Original Article

Effects of different dark chocolate diets on memory functions and brain corticosterone levels in rats under chronic stress

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Abstract

Introduction: Stress influences cognitive behavior adversely, whereas dark chocolate exhibits positive effects on memory processes. This study investigated the effects of different dark chocolate diets on various aspects of brain functions in rats under chronic stress

Methods: Thirty-five rats were randomly allocated into five groups: control, stress, stress with different (compulsory, optional and restricted) dark chocolate diets. Latency, dark stay (DS) time and the number of entrance to the dark compartment were respectively evaluated as memory, memory consolidation and locomotor activity by passive avoidance test.

Results: There were significant differences between initial latency and latency after 1 day in all groups. In the stress-compulsory and restricted dark chocolate diet groups, latency after 1 day increased significantly. Moreover, the DS time was not significantly higher in the stressed group than the control group. The DS time and number of entrance to dark compartment decreased significantly in the stress-compulsory dark chocolate diet group compared to the stressed group. Furthermore, the number of entrance to dark compartment was significantly higher for the stress- optional dark chocolate diet compared to those with the compulsory diet. Additionally, serum and hippocampal corticosterone levels, except in the frontal cortex, were significantly lower only in the stress-compulsory dark chocolate diet group compared to the stressed group.

Conclusion: Different dark chocolate diets had various effects on brain functions under chronic stress. Respectively, the compulsory and optional dark chocolate diets had the best and least effects on brain function improvement. Only the compulsory dark chocolate diet could improve brain functions such as memory, memory consolidation and locomotor activity.

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Introduction

Stress is the state of psychological response to actual or potential stressors and activates the hypothalamic-pituitary-adrenal axis and glucocorticoids (the stress hormones) secretion (Dedovic et al., 2009; Radahmadi et al., 2015). Also, stress impairs some

brain functions such as learning, memory, memory consolidation and behavioral activities by glucocorticoid secretion (Dastgerdi et al., 2018; Miranda and Oliveira, 2015). Hippocampus and frontal cortex are structures that have glucocorticoid receptors (Allan et al., 2014; Barrientos et al., 2015) and are known to be responsible for memory, moods, cognition and stress-responsive neuroendocrine systems (Cerqueira et al., 2005; Kim and Han, 2016; Radahmadi et al., 2014; Sugimoto et al., 2017). Hence, the functional changes in both hippocampus and frontal cortex under stressful conditions could impair brain activities (Holtzer et al., 2017; Li et al., 2011; Radahmadi et al., 2014).

In recent years, herbal drugs, their products and bioactive substances are considered to be alternative remedial strategies for some disorders due to their easy access and lower side effects (Bandegi et al., 2014). As such, chocolate, derived from cocoa beans, is a worldwide product that has been promoted as a functional food during the past decade (Franco et al., 2013). Besides, there is an increasing in the potential benefits of cocoa and chocolate for health (Steinberg et al., 2003). Cocoa and kinds of chocolate have antioxidants and phytochemicals like flavonoids that participate in improving deficits in brain function (Crichton et al., 2016). Cocoa flavonoids can exhibit beneficial impacts on cognition, mood and behavior (Sokolov et al., 2013). There were different kinds of chocolate including white, milk and dark chocolates, based on the amount of cocoa (Crichton et al., 2016; Kemsawasd et al., 2016); however, among the various kinds, dark chocolate is most effective type on brain functions and glucocorticoid levels (Martin et al., 2009; Sokolov et al., 2013). Correspondingly, the consumption of dark chocolate may optimize cognitive functions over the life span of an individual (Crichton et al., 2016), especially under stress conditions (Simadibrata, 2011). Furthermore, dietary changes are major lifestyle factors that can influence the progression of chronic disorders (Shah et al., 2017). Even though. the positive effects of dark chocolate consumption in reducing stress responses have been discussed before (Martin et al., 2009), none of the former studies have indicated how dark chocolate consumption may affect different aspects of brain function under stress. Therefore, this study aims to investigate the impacts of three different dark chocolate dietary patterns on some of the brain functions, such as learning, memory, memory consolidation and locomotor activity in rats under chronic stress, so the dark chocolate diet for the protection of brain corticosterone levels in serum, hippocampus, and frontal cortex under stress conditions could be introduced.

Materials and methods

Experimental animals

Thirty-five male Wistar rats (initial weight 250–300g) were obtained from Pasteur Institute of Tehran, Iran. The rats were housed under controlled humidity (50±5%) and light conditions (12h light/dark; lights on 07:00–19:00), in the cages with similar size among all groups. The room temperature was set to 23±2°C and water was made available *ad libitum*. All behavioral experiments were performed at 13:00-14:00 and the experiment lasted for a period of 14 days. The Ethics Committee of Animal Use at the Isfahan University of Medical Sciences approved the study (IR.MUI.MED.REC.1398.036) and all experiments were conducted in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 2011). After a period of 2 weeks for adaptation, the animals were randomly divided equally into five groups (n=7): 1- control group, the rats were maintained in the cage with no special treatment for 14 days and only received rodent's food (chow); 2- stress (St) group, the rats were subjected to isolation stress. Each rat was placed inside a cage alone and only received chow for 14 days; 3- stress with compulsory dark chocolate diet (St-Compulsory Choc) group, the rats were subjected to isolation stress. Each rat was placed inside a cage alone and only received dark chocolate for 14 days; 4- stress with optional dark chocolate diet (St-Optional Choc) group, the rats were subjected to isolation stress. Each rat was placed inside a cage alone and freely received dark chocolate and chow for 14 days. Therefore, rats could optionally eat dark chocolate and/or chow; 5- stress with restricted dark chocolate diet (St-Restricted Choc) group, the rats were subjected to isolation stress. Each rat was placed inside a cage alone and freely received chow and only 4g/rat/day of dark chocolate for 14 days. Hence, rats could limitedly eat dark chocolate.

