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Research Letter

Psychoneuroendocrinoimmunology-based meditation (PNEIMED) training reduces salivary cortisol under basal and stressful conditions in healthy university students: Results of a randomized controlled study

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ABSTRACT

Background: Meditation represents an effective and safe practice to lower distress and promote well-being. PsychoNeuroEndocrinoImmunology-based Meditation (PNEIMED) is a validated method that can reduce stress-related symptoms and salivary cortisol secretion. To date, few randomised controlled trials (RCTs) have assessed cortisol levels through salivary samples, collected both in the morning phase and during acute mental stress elicitation, in healthy young subjects following brief meditation training.

Aim: The present study aims to investigate, in healthy young undergraduate students, the effects of a brief PNEIMED training course on HPA axis by measuring salivary cortisol levels.

Methods: Forty students attending the Faculty of Psychology, without comorbidities and previous experience of meditation, were enrolled in the study. Twenty subjects were randomly assigned to 30 h of PNEIMED training (intervention group, IG), and twenty subjects were randomly assigned to 30 h of academic lessons (control group, CG). Salivary cortisol measures included basal morning (t0 = baseline time, collected 30 min after waking) and under stress-eliciting task values. Cortisol measurement under the stress-eliciting task was provided through the Subtraction Stress Task (SST) at scheduled time intervals (t1 = 5 min pre-SST, t2 = 10 min post-SST, t3 = 30 min = post-SST). Salivary cortisol was measured among all subjects (IG + CG) at the beginning (pre-test) and at the end (post-test, four days later) of the study.

Results: ANOVA between-group analysis of basal diurnal salivary cortisol showed a significant hormone deflection in the IG at the end of the PNEIMED course (post-test) when compared to the CG (IG post-test 5.64 ± 4.2 vs CG post-test 9.44 ± 4.9; $F_{1,38} = 6.838$; $p = 0.013$). RM-ANOVA within-group analysis for the IG also showed that time and condition effects were statistically significant, with *F*time = 5.438; $p = 0.002$ and *F*condition = 10.478; $p = 0.004$, respectively. The IG group presented a significant reduction in basal morning cortisol at the end of the PNEIMED course (post-test) compared to the salivary concentration at baseline (pre-test) (IG pre-test 9.42 ± 6.0 vs IG post-test 5.64 ± 4.2; $F_{1,38}$ 8,354; $p = 0.009$). RM-ANOVA for the control group showed only the main effect of time ($F_{1,38} = 40.348$; $p < 0.001$). Regarding cortisol measures under the SST-stress eliciting task, ANOVA between-groups analysis showed higher cortisol levels in the IG than in the CG before the PNEIMED course, with significant differences between groups at time t2 and time t3. After the PNEIMED course, the cortisol levels in the IG had decreased, although the differences between groups were not significant. Interestingly, ANOVA within-groups analysis showed that in the IG, the cortisol levels post-test (after the PNEIMED course) were lower than at pre-test (before the PNEIMED course), showing a significant difference of cortisol salivary concentration between conditions at t3 ($F = 5.326$; $p = 0.032$). In the control group, the post-hoc analyses for pair-wise comparisons between conditions (pre-test vs post-test) did not show significant differences.

Conclusion: Although the low number of subjects enrolled in the study does not allow for definitive conclusions to be drawn, the present findings confirmed the capability of the PNEIMED method to lower stress hormone secretion both at baseline and under acute mental stimulation in a group of young naïve practitioners and make a contribution to the existing literature by increasing the number of published RCTs about the topic.

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Introduction

Neuroendocrinology of stress response in mammals

Stress in mammals is an adaptive process in response to a real or perceived threat that induces integrated neuro-endocrine, metabolic and immunologic cascades to achieve stability (homeostasis, according to Cannon[1]) and successfully adapt to such challenges through behavioral coping strategies, thus adjusting physiological parameters for both predictable and unpredictable events and thereby obtaining a new steady state (allostasis,[2] namely, maintaining stability through change). Stressors of different origin, both physical and psychosocial, can elicit stress response activation. The hypothalamic–pituitary–adrenal (HPA) axis and the autonomic, predominantly sympathetic, nervous system (ANS) provide the afferent and efferent limbs of the stress response in all vertebrates, including humans, first described by Hans Selye[3] in 1930s. Both arms of the stress response transmit the output of central nervous activity (arousal state) and result in the release into the blood stream of glucocorticoids (cortisol or corticosterone) and monoamines (adrenaline and noradrenaline), which represent the final mediators of the HPA and ANS systems, respectively. The hypothalamic–pituitary–adrenal (HPA) axis,[4] starting from the hypothalamic paraventricular nucleus, secretes the corticotropin-releasing factor (CRF) that mediates neural control of adrenocorticotropic hormone (ACTH) release from the pituitary gland. Cortisol is the active form of the hormone released from the adrenal cortex in response to ACTH in humans and elicits negative feedback from the hypothalamic CRF and pituitary ACTH secretion after termination of a stressful stimulus. Glucocorticoids and monoamines act synergistically to modify cardiovascular activity and metabolic assets (increasing of blood pressure, cardiac output, skeletal muscles perfusion, glucose mobilization), enabling the individual's classic "fight or flee" response, as observed by Selye[5] in humans and experimental animals. The endocrine and autonomic arms of stress response are interconnected with the central nervous system areas of the limbic system (hypothalamus, amygdala, hippocampus, basal ganglia, thalamus, and cingulate gyrus), prefrontal cortex, locus coeruleus, and other regions of the forebrain and hindbrain.[4] Repeated and/or protracted exposure to physical or psychosocial stress may threaten the body's regulatory systems and result in allostatic overload,[6,7] a maladaptive condition observed in several diseases (mood and behavioral disorders,[8] metabolic disturbances and cardiovascular diseases[9]), often characterized by low quality of life.[10]

Cortisol as biological marker of life stress

Through the HPA axis activation, cortisol is secreted by the adrenal glands into the bloodstream following a morning circadian rhythm, as well as in response to both acute and chronic daily life stress. Although cortisol is a fundamental hormone that plays a physiological role in regulating human metabolism, immune and endocrine systems as well as many neural activities, it is also regarded as a valuable biological marker of chronic stress activation when is overproduced. Salivary cortisol assay is a sensitive, specific and pain-free procedure frequently used to evaluate the effectiveness of interventions intended to reduce stress, including anti-stress practices and meditation.[11] Sustained cortisol response is thought to be caused mainly by situational factors, such as work overload[12] or social inequality.[13] Indeed, epidemiological evidences confirm that higher morning cortisol levels are associated with an increased risk of mortality and chronic diseases[14] in the older population.

