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IMPACT OF BIOACTIVE MOLECULES ON ADAPTIVE, BEHAVIORAL AND IMMUNE RESPONSES IN WISTAR RAT

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For My beloved, Parents & Grandparents. If it were not for you, i would never have made it this far.

ABSTRACT

This study aims at evaluating the behavioral and physiological effects of chronic administration of β-carboline Harmine and Amitriptyline in rats exposed to CMS procedure. After 30 days of exposure to CMS procedure, rats were treated with Harmine (15 mg/kg/day, ip.) or Amitriptyline (20 mg/kg/day, ip.) for 7 days. Afterwards, rats were tested with a battery of behavioral tests: Annedonia, Open Field (OF), Elevated plus Maze (EPM) and Light and Dark Box (LDB).

The findings demonstrate that CMS shows behavioral changes during the different tests; a decrease in the consumption of sweet solutions compared with the control group.

Concerning the data of the effects of CMS in the EPM test, CMS rats demonstrated a decrease in the number of entries and the time spent in the open arms compared to non-stressed rats. On the other hand the results show no significant differences on the number of entries and the time spent in closed arms on CMS rats compared with the control rats.

However, in the OF parameters, we notice a decrease of the time spent in the field center, a decrease of grooming time, without affecting defecation, crossing and rearing activity in the stressed group compared with the control group.

The data of the CMS effects on the LDB parameters show a decrease in the number of entries and the time spent in the light compartment compared with the control group.

Regarding the physiological parameters; the results reveal an increase of ACTH level compared with the control group. Moreover, CMS alters the immunological parameters by increasing of IgG, without affecting IgM circulating levels.

The results demonstrate also how that CMS induces a decrease in brain weight and hypertrophy of adrenal gland.

The chronic treatment with Harmine or Amitriptyline for 1 week reverse anhedonia, OF, EPM and LDB behaviors, brain and adrenal gland weights, normalized ACTH and immunoglobulin G (IgG) circulating levels .

In conclusion, treatment with Harmine or Amitriptyline has robust effects in reverting mainly physiological alterations reliable as indicators of animal depressive disorders.

Keywords:

CMS, Harmine, Amitriptyline, Behavioral changes, ACTH, IgG, IgM, depression.

RESUME

La présente étude vise à évaluer les effets comportementaux et physiologiques de l'administration chronique d'Harmine β-carboline et d'Amitriptyline chez des rats exposés à la procédure de CMS.

Après 30 jours d'exposition au stress chronique modéré (CMS), les rats ont traités avec l'Harmine (15mg/ kg/ j, ip.) ou l'Amitriptyline (20mg/kg/j, ip.) pendant 7 jours. Ensuite, les rats ont été soumis à une série de tests comportementaux: solutions sucrées, Open Field (OF), labyrinthe en croix surélevé (EPM) et la Boite claire obscure (LDB).

Les résultats ont montré que le CMS induit des changements comportementaux au cours des différents tests; une diminution de la consommation des solutions sucrées par rapport au groupe témoin.

Les résultats montrent également les effets du CMS sur les paramètres d'EPM; les rats stressés ont montré une diminution du nombre d'entrées et du temps passé dans les bras ouverts par rapport à des rats non stressés. Bien qu'aucune différence significative n'a été détectée chez les rats stressés par rapport aux rats témoins sur le nombre d'entrées et le temps passé dans les bras fermés.

En outre, dans le champ ouvert, nous avons constaté une diminution du temps passé dans le centre du champ, la diminution du temps de toilettage, sans affecter la défécation, l'activité locomotrice et l'escalade dans les groupes stressés par rapport au groupe témoin.

Les résultats montrent également les effets du CMS sur les paramètres de LDB. Nous avons constaté une diminution du nombre d'entrées et du temps passé dans la boite claire par rapport au groupe témoin.

En ce qui concerne les paramètres physiologiques; les résultats d'augmentation du niveau de l'hormone ACTH sont affiché par rapport au groupe témoin. Pour les paramètres immunologiques, les résultats ont révélé que le CMS a induit une augmentation d 'IgG, sans affecter les taux circulants d'IgM.

Les résultats ont montré que le CMS a induit une diminution du poids du cerveau et une augmentation du poids de la glande surrénale.

Le traitement chronique avec l'Harmine et l'Amitriptyline pour 1 semaine a inversé les comportements anhédoniques, EPM, OPF et LDB, Le poids du cerveau et des glandes

surrénales. La normalisation des taux circulants d'hormone ACTH et aussi des taux circulants de l'immunoglobuline IgG .

Les traitements avec l'Harmine et l'Amitriptyline ont des effets puissants en revenant des altérations physiologiques qui sont principalement des indicateurs fiables des troubles dépressifs des animaux.

Mots clés :

CMS, Harmine, Amitriptyline, changements comportementaux, ACTH, IgG, IgM, dépression.

الملخص

تهدف هذه الدراسة إلى تقييم الآثار السلوكية والفيزيولوجية الناتجة عن استخدام بيتا كاربولين حارمين والأميتريبتيلين على الفئران التي تم تعريضها للإجهاد المزمن المعتدل .

بعد 30 يوما من التعرض لإجراءات الإجهاد المزمن المعتدل ، تمت معالجة الفئران بجرعات متفاوتة من حارمين (15 ملغ/كغ/يوم,ح ب) والأميتريبتيلين (20ملغ/كغ/يوم,ح ب) لمدة 7 أيام . وبعد ذلك، تم اخضاع الفئران لمجموعة من الاختبارات السلوكية مثل اختبار المحلول الحلو، المتاهة (EPM) بالإضافة إلى الحقل المفتوح (OF)وصندوق الضوء و الظلام (LDB) .

بينت النتائج التي توصلنا إليها أن الإجهاد المزمن المعتدل قد أظهر تغييرات سلوكية من خلال إجراء اختبارات مختلفة انخفاض في نسبة استهلاك محلول السكروز مقارنة مع المجموعة الشاهدة.

أما بيانات اختبار المتاهة، فقد أظهرت النتائج انخفاض في عدد مرات الولوج و الوقت الذي قضته الفئران المجهدة في المجالات المفتوحة مقارنة مع الفئران غير المجهدة. بينما لم تتم ملاحظة فروق ذات دلالة إحصائية بالنسبة للفئران المجهدة مقارنة مع المجموعة الشاهدة فيما يخص عدد مرات الولوج والوقت الذي قضته الفئران في المجالات المغلقة.

أما بالنسبة للحقل المفتوح، فقد لاحظنا انخفاضا في الوقت الذي تقضيه الفئران في وسط الميدان، و انخفاضا في وقت التنظيف دون أن يؤثر ذلك على الصفق او النشاط الحركي للفئران المجهدة مقارنة مع المجموعة الشاهدة.

من خلال البيانات الإحصائية لاختبار صندوق الضوء و الظلام، لاحظنا انخفاضا في عدد مرات الولوج وأيضا في الوقت الذي قضته الفئران المجهدة في الحجرة المنيرة مقارنة مع المجموعة الشاهدة.

وفيما يتعلق بالمؤشرات الفسيولوجية، أظهرت النتائج الهرمونية زيادة في مستوى الهرمون الموجِّه لقشر الكظر(ACTH) مقارنة مع المجموعة الشاهدة.

أظهرت نتائج القياسات المناعية أن الإجهاد المزمن المعتدل سبب ارتفاعا في مستوى الغلوبولين المناعي G دون أن يؤثر ذلك على مستويات الغلوبولين المناعي M. كما بينت النتائج أن الإجهاد المزمن المعتدل يسبب انخفاض في وزن المخ و ارتفاع وزن الغدة الكظرية .

عكست المعالجة طويلة المدى بالحارمين والأميتريبتيلين لمدة اسبوع انعدام التذوق و مختلف السلوكيات : (EPM) (OF) و(LDB)، وزن كلا من المخ و الغدة الكظرية . تعديل مستويات الهرمون الموجِّه لقشر الكظر بالإضافة إلى الغلوبولين المناعيG عند الفئران المجهدة مقارنة مع المجموعة الشاهدة.

وفي الختام نستنتج أن المعالجة بالحارمين والأميتريبتيلين لها تأثيرات قوية و فعالة في احداث التعديلات خاصة الفسيولوجية منها و التي هي مؤشرات موثوقة عن الاضطرابات الاكتئابية لدى الحيوان.

الكلمات المفتاحية:

الإجهاد المزمن المعتدل ، حارمين، الأميتريبتيلين، التغيرات السلوكية، الهرمون الموجِّه لقشر الكظر، الغلوبولين المناعي G ، الغلوبولين المناعي M ، الاكتئاب.

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ACTH : Corticotropin (Adreno-Cortico-Tropic Hormone)
ANOVA : Analysis of variance
AVP : Arginine-Vasopressin
BDNF : Brain-Derived-neurotrophic Factor
β-CA : β –carboline
Bw : Body Weight
CAM : Complementary Alternative Medicine
CMS : Chronic Mild Stress
CNS : Central Nervous System
CRH : Corticotrophin Releasing Hormone
DHEA : Dehydroepiandrosterone
DYRK1A : Dual specificity tyrosine-phosphorylation-regulated kinase 1A
EDTA : Ethylene Diamine-Tetraacetic Acid-
EPM : Elevated Plus -Maze
EU-phytomedicines : European phytomedicines
FST : Forced swim test
G : Group
GnRH : Gonadotropin-releasing hormone
5-HT : 5-hydroxytryptamine serotonin
HLL : Tetrahydroharmine
HML : Harmine
HMR : Harmaline
HPA : Hypothalamic–pituitary–adrenal axis
IgG : Immunoglobuline G
IgM : Immunoglobuline M
IL-1β : Interleukin -1β
IL-6 : Interleukin 6
IMAO : Monoamine oxidase inhibitors
LC/NE : Locus Coeruleus Norepinephrine
LDB : Light Dark Box

mg/ml/Kg : milligramme per millilitre per kilogramme

NA : Noradrenaline

NHPs : Natural Health Products

NMDA : N-methyl-D-aspartate

NO : Nitric oxide

OF : Open Field

P : Probability value

PVN : Paraventricular Nucleus

SOD : Superoxide Dismutase

SSRIs : Selective Serotonin-Reuptake Inhibitors

TNF-a : Tumor Necrosis Factor

$\bar{x}$: Mean

SUMMARY

Introduction

1. INTRODUCTION

Humans have been created with a complex range of metabolic machinery intended to maintain normal homeostasis. This physiologic state of balance is susceptible to various perturbations by intrinsic and extrinsic events, whether actual or perceived (Guilliams & Edwards, 2010). The term "stress" has been coined to describe a "state of threatened homeostasis or disharmony" that must then be counteracted by an "adaptive stress response," a complex array of physiologic and behavioral responses intended to re-establish homeostasis. (Kirschbaum & Hellham, 1994). Stress may be acute (single or short exposure to stress) or chronic (long-term exposure to stress) (Nagaraja & Jeganathan, 1999).

Chronic stress may cause depression (Sakakibara *et al*., 2005). It has become the most prevalent psychiatric disorder and imposes a substantial societal burden (Lépine & Briley, 2011; Hidaka, 2012). Many signaling pathways have been shown that depression constitutes a serious and recurrent disorder often manifested with psychological, behavioral and physiological symptoms: depressed mood, loss of interest or pleasure, decreased energy, feelings of guilt or low self-worth, disturbed sleep or appetite (Armario, 2006; Wei-Wei *et al*., 2014). Several studies have also indicated that depression is related to structural and functional changes in speciefic brain regions (Vyas *et al*., 2002; Stockmeier *et al*., 2004; Oh, 2012). These disabilities appear through a disturbance of the hypothalamic-pituitary-adrenocortical (HPA) axis (Howell & Muglia, 2006); the key components of the "stress system" and one of the primary systems moderating the physiological response to psychological and physiological stressors in mammals (Sapolsky *et al*., 2000), including the release of glucocorticoids. The principal end products of the HPA axis regulate many physiological functions and play an important role in affective regulation and dysregulation (Maric & Adzic, 2013). Obviously, this dysfunction is not only affecting regulation, but it can also lead to serious metabolic, immune, cardio vascular and psychological dysfunction. Newly studies focused on the interactions between the central nervous system (CNS), the endocrine system and the immune system which have given rise to the field of psycho-neuro-immunologic researches (Fleshner *et al*.,2011) and the impact of these interactions on health are the two major pathways through which immune function can be altered, especially induces a decrease or a disruption of immunity cells , a decrease of the different subsets of

lymphoid cells in secondary lymphoid organs that correlates with a decrease of antibody levels (Moazzam *et al.*, 2013) as well as a disruption of cytokine secretion (McEwen, 2008).

Dysfunction of HPA and immune systems is in line with an illness model of depression in which the stimulus (i.e. time-dependent risk factor) continues to have an impact even when no longer present. In this model there is an immediate increase in the risk of illness following application of a stimulus; once ill. However, removing the risk factor does not necessarily lead to restitution (Bottomley *et al.*, 2010). Therefore, we need to look for treatment strategies that provide HPA functional restitution and promote resilience. Resilience is defined as the ability to maintain a state of normal equilibrium in the face of extremely unfavorable circumstances.

One of the most elegant long-term models of depression is the chronic mild stress (CMS) procedure that was first used by (Katz, 1982). In the CMS model, chronic sequential exposure of rats to a variety of mild model has been shown to induce lower consumption of sucrose postulated to reflect anhedonia (the loss of interest or pleasure) in animals. One of the core symptoms required for diagnosis of a major depressive episode in humans is reflected by a reduced consumption and/or preference of sweetened solutions. Exposure to chronic mild stress also induces significant changes in behavioral parameters (Farhan *et al.*, 2014) such as locomotive and explorative behavior; a decline in food and water intakes. It can also lead to hyper-activity of the HPA axis including adrenal hypertrophy and corticosterone hyper-secretion and loss of body weight (Jankord & Herman, 2008; Lucca *et al.*, 2009). All these symptoms are consistent with human depression (Harro *et al.*, 2001); although it has been extensively reported that stress may reduce body weight in rodents (Matuszewich & Yamamoto, 2003; Lin *et al.*, 2005).

The complexity of daily life in modern societies frequently leads to various degrees of anxiety and depression. Mood, depression and anxiety disorders have been found to be associated with chronic pain amongst medical patients in both developed and developing countries (Rupesh *et al.*,2011). Currently many experimental studies focusing on the pathophysiology of depression have examined the effects of stress and/ antidepressant in male subjects (Palanza, 2001). Therefore, the development of new drugs for the treatment of depression disorders is an important goal of neuro-psychopharmacology researches.

Most abnormal effects of CMS can be reversed by antidepressant treatments, suggesting a strong predictive validity (Zhang *et al*., 2010).

However, the exact molecular and cellular mechanism underlying the development of depression and therapeutic actions of antidepressants remain poorly understood. In recent years, researchers have focused on the interactions between the monoamine neurotransmitters and their reuptake and the receptor proteins.

Most antidepressant drugs used clinically are synthetic compounds, such as selective serotonin-reuptake inhibitors (SSRIs), tricyclic antidepressants, monoamine oxidase inhibitors, and norepinephrine reuptake inhibitors (Ping *et al*., 2014). Although these antidepressant agents can produce a rapid increase of serotonin (5-HT) and/or noradrenaline (NA) at synaptic levels, it usually takes at least 3 to 4 weeks to obtain an appreciable clinical effect (Wong & Licinio, 2001; Santarelli *et al*., 2003).

Since these applied agents present in many cases adverse effects on patients (Dhawan *et al*., 2001; Nutt, 2002; Wei-Wei *et al*., 2014). The usage of complementary and alternative medicine (CAM) is required.

Nowadays, plants are being used as a source of medicine in alternative therapy of depression (Kessler *et al*., 2001). There are over 10 medicinal plants used commercially as regulated Natural Health Products (NHPs), or EU- phytomedicines to treat mood disorders related to anxiety (Cayer, 2011). There is a growing interest in these products among some researchers because patient compliance is high and evidence of efficacy and fewer drawbacks are available.

Medicinal plants have been an important source for the discovery of new bioactive compounds which served and continue to serve as lead molecules for the development of new drugs. (*Banisteriopsis capi*) and (*Peganum harmala*) are most widely used as medicinal plants; this may be due to the high level of pharmacologically active alkaloids produced mainly of β-carboline (β-CA) as Harmine (HMR) harmaline (HML) and tetrahydroharmine (THH) (McKenna, 2004). These medicinal plants have been studied pharmacologically, phytochemically, extensive animal behavior and clinical researches supporting their efficacy are found.

In this regard, Harmine a naturally occurring β-carboline alkaloid has long been used in folk medicine in the Middle East and in Asia (Sourkes, 1999) and as a hallucinogenic drug (Wink & Van Wyk, 2008). Harmine present the most abundant and active ingredients of several plants such as Grevia bicolor (Malvaceae), Passiflora incarnate (Passifloraceae), *Banisteriopsis capi* and *Peganum harmala* (Zygophyllaceae) (Kartal *et al*., 2003; Cao *et al*., 2007). Harmine was first isolated from the seeds of *Peganum harmala L* in 1874 (Zhao & Wink, 2013).

Harmine possesses a broad range of pharmacological activities such as anxiolytic and behavioral effects (Brierley & Davidson, 2012). Some studies demonstrated that Harmine contains significant anti-tumor potential both in vitro and in vivo (Martín *et al*., 2004; Cao et al., 2013). Hypothermic, antibacterial (Arshad *et al*, 2008), antioxidant, hallucinogenic, cytotoxic, (Cao *et al*., 2007; El Gendy & El-Kadi, 2012). Insecticide (Bouayad *et al*., 2012), to activities in complex processes of bone regeneration (Yonezawa *et al*., 2011). Furthermore, antiplasmodial activity (Astulla *et al*., 2008), vascular (Berrougui *et al*., 2006) muscle, antimutagenic, antiplatelet properties, and antigenotoxic activities (Moura *et al*., 2007, Im *et al*., 2009). Similarly analgesic, powerful anti-inflammatory (Monsef *et al*., 2004; Shahverdi *et al*., 2005), is an antidiabetic agent (Vollmayr & Henn, 2003), cholesterol lowering, with hepato-protective effects (Hamden *et al*., 2007) and immune modulator influences (Wang *et al*., 1996). But the main studies are focused on the activity of the substance on the nervous system, that's why some of these properties may explain why Harmine was found to have significant antidepressant actions (Kim *et al*., 1997; Glennon *et al*., 2000; Husbands *et al*.2001; Halberstadt *et al*., 2008). Altogether, many researches have been reported that Harmine have a wide range of neurotrophic and neuroprotective effects (Li *et al*., 2011, Zhong *et al*., 2015).

In fact, systemic administration of Harmine has been shown to induce antidepressant- like actions in animals subject to forced swimming test (Farzin & Mansouri, 2006; Fortunato *et al*., 2009).

Aims of the study:

The overall objectives of the thesis are to assess:

- The construct validity of the CMS model that we introduced a few modifications in our laboratory.

a) Administration of stressors.

b) Assessment of anhedonia (sucrose/saccharine intake).

c) Evaluation of pathophysiological and neurobiological aspects of depression in the CMS model:

  - Exploration activity and emotionality in various behavioral tests.
  - Plasma ACTH.
  - Plasma IgG and IgM .

- We examined if there was any behavioral measure which correlates with the changes of the hedonic reactivity due to CMS.
- The ability of chronic administration of Harmine and Amitriptyline to regulate disorders after CMS in a battery of animal behavior paradigms.
- The effects of Harmine or Amitriptyline chronic treatment on the altered physiological responses mainly ACTH circulating levels and humoral immune response of wistar rats after CMS.