Experimental procedures

Stress paradigm

In this study to induce isolation stress in rats, they were kept inside separate cages without any neighbors (individual housing) for 14 days (Iglesias et al., 1992; Mills and Ward, 1984).

Chocolate consumption

Each rat consumed the specified amount of solid 85% dark chocolate (588 kcal/100g, 70.8% fat, 21.6% protein, 10.98% carbohydrate and other minerals; Shirin-Asal Co., Iran). The stressed rats in the compulsory and optional dark chocolate diets were respectively fed with only dark chocolate in the compulsory diet, and chow/chocolate in the optional diet while placed in the separate cages. Also, the stressed rats with a restricted dark chocolate diet were fed with chow and only the specified amount of dark chocolate (4g/rat/day) in their separate cage, calculated based on the human equivalent dose (Shin et al., 2010). However, another study has reported a respective chocolate consumption of 5g/rat/day (Dailey et al., 2012).

Behavioral Paradigms

To assess learning, memory, memory consolidation and locomotor activity involving the cognitive memory, the passive avoidance (PA) test was used (Dastgerdi et al., 2018; Vohora et al., 2000). The PA apparatus (shuttle-box 64×25×35cm) was divided into two identical compartments (32×25×35cm) with sliding guillotine doors and grid floors. On day 12, each rat was placed in the apparatus for 300s for habituation. On the following day (Day 13), a single learning trial was performed and on day 14, the passive avoidance memory trial was conducted. There were no foot shocks in both habituation and memory trials. In the learning trial, the rats were individually placed in the light compartment for 60s before the guillotine door was raised. When the rat entered the dark compartment, the door was closed and a single electric shock (0.5mA, 50v and 2s; once) was delivered to the animal's foot through the grid floor using an isolated stimulator (Huang et al., 2013). The initial latency time to enter the dark compartment was recorded before inducing the electric shock. In the memory trial, the latency time of entry to the dark compartment was measured after one day (up to a maximum delay of 300s). If the rat did not enter the

dark compartment within 300s, the memory trial was terminated. In this experiment, the difference between the initial latency and the latency after 1 day was translated as the occurrence of learning (Dastgerdi et al., 2018). Moreover, the total dark stay (DS) time was assigned as either memory consolidation and/or storage of new information (Dastgerdi et al., 2018). Similarly, the number of entrance into the dark compartment was recorded as the locomotor activity (Divsalar, 2012; Vohora et al., 2000). The animal's ability to remember the received foot-shock was determined by the PA test. Avoidance to enter the dark compartment or even a longer duration of stay in the light compartment was interpreted as a positive response (Dastgerdi et al., 2018).

Assessment of corticosterone levels in the serum, hippocampus and frontal cortex

On day 15, the rats were anesthetized with Urethane (1.5g/kg IP, Sigma-Aldrich Chemical Co.) and sacrificed between 14:00-16:00. Subsequently, the serum was separated from blood samples and the hemi-hippocampus and hemi-frontal cortex were immediately dissected to be kept on dry ice. Furthermore, they were separately immersed in Problock™-50, EDTA free (GoldBio Co., USA) and in phosphate buffer solution (0.01M, pH 7.4) (Dastgerdi et al., 2017). The hippocampus and frontal cortex were homogenized and centrifuged at 10,000g for 20min at 4°C. The supernatant fluids were collected, subsequently both serum and supernatants were stored at −80°C until the assessment time. The commercial Enzyme-Linked Immuno-Sorbent Assay kit (ZellBio GmbH Co., Marburg, Germany) was used to assess the serum, hippocampal and frontal cortical corticosterone levels.

