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Is Riparin III a promising drug in the treatment for depression?

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ABSTRACT

Stress is crucially related to the pathophysiology of mood disorders, including depression. Since the effectiveness and number of the current pharmacological options still presents significant limitations, research on new substances is paramount. In rodents, several findings have indicated that corticosterone administration induces the manifestation of behavioral and neurochemical aspects of depression. Recently, riparin III has shown antidepressant-like properties in trials performed on animal models. Thus, our goal was to investigate the effects of riparin III on behavioral tests, monoamines levels, oxidative stress and cytokines levels in chronic corticosterone-induced model of depression. To do this, female swiss mice were treated with subcutaneous administration of corticosterone for 22 days. In addition, for the last 10 days, riparin III or fluvoxamine were also administered *per os* in specific test groups. Control groups received subcutaneous saline injections or distilled water *per os*. At the end of the timeline, the animals were killed and their hippocampi, prefrontal cortex, and striatum dissected for neurochemical analysis. Brain changes following corticosterone administration were confirmed, and riparin III could reversed the most abnormal behavioral and neurochemical corticosterone-induced alterations. These results suggest the potential antioxidant, anti-inflammatory and antidepressant effects of riparin III after a chronic stress exposure.

1. Introduction

Stress is crucially related to the pathophysiology of many mood disorders (Gupta et al., 2015). One particular adaptive response to stress involves stimulation of the hypothalamic–pituitary–adrenal (HPA) axis, leading to the release of glucocorticoids (GC) to the systemic circulation. Excessive release of GCs can promote psychological dysfunctions such as depression and anxiety (Nestler et al., 2002; Zunszain et al., 2011).

In rodents, several findings have indicated that repeated corticosterone (Cort) injections induce the manifestation of neurobiological aspects of depression, including effects on neurogenesis, brain monoamine metabolism, alterations of cytokines levels and oxidative stress (Cai et al., 2015; Lussier et al., 2013; Pitta et al., 2013; Vargas et al., 2013). This, alongside the fact that this pathology demonstrates high levels refractoriness to pharmacological treatment, led to prolonged Cort administration in rodents being suggested as an animal model of

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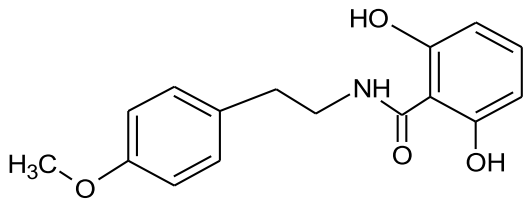

Fig. 1. Chemical structure of riparin III (Catão, 2005).

treatment-resistant depression, useful to evaluate the activity of new drugs for depression treatment (NIMH, 2006).

Recently, anxiolytic, antidepressant and anticonvulsant-like properties of riparin III (Rip III) have been demonstrated in acute and chronic animal models. The substance's organic nomenclature is N-2,6-dihydroxy-benzoyl tyramine methyl ether (Sousa et al., 2004; Vasconcelos et al., 2015) (Fig. 1).

In this study, the antidepressant-like properties of Rip III treatment were further evaluated in a female mice model of depression induced by Cort to determine its effects in behavioral and neurochemical tests, such as monoamines levels, oxidative stress and cytokines levels.

2. Methods

2.1. Animals

Swiss female mice (*Mus musculus*) from the university central animal housing, Drug Research Development Center – Federal University of Ceará, and weighing between 18 and 22g (age: 8–10 weeks), were kept under a light/dark cycle, 12 hours in each stage, the animals received a regular diet (Purina®) and water *ad libitum*. All procedures were performed according to the recommendations of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Experimental protocols were approved by the Animal Research Ethics Committee of the UFC (13/2014 and 06/2017).

2.2. Drugs

Corticosterone (Cort; Sigma-Aldrich, St. Louis, MO, USA) was suspended in vehicle 1 (saline solution containing 0.1% dimethyl sulfoxide (DMSO; VETEC™, USA) and 0.1% polysorbate 80 (Tween®; (Sigma-Aldrich, St. Louis, MO, USA), totaling a 4mg/mL solution.

Corticosterone 20 mg/kg was administered through a single daily subcutaneous injection, from 09:00 to 11:30 A.M. for twenty-two consecutive days. The dosage and route of administration for Cort were selected based on previous studies (Silva et al., 2013; Zhao et al., 2008).

Rip III was provided by Laboratory Chemistry of Bioactive Natural and Synthetic Products, FederalUniversity of Piauí, Teresina, PI, Brazil. Rip III was suspended in vehicle 2 (water solution containing 2% Tween-80), obtained a concentration of 5mg/mL, and administered *per os* by gavage for 10 consecutive days at dose of 50 mg/kg.

Fluvoxamine (Flu; Abott®, New Jersey, USA) was suspended in vehicle 2, obtained a concentration of 5mg/mL) and administered *per os* by gavage for 10 consecutive days at the dose of 50 mg/kg.

2.3. Treatment

The animals were randomly divided into one of the following four experimental groups (n = 20 animals per group), with 14 animals in each group for the oxidative stress dosages, and 6 animals to determine monoamines levels in the striatum and cytokines levels in hippocampus (Fig. 2).

Control group: mice received a daily injection of saline solution containing 0.1% DMSO and 0.1% Tween-80 by subcutaneous injections (s.c.), for 22 consecutive days. In the last seven days, the animals additionally received vehicle 2 by gavage (*per os*), without previous experimental manipulation.

Cort-induced depression model: mice in this group received daily subcutaneous injections of Cort (20 mg/kg, s.c.) once a day, between 09:00 AM and 11:30 AM., for 22 days (Zhao et al., 2008). In the last seven days, the animals additionally received vehicle 2 by gavage (*per os*), without previous experimental manipulation.

Treated groups with two associated drugs: animals in this group received a repeated injection of Cort (20 mg/kg, s.c.) for 14 days. From 15th day, the mice received Cort + Rip III (50 mg/kg, *per os*) or Cort + Flu (50 mg/kg, *per os*).

Twenty-four hours after the last drug administration, the animals were decapitated and the brain areas prefrontal cortex (PFC), hippocampus (HC) and striatum (ST) were dissected for the neurochemical tests.