Materials & Methods

2. MATERIALS AND METHODS

2.1. Animals and Housing

Adult male albino *Wistar* rats were purchased from the Pasteur institute of Kobba (Algiers) with body weight 200-331 g. The laboratory rats, *Rattus norvegicus* belongs to the order Rodentia and family Muridae (Fallon, 1996).Unlike wild ones, they are year round breeders, omnivorous and will burrow if given the chance. They are nocturnal animals (Würbel *et al*., 2009; Lennox & Bauck, 2011) but adapt to their environments.

Laboratory rats are shorter lived two to four years (Ballam *et al*., 2000; Bulliot, 2004) dependent genetic and environmental factors. Puberty occurs between (45 –75) days of age for male rats (4 ~ 8 weeks) (Wu & Gore, 2010) with some inbred strains maturing sexually at 3-4 months of age. Laboratory rats are docile, very adaptable, curious intelligent and easily trained. Rats are most commonly housed in solid cages (50× 60×53 cm^3) constructed of durable plastic and bedding in special circumstances such as stainless steel cages. A contact bedding material such as wood chips, particles should be placed in the bottom of cages. The study was carried out in the animalery of the University Badji Mokhtar-Annaba.

The animals were housed ten per cage with access to food and water *ad libitum*. They were maintained in under natural conditions of temperature, photoperiod and relative humidity (Fig.1). Animals were weighted and divided into six experimental groups as follows: (1) Control - Saline, (2) Control - Amitriptyline ; (3) Control - Harmine, (4) Stressed - Saline; (5) Stress - Amitriptyline ,(6) Stress - Harmine .They were allowed to acclimatize for a period of 30 days. Animals in the control group were reared in single cages without any environmental stresses unlike animals in CMS were entered into the CMS procedure.

Figure 1. Rat housing conditions.

2.2. Drug and Treatment

The drugs used in this study are:

- Harmine (C13H12N2O) is a tricyclic beta-carboline alkaloid .It was purchased from Cayman Chemical (USA) (CAS 343-27-1, approx. 98% purity) (Fig.2).The dose was chosen based on (Garcia *et al*., 2008a, b).

- Amitriptyline (C20H23N) is a tricyclic standard antidepressant (for many years it has been considered as one of the reference compounds for the pharmacological treatment of depression) was purchased from pharmaceutical industry Gencopharm (ZI Rouiba, Route C BP 73, Algeria) (Fig.3).

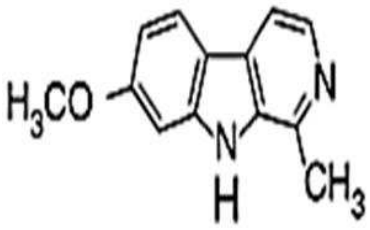


Figure 2. Chemical structure of Harmine (7-methoxy-1-methyl-9H-pyrido [3, 4-b] indole) (Wang *et al*., 2015).

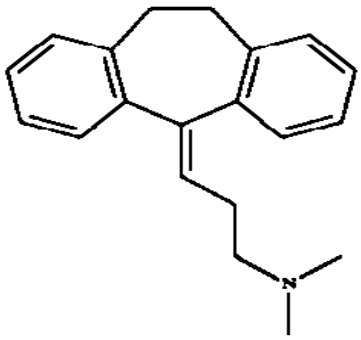


Figure 3. Chemical structure of Amitriptyline [3-(10, ll-dihydro-5H-dibenzol [a,d] cyclohept-5-ylidene) propyldimethylamine] (Soni *et al*.,2012).

To develop this study we used 60 animals (n=10 animals in each group) separated into six groups, as follows:

- ✓ G1: Control + Saline; Group treated with saline 1ml / kg bw.
- ✓ G2: Control + Amitriptyline; Group treated with 20 mg/1ml / kg bw.
- ✓ G3: Control + Harmine; Group treated with 15 mg/1ml / kg bw.

G1; G2; G3: served as a control group

- ✓ G4: Stressed + Saline; Group treated with saline 1ml / kg bw.
- ✓ G5: Stressed + Amitriptyline; Group treated with 20 mg/1ml / kg bw.
- ✓ G6: Stressed + Harmine; Group treated with 15 mg/1ml / kg bw.

All groups were treated intraperitoneally (i.p.) with saline, Harmine or Amitriptyline respectively according to body weight (Carbajal *et al*., 2009) (Fig.4).

All the treatments were administered in a volume of 1 ml/kg.

The chronic treatment was performed during the anhedonia test once a day during 7 days, 30 min before the behavioral evaluation. Physiological Saline (0.9%) was used as a control vehicle for this experiment. The drugs were freshly prepared and the unused portions of drug solutions were discarded after 24 h.

Figure 4. Drug was administered intraperitoneally (i.p) at (1 mg/kg/day) on each rat.

2.3 Experimental Protocol

The CMS used was developed on the basis of the previous experiments with minor modifications (Willner *et al.*, 1992; Xiong *et al.*, 2011). This model used to achieve depressive-like symptoms in wistar rats (Willner *et al.*, 1987; Jayatissa *et al.*, 2006). Also it is used for screening novel antidepressant treatments and investigating the neurobiology of depression and its relation to other diseases (Fawcett *et al.*, 1983; Willner *et al.*, 1987). During this experiment, the rats in the control group were left undisturbed in their home cages in a separate room, without any stress, receiving only ordinary daily care with supports of food and water, whereas the rats in CMS groups exposed to CMS procedure. Rats were subjected once a day for 30 days to one of the following stressors such as (i) forced swimming for a duration of 10 or 15 min on days 1, 15, 21, 25 ;(ii) 24 h water deprivation on days 5, 10, 14, 19,30 ; (iii) 24 h food deprivation was applied on days 6, 13, 20,26; (iv) 1–1,5 h restraint on days 2, 3, 4, 7, 8, 9 ; and 3-4 h restraint on days 16- 29 13, 26 (v) and no-stress on days 11-12-27. Stressor stimuli were applied at different times every day, in order to minimize its predictability. The Restraint test was model described by (Bardin *et al.*, 2009). Animals were restrained by gentle wrapping of their upper and lower limbs with masking tape, then returned to their cages. The Forced swimming was carried out by placing the animal in a glass tank measuring 50×47 cm with 30 cm of water at 23±2°C (Porsolt *et al.*, 1977). After 30 days of chronic mild stress protocol, one group of rats was treated with Harmine (15 mg/kg/day) and another group was treated with Amitriptyline (20 mg/kg/day) once a day across 7 days. The body weight was determined throughout the experiment.

At the end of the experimental procedure, rats were killed by decapitation and the blood was collected. Then, the organs of each animal of the different groups were removed and weighed. The stressed group was exposed to water and food deprivation prior to sucrose intake, in all other cases, food and water were freely available in their home cages (Willner *et al*.,1992 ;Gronli *et al*., 2005).Individual stressors and length of time applied each day are listed in Table 1.

Table 1

❖ The protocol and stress procedures for chronic mild stress

Day of treatment	Stressor used	Duration
Day 1	Forced swimming	10 min
Day 2	Restraint	1h
Day 3	Restraint	1h
Day 4	Restraint	1h
Day 5	Water deprivation	24h
Day 6	Food deprivation	24h
Day 7	Restraint	1.30
Day 8	Restraint	1.30
Day 9	Restraint	1.30
Day 10	Water deprivation	1.30
Day 11	No stressor applied	-
Day 12	No stressor applied	-
Day 13	Food deprivation	24h
Day 14	Water deprivation	24h
Day 15	Forced swimming	15min
Day 16	restreint	2h
Day 17	restreint	2h
Day 18	restreint	2h
Day 19	Water deprivation	24h
Day 20	Food deprivation	24h
Day 21	Forced swimming	10 min
Day 22	restreint	3h
Day 23	restreint	3h
Day 24	restreint	3h
Day 25	Forced swimming	15 min
Day 26	Food deprivation	24h
Day 27	No stressor applied	-
Day 28	restreint	4h
Day 29	restreint	4h
Day 30	water deprivation	24h

2.4. Behavioral Assessment

Organism reaction to stressor or any environmental change is an important adaptive response that mobilizes the organism and re-establishes homeostasis. As known, Laboratory rats tend to be social animals, docile and curious but within the stressful procedures that may result in either adaptive or maladaptive reactions. Therefore, there are numerous behavioral paradigms using several conflict procedures, social interaction or exploration of novel environments have been proposed as animal models of anxiety. In this study, we carried out on using the following employed paradigms to assess anxiety-like and depression related behavior modification by pharmacological agents in rodents: *Anhedonia test*, *Elevated Plus Maze* (*EPM*), *Open Field* (*OF*) and *Black & White Box test*. These tests were performed at the end of CMS procedures or treatment.

2.4.1. Sucrose Preference Test (Anhedonia Test) (Willner *et al.,* 1987)

The sucrose preference is considered as an index of anhedonia a core symptom of depression (Wann *et al*., 2010; Shi *et al*., 2012). The test is a two-bottle choice paradigm performed according to the procedure as described (Bolanos *et al*., 2008, Shi *et al*., 2012). Briefly, rats were given access to two bottles (water and 1 % sucrose solution) freely for 7 days. The position of the 250-mL bottles containing sucrose solution or tap water was alternated each day, to prevent location preference. The sucrose consumption test was performed (1st day of behavioral test) by presenting both sucrose solution and tap water in the morning (08:00 am). The bottles were weighed after 24 h (the next morning). The rats are typically subjected to an acclimatization period (Dagytė *et al*., 2011) where they are trained to consume the sucrose solution (Wang *et al*., 2013) and then deprived of food and water prior to testing so as to motivate them to drink.

Sucrose preference was calculated according to the following formula:

Sucrose preference was calculated as the percentage of sucrose solution ingested relative to the total amount of liquid consumed (Willner *et al*., 1987, Sclafani & Ackroff, 2003). Behavioral changes were measured in the following tests;

- Elevated plus-maze (EPM**).**
- Open Field (OF).
- Light dark box (LDB).

2.4.2. Elevated Plus Maze (EPM) (Montgomery, 1955)

The elevated plus maze has strong claims to validity as an animal model of anxiety, is based on a rat's natural fear of open, unprotected, and elevated spaces (Pellow *et al.*, 1985; Parihar *et al.*, 2011). The apparatus and the testing procedure were carried out as originally described by (Pellow *et al.*, 1985). Frequently, it was used to measure the exploratory and anxiety levels in rodents and to screen potential anxiolytic drugs. The EPM apparatus was made of wood and consisted of two opposite open arms (50×10 cm) had no walls and the other two closed arms (50×10 cm) had 45 cm high walls made of clear Plexiglas. The open and closed arms were connected by a central square (10×10 cm) (Montgomery, 1955; Roy, 2002) and were elevated 50 cm above the floor. Rats from each group were placed in the central square of the EPM facing an open arm and then were allowed to freely explore the apparatus. And their activity was videotaped for 5 min. The following behaviors were scored during the test:

- ✓ The number of entry in each arm, which was mostly, used as measures of general motor activity (Dawson *et al.*, 1995; Weiss *et al.*, 1998; Costa *et al.*, 2012).
- ✓ The time spent exploring both open and closed arms were recorded (s).

An individual entry was recorded when the animal entered the arm with at least two front paws and half of its body. The shorter is the time spent in opens arms, the higher is anxiety and vice versa (Ho *et al.*, 2002; Mechan *et al.*, 2002). After 5 minutes, rat was removed from the maze by the base of their tails and returned to their home cage. The maze was than cleaned with a solution of 30% ethanol and soft paper permitted to dry between tests.

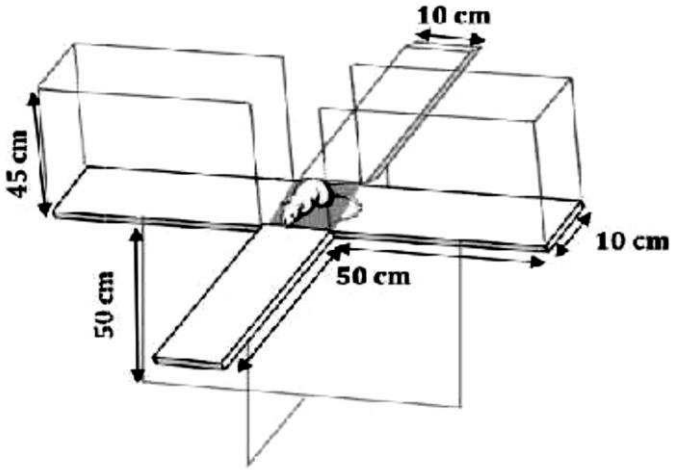


Figure 5. Elevated plus-maze test (EPM) (Handley & Mithami, 1984).

2.4.3. Open Field Test (OFT) (Hall, 1934)

The open field test has been performed as previously reported (Hallam *et al*., 2004). The test has been used to assessing the emotionality, the spontaneous locomotor activity, the exploratory and anxiety behavior of animals (Pellow *et al*., 1985; Swiergiel & Dunn, 2007; Calabrese, 2008; Mesquita *et al*., 2008; De Paiva *et al*., 2010). It was performed after the EPM test. When the elevated plus maze test was finished, the rat was immediately placed into the open field.

The rats were treated with Harmine (15 mg/kg), Amitriptyline (20 mg/kg) and saline 60 min before the exposure to the open field apparatus, in order to assess possible effects of drug treatment on spontaneous locomotor activity. The test was performed between 08:00 and 11:00 h. The open-field apparatus is circular arena 70 cm×70 cm surrounded by 40 cm high walls made of white plywood with a frontal glass wall. The floor of the open-field was divided into 9 rectangles (10 cm×10 cm each) by black lines (Frey *et al*., 2006). At the start of the test, each animal were gently placed in the central square of the open field and rats received one 10 minute exploration session to help them habituate to the apparatus and assess overall activity level .The following behaviors were recorded by a video camera for 5 min:

- ✓ The time spent (s) in the center was measured as an anxiolytic indicator (Prut & Belzung, 2003; Grivas *et al*., 2013).
- ✓ The time spent in the peripheral zone of the arena (s).

- ✓ The number of rearing's (standing on the hind paws)
- ✓ Time spent (s) in rearing in each of the open field zones.
- ✓ The number of crossing.
- ✓ Grooming time (s) (rubbing the body with paws or mouth and rubbing the head with paws). (Prut & Belzung 2003).
- ✓ Defecation: The number of (fecal pellets) excreted during open field exploration (Hall, 1934; Qi, 2006)

After each trial, the plate was cleaned each time after testing a rat to minimize circadian differences in behavior.

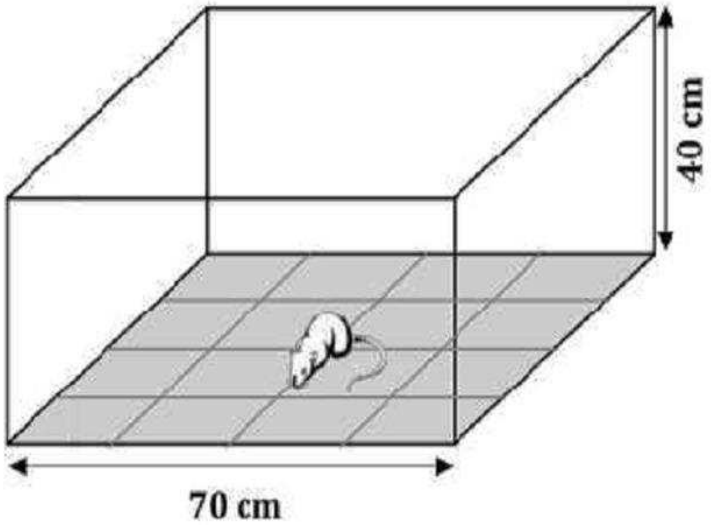


Figure 6. Open Field Test (Hall, 1934).

2.4.4. Light Dark Box Test (Shimada *et al.*, 1995)

Light dark box allows easy and quick evaluation of an animal's anxiety as reflected in their behavior. The test identifies behavioral modifications resulting from pharmacological agents.

Activity in LDB is used to assess anxiety behavior in rodents (Shimada *et al.*, 1995). Briefly, the apparatus consisted of clear Plexiglas cage (70×70×40 cm) separated in two compartments with an access (10x10cm) at floor level. The open compartment was open topped, transparent and brightly illuminated by a 60 Walt desk lamp overhead and the other was made of black Plexiglas. It was cover on top also by black Plexiglas. Animals were placed in the dark box

facing the opening and were allowed 10 min of free exploration. The following behaviors were recorded using a video camera:

- ✓ The number of entries (all four paws) into the light area, which is defined as the placement of all four paws in the compartment of the activity box (Bourin & Hascoët, 2003).
- ✓ The time spent in the light area (s).

After performing tests the rat is returned to home cage and the apparatus was cleaned thoroughly between subjects (Bailey & Crawley, 2009).

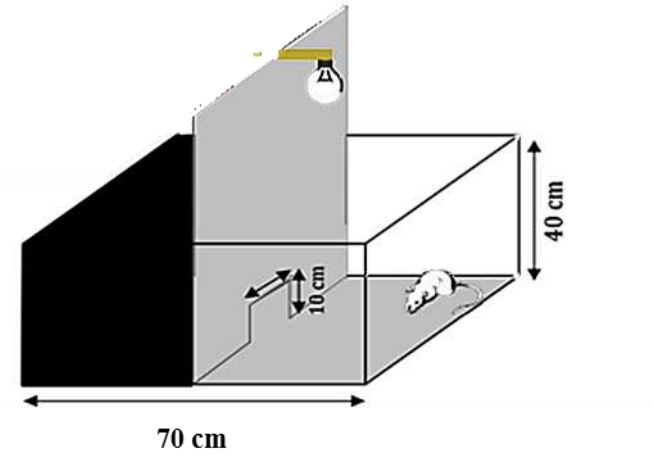


Figure 7. Light dark box test (Shimada *et al*., 1995).

2.5. Methods

2.5.1. Body Weight

Animal body weight was measured at the beginning (1st day) of the chronic mild stress protocol, and 7 days after completing treatment (37th day).

2.5.2. Blood Analysis:

On the seventh day of consumption of sweet water, immediately after the last testing session, under ether anesthesia, blood samples taken in the morning between 8:00 am and 10:00 am from jugular vein (Toft *et al*.,2006) , then was collected in tubes containing;

- Ethylenediaminetetraacetic acid (EDTA) for determination of adrenocorticotropic ACTH plasma levels (Katz, 1981; Gamaro *et al*., 2003).

- However blood collected in tubes containing heparin, used for determination of immunological parameters.

Figure 8. Photographs illustrating (A) Anesthesia; (B) Blood collection; (C) Dissection; (D) Organs removed.

2.5.3. Organ Weight

Immediately after decapitation, the following organs were removed and dissected out through laparotomy, carefully pruned from surrounding fat and tissues and slowly cleaned using filter paper saturated in 0.9% NaCl solution and weighed in precision scale (SCALTEC SBC 51) (Bikas *et al.,* 2002).

- Adrenal gland and brain weight were used in this study as an indirect parameter of hypothalamic-pituitary-adrenal axis activation (Katz, 1981; Gamaro *et al.,* 2003)**.** When the left and right adrenals were weighed together.
- Thymus (the immune system organs).
- Liver (the digestive system organs).
- Heart (the circulatory system organs).

The relative weight of organs is calculated using the formula (Talip *et al*., 2013):

Relative weight (mg /g BW) = (organ weight/ body weight)

All values represent relative (organ weight/ body weight (mg/ g)).

