The effect of AdrenoState® on salivary cortisol levels and perceived levels of stress in males

A research dissertation presented to the

Faculty of Health Sciences, University of Johannesburg,
in partial fulfilment of the Degree of Masters of Technology in Homoeopathy by

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DECLARATION

I, Kelly Edith Joffe, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Technology in Homoeopathy at the University of Johannesburg. It has not previously been submitted for any other degree or examination in any other Technikon or University.

..................
Kelly Edith Joffe

......Day of ...................... 2011
This serves to confirm that I, Kelly Edith Joffe, ID Number 8502150092085, Student number 802041894, enrolled for the Qualification M Tech Homoeopathy in the Faculty of Health Sciences herewith declare that my academic work is in line with the Plagiarism Policy of the University of Johannesburg which I am familiar with.

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Signature__________________________________ Print name_________________________
ABSTRACT

Stress is a normal physiological response to stressful stimuli. If, however, one is exposed to stress for long periods of time it can be harmful to one’s body. There is a lack of research on the treatment of stress both pharmaceutically and naturally. Many undesirable side-effects are associated with the current pharmaceutical treatment of stress. Adrenostate® is a nutritional supplement indicated for people who live a stressful lifestyle.

The aim of the study was to determine whether Adrenostate® would have an effect on salivary cortisol and perceived levels of stress in men.

Thirty eight male participants, who scored in the eligible range in the screening questionnaire and who fitted the criteria, completed the study. The study was a double blind placebo controlled study. The participants were placed into either group A or B, with equal distribution of age and levels of physical exercise. The study revealed that Group A was the placebo group and B the experimental group. The study was conducted over six weeks. Salivary cortisol, perceived stress levels, blood pressure and heart rate were measured and obtained at the first consultation (0 weeks), second consultation (3 weeks) and final consultation (6 weeks). The salivary cortisol was measured by means of an enzyme-linked immunoassay (ELISA).

Adrenostate® appeared to cause a statistically significant decrease in perceived stress levels, however, this decrease was also observed in the placebo group. There was no statistically significant change in salivary cortisol, heart rate or blood pressure, although a majority of the participants fell into the normal range of measurements.

Further research is required in this area and should be conducted with participants who already have high levels of cortisol and who live under more stringent conditions.
DEDICATION

I dedicate this dissertation to my dad, Louis Joffe and, in loving memory, to my mom, Carol Joffe, for their support both financially and emotionally throughout my studies and who taught me to never give up. I further dedicate this dissertation to Gil Sperling for his unconditional love, encouragement and belief in me.
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Abby and Lady - thank you for being in my life.

To my dad, thank you for everything!
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CHAPTER ONE
INTRODUCTION

1.1 Background
Although there are no related statistics in South Africa, the United Kingdom Health and Safety Executive (HSE) estimate that 60% of all recorded cases of absenteeism from the workplace are caused by stress-related illnesses (Clarke and Cooper, 2004). Repeated exposure to stressors results in disturbances to almost all of the systems in the body, for example hypertension, depression, headaches, fatigue and sleep disturbances (Bao et al., 2007). Workplace-related stress is suggested to increase the risk of cardiovascular complaints sixfold (Brotman, et al., 2007). Cortisol is the main stress hormone and is secreted in response to a stressful situation (Bao et al., 2007). Increased levels of cortisol over an extensive period of time can cause a suppressed immune response thereby escalating the susceptibility to infection, neoplasms as well as autoimmune diseases (Plotnikoff et al., 1999).

There is a lack of experimental data on the effect of different pharmaceutical products on the response by the body’s cortisol levels to acute psychological stress. In addition, a variety of the conventional pharmaceutics employed for this purpose cause undesirable side-effects such as drowsiness, headaches, sedation and tachycardia (Kudielka et al., 2007). AdrenoState® is a nutritional supplement containing a variety of eight vitamins and eight different herbs indicated for people who live a stressful lifestyle and who suffer from the symptoms of stress. No research to date has been done on this product (Pringle, 2008).

1.2 Aim of the study
The aim of this study was to determine if AdrenoState® had any influence on salivary cortisol levels as well as perceived stress levels in males who considered themselves as having high levels of stress. Heart rate and blood pressure were also measured to determine whether AdrenoState® would have any effect.

