Effects of Laughing Training on Serum Cortisol and Nitrite Levels in Thai Private Office Workers

Panaree Busarakumtragul PhD*,
Jittra Dusadeemethagul PhD**, Uayart Chuchuen PhD***

** Department of Physiology, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand*
*** Graduate School, Srinakharinwirot University, Bangkok, Thailand.*
**** Department of Psychiatry Nursing, Faculty of Nurse, Srinakharinwirot University, Nakhon Nayok, Thailand*

Objective: *To evaluate the effects of the laughing training on serum cortisol and nitrite levels in Thai private office workers.*
Material and Method: *Forty volunteers age 25 to 60 years from private offices were recruited. Thirty eight remaining volunteers were divided into two groups: 20 for the experimental group and 18 for the control group. They were randomly assigned to participate in the training by matching up pairs of subjects with the same level of mean total scores from the Suanprung Stress Test-60 (SPT-60). The program ran and continued from June to July, 2013. The control subjects took a rest in the same room as the experimental group throughout the experiment. The experimental subjects participated in laughing training program for 3 days a week, 1 hour a day for 8 consecutive weeks. At 8.00 AM, eight milliliters of venous blood was drawn from individuals in both control and experimental groups at the beginning and at the end of the training program. Serum cortisol and nitrite were assayed by electro chemi-luminescense immunoassay and enhance chemiluminescense using high sensitivity nitrite, respectively.*
Results: *No significant difference was found in the mean of pre-post level of serum cortisol within control and experimental groups. Additionally, pre-level of serum nitrite in the experimental group revealed significant difference from post-level of serum nitrite in control and experimental groups at $p<0.05$.*
Conclusion: *Laughing training is supposed to promote good health by reducing acute or chronic stress during work. In this present study, laughing training for 8 weeks had significant effect on serum nitric oxide level indirectly determined by serum nitrite level. However, this laughing training program had no significant effect on the serum cortisol concentration.*

Keywords: *Laughing training, Cortisol, Nitrite*

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Nitric oxide (NO) or endothelial-derived relaxing factor is an important protective molecule in the vasculature, and endothelial nitric oxide synthase (eNOS) is responsible for most of the vascular NO production[1]. NO production is a calmodulin-dependent process, preceded by the elevation of intracellular Ca^{2+} concentration, and different receptors including nicotine and acetylcholine receptors may mediate the increase in Ca^{2+}. Vascular nitric oxide dilates all types of blood vessels by stimulating soluble guanylyl cyclase and upregulating cyclic guanosine monophosphate in smooth muscle cells[2]. Healthy endothelial cells secrete vasoactive chemicals which are released into the vascular lumen. NO is also a potent inhibitor of platelet aggregation and adhesion. Michael, 2009[3] reported that humans who have been exposed to negative emotion including mental stress and depression have been associated with reduced endothelial vasoreactivity as measured by brachial artery reactivity test. However, mirthful laughter, positive emotion, activates the pituitary release β-endorphins which upregulates nitric oxide synthase to enhance NO production. Stress hormones response to mental stress participate in endothelial dysfunction possibly via down regulation of endothelial nitric oxide synthase (eNOS) expression causing eNOS inactivation, and decreased NO actions, and increased NO degradation[4]. eNOS is an enzyme which usually generates the vasoprotective molecule, NO. Stress induced significant neurohormonal changes including nitrite, nitrate, cortisol and adrenocorticotrophic hormone (ACTH) which can be considered as part of a

Correspondence to:
Busarakumtragul P, Department of Physiology, Faculty of Medicine, Srinakharinwirot University, 114 Sukhumvit 23, Wattana, Bangkok 10110, Thailand.
Phone: +66-84-0918989, Fax: +66-2-2601533
E-mail: panareep@gmail.com

complex mosaic model of the neuroendocrine system during academic stress[5]. Cortisol produced a dose-dependent decrease in NO release. Increase in NO concentration causes oxidative stress[6]. The stress hormone, cortisol, has direct effects on nitric oxide release[7]. Plasma levels of cortisol, ACTH, nitrite and nitrate increased during academic stress periods as compared to baseline levels[5]. NO is an important signaling molecule contributing to regulation of the hypothalamo-pituitary-adrenocortical (HPA) axis. NO has the ability to substantially affect the dopaminergic, serotonergic, and noradrenergic neurotransmission. It is involved in central muscarinic, histaminergic, and adrenergic stimulation of HPA axis. NO is involved in stress physiology and stress-related disease processes. NO is involved in immunological, cardiovascular, and neurodegenerative diseases/ mental disorders[8]. Stress and depression have been linked to a reduction in serum NO[9]. Relocated nursing home residents demonstrated significant differences in salivary cortisol and mood compared with randomly selected group of residents that had not yet moved. Relocation resulted in significantly higher cortisol levels 1 week after the move ($p = 0.005$)[10].