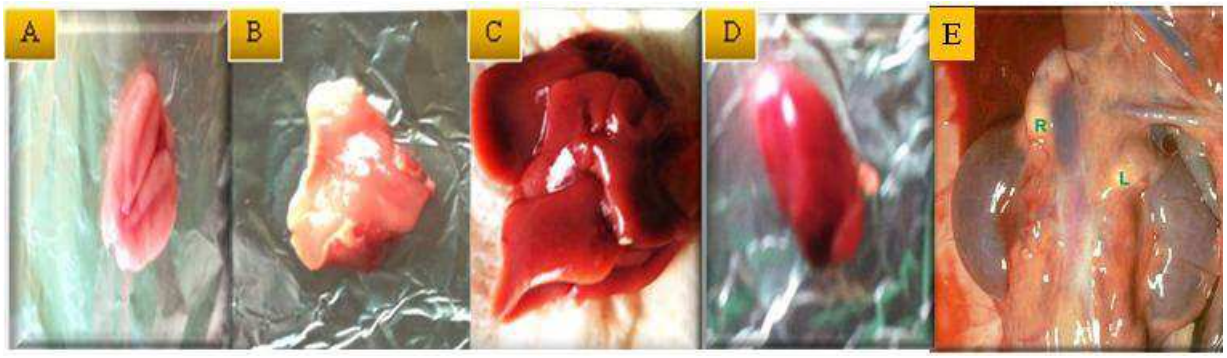


Figure. 9 Photographs illustrating the removed organs (A) Brain; (B) Thymus; (C) liver; (D) Heart; (E) Adrenal gland.

2.5.4. ACTH, IgG and IgM Assays

At the end of the experiment, ten rats from each group were randomly chosen for blood analysis. Blood samples were taken in the morning. The tubes were kept at room temperature for approximately half-hour to allow clotting of blood. Then serum was centrifuged with 5000 tr/min during 15 mn, the plasma separated was used for determination of ACTH, IgG and IgM levels. The plasma of each parameter concentration were measured using commercially available radioimmunoassay kits (both from sigma - Aldrich, USA).

2.5.4.1. Measurement of Plasma ACTH Level (Katz, 1981)

Plasma ACTH was measured by (Katz, 1981) (following the technical bulletin).

2.5.4.1.1. Assay Procedure

1. Secure the desired number of coated wells in the holder.

2. Add 200 µL of standards or calibrators, specimens and controls into appropriate wells. Freeze (-20°C) the remaining calibrators and controls as soon as possible after use.

3. Add 25 µL of Reagent 1 (Biotinylated Antibody) to each well.

4. Add 25 µL of Reagent 2 (Enzyme Labeled Antibody) to each well.

5. Cover the plate with aluminum foil to avoid exposure to light and incubate for 2 hours at room temperature (20-25°C) with shaking.

6. Remove liquid from all wells. Wash wells five times with 300 µL of 1X wash buffer. Blot on absorbent paper towels.

7. Add 100 µL of luminol substrate to all well.

- Read the relative light units in each well using Luminometer (0.2-1 second integration time) within 5 minutes of substrate addition.

2.5.4.2. Quantitative Determination of IgG and IgM (Whicher *et al.*, 1982)

2.5.4.2.1. IgG Assay Procedure

1. Secure the desired number of coated wells in the holder.

2. Dispense 100 ml of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).

3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.

4. Aspirate the contents of the microtiter wells and wash the wells 5 times with 1x wash solution using a plate washer (400 ml/well). The entire wash procedure should be performed as quickly as possible.

5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash buffer.

6. Add 100 ml of enzyme conjugate reagent into each well.

7. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.

8. Wash as detailed in 4 to 5 above.

9. Dispense 100 ml of TMB Reagent into each well.

10. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.

11. Stop the reaction by adding 100 ml of Stop Solution to each well.

12. Gently mix. It is important to make sure that all the blue color changes to yellow.

13. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

2.5.4.2.2. IgM Assay Procedure

1. Secure the desired number of coated wells in the holder.

2. Dispense 100 µl of standards and diluted samples into the wells (we recommend that samples be tested in duplicate).

3. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.

4. Aspirate the contents of the microtiter wells and wash the wells

5 times with 1x wash solution using a plate washer (400µl/well). The entire wash procedure should be performed as quickly as possible.

5. Strike the wells sharply onto absorbent paper or paper towels to remove all residual wash buffer.

6. Add 100 µl of enzyme conjugate reagent into each well.

7. Incubate on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 45 minutes.

8. Wash as detailed in 4 to 5 above.

9. Dispense 100 µl of TMB Reagent into each well.

10. Gently mix on an orbital micro-plate shaker at 100-150 rpm at room temperature (18-25°C) for 20 minutes.

11. Stop the reaction by adding 100 µl of Stop Solution to each well.

12. Gently mix. It is important to make sure that all the blue color changes to yellow.

13. Read the optical density at 450 nm with a microtiter plate reader within 5 minutes.

2.5.5. Statistical Analysis (Daniel & Cross, 2010)

Data and interactions (body and organs weight, sucrose preference test, open field, elevated plus maze, light dark box tests, ACTH, IgG and IgM plasma level) were evaluated by one-way analysis of variance ANOVA was performed for multiple comparisons followed by Tukey post hoc test. Student t test and student t test for paired data. All results are expressed as mean ± standard error of the mean (SEM).

All analysis was performed using Statistic Minitab version 16.0 for windows. The level of probability was set at ($p < 0.05$) as statistically significant.

2.5.5.1. The Multiple Comparison Method

2.5.5.1.1. Tukey's Method

The Tukey (also called Tukey-Kramer in the unbalanced case) method is extensions of the methods used by one-way ANOVA. The Tukey approximation has been proven to be conservative when comparing three means. "Conservative" means that the true error rate is less than the stated one. In comparing larger numbers of means, there is no proof that the Tukey method is conservative for the general linear model.

Tukey method used in ANOVA to create confidence intervals for all pairwise differences between factor level means while controlling the family error rate to a level you specify. It is important to consider the family error rate when making multiple comparisons because your chances of making a type I error for a series of comparisons is greater than the error rate for any one comparison alone. To counter this higher error rate, Tukey's method adjusts the confidence level for each individual interval so that the resulting simultaneous confidence level is equal to the value you specify.

The ANOVA resulted in a p-value of 0.01, leading you to conclude that at least one of the manufacturer means is different from the others.

Tukey' confidence intervals provide less precise estimates of the population parameter but limit the probability that one or more of the confidence intervals does not contain the true difference to a maximum of 5%. Understanding this context, you can then look at the confidence intervals to see if any do not include zero, suggesting a significant difference.

Some characteristics of the multiple comparison methods are summarized below:

Table 2 The characteristics of the multiple comparison methods

Comparison method	Properties
Tukey	all pairwise differences only, not proven to be conservative

2.5.5. 2. Sample T-Test or Student T Test

A hypothesis test for two populations means to determine whether they are significantly different. This procedure uses the null hypothesis that the difference between two population means is equal to a hypothesized value (H_0: $U_1 - U_2 = U_0$), and tests it against an alternative hypothesis, which can be left-tailed ($U_1 - U_2 < U_0$), right-tailed $U_1 - U_2 > U_0$), or two-tailed $U_1 - U_2 \neq U_0$).

The 2-sample t-test analyzes the difference between these two means to determine whether the difference is statistically significant. The hypotheses of a two-tailed test would be:

- H_0: $U_1 - U_2 = 0$ (seatbelt strengths from both companies are equal)
- H_1: $U_1 - U_2 \neq 0$ (seatbelt strengths from both companies are different)

If the test's p-value is less than our chosen significance level, we should reject the null hypothesis. To conduct a 2-sample t-test, the two populations must be independent; in other words, the observations from the first sample must not have any bearing on the observations from the second sample.

2.5.5. 3. Paired T-Test

A hypothesis test for the mean difference between paired observations that are related or dependent. The paired t-test is useful for analyzing differences between twins, differences in before-and-after measurements on the same subject, and differences between two treatments given to the same subject. This procedure tests the null hypothesis that the true mean difference within pairs ($\mu_d = \mu_1 - \mu_2$) is equal to a hypothesized value (H_0: $\mu_d = \mu_1 - \mu_2 =$

μ_0). The alternative hypothesis can be left-tailed ($\mu_d < \mu_0$), right-tailed ($\mu_d > \mu_0$), or two-tailed ($\mu_d \neq \mu_0$).

A paired t-test can be more powerful than a 2-sample t-test because the latter includes additional variation arising from the independence of the observations. A paired t-test is not subject to this variation because the paired observations are dependent. Also, a paired t-test does not require both samples to have equal variance. Therefore, if you can logically address your research question with a paired experimental design, it may be advantageous to do so, in conjunction with a paired t-test, to gain more statistical power.

- **p-value (P)**

Used in hypothesis tests to help you decide whether to reject or fail to reject a null hypothesis. The p-value is the probability of obtaining a test statistic that is at least as extreme as the actual calculated value, if the null hypothesis is true. A commonly used cut-off value for the p-value is 0.05. For example, if the calculated p-value of a test statistic is less than 0.05, you reject the null hypothesis.

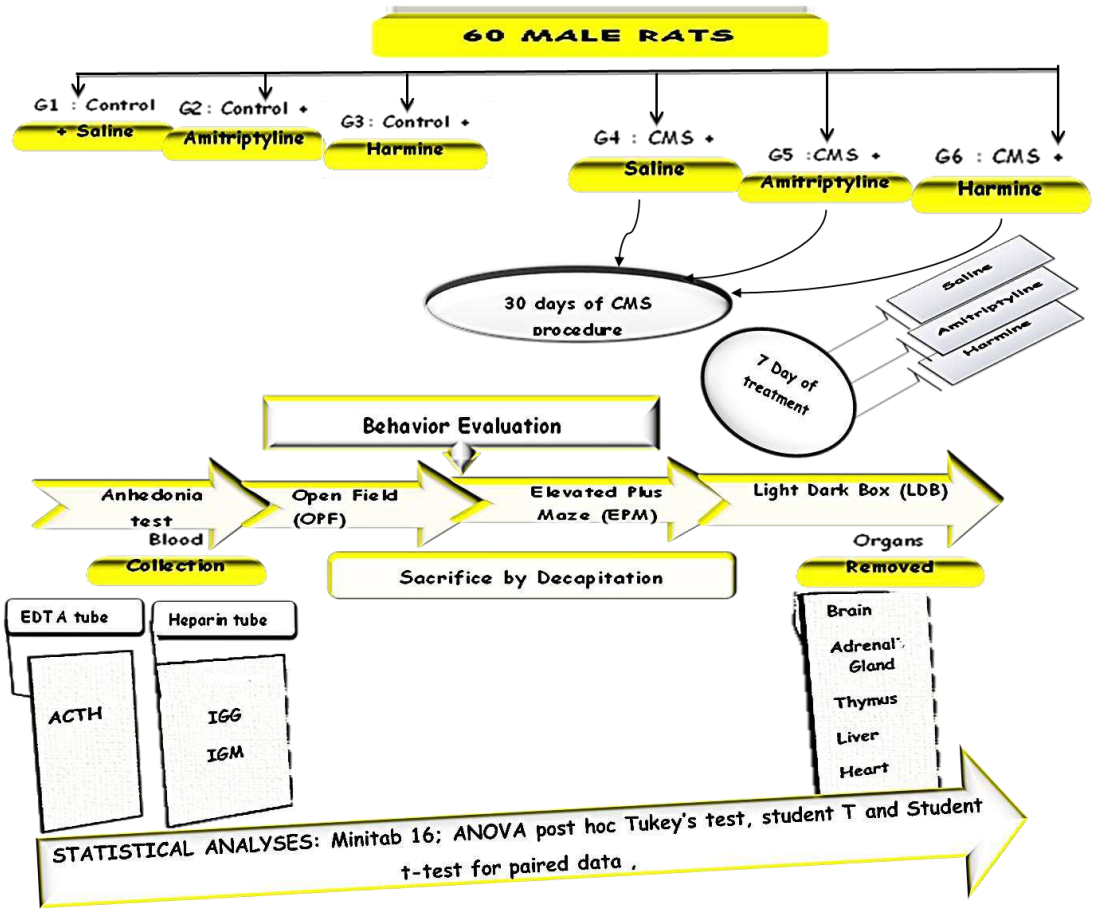


Figure10. Schematic representation of the experimental protocol; CMS procedure and treatments using Harmine or Amitriptyline.

Results

3. RESULTS

3.1. Body Weight Gain

The effects of the CMS paradigm in body weight are illustrated in Fig. 11. It should be mentioned that at the beginning of the experimental procedure, no body weight differences were observed between groups (F=0.04; p=0.999). However, CMS rats treated with saline failed to gain weight (t=-1.26; df=18; p=0.225) during the 30 days of observation. Also, CMS animals failed to gain weight after the treatment with Harmine or Amitriptyline compared with the rats' body weight assessed at the beginning of the experiment (t=-1.86; df=18; p=0.080) (t=-2.05; df=18; p=0.055).

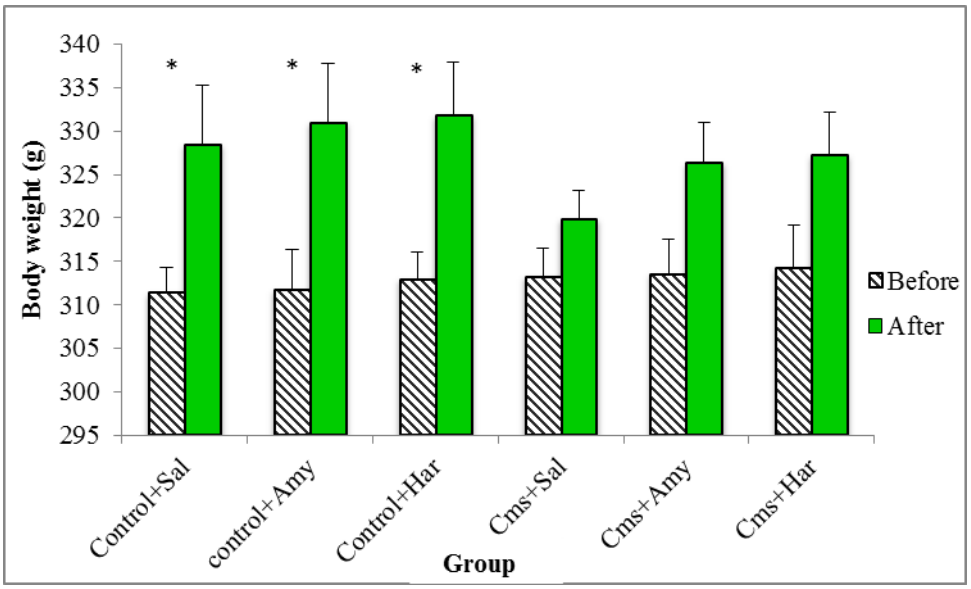


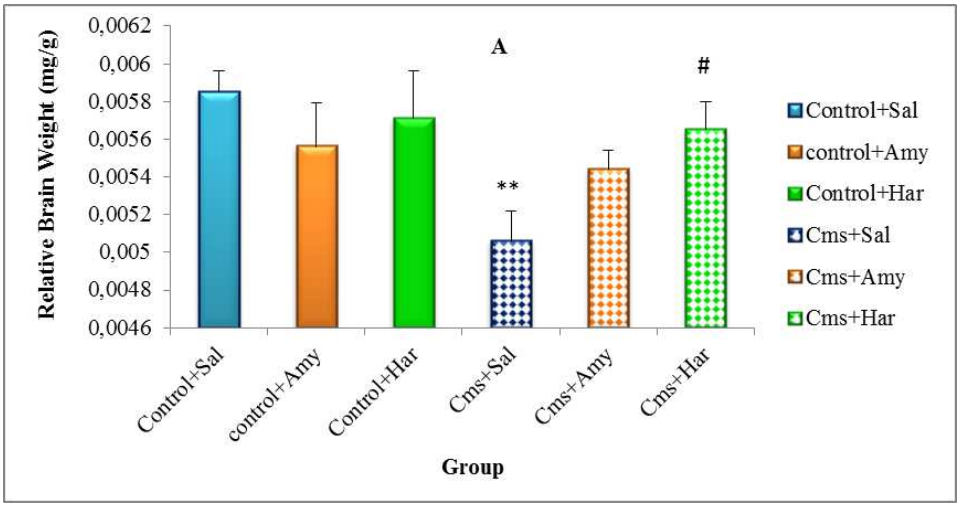
Figure 11. Effects of CMS procedure on body weight of rats repeatedly treated with Harmine (Har; 15 mg/kg i.p.) and Amitriptyline (Amy; 20 mg/kg i.p.) on body weight gain in rats. 30 days of CMS were followed by 1 week of drug treatment. Bars represent means ± SEM (n=10 per group). Ns: No significant difference p >0, 05 vs. control saline, according to the Student t-test for paired data.

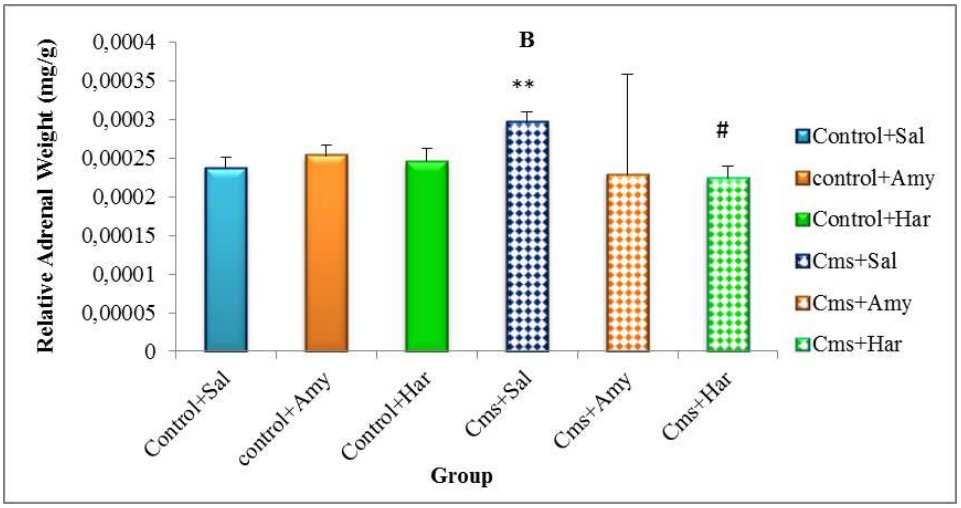
3.2. Variation of Organs Weight

The effects of the CMS protocol in organs weight are summarized in the Fig. 12. As compared to the mean values of stressed rats treated with saline to non-stressed rats injected with saline, significantly lower mean relative brain weight (F=2.51; p = 0.041; Fig. 12A). Also, CMS procedure induced an increase of relative adrenal gland weight in stressed rats

treated with saline compared with non-stressed rats (F = 3.38; p =0.010; Fig. 12B) and were statistically different from all other treated groups.

Whereas no statistical significant alterations were observed in thymus , liver and heart weight (F= 0.29; p= 0.914; Fig. 12C),(F= 1.51; p =0.186; Fig. 12D),(F= 1.60 ; p =0.175; Fig. 12E) .Interesting enough, the treatment with Harmine or Amitriptyline for 1 week re-established to a normal range the brain and adrenal gland weight in stressed rats.