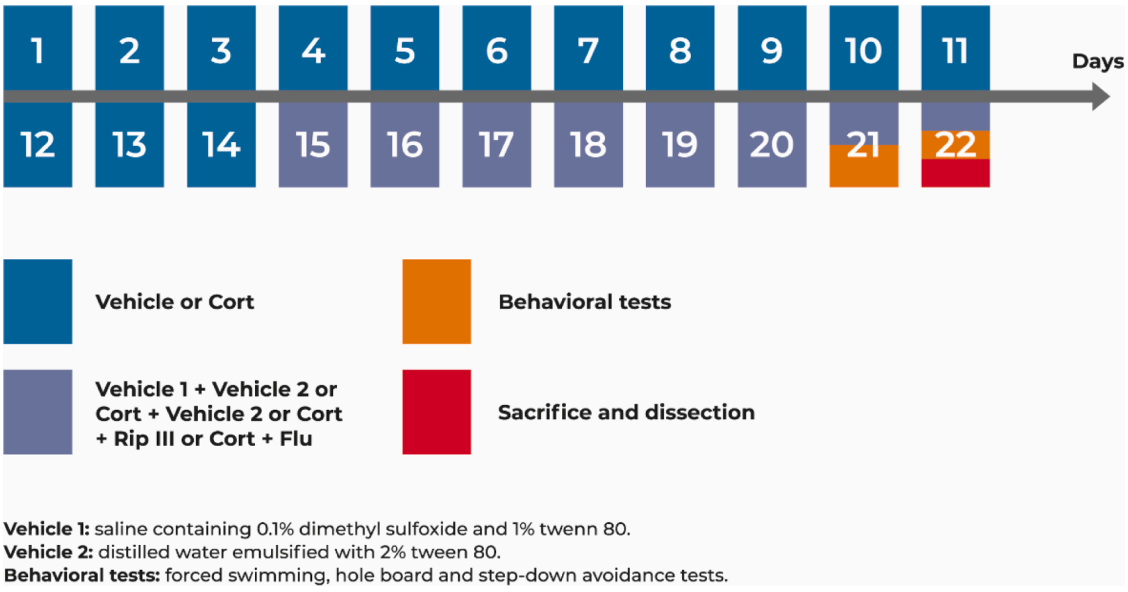

Fig. 2. Treatment scheme.

2.4. Behavioral tests

2.4.1. Forced swimming test

This test is indicative of depression-like behavior and was performed according to the protocol described in (Porsolt et al., 1927) with minimum modification. Mice were placed individually in the cylinder tank filled with water (25°C ± 1°C) to a depth of 25cm, such that the mice were not be able to touch the bottom of the tank, either with their feet or their tails, during the swimming test. Animal behavior was analyzed by an independent researcher who did not know the experimental groups. The immobility and climbing times during a five minute period were recorded. Immobility was defined as the animal floating in the water with the absence of any movement except for those necessary for keeping the nose above water. Climbing occurs when quick movements of the forelimbs are observed such that the front paws break the surface of the water. An increase in the duration of immobility and decrease of climbing is indicative of depressed-like behavior (Yankelevitch-Yahav et al., 2015).

2.4.2. Hole-board test

The hole-board test is based on the concept that rodent exploratory activity is inversely proportional to the animal's anxiety level and was carried out according to the protocol described by Boissier and Simon (1962). The apparatus, 60 × 30 cm, was consisted of gray acrylic panels with sixteen equidistant holes 3 cm in diameters on the floor. The apparatus has no walls and was elevated to a height of 18 cm above floor level. Each mouse was placed singly in the center of the board facing away from the observer and for 5 min the total number of head-dips was counted and recorded.

2.4.3. Step-down avoidance test

The step-down avoidance test was performed with the objective of evaluating the memory parameter, being and previously described by Joshi and Parle (2006). The apparatus consisted of a box (31 × 27 × 24 cm) with three walls of wood and the front wall of Plexiglas. The floor consisted of a series of parallel stainless-steel bars, spaced 0.8 cm apart, and connected to a generator. The left extremity of the grid was covered by a high non-conductive formic platform (safety platform).

Training was performed in two similar sessions. First, each mouse was placed on the safety platform and the amount of time for the animal to stepdown on the grid was recorded (step-down latency time). Then, a 0.5 mA shock was applied, and the animal was removed from the apparatus. The second session was carried out 90 min after the first test. For the evaluation of the short-term memory and long-term memory, the animals were submitted to the test session at 90 min (second session) and at 24 h (retention) after the training session, respectively, with an upper cutoff time of 300 s.

2.5. Neurochemicals tests

2.5.1. Determination of monoamine levels

The analysis of monoamines was performed in the striatum as described by (Hallman and Jonsson, 1984) using CLC-ODS (M) with a length of 25 cm, 4.6 mm diameter and 3 mm particle diameter (Shimadzu, Japan). Dosage of noradrenaline (NE), dopamine (DA), serotonin (5-HT) and the metabolites 3,4-dihydroxyphenylacetic (DOPAC), homovanillic acid (HVA) and 5-hydroxuindoleacetic acid (5-HIAA) were performed. Then, monoamine levels were electronically measured using an amperometric detector (Model L-ECD-6A from Shimadzu, Japan).

2.5.2. Oxidative stress

For the oxidative stress, the brain areas ST, PFC and HC were homogenized in ten volumes (1:10 w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl in a Teflon-glass homogenizer and centrifuged at 750 × g for 10 min at 4°C. Finally, the supernatant was separated and immediately used for evaluation of superoxide dismutase (SOD) and catalase (CAT) activity and quantification of nitrite/nitrate (NIT), reduced glutathione (GSH) and malondialdehyde (MDA) levels.

2.5.2.1. Determination of superoxide dismutase (SOD). Determination of SOD activity was performed according to a previously described method (Sun et al., 1988). In this assay, the photochemical reduction of riboflavin generates superoxide (O^{2-}) that reduces the nitro-blue tetrazolium (NBT) to produce formazan salt. This reduction is inhibited in the presence of SOD. Briefly, supernatants were centrifuged (20 min, 12,000 rpm, 4°C) and mixed with NBT and riboflavin. Measurement was performed after exposure of the resulting solution to fluorescent light (15 W) for 15 min in a spectrophotometer at 560 nm. The results are expressed as the quantity of SOD in units of the enzyme per µg of protein (U/µg protein).