Laughing training is not only used to relieve stress but also use to control the rhythm of breathing. Aerobic exercise, a physical stressor, can activate the HPA axis, and regular exercise training can impact how the HPA axis responds to stress[11]. They reported that NOS activity modulates the response of the neuroendocrine component of the HPA axis during exercise stress. The effects of viewing a 30-minute segment of two films inducing laughter or stress were assessed. Laughter decreased cortisol levels[12]. Cortisol, through activation of glucocorticoid receptors, suppresses NO release by down regulating eNOS proteins and inhibiting intracellular Ca^{2+} mobilization. Decreased NO is likely to result in an increase in contraction of coronary arteries leading to a decrease in coronary blood flow[7]. The generation of NO contributes to the systemic response to the organism to stress. In the vascular system, increased generation of NO may attenuate the vasoconstrictor and platelet aggregation effects of catecholamines and other mediators of stress[13].

Up to now, no evidence has been shown the effect of laughing training on serum cortisol or nitrite levels. Therefore, the purpose of this study was to investigate the beneficial effects of laughing training on serum cortisol and nitrite levels in Thai private office workers who usually have stresses during work.

Material and Method

Subjects

This research was performed after the project was approved by an ethic research committee, Faculty of Medicine, Srinakharinwirot University (No. SWEEC/EX21/2556). Thirty eight volunteer subjects consisted of 15 male and 23 female workers from private office company in Bangkok, Thailand. Their age ranges were 25-60 years. The experimental and control groups were 20 and 18 subjects, respectively. They had no history of hypertension, diabetic mellitus and asthma. They also had no history of smoking and drinking alcohol 1 month before starting training.

Laughing training program

Laughing training consisting of laughing and aerobic exercise is a kind of conventional medicine which considered to emphasize diaphragmatic breathing exercise especially abdominal muscles, active exercise of facial muscles, and active exercise of upper and lower limbs muscles. All experimental subjects performed training program 3 days a week, 60 minutes a day. Laughing program continued consecutively for 8 weeks from June to July, 2013.

Blood chemistry analysis

Cortisol assay

At 8.00 AM eight milliliters of venous blood was drawn from antecubital vein. Collected blood tubes were left at room temperature until blood became clotted. After that, the clotted blood was centrifuged at 1,500 g for 10 min. Then supernatant was collected and kept in -80°C until it was assayed. Serum cortisol was assayed by Electro Chemi-Luminescense Immunoassay (ECLIA) (Roche Diagnostics, USA; Elecsys 2010 and cobas e 411 analyzers). Cortisol in each sample competed with horseradish peroxidase (HRP)-labeled cortisol on a mouse monoclonal antibody. During the incubation, the monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. After washing to remove excess of conjugate and unbound cortisol, a substrate solution is added to the wells to determine the unbound enzyme activity. The color development is stopped and the absorbance is read at 450 nm. The intensity of the color development is inversely proportional to the concentration of cortisol in the sample.

Nitric oxide assay

The supernatant from venous blood collection at the same time by the same procedure as serum

preparation for cortisol assay was kept in -80°C until it was assayed. Total serum nitric oxide is indirect assayed by measuring total amount of serum nitrite which is converted from nitrate. They were assayed by Enhance Chemiluminescense using high sensitivity nitrite assay kit (M36051, Invitrogen European Limited).

Statistical analysis

Dependent t-test was used to analyze pre-post serum cortisol levels within the experimental and control groups. Moreover, pre-post serum nitrite levels within the experimental and control groups were also analyzed by t-test. Statistical significant difference was determined at $p<0.05$. However, serum cortisol and nitrite concentration at the beginning and the end of the program were compared between the experimental and control groups using Repeated Measured Anova. Differences were considered significant at $p<0.05$.

Results

Pre-post values of serum cortisol concentration in control group were 405.60±33.67 nmol/L and 404.47±43.95 nmol/L, respectively. In experimental group, pre-post values of serum cortisol concentration were 344.76±25.98 nmol/L and 382.21±38.02 nmol/L. No significant difference was found between pre-post values of serum cortisol concentration in control and those in experimental groups. Although post-value of serum cortisol concentration in experimental group has a slightly increase after training for 8 consecutive weeks, but no significant difference was observed.