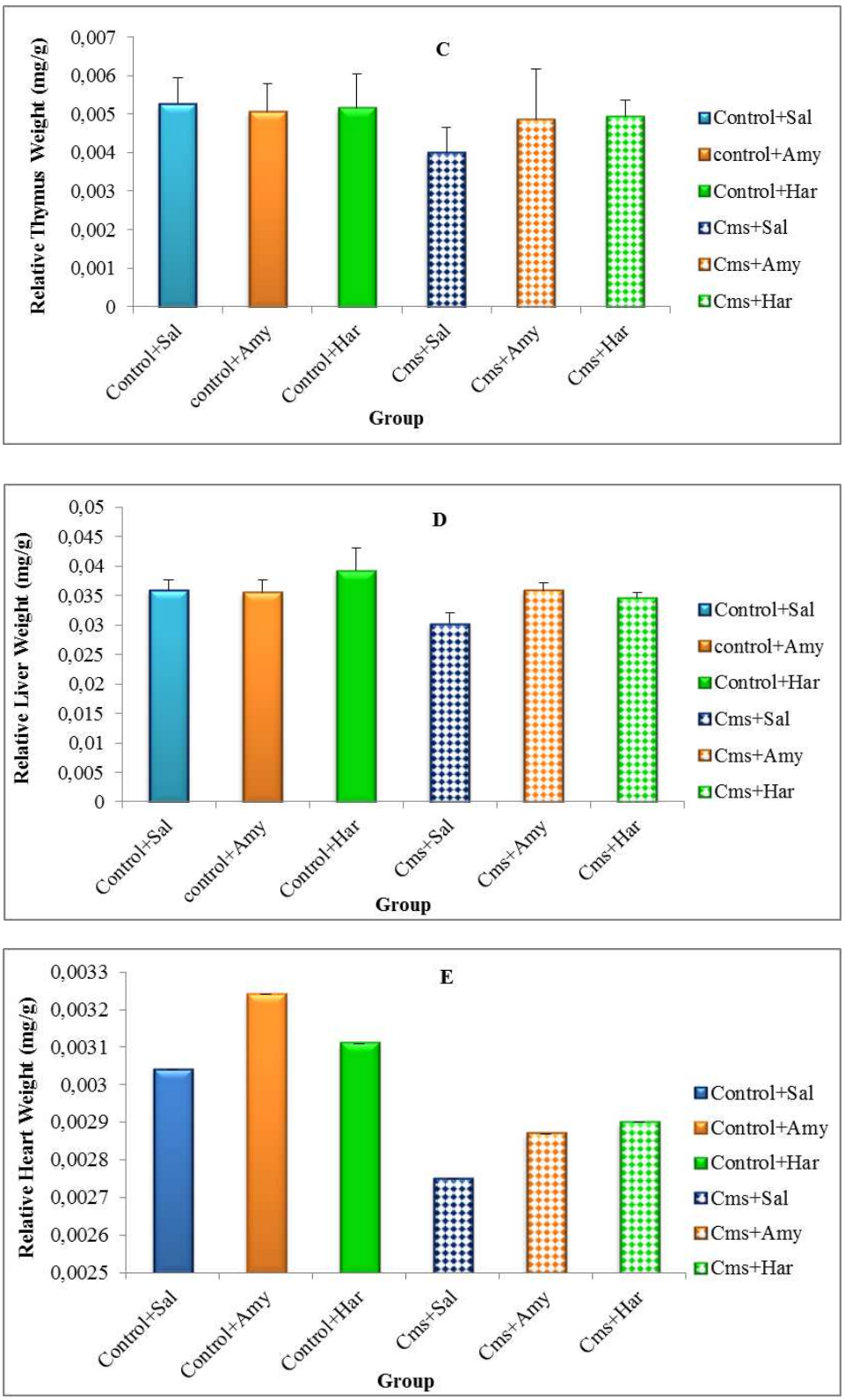


Figure 12. Effects of CMS procedure on the relative weight of (A) the brain, (B) adrenal gland, (C) thymus, (D) liver and (E) heart in rats repeatedly treated with Harmine (Har; 15 mg/kg, i.p.) and Amitriptyline (Amy; 20 mg/kg, i.p.).Bars represent means ±S.E.M. Ns: No significant difference p >0, 05.**p<0 .01 vs. control saline; #p < 0.05 vs. CSM saline, according to ANOVA post hoc Tukey's test.

3.3. Behavioral Assessment

The results of various employed paradigms have been used in this study *Anhedonia, Elevated plus maze (EPM), Open Field (OPF) and Light Dark Box test are* discussed below:

Table 3. Effect of Harmine and Amitriptyline on Gross behavioral parameters.

Parameters	CMS	Amitriptyline	Harmine
Grooming	-	+	+
Crossing over	Normal	Normal	Normal
Defecation	Normal	Normal	Normal
Urination	Normal	Normal	Normal
Lacrimation	Normal	Normal	Normal
Dilates pupil of eye	Normal	**+**	Normal
Hair follicles	Piloerection	Normal	Normal

3.3.1. Sucrose Preference Test

CMS gradually reduced the consumption of the sucrose solution. As compared in the baseline test, 1 week later the sucrose intake was reduced in the stressed animals. The difference in sucrose intake between the control and stressed groups treated with saline ($p< 0.05$). As shown in Figure.13, unstressed rats treated with Har, Amy and saline presented no significant variation in their preference for sucrose consumption. But in the stressed animals, when compared at this week, to the treatment with Har or Amy caused a gradual recovery of the sucrose intake. Stressed animals repeatedly treated with Har for one week showed significant improvement and there was no significant difference between the drug-treated stressed and vehicle-treated control animals.

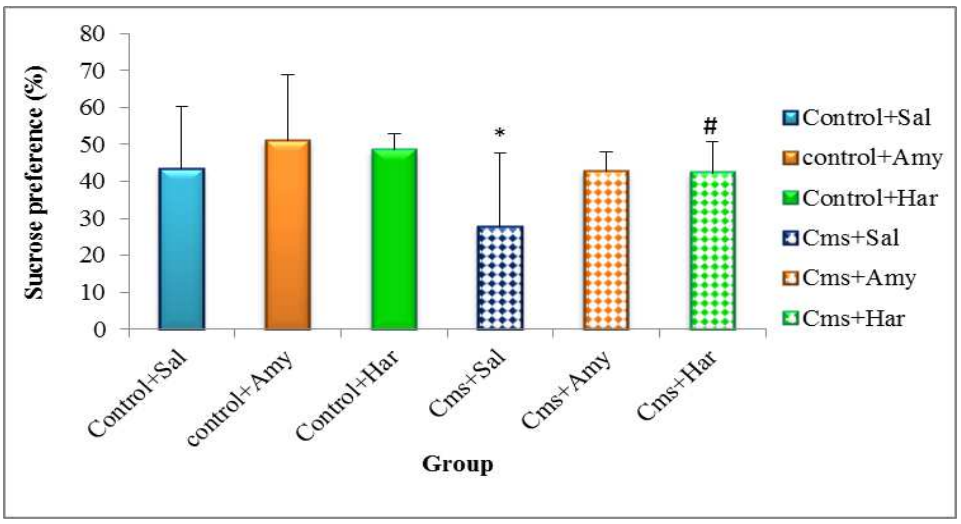
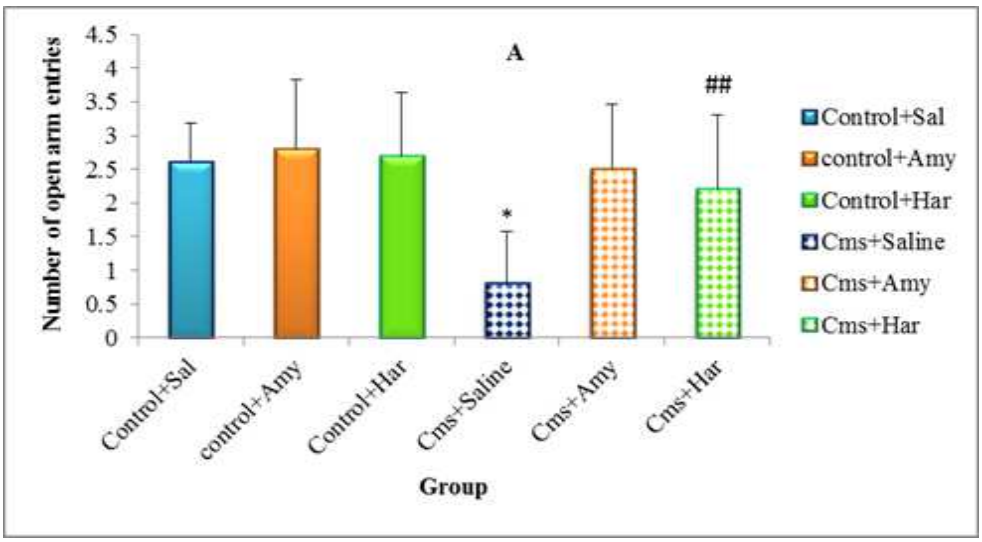
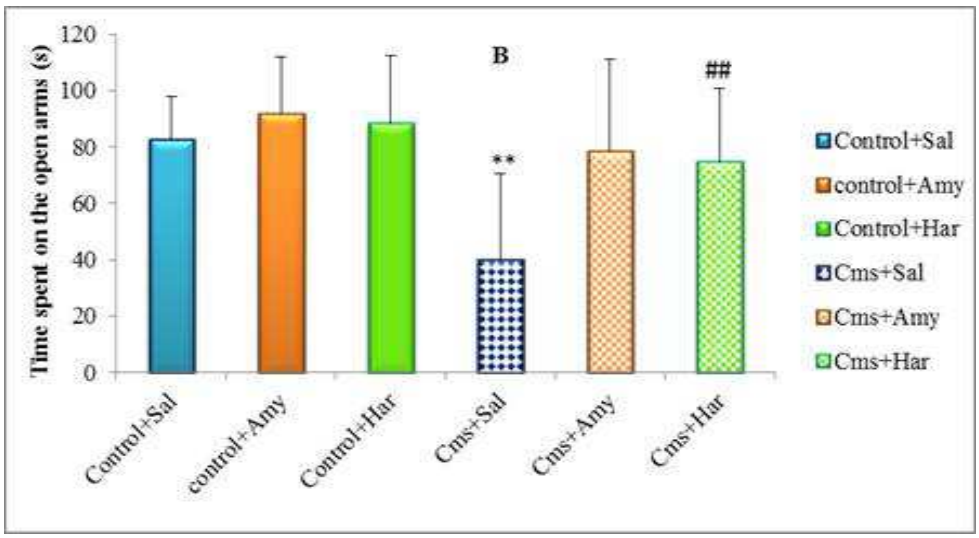


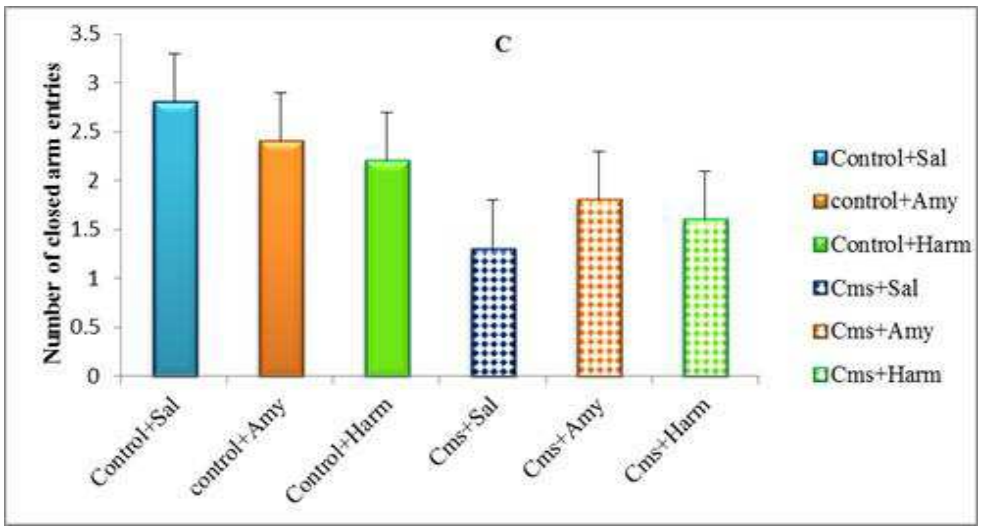
Figure 13. Effects of chronic mild stress (CMS) and chronic treatment with Harmine (Har; 15 mg/kg, i.p.) and Amitriptyline (Amy; 20 mg/kg ,i.p.) on 1% sucrose consumption in rats. 30 days of CMS was followed by 7 day of drug treatment. Bars represent means ±SEM (n = 10). *p< 0.05 vs. control saline; #p < 0.05 vs. CSM saline, according to ANOVA post hoc Tukey's test.

3.3.2. Anxiolytic Activity in Elevated Plus Maze (EPM) Test

Data regarding to the effect of chronic administration of Harmine at dose of 15 mg/kg and Amitriptyline at dose of 20 mg/kg on the behavioral changes during the elevated plus- maze were outlined in (Figure 14). CMS rats displayed a decrease in the number of entries and in the time spent in the open arms compared to non-stressed rats (F= 2.62 ; p =0.034; Fig. 14A), (F= 4.140 ; p =0.003; Fig. 14B); Moreover, No significant differences were detected for CMS rats compared with control rats in the number of entries and in the time spent in the closed arms (F= 1.17 ; p =0.334; Fig. 14C), (F= 2.11 ; p =0.078; Fig. 14D).The number of entries and the time spent in the open arms were significantly affected by Harmine or Amitriptyline treatment.


A
Number of open arm entries
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
*
##
Control+Sal
control+Amy
Control+Har
Cms+Saline
Cms+Amy
Cms+Har
Group


B
Time spent on the open arms (s)
120
100
80
60
40
20
0
**
##
Control+Sal
control+Amy
Control+Har
Cms+Sal
Cms+Amy
Cms+Har


C
Number of closed arm entries
3.5
3
2.5
2
1.5
1
0.5
0
Control+Sal
control+Amy
Control+Harm
Cms+Sal
Cms+Amy
Cms+Harm

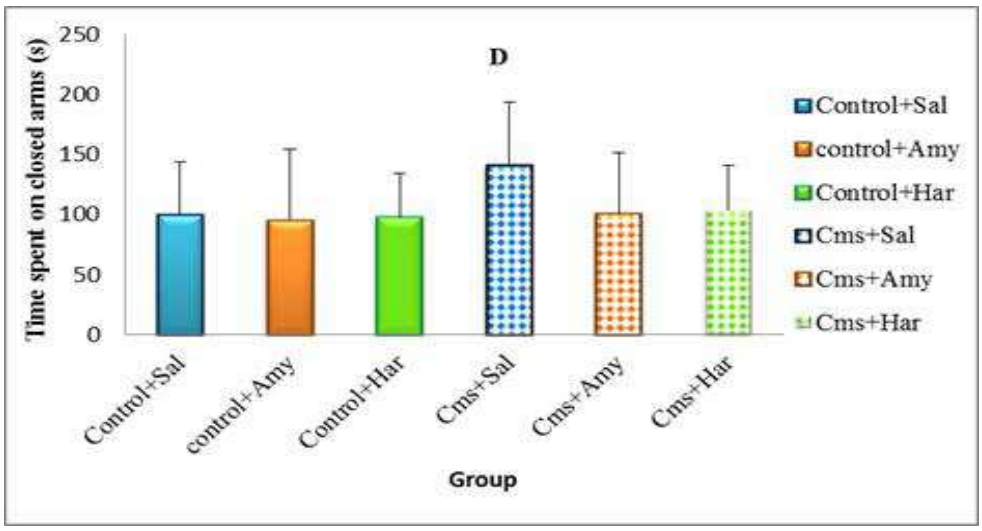


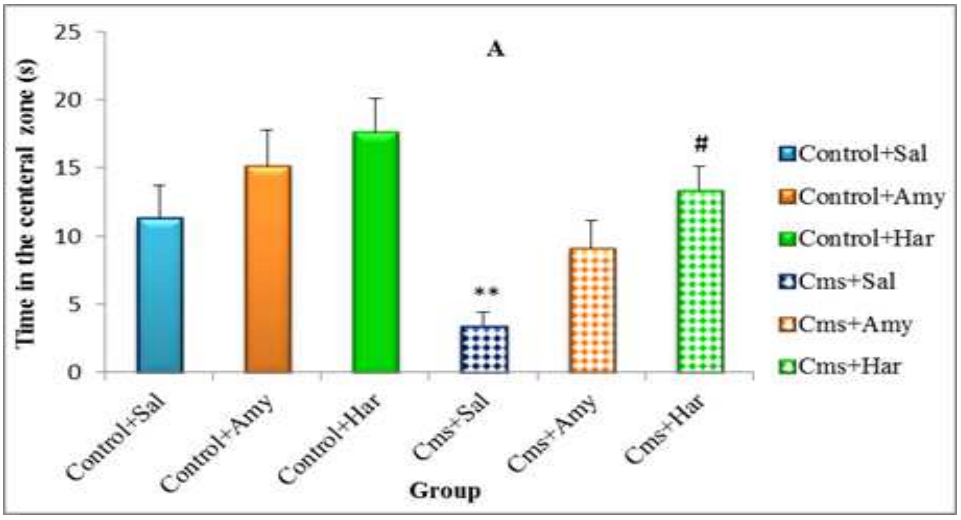
Figure 14. Effects of CMS procedure on the number of entries into the open arms (A) the time spent in the open arms (B) the number of entries into the closed arms (C) and the time spent in the closed arms (D) in the elevated plus maze test in rats repeatedly treated with Harmine (Har; 15 mg/kg, i.p.) and Amitriptyline (Amy; 20 mg/kg, i.p.). Bars represent means± SEM (n = 10). Ns: No significant difference p >0, 05,* p<0, 05, ** p<0, 01 vs. control saline; # p<0.05, ## p<0.01 vs. CMS saline, according to ANOVA post-hoc Tukey test.

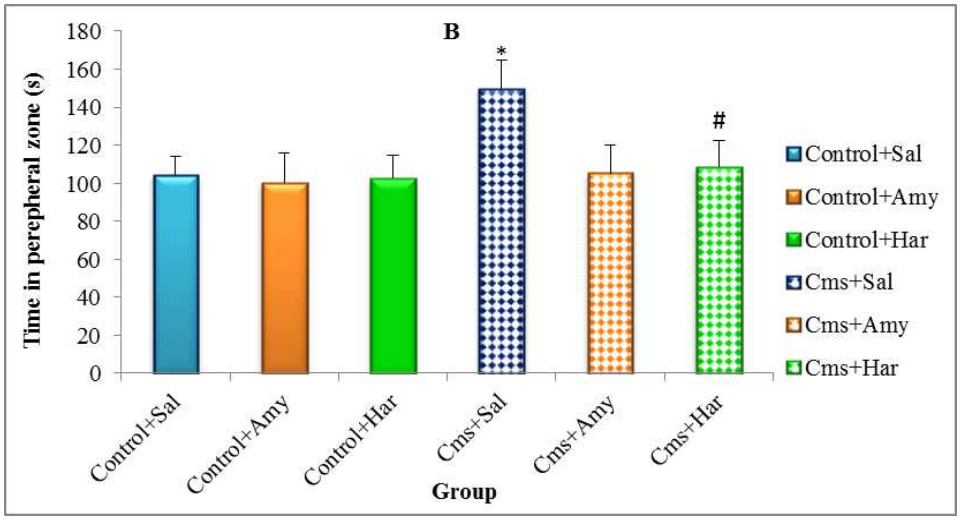
3.3.3. Anxiolytic Activity in Open Field (OPF) Test

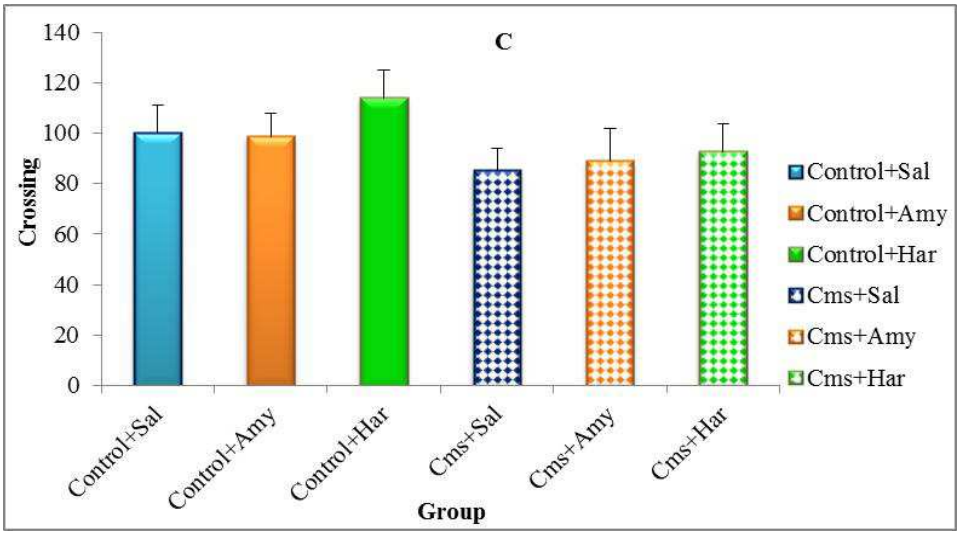
Fig. 15 showed the behavior of rat in the open Field test. Statistical analysis revealed that CMS rats spent a few time in the center of the field compared with the control (F= 5.10; p =0.001; Fig. 15A). By contrast, in the peripheral part of the arena, CMS rats treated with saline spent significantly more time compared to control group (F= 2.47; p =0.044; Fig. 15B).