Summary of scientific evidence for mind-body therapies

Stress management interventions, including meditation and other mind-body techniques (MBTs), are regarded as effective and safe strategies to reduce allostatic load and promote psychological and physical well-being. The effects of meditation practices and stress management programs have been widely studied since 1970, with Wallace's seminal study on Transcendental Meditation.[15] In practitioners, meditation has been found to improve cognitive performances,[16] mood,[17] and affective processes[18] of emotional regulation.[19] Other evidence links regular meditation practices (mindfulness, yoga, Tai Chi, Qigong, cognitive-behavioral stress management, relaxation and breath regulation) to the enhancement of immune system functioning, as observed in oncology patients,[20] and the reduction of inflammatory cascade[21] through the epigenetic downregulation of nuclear factor kappa B pathway.[22] Traditional meditation programs often require long and expensive training periods. However, brief courses in stress management yield positive results in reduction of pain,[23] fatigue, and stress-related mood disorders. Brief meditation practice can influence the autonomic regulation of cardiovascular parameters (i.e., blood pressure and heart-rate variability), leading to the reduction of classical cardiovascular risk factors, as observed in both primary and secondary cardiovascular prevention studies.[24,25] Evidence from both mindfulness-based stress reduction programs and other integrative MBTs have shown that four to seven days of meditation practice for few minutes a day can enhance attention and self-regulation,[26,27] reduce anxiety, fatigue and cardiovascular parameters[28] and regulate adrenergic tone, increasing heart rate variability (HRV).[29,30]

MBT$_S$-lowering cortisol techniques: overview

Long-term practice of mindfulness-based meditation and other traditional techniques have been reported to lower plasma cortisol levels both in healthy subjects[31] and in cancer patients[32,33] and to reduce awakening salivary cortisol in PTSD patients[34] as well as in substance abusers.[27] The beneficial regulatory effects on daily life stress as well as on basal cortisol secretion can be obtained with brief meditation training formats. Recent reports[35,36] also have shown that healthy subjects practicing combined meditation techniques experience a consistent reduction of morning plasma cortisol after few hours of training[37] and reduce, in general, the main physiological markers of stress.[38,39] Most studies have focused on physiological adaptations to meditative status, including the improvement of attention and self-regulation,[26,27] harmonization of the autonomic nervous system,[29,30] structural and functional modifications in brain areas involved in emotion regulation,[40,41] positive effects on psychological well-being and quality of life,[42] modulation of pain[43] and long-term biological effects on the immune system.[44] Previous studies have focused on hormonal changes during meditative status and demonstrated lower cortisol secretion in response to acute stressors in Transcendental Meditation experts.[45] More recently, even a brief meditation training was proven effective in reducing physiological stress modifications, including increased cortisol, and psychologic stress markers through a physiological relaxation response to acute experimental stress (i.e., a computer game) in naïve young adults.[46] Practicing integrative body-mind training (IBMT) can reduce both acute endocrine stress response, with the decrease of salivary cortisol amount if the training session is given immediately after acute stress, and basal endocrine stress system with decreased basal cortisol level after 4 weeks of training in Chinese students, compared to a control group receiving only relaxation training.[47] Mindfulness practice has also been tested in numerous oncology patients to mitigate distress and ameliorate their quality of life; Black[48] et al. demonstrated in a randomized clinical trial the capability of this type of meditation to lower acute cortisol release during chemotherapy administration in colorectal cancer patients. A single session of Hatha yoga[47] can also regulate physiological responses to acute psychological stressors, accelerating blood pressure recovery and salivary cortisol reactivity in 24 healthy young adults.

Background of the psychoneuroendocrinoimmunology-based meditation (PNEIMED) method

The PsychoNeuroEndocrinoImmunology-based meditation (PNEIMED) method is a meditation practice developed by Carosella and Bottaccioli[49-52] in 1998 in Italy and combines modern scientific

concepts with ancient Buddhist philosophy and meditative approaches to real life. The PNEIMED method promotes the importance of the mind–body relationship through the modern vision of the human organism as a psycho-neuro-endocrine-immunology (PNEI) network,[53,54] associating scientific sessions with philosophical lectures and meditative and stress-control practices from the Buddhist Mahayana tradition, integrated with modern theories (i.e., psychosynthesis according to Assagioli[55]). In a previous prospective, non-randomized, cohort-controlled study,[56] four days (30 h) of PNEIMED training was able to reduce stress-related symptoms and salivary cortisol secretion in healthcare workers. The present study aims to investigate, in healthy young undergraduate students, the effects of a brief PNEIMED training course on the HPA axis by measuring salivary cortisol levels. We hypothesize that a brief PNEIMED training may have a positive effect on biological stress reactions, lowering cortisol secretion both under basal conditions (30 min after waking) and in response to an acute mental stress task.