1.3 Potential outcomes
The expected outcome was an effect on the participants’ perceived stress levels. Another expectation was that AdrenoState® would have an effect on salivary cortisol levels, blood pressure and heart rate in men. A further possible outcome was that either the salivary cortisol levels or the perceived stress levels would remain unchanged. The finding of this research will provide a foundation for further investigation in the field of complementary medicine in the treatment of stress. It will also benefit people in the workplace by helping them to become more
aware of stress and its harmful effects. This knowledge will enable people to more effectively combat stress and consequently maintain a balanced and healthy lifestyle.
2.1 Stress
Stress can be defined as a physiological response that serves as a mechanism of mediation linking any given stressor to its target-organ affect (Everly, 2002). Stressors or stressful stimuli include:

1) environmental stimuli such as: noise, temperature, pollution and radiation. Excessive noise has shown to decrease short term memory and learning ability, whilst increasing blood pressure, adrenal medullary function and adrenal cortical function. Temperature also contributes to stress: temperatures that are too high or too low can decrease productivity in the work-place;

2) physiological stimuli such as: ageing, illness, pain, trauma, exercise, and hypoglycaemia. Physiological stress is a direct cause of the release of stress hormones and their subsequent harmful effects;

3) biological stimuli such as: viruses, bacteria or parasites that cause infections and leave the body in an immunologically compromised state;

4) psychological stimuli such as: financial problems, emotional worry, relationships, performance stress and unemployment. In this case, the stimulus is relative to the individual and the degree to which they will react is self initiated and self propagated.

Although the stress response of the body is meant to maintain homeostasis and help the body to adapt to emergencies, long-term activation of the stress system can have a harmful effect on the body by increasing the risk of obesity, heart disease, diabetes, amenorrhoea, infertility, depression, dementia, psychosis and a variety of other illnesses (Bao et al., 2007).

Although physiological pathways exist through which chronic stress could result in cardiovascular disease (such as increased blood pressure), behavioural changes (such as medical non-compliance, smoking, or a sedentary lifestyle) might also accompany chronic psychological stressors (Brotman, et al., 2007).

Selye, H., founder of the General adaptation syndrome (1998) differentiated three stages which the body goes through when responding to stress:

1) Alarm reaction, in which the body prepares itself for “fight or flight”;
2) Adaptation (provided the organism survives the first stage), is one in which a resistance to the stress is built. This process allows the body to continue to fight the stressor as long as there is sufficient “adaptation energy”;

3) If the duration of the stress is sufficiently long, the body enters a stage of exhaustion, a sort of aging, due to ‘wear and tear’. This usually occurs when the body’s resources become depleted (adaptation energy is exhausted) or when the resistance phase can no longer be maintained due to the consequences of the prolonged exposure to the hormones that are released (Figure 2.1a and b).

![Figure 2.1a The General Adaptation Syndrome depicting the initial alarm phase](image-url) (Martini, 2001).
According to Seyle, (1998) there is only a finite amount of adaptation energy that the body possesses and which declines with increasing or continuous exposure to stressors. One of the results of this is faulty adaptation and disease.

2.1.1 Symptoms of stress

During periods of stress, the sympathetic nervous system responsible for the “fight and flight” response stimulates the medulla of the adrenal gland. The adrenal gland then releases adrenalin and noradrenalin, which stimulate the $\beta_1$ receptors in the heart causing an increase in heart rate, cardiac muscle contraction and a raised blood pressure (Martini, 2001). Acute stressors, either physical or emotional, are well known triggers of cardiovascular events.

Figure 2.1b The General Adaptation Syndrome depicting the resistance phase and ending in the exhaustion phase (Martini, 2001).
Symptoms of stress may appear in various parts of the body and are generally divided into psychological and physical symptoms.