2.5.2.2. Determination of reduced glutathione (GSH). The levels of reduced glutathione GSH were determined to estimate endogenous antioxidant defenses. Described by Sedlák and L'Hanus (1982), the method was based on Ellman's reagent (DTNB) reaction with free thiol groups. The samples were mixed with 0.4 M Tris–HCl buffer (pH 8.9) and 0.01 M DTNB. The GSH levels were determined using a microplate reader set at 412 nm and calculated based on a standard glutathione curve and expressed as ng of GSH/g wet tissue.

2.5.2.3. Determination of malondialdehyde. The rate of lipoperoxidation was estimated by determination of malondialdehyde (MDA) using the Thiobarbituric Acid Reactive Substances (TBARS) test (Draper and Hadley, 1990). The brain areas were homogenized to 10% tissue with 50 mM potassium phosphate monobasic buffer pH 7.4. Then, 63 µL of the homogenate was mixed to 100 µL of 35% perchloric acid. The samples were centrifuged (5000 rpm/10 min), the supernatants were removed, mixed with thiobarbituric acid 1.2%, and then heated in a boiling water bath for 30 min After cooling, the lipid peroxidation was determined using a microplate reader set at 535 nm and expressed as µmol MDA/g wet tissue.

2.5.2.4. Determination of nitrite/nitrate. The production of nitric oxide (NO) was determined based on the Griess reaction, described by Green et al. (1982). Mouse brain areas homogenates (10% w/v) were prepared in 1.15% chloride solution of potassium (KCl). After centrifugation, supernatants were collected and incubated with the 100 µL Griess reagent at room temperature for 10 min. The absorbance was measured at 546 nm via a microplate reader. The amount of nitrite was calculated from a $NaNO_2$ standard curve, and it was expressed as nmol/g of wet tissue.

2.5.2.5. Determination of catalase (CAT) activity. The CAT activity has as principle the measurement of the rate of production of O2 and H2O to the extent that H2O2, used as substrate, is hydrolyzed, according to Chance and Maehly (1955), Maehly and Chance (1954). The enzyme activity is measured by reading the absorbance variation (at 230 nm) per minute for 6 minutes and the results are expressed in µM/min/µg protein.

2.5.2.6. Determination of cytokines levels. For cytokine analysis, was used the BD Cytometric Bead Array (CBA) Th1/Th2/Th17 cytokine kit (BD, New Jersey, USA). The dissected hippocampi were homogenized with Assay Diluent and then centrifuged (10000 rpm, 5 min, 4°C). The concentrations of cytokines were determined by flow cytometry according to the manufacturer and expressed in pg/ml, as follows: tumor necrosis factor-α (TNF-α), interferon -γ (IFN-γ), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-17A (IL-17A). The results were extracted using the FCAP Array software.

2.6. Statistical analysis

Results were analyzed using GraphPad Prism 8.0.1 software (San Diego, CA, USA). Initially, the results were submitted to the Shapiro-Wilk test to verify the normality of the sample (parametric or nonparametric). Statistical analysis was performed by one-way ANOVA followed by Tukey's *post hoc*, for parametric results, or Kruskal-Wallis followed by Dunn's test, for nonparametric. Data are expressed as mean ± standard error of the mean (SEM), and differences were considered significant when $p \leq 0.05$.

3. Results

The results showed that Rip III has antidepressant, anxiolytic and memory preservation activity, and that its mechanism of action possibly involves varied pathways described in different hypothesis of the pharmacological treatment of depression, including modulation of HHA axis, restored monoamine levels, antioxidant and anti-inflammatory.

3.1. Behavioral tests

3.1.1. Forced swimming test

Through the forced swimming test (Fig. 3) it was possible to assess the antidepressant activity of Rip III utilizing the parameters of immobility time (Fig. 3a) and climbing time (Fig. 3b). Corticosterone treatment increased immobility time ($F_{3,24} = 107.3$, n=28, $p < 0.0001$) and decreased climbing time ($F_{3,24} = 39.1$, n=28, $p = 0.0056$). Rip III ($p < 0.0001$) and Flu ($p < 0.0001$) were able to reverse these changes, however.

3.1.2. Hole board test

The hole board test was performed to evaluate the anxiolytic effects of riparin III. Cort treatment decreased the number of head dips ($F_{3,24} = 9.479$, n=28, $p = 0.0012$) and administration of riparin III reversed this parameter (Fig. 4).

3.1.3. Passive avoidance test

Through the step-down avoidance test it was possible to verify that corticosterone promoted short- (Fig. 5a; n=28, $p = 0.0115$) and long-term (Fig. 5b; n= 28, $p = 0.0098$) memory impairment in the animals, and that the treatments with Rip III ($p = 0.0006$) and Flu ($p = 0.012$) reversed the short-term memory deficit, demonstrated by the increase of the animal's step-down latency towards the region causing electric shock.

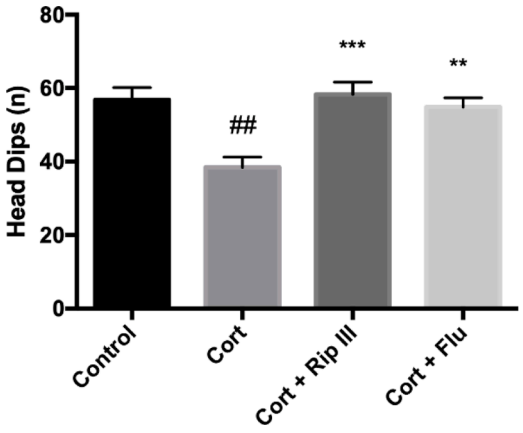

Fig. 4. Effect of oral Riparin III (50mg/kg) and Flu (50mg/kg) on the number of head dips in the hole board test.
Results are expressed as the mean ± SEM (n = 7 animals / group). Statistical analysis was determined by one-way ANOVA, followed by Tukey's post hoc test. Significant values: ##p <0.01 vs control, **p <0.01, ***p <0.001 vs Cort.

3.2. Neurochemical tests

3.2.1. Stress

Corticosterone treatment promotes a significant increase in nitrite/nitrate levels in the PFC ($F_{3,28} = 46.98$, n=32, $p = 0.0001$) and ST ($F_{3,28} = 10.19$, n=32, p= 0.0025) brain areas, showed in Fig. 6. In contrast, Rip III and Flu treatment was able to reduce these levels in the PFC ($p = 0.0004$; $p = 0.0018$, respectively) and ST ($p < 0.0001$; $p < 0.0001$, respectively).