Statistical analysis

All passive avoidance test data, corticosterone levels in serum, hippocampus and frontal cortex were analyzed by analysis of variance (ANOVA) test, followed by LSD post-hoc testing for multiple groups. The comparisons between the initial latency and latency after 1 day (within groups) were analyzed using the paired sample t-test. Furthermore, all data were estimated as mean±SEM. A P-value less than 0.05 was considered statistically significant. Notably, the calculations were performed using
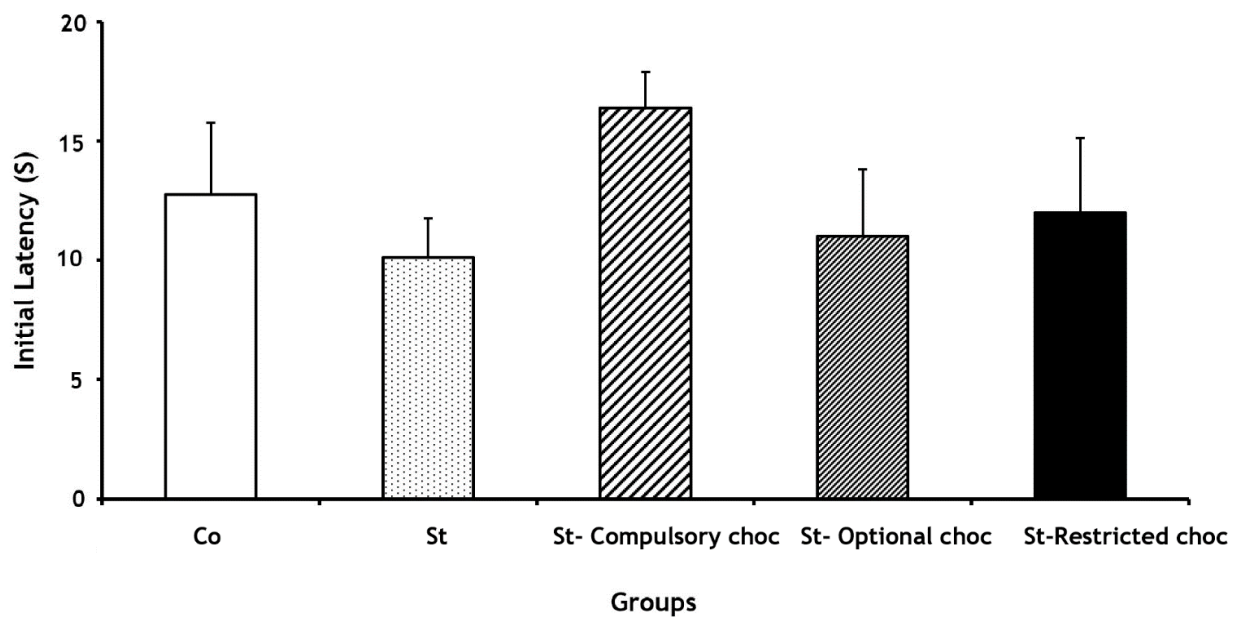
Fig.1. Initial latency to entrance into the dark room of the passive avoidance apparatus for all the groups before receiving a foot shock (n=7). Results are expressed as mean±SEM. No significant differences were observed among the groups. Co: control group, St: stress group, St- Compulsory Choc: stress with compulsory dark chocolate diet group, St-Optional Choc: stress with optional dark chocolate diet group, St-Restricted Choc: stress with restricted dark chocolate diet group.

SPSS v21.0 (SPSS Inc., Chicago, IL, USA).

Results

In the passive avoidance test, ANOVA indicated no significant differences of the initial latency [$F_{(4, 35)}$= -0.915, P>0.05; Fig. 1], total dark stay time [$F_{(4, 35)}$= -1.847, P>0.05; Fig. 4], and the number of entrance into the dark compartment [$F_{(4, 35)}$= 1.833, P>0.05; Fig. 5]. Whereas, there was a significant latency difference after 1 day [$F_{(4, 35)}$= 2.661, P<0.05; Fig. 2].

Assessment of memory functions

Figures 1 and 2, respectively, show the initial latency and latency after 1 day for all groups. No significant differences were observed in initial latency values across all groups (Fig. 1). In the stress (St) group, the latency after 1 day was significantly (P<0.05) lower than that value recorded for the control group, indicating the memory declination as a result of chronic isolation stress (Fig. 2). The latency showed significant enhancements in St-Compulsory Choc and St-Restricted Choc groups (respectively, P<0.01 and P<0.05) in comparison with the St group, representing the improving effects of compulsory and even restricted dark chocolate diets on memory deficit caused by stress after 1 day. However, the optional dark chocolate diet had no major impact on memory improvement in stressed rats (Fig. 2).

As shown in Figure 3, the initial latency and latency after 1 day were analyzed using a paired sample t-test to evaluate within-group latency changes. There were significant differences between initial latency and latency, determined by the PA test in the control [$t_{(7)}$= -7.169, P>0.01], St [$t_{(7)}$= -3.859, P>0.01], St-Compulsory Choc [$t_{(7)}$= -4.387, P>0.01], St-Optional Choc [$t_{(7)}$= -2.787, P>0.05] and St-Restricted Choc [$t_{(7)}$= -4.303, P>0.01] groups (Fig. 3). Therefore, significant differences were detected between the initial latency and latency after 1 day in all groups. It indicated that the occurrence of learning in all groups, nevertheless at different levels. For instance, the learning happened at the lowest level in the St group and at the highest level in the St-Compulsory Choc group.

The total DS time in the St group was not significantly higher than what was recorded for the Co group. However, the total DS time in the St-Compulsory Choc group was significantly (P<0.05) lower than the DS in the St group, indicating that compulsory dark chocolate diet improved the memory consolidation. Whereas, the restricted and optional dark chocolate diet groups were not significantly different compared to the control and St groups (Fig. 4).