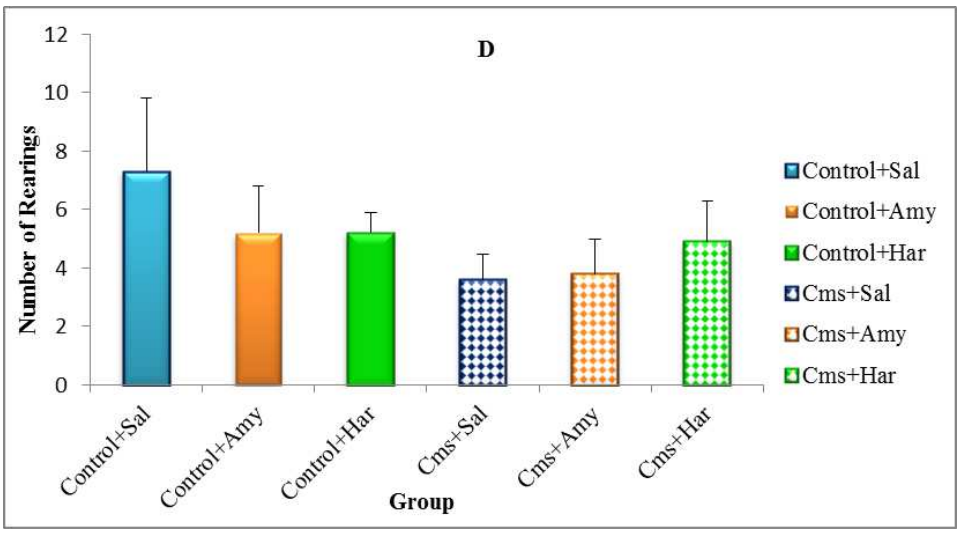
By contrast Fig. 15C, D, E and G showed that 30-day of chronic stressful stimuli did not alter the number of crossings (F= 0.90; p =0.485; Fig. 15C), neither the number (F= 0.78; p =0.567; Fig. 15D) nor the time of rearings (F= 1.39; p =0.242; Fig. 15E) and defecation (F= 0.83; p =0.537; Fig. 15G) displayed by all groups. This indicated that neither CMS nor Har or Amy treatments affected horizontal and vertical activity.

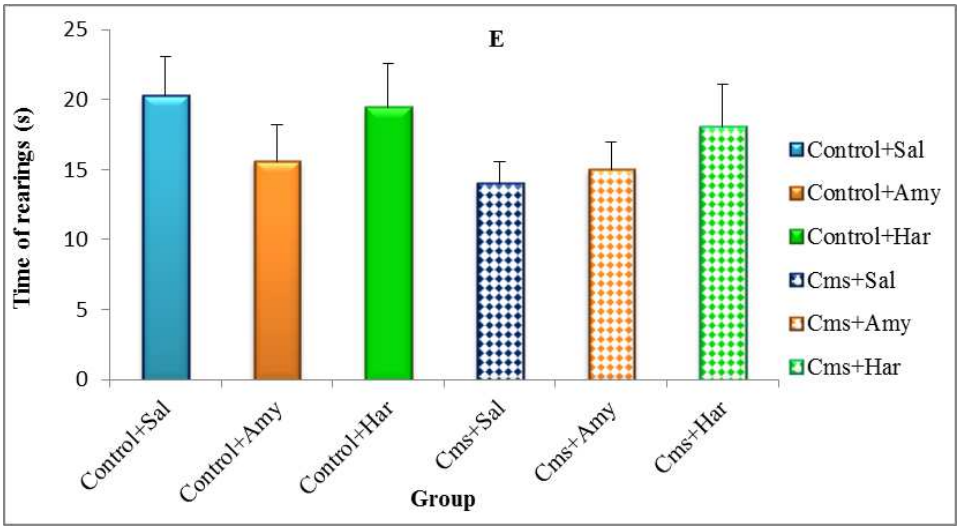
Self-grooming time illustrated in (Fig. 15F) respectively. Rats subjected to the CMS paradigm and treated with saline, displayed decreased grooming time (F= 2.49; p =0.042; Fig. 15F) compared with non-stressed rats injected with saline. Interestingly, CMS rats repeatedly treated with Harmine or Amitriptyline reversed the decrease of self-grooming time.

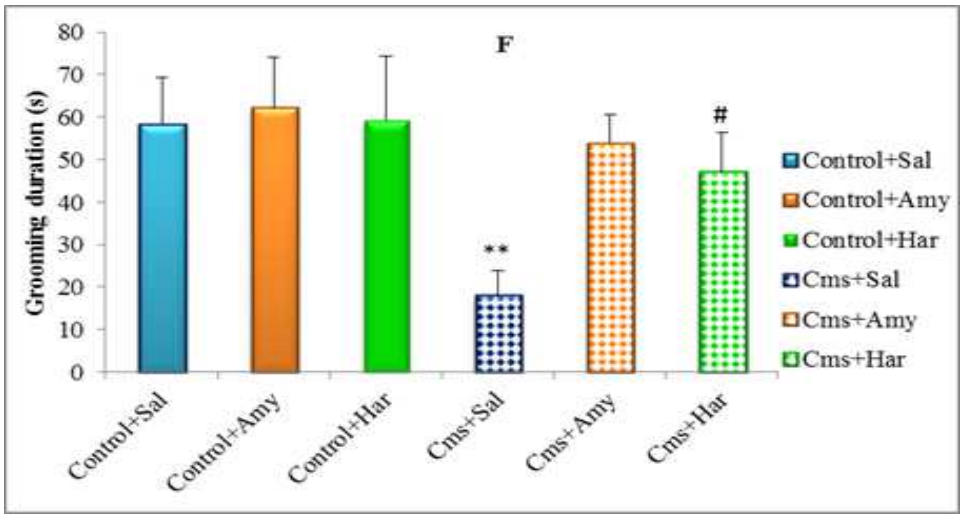

A
Time in the centeral zone (s)
25
20
15
10
5
0
**
#
Control+Sal
Control+Amy
Control+Har
Cms+Sal
Cms+Amy
Cms+Har
Group


B
Time in perepheral zone (s)
180
160
140
120
100
80
60
40
20
0
*
#
Control+Sal
Control+Amy
Control+Har
Cms+Sal
Cms+Amy
Cms+Har
Group


C
Crossing
140
120
100
80
60
40
20
0
Control+Sal
Control+Amy
Control+Har
Cms+Sal
Cms+Amy
Cms+Har
Group


D
Number of Rearings
12
10
8
6
4
2
0
Control+Sal
Control+Amy
Control+Har
Cms+Sal
Cms+Amy
Cms+Har
Group


E
Time of rearings (s)
25
20
15
10
5
0
Control+Sal
Control+Amy
Control+Har
Cms+Sal
Cms+Amy
Cms+Har
Group


F
Grooming duration (s)
80
70
60
50
40
30
20
10
0
**
#
Control+Sal
Control+Amy
Control+Har
Cms+Sal
Cms+Amy
Cms+Har

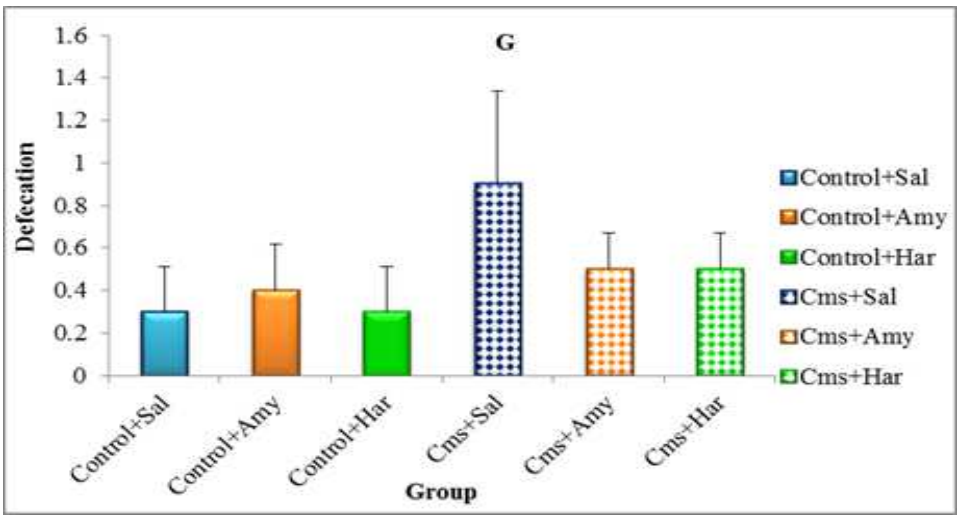


Figure 15. Effects of CMS procedure on emotional rat behaviors repeatedly treated with Harmine (15 mg/kg, i.p.) and Amitriptyline (20 mg/kg, i.p.) in the open-field on test. (A) the time spent in the central zone;(B) the time spent in the peripheral zone; (C) crossings number; (D) rearings number; (E) rearings time (F) grooming time; (G) defecation . Bars represent means ± SEM, (n=10). Ns: No significant difference p >0, 05,*P<0.05, **P<0.01 vs. control saline; $^{\#}$P<0.05 vs. CMS saline, according to ANOVA post-hoc Tukey test.

3.3.4. Anxiolytic Activity in Light Dark Box (LDB) Test

As depicted in (Fig. 16) stressed rats treated with saline decreased the number of entries (F= 2.60; p =0.035; Fig. 16A), also the time spent in the light compartment compared with control rats treated with saline (F= 2.47; p =0.044; Fig. 16B).Statistical analysis revealed that CMS rats chronically treated with Harmine or Amitriptyline reversed in the same manner the decrease of the number and the time spent in the light compartment induced by 30 days of chronic mild stress in rats. Altogether, these effects are indicative of anxiolytic-like behavior of Harmine on CMS rat.

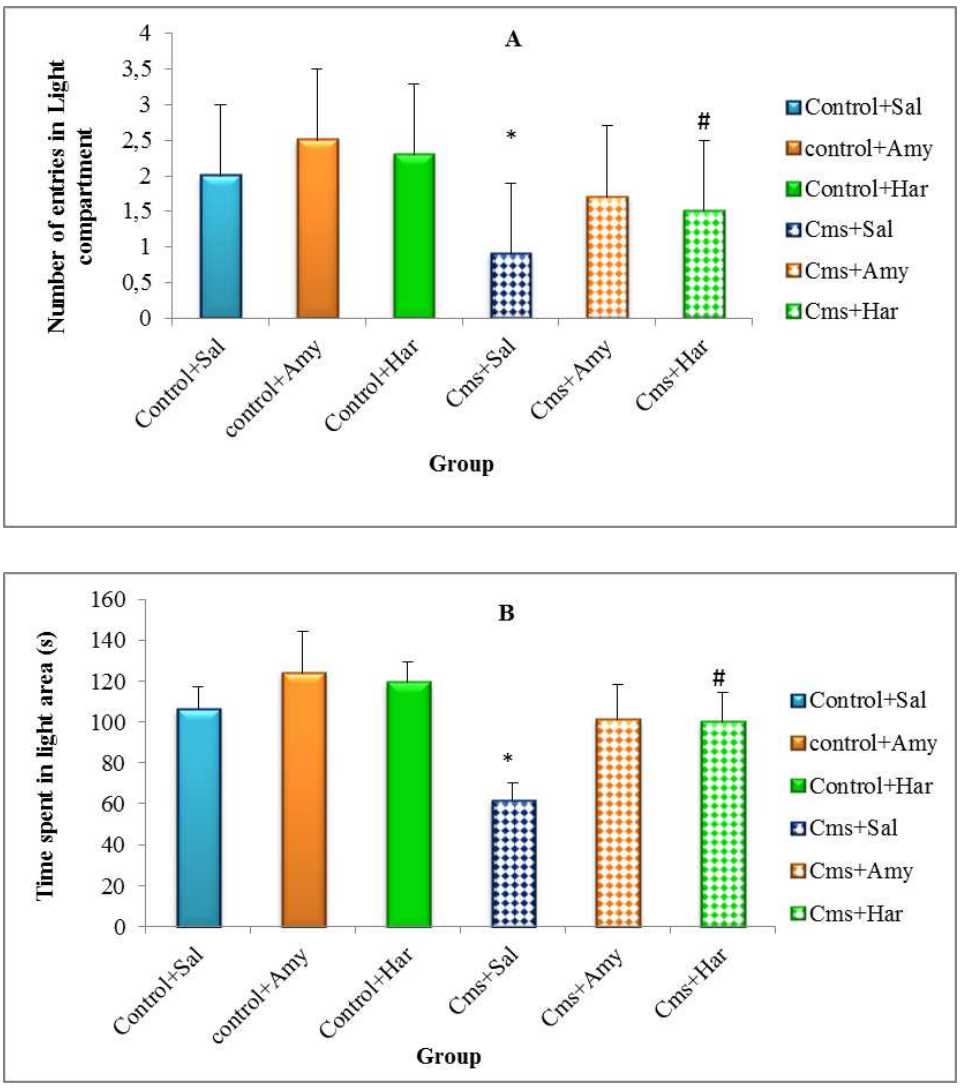


Figure 16. Effects of the chronic administration of Harmine (15 mg/kg, i.p.) and Amitriptyline (20 mg/kg, i.p.) on (A) the number of entries in light compartment and (B) the time spent in light area (sec) in rats subjected to the Light Dark Box test .Bars represent means ± SEM (n =10).* P <0.05 vs. control saline; # P<0.01 vs. CMS saline, according to ANOVA post hoc Tukey's test.

3.5 Variation of Hormonal Parameters

3.5.1 Plasma ACTH Level

The stress was assessed by plasma ACTH hormone levels which illustrated in Fig. 17. Rats subjected to the CMS procedure, and treated with saline, displayed increased ACTH (F=3.32; p =0.011) hormone levels compared with non-stressed rats injected with saline. The treatment with Harmine or Amitriptyline reversed the increase of circulating ACTH hormone levels in CMS rat.

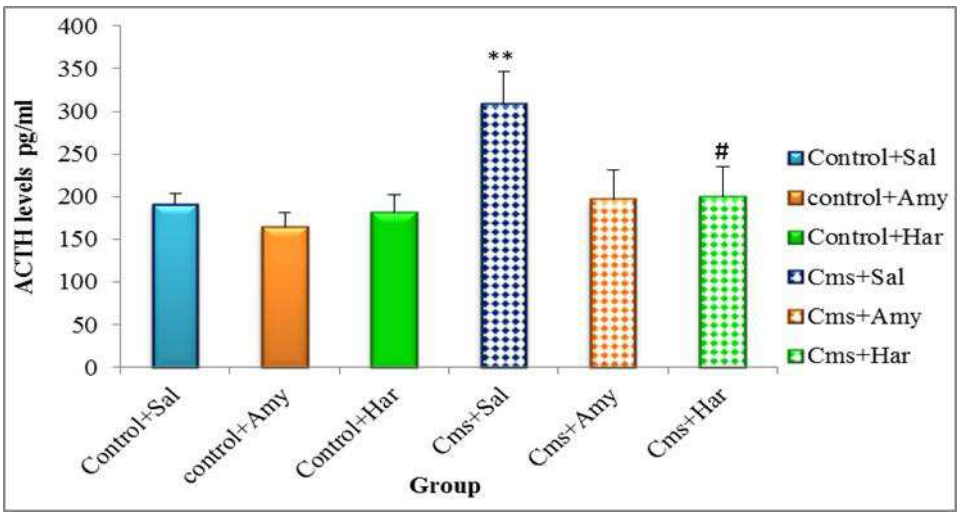


Figure 17. Effects of CSM procedure on adrenocorticotropic hormone (ACTH) circulating levels in rats repeatedly treated with Harmine (15 mg/kg, i.p.) and Amitriptyline (20mg/kg, i.p.). Bars represent means ±SEM. (n =10). ** P <0.01 vs. control saline; # P<0.05 vs. CMS saline, according to ANOVA post hoc Tukey's test.

3.5.2 Immunoglobulin's Level

Comparison of immunoglobulin levels between all the groups in (Fig .18). It was also found to be significantly ($p<0.05$). Rats subjected to the CMS paradigm, and treated with saline, displayed increased of IgG ($F=2.55$; $p=0.038$; Fig .18A) without affecting the IgM ($F=0.89$; $p=0.496$; Fig .18B) immunoglobulin levels compared with non-stressed rats injected with saline. However, the treatment with Harmine or Amitriptyline reversed the increase of IgG circulating levels in CMS rats.