Corticosterone treatment promoted a significant increase in MDA levels in the PFC ($F_{3,28} = 46.98$, n=32, $p < 0.0001$) and HIP (n= 32, $p = 0.0238$) brain areas. Treatment with Rip III showed a significant reduction of these levels in PFC ($p < 0.0001$), HIP ($p < 0.0001$) and ST ($p = 0.0418$); and treatment with Flu, only in PFC ($p < 0.0001$) and ST ($p = 0.0005$) (Fig. 7).

Corticosterone treatment promoted a significant decrease in GSH levels in PFC ($F_{3,28} = 6.134$, n=32, $p = 0.0039$), HIP (n = 32, $p < 0.0001$) and ST (n= 32, $p < 0.0001$) (Fig. 8). Rip III (PFC: $p = 0.0384$; HIP: $p = 0.0199$; ST: $p = 0.0283$) and Flu (PFC: $p = 0.0099$; HIP: $p = 0.0429$; ST: $p = 0.0111$) reversed changes in the three cerebral areas.

The results of catalase and SOD were not statistically significant, suggesting that Rip III has no effect on these antioxidant systems.

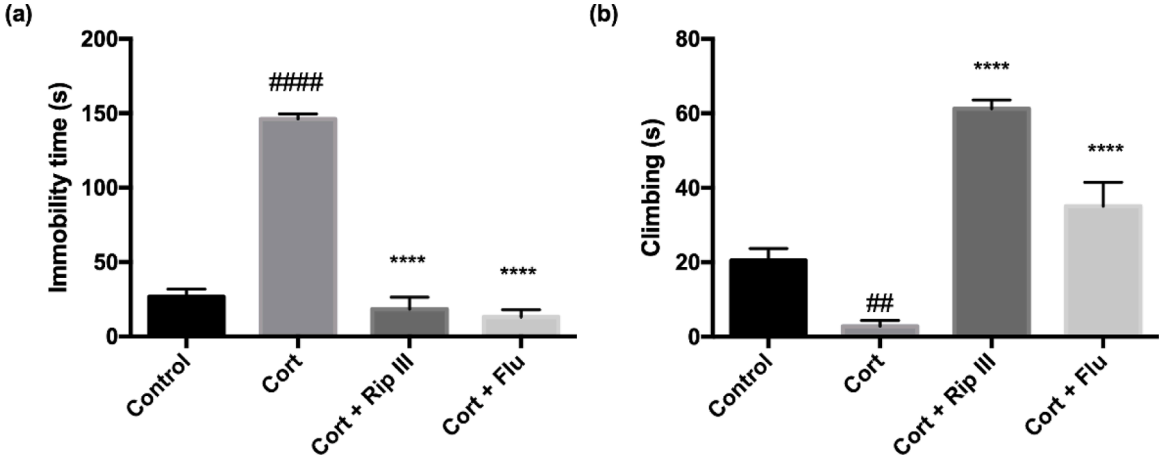

Fig. 3. Effect of Rip III (50mg/kg) and Flu (50mg/kg), orally, on (a) immobility time and (b) climbing time in mice in the forced swimming test.
Results are expressed as the mean ± SEM (n = 7 animals/ group). Statistical analysis was determined by one-way ANOVA, followed by Tukey's *post hoc* test. Significant values: ##p <0.001; ####p <0.0001 vs control, ****p <0.0001 vs Cort.

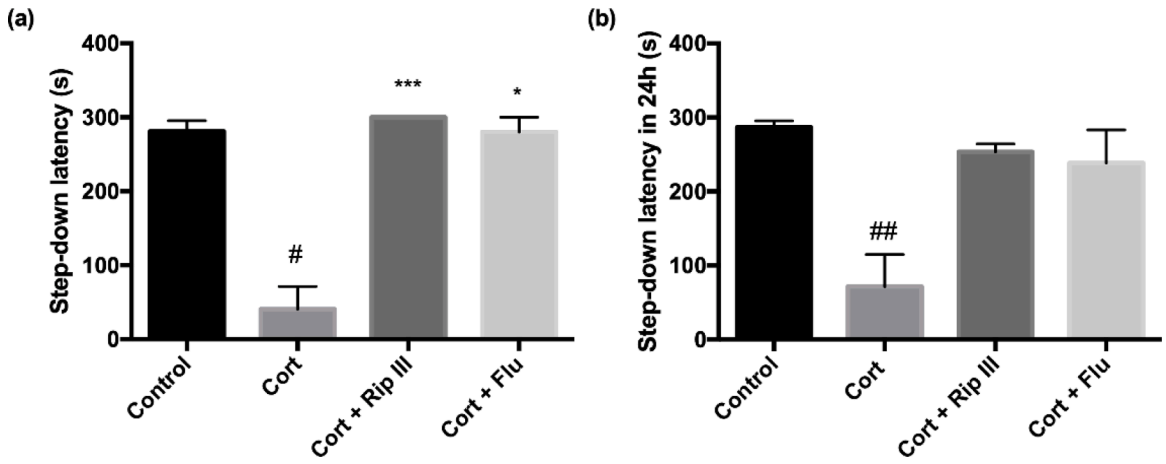

Fig. 5. Effect of oral Rip III (50mg/kg) and Flu (50mg/kg) on reversal of Cort-induced (a) short- and (b) long-term memory loss.
Results are expressed as the mean ± SEM (n = 7 animals / group). Statistical analysis was determined by Kruskal-Wallis, followed by Dunn's test. Significant values: #p <0.05, ##p <0.01 vs control, *p <0.05, ***p <0.001 vs Cort.