The number of entrance into the dark compartment showed no significant differences in St group compared to that recorded for the control group. Also,
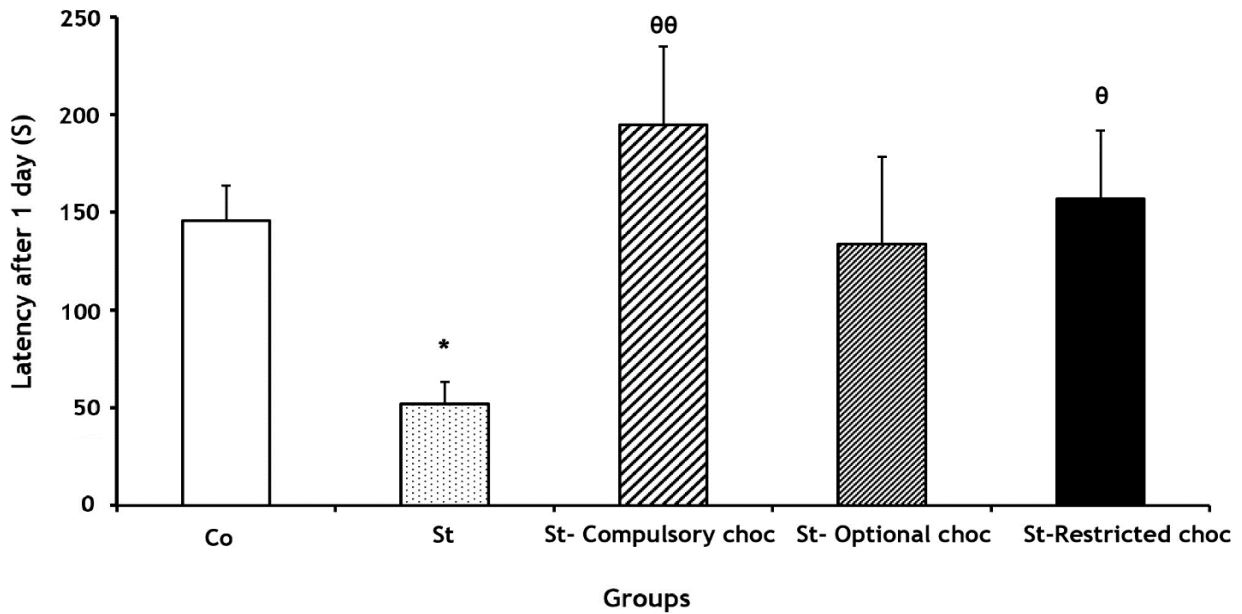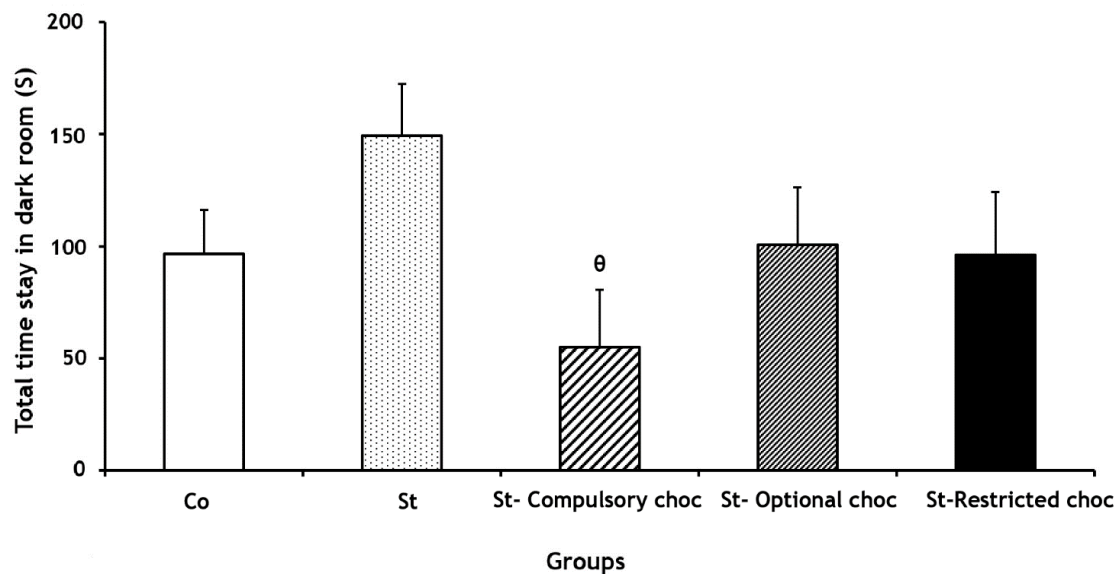
Fig.2. Latency after 1 day to entrance into the dark room of the passive avoidance apparatus for all the groups 1 day after receiving the foot shock (n=7). Results are expressed as mean±SEM. $^*P<0.05$ compared to control group; $^\theta P<0.05$ and $^{\theta\theta}P<0.01$ compared to stress group. Co: control group, St: stress group, St- Compulsory Choc: stress with compulsory dark chocolate diet group, St-Optional Choc: stress with optional dark chocolate diet group, St-Restricted Choc: stress with restricted dark chocolate diet group.
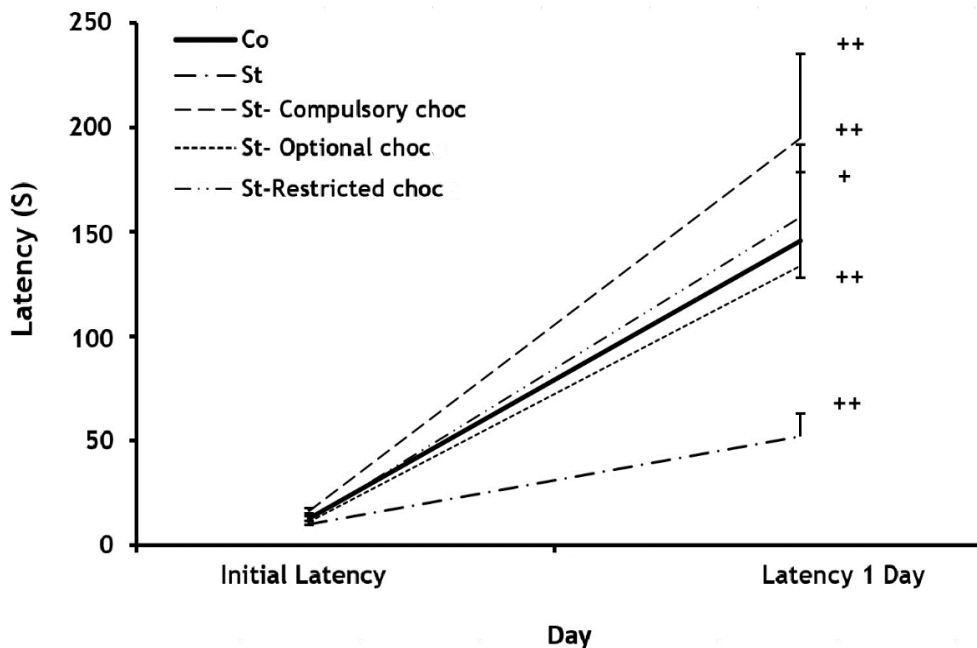