Psychological symptoms may include: (Johnson, 2003)

- Anxiety
- Depression
- Agitation
- Forgetfulness
- Mood swings
- Relationship problems (work or personal)
- Difficulty concentrating
- Feeling overwhelmed
- Feeling detached
- Excessive worrying
- Loneliness

Physical symptoms may include:

- Headaches
- Muscle tension, lower back and shoulder pain
- Clenching teeth
- Nausea
- Bowel disturbances
- Sleep disturbances or insomnia
- Fatigue
- High blood pressure
- Appetite disturbances
- Numbness and tingling
- Rash, hives or shingles
- Use of cigarettes, alcohol or other drugs to cope
2.1.2 Work Related Stress
A potential factor for the experience of stress would be the lack of adequate appreciation shown to a working individual for her or his efforts in the work-place. Overcommitted individuals tend to repeatedly exaggerate their efforts at work while at the same time overtaxing their resources. This diminishes their potential to sufficiently recover from the demands of their job which eventually results in exhaustion and poor health (Bellingrath, and Kudielka, 2008). Upcoming deadlines in the work-place are associated with a six fold increase in myocardial infarction, and it is also suggested that chronic work-related stress could carry a two to three times higher risk of cardiac events, especially when employees perceive little or no control over their work environment (Brotman et al., 2007).

2.2 Cortisol
Cortisol is a major stress hormone that acts on many organs and areas of the brain (Plotnikoff et al., 1999). Stress causes an immediate increase of Adrenocorticotropic hormone (ACTH) secretion and consequently an increase of cortisol as well (Guyton and Hall, 2006). Cortisol normally exerts a negative feedback effect to shut down the stress response after the threat has passed (Bao et al., 2007). Bergdahl et al., (2005) found that reduced stress may decrease the activation of the hypothalamic-pituitary-adrenal (HPA) axis resulting in a lowered secretion of glucocorticoids.

Cortisol levels rise dramatically during times of stress. This elevation protects key metabolic functions and puts a significant halt on potentially damaging inflammatory responses to infection and injury (Boon et al., 2006).

2.2.1 Cortisol and Endocrine relationships
2.2.1.1 Hypothalamus and pituitary gland
Cortisol is one of the intrinsic hormones and plays a vital role in the endocrine system (Martini, 2001). The core of the neuroendocrine system is represented by the hypothalamic-pituitary complex (Griffin and Ojeda, 2000). The pituitary gland is located in a small depression in the sphenoid bone, the sella turcica, just underneath the hypothalamus and is connected to it by the infundibulum. The pituitary gland is divided into an anterior pituitary or adenohypophysis and the posterior pituitary or the neurohypophysis. The hypothalamus controls the pituitary gland’s release of hormones by secret ing either releasing or inhibiting hormones. The rate that these hormones are released is controlled by a negative feedback system (Martini, 2001).
The hypothalamus secretes the following releasing hormones with their respective effects:

- Corticotropin-releasing hormone (CRH) causes a release of adrenocorticotrophic hormone (ACTH) from the pituitary gland which then causes the adrenal cortex to release cortisol;
- Growth hormone-releasing hormone (GHRH) leads to growth hormone (GH) secretion by the pituitary gland;
- Thyrotropin-releasing hormone (TRH) which causes secretion of thyroid stimulating hormone (TSH) from the pituitary gland and subsequently thyroid hormone from the thyroid gland; and
- Gonadotropin-releasing hormone (GnRH) which is responsible for the consequent secretion of follicle stimulating hormone (FSH) and luteinising hormone (LH).

The pituitary gland secretes the following seven hormones:

- Thyroid stimulating hormone (TSH);
- Adrenocorticotrophic hormone (ACTH);
- Gonadotropins- Follicle stimulating hormone (FSH) and luteinising hormone (LH);
- Prolactin (PRL);
- Growth hormone (GH) and
- Melanocyte-Stimulating hormone (MSH) (Martini, 2001).