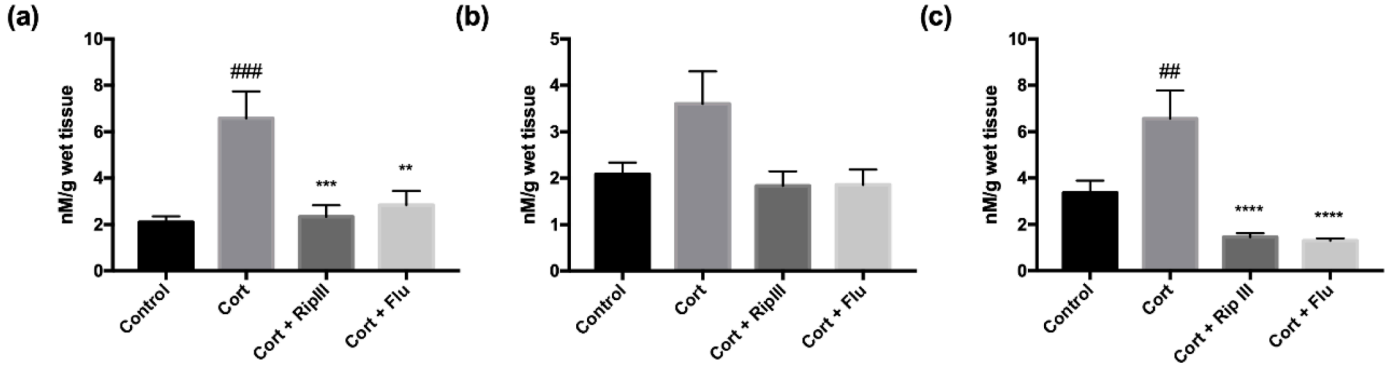

Fig. 6. Effect of Riparin III on nitrite/nitrate levels in (A) prefrontal cortex, (B) hippocampus and (C) striatum after chronic administration of Cort, Cort + Rip III and Cort + Flu.
Results are expressed as the mean ± SEM (n = 8 animals / group). Statistical analysis was determined by one-way ANOVA, followed by Tukey's test for PFC and ST. Statistical analysis was determined by Kruskal-Wallis, followed by Dunn's test for HIP. Significant values: $^{\#\#}$p <0.01, $^{\#\#\#}$p<0.001 vs control, **p <0.01, ***p <0.001, ****p <0.0001 vs Cort.

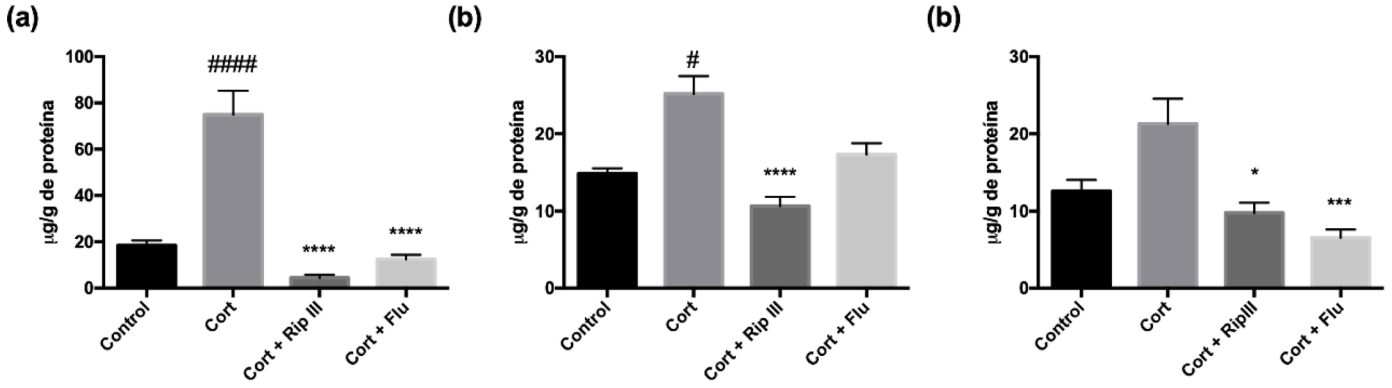

Fig. 7. Effect of Riparin III on MDA levels in the (A) PFC, (B) HC and (C) ST after chronic administration of Cort, Cort + Rip III and Cort + Flu.
Results are expressed as the mean ± SEM (n = 8 animals / group). Statistical analysis was determined by one-way ANOVA, followed by Tukey's post hoc test for the PFC. Statistical analysis was determined by Kruskal-Wallis, followed by Dunn's test for the HIP and ST. Significant values: $^{\#}$p <0.05, $^{\#\#\#\#}$p <0.0001 vs control, *p <0.05, ***p <0.001, ****p <0.0001 vs Cort.

3.2.2. *Cytokines levels*

Fig. 9 shows that corticosterone administration resulted in increased pro-inflammatory cytokines with Th1/Th17 profile in the hippocampus of animals, being significant in IL-17 ($F_{3,16}$ = 9.111, n = 20, p= 0.0012). Treatment with Rip III reduced all cytokines studied, being statistically significant in IL-2 ($F_{3,16}$ = 4.492, n=20, p= 0.044) and IL-17 (p= 0.0029), whereas in Flu only for IL-2 (p= 0.0235).

Corticosterone administration resulted in alterations in the levels of Th2-profile cytokines in the hippocampus, with an increase in IL-6 and a reduction in IL-4 and IL-10 with significance in IL-4 (p= 0.013) and

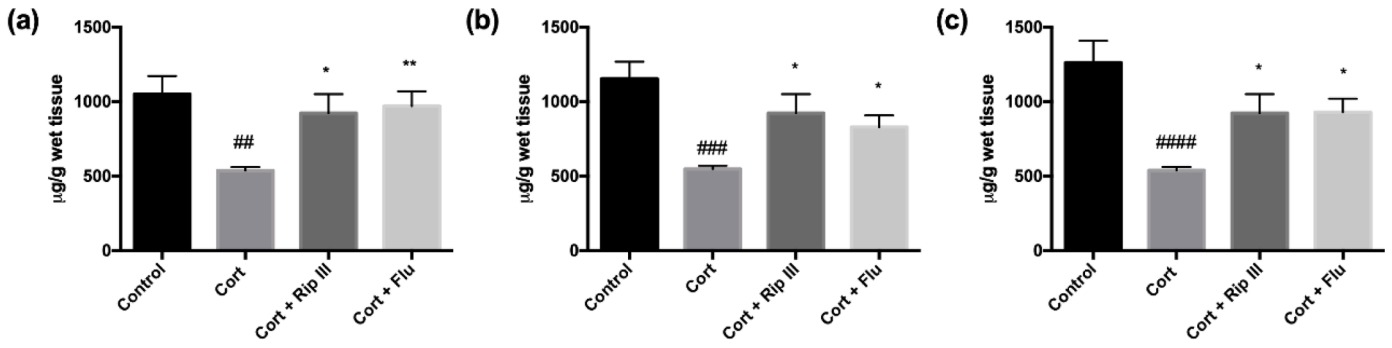

Fig. 8. - Effect of Rip III on GSH levels in (A) PFC, (B) HC and (C) ST after chronic admission of Cort, Cort + Rip III and Cort + Flu.
Results are expressed as the mean ± SEM (n = 8 animals / group). Statistical analysis was determined by one-way ANOVA, followed by Tukey's post hoc test for PFC. Statistical analysis was determined by Kruskal-Wallis, followed by Dunn's test for HIP and ST. Significant values: $^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$ vs control, $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs Cort.

