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Effects of sugar-free, αs1-casein–enriched chocolate on stress: based on salivary cortisol measurement and questionnaire data collection

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ABSTRACT

Objective: Some food-derived bioactive peptides have demonstrated positive effects on stress reduction. This study was an attempt to evaluate the effectiveness of daily consumption of 12 g of sugar-free chocolate containing 150 mg of αs1-casein (91-100) peptide in alleviating stress in healthy, normal-weight participants.

Methods: Salivary cortisol concentration and self-report questionnaire data were obtained before and after chocolate consumption. Sixty participants completed the Cattle Anxiety Scale (CSI), the Beck Depression Inventory (BDI), and the Depression Anxiety Stress Scales 21 (DASS-21).

Results: Results from the CAS and DASS-21 questionnaires showed that consumption of chocolate containing bioactive peptide reduced anxiety and stress ($p < 0.05$). Salivary cortisol measurement showed more than 30% reduction in cortisol level in the intervention group. The peptide had a 95% recovery rate and also demonstrated thermal and mechanical stability during the production process.

Conclusion: Findings confirmed the undenatured structure of the αs1-casein peptide in chocolate and its resistance to the chocolate processing condition.

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Introduction

Stress generally refers to any physical or physiological stimulus that disrupts homeostasis [1]. Anxiety is a normal reaction that is activated in animals and humans under stress conditions [2]. Stress could contribute to the progression of psychological disorders including sleep disturbance, memory deficit [3], and nutritional behavior problems [4]. It could also be implicated in gastrointestinal secretions and movements leading to gastric and duodenal ulcer [5]. Several studies have reported a positive relationship between stress-induced eating and the development of obesity in [6]. Effective stress management is essential for reducing vulnerability and susceptibility to diseases, as well as improving the quality of life [7].

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Numerous studies have attributed beneficial health effects to dark chocolate consumption. Chocolate is a source of biologically active substances such as theobromine alkaloids, phenylethylamine, polyphenols, and tocopherols. There is strong evidence proving that chocolate consumption is beneficial to brain health and for alleviating stressful situations [8, 9]. Chocolate is a functional food, and there are increasing efforts to further enhance its functionality [10]. Replacing sucrose with low-calorie sweeteners would increase the health benefits of chocolate. The potential effects of bioactive peptides have sparked scientific interest over the last few years [11].

Milk-derived peptides, which are usually produced by the proteolysis of casein (α-, β-, γ-, and κ-casein) and whey proteins, with various proven health effects, such as hypertension prevention, immune system regulation, mineral transfer function, and stress reduction, are considered potential ingredients of health-promoting functional foods [12].

Considering today's lifestyle, the necessity of producing functional foods containing specific ingredients to alleviate stress is felt more than ever [13]. The aim of this study was therefore to (1) investigate the effect of αs1-casein–enriched chocolate consumption on stress in a sample of Iranian population; (2) determine the possible recovery of the peptide and its resistance to the harsh processing conditions such as thermal and mechanical stress. This research was an attempt to open a new horizon for using bioactive peptides in food formulation to increase functionality.

Methods

The materials for chocolate production were supplied by Rezvan Chocolate Company (Baraka, Tehran, Iran). They included cocoa powder (12% fat) and cocoa butter (Cargill, Denmark); soybean lecithin and polyglycerol polyricinoleate (PGPR) (Palsgaard, Denmark); inulin (Cosucra, Belgium); and isomalt and maltitol (BENEO, Germany). Isomalt, maltitol, and inulin are low-calorie sweeteners used as sugar substitutes. The αs1-casein peptide was purchased from Ingredia Company in France, and all other reagents were purchased from Merck Company in Germany.

Chocolate preparation

The raw materials for making the regular chocolate—cocoa powder (30%); isomalt, inulin, and maltitol (38%); cocoa butter (31.4%); lecithin (0.5%); and PGPR (0.1%)—were mixed and transferred to a laboratory ball mill (Armankherad Co., Mashhad, Iran). Mixing, milling, and particle size reduction were carried out at 100 rpm at 60°C. The samples were conched for 60 minutes at 60°C and tempered before molding and cooled down to 4°C for 30 minutes. The chocolate samples were kept in plastic containers at ambient temperature for further experiments. Another batch of chocolate was made using the same composition plus 150 mg of αs1-casein peptide added to the chocolate mix before the conching process.