Theory and practice of psychoneuroendocrinoimmunology-based meditation (PNEIMED)

The PNEIMED method finds its philosophical roots both in the teaching of the 14th current Dalai Lama Tenzin Gyatso[57] and in the classic Buddhist Mahayana tradition (or “Great Vehicle”), which find in the individual’s constant practice of concentration (*samatha*, in the Pāli language) and deep vision (*vipassana*) the way to achieve a perfect state of conscious well-being called enlightenment.[58] Based on a critical and non-religious approach, PNEIMED method embraces the philosophical knowledge and practice of meditation while joining it with the modern scientific understanding of neuroscience. According to Gyatso,[59] the Mahayana tradition is “the Buddhism of knowledge, the 21st-century Buddhism based on an extremely solid knowledge which includes new scientific progress”. Interpretation of reality through the scientific method is not in opposition to the meditative and contemplative way but rather serves as a fundamental tool to achieve full awareness and, finally, the “liberation” of the individual from suffering and pain. PNEIMED combines meditative teachings with scientific knowledge regarding the influences of the emotions on brain functions and the reciprocal interconnections among the psyche-brain system and the other main biological systems, according to PNEI research. Scientific knowledge is transmitted through face-to-face learning in a classroom with the support of slide presentations. Participants become aware of the biological concept of stress, the different stress responses and their influences on emotions, cognition and behavior, including their biological correlates.

Similarities to and differences from the mindfulness method

PNEIMED shares some similarities with the mindfulness method, a technique developed in the USA by Kabat-Zinn and co-workers[60] in the 1970s, but also differs in other aspects. Both PNEIMED and mindfulness methods aim to make the tradition of Buddhist meditation more accessible to Westerners, favoring a laic and rational approach to the meditative way. While mindfulness refers to the Theravada tradition, PNEIMED mainly concerns the Mahayana tradition. Similarities are shown among meditation exercises (i.e., sitting meditation, body scan, and observing one’s own thoughts, images and sensations without holding or judging them). However, the evocation of “emotional words” is a particular technique used in PNEIMED courses to improve concentration and attention, helping the learners to recognize the emotion and detach from it. Mindfulness was designed as an easy and reliable technique for all individuals who must face stressful life events in different social contexts (i.e., health care, education, business economy, security and defense policy, criminal justice). The Mindfulness-Based Stress Reduction program (MBSR), the first and most famous protocol developed by Kabat-Zinn, has been successfully adapted for several uses. Mindfulness-based Mind Fitness Training (MMFT, or M-fit), taught to US cohorts of soldiers prior to deployment to achieve operational effectiveness in war scenarios and protect them against cognitive stressors, provoked some criticisms among meditation practitioners in 2014.[61] In mindfulness training, acceptance of oneself and non-critical judgment toward one’s thoughts, feelings, and behaviors are encouraged. However, this level of non-judgment and acceptance may not be as beneficial for people suffering from high levels of impulsivity and aggression. In a recent study involving 259 jail inmates, the mindfulness-based non-judgment of self-scale showed a direct positive relation to criminogenic thoughts and behaviors, suggesting a competing iatrogenic effect.[62] PNEIMED practitioners are encouraged to explore their mind, biological processes, and the interaction between their biological and psychological systems as a complex network, becoming gradually aware of their inner mental automatisms (i.e., the wandering mind) generating prejudice and greed, through both introspective meditative practice and solid rational knowledge about cognitive neuroscience and body regulation systems in physiological and pathological conditions. The PNEIMED represents not only a pragmatic method to lower distress but a life-transforming opportunity, a modern, non-religious, ethical vision of life derived from deep knowledge of ancient Buddhadharma teachings on kindness, non-violence and compassion.

Materials and methods

PNEIMED course

The PNEIMED course offers 30 hours of face-to-face learning (over four consecutive days) organized in alternate sessions of one and a half hours of informative scientific lessons regarding the principles of PNEI and meditative theory and practice, respectively. PNEI lessons account for 15 h in total, and meditation instruction consists of 15 h in total. However, PNEI scientific content is targeted to the cultural and educational background of the audience. PNEI topics are organized following a precise didactic program: the first day provides an historical overview of modern scientific theories about mind–body relationships, from Descartes’s scientific and philosophic contributions to the birth of modern physiopathology of the XIX century, through to DNA discovery and the establishment of genetic reductionism and the subsequent shift towards the revolutionary epigenetic theory. The second day focuses on the neurobiology and neuroscience of emotions and the link between emotions and consciousness. The lessons of the third day deal with neurobiology and the psychology of stress, with specific regard to the history of stress research (from Cannon and Selye’s seminal work to contemporary research), illustrating the main characteristics of neuroendocrine stress pathways and the pathological consequences of chronic stress on brain functions and immune and metabolic systems. The lessons of the last day provide an update on scientific evidence regarding the effects of mind-body therapies, nutrition, and physical activity on human health, underlining the importance of changing lifestyle to the audience. To allow for considerable attention to each participant during exercise sessions, the class size does not exceed twenty practitioners. The techniques are based on the exercises included in the texts of Carosella and Bottaccioli.[49–52] The philosophical origins as well as the actual purposes of the exercises are explained in light of the PNEIMED method and their use in everyday life, with specific details on the position during meditation sessions and how to begin and terminate the exercise without trouble. The first day is focused on the body relaxation exercise (from feet to head in ascending order). At this early stage, guided relaxation is not particularly profound to allow participants to become confident with their new physiological status, characterized by parasympathetic tone predominance (i.e., lower heart and respiratory rate and reduced blood pressure from the norm). Moreover, guided exercises on geometric shapes (triangles, circles, and squares) visualizations are provided. On the second day, learners experience an intermediate-level relaxation exercise, including visualization exercises where they visualize their own names on a white board and

then gradually delete them. In this setting, basic breathing exercises are also taught. On the third day, participants are instructed to observe their own bodies from different points of view (from the front, the side, above, and behind) and visualize on the white board some emotive words, such as "patience," "calm," "peace," "serenity," "courage," and "compassion". Participants are encouraged to replicate exercises at home, using instruments such as mirrors, to better visualize and memorize their own bodies. On the final day, participants begin to practice deep relaxation with the visualization of more complex scenes (e.g., the lake exercise) and the observation of one's thoughts and of oneself.