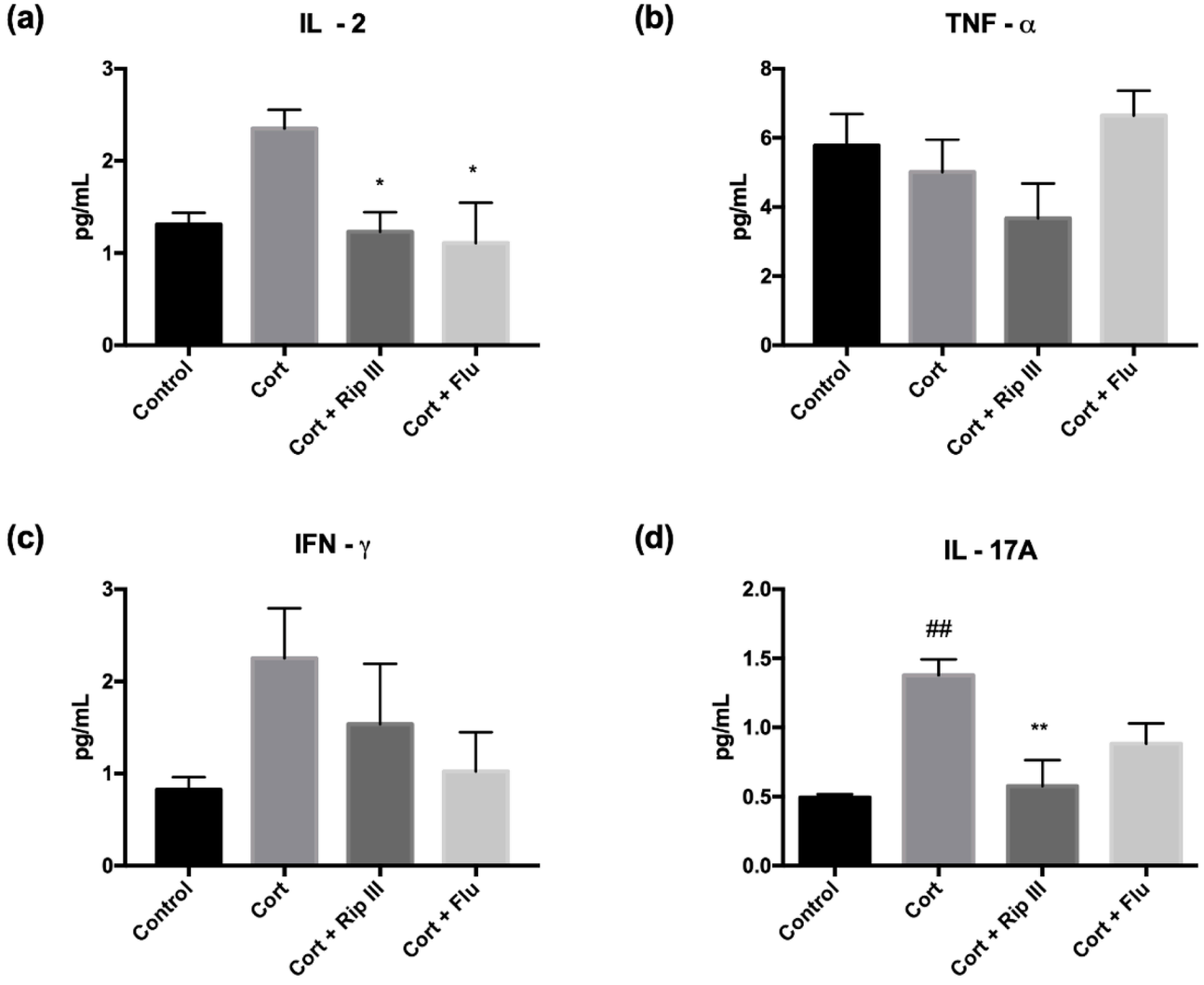

Fig. 9. Effect of Rip III on cytokines Th1/Th17 profile levels in HC after chronic admission of Cort, Cort + Rip III and Cort + Flu.
Results are expressed as the mean ± SEM (n = 5 animals / group). Statistical analysis was determined by one-way ANOVA, followed by Tukey's *post hoc* test for IL-2, IL17, TNF-α and IFN-γ. Significant values: $^{\#\#}p < 0.01$ vs control, $^{*}p < 0.05$, $^{**}p < 0.01$. IL-2: $F_{(3,16)} = 4.492$; TNF-α: $F_{(3,16)} = 1.942$; IFN-γ: $F_{(3,16)} = 1.749$; IL-17: $F_{(3,16)} = 9.111$. Tumor Necrosis Factor-α (TNF-α), Interferon-γ (IFN-γ), Interleukin-2 (IL-2), Interleukin-17 A (IL-17A).