Fig.3. Initial latency and latency after 1 day to entrance into the dark room of the passive avoidance apparatus before and after the foot shock (within groups) (n=7). Results are expressed as mean±SEM. $^+P<0.05$ and $^{++}P<0.01$ Initial latency relative to the latency after 1 day. Co: control group, St: stress group, St- Compulsory Choc: stress with compulsory dark chocolate diet group, St-Optional Choc: stress with optional dark chocolate diet group, St-Restricted Choc: stress with restricted dark chocolate diet group.

in the St-Compulsory Choc group, it was significantly ($P<0.05$) lower than the St group, indicating a reduced locomotor activity due to a compulsory dark chocolate diet under stress conditions. As such, the number of entries to the dark compartment were not significantly different in the restricted and optional dark chocolate diet groups compared to the St group (Fig. 5). In addition, the number of entrance into dark

Fig.4. Total stay time in dark room of the passive avoidance apparatus for all the groups 1 day after receiving the foot shock (n=7). Results are expressed as mean±SEM. $^{\ominus}P<0.05$ compared to stress group. Co: control group, St: stress group, St- Compulsory Choc: stress with compulsory dark chocolate diet group, St-Optional Choc: stress with optional dark chocolate diet group, St-Restricted Choc: stress with restricted dark chocolate diet group.
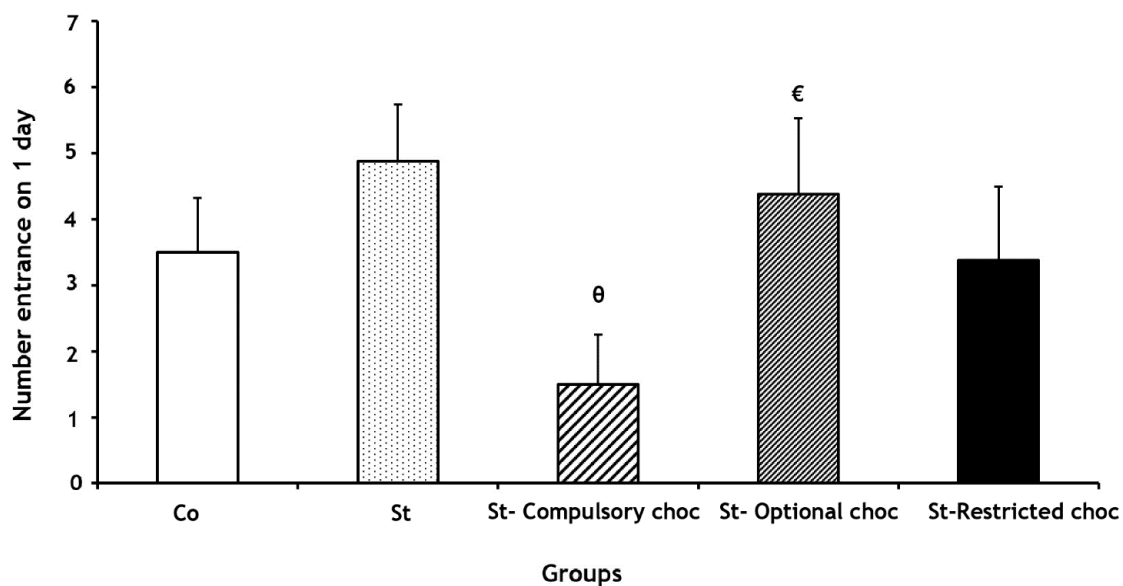

Fig.5. The number entrance to dark room of the passive avoidance apparatus for all the groups 1 day after receiving the foot shock (n=7). Results are expressed as mean±SEM. $^{\ominus}P<0.05$ compared to stress group; $^{\epsilon}P<0.05$ compared to stress-compulsory choco group. Co: control group, St: stress group, St- Compulsory Choc: stress with compulsory dark chocolate diet group, St-Optional Choc: stress with optional dark chocolate diet group, St-Restricted Choc: stress with restricted dark chocolate diet group.

compartment in the St-Optional Choc diet group increased significantly ($P<0.05$) in comparison with the St-Compulsory dark chocolate diet group, displaying an elevated locomotor activity in this group (Fig. 5).