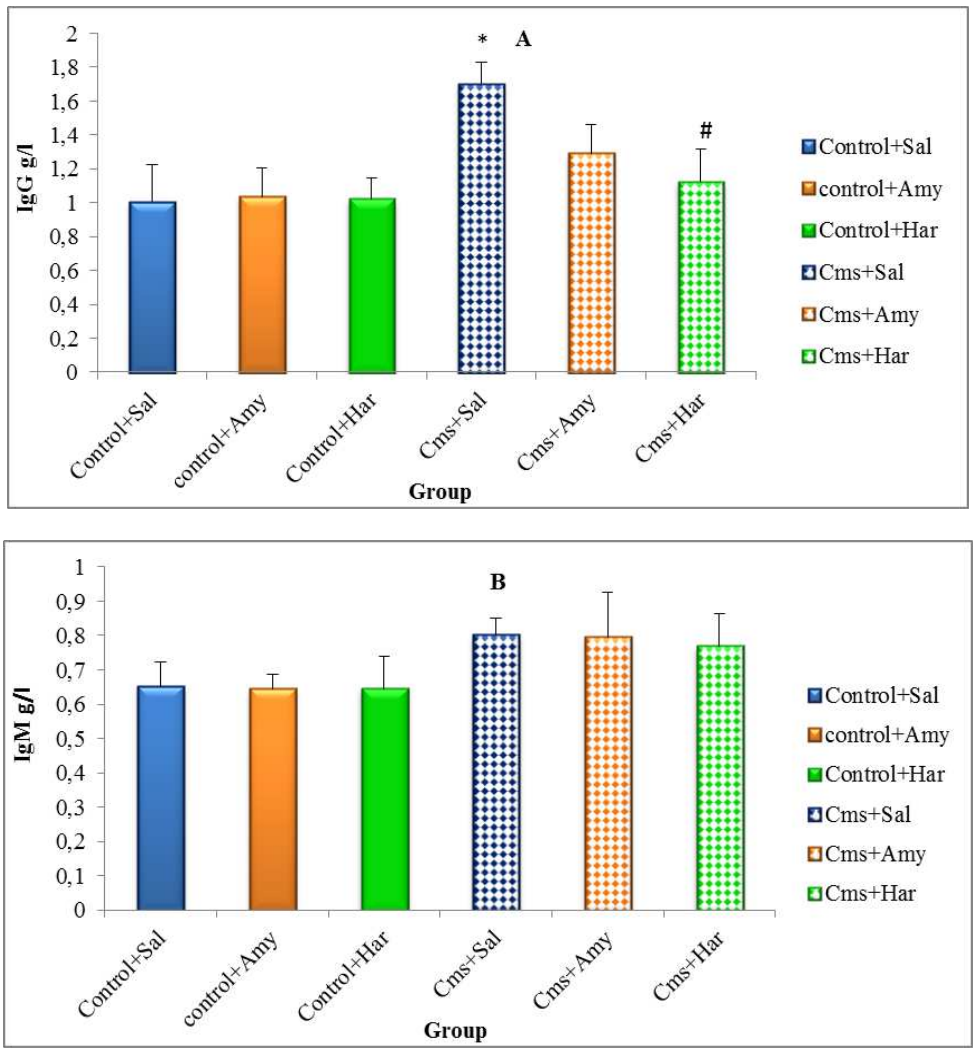


Figure 18. Effects of CSM procedure on immunoglobulin's (A) IgG, (B) IgM levels in rats repeatedly treated with Harmine (15mg/kg, i.p.) and Amitriptyline (20 mg/kg, i.p.). Bars represent means ±SEM. (n =10).*P <0.05 vs. control saline; # P<0.05 vs. CMS saline, according to ANOVA post hoc Tukey's test.

Discussion

4. DISCUSSION

Environmental factors, such as stress, can impact the neurobehavioral profile of an organism and precipitate a depression-like syndrome. Numerous studies have shown that similar alterations accompany depression in humans as well as in rodents after exposure to a variety of mild stressors (CMS). (Connor & Leonard, 1998**;** Grippo & Johnson, 2002; Peng *et al.*, 2000)(Fig. 19).

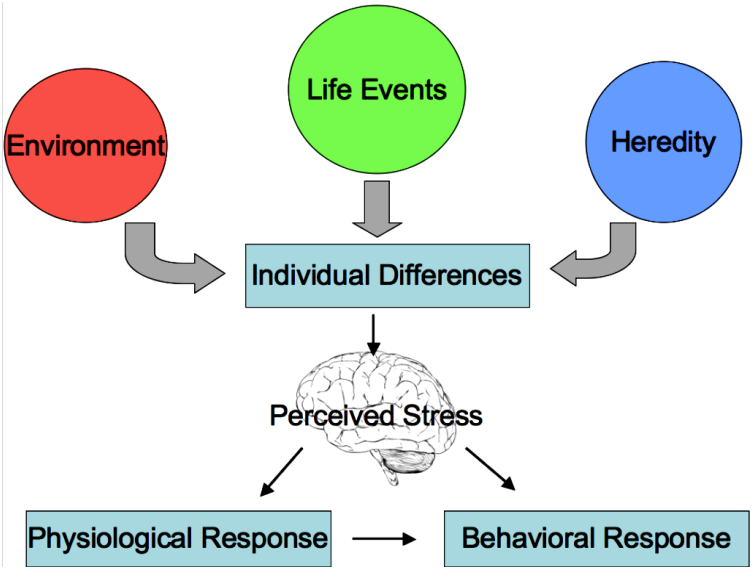


Figure 19. Individual differences in stress. Stress does not affect each individual the same way. A stimulus that may be stressful to one individual may not be stressful to another. Environment, life events, and genetics play a role in an individual's tolerance for stress. When an individual perceives a stimulus as stressful a physiological and behavioral response will be displayed. (Atchley, 2011).

In the present study, we assessed the potential antidepressant- like activity of Harmine using a well-validated animal model of depression, the CMS model in rats (Papp *et al.*, 2003; Liu *et al.*, 2013). CMS is often associated with disruption in appetite or weight gain, which is easily measured in rodents. This is in accordance with the data of (Liu *et al.*, 2013).

In this research, the body weight gain of stressed animals did not maintain a marked reduction and did not display a significant change. Several studies have also reported a lack of significant

alternations in body weight and food intake between stressed and control animals (Piato *et al*., 2008; Henningsen *et al*., 2009; Schmidt *et al*., 2010; Ramanathan *et al*., 2011).

Other studies have demonstrated that chronic exposure to stressors may alter rat body weight (Nielsen *et al*., 2000; Gamaro *et al*., 2003; Bekris *et al*., 2005; Strekalova *et al*., 2008; Bayramgurler *et al*., 2013).

The difference from previous studies may result from possibly milder stress applied in our CMS protocol and experimental procedures (Day-night phase of the application of stress strain, age and sex of the animal).

Exposure to unpredictable mild stressors have been produced a significant number of behavioral and molecular changes in rodent a conditions that could be reversed by the acute or chronic administration of classical antidepressant drugs as well as dopaminergic agonists that are reversed by treatment with antidepressant drug . In contrast, the efficacy of chronic Harmine or Amitriptyline candidates can be evaluated through behavioral tests like the sucrose preference, open field, elevated plus-maze and black & white box tests.

CMS in rats models depression in humans distinguish by decreasing sensitivity to rewards (anhedonia) and by inducing other changes as symptoms of depression (Di Chiara *et al*., 1999**;** Wiborg 2013, Wu *et al*., 2015).The data presented in this study were especially, focused on consumption of sweet solutions as central readout measure for hedonic/motivational behavior in mice**,** in addition to several behavioral changes were observed as a consequence of CMS which mentioned later. In the CMS model, both preference for sucrose intake, as well as decreased intracranial self-stimulation behavior, serve as markers of generalized decrease in sensitivity to reward and they are quite related to anhedonia (Gamaro *et al*., 2003; Bekris *et al*., 2005; Waki *et al*.,2007). Although some authors have not observed a reliable decrease in sucrose consumption after chronic mild stress , possibly because of different sensitivity to stress by different rat strains (Matthews *et al*., 1995; Nielsen *et al*., 2000).

According to the literature, the present data confirm that rats subjected to CMS procedure and treated with saline consume less sweet solution compared to non-stressed rats treated with saline (Allaman *et al*., 2008; Lucca *et al*., 2008,2009**;** Garcia *et al*., 2009; Darwish *et al*., 2013).

Also, it was very similar to symptoms of depressed patients: for example, weight loss, lack of pleasure, and low energy (Nina *et al*., 2011).

Matching to our results at least in part, regarding in reactivity to reward, (Dalla *et al*., 2005, 2008) and (Kamper *et al*., 2009) who demonstrated that the decrease in sucrose intake during the CMS procedure is more pronounced in males than in females. These findings suggest that, under our experimental conditions, the CMS procedure induced anhedonic-like behavior in our rats.

The present findings revealed that repeated administration of Harmine or Amitriptyline reversed the anhedonic-like behavior in CMS rats. These results are consistent with the clinical observations, consonant with a number of findings from studies showing the reversal of CMS-induced preference behavior by treatment with the antidepressant such as fluoxetine (Chen *et al*., 2015).

Open field is an exploratory model that can provide information on 1) anxiety - when anxiety stimuli are sufficient to indicate danger; 2) exploration - if the anxiety stimuli are not so excessive as to inhibit the behavior of the animal; and 3) locomotor activity after periods of habituation - when the arena is no longer (Prut & Belzung, 2003). Also, the open field test is considered as an indicator of the emotional state of the animal (Bagdy *et al*., 2007; Passos *et al*., 2011).

The central square is used because some mouse strains have high locomotor activity and cross the lines of the test chamber many times during a test session. Also, the central square has sufficient space surrounding it to give meaning to the central location as being distinct from the outer locations (Carrey *et al*., 2000). Rodents that spend significantly more time exploring the unprotected center area demonstrate anxiolytic- like baseline behavior.

The center area of the chamber can be defined by the experimenter as a proportion of the overall arena size. Many software systems allow the researcher to designate this center area, as well as multiple other regions of the test chamber, to track exploratory activity (Olanrewaju, 2015).

Peripheral movement or thigmotaxis is related to attempts to escape from a novel environment, and is an important anxiety-related behavior in open-field testing (Crawley, 1999), which is sensitive to the action of anxiolytic drugs such as benzodiazepines (Schmitt & Hiemke 1998). Meanwhile, the treatment with Harmine or Amitriptyline had no effect on peripheral movement.

In rat, exploratory behavior including locomotion , rearing, increased defecation in a novel environment have been taken as an indicator of anxiety-like state in rats (Hall, 1934; Prut & Belzung, 2003; Ducottet & Belzung, 2004). Crossing and Rearing is an aspect of exploratory behavior and generally decreased when an animal is placed in a novel or stressful environment, and may increase when anxiolytic drugs are given (Johansson & Ahlenius, 1989; Prut & Belzung, 2003).

Defecation is also a good indicator of emotionality in animals, and research shows that high emotionality is related to an increase in defecation, with anti-anxiety drugs reducing defecation (Angrini *et al*., 1998). In this study, no significant differences were found in these parameters between the CMS and control animals. Whatever the reason for inconsistent reports of the effects of Harmine or Amitriptyline on crossing and rearing, the results obtained here are consistent with the hypothesis that an anxiolytic effect also occurs even there were no significant change.

Self-grooming in rodents is stereotypically sequenced and naturally occurs after stress, which provokes a disorganization of grooming sequences (Komorowska & Pellis, 2004; Audet *et al*., 2006).

Beside the recent evaluation of the condition of fur animals due to grooming deficit was introduced as the new measurable parameter**.** Several studies have been shown to reduce animal grooming (Yalcin *et al*., 2007; Piato *et al*., 2008; Wang *et al*., 2013). Contrary to this, (Kalueff & Tuohimaa, 2005b) reported an increased number and duration of grooming actions in stressed rats, while port the idea that mice display abnormally high or low levels of grooming, that may be a strain-specific phenomenon (Kalueff & Tuohimaa, 2005).

Our results showed that CMS led to reduced duration of grooming , this is in accordance with the data of (Lin *et al*., 2005), who found that rats exposed to chronic unpredictable mild

stress for a period of 3 weeks experience was associated with a reduced anxiety-like behavior as reduced open-field exploration, fewer rearings, and grooming indicative of lethargy, apathy , and bodily neglect also lower weight gain. However, most studies have reported either no change or a reduction of locomotor activity after treatment (Reus *et al*., 2011; Yang *et al*., 2012; Akinfiresoye & Tizabi, 2013). The results reported here indicate also that rat exposed to different stress conditions use different behavioral strategies to cope with external challenges.

However, some data indicates that higher stress or anxiety in animals does not necessarily translate into their increasing grooming activity (Boccalon *et al*., 2006; Bouwknecht *et al*., 2007). Thus, increased grooming behavior is indices of anxiolytic activity (Li *et al*., 2014). Regulation of grooming behavior is mediated by multiple brain regions (especially the basal ganglia and hypothalamus) (Aldridge *et al*., 2004), as well as by various endogenous agents (neuromediators) (Berridge *et al*., 2005), and psychotropic drugs (Rupniak *et al*., 2001; Audet *et al*., 2006). Combined with the effect on peripheral movement, crossing, rearing and defecation the consistent effect of Harmine or Amitriptyline on grooming is strong evidence for an anxiolytic effect.

Allowing the assessment of the Protective effects of some drugs on the behavioral changes in chronic variable Stress-induced rats using the OF assay (Dai *et al*., 2010; Browne & Lucki, 2013, Ping *et al*., 2014). Several recent studies demonstrated that treatment with harmane, norharmane and Harmine dose-dependently reduced the immobility time in the mice forced swimming test (Farzin & Mansouri, 2006). Moreover, recent findings showed that Harmine in different doses and manner of treatment reduced the immobility time in the rats forced swimming test (Fortunato *et al*., 2009). In our study, Harmine or Amitriptyline-treated rats clearly showed increase in grooming and time spent in the center. The behavioral effects induced by Harmine in rats reported in this study are in agreement with literature data, which support an antidepressant action for β-carboline Harmine or other antidepressant without affecting spontaneous locomotor activity (Fortunato *et al*., 2010, Réus *et al*., 2012). Therefore, the antidepressant-like effects of β-carboline Harmine could be due to interactions with several receptor systems involved in the modulation of behavioral and molecular actions of antidepressants.

Across this experiment, the effects of Harmine are according to those seen in many similar studies with anxiolytic drugs. This confirms the anxiolytic behavioral effects of this drug in this strain of rat at the used doses, and suggests that the open field may be a valid model for comparing the anxiolytic effects of this drug with Amitriptyline intraperitoneally administered (the reference antidepressant drug in this study).

Until now, there have been no reports regarding the activity of Harmine on EPM test in normal or stressed rats. Therefore herein we mentioned second focus to address additional indicators of anxiety- and depressive-like behavior. In the test of elevated plus-maze, a significantly decreased in the number of entries and in the time spent in the open arms was observed in CMS rats compared to non-stressed rats. Meanwhile, no significant differences were detected for CMS rats compared with control on the number of entries and the time spent in closed arms.

The result concerning the alteration of the elevated plus-maze test is based on the spontaneous exploratory behavior of rodents and their natural aversion to the open arms caused by fear and anxiety (Walf & Frye, 2007) Which, an increase in the number of entries added to the lengthy time spent in the open arms apparatus demonstrate a lower level of anxiety (Hogg, 1996). This finding suggests that seven days of treatment with Harmine (15 mg/kg) or Amitriptyline (20 mg/kg) is significantly caused anti-anxiety-like effect, which is consistent with a previous study (Miao *et al*., 2014). On the other hand, both of Harmine and Amitriptyline did not enhance the change in locomotor activity in the closed arms. Whereas other antidepressant such us venlafaxine treatment significantly caused anti-anxiety-like effect and also improved locomotor activity (Darwish *et al*., 2013).

But, the most of behavioral tests have limitations. Sometimes, different models of animal behaviors based upon approach/avoidance conflict but do not produce identical behavior can present contradictory results. It will therefore be necessary to find other possible interpretations of the observed results, since rats display different strategies of defense while exploring the environment. So in addition to, sucrose intake, OF, EPM and stress effects on the reward system, the sensitivity to changes in activity due to the CMS procedure in other test are needed.

It has been suggested that the LDB test measures provide the best comparison of anxiety-like and locomotor behavior (Arrant *et al*., 2013) and differentially susceptible to drug treatments.

In the present study, group of stressed rats showed significant decreases in locomotor and exploratory activities as compared with the control group. In stressed animals, we observed a decrease in the number of entries and the time spent in light box as well as in open arm of elevated plus maze. It has been demonstrated that in exploration-based tests for anxiety-like behaviors, such as the light–dark exploration or emergence tests, animals with anxiety would rather spend more time in the dark compartment (Holmes *et al*., 2003).

Reduction in the natural aversion to light and time spent in the light compartment will increase in case of anxiolytic agents. (Kafeel & Rukh, 2015).

According to the literature, the present data confirm that CMS induced hypolocomotive effects could be due to the decrease in serotonergic function "central 5-HT neurotransmission" resulting in the development of depressive symptoms (Joca *et al*., 2003). This can be reversed by antidepressant drugs (Froger *et al*., 2004).

Serotonergic mechanisms play an important role in the modulation of locomotor activity at a number of levels in the neuroaxis including the spinal cord, the basal ganglia, limbic structures, and in the frontal cortex (Brocco *et al*., 2002).

The present findings revealed that repeated administration of Harmine or Amitriptyline induced higher activity in LDB test. Rats showed more transitions between light and dark compartments and spent more time in the light compartment than CMS rats.

The biological actions of Harmine are complex and include some effects that can be related to affective and anxiety disorders, such as modulation of the HPA axis and the monoaminergic system.

Monoamine oxidases are mitochondrial outer membrane-bound flavor-enzymes that catalyze the degradation of biogenic amines, more specifically the oxidative deamination of several important neurotransmitters, including 5-hydroxytryptamine (5-HT) (or serotonin), histamine, and the catecholamines dopamine, noradrenaline, and adrenaline. They play an important role in motor and mood control, as well as in the regulation of motivation and other brain functions (Passos *et al*., 2014).

As low levels of MAO-A were detected in serotonergic neurons, selective MAO-A inhibitors were shown to increase brain 5-HT and to exert an antidepressant effect (Passos *et al.*, 2014).

Regarding their biological effects, Harmine have been extensively investigated for their effects on MAO-A and –B, the b-carboline alkaloids may interact selectively with specific enzymatic targets leading to a variety of pharmacological activities (Cao *et al.*, 2007), that increase 5-HT availability in terminal region. The subtle variations in activity reported here could suggest that Harmine is acts as agonists at serotonin receptors (Song *et al.*, 2006); by direct interaction with specific receptors stimulating the central nervous system or by inhibiting the metabolism of amine neurotransmitters that can be determined at different levels **(Fig.20).**

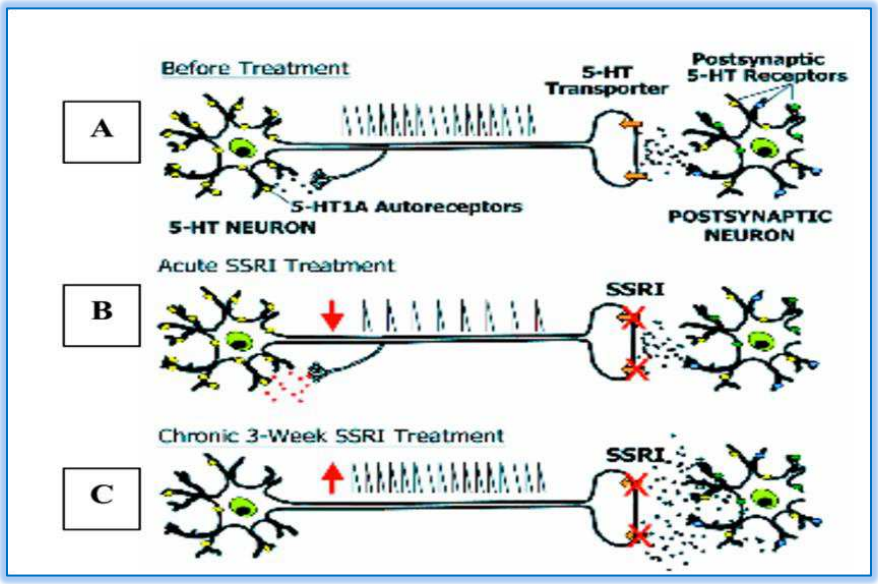


Figure 20. Schematic representation of serotonergic neuron (A) before treatment, (B) in the acute treatment with an SSRI (inhibition of neuronal firing due to the activation of 5-HT1A autoreceptor) and (C) in chronic treatment (desensitization of 5-HT1A autoreceptor / 1B / 1D). (Pineyro & Azzi, 2005)**.**

Data from this study show that chronic mild stress, leads to increased anxiety and depressive-like behaviors, accompanied by higher levels of ACTH levels, all traits indicative of a disrupted emotional state. This confirms some previous studies (El Fazaa *et al.*, 2000). (Atchley, 2011) has also reported that stressful situations are associated with activation of the hypothalamic-pituitary-adrenal axis (HPA axis) as a marker of the stress response. Furthermore,

an extensive body of studies has found that enhanced HPA axis activities also impair cognitive functions both in rodents and in humans (Song *et al*., 2006; Csernansky *et al*., 2006; Maccari & Morley-Fletcher, 2007; Aisa *et al*., 2007).

