IFA+lig20 and a significant decrease of estradiol in the same subjects, allowing us to assume that it is innate immunity who heckled the neurophysiological mechanisms leading exacerbation hence mitigating these depressive disorders. This means that testosterone plays an antidepressant-like effect while estradiol would have a depressant-like effect.

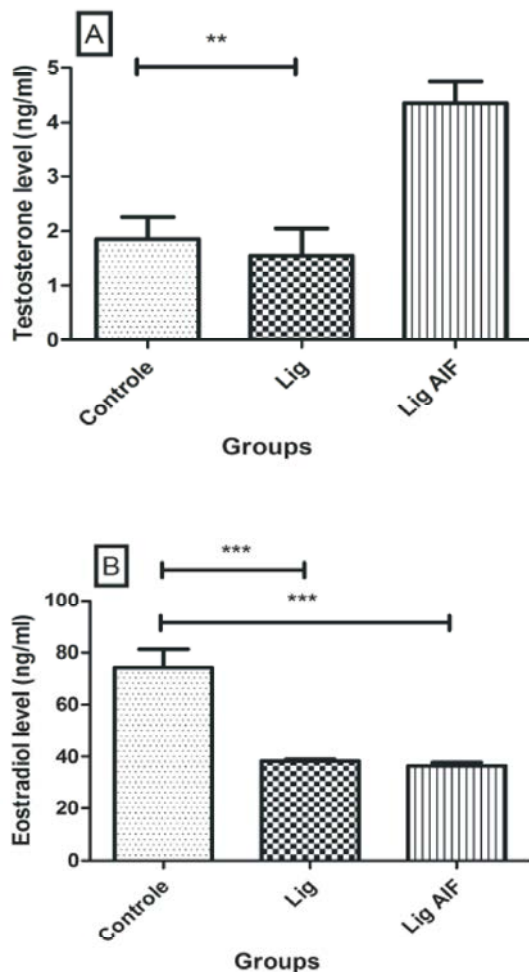


Fig. 2: Change in hormonal level testosterone (ng/ml)(A) and estradiol (ng/ml) (B) in the control rats, ligated and treated with AIF. One-way ANOVA and Newman-Keuls test multiple comparison test post hoc test were used to compare groups to another (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The participation of sex steroids in mood disorders has often been documented and disruptions in the circulating levels of ovarian hormones produced behavior of depression [38], more behavioral changes associated with the estrous cycle, clearly showed a reduction of immobility and active behaviors highest active behavior during proestrus -estrus [39]. From these observations, it would appear that estrogen would have a depressing effect [40]. In these categories, the sources of estradiol may be gonads from the ovaries and testes that have a high content of aromatase (an enzyme that converts androgens into estrogen). The concentrations of estradiol in adult females and prepubertal rats could be related to

the reduction of immobility and its increase observed during the test, revealing activation of the serotonin system [41].

The administration of 17 β -oestradiol in the female or male rats increased the swimming and reduce immobility in the FST, the antagonist of the 5-HT_{1A} receptor, blocks the antidepressant effect of estradiol in the FST [42, 43]. Other studies on the effect of estrogen antagonists on the swimming behavior should be conducted to determine the role of estrogen in the FST in young and adult rats. Also, it is well known that estrogens also have anti-inflammatory properties.

Studies in rats and mice have demonstrated the ability of estradiol to reduce tissue damage, while decreasing the expression of Th1 cytokines involved in the acute inflammatory response, such as Macrophage Migration Inhibitory Factor (MMF), TNF α , IL-1 β and IFN- γ [43, 44].

By cons, testosterone would anxiolytic and antidepressant-like effects [45, 46]. Recent reports show that in adult males, stress increases testosterone possibly by stimulating the secretion of gonadotropin. However, current data argue against this observation since the levels of this hormone are similar between the control animals and those exposed to FST. During inflammation, circulating cytokines suppress reproductive function by activating the secretion of hypothalamic CRH and therefore inhibiting ovarian and testicular steroidogenesis [47]. Activation of pro-inflammatory cytokines by altering neurotransmission of serotonin, is a component of the depression, although the anti-inflammatory cytokines regulate the inflammatory response by inhibiting the production and action of inflammatory cytokines [48].

CONCLUSION

Our study includes an experimental investigation to assess and explore the effect of sciatic nerve injury and injection AIF intradermally. The results obtained are consistent with those of some authors to know the inflammatory effect of sciatic nerve ligation and depressive-like effect of AIF in the forced swimming test.

Through these results we observed that:

- The males included in our experiment may acquire depressogenic-like post sciatic nerve ligation (Neuro-inflammation) and this is revealed by the forced swimming test.

- The ligated subjects have shown an adaptative situation to neuro-inflammation is justified by the reduction of level of depression twenty days following ligation.
- Neuro-Inflammation can last in time and become chronic.
- We found through the results of the forced swim that administration of AIF would improve negative changes caused by sciatic nerve ligation (effect after 20 days).
- Sex steroids have an effect on the fluctuations of depressive behavior with antidepressant-like effect of testosterone and depressinogenic-like effect of estradiol.

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