Assessment of corticosterone levels in the

serum, hippocampus and frontal cortex

In the assessment of corticosterone levels, the ANOVA test indicated a significant difference in serum corticosterone levels [$F_{(4, 35)}= 4.309$, $P>0.01$; Fig. 6]. Whereas, there were no significant differences in hippocampal corticosterone levels [$F_{(4, 35)}= 1.587$, $P>0.05$; Fig. 7] and in the frontal cortical
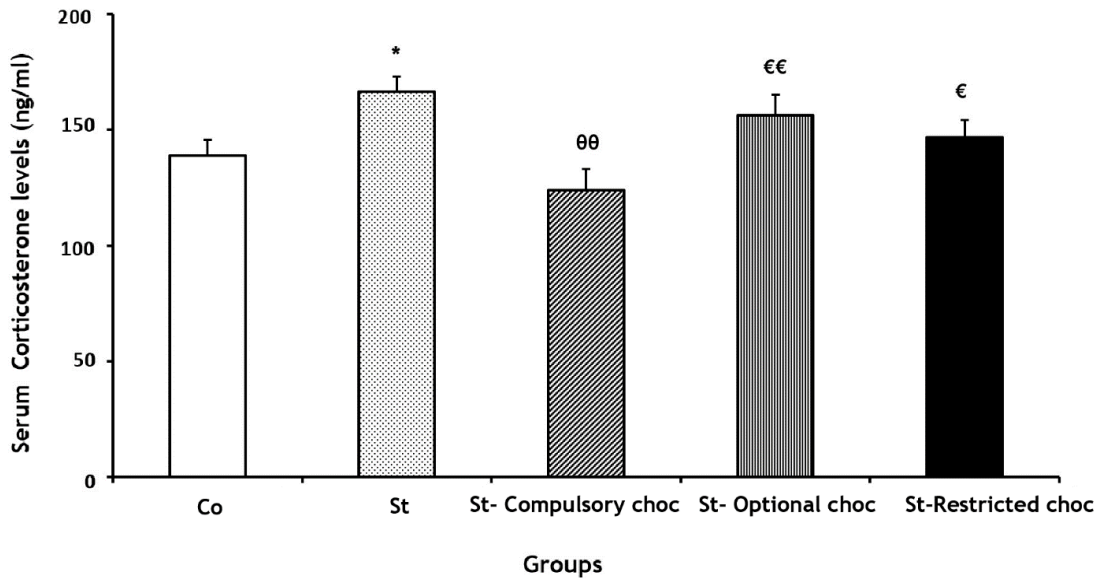
Fig.6. Effects of different dark chocolate diets on serum corticosterone levels (ng/ml) in different groups under isolation stress (n=7). Results are expressed as mean±SEM. $^*P<0.05$ compared to control group, $^{\theta\theta}P<0.01$ compared to stress group, $^{\text{€}}P<0.05$ and $^{\text{€€}}P<0.01$ compared to stress- compulsory choco group. Co: control group, St: stress group, St-Compulsory Choc: stress with compulsory dark chocolate diet group, St-Optional Choc: stress with optional dark chocolate diet group, St-Restricted Choc: stress with restricted dark chocolate diet group.
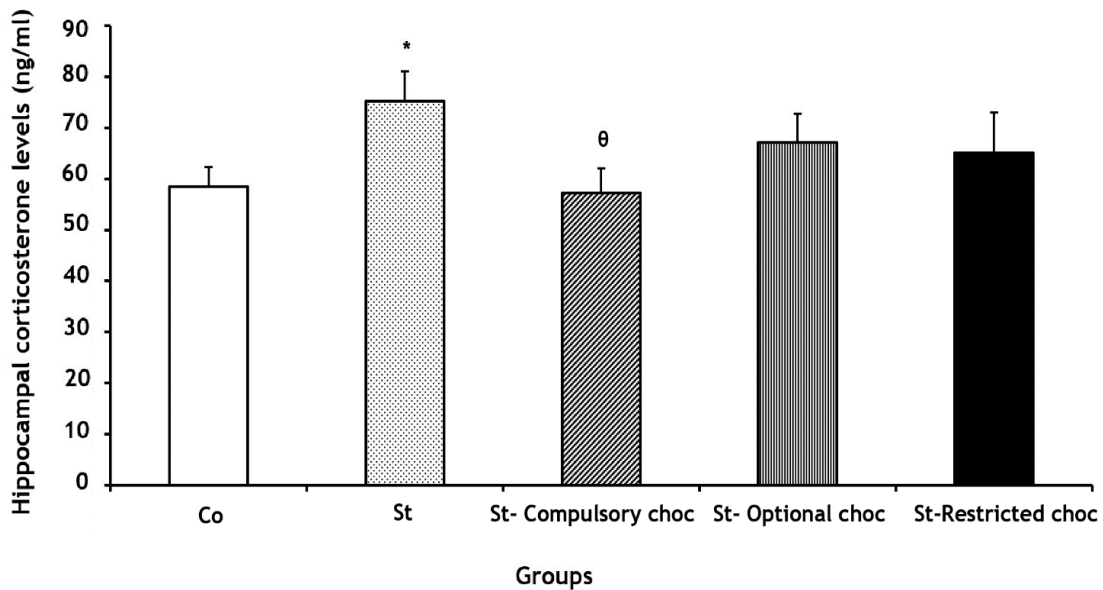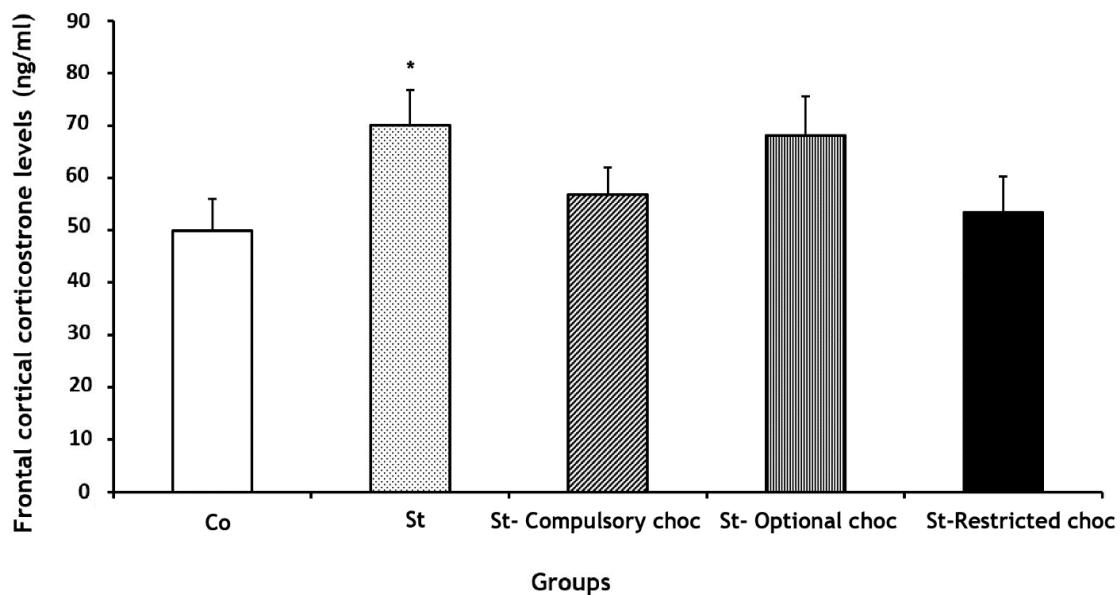