Pre-post values of serum nitrite concentrations in the control group were 13.17±1.05 pmole/μL, and 16.30±0.81 pmol/μL, respectively. Moreover, pre-post

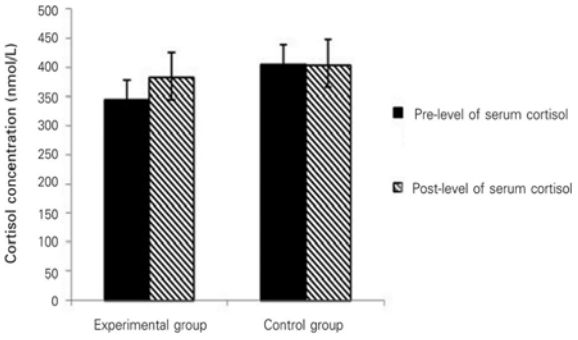

Fig. 1 This figure showed pre-post level of serum cortisol concentrations at the beginning (week 0), and the end of program (week 8) in both experiment (n = 20) and control (n = 18) groups, respectively. Data are mean ± SEM.

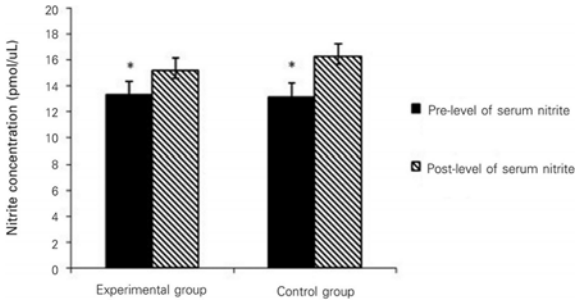

Fig. 2 This figure showed pre-post level of serum nitrite concentrations at the beginning (week 0), and at the end of program (week 8) in both experiment (n = 20) and control (n = 18) groups, respectively. Data are mean ± SEM. * Represents significant difference at $p<0.05$.

Table 1. Physical and physiological characteristics of subjects

Subject characteristics	Experimental group (mean ± SD) (n = 20)	Control group (mean ± SD) (n = 18)
Age (yr)	38.40±4.95	38.77±5.97
Height (cm)	162.25±7.77	164.44±6.60
Body weight (kg)	59.50±15.90	61.72±13.23
BMI (kg/m^2)	22.35±3.92	22.69±3.80
Male	8 (40%)	7 (39%)
Female	12 (60%)	11 (61%)
Resting axillary temperature (^{o} C)	36.30±0.46	36.42±0.66
Resting heart rate (bpm)	82.15±10.00	80.50±10.02
Resting systolic blood pressure (mmHg)	117.05±16.46	126.00±20.23
Resting diastolic blood pressure (mmHg)	75.15±10.81	80.22±11.48

Data were recorded at rest before all subjects enrolled the program

level of serum nitrite concentrations in the experimental group was 13.31±0.98 pmol/μL, and 15.20±0.61 pmol/μL. When pre-post values of serum nitrite concentrations in the experimental and control groups were compared, a significant difference was found at $p<0.05$. Total amount of serum nitrite concentration could be used as an indirect indicator of the total amount of serum nitric oxide.

Discussion

The results of this study showed no significant difference in pre-post serum cortisol level within or between the control and experimental groups after finishing the laughing program. However, previous study reported that stress reaction, and exercise could activate HPA axis resulting in a significant increase in plasma cortisol[13]. In the present study, the basal cortisol level in the experimental subjects is less than that level in control subjects. This may be due to the different basal level of stress leading to different basal serum cortisol concentration in control and experiment groups. Other studies found that serum cortisol levels increased with an increase of the intensity of perceived examination stress. Mild to moderate stress enhanced performance but severe stress decreased performance[14]. According to the pre-post serum cortisol concentrations in this study, subjects in experimental group seemed to have less stress than that in control group before joining the laughing training program. Because control subjects did not perform laughing training, they would rather have a higher post cortisol level than that level in experimental group. The higher post serum cortisol level in the experimental group may be that the exercise intensity using in this study is 60% maximum oxygen consumption (VO_2 max) that can trigger cortisol release[15]. Although we recruited subjects who worked in the same department/environment and expected to have the same basal stress level subjects to gain nearly the same basal serum cortisol level. We could not control for exogenic and endogenic stress factors which may have influenced the basal stress level. Additionally, the basal, non stressful secretion of cortisol follows a circadian rhythm, beginning with a distinct sharp rise of cortisol at the time of awakening, followed by a steady decline during the day, with the lowest levels at night[16].