Study design

A randomized, controlled study was conducted in accordance with the 2017 Updated CONSORT Statement (extension version for nonpharmacological trial; see the schematic flow chart for the details, Fig. 1). The study took place from 11 to 14 May 2015. The study population consisted of 4th- and 5th-year students in the Psychology course at L'Aquila University, Italy, without previous experience in meditation. A necessary sample size of forty participants was determined. Participants were selected using inclusion criteria (4th- and 5th-year students in the Psychology course at L'Aquila University, Italy, who have never experienced meditation training before) and exclusion criteria (see below). Study participation was voluntary, and subjects were enrolled after a complete presentation of the research and their provision of informed verbal consent. The project was carried out in compliance with the Helsinki Declaration. The privacy rights of human subjects were always observed. The exclusion criteria were: active cigarette smoking, body mass index (BMI) $\geq$25, diagnosis of psychiatric diseases, presence of mood disorders such as anxiety and depressive symptoms, insomnia or poor sleep quality, hormonal treatments at the time of interview, presence of oral or dental pathologies and inadequate knowledge of the Italian language. Participants were screened for depression, anxiety and sleep disorders through the Beck Depression Inventory-II (BDI-II),[63] the State-Trait Anxiety Inventory-2 (STAI-Y2)[64] and the Pittsburgh Sleep Quality Index (PSQI).[65] Subjects with STAI-Y2 score > 50, BDI score > 20 and PSQI score > 5 were excluded from the randomization.

The experimental sample consisted of forty (n = 40) participants. Twenty (n = 20, 8 males and 12 females) were assigned using computerized random numbers procedure to the PNEIMED training (intervention group, IG) and twenty (n = 20, 6 males and 14 females) were included with the same standardized procedure in the control group (CG). The IG was tested at the beginning (pre-test) and at the end (post-test) of the PNEIMED course (4 days, 30 h in total), and the CG was tested at the beginning (pre-test) and at the end (post-test) of university activities (academic lessons). The time interval between pre-test and post-test (Tf) was four days (see the flow chart of the study). Salivary cortisol detection was performed 30 min after waking (t0 = baseline time) and under an acute mental stress task (Subtraction Stress Task, SST). Cortisol assessment under the SST was collected at t1 = five minutes pre-stress task, t2 = ten minutes post-stress task, and t3 = 30 min post-stress task. Salivary cortisol samples were collected prior to the PNEIMED course (pre-test) and after the PNEIMED course (post-test, four days after) in the IG and at the beginning (pre-test) and at the end of university activities (post-test, four days after) in the CG. The control group was considered as active, since the students attended academic lessons regarding psychological and behavioral processes in health and illness in a classroom close to the IG experimental setting, in the same time period and for the same number of hours (30 h in total).

Measurements

Salivary cortisol test

To investigate cortisol excretion both at baseline and in response to SST-elicited acute mental stress, four salivary samples were collected at the beginning of the study (pre-test) and a further four salivary samples at the end of the study (post-test) for each subject of the study population at scheduled interval times (t0, t1, t2 t3). All participants were instructed to collect salivary samples and to observe several requirements to optimize and standardize sample collection as follows: in the two hours preceding the sample collection, do not eat food or beverages (except water); ten minutes before each saliva sample collection, rinse the mouth with a glass of water. The first sample (t0) was taken 30 min after waking to assess basal diurnal cortisol. The second was taken 5 min prior to the SST, and the last two were taken 10 min and 30 min, respectively, after the SST test. Except for the basal sample (collected at home by each participant), all other samples were collected with the assistance of laboratory personnel near the classroom where the meditation sessions were performed.

Sample collection and processing

Saliva samples were collected with a Salivette tube (Sarstedt, Numbrecht, Germany) according to the manufacturer's instructions, and a volume of at least 1 ml of saliva was recovered in each case (average, 1.15 ml). Saliva samples were centrifuged at 1000 g for two minutes, and supernatant was collected, aliquoted in at least four 200-μl parts in microfuge tubes (Clicklock microfuge tubes, Simport, Quebec, Canada) and stored at −20 °C until the following assays were performed.

Cortisol ELISA test

Determination of cortisol in saliva was performed using an enzyme-linked immunosorbent assay (cortisol ELISA assay kit, Arbor Assays, Ann Arbor, Michigan, USA). Fifty microliters of saliva were deposited per assay well, following the kit procedure and using the kit reagents, and readings of the absorbance of the resulting reaction were conducted in a 96-well micro-ELISA reader (Gentaur/GDMS, Kampenhout, Belgium) connected to a computer to record each reading value. Each sample was tested at least in triplicate.

Statistical analysis

For all variables, descriptive statistics (proportions, means and standard deviations) were elaborated. The sample size was composed of n = 40 subjects. N = 20 (8 males and 12 females) were randomly assigned to the PNEIMED training (intervention group, IG) and n = 20 (6 males and 14 females) were included in the control group (CG). To investigate the effects of the intervention, we conducted a mixed ANOVA model with group (intervention group, IG/control group, CG) as the between factor and two within factors: condition (composed of two levels: pre-test/post-test) and time (divided into four levels: t0 = baseline, t1 = five minutes pre-stress task, t2 = ten minutes post-stress task, t3 = 30 min= post stress task). The Shapiro–Wilk test was used to study the normality and the Greenhouse–Geisser epsilon was used to measure sphericity if necessary. We conducted separate repeated-measures ANOVAs (RM-ANOVA) for the IG and the CG to investigate changes over the condition and the time within groups, considering two repeated factors of time (four levels) and condition (two levels). Then, repeated-measures ANOVA models were used to assess differences between the two groups at each time point. Post-hoc analyses were performed using the Bonferroni correction for multiple comparison. All analyses were conducted using STATA 14 Software, with alpha set to 0.05.