2.2.1.2 Adrenal Glands

Cortisol is produced in the adrenal glands (Martini, 2001). The adrenal glands (Figure 2.2) are bilateral and located in the retroperitoneum superior to the kidneys and are encapsulated by a fibrous capsule. The adrenals have a very rich vascular supply. The adrenals are comprised of an outer cortex, which makes up more than three quarters of the adrenal mass, and an inner medulla region (Griffin and Ojeda, 2000). The adrenal medulla synthesises and secretes the catecholamines epinephrine and norepinephrine (Guyton and Hall, 2006). Histologically the cortex is comprised of three zones: an outer zona glomerulosa, a zona fasciculata and an inner zona reticularis (Guyton and Hall, 2006). The zona glomerulosa produces aldosterone, and lacks a defined structure, and the small lipid-poor cells are scattered beneath the adrenal capsule. The zona fasciculata is the thickest zone and produces cortisol and androgens. The inner zona reticularis also produces cortisol and androgens. The zona fasciculata and zona reticularis are regulated by ACTH (Guyton and Hall, 2006).
2.2.2 Actions of cortisol
Cortisol has widespread effects throughout the body (Figure 2.3), the most important of which are its anti-inflammatory actions, maintenance of the body’s blood sugar level, blood pressure, and muscle strength, and its assistance with controlling the body’s salt and water balance (Beers et al., 2003).
Cortisol has effects on carbohydrate, protein and fat metabolism as well as a function in the inflammatory response. Cortisol is best known for its ability to stimulate gluconeogenesis by firstly increasing the enzymes required to convert amino acids into glucose in the liver. Secondly, cortisol causes mobilisation of amino acids from the extrahepatic tissues. Therefore more amino acids become available in the plasma to become a part of the gluconeogenesis process in the liver. Cortisol also causes a decrease in the rate of glucose utilisation in the cells. The effect of cortisol on protein metabolism is to reduce protein stores in all of the tissues except the liver. This is caused by a decrease in protein synthesis and an increase in catabolism of protein in the cells. This decrease in protein in the cells ultimately results in an increase of protein in the liver and plasma by depressing transport of amino acid to extrahepatic cells and enhancing transport into the liver. Cortisol has shown to increase:

- The rate of deamination of amino acids by the liver;
- protein synthesis in the liver;
- the formation of plasma proteins by the liver; and
- the conversion of amino acids to glucose.

The effects on fat metabolism are similar to those of protein metabolism in the manner in which cortisol causes mobilisation of the fatty acids from adipose tissue. In addition, cortisol also causes increased oxidation of the fatty acids. This process however takes a long period of time and it is not as rapid and optimal as protein metabolism. It is, however, used as a long term conservation of glucose and glycogen (Guyton and Hall, 2006).

Cortisol acts as a powerful anti-inflammatory by causing stabilisation of the intracellular lysosomal membranes. This, in conjunction with the ability of cortisol to make glucose available for healing in the particular area, is why cortisol is administered so widely as an anti-inflammatory drug (Guyton and Hall, 2006).

Raised levels of cortisol over an extended period of time can cause a suppressed immune response thereby increasing the susceptibility to infection, neoplasms as well as to autoimmune diseases (Plotnikoff et al., 1999).

### 2.2.3 Cortisol levels

Normal cortisol levels range between 1.2-14.7ng/mL (Lac, 2001). Diseases associated with hypofunction (Addison’s disease) are relatively rare whereas those of hyperfunction (Cushing’s...
disease) are more common (Griffin and Ojeda, 2000). Addison’s disease results from failure of the adrenal cortices to produce adrenocortical hormones. This is usually caused by primary atrophy of the cortices which results from autoimmunity, tuberculosis destruction or invasion by cancer. Cushing’s disease is caused by either a cortisol-secreting tumour or general hyperplasia of the adrenal cortices (Guyton and Hall, 2006). The effects and clinical expressions of cortisol excess and deficiency are shown in table 2.1.

Table 2.1 Cortisol deficiency and excess (Griffin and Ojeda, 2000).