Pre-post serum nitrite concentrations in experimental and control groups showed significant difference at $p<0.05$. Serum nitrite level indirectly represents nitric oxide level because nitrite is a nitric oxide metabolite. NO activates HPA axis by cholinergic, adrenergic, and histaminergic systems and by corticotrophin-releasing hormone and arginine vasopressin in vivo under basal condition[17]. Due to a significant increase in nitrite level in the experimental group in this study, NO might regulate the secretion of hypothalamus (CRH), pituitary hormones (ACTH), and consequently, glucocorticoid including corticosterone/cortisol, respectively. The role of endogenous NO in regulating the activity of HPA axis is not well characterized, since both a stimulatory and inhibitory effect of NO on the stimulated release of CRH was observed in the cultured hypothalamic tissue. The levels of NO were increased up to 1 h after exercise and subsequently lowered to basal level after 24 h. Acute exercise may induce high levels of NO[18].

In this study, after terminate training venous blood samples collected immediately resulting in sustained high level of post serum nitrite concentration was observed in the experimental group. Thus NO may act as a stimulatory neurotransmitter to activate CRH release leading to an increased post level cortisol via signaling messenger; cAMP and IP_3.

Conclusion

In conclusion, laughing training revealed a significant effect on serum nitrite concentration indirectly determined by serum nitric oxide concentration. But it had no significant effect on serum cortisol level. This may be implied that laughing training has potential to upregulate nitric oxide indirectly to enhance vasoprotective effect. We still need to investigate further study by applying this laughing training to other patients with clinical signs and symptoms such as hypertension, anxiety, and depression including cognitive impairment for beneficial effects. In addition, the duration and intensity of the training course may be adjusted to make it much more effective.

What is already known on this topic?

Mirthful laughter, positive emotions, had a significant impact on lowering blood pressure. No previous study concerning about the effect of laughing training on serum cortisol and nitrite was reported.

What this study adds?

This study found that laughing caused a positive effect due to the significant difference between pre-post serum nitrite concentrations in experimental and control groups. This may be summarized that

laughing training caused a significant increase in serum nitrite, an indirect biomarker of nitric oxide levels, a vasoprotective effect resulting in vasodilation and atherosclerosis protection.

Acknowledgements

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Potential conflicts of interest

None.

References

1. Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. Circulation 2006; 113: 1708-14.
2. Forstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, et al. Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. Hypertension 1994; 23: 1121-31.
3. Miller M, Fry WF. The effect of mirthful laughter on the human cardiovascular system. Med Hypotheses 2009; 73: 636-9.
4. Toda N, Nakanishi-Toda M. How mental stress affects endothelial function. Pflugers Arch 2011; 462: 779-94.
5. Al Ayadhi LY. Neurohormonal changes in medical students during academic stress. Ann Saudi Med 2005; 25: 36-40.
6. Delsouc MB, Della Vedova MC, Ramirez D, Anzulovich AC, Delgado SM, Casais M. Oxidative stress and altered steroidogenesis in the ovary by cholinergic stimulation of coeliac ganglion in the first proestrous in rats. Implication of nitric oxide. Nitric Oxide 2016; 53: 45-53.
7. Rogers KM, Bonar CA, Estrella JL, Yang S. Inhibitory effect of glucocorticoid on coronary artery endothelial function. Am J Physiol Heart Circ Physiol 2002; 283: H1922-8.
8. Esch T, Stefano GB, Fricchione GL, Benson H. The role of stress in neurodegenerative diseases and mental disorders. Neuro Endocrinol Lett 2002; 23: 199-208.
9. Trueba AF, Smith NB, Auchus RJ, Ritz T. Academic exam stress and depressive mood are associated with reductions in exhaled nitric oxide in healthy individuals. Biol Psychol 2013; 93: 206-12.
10. Hodgson N, Freedman VA, Granger DA, Erno A. Biobehavioral correlates of relocation in the frail elderly: salivary cortisol, affect, and cognitive function. J Am Geriatr Soc 2004; 52: 1856-62.
11. Jankord R, McAllister RM, Ganjam VK, Laughlin MH. Chronic inhibition of nitric oxide synthase augments the ACTH response to exercise. Am J Physiol Regul Integr Comp Physiol 2009; 296: R728-34.
12. Vlachopoulos C, Xaplanteris P, Alexopoulos N, Aznaouridis K, Vasiliadou C, Baou K, et al. Divergent effects of laughter and mental stress on arterial stiffness and central hemodynamics. Psychosom Med 2009; 71: 446-53.
13. Leza JC, Salas E, Sawicki G, Russell JC, Radomski MW. The effects of stress on homeostasis in JCR-LA-cp rats: the role of nitric oxide. J Pharmacol Exp Ther 1998; 286: 1397-403.
14. Inam QU, Shireen E, Haider S, Haleem DJ. Perception of academic examination stress: effects on serum leptin, cortisol, appetite and performance. J Ayub Med Coll Abbottabad 2011; 23: 97-9.
15. Genc A, Tur BS, Aytur YK, Oztuna D, Erdogan MF. Does aerobic exercise affect the hypothalamic-pituitary-adrenal hormonal response in patients with fibromyalgia syndrome? J Phys Ther Sci 2015; 27: 2225-31.
16. Dedovic K, Duchesne A, Andrews J, Engert V, Pruessner JC. The brain and the stress axis: the neural correlates of cortisol regulation in response to stress. Neuroimage 2009; 47: 864-71.
17. Gadek-Michalska A, Bugajski J. Role of nitric oxide in the nicotine-induced pituitary-adrenocortical response. J Physiol Pharmacol 2004; 55: 443-55.
18. Rahman ZA, Abdullah N, Singh R, Sosroseno W. Effect of acute exercise on the levels of salivary cortisol, tumor necrosis factor-alpha and nitric oxide. J Oral Sci 2010; 52: 133-6.
19. Fonzi L, Matteucci G, Bersani G. Laughter and depression: hypothesis of pathogenic and therapeutic correlation. Riv Psichiatr 2010; 45: 1-6.