Results

Demographic characteristics

Participant characteristics are reported in Table 1. All subjects were 4th- and 5th-year university students attending the course in

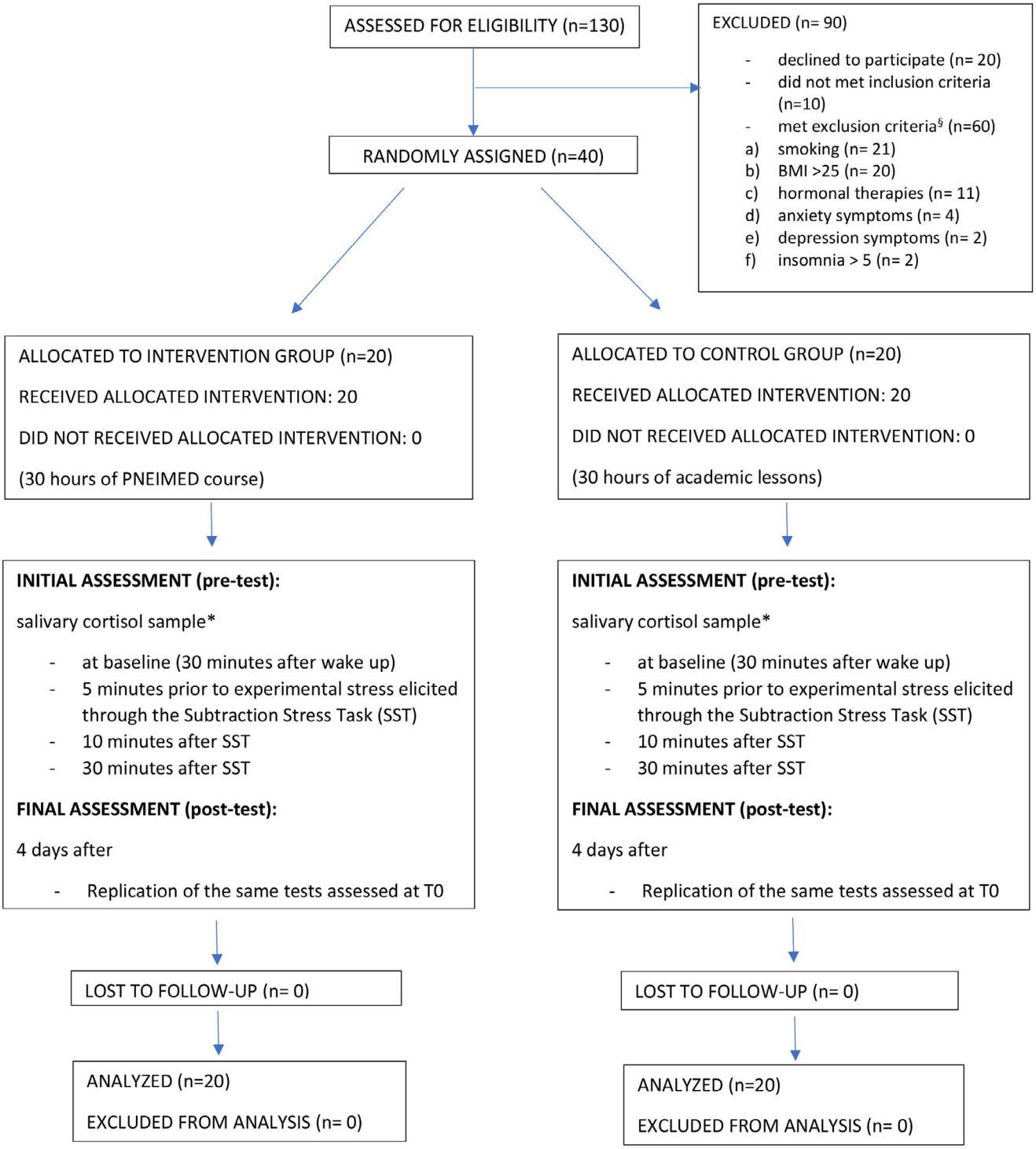

Fig. 1. Flow chart of the study.

*ELISA assay.

§Exclusion criteria = active cigarette smoking, overweight (BMI > 25), oral contraceptives or hormonal therapies assumption, oral or dental pathologies, inadequate knowledge of the Italian language, mood disturbances: anxiety and depressive symptoms, insomnia (screening tests cut-off scores: Beck Depression Inventory >20; State-Trait Anxiety Inventory-2 > 50; Pittsburgh Sleep Quality Index > 5). BDI = Beck Depression Inventory; STAI-Y2 = State-Trait Anxiety Inventory-2; PSQI = Pittsburgh Sleep Quality Index.

the Faculty of Psychology at the University of L'Aquila (Italy) without previous experience in meditation, homogeneously selected through a computerized randomization procedure and allocated to either the intervention group (IG) or the control group (CG). The IG (n = 20) was composed of 8 males and 12 females, mean age ± SD: 25.2 ± 4.07; the CG (n = 20) was composed by 6 males and 14 females, mean age ± SD: 24 ± 2.7.

Screening tests

The study's whole population included healthy young subjects without clinical diagnosis of mood or sleep disturbances. Screening tests included the Beck Depression Inventory[63], the State-Trait Anxiety Inventory-2[64] and the Pittsburgh Sleep Quality Index[65], which were performed prior to the beginning of the study in each group.

Table 1
Demographic characteristics of the population enrolled in the study. Data are summarized as mean ± standard deviation.

	Sample (N = 40)	
	IG (n = 20)	CG (n = 20)
Demographic characteristics	Mean ± SD	Mean ± SD
Age (years)	25.2 ± 4.07	24 ± 2.7
Gender		
male	8	6
female	12	14

IG = intervention group; CG = control group; SD = standard deviation.

Table 2
Screening tests for mood and sleep disorders performed in the population study. Data are summarized as mean ± standard deviation.

	Sample (N = 40)			
Screening tests	IG (n = 20)	CG (n = 20)		
	Mean ± SD	Mean ± SD	t(df = 38)	p^* value
BDI	6.45 ± 4.54	6.80 ± 4.36	0.248	0.805
STAI-Y2	36.6 ± 8.53	38.25 ± 7.29	0.657	0.514
PSQI	4.25 ± 1.52	4.1 ± 1.52	0.312	0.756

SD = standard deviation. BDI = Beck Depression Inventory; STAI-Y2 = State-Trait Anxiety Inventory; PSQI = Pittsburgh Sleep Quality Index; df = degree of freedom. * Comparison of IG vs CG; P-values were calculated with Student's t-test.