The HPA axis is governed by several brain regions, including the amygdala and hippocampus; that control the release of corticotrophin releasing hormone (CRH) and arginine-vasopressin (AVP) from the paraventricular nucleus (PVN) of the hypothalamus (Antoni, 1993). When the hypothalamus is triggered by a stressor, CRH-aka CRF, and arginine vasopressin (AVP) are secreted, eliciting both the production of adrenocorticotropin hormone (ACTH) from the anterior pituitary gland and the activation of the noradrenergic neurons of the locus caeruleas/norepinepherine (LC/NE) system in the brain. The LC/NE system is primarily responsible for the immediate "fight or flight" response driven by epinephrine and norepinephrine, while ACTH drives the production of cortisol from the adrenal cortex. ACTH in turn stimulates the production of glucocorticoids hormones (cortisol in humans and corticosterone in rodents) (Zhu *et al*., 2001; Haghparast *et al*., 2013) in the adrenal cortex and their release into the blood stream .Glucocorticoids have multiple functions in almost every tissue of the human body, such as regulation of energy metabolism (through increased gluconeogenesis, lipolysis and protein degradation), regulation of immune functions, sexuality and mood **(Fig. 21).**

Albeit produced in the periphery, glucocorticoid hormones can act back on the hippocampus, the PVN and the anterior pituitary, to exert GR-mediated negative feedback inhibition on the HPA axis and to inhibit the synthesis and secretion of CRH and ACTH. Ultimately, this regulatory feedback loop maintains low glucocorticoid levels under normal physiological conditions (De Kloet *et al*., 2007). So the important function of the hippocampus is its regulation of the negative feedback system in the HPA axis.

High levels of glucocorticoids in the hippocampus lead to down regulation of receptors, which inhibits the ability of the hippocampus to regulate the HPA axis (Gregus *et al*., 2005).

Another hormone dehydroepiandrosterone (DHEA) is also produced in the adrenal cortex; and while its secretion is affected by pituitary ACTH secretion, additional regulatory activities and aging result in an almost complete loss of the diurnal rhythm in elderly subjects (Fries *et al*.,

2009; Chida & Steptoe 2009; Kudielka & Wüst, 2010). DHEA, a glucocorticoid antagonist, serves not only to prevent excessive systemic inflammation, but also to protect the neurologic machinery, particularly the hippocampus, from the damaging effects of cortisol (Cardounel *et al*., 1999) a phenomenon that may also be true of its neurosteroid precursor pregnenolone (Gursoy *et al* ., 2001).

Also, the HPA axis is found to be a mediator of changes in brain monoamines, e.g. locus coeruleus noradrenaline (Mello *et al*., 2003) and raphe serotonin (Chaouloff, 2000). Antidepressants, both individually and in conjunction with anti-glucocorticoid agents, reverse a number of the HPA-axis abnormalities (Anand *et al*., 1995).

According to this view, our findings showed that ACTH circulating levels were increased in stressed rats compared with non-stressed animals. Importantly, administration of Harmine or Amitriptyline reversed these hormonal alterations in CMS rats. This is in line with vitro studies either have shown these patterns of changes induced by stress and by antidepressants (Fortunato *et al*., 2010; Garcia *et al*., 2009), that suggest suitability of these doses to assess the balances effect of the organism. Especially reinforcing the relevant role played by Harmine in the mediation of physiological aspects of stress.

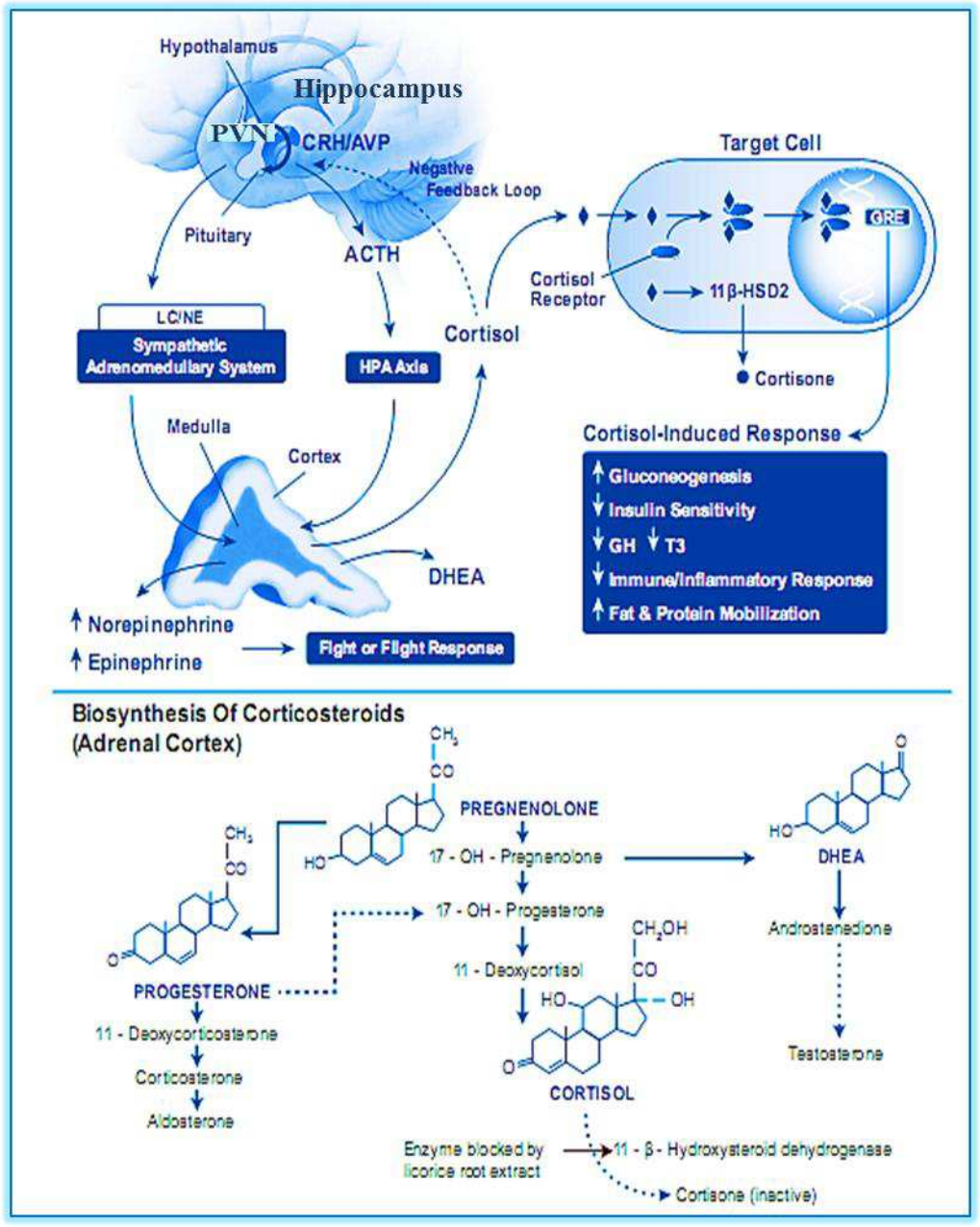


Figure 21. The HPA Axis and Stress Response System (Guilliams & Edwards, 2010).

Our findings demonstrate an increase in adrenal gland and a decrease in brain weight in CMS rats compared with non-stressed rats. However, there were no significant differences in the weight of other organs among the groups. These alterations in organ weight due to the actions of stress hormones, particularly glucocorticoids. However, stress hormones, and glucocorticoids, in particular, contribute to impairment of cognitive function and promote damage to brain

structures such as the hippocampus through decreased neurogenesis (McEwen & Sapolsky, 1995). Although impairments in hippocampal neurogenesis have not yet provided functional mechanism for the pathophysiology of depression, this phenomenon gained an enormous interest among researchers (Dagyte *et al*., 2011). In accordance with the literature, present data confirm that CMS, can influence brain structure, and a number of studies have demonstrated that stress and depression lead to reductions in the total volume of the hippocampus, as well as atrophy and loss of neurons in this structure (Ashwani & Preeti, 2012) **(Fig.22).**

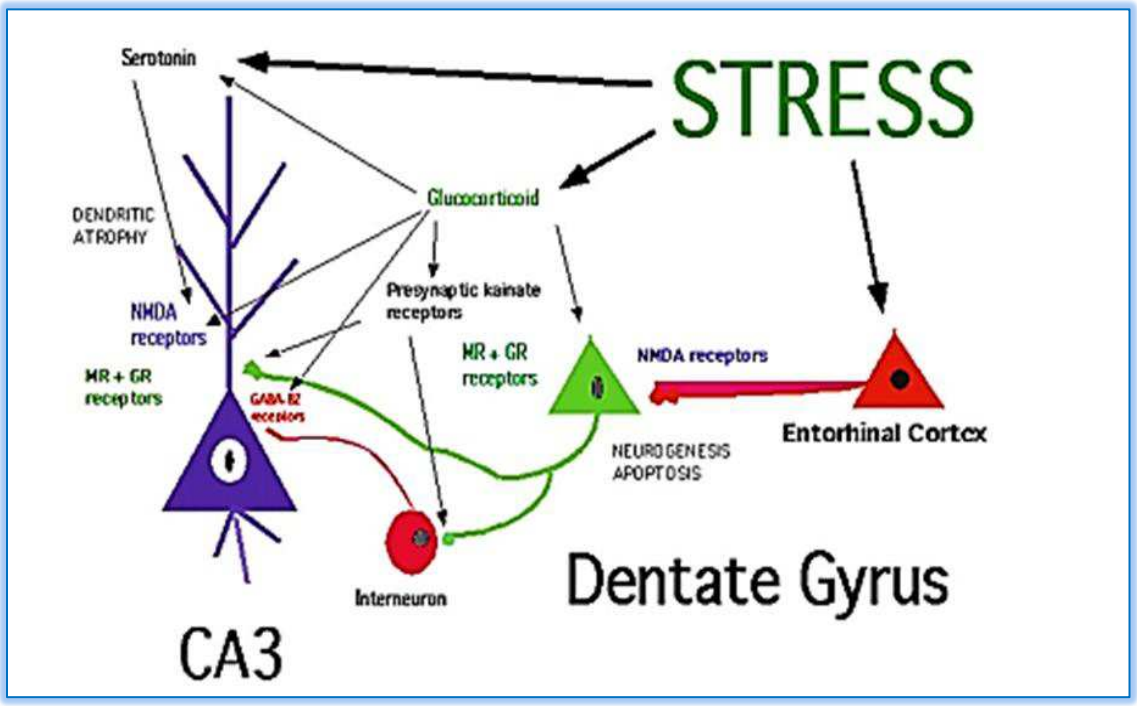


Figure 22. Shematic diagramme of the role of neurotransmitters and glycocorticoides in regulation neurogenesis and dendritic remodeling in the dentate gyrus-CA3 system of the hippocampal formation (McEwen, 2000).

It appears that animals submitted to chronic variable stress may present other distinct physiological evidence, such as increased adrenals weight. Distinct authors have already suggested an increase of rat adrenal weight after 14 (Harro *et al*., 2001), 28 (Konarska *et al*., 1990) ,40 days (Lucca *et al*., 2008,2009; Garcia *et al*., 2009) or 42 days (Lin *et al*., 2008) of CMS paradigm. This hypertrophy of adrenal gland could be mediated by the increase of

glucocorticoids as well as ACTH circulating hormone, which is released in high concentrations during stressful situations by anterior pituitary gland, which is often associated with a hyperactive HPA axis (O'Connor *et al*., 2000; Mondal, 2011; Berry *et al*., 2012). As we have been mentioned before, this enlargement is in association with increased glucocorticoids levels. Whereas in another study, CMS induced an increase in the CRH mRNA levels in the paraventricular nucleus (PVN), without any alteration in the adrenal weight, indicating that these changes are associated with the nonhypophysiotropic compartment of the PVN (Dunčko *et al*., 2001).

These observations could indicate that daily intraperitoneally treatments with Harmine or Amitriptyline modulate the contents of extracellular fluids in this organ.

Recently, several studies have drawn special attention to the role of the immune system in depression. Immunologic abnormalities in depression have been described for over two decades (Abelaira *et al*, 2013) but it is still unclear whether these abnormalities play a role in the pathogenesis of depression. There is still great difficulty in understanding the complexity of the communication between the immune system and the brain. Nevertheless, a few studies have addressed this issue (DellaGioia & Hannestad, 2010). (Tagliari *et al*, 2011; You *et al*.,2011) have demonstrated that CMS could induce immune alterations in both peripheral tissues and CNS. (Girotti *et al*., 2011, Kubera *et al*., 1995) reported that rats subjected to chronic mild stress display increased of the hippocampal levels of pro-inflammatory cytokines such as IL-1β, IL-6 in peripheral circulation (Goshen *et al*., 2008) **(Fig.23).**

In the present study, data demonstrate that chronic mild stress increases IgG production without affecting IgM production. So it has been assumed to represent a link between pro-inflammatory cytokines and the neurochemical or neuroendocrine alterations that may be responsible for this impairment.

Immunoglobulins comprise five major classes when the immunoglobulin IgG and IgM will be considered in more detail here, as these are by far the most frequently utilized antibodies in immunohistochemistry. IgG is the most abundant immunoglobulin in serum, which would be released in blood stream, the only ones that are able to pass from mother to child during pregnancy through the placenta.

A Growing body of evidence has been shown that stress has opposing effects on antibody responses. Fernandes (2012) analyzed the natural antibodies of mice subjected to the chronic stress of forced swimming for twenty-five days and found an decrease in IgG levels without affecting the production of antibody classes IgM (Fernandes *et al*., 2012).

Friedman (2001) demonstrated that the central doses of CRH induced decreases of cellular and humoral immune responses, with sympathetic effector mechanisms mediating the action of central CRH on the immune system **.**These data implicated central CRH as being a key neuropeptide that might coordinate and induce immune suppression .Further studies showed that Cortisol exerts diverse effects on a wide variety of physiological systems, and also coordinates the action of various cells involved in an immune response by altering cell distribution and the production of cytokines or immune messengers (Strausbaugh & Irwin, 1992; Friedman & Irwin, 2001).

Taken together, these findings could be concerned by the effect of stress mainly on humoral response; thought to be produced by mature B lymphocytes, but recently has been shown to be produced by the neuronal cells (Huang *et al*., 2008).

Dopamine neuronal functioning is essential in sustaining a wide variety of pleasurable and rewarding experiences. Especially dopaminergic neurons, projecting from the ventral tegmental area to the prefrontal cortex, basolateral amygdala, and nucleus accumbens are essential in reward processes (Wise, 2002). which explained the high production of IgG, for inducing neuroprotective effects .The human immunoglobulin (IgG) is the main component of protection against lesions of dopaminergic neurons from 6-hydroxydopamine (6-OHDA) (Zhang *et al*., 2012).

Huang (2008), demonstrated that the IgG can be produced by the central neurons in rats and previously has been shown that IgG is produced extensively by the neurons of the cerebral cortex, hippocampus, dentate gyrus, cerebellum, the pons, medulla and spinal cord, it is synthesized by the same intraocular eye cells in humans (Niu *et al*., 2011).

Although the function of IgG neurons is poorly understood, it has been suggested that neurons are involved in IgG in maintaining the stability of the nervous system.

Treatment with Harmine reversed the increase of IgG level that could be explained by his immune modulator and anti-inflammatory effects. Also, it has a central pharmacologic effect that already identified. These include neurotrophic and neuroprotective effects especially on dopaminergic neurons. To gain some insight into the specificity of Harmine's ability to crosses the blood brain barrier (BBB). As described previously, Harmine penetrates the brain very rapidly, most likely due to its highly lipophilic structure (Li *et al*., 2011). This permitted to display a modulation of antibody level.

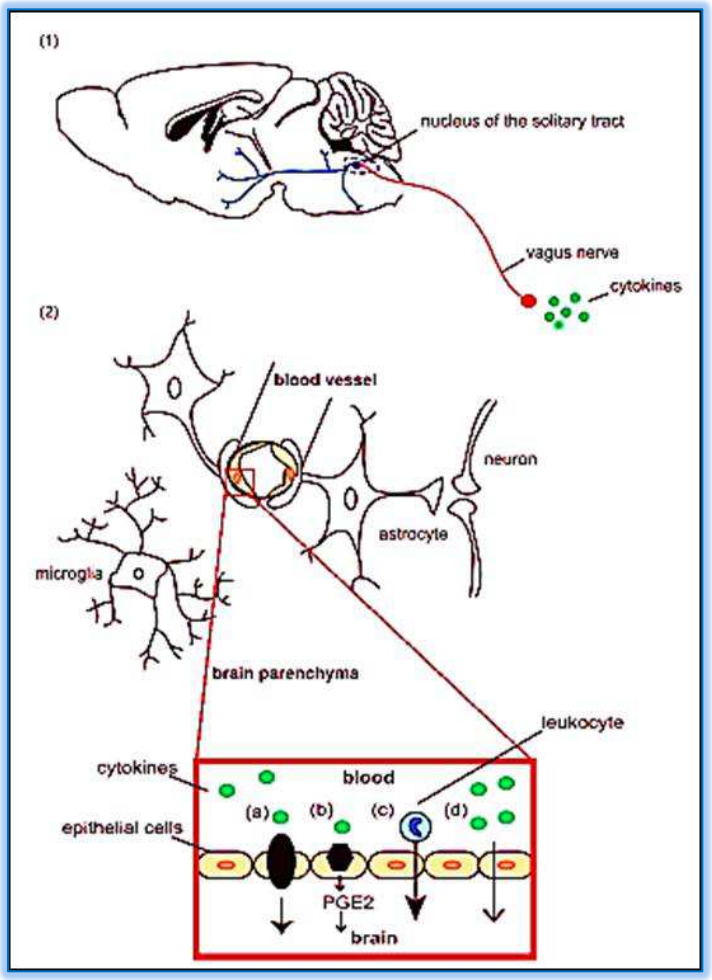


Figure 23. The interface of the immune and central nervous systems. Peripheral immune cells and signals reach the CNS via two primary routes: the neural pathway and the humoral pathway. (Pfau & Russo, 2015).

Conclusion & Perspectives

5. CONCLUSION

In the present study, we assessed the potential antidepressant - like activity of Harmine and Amitriptyline using a well-validated animal model of depression, the CMS model in rats.
From the preceding discussion, it is evident that the modified CMS protocol can induce a complex array of adaptive stress, behavioral and physiological responses similarly to the original version. The repeatedly treatment with Harmine or Amitriptyline reverted these changes induced by CMS. The results can be summarized in following points:

CMS experience induces behavioral changes in rats, expressed by decreasing sensitivity to rewards (anhedonia) that showing a positive correlation between the others behavior as a deficit in open arm exploration of an elevated plus-maze. Also disturbance of emotional state of animal in Open field and reduced locomotor activity in the light and dark box test.

The CMS have been shown to causes disturbance in physiological function were manifested by alteration of ACTH hormone and immunoglobulin levels. Also, CMS induced hypertrophy of adrenal glands and a decrease of brain weight.

Actually, we found that repeated administration of Harmine or Amitriptyline reversed the reduction in sweet solution besides the other behavior deficits including elevated plus-maze, Open field and light and dark box test.

Additionally, our findings revealed that chronic administration of Harmine or Amitriptyline reversed the hypertrophy of adrenal glands, ACTH and immunoglobulin circulating levels, which could suggest a participation pathway activation of Harmine in the mediation of physiological changes in rats subjected to stressful situations. Taken together, these findings support a rapid and robust reversion of anhedonic behavior and physiological alterations induced by chronic mild stress.