ผลของการฝึกหัวเราะต่อปริมาณคอร์ติซอลและไนไตรท์ในเลือดของพนักงานบริษัทเอกชน

ภนารี บุษราคัมตระกูล, จิตรา ดุษฎีเมธากุล, เอื้อญาติ ชูชื่น

วัตถุประสงค์: *เพื่อศึกษาผลของการฝึกหัวเราะต่อปริมาณคอร์ติซอลและไนไตรท์ในเลือดของพนักงานที่ทำงานในบริษัทเอกชนแห่งหนึ่ง*

วัสดุและวิธีการ: *อาสาสมัครเพศชายและหญิงที่มีอายุระหว่าง 25 ถึง 60 ปี จำนวน 40 คนจากบริษัทเอกชนแห่งหนึ่ง ผ่านการคัดเลือกเข้าร่วมโครงการวิจัยนี้จำนวน 38 คน ถูกแบ่งออกเป็น 2 กลุ่ม ได้แก่ กลุ่มทดลองจำนวน 20 คน กลุ่มควบคุมจำนวน 18 คน โดยกลุ่มควบคุมพักผ่อนอยู่ในห้องเดียวกับกลุ่มทดลอง ขณะที่เข้าร่วมการฝึกหัวเราะ กลุ่มทดลองได้รับการฝึกหัวเราะ 3 วัน/สัปดาห์ ครั้งละ 60 นาที เป็นเวลาต่อเนื่อง 8 สัปดาห์ การฝึกเริ่มตั้งเดือนมิถุนายน ถึง กรกฎาคม พ.ศ. 2556 กลุ่มควบคุมและกลุ่มทดลองได้รับการเจาะเลือดก่อนการฝึกหัวเราะ (สัปดาห์ที่ 0) และทันทีที่สิ้นสุดการฝึกหัวเราะ (สัปดาห์ที่ 8) ปริมาณคอร์ติซอลในเลือดถูกวิเคราะห์ด้วยวิธี electro chemi-luminescense immunoassay และปริมาณไนไตรท์ในเลือดถูกวิเคราะห์ด้วยวิธี enhance chemiluminescense*

ผลการศึกษา: *จากการศึกษาพบว่าปริมาณไนไตรท์ในเลือดก่อนและภายหลังการฝึกหัวเราะระหว่างกลุ่มควบคุมและกลุ่มทดลอง มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) นอกจากนี้ยังแสดงให้เห็นว่าการฝึกหัวเราะไม่มีผลต่อการเปลี่ยนแปลงปริมาณคอร์ติซอลในเลือด*

สรุป: *การวิจัยนี้พบว่าฝึกหัวเราะอย่างต่อเนื่องเป็นเวลา 8 สัปดาห์ มีผลทำให้ปริมาณไนตริกออกไซด์ ซึ่งแสดงด้วยปริมาณไนไตรท์ในเลือดเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติที่ระดับ $p<0.05$ แต่ไม่มีผลทำให้ปริมาณคอร์ติซอลในเลือดเปลี่ยนแปลง*