Clinical trial

Participants: Totally, 75 volunteers were recruited from different universities and institutes in Mashhad. Inclusion criteria were taking no antianxiety or and antistress medications during the previous year, having no specific disease based on medical history, and accepting to follow a normal diet during the trial period. This study was conducted under the approval of the Ethics Committee of Mashhad University of Medical Sciences (IR.MUMS.REC.1394,615).

Samples: Two types of chocolate with identical appearance and manufacturing process were prepared. Chocolates were packed in coded containers for delivery to participants.

Procedure: A double-blind, randomized controlled trial was conducted [8]. Coded samples were distributed randomly among the participants. People involved in sample distribution and data analysis were not aware of the codes assigned to each type of chocolate. Accordingly, participants were randomly divided into two groups: group C (receiving chocolate without peptide) and group I (receiving chocolate containing the peptide). Demographic data were collected at the beginning of the trial. Both groups received 12 g (2 × 6-g squares) of chocolate per day for 14 days [8].

Table 1. Applied gradient in recovery of peptide from chocolate

Time (min)	Flow (ml.min^{-1})	Eluent		ACN (%)
		A	B	
0	0.50	82	18	**28.1**
5	0.50	0	100	**65.0**
10	0.50	0	100	**65.0**
15-45	0.50	82	18	**28.1**

ACN: acetonitrile

Cortisol assay: Saliva samples were obtained from the participants one day before and after the intervention, exactly at 11:00 AM, and frozen immediately for later analysis. Salivary cortisol concentrations were measured using a commercial enzyme immunoassay kit (ZellBio, Germany) according to the manufacturer's instructions.

Table 2. Baseline characteristics of the subjects (N = 60)

		Group I	Group C	P value
Age, mean ± SD, y		24.7 ± 6.3	26.3 ± 4.2	0.322
Gender	Male, n (%)	15 (51.73)	16 (51.62)	
	Female, n (%)	14 (48.27)	15 (48.38)	0.621
Weight, mean ± SD, kg		67 ± 11.4	71 ± 6.4	0.722

Stress and anxiety assessment

Stress and anxiety were assessed using the following standard questionnaires:

(a) Cattle Anxiety Scale (CAS). This is a 40-item self-report tool to measure anxiety. We used the validated Persian version of the scale. Scores can range from 0 to 80 [14]. Classification of anxiety level is as follows: without anxiety (0-27), moderate anxiety (28-40), neurotic anxiety (41-49), and severe anxiety (50-80).

(b) Beck Depression Inventory (BDI). The Persian adaptation of the Beck Depression Inventory, containing 21 items rated on a scale of 0 to 3, was used in the study. The scale is widely used for measuring the intensity of depression. Each item describes a specific behavioral manifestation of depression. Total scale scores can range from 0 to 63. A total score of 17 or above may indicate clinical depression. The subject is classified as having no depression (0-16), mild depression (17-27), moderate depression (28-34), or severe depression (35-63) [15].

(c) Depression Anxiety Stress Scales 21 (DASS-21). The DASS-21 comprises 3 subscales for measuring depression, anxiety, and stress, each consisting of 7 questions rated on a scale of 0 (did not apply to me at all) to 3 (applied to me very much). The test used was a translated and validated Persian version of the scale. The DASS-21 is the shorter form of the original scale (42 questions), therefore the final score of each subscale must be doubled. The severity of symptoms could be determined by referring to the predefined table [16].

Table 3. Mean cortisol levels (ng/ml) in subjects before and after the intervention

Subjects	Before	After
Group I*	0.148 ± 0.01	**0.101 ± 0.02**
Group C	0.142 ± 0.01	**0.139 ± 0.02**

***Indicate a significant difference ($p < 0.05$).**

Evaluation of thermal and mechanical stability of the peptide

To evaluate the thermal and mechanical stability of the peptide, we exposed chocolate samples to indirect heat treatment at 80°C for 80 minutes and the conching process (as mechanical stress) before analysis. After thermal and mechanical treatments, peptides were extracted from chocolate according to the standard method provided by the manufacturer of the peptide. The instructions included adjusting the pH to 4 using lactic acid, followed by centrifugation at 14000 rpm for 10 minutes. The supernatants containing αs1-casein peptides were frozen, dried, and analyzed for structural changes by Fourier-transform infrared (FTIR) spectroscopy.