6. PERSPECTIVES

We look in the next work to achieve:

- ✓ The effect of Harmine Drug in female rat.
- ✓ The effect of Harmine on sexual activity especially, studying how this drug affect fertility and sperm quality as a part of infertility investigations a purpose for more understand the therapeutic effects at molecular level.
- ✓ See the long-term effects of treatment and whether these effects disappear by time or develop other diseases.
- ✓ Determination of GnRH and testosterone serum level.
- ✓ The molecular mechanisms by which Harmine induces antidepressant-like effects.
- ✓ Determination of BDNF and DYRK1A.
- ✓ Determination of pro-inflammatory cytokines: IL-1, IL-6, TNF-a.
- ✓ Determination of neurotransmitters 5-HT and Dopamine.
- ✓ Determination of oxidative stress parameters (Acetyl cholinesterase, MDA, NO and SOD).
- ✓ Make histological study in different organs mainly in the brain.

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Annex

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Anxiolytic Effects of Harmine Injection on Elevated Plus-Maze Behavior in Male Wistar Rats

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Abstract: The aim of this study is to determine the effect of acute administration of β carboline harmine on the behavioral of rats exposed to chronic mild stress (CMS) procedure. To develop this study we used 60 animals divided into six groups as follows: (G1) non- stressed- Saline. (G2) non-stressed-Amitriptyline; (G3) non- stressed - Harmine; (G4) stressed- Saline; (G5) stressed-Amitriptyline; (G6) stressed- Harmine. After 30 days of exposure to CMS, rats were treated with harmine (15 mg/kg/day) and amitriptyline (20 mg/kg) for 7 days. After treatment period, the rats were subjected to the elevated plus- maze test and sucrose preference test (SPT). Our results demonstrated that CMS procedures induced anhedonia, a decrease in the number of entries and time spent in the open arms without affecting the number of entries either the time spent in closed-arms on EPM apparatus. Harmine and Amitriptyline treatment reversed anhedonia, increased the number of entries and the time spent on open arms. Finally, we concluded that harmine could be used to improve depressive disorders.

Key words: Harmine · Cms · Anhedonia · Epm

INTRODUCTION

Anxiety is a frequent consequence of chronic stress. In humans, stressful life events may lead to anxiety even in the absence of a chronic physiological stress response [1]. Many studies have been conducted to identify the role of different factors that contribute to the development of depression and anxiety [2, 3].

It has been reported that anxiety related behavioral alterations which induced in humans and animals, so it could be explained by change in the neuroendocrine mechanisms [4]. Exposure to stress is a main environmental risk factor associated with the occurrence of depression [5-7]. It's may be the most important environmental factor affecting feeding behavior, metabolism and neuroendocrine functions [8, 9]. Among trusty models of depression, chronic mild stress (CMS) model of depression in rodents has been proposed to model some of the environmental factors that contribute to the introduction of depressive disorders in humans [10]. In the present protocol, CMS sequential exposure of rats to a variety of mild stressors causes behavioral changes which can be related with a modifications of the HPA axis [11-14]. The sucrose-intake deficits is a condition which could be reversed by the chronic administration of classical antidepressant drugs at the same time as dopaminergic agonists the chronic administration of classical antidepressant [15-18]. These changes develop gradually over time with combination of mild, unpredictable stressors and suggest improved face validity of this compared with the more acute stress models. Construct validity for CMS is largely based on the development of reduced sucrose preference, which is interpreted to reflect anhedonia, a core symptom of

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depression [19]. Therefore a many studies concerning to identify a new substance that can potentially treat anxiety disorders and depression [20, 21]. Studies have proved that β-carboline alkaloids, like harmane, norharmane, harmine and others, display antidepressant actions in mouse subjected to forced Swimming test as an animal model of depression [22]. The B carboline harmine was first isolated in 1847 from seeds of *Peganum harmala* and *Banisteriopsis caapi*, both of which have traditionally been used for ritual and medicinal preparations in the Middle East, Central Asia and South America [23]. There are many reports showing the antioxidative action [24, 25] and the inhibition of monoamine oxidase [24].The results of above studies suggest a possible importance of β-carbolines in control of depressive states. Considering this background, the current study was carried out to investigate the antidepressant effects of β-carbolines harmine in rats exposed to chronic mild stress (CMS), generally used behavioral model to induce depressive like state [26, 27] also to investigate the impact of this dose on rats behavior.

MATERIAL AND METHODS

Animals: Male Wistar rats were obtained from the Pasteur institute of Algiers, that weighed 200-340 g they were housed ten per cage with food and water available *ad libitum* and they were maintained at natural conditions (Temperature and a relative humidity). Before the initiation of the experiment. Animals were weighted and separated into six groups, as follows: (1) control-saline (1ml/kg), (2) control-amitriptyline (20mg/kg); (3) control- harmine (15mg/kg); (4) stressed - saline (1ml/kg); (5) stressed-amitriptyline (20mg/kg); (6) stressed- harmine (15mg/kg). Animals in the control group were reared in single cages without any environmental stresses unlike animals in CMS were entered into the CMS procedure.

Drugs: Harmine was obtained from Cayman Chemical (USA) in dose of 15 mg / kg and amitriptyline, the standard antidepressant, from Gencopharm (ZI Rouiba, Route C BP 73, Algeria).was injected intraperitoneally once per day during 7 days. After CMS procedure, different groups of rats (n=10 each) were administered intraperitoneally (i.p.) with saline, harmine (15 mg/kg) or amitriptyline (20 mg/kg) before 30 min of the test session elevated plus maze to assess the behavior of rats and the possible effects of treatment. All treatments were administered in a volume of 1mL/kg.

Chronic Mild Stress Procedure: The chronic mild stress protocol was adapted from a several studies [26] with some modification. This model used to achieve depressive-like symptoms in Wistar rats [28, 29]. Also it is used for screening novel antidepressant treatments and investigating the neurobiology of depression and its relation to other diseases [29, 30]. During this experiment, the rats in the control group were kept unperturbed, in their home cages without any stress, receiving only ordinary daily care with daily supports of food and water, whereas the CMS groups were subjected to different mild stressors, applied randomly for 30 days. Briefly, The following stressors were used: (i) forced swimming for a duration of 10 or 15 min on days 1, 15, 21, 25 ;(ii) 24 h water deprivation on days 5, 10, 14,19,30 ; (iii) 24 h food deprivation was applied on days 6, 13, 20,26; (iv) 1–1,5 h restraint on days 2,3,4,7,8,9 and 2-3-4 h restraint on days 16-29,(v) and no-stress on days 11-12-27. Restraint stress is based on that of Bardin *et al*. [31] FST is formed on that of Porsolt *et al*. [32].

Elevated plus Maze (EPM): The elevated plus maze has strong claims to validity as an animal model of anxiety, The apparatus and the testing procedure were carried out as originally described by Pellow and associates [33]. The EPM apparatus was made of wood and consisted of Two opposite open arms (50×10 cm) had no walls and the other two closed arms (50×10 cm) had 50 cm high walls made of clear Plexiglas. The model is based on rodents' aversion of open spaces. The open and closed arms were connected by a central square (10×10 cm) and were elevated 50 cm above the floor. Rats from each group were placed in the central square of the Plus-Maze facing an open arm and were then allowed to explore the apparatus. And their activity was videotaped for 5 min. The following behaviors were scored during the test: the number of entry in each arm, Time spent in the open and closed arms, while the numbers of entries into the open and closed arms were mostly used as measures of general activity [34, 35]. An individual entry was recorded when the animal entered the arm with at least two front paws and half of its body. The shorter is the times spent in open arms, the higher is anxiety and vice versa [36, 37]. After 5 minutes, rat were removed from the maze by the base of their tails and returned to their home cage. The maze was than cleaned with a solution of 30% ethanol and soft paper permitted to dry between tests.

Sucrose Preference Test (Anhedonia Test): Sucrose preference (SP) test is a measure to evaluate anhedonic effect of CMS [29, 38]. In this test, rats were trained access to two bottles (Water and 1% sucrose solution) freely for 7 days. The position of the 250-mL bottles containing sucrose solution or tap water was changed every day. Sucrose preference was expressed as percent of the volume of sucrose solution of a total volume of fluid (Sucrose plus regular water).

Data Analysis: The Statistical Minitab 16.0 was used for statistical analyses. All data are presented as mean±S.E.M. Differences among experimental groups were determined by one-way ANOVA followed by Tukey's post hoc test. *p* Values less than (0.05) were considered statistically significant.

RESULTS

The Results of Various Tests Are Discussed Below: Data regarding to the effect of acute administration of harmine on the behavioral changes during the elevated plus- maze were outlined in Figure (1.A, B). The number of entries and time spent in open arms were significantly affected by amitriptyline and harmine treatment (P<0.05, P <0.01). Which CMS rats displayed decrease in the number of entries and in the time spent in the open arms compared to non-stressed rats Figure 1A,B [F(2,70-4,14), P =(0.030-0.003)]; Moreover, No significant differences were detected for rats treated with amitriptyline or harmine compared with control rats on the number of entries and the time spent in closed arms ([F(2,15-1,36), (P=0.074-0,252]) Figure (1C ;D) (P >0.05).

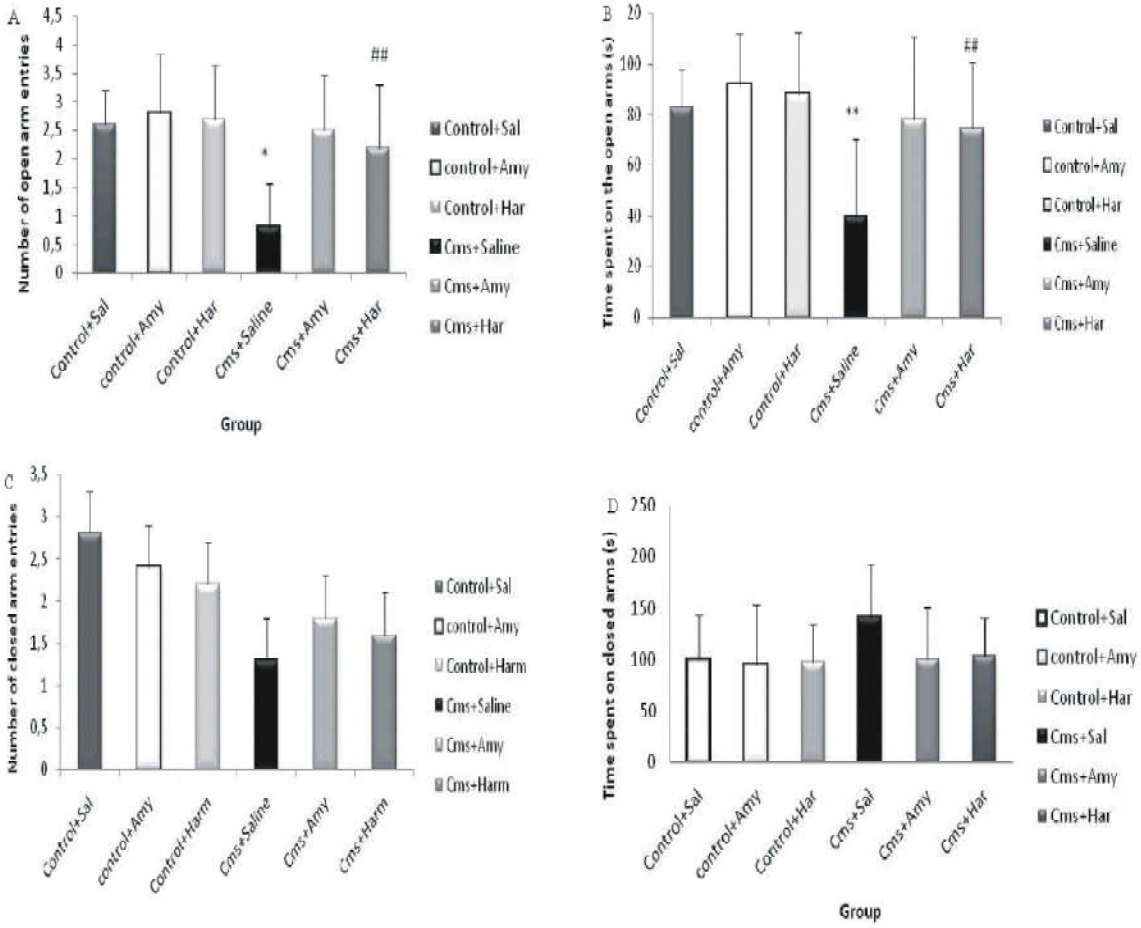

Fig. 1: Effects of CMS procedure on the number of entries in open arm (A) On the Time spent in open arms (B) on the number of entries in closed arms (C) and on the time spent in closed arms (D) in the Elevated plus maze test in rats repeatedly treated with amitriptyline or harmine. Bars represent means±S.E.M. * p<0, 05 vs. control saline; # p<0.05 vs.CMS saline, according to ANOVA post-hoc Tukey test.

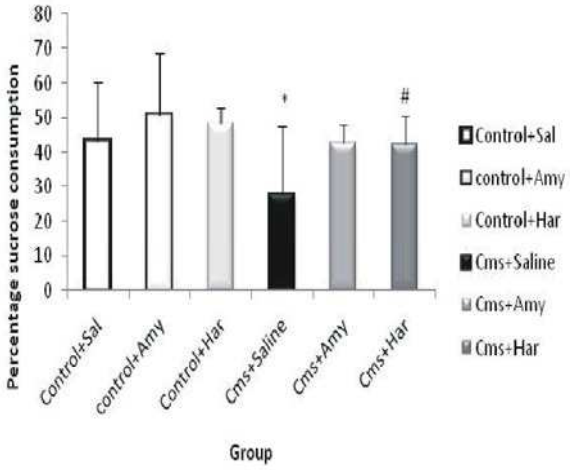

Fig. 2: Effects of CMS procedure on sweet food consumption in rats treated with amitriptyline or harmine. Bars represent means±S.E.M. * p<0, 05 vs. control saline; # p<0.05 vs.CMS saline, according to ANOVA post-hoc Tukey test.

As shown in Fig. 2, CMS rats treated with saline reduced sucrose solution consumption compared with non-stressed rats treated with saline (P <0.05). Statistical analysis revealed that CMS rats treated with harmine reverted the reduction of sucrose solution consumption induced by chronic mild stress ([F=3, 27), P =0.012]).

DISCUSSION

The present data clearly suggest that intraperitoneally injection of harmine decreased the level of anxiety that appeared in the behavior change induced by chronic stressful stimuli.

When behaviors were assessed, we found that the number of entries and the time spent on the open arms were significantly changed by stress, so administration of harmine reversed the behavior induced by chronic stressful stimuli that induced an increase of the number of entries and the time spent in the open arms apparatus. According to these results, in rats application of chronic mild stress (CMS) procedures resulted in a variety of behavioral, neurochemical, neuroendocrine and neuroimmune alterations resembling some of the dysfunctions observed in human depression [39-42].

The elevated plus-maze test is based on the spontaneous exploratory behavior of rodents and their natural aversion to the open arms caused by fear and anxiety [33, 43]. Which, an increase in the number of entries added to the lengthy time spent in the open arms apparatus demonstrate a lower level of anxiety [43, 44].

This finding suggests that seven days of treatment with harmine and amitriptyline (15-20 mg/kg) are significantly caused anti-anxiety-like effect, which is consistent with a previous study [45-49] on the other hand both of harmine and amitriptyline did not enhance the change in locomotor activity in the closed arms. Whereas other antidepressant such us venlafaxine treatment significantly caused anti-anxiety-like effect and also improved locomotor activity [50].

The difference from previous studies may result from possibly milder stress applied in our CMS protocol, experimental procedures (Day-night phase of the application of stress and age of the animal) may affect the behavior of the animal.

The CMS paradigm is a model of depression which induces by chronic mild and unpredictable stressors [33]. In the CMS model, both of sweet food consumption and preference sucrose intake, as well as decreased intracranial self stimulation behavior, serves as markers of generalized decrease in sensitivity to reward and they are quite related to anhedonia [27, 51, 52]. In accordance with the literature, present data confirm that rats subjected to CMS procedure consume less sweet food compared to non-stressed rats treated with saline [53-57]. These findings suggest that, under our experimental conditions, the CMS procedure induced anhedonic-like behavior in our rats.

The present findings demonstrate that repeated administration of harmine reversed the anhedonic-like behavior in CMS rats and increased sweet food consumption in non-stressed rats. In this study, The behavioral effects induced by harmine in rats are in coincidence with the literature data, which support an antidepressant action for harmine in basic studies that could be due to interactions of harmine and related alkaloids with several receptor systems act as agonists at serotonin receptors [58-60] involved in the modulation of behavioral and molecular actions of antidepressants [25, 59, 61, 62]. ß carbolines, mainly harmine and harmaline, inhibit MAO activity [63] Furthermore, as MAO inhibitors, ß-carbolines can increase the level of serotonin in the brain [64] and are capable of inducing direct psychoactive effects [65,66]. Brierley *et al*. [67] also suggested that harmine increase dopamine efflux via a novel shell-specific, presynaptic 5-HT2A receptor dependent mechanism, independent of MAO inhibitory activity.

Consistent with previous studies in which 40 day of CMS and in the forced swimming test significantly induced depressive-like behavior in rats [22, 68] the results of our study also showed that chronic mild stress induced significant depressive-like behavior, including decreased sucrose solution intake.

CONCLUSION

From the above observations we can conclude that acute administration of harmine develops anxiolytic activity & Anti depressant at both the dose level which is comparable with the standards. Which prevents the development of anxiety/depressive-like behavior in CMS rats. However further studies are required to know the exact mechanism action of harmine as anxiolytic.

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ABSTRACT

This study aims at evaluating the behavioral and physiological effects of chronic administration of β-carboline Harmine and Amitriptyline in rats exposed to CMS procedure. After 30 days of exposure to CMS procedure, rats were treated with Harmine (15 mg/kg/day, ip.) or Amitriptyline (20 mg/kg/day, ip.) for 7 days. Afterwards, rats were tested with a battery of behavioral tests: Annedonia, Open Field (OF), Elevated plus Maze (EPM) and Light and Dark Box (LDB).

The findings demonstrate that CMS shows behavioral changes during the different tests; a decrease in the consumption of sweet solutions compared with the control group.

Concerning the data of the effects of CMS in the EPM test, CMS rats demonstrated a decrease in the number of entries and the time spent in the open arms compared to non-stressed rats. On the other hand the results show no significant differences on the number of entries and the time spent in closed arms on CMS rats compared with the control rats.

However, in the OF parameters, we notice a decrease of the time spent in the field center, a decrease of grooming time, without affecting defecation, crossing and rearing activity in the stressed group compared with the control group.

The data of the CMS effects on the LDB parameters show a decrease in the number of entries and the time spent in the light compartment compared with the control group.

Regarding the physiological parameters; the results reveal an increase of ACTH level compared with the control group. Moreover, CMS alters the immunological parameters by increasing of IgG, without affecting IgM circulating levels.

The results demonstrate also how that CMS induces a decrease in brain weight and hypertrophy of adrenal gland.

The chronic treatment with Harmine or Amitriptyline for 1 week reverse anhedonia, OF, EPM and LDB behaviors, brain and adrenal gland weights, normalized ACTH and immunoglobulin G (IgG) circulating levels .

In conclusion, treatment with Harmine or Amitriptyline has robust effects in reverting mainly physiological alterations reliable as indicators of animal depressive disorders.

Keywords:

CMS, Harmine, Amitriptyline, Behavioral changes, ACTH, IgG, IgM, depression.