T-test results showed no statistical differences between the IG and the CG (see Table 2).

Salivary cortisol

The general mixed ANOVA model showed a main effect for the time ($F_{1,38}$ = 31.79, $p < 0.001$) and the effect of the condition ($F_{1,38}$ = 35.07; p = 0.030) but did not show a group effect ($F_{1,38}$ = 0.064 p = 0.0801). The interactions time × group and condition × group were significant, with $F_{1,38}$ = 7.611; $p < 0.001$ and $F_{1,38}$ = 5.803, p = 0.021, respectively. There was no statistical significance for the following interactions: time × condition ($F_{1,38}$ = 2.908; p = 0.059) and time × condition × group ($F_{1,38}$ = 0.171; p = 0.850).

Basal salivary cortisol

Basal salivary morning cortisol was detected after 30 min after waking in both groups.

Between-group analysis

At baseline time, before the PNEIMED course (pre-test), there was no significant difference between the IG and CG groups, whereas there was a significant hormone deflection in the IG at the end of the PNEIMED course (post-test) when compared to the CG (IG post-test 5.64 ± 4.2 vs CG post-test 9.44 ± 4.9; $F_{1,38}$ = 6.838; p = 0.013) (see Table 3).

Within-group analysis

RM-ANOVA analysis for the IG showed that time and condition effects were statistically significant, with Ftime = 5.438; p = 0.002 and Fcondition = 10.478; p = 0.004, respectively. The IG presented a significant reduction in morning cortisol at the end of the PNEIMED course (post-test) compared to the salivary concentration at baseline (pre-test) (IG pre-test 9.42 ± 6.0 vs IG post-test 5.64 ± 4.2; F1,38 8,354; p = 0.009). The RM-ANOVA for the control group showed only a main effect of time ($F_{1,38}$ = 40.348; $p < 0.001$), as reported in Fig. 3.

Table 3
Mean and standard deviations of salivary cortisol, pre/post PNEIMED course, at baseline and under SST acute stress elicitation between the intervention group (IG) and the control group (CG) – N = 40.

Basal salivary cortisol

		IG (n = 20)	CG (n = 20)	
Time	Condition	Mean ± SD	Mean ± SD	F; (p)*
Baseline time	Pre-test	9.42 ± 6.0	11.64 ± 12.1	1.593 (p = 0.215)
	Post-test	5.64 ± 4.2	9.44 ± 4.9	6.838 (p = 0.013)

Salivary cortisol under stress-eliciting task

		IG (n = 20)	CG (n = 20)	
Time	Condition	Mean ± SD	Mean ± SD	F; (p)*
5 min pre-stress task	Pre-test	4.57 ± 3.7	3.45 ± 1.8	1.492 (p = 0.229)
	Post-test	3.13 ± 2.2	4.62 ± 4.8	1.576 (p = 0.217)
10 min post-stress task	Pre-test	6.32 ± 5.5	3.11 ± 1.9	6.163 (p = **0.018**)
	Post-test	4.55 ± 3.8	4.34 ± 2.8	0.039 (p = 0.844)
30 min post-stress task	Pre-test	6.42 ± 3.9	3.37 ± 2.4	8.683 (p = **0.005**)
	Post-test	4.53 ± 2.3	3.46 ± 3.6	1.238 (p = 0.273)

Condition: pre-test = initial period of the study (before the PNEIMED course); post-test = final period of the study (after the PNEIMED course); *RM ANOVA: between-group analysis.

Salivary cortisol under stress-eliciting task

Salivary cortisol was measured immediately before (t1 = five minutes pre-stress task) and during the TSST stress-eliciting task (t2 = ten minutes post-stress task; t3 = thirty minutes post-stress task), both in the IG and the in CG, to evaluate the dynamic trend of stress hormone release in response to acute psychological stressors.

Between-groups analysis

As reported in Table 3, the analysis showed higher cortisol levels in the IG than the CG before the PNEIMED course, with significant differences between groups at time t2 and time t3.

After the PNEIMED course, the IG demonstrated decreased cortisol levels, meaning that the differences between groups were not significant.

Within-groups analysis

As reported in Fig. 2, in the IG, the cortisol levels post-test (after the PNEIMED course) were lower than at pre-test (before the PNEIMED course), showing a significant difference in cortisol salivary concentration between conditions at t3 (F = 5.326; p = 0.032), recognized as the recovery phase of the stress activation response. These results confirmed the capability of the PNEIMED method to lower stress hormone secretion both at baseline and under mental stimulation in naïve practitioners.

In the control group, the post-hoc analyses for pairwise comparisons between conditions (pre-test vs post-test) did not show significant differences (see Fig. 3).

Discussion

The meditation research field has grown considerably in the last two decades, and this randomized study adds further evidence for the multiple benefits of meditation on health and increases the exiguous number of RCTs that have investigated the ability of a brief session of meditation to reduce stress, measured through salivary cortisol assessment, in a group of healthy young subjects in their first meditation experience. The main results of the study demonstrate that a brief PNEIMED training, in young university students without any experience in meditation,