Preparation of standard peptide solution

A standard peptide solution was combined with a mixture of water/acetonitrile/trifluoroacetate to determine the rate of peptide recovery from the chocolate. High-performance liquid chromatography (Smartline, Knauer, Germany) with a solid-phase sorbent C18 column (250 × 4.6 mm, 5 µm particle size, 100 Å pore size). The injection volume was 50 µl. The solvents used for separation were solvent A (main solution; 80% water, 20% acetonitrile, and 0.1% trifluoroacetate) and solvent B (subslution; 35% water, 65% acetonitrile, and 0.1% trifluoroacetate). The elution profile included 45 minutes with solvent A at a flow rate of 0.5 ml/min, with a linear

Table 4. Results of questionnaire studies (N = 60)

	BDI		CAS		DASS-21	
	Baseline	**Final**	**Baseline**	**Final**	**Baseline**	**Final**
Group I	11.37[a]	10.31[a]	35.04[a]	28.77[b]	17.56[a]	**12.60[b]**
Group C	10. 99[a]	10.287[a]	34.16[a]	33.11[a]	16.83[a]	**15.68[a]**

BDI: Beck Depression Inventory; CAS: Cattle Anxiety Scale; and DASS-21: Depression Anxiety Stress Scales 21. Different letters in each column indicate a significant difference ($p < 0.05$).

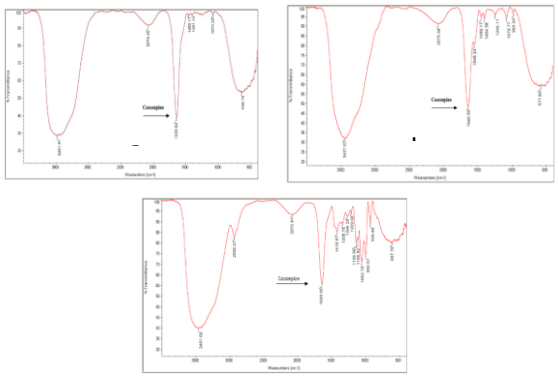

Figure 1. a) Extraction of control peptide at ambient temperature; b) extraction of peptide after thermal treatment at ambient temperature; c) extraction of peptide after mechanical treatment at ambient temperature.

gradient. The absorbed wavelength was detected at 280 nm (Table 1).

Statistical analysis

Independent-samples t tests ($\alpha = 0.05$) were used for comparing variables between the groups. Data analysis was carried out using Minitab software, version 16. All measurements for thermal and mechanical treatments and peptide recovery were carried out in triplicate and the mean and standard deviation of the replications were reported.

Results and discussion

Differences in general characteristics between the two groups were not significant (Table 2).

Cortisol measurement and questionnaire analysis

Among participants, 15 volunteers could not continue chocolate consumption regularly and were eliminated from the investigation.

The mean values of cortisol level before and after chocolate intake in the two groups are shown in Table 3. It should be considered that no significant difference ($p > 0.05$) existed between group C and I in cortisol level at baseline.

The cortisol level in group I decreased significantly after 14 days ($p < 0.05$). Data from the questionnaires also identified stress reduction in subjects who consumed peptide-containing chocolate after 14 days ($p < 0.05$) (Table 4). Good correlation was observed between cortisol measurement and the results obtained from questionnaires. There were significant reductions in stress and anxiety ($p < 0.05$) as assessed with the DASS-21 and CAS in group I at the end of the trial. However, no significant difference from baseline to post intervention was observed in either group according to BDI test results (Table 4). It should be mentioned that the cortisol level in group C also showed a slight decrease ($p > 0.05$). This slight reduction might be due to the consumption of chocolate. Martin et al reported that bitter chocolate intake for two weeks decreased secretion of stress-related hormones and balanced the metabolic effects of systemic stress [8].

Salivary cortisol level demonstrates a close relationship with plasma cortisol level. Salivary sampling is an effective tool for cortisol measurement because it is noninvasive, easy, and repeatable. There are two basic systems for stress response regulation including the sympathoadrenal-medullary system (SAM) and the hypothalamic-pituitary-adrenal (HPA) axis. The SAM results in the release of epinephrine and norepinephrine from the adrenal medulla. Activation of the HPA axis increases cortisol secretion from the adrenal cortex in response to stress. Most of the physiological stressors activate HPA and increase salivary cortisol levels [17]. Takai et al confirmed that the cortisol level increases under stress situation and indicated salivary cortisol to be a useful index for measuring stress [18].