Fig.7. Effects of different dark chocolate diets on hippocampal corticosterone levels (ng/ml) in different groups under isolation stress (n=7). Results are expressed as mean±SEM. $^*P<0.05$ compared to control group, $^{\theta}P<0.05$ compared to stress group. Co: control group, St: stress group, St- Compulsory Choc: stress with compulsory dark chocolate diet group, St-Optional Choc: stress with optional dark chocolate diet group, St-Restricted Choc: stress with restricted dark chocolate diet group.

corticosterone levels [F(4, 35)= 1.882, $P>0.05$; Fig. 8].

As depicted in Figure 6, the serum corticosterone levels represented significant ($P<0.05$) enhancements in the St group in comparison with the control group. Moreover, the results of serum

corticosterone levels showed some decreases in all three dark chocolate dietary patterns, but the serum corticosterone levels decreased significantly ($P<0.01$) only in the St-Compulsory Choc group compared to the St group. Furthermore, the serum corticosterone levels increased significantly in the St-Optional and St-

Fig.8. Effects of different dark chocolate diets on frontal cortical corticosterone levels (ng/ml) in different groups under isolation stress (n=7). Results are expressed as mean±SEM. $^*P<0.05$ compared to control group. Co: control group, St: stress group, St- Compulsory Choc: stress with compulsory dark chocolate diet group, St-Optional Choc: stress with optional dark chocolate diet group, St-Restricted Choc: stress with restricted dark chocolate diet group.

Restricted Choc diet groups (respectively, $P<0.01$ and $P<0.05$) in comparison with the St-Compulsory Choc group (Fig. 6).

The hippocampal and frontal cortical corticosterone levels showed significant enhancements ($P<0.05$, in both them) in the St group compared to the control group. The hippocampal corticosterone levels decreased significantly ($P<0.05$) only in St-Compulsory Choc group in comparison with that in the St group (Figs. 7 and 8). The frontal cortical corticosterone levels did not show any significant difference in a similar comparison (Figs. 7 and 8), indicating that under stress conditions, there is a higher sensitivity in the hippocampus on corticosterone levels compared to the frontal cortical corticosterone levels.

Discussion

The effects of different (compulsory, optional and restricted) dark chocolate diets were investigated on brain functions such as learning, memory, memory consolidation and locomotor activity as well as the serum, hippocampal and frontal cortical corticosterone levels in rats under chronic isolation stress to determine which of the dark chocolate diet patterns may affect the brain function improvement under stress condition.

Regarding the current findings, there was occurrence of learning in all experimental groups at different

levels, even in the chronically stressed group a low level of learning was seen. Accordingly, some studies have confirmed that even though stress accelerates the onset and severity of cognitive impairments such as learning (Ayoub, 2009; Gulpinar and Yegen, 2004; Jeong et al., 2006), learning was still formed under stress conditions (Dastgerdi et al., 2018; Joëls et al., 2006). Memory impairment was observed in chronic isolation stress as well. In some investigations, the simultaneous occurrence of memory deficits and chronic stress was seen, especially when the stress was uncontrollable, repeated and prolonged (Corcoba et al., 2017; Dastgerdi et al., 2017). In contrast to current findings, McEwen et al. (2001) reported a state of chronic stress habituation for the animals (McEwen, 2001) in which the observed differences may depend on the types of stress and memory tasks (Dastgerdi et al., 2018). In the current study, corticosterone levels increased in the serum, hippocampus and frontal cortex of the stressed group. Furthermore, chronic stress may alter the learning and memory by stress hormones (such as corticosterone) and other neurochemical factors (Corcoba et al., 2017; Seo et al., 2016).

According to other present findings, dark chocolate dietary patterns affected different aspects of brain functions under chronic stress. The compulsory and even restricted dark chocolate diets improved memory deficits induced by chronic stress. Whereas,

the optional dark chocolate diet did not have any significant effects on memory improvement. The types of diet seem to play an important role in some brain functions such as memory (Mattson, 2000). Additionally, previous studies have demonstrated that cocoa-derived products and dark chocolate consumption may probably improve stress-related memory deficits (Swinton et al., 2018) and cognitive functions such as the visuospatial working memory in humans (Crichton et al., 2016; Ramirez-Sanchez et al., 2017). Furthermore, moderate and high doses of flavonoids and/or a dose of chocolate displayed memory improvement (Field et al., 2011; Scholey et al., 2010; Vauzour, 2014). Whereas, another study has emphasized the negative impact of cocoa on cognitive performance (Pase et al., 2013). These could vary depending on the chocolate's antioxidant status (flavonols) and bioavailability, the varieties of cocoa beans, participants' conditions (healthy or clinical), production stages, research type (Allgrove and Davison, 2014; Crichton et al., 2016) and even based on the current study, the dietary patterns is effective on brain functions. Overall, the aforementioned factors might affect the cocoa consumption efficacy in studies related to humans and animals (Allgrove and Davison, 2014; Crichton et al., 2016).

In this study, other findings indicate an improvement in memory consolidation and locomotor activity by compulsory dark chocolate diet under isolation stress conditions. In a previous study dark chocolate and its components are reported to consolidate memory (Fernell et al., 2016) and to improve neurocognitive status (Crichton et al., 2016). Nevertheless, the sustained consumption of cocoa-based food, enriched with dark chocolate, promoted locomotor activity and improved motor coordination (Cicvaric et al., 2018). Even though caffeine withdrawal decreased locomotor activity (Strain and Griffiths, 1995), Yamada et al. (2009) demonstrated that cocoa mass administration did not affect the locomotor activity in the open field test (Yamada et al., 2009). Such paradoxical results with dark chocolate diets may relate to factors including the stress type and its duration, type of behavioral tasks, rat's age and gender, and probably the variety of cocoa and/or chocolate and duration of their consumption (Engler et al., 2004; Ranjbar et al., 2016).

Notably, the compulsory dark chocolate diet could improve brain functions such as memory, memory consolidation and locomotor activity; but the brain responses seem to act differently in various dark chocolate diets. For instance, the compulsory dark chocolate diet appears to be the most effective type of dark chocolate diets under stress conditions. The authors, however, did not find any evidence about the memory processing in dark chocolate dietary patterns under stress. Some previous studies confirmed that cocoa and cocoa-derived products such as dark chocolate are the richest sources of different antioxidant compounds such as flavonoids (Small et al., 2001; Sokolov et al., 2013). Therefore, the compulsory dark chocolate diet may have had more flavonoids compared to other types of dark chocolate diets that could have acted more effectively on brain functions. However, the underlying effects of dark chocolate diet mechanism(s) have remained unexplored.

All in all, the current findings about hormonal changes represent the important role of the compulsory dark chocolate diet. Results in current study demonstrated that under stress conditions, compulsory dark chocolate diet reduced the corticosterone levels only in the serum and hippocampus, except in the frontal cortex. In addition, in previous studies, the decrease of stress hormones due to the consumption of dark chocolate were reported as well, albeit without considering the diet type (Almoosawi et al., 2012). Additionally, another study on the effects of dark chocolate indicated the reduced salivary cortisol levels in adults recruited from health-care and social settings (Tsang et al., 2019). In the compulsory dark chocolate diet, the changes of corticosterone levels in the hippocampus in comparison with the frontal cortex proposed a probable higher sensitivity in the hippocampus against dietary patterns of dark chocolate and its flavonoids. As such, Fowler et al. (2014) reported a differential sensitivity in hippocampus and prefrontal cortex towards the toxicity caused by alcohol.

Conclusion

To sum up, the different types of dark chocolate diets had various effects on brain functions under stress conditions. The restricted dark chocolate diet and particularly the compulsory one positively improved memory under stress conditions. Whereas, the memory consolidation, locomotor activity, serum and

hippocampal corticosterone levels except the frontal cortical corticosterone level, improved only in compulsory dark chocolate diet. It seems that the compulsory and optional dark chocolate diets, respectively, had the best and least effects on the improvement of brain functions. Finally, concerning the changes in corticosterone levels due to different dark chocolate dietary patterns under stress conditions, it seems that, compared to the frontal cortex, the hippocampus shows more sensitivity to these diets. However, further cellular, structural and biochemical studies are needed to clarify the underlying physiological mechanism in different dark chocolate diets.

Acknowledgments

Conflict of interest

The authors declare that they have no conflict of interest.

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