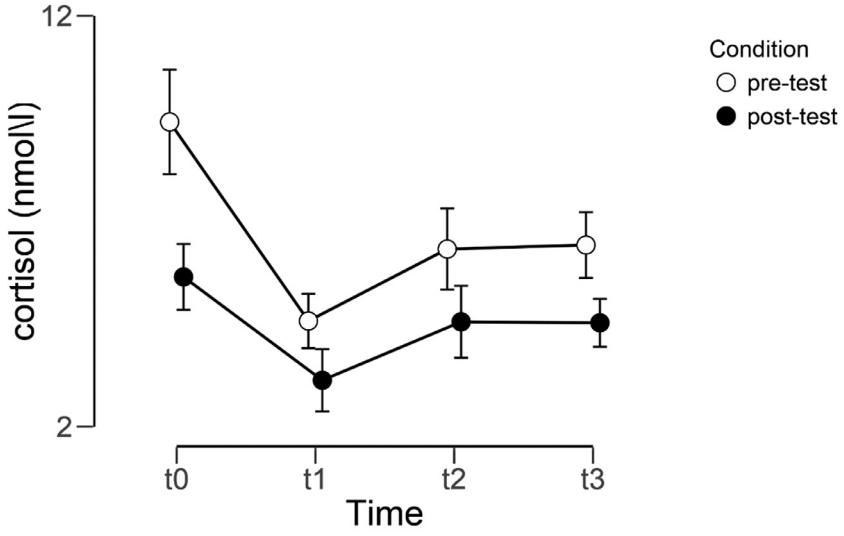

Fig. 2. Intervention group (IG): Salivary cortisol, pre/post PNEIMED course, at baseline and under SST acute stress elicitation over time (0, 1, 2, 3 time intervals).
RM-ANOVA: *F*time = 5.438; $p = 0.002$; *F*condition = 10.478; $p = 0.004$; *F*time × condition = 0.929; $p = 0.433$. Post hoc analyses: # Indicates significant difference between conditions; $p < 0.05$ with Bonferroni correction; * Indicates significant difference from baseline score; $p < 0.05$ with Bonferroni correction. Error bars displaying 95%CI.

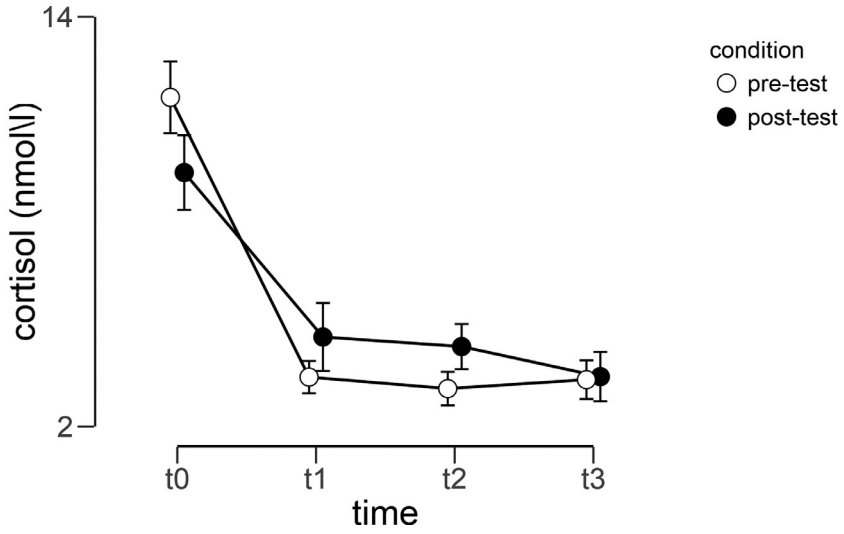

Fig. 3. Control group (CG): Salivary cortisol, pre/post PNEIMED course, at baseline and under SST acute stress elicitation over the time. (0, 1, 2, 3 time intervals).
RM-ANOVA: *F*time = 40.348; $p < 0.001$; *F*condition = 0.012; $p = 0.912$; *F*time × condition = 2.145; $p = 0.134$. Post hoc analyses: there were no significant differences. Error bars displaying 95%CI.

is able to induce a significant reduction of both basal diurnal and stress-elicited salivary cortisol after only four days of intensive practice. The ANOVA between-group analysis of basal diurnal salivary cortisol showed that there was a significant hormone deflection in the IG at the end of the PNEIMED course (post-test) when compared to the CG. RM-ANOVA within-group analysis for the IG also showed that time and condition effects were statistically significant. The IG group presented a significant reduction in basal morning cortisol at the end of the PNEIMED course (post-test) compared to the salivary concentration at baseline (pre-test). The RM-ANOVA for the control group showed only a main effect of time. Regarding cortisol measures under the SST stress-eliciting task, ANOVA within-groups analysis showed that in the IG group, the cortisol levels post-test (after the PNEIMED course) were lower than at pre-test (before the PNEIMED course), showing a significant difference of cortisol salivary concentration between conditions at t3, which is recognized as the recovery phase of stress response. In the control group, the post-hoc analyses for pairwise comparisons between conditions (pre-test vs post-test) did not show significant differences.

The present study enrolled a homogeneous sample comprising forty undergraduate psychology students without comorbidities or previous experience in meditation. Twenty were randomly assigned to the IG and took part in the PNEIMED training, which lasted 4 consecutive days (30 h in total); the other twenty subjects were randomly assigned to the CG and attended 30 h (4 consecutive days) of academic lessons. The PNEIMED course includes 15 h of face-to-face, informative scientific lessons on mind-body relationships according to the principles of PNEI and 15 h of meditative theory and practice. Both the IG and the CG were tested at the beginning (pre-test) and at the end (post-test) of the study through salivary cortisol assessment.

The effects of meditation on cortisol levels in healthy subjects are still under scientific investigation due to the limited number of published randomized controlled studies in the existing literature. In a systematic review published in 2016,[66] only 6 studies examining mindfulness intervention effects on cortisol were included, but significant changes were not observed in randomized controlled trial designs, and findings were overall inconclusive. In 2017, Pascoe et al. published two systematic

reviews and meta-analyses regarding the effects of mind-body interventions on stress-related physiological measures, including cortisol. In the first meta-analysis,[67] out of a total of 42 studies included, cortisol was measured in 12 studies, of which 8 studies used salivary samples. In the second meta-analysis,[68] of a total of 45 studies analyzed, cortisol was measured in 18 studies, of which only 9 studies used salivary samples. The existing literature on the effects of MBIs on salivary cortisol regarding healthy adult populations (>18 years) is very limited. In fact, there is one meta-analytical review published in 2016[70] that included only 5 RCTs (190 participants in total) with moderately low overall ES. Moreover, current literature about RCTs that tested the efficacy of MBIs to lower salivary cortisol in young subjects (i.e., students) also restricted the total number of RCTs. In the two meta-analyses conducted by Pascoe et al. cited above,[67,68] only 3 RCTs were included this target population.