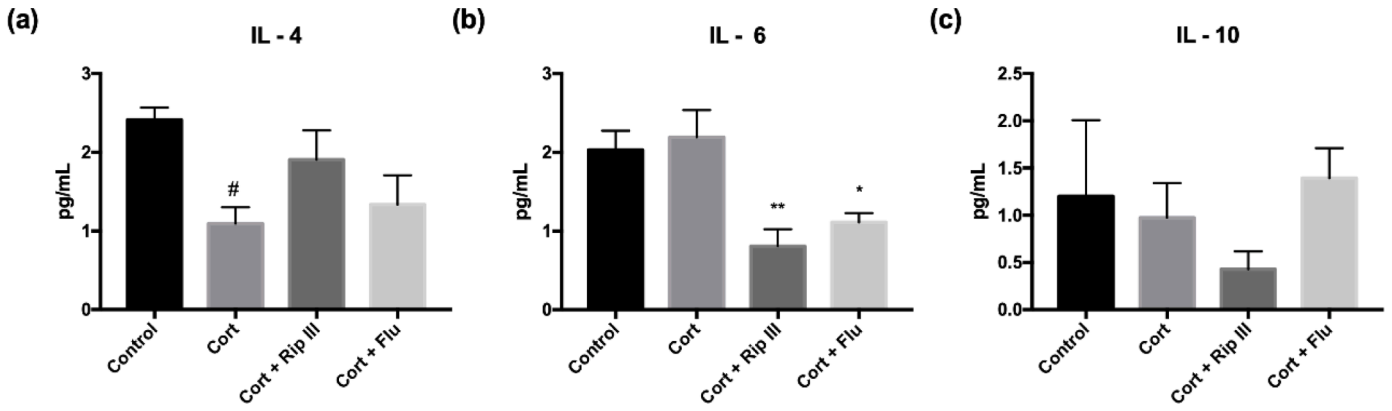

Fig. 10. Effect of Rip III on **cytokines Th2 profile** levels in HC after chronic admission of Cort, Cort + Rip III and Cort + Flu.
Results are expressed as the mean ± SEM (n = 6 animals / group). Statistical analysis was determined by one-way ANOVA, followed by Tukey's test for IL-4 and IL-6. Statistical analysis was determined by Kruskal-Wallis, followed by Dunn's test for IL-10. Significant values: $^{\#}p < 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$. IL-4: $F_{(3,16)} = 5.039$; IL-6: $F_{(3,16)} = 7.769$. Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10).

Table 1
Effect of Riparin III (50mg/kg) and Flu (50mg/kg), orally, on the modulation of monoamine levels.

Brain areas	Monoamine/Metabolyte	Control	Cort	Cort + Rip III	Cort + Flu
Striatum	Noradrenaline	4.3±0.6	7.3±0.67$^{\#}$	2.1 ±0.74**	4.6±1.38
	Dopamine	1.7±0.16	6.9±0.60$^{\#\#}$	2.7±0.84	2.0±0.51*
	DOPAC	4.9±0.68	1.8±0.13$^{\#}$	6.3±0.47*	2.5±1.0
	HVA	5.8±0.66	1.9±0.22$^{\#\#}$	6.3±0.12*	2.7±1.2
	Serotonin	3.1±0.37	1.5±0.32$^{\#}$	4.6±0.65***	3.6±0.33*
	5-HIAA	4.7±0.19	4.1 ±0.59	7.2 ±0.62	7.3±2.12

treatments with Rip III ($p = 0.005$) and Flu ($p = 0.0292$) have been shown to reverse the Cort administration effects by being significant in IL-6.

3.2.3. *Monoamines levels*

Through the measurement of monoamines in the striatum of the animals, it was verified that corticosterone promoted a significant increase of NE ($p = 0.0443$), DA ($p = 0.0013$) and reduced 5-HT ($p = 0.0487$), DOPAC ($p = 0.0472$), and HVA ($p = 0.0076$). In contrast, Rip III treatment was effective in modulating NE ($p = 0.0037$), DOPAC ($p = 0.0299$), HVA ($p = 0.0233$), and 5-HT levels ($p = 0.0008$); and Flu treatment, 5-HT ($p = 0.0151$), and DA ($p = 0.0407$) levels.

Table 1. Results are expressed as the mean ± SEM (n = 6 animals/ group). Statistical analysis was determined by one-way ANOVA, followed by Tukey's test for noradrenaline, serotonin. Statistical analysis was determined by Kruskal-Wallis, followed by Dunn's test for dopamine, DOPAC, 5-HIAA, HVA. Significant values: $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs control, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs Cort. NA: $F_{(3,20)} = 5.852$; 5-HT: $F_{(3,20)} = 8.07$.

4. Discussion

The etiology of major depression is multifactorial and may be related to a wide array of neurochemical alterations, such as reduction of monoamine levels (Hamon and Blier, 2013), changes in neurotrophin levels (Lussier et al., 2013; Melo et al., 2011; Perito and Fortunato, 2012), changes in cytokine levels due to a possible inflammatory component (Cai Songa et al., 2015; Cai et al., 2015; Mondin et al., 2016), and an increase of oxidative stress represented by a greater production of free radicals (Cai et al., 2015; Kapczinski et al., 2008; Lee et al., 2009; Lopresti et al., 2014; Mondin et al., 2016). In the present study, our results have shown that Rip III has the pharmacological potential of restoring the alterations promoted by each of these factors.

Considering the behavioral tests performed, Rip III has displayed antidepressant activity represented by an increase in active behavior (climbing and swimming) in the forced swimming test (Fig. 3). Such behaviors indicate attempts to escape harm and thus lead to a decrease in the stress experienced by the mice. It is important to highlight the increase of immobility time induced by Cort, which demonstrates that the subjects developed an inability of maintaining effort (Yankelevitch-Yahav et al., 2015). The anxiolytic potential of Rip III has also been demonstrated through the increased number of head dips seen in the hole board test (Fig. 4), a result similar to such seen in the use of benzodiazepines (Mendonça Netto et al., 2009). In addition, Rip III also promoted preservation of memory in the passive avoidance test (Fig. 5), demonstrated by the step-down avoidance learning rates in the context of electrical shocks (Ogren, Sven & Stiedl, 2013).

Regarding monoamine levels, Rip III was effective in modulating noradrenergic, dopaminergic and serotonergic systems (table 1). Through HPLC, it was revealed that Cort led to an increase of NE and DA levels. The association between corticosteroid administration and increased NE levels has already been documented (McReynolds et al., 2010). Such increase, caused by stress, including that induced by Cort, leads to significant consequences in the form of neurogenesis reduction with loss of memory and decreased social interaction (Vasconcelos et al., 2015). Rip III also recovered serotonin levels which were reduced following Cort administration. The association between the serotonin system and stress has also been previously reported. Hypercortisolemia knowingly inhibits 5-HT_1 receptor function and alters 5-HT_2 receptor density. Considering this, drugs that influence such pharmacological effects may present antidepressant or anxiolytic action through serotonergic transmission modulation (Dubey *et al.*, 2015). In addition, reductions in serotonin levels may result in an increased dopaminergic function, promoting hypervigilance in stressful contexts (Margis et al., 2003).

The association between depression and oxidative stress has also been studied. Recent evidence suggests that reactive oxygen species can initiate signaling cascades in response to stress, modifying specific redox-sensitive areas as a regulatory mechanism . It is thus important to study pharmacologically active drugs in search for an antioxidant that promotes a reduction of injurious effects . Pre-clinical and clinical

evidence indicates that oxygen reactive species prevail over the defensive systems in the brain in the context of psychiatric ailments (Vargas et al., 2013; Smaga et al., 2015). In the present study, Cort promoted brain damage through increased levels of nitrite, nitrate (fig 6), and malondialdehyde (fig 7), as well as through reduction of GSH levels (fig 8). Rip III in turn was able to revert these mechanisms, potentially working as a drug with antioxidant properties. It is known that exogenous antioxidants may be used as adjuvants in the treatment of major depression, considering too that many of the synthetic antidepressants currently available show relatively low response rates, high rates of disease relapse and grievous adverse effects (Bilici et al., 2001; Cumurcu et al., 2009; Nestler et al., 2002).