<table>
<thead>
<tr>
<th>EFFECT</th>
<th>CLINICAL EXPRESSION</th>
<th>CORTISOL DEFICIENCY</th>
<th>CORTISOL EXCESS</th>
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<tbody>
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<td>Carbohydrate metabolism</td>
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<td>decreased protein structure</td>
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<td>of bone, skin and muscle</td>
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<td>Protein metabolism</td>
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<tr>
<td>Decreased extrahepatic amino acid</td>
<td>hypoglycemia</td>
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<td>Distribution of fat</td>
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<td>Maintain capillary integrity</td>
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<td>Inflammatory and immune responses</td>
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<td>Stabilise lysosomes</td>
<td>propensity toward autoimmune disease</td>
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<td>decreased inflammatory response</td>
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<td>Suppress synthesis of antibodies</td>
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<td>increased ACTH secretion</td>
<td>decreased ACTH secretion</td>
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<tr>
<td></td>
<td>pigmentation</td>
<td>pigmentation</td>
<td></td>
</tr>
</tbody>
</table>

if excess is secondary to hypothalamic-pituitary drive, ACTH increased
2.2.4 Other influences on cortisol

2.2.4.1 Circadian rhythms

Cortisol circadian rhythms characteristically rise during the night, peak at the first hour after awakening, drop quickly during the morning hours, increase slightly after midday and then decline slowly to a low at night as seen in Figure 2.4 (Elverson and Wilson, 2005). It is therefore recommended that samples be taken in the first hour after awakening (Kudielka et al., 2005).

![Figure 2.4](image)

**Figure 2.4** Circadian rhythms of cortisol fluctuate throughout the day being highest the first hour after awakening and lowest after midnight (Weitzman, 1971).

2.2.4.2 Gender

Males and females react differently to stress. Adult men appear to show a greater and more acute hypothalamic pituitary axis (HPA) and autonomic response as compared to adult women (Wang et al., 2007). In women the cortisol rhythm is blunted in the luteal phase compared with the follicular phase of the menstrual cycle which would lead to possible fluctuations in cortisol in the various phases of the menstrual cycle (Baker and Driver, 2006). Furthermore according to Meulenberg et al., (2003), salivary cortisol levels are raised with use of the oral contraceptive pill.

2.2.4.3 Environmental and psychosocial influences

Environmental factors such as quality of sleep, diet and physical exercise as well as genetic factors and individual temperaments play a role in the extent of cortisol released (Kupper et al., 2005). Due to the major psychological component of stress, the awareness of a person to their stress levels and a conscious effort to improve one’s stress levels can have a positive effect on their stress levels (Eller et al., 2006).
2.3 Lifestyle factors and treatment of stress

2.3.1 Lifestyle factors

Cigarette smoking is said to provide short term relief from stress but the long term effects outweigh the short term benefits. Initial cessation of smoking can increase stress short term but long term cessation helps to alleviate stress (Hiramatsu et al., 2006).

Sleep and stress are very closely linked where research confirms that insomnia is often caused by stress and conversely that insomnia leads to stress and fatigue. Chronic stress, burnout and insomnia are commonly and closely related. For example, an individual who suffers from insomnia, non-refreshing sleep, and who wakes up feeling exhausted and who is also exposed to work and life stresses may experience a reduction in their resources necessary for coping with stress. These problems may also exacerbate symptoms of mental and physical fatigue among those who are already burned out, and lead to sustained burnout or the development of new cases of burnout. The same line of interpretation can be applied to the exacerbation of insomnia symptoms or the onset of new insomnia cases among burned-out individuals. Likewise, activation of both the HPA axis and the sympathetic nervous system was also observed in cases of insomnia. In most studies, insomnia and poor sleep patterns were consistently found to be associated with elevated evening cortisol levels, as observed in some burnout studies (Armon et al., 2008). Sleep deficiency, either in quantity or quality, results in irritability, anxiety, depression, disturbed thinking and physical disorders (Schafer, 1998).

Exercise is a powerful anti-stress activity (Nguyen-Michel et al., 2006) and has shown in many studies to reduce anxiety, feelings of helplessness, depression and hostility (Balch et al., 2008). Regular exercise increases contractility of the heart, lowers blood pressure and reduces levels of low density lipoproteins and thereby reduces the risk of myocardial infarction and strokes (Beers et al., 2003). Many studies prove that frequent exercise helps to control stress and adds to a positive quality of life (Schafer, 1998). Exercise relieves stress in the following ways: (Schafer, 1998)

- Release of pent-up emotions;
- Enhanced self-esteem and self-acceptance;
- Heightened internal control;
- Feelings of well-being and calmness;
- Mood stabilisation;
- Release of muscle tension;
• Burning off of stress-induced adrenaline, which leaves the bloodstream and is consumed by muscles;
• Post-exercise reduction in adrenaline production;
• Post-exercise relaxing of the sympathetic nervous system;
• Production of beta-endorphins which creates a feeling of well-being;
• Faster recovery from acute stress; and
• The body also becomes familiar with and habituated to physiological arousal.