All the studies included in the previously cited systematic reviews had an active control group, the most accepted comparison group type in this research field. In the present RCT study, the control group was considered as active, since the students attended academic lessons regarding psychological and behavioral processes in health and illness in a classroom close to the IG experimental setting, in the same time period and for the same number of hours (30 h in total). The topics of these academic activities were similar to PNEI lessons: the difference between the IG and the CG was the meditative practice (15 h, half of the total number of hours in the PNEIMED course) experienced in the IG. These results increase scientific evidence[36,47] for the capability of brief meditation trainings (<7 days) to positively regulate HPA stress response and confirm our findings presented in the published non-randomized, controlled, before-and-after PNEIMED study.[56] In the previous non-randomized PNEIMED study, both training group and control group underwent salivary cortisol assessment at the beginning (T0) and at the end (Tf) of the course. We reported a significant improvement of adrenocortical activity (decreased basal morning and under acute stress-eliciting task cortisol levels) in the PNEIMED experimental group at Tf. In contrast, no differences were found in the control group at the end of the study. Notably, this PNEIMED non-randomized study differed significantly from the present study in many aspects: study design (before-and-after controlled study vs randomized-controlled trial), mean age of participants (middle-aged health workers vs young university students), characteristics of control group (wait-list vs active group), and type of stress-eliciting task (the Raven Progressive Matrices vs Subtraction Stress Task), but in both studies, the PNEIMED method demonstrated its efficacy in modulating the neuroendocrine stress pattern in treated subjects. The reduction of cortisol after the PNEIMED training may thus ameliorate the situation-dependent adaptation to daily life stress among different populations in this study.

Strengths and limitations of the study

The quality of the present study is based mainly on five main aspects: (1) the randomized-controlled design; (2) the homogeneous characteristics of the sample; (3) the presence of an active control group; (4) the use of a simple and non-invasive stress marker; and (5) the standardized measurement of salivary cortisol. Although there are still few randomized-controlled trials assessing the effect of meditation intervention on salivary cortisol concentration in healthy adult subjects[69], the present study demonstrated that PNEIMED intervention reduced both morning basal and stress-elicited salivary cortisol in naïve participants. Some limitations of the present study must be noted. First, although the design study is a randomized controlled trial, the exiguous number of subjects enrolled limits the statistical power and does not allow definitive conclusions to be drawn. Second, basal morning cortisol secretion was assessed through a single salivary sample, although multiple measures could provide more complete information. In fact, basal cortisol secretion is affected by gender, age, sleep duration and time of awakening, but in healthy people, the cortisol awakening peak is generally reached around a half hour after waking.[71,72] In the present study, to avoid major biases in within-subjects comparisons, basal morning salivary cortisol was rigorously collected 30 min after awakening (t0 = baseline time), prior to the PNEIMED course (pre-test) and after the PNEIMED course (post-test, four days after) in the IG, as well as at the beginning (pre-test) and at the end of university activities (post-test, four days after) in the CG. Based on a recent systematic review[73] by Ryan et al., salivary diurnal cortisol might be collected two days both before and at least once after the intervention to optimize the reliability and validity of the cortisol awakening response (CAR) as a biomarker within RCTs. Notably, the present study was conducted in 2015, prior to the recommendations published one year later; according to the meta-analyses published by Pascoe et al. (cited above),[67,68] only 6 RCTs have investigated the effects of meditation on diurnal cortisol with pre-/post-intervention time assessments using multiple salivary specimens across multiple days in healthy subjects. Indeed, further RCTs should be conducted to supplement the extant literature, and we will apply this recommendation for the next PNEIMED RCT, scheduled for the end of 2019. Third, the habituation phenomenon to repeated mental tasks could somewhat explain the observed reduction of salivary cortisol following the acute stress task after the PNEIMED session. In fact, salivary cortisol samples were collected under the acute mental stress task (Subtraction Stress Task, SST) at t1 = five minutes pre-stress task, t2 = ten minutes post-stress task, t3 = 30 min post-stress task both in the IG and in the CG and at pre-/post-test for each group. However, such a contribution seems to be of less concern, since the SST mental task was able to increase cortisol levels in both groups even though it was reintroduced on the last day of the study period without modifications compared to the administration at the beginning of the study.[71] It is worth underlining that the PNEIMED intervention led to a drop in cortisol levels after repeated mental stress only in the experimental group, whereas in the control group, cortisol response to the mental task remained unchanged from the initial assessment to the end of the study. Moreover, the intervention and control groups shared the same setting (i.e., university lessons) and rhythms of diurnal activities, although various potential confounding factors (i.e., frequency and type of physical exercise, work load, lifestyles, environmental stress) cannot completely be ruled out. Finally, the study population of the present study markedly differs from our previously published non-randomized PNEIMED study in both demographic characteristics (young students vs middle-aged health practitioners) and in the control group (active vs passive); however, the positive results in the intervention group regarding stress reduction have been replicated.

Conclusions and perspectives

In conclusion, the present findings show that the PNEIMED training course was effective in regulating the neuroendocrine pathway of stress response both at baseline and under acute mental stimulation in a group of healthy young naïve practitioners. Although the low number of subjects enrolled does not allow definitive conclusions to be drawn and underlines the need for further randomized studies to confirm these results, present study makes a contribution to the existing literature about the topic by increasing the number of published RCTs which have assessed salivary cortisol levels in healthy young subjects following brief meditation training. The positive effects observed after a few hours of training in young naïve practitioners confirm that constant practice of PNEIMED meditation could represent a safe and reliable tool to reduce life stress and improve well-being in the general population.

CRediT authorship contribution statement

Anna Giulia Bottaccioli: Writing - original draft, Writing - review & editing. **Francesco Bottaccioli:** Writing - original draft. **Antonia Carosella:** Writing - original draft. **Vincenza Cofini:** Data curation, Formal analysis, Writing - original draft. **Paola Muzi:** Investigation, Methodology. **Mauro Bologna:** Investigation, Methodology, Writing - original draft.

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