Dela Pena and colleagues studied the effect of oral intake of αs1-casein at various concentrations (75, 150, 300, and 500 mg/kg body weight) on stress-induced sleep disorders in rats [19]. Sleep-promoting effect of 150 mg/kg αs1-casein was highlighted by improvements in slow-wave (deep) sleep [19]. The effectiveness of αs1-casein in reducing stress was shown in human subjects who took 2 capsules of 200 mg of αs1-casein hydrolysate on 3 occasions at 12-h intervals [20]. Stress-reducing effect of oral intake of αs1-casein in females with stress-related symptoms was examined using a self-report questionnaire. Based on the results, a 30-day period of consuming this peptide reduced stress in women who suffered from behavioral symptoms of stress [7].

Stress-reducing effect of αs1-casein has also been shown in various animal studies. Kato et al investigated the effects of a diet supplemented with αs1-casein tryptic hydrolysate on anxiety-related behaviors in privately owned anxious dogs. After 7 weeks, the owners of the dogs were

asked to fill out a questionnaire measuring 4 anxiety-related behavioral parameters. Questionnaire data analysis and urinary cortisol measurement showed that dietary strategies could be a solution for anxiety management in dogs [21]. Anxiety levels after oral administration of αs1-casein in mice were investigated by Violle et al [22]. According to their results, 15 mg/kg of αs1-casein showed a similar effect to that of 3 mg/kg diazepam. The authors stated that the mechanism of αs1-casein action was probably different from diazepam.

Thermal and mechanical stability of the peptide

During food processing, the ingredients are exposed to stress-inducing conditions and occasionally undergo unpleasant changes. Proteins and peptides are more sensitive and may lose their potential properties. In order to use and commercialize bioactive peptides as ingredients in chocolate, their stability should be approved in thermal (maximum 80°C) and mechanical conditions such as refining and conching processes.

FTIR spectroscopy is an effective method for characterization of the secondary structure of a protein or peptide. The most intense absorption band detected in proteins with a secondary structure is amide I, with a frequency of 1600 to 1700 cm-1, which is mainly related to stretching vibrations of the C=O (75%-80%) group, with a minor contribution from the C-N group. Any small variation in molecular structure and hydrogen bonding patterns would produce characteristic electron densities in the amide C=O groups, resulting in variation in amide I frequencies [23]. The absorbance peak at 1640 cm-1 frequency reveals the exact location of the folding or looping C=O within the molecular structure. IR spectra of proteins in the amide I region indicated no structural relocation in the peptide (Figure 1). Thermal-related amide I area for the peptide recovered from the chocolate highly corresponded with that of the control peptide at 1640 cm-1 frequency, which indicates high thermal resistance of αs1-casein during chocolate processing (Figure 1). The spectrum at the frequency of 1639.5 cm-1 for the peptide exposed to mechanical stress was also similar to that of the standard peptide, suggesting no structural change in αs1-casein during chocolate processing (Figure 1). Many studies have focused on the thermal resistance of proteins and peptides in terms of denaturation and activity [24-26]. Previous research has shown heat stability of casein peptides at different pH values (75°C, 45 min at pH 6, 7, and 8) [27]. D'Hondt et al demonstrated that casein peptides were stable for more than 3 minutes at 180°C [28].

Peptide recovery from chocolate

Standard peptide absorbance peak revealed retention time at the 17th minute, which was consistent with the obtained peak for the standard synthetic αs1-casein fraction of 91-100 (Neosystem Company, France) (Figure 2). The recovery rate of the peptide from the chocolate was more than 95%. This experiment verified our previous results indicating αs1-casein resistance against thermal and mechanical stresses during chocolate processing.

Conclusion

Salivary cortisol measurement and the data from questionnaires indicated that consumption of chocolate containing αs1-casein bioactive peptide is effective in reducing stress in a sample of the Iranian population. Moreover, FTIR analysis verified heat and mechanical stability of the αs1-casein peptide during the chocolate processing conditions. The bioactive peptide recovery rate was more than 95%. Consequently, the incorporation of stress-reducing bioactive peptides into chocolate formulation was successful. The peptide has a great potential to be used as a functional ingredient in other food formulations. However, long-term clinical trials with more participants are needed to reach a definitive conclusion as to the effectiveness of the αs1-casein peptide in reducing stress.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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