Stress can lead to nutritional deficiencies. The stress hormones (adrenalin and cortisol) cause an increase in the metabolism of proteins, fats and carbohydrates. This response causes the body to use up more amino acids, potassium, and phosphorus and causes a depletion of the body’s store of magnesium and calcium. As a result of a combination of physical responses the body does not absorb nutrients optimally when under stress. Many of the disorders that arise from stress are the result of nutritional deficiencies. A balanced diet, therefore, and the combination of nutrients found in fresh fruit and vegetables are very important for people with a stressful lifestyle (Balch, 2006). The risk of developing metabolic syndrome is increased in patients with persistent work-related stress (Brotman et al., 2007).

2.3.2 Conventional Treatment
Primary conventional treatment involves counselling as well as symptomatic treatment of stress such as depression, anxiety, insomnia, muscle pain and headaches, with the use of a variety of drugs (Boon et al., 2006). The conventional drugs that are used are tabulated with their therapeutic uses and adverse effects in table 2.2.

Drugs that are used for muscle pain and headaches include: antipyretic analgesics and non-steroidal anti-inflammatory drugs. Side effects associated with these drugs may include: gastric irritation, antithrombotic action, renal disturbances, and allergic reactions (Dreyer, 2004).
Table 2.2 Conventional drugs used for depression, anxiety and insomnia (Dreyer, 2004).

<table>
<thead>
<tr>
<th>GROUP OF DRUG</th>
<th>DRUG</th>
<th>THERAPEUTIC USES</th>
<th>ADVERSE EFFECTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anxiolytics and sedative hypnotics</td>
<td>Benzodiazepines</td>
<td>Anxiety, Insomnia, Skeletal muscle relaxation, Pre-anaesthetic medication, Delirium tremens</td>
<td>Drowsiness, over-sedation, Disorientation, confusion, ataxia, blurred speech, Paradoxical hyper-excitability and aggression, Residual sedation, “hangover”, Dependency and withdrawal</td>
</tr>
<tr>
<td>Antidepressants</td>
<td>Tricyclic antidepressants</td>
<td>Major depression, Depression with anxiety, insomnia, panic disorders, phobias, obsessive compulsive disorder (OCD)</td>
<td>Cardiovascular effects: changes in blood pressure, tachycardia, arrhythmias and syncope, Sedation, Anticholinergic effects, Excessive perspiration, Muscular tremors, Restlessness, Weakness, Disorientation, Sexual dysfunction, Mania</td>
</tr>
<tr>
<td></td>
<td>Tetracyclic antidepressants</td>
<td>Depression</td>
<td>Bone marrow depression, Blood sugar concentration and weight gain, Drowsiness, Dry mouth</td>
</tr>
<tr>
<td></td>
<td>Bicyclic antidepressants</td>
<td>OCD</td>
<td>Sedation, Excessive perspiration, Weight loss due to appetite suppression</td>
</tr>
<tr>
<td></td>
<td>Atypical antidepressants</td>
<td>OCD, Mood stabiliser, Mania, Depression</td>
<td>Sedation, Headaches, Dizziness, Restlessness, Weight loss, Toxic overdose leads to coma, convulsions and death</td>
</tr>
<tr>
<td>Psychostimulants</td>
<td>Caffeine, Cocaine, Ritalin, D-norpseudoephedrine Thinz</td>
<td>Feeling of Psychological wellbeing, Increased energy and alertness, Appetite suppression</td>
<td>After effects are even more depressants than before taking them, Dependency, Tachycardia, arrhythmias, tremors, Rise in blood pressure, Nervousness, restlessness and irritability</td>
</tr>
</tbody>
</table>
2.4. Homoeopathy in South Africa

Homoeopaths are registered with the Allied Health Professions Council and their practise is controlled and regulated as diagnostic professionals with an appropriate therