Influence of the Phosphodiesterase Type 5 Inhibitor, Sildenafil, on Some Behavioral and Central Biochemical Changes on Chronic Restraint Stress in Rats

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Abstract

Stress and anxiety are now considered as major health problems that may impair the brain ability to regulate physiological and behavioral responses to stress. Disturbed serotonin and oxidative stress have been linked to anxiety and depression. This study aimed to demonstrate the effect of sildenafil (phosphodiesterase 5 inhibitor) on anxiety like behavior in chronic restrained rats and to elucidate the rule of hippocampal serotonin and oxidative stress. Fifty adult male albino rats of the same age were divided into five equal groups: Control group, chronic restraint-stressed group (CRS): Stress was induced by repeated restraint of rats in plastic tubes 2h/21 days, Sildenafil-treated group: CRS rats were treated by sildenafil, Atropine-treated group: In which CRS rats were treated by atropine, Combined treated group: CRS rats were treated by both sildenafil and atropine. One day after the final restraint stress, locomotor activity and behavior were tested using open field and elevated plus maze tests. After behavioral assessment, rats were sacrificed by cervical decapitation. Fresh brains were taken hippocampus was identified and dissected then used for preparation of homogenate. These homogenates were used for estimation of serotonin level, malondialdehyde (MDA) and super oxide dismutase (SOD). CRS was found to decrease brain serotonin level, produce oxidative stress as indicated by increased level of MDA and decreased level of SOD. Treatment with sildenafil alone or combined treatment with sildenafil and atropine were found to increase the brain serotonin levels and SOD with decrease in MDA levels. Treatment with atropine alone did not cause significant changes in the measured parameters. It was found that CRS induced a state of anxiety with oxidative stress response. Sildenafil and combined treatment had an anxiolytic and antioxidant effects on CRS.

Key Words: Chronic restraint stress – Phosphodiesterase 5 inhibitor – Sildenafil – Serotonin and anxiety.

Introduction

STRESS and anxiety are now considered as major health problems. They may be considered as main risk factors for many diseases including cardiovascular, metabolic and neuropsychiatric disorders [1]. Excessive stress induces abnormal physiological functions of the brain that may impair its ability to regulate physiological and behavioral responses to stressors [2]. Previous studies have shown that repeated restraint stress led to susceptibility to depression, anxiety or aggression [3]. Several animal studies using chronic stress revealed morphological changes in different brain areas as (hypothalamus, hippocampus, and amygdale) [4].

Most of the researchers have focused primarily on the response of the adrenergic and serotonergic systems in anxiety [5]. Serotonin (5–hydroxytryptamine, 5-HT), is a neurotransmitter present both central and peripheral. It is involved in many physiological functions as regulation of sleep, reproduction, feeding behavior and emotions. Central serotonin has been linked to mood. Disturbed 5-HT signaling has been linked to anxiety and depression [6].

Seven families of 5-HT receptors have been identified. Six of which are mainly G protein-coupled receptors and one is ligand-gated ion channel. Modulation of extracellular 5-HT can be done by several means [7]. It can be reduced either by reducing its precursor amino acid tryptophan or by inhibition of tryptophan hydroxylase enzyme, which is involved in its synthesis [8]. On the other hand its level can be increased by administration of selective serotonin receptor inhibitors (SSRIs). Modulation of 5-HT can be used to control certain stress-induced depression, anxiety or aggression [9]. Treatment of anxiety disorders include different classes of antidepressants, SSRIs, serotonin and norepinephrine reuptake inhibitors (SNRIs), as well as benzodiazepines, anticonvulsants and anti-schizophrenic drugs. These drugs primarily treat the symptoms rather than the underlying neuropathology or to reverse compromised neuroplasticity [10].
The glutamate/NO/cGMP signal-transduction pathway has been demonstrated to play a role in neuroplasticity and the neurobiology of anxiety-related disorders [11]. Inhibitors of the phosphodiesterase type 5 (PDE5) enzymes such as sildenafil, or more commonly known by its commercial name Viagra, promote cGMP accumulation in this pathway. These drugs are already in clinical use for erectile dysfunction, but have also been shown to exert central effects in rodents and humans. Also, it intervenes with the neuropathology of anxiety-related disorders [12-14]. But it has been found to have a pro cholinergic effect that may cause worsening of depressive symptoms. So, its anxiolytic and antidepressant effects can only occur if it is combined with muscarinic acetylcholine receptor antagonist [12].

Also, stress is one of the conditions associated with release of excessive amounts of free radicals. Restraint is one of stress models used which combines both emotional and physical components of stress. It is associated with production of reactive oxygen species and consequent oxidative damage with decrease in vivo antioxidant defenses [15]. Oxidative stress and the impairment in the antioxidant system have been demonstrated to exert an important role in the pathogenesis of depression or anxiety [16].

The brain is particularly vulnerable to oxidative stress due to its high rate of oxygen consumption and elevated level of polyunsaturated fatty acids [17]. Recent reports suggest that sildenafil may also, have antioxidant effects. It can prevent oxidative stress induction and lipid peroxidation [18].

The aim of the present investigation was to evaluate the effect of sildenafil as a phosphodiesterase 5 enzyme inhibitor on anxiety-like behavior, hippocampal serotonin level, and some brain oxidant and antioxidant parameters in repeated restraint stressed Wister albino rats.

Material and Methods

Animals:

Fifty adult male Wister albino rats of the same age, weighing 200-250gm were used in the present experiment. All rats were housed in groups of five per cage in standard rat cages under controlled temperature (22-24 °C), humidity (30-40%) and artificial light/dark cycle with free access to food and water ad libitum. Animals were acclimatized to these conditions for at least 1 week prior to the experiment.

All animals received appropriate care in compliance with the Public Health Service Policy on Use of Laboratory Animals published by the National Institutes of Health and was approved by the Ethical Committee of the College of Medicine, Menoufia University, Egypt.

The animals were divided into 5 equal groups (n=10 each): I- Control group: Non stressed rats daily handled and treated with saline (0.9% NaCl), II- Chronic restraint-stressed group (CRS): Restraint-stressed rats daily treated with saline, III- Sildenafil-treated group: CRS rats treated with 10mg/kg sildenafil, IV- Atropine-treated group: CRS rats treated with 1mg/kg atropine and V- Combined treated group: CRS rats treated with both 10mg/kg sildenafil and 1mg/kg atropine. All drugs were administrated intra-peritoneally (i.p.) in a volume of 1 ml one week after beginning of restraint procedure for 14 successive days. The selected dose of sildenafil and atropine were based on previous studies in rats necessary for evoking centrally mediated responses [19-23]. Work was held from November 2013 to May 2014.

Repeated restraint stress procedure:

Each rat was placed in a transparent plastic tube (20x7cm) for 2 hours from 10:00a.m. to 12:00p.m. for 21 days. One end of the restrainer is conical and has several 3-mm holes for breathing. The animals had ample air but were unable to move within the tubes [24].

Chemicals and reagents:

Atropine was purchased from Sigma-Aldrich Co. (St. Louis, MO), dissolved in saline and administered (1mg/kg, ip). Sildenafil citrate (Viavage, Hi Pharm, Egypt), dosing solutions were prepared following methods similar to those previously reported (Hotchkiss et al., 2005). Briefly, sildenafil citrate (25mg) tablets were ground into a fine powder using a mortar and pestle. The resulting powder was then mixed with saline and passed through 40 micron filters (PTFE Filters, Cole-Parmer, General control, Milan, Italy) to eliminate residues of excipients. The resulting solution was kept chilled at 4°C. Dosing solutions were brought to room temperature prior to injections. Kits for estimation of serotonin was ordered from Glory Science Co., Ltd (USA). Kits for estimation of MDA and SOD were supplied from Boidiagnostic Co., Egypt.

Behavioral assessment:

One day after the final restraint stress, locomotor activity and behavior in open field test and elevated plus maze were measured. Following behavioral testing rats were euthanized to collect brain samples. All tests took place between 10:00a.m. and 2:00p.m. in a dimly lit room.
Open Field Test (OFT):

Open field test was used to assess the locomotor as well as the exploratory behavior in rats. The open field apparatus consisted of a square area (100cmx100cmx50cm) with a dark surface covering the inside walls. The floor of the area was divided equally into 25 squares marked by white lines [25]. In this test, a single rat was placed in the center of the arena and allowed to explore freely for 5 min. Rats were observed via video-camera by an observer located in the same room. The latency to move from the Centre square (in seconds), horizontal locomotion (number of crossed squares), the number of rears (posture sustained with hind-paws on the floor) and the frequencies of grooming (including washing or mouthing of forelimbs, hind-paws, face, body and genitals) were counted manually. After each test the arena was cleaned with 90% alcohol solution.

Elevated Plus-Maze (EPM) test:

The apparatus is based on that described by [26]. The maze was elevated to a height of 70cm with two open (50x10cm) and two enclosed arms (50x10x50cm), arranged so that the arms of the same type were opposite each other, and connected by an open central area (10x10cm). Experiments were performed under dim light conditions to encourage exploration. At the beginning of the experiment, rats were placed in the center of the maze, facing one of the enclosed arms, and observed for 4 minutes rats were observed via video-camera by an observer located in the same room. At the end of the 4-minutes test, rats were removed from the plus maze and placed in a transport cage. Elevated plus maze was cleaned with alcohol and dried with paper towels before testing another rat. Proportion of entry into the open arm, time spent in the open arm, frequencies of rearing, frequencies of grooming were measured. Entry into an arm was defined as having occurred when the animal placed two limbs onto the arm. Reduction in anxiety level was indicated by an increase in the time spent in the open arms, and an increase in the percentage of entries into the open arms (entries into open arms/total entries into open and closed arms).

Sample preparation and analysis:

After behavioral assessment, rats were sacrificed by cervical decapitation. The technique used was essentially the same as described earlier [27]. A fresh brain was rinsed with ice cold saline and placed in a Petri dish kept on ice then hippocampus was identified, dissected and weighed. The samples were then homogenized by hand in PBS (PH 7.2-7.4), then centrifuged for 20min at 4ºC at speed of 2000-3000rpm. Supernatant was taken and if any precipitation found centrifuged again. Then samples were kept at –80 till the time of biochemical analysis.

Serotonin level in hippocampus:

The level of serotonin was estimated using Serotonin Elisa kit from tissue homogenate of the hippocampal region as per manufacturer protocol. Briefly, standard, blank and samples were pipetted into the respective wells of the microliter plate. After 30min of incubation at 37ºC, the plate was washed 5 times with diluted wash solution followed
by addition of freshly prepared HRP enzyme conjugate. The plate was then covered and incubated for 30 min at 37°C followed by washing 5 times with diluted wash buffer. Chromogen solution A&B was then added into each well and incubated 15 min at 37°C. The reaction was then stopped by adding stop solution into each well. Optical density was measured with Elisa plate reader at 450 nm within 15 min. The concentration of serotonin in the tissue sample was calculated by multiplying with dilution factor and expressed in pg/ml.

**MDA level in hippocampus:**

Colorimetric method for estimation of malondialdehyde (MDA) was done by using the protocol described in [28] by using thiobarbituric acid reactive substance for measuring the peroxidation of fatty acids as oxidative stress marker.

**SOD level in hippocampus:**

Colorimetric method for estimation of superoxide dismutase (SOD) as described by [29] depend on the ability of SOD to inhibit the initial rate of photoactivated phenazine methosulfate mediated reduction of O$_2$ to O$_2$ which then reduce nitroblue tetrazolium dye.

**Statistical analysis:**

In this investigation, the results were expressed as mean±standard error of mean (X±S.E.M) (n=10). The data were analyzed statistically utilizing the computer software GraphPad Prism (version 4.01 for Microsoft Windows, GraphPad Software, San Diego, CA, U.S.A.), by one way analysis of variance (1-ANOVA) and different group means were compared by Newman-Keuls test, while $p<0.05$ was considered statistically significant in all cases.

**Results**

**Behavioral tests:**

1- **Open field test:**

This study showed the effect of chronic restraint stress and treatment with sildenafil on locomotor activity and exploratory behavior among rats. Chronic restraint stress significantly increased the latency of rats to start movement from the central square compared to control (4.4±0.45 seconds vs 1.2±0.33 seconds, respectively). However, treatment with sildenafil alone or sildenafil combined with atropine caused significant decrease in this latency (1.5±0.30 and 1.3±0.36 seconds) compared with corresponding values in restrained stressed group (4.4±0.45 seconds) as seen in Fig. (2-A).

There was no significant difference in the number of crossings and rearing behavior in all studied groups as seen in (Fig. 2-B,C). On the other hand, co-administration of sildenafil and atropine significantly increased the grooming frequency (9.0±1.17) compared to CRS group (5.4±0.77) (Fig. 2-D).

2- **Elevated plus maze:**

The time spent in open arm by CRS rats did not significantly change when compared to control. However, combined treatment of the CRS rats with sildenafil and atropine significantly increased the time spent in open arm (41.80±2.80) when compared to CRS group (23.30±4.38) and sildenafil-treated group (29.00±3.08) as shown in Fig. (3-A).

The percentage of entry into open arm of the EPM did not differ significantly between CRS rats and controls (23.88±4.74 vs 28.67±8.13, respectively). In addition, treatment with sildenafil alone significantly increased the percentage of entry into open arm of the EPM of CRS rats (38.73±3.91 vs 23.88±4.74, respectively). Also, combined treatment of sildenafil with atropine significantly increased the percentage of entry into open arm compared with CRS rats (50.41±3.45 vs 23.88±4.74, respectively). On the other hand, treatment with atropine alone did not significantly increased the percentage of entry into open arm compared with CRS rats (29.90±5.24 vs 23.88±4.74, respectively) (Fig. 3-B).

There was no significant change in rearing between CRS group and other treated groups, but all were significantly more than rearing behavior in control rats (Fig. 3-C). Regarding grooming, CRS significantly elevated grooming behavior when compared to control group (3.70±0.47 vs 1.30±0.39). Treatment with sildenafil significantly increased grooming behavior when compared to CRS group (5.60±0.70 vs 3.70±0.47) while atropine significantly decreased grooming behavior when compared to CRS group (1.40±0.45 vs 3.70±0.47).

**Biochemical analysis:**

1- **Hippocamal serotonin level:**

CRS significantly decreased serotonin level in the hippocampus when compared to its correspond-
ing value in control group (46.50±1.36pg/ml vs 83.84±1.99pg/ml). Treatment with sildinafil alone (65.27±1.53pg/ml) and combined treatment with sildinafil and atropine (76.06±1.76pg/ml) significantly increased the serotonin level when compared to its corresponding value in CRS rats (46.50±1.36 pg/ml). Atropine administration alone did not cause any significant change in the serotonin level when compared to the corresponding value in CRS (Fig. 4).

Fig. (2): Effect of sildenafil treatment (10mg/kg/day, I.P., 14 days), alone or in combination with atropine, on the locomotor exploratory activity of CRS rats (2h/21 days) by Open field test. A- Latency to move from the centre. B. Number of crossed squares. C. Rearing frequency. D- Grooming frequency. Bars represent means ±SEM from 10 rats. One way ANOVA and Newman-Keuls test: *p<0.05, vs control; #p<0.05, vs chronic restraint stress groups; ~p<0.01, vs sildenafil-treated group group.
Fig. (3): Effect of sildenafil treatment (10mg/kg/day, I.P., 14 days), alone or in combination with atropine, on the time spent by CRS rats in the open arms of the elevated “plus” maze (A) and percentage of open arm entry to total arm entries therein (B). Rearing frequency (C). Grooming frequency (D). Bars represent means ±SEM from ten rats. One way ANOVA and Newman-Keuls test: *p<0.05, vs control; #p<0.05, vs chronic restraint stress groups; ~p<0.01, vs sildenafil-treated group.

2- Hippocampal levels of MDA and SOD:

Table (1) showed the level of MDA and SOD in brain homogenate. The MDA in the CRS was significantly higher than its corresponding value in control group (25.88±1.08 vs 13.94±1.09 nmol/gm tissue respectively). Treatment with sildenafil and combined treatment with sildenafil and atropine significantly decreased the MDA level (15.79±1.08 and 15.78±0.83nmol/gm tissue) when compared to the corresponding value in CRS (25.88±1.08nmol/gm tissue). Atropine administration alone did not cause any significant change in MDA level when compared to the corresponding value in CRS.

The SOD in the CRS (6.81±0.38U/gm tissue) was significantly lower than its corresponding value (14.08±0.66U/gm tissue) in control group.
Treatment with sildenafil alone and combined treatment with sildenafil and atropine caused significant elevation in SOD levels (11.83 ± 0.61, 12.97 ± 0.69 U/gm tissue) compared with its corresponding value in CRS (6.81 ± 0.38 U/gm tissue). Atropine administration did not cause any significant change in SOD level when compared to the corresponding value in CRS.

Table (1): Effect of sildenafil on MDA and SOD levels in the hippocampus of chronic restrained stressed rats.

<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>Restraint stressed group</th>
<th>Sildenafil treated group</th>
<th>Atropine treated group</th>
<th>Combined treated group</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (nmol/gm tissue)</td>
<td>13.94 ± 1.09</td>
<td>25.88 ± 1.08*</td>
<td>15.79 ± 1.08#</td>
<td>24.99 ± 0.74~</td>
<td>15.78 ± 0.83#</td>
</tr>
<tr>
<td>SOD (U/gm tissue)</td>
<td>14.08 ± 0.66</td>
<td>6.81 ± 0.38*</td>
<td>11.83 ± 0.61#</td>
<td>6.71 ± 0.39~</td>
<td>12.97 ± 0.69#</td>
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</table>

Data are expressed as mean ± S.E.M. (n=10). One way ANOVA and Newman-Keuls test: *p < 0.05, vs control; #p < 0.05, vs chronic restraint stress groups; ~p < 0.01, vs sildenafil-treated group.

**Discussion**

Our study showed that CRS resulted in decrease in the level of serotonin in the hippocampus with oxidative stress as indicated by the imbalance in the oxidant and antioxidant system (elevated level of MDA and decreased level of SOD). Treatment of chronic restrained rats by sildenafil alone or sildenafil in combination with atropine significantly elevated serotonin level and improved the oxidative stress by decreasing the MDA levels and increasing the SOD levels in the hippocampus. Treatment with atropine alone did not significantly affect either the serotonin level or the oxidative stress in the hippocampus of CRS rats.

Several studies indicated that long lasting stress affects synaptic plasticity, dendritic morphology and neurogenesis in animals and induces both clinical and anatomical features of neurotoxic damage. Restraint is a model which was an established method of inducing stress. It is found to induce oxidative damage in rat brain, which is a major internal threat to cellular homeostasis [15].

The brain is uniquely vulnerable to oxidative injury, due to high metabolic rate, high rate of oxygen use and high levels of polyunsaturated lipids, which are the target of lipid peroxidation [18]. Most of the oxygen used in brain tissue is converted to CO2 and water, but small amounts of oxygen forms ROS and consequently tissue damage. Moreover, the brain has a little antioxidant defenses [30].

There are numerous studies indicating that ROS induced neuronal damage and has an important role in the pathophysiology of depression and anxiety [31]. However, with stress, the loss of efficiency of antioxidants mechanisms and the alterations in the proinflammatory cytokine system result in increases in the free radical formation due to the activation of phagocytic cells [32] these previous studies support our result that, restraint stress caused oxidative stress in rat brain.

Restraint stress is one of stress models which were found to affect central neurotransmitters as catecholamines and serotonin and also affects hypothalamic-pituitary adrenal axis. Our results are consistent with studies demonstrating decrease in 5HT in the hippocampus in response to chronic restraint stress [33]. Also, 5-HT depletion has been linked to inducing anxiety-like behavior [34]. Moreover, decrease in serotonergic neurotransmission is involved in restraint-induced behavioral deficits [35]. The serotonin levels were reduced in the brain of chronic restraint stressed rats, which may contribute to increasing neuronal excitability in the brain and anxiety in the stressed rats [36,37], revealed that genetic mice with reduced level of 5-HT had anxiety like behavior while mice over-expressing 5-HT exhibited weak- anxiety phenotype. Also, they found that serotonin knocked out mice develop anxiety-like behavior with decreased exploratory behavior in several behavioral tests (elevated plus-maze, open-field). Psychological outcomes in stress related disorders are improved by drugs that target the brain 5-HT system, such as 5-HT reuptake inhibitors [38,39] showed that 5-HT agonist (buspirone) induced anxiolytic effect on anxiety-like behavior, by facilitating 5-HTA1 receptor mediated transmission.

Sildenafil was used for treating erectile dysfunctions (ED) in men. Studies had shown that it had an antidepressant action in these patients, because depression and ED are usually interrelated. Chronic treatment with sildenafil was found to reduce anxiety-like behavior of rats in the open
field test [40]. Its antidepressant and anxiolytic properties were revealed only after central muscarinic acetyl choline receptor (mACh) blocker as atropine. That is because it has a pro-cholinergic effect, an effect that is believed to be depressogenic. The glutamate/NO/cGMP/PK-G pathway has also been implicated to play a major role in anxiety-like behavior [41]. Studies indicated that the inhibition of the NO/cGMP/PK-G pathway may have an antidepressant action. Also, inhibition of PDE5 enzyme with Sildenafil had antidepressant properties when combined with mACh receptor blockers [12].

Although cholinergic theory may be involved in the pathogenesis of depression, the use of anti-cholinergic drugs alone failed to treat depression or anxiety. The mACh receptor blockers are only used to augment the antidepressant effect of other antidepressant [42].

Locomotor activity and behavioral assessment of chronic restrained rats were done by open field test and elevated plus maze. It showed that there was significant increase in the latent period after which restrained rats start to move from the central square in the OFT, when compared to control rats. Sildenafil treatment alone or combined with atropine significantly decreased this latent time. Increase latency to moving indicating a decrease in the motivation of the animals [43], which was considered as a major symptom of depression [44]. Sildenafil treatment alone or combined with atropine improved this depressive like behavior which may be correlated to our results of increased hippocampal serotonin level and improved oxidative imbalance.

EPM is a rat model of anxiety that was used for the screening of compounds with anxiolytic potential and used as a general research tool in neurobiology of anxiety and depression research [45, 46]. Evidence suggesting that repeated restraint stress produces an increase in anxiety behavior, when exposed to an elevated plus maze, stressed rats tend to avoid the open arms and prefer to stay in the enclosed arms [47] also [48] observed that non anxious selected rats showed more rearing behavior and less freezing time. On contrast, our present findings showed insignificant increase in the percentage of open arm entries, time spent in open arm and rearing in CRS compared to control; this may be explained by adaptation of restrained rats to the stress situation. Rats have been reported to be able to adjust to any mild stressor within a period of about 3 days [49]. The results of chronic stressed rat models differ from acute stress application and also they differ according to the duration and the nature of stress. This was interpreted by [50], who found that restrained rats choose to enter open arms more frequently than controls, as a counter reaction for being confined to a small space during restraint.

In the present study, increase in the percent of open arm entries and increase in time spent in open arm caused by sildenafil alone or when combined with atropine were taken as best index of reduced anxiety levels [47]. Serotonergic neurons are integrally involved in the mediation of anxiety responses [51]. This may explain the reduced anxiety level associated with increased hippocampal serotonin level in sildenafil treated groups in this study. This was in agreement with [52] who found that sildenafil crosses the blood brain barrier to inhibit intracellular PDE5 and thus to increase cGMP, leading to enhanced biological effects in the brain such neuromodulation, synaptic plasticity, neurogenesis, antinociception and effects on anxiety and mood.

Sildenafil significantly increased grooming behavior in rats either when given alone or when combined with atropine in OFT and EPM. Grooming is a multifunctional behavior in rodents that acts as replacement behavior, which maintains body hygiene, serves for communication and plays a very important role in coping with stressful situations as well as in relaxation [53]. Increased grooming in a low stress situation is interpreted as a sign of increased anxiety. However, similar changes of grooming may reflect anxiolytic effects, i.e. to comfort [54]. Anxiolytics and antidepressants increase grooming activity in mice [55-57]. Also, this increased grooming behavior with sildenafil treatment may possibly be through its effect on genital system as it increases the number of ex copula erection, as well as genital grooming [19].

In conclusion, this investigation provides evidence indicating that phosphodiastase 5 inhibitors as sildenafil exhibit anxiolytic-like effects in chronic restraint stress behavioral models especially when combined with atropine. It is also suggested that the anxiolytic-like effects of sildenafil seem most likely to be mediated through antioxidant effect and serotonergic systems which was more obvious after central muscarinic receptor blockade.

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