Other neurochemical parameters contribute to the hypothesis of an antioxidant potential from Rip III. An association between nitrite levels and neurotoxicity has been suggested previously, specifically a rise in such levels along with learning and memory impairment in rodents (Cha et al., 2016; Hassan et al., 2010). Considering the results on malondialdehyde, Rip III may exert an antioxidant protection over lipid biomolecules (Serafini et al., 2011). The results on GSH are of extreme relevance, as this is considered one of the most important agents in antioxidant defense. It is believed that alterations in this system are responsible for the development of neuropathological conditions, including depressive behavior, since GSH takes part in the removal of free radicals, such as H_2O_2, superoxide anions and alcoxi radicals, acts in the maintenance of membrane protein thiols, and serves as a substrate for glutathione-peroxidase and glutathione-redutase (Naik et al., 2011). Lastly, Rip III didn't restore SOD and CAT activity but the results were not statistically signicant, probably due to an excellent functioning of other antioxidant systems (Thakare et al., 2016). In contrast, a study conducted by Lucca et al. (2009) revealed a decrease in CAT and SOD activity in the PFC and HIP of stressed mice, leading to the conclusion that alterations in the endogenous antioxidant defense system are responsible for induction of depressive behavior in these animals.

Another important aspect of major depression is its relation to inflammation, raising the hypothesis that depression is frequently related to immune system disorders, to activation of the inflammatory response system with an increase in pro-inflammatory cytokines under stressful conditions, with IL-2, IL-6 and TNF-α (fig 9 and 10) amongst the most relevant. These substances induce resistance to glucocorticoids and excitotoxicity, and may reduce the number of synapses and BDNF expression, as well as may affect monoamine systems (Cai et al., 2015; Li et al., 2013; Pan et al., 2013). Besides this, the use of anti-inflammatory cytokines has been shown to be effective as an adjuvant in the treatment of depression (Bilici et al., 2001; Cumurcu et al., 2009; Nestler et al., 2002).

IL-6 is necessary at a hypothalamic level for induction of the cortisol-related chronic stress effects, and deserves highlights due to its increase in patients with melancholic manifestations, including anhedonia, weight loss and insomnia (Dunjic-Kostic et al., 2013). In addition, in patients with increased IL-6 levels, monoaminergic antidepressants did not revert such symptoms (Sukoff Rizzo et al., 2012). Regarding TNF-α, its association with altered monoamine levels has been described, with increases in 5-HT metabolization and reduction in NE levels, as well as with inhibition of NE release. Moreover, a combination of both NE and 5-HT (table 1) alterations and the rise in glutamate toxicity may consist a important element in translating neurochemical inflammatory changes into depressive behavior in humans (Clement et al., 1997; Fasick et al., 2015). Lastly, increased IL-2 levels may lead to apoptosis, diminished neuronal differentiation, suppressed synaptic transmission and inhibition of the induction and maintenance of long-term potentiation, which is behaviorally expressed in learning deficits and, ultimately, in depression (Lopresti et al., 2014).

Previous studies demonstrated that, in acute models, Rip III presented action over the central nervous system, with anxiolytic activity being observed through the hole board and elevated plus maze tests (Melo et al., 2006; Sousa et al., 2004), with antidepressant action in the forced swimming and tail suspension tests (Sousa et al., 2004), along with a possible sedative-hypnotic and anticonvulsant effect in a pentobarbital-induced sleep time and pentylenetetrazol-induced convulsion model (Sousa *et al.*, 2004). In another previous study that also relied on a Cort model, Rip III displayed antidepressant effects in the forced swimming, tail suspension, anhedonia and social interaction tests, anxiolytic effects in the elevated plus maze test, and memory recovery in the Y-maze test, without sedative or stimulant activity in the open field test (Vasconcelos *et al.*, 2015).

In addition, the antidepressant effects from Rip III have been analyzed to determine its mechanism of action. With the use of specific antagonists, antidepressant effects were observed in the forced swimming test. These results appear to be related to the modulation of alpha 1 and 2 noradrenergic systems, the serotonergic system and the D2 dopaminergic system (Melo et al., 2011; Sousa et al., 2004). Also, Rip III was capable of reverting a reduction of BDNF levels, demonstrating activity in inducing neurogenesis (Vasconcelos et al., 2015).

Despite the wide array of antidepressants available commercially, the selective serotonin reuptake inhibitions (SSRI) have been amply used due to their efficacy, safety and tolerability. The therapeutic potential of fluvoxamine in depression is well described, being effective even in severe depression (Vasconcelos *et al.*, 2015), its pharmacological action resulting in restoration of monoamine levels (Dear and Bateman, 2016), in the stimulation of neurotrophin activity, such as BDNF (Cai, Huang, Hao, 2015) and in antagonizing oxidative stress (Dallé et al., 2017; Moura et al., 2015). Such findings were seen in the corticosterone-induced depression model as well (Lopes et al., 2018), justifying its use in this study. We also highlight that, in this work, the anti-inflammatory activity of Flu has been demonstrated through the reduction of inflammatory cytokines.

Considering that the current therapy for depression is insufficient in some cases, the use of other agents must be considered. Rip III thus appears as a promising substance in the treatment of this ailment, given its restorative properties on monoamine and BDNF levels, in addition to the reduction of damage from oxidative stress and inflammation. However, further studies are needed to confirm and expand the knowledge of its effects.

Experiment limitation

The study has important limitations in addition to the following: we did not consider riparin III administration alone in this model, so our data might not be conclusive.

Significance Statement

Depression has been reported as one of the leading cause of disability in the world and, even with advances in the field of antidepressant drugs, there is a need for the development of new substances that may have better efficacy, decreased therapeutic effect latency, decreased relapses and fewer undesirable effects. Considering that Rip III triggered important antidepressant effects in acute and chronic stress models, even corroborating with different theories of depression, this work aims to provide subsidies for the expansion of the therapeutic arsenal for the treatment.

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the European Journal of Phamaceutical Sciences.

CRediT authorship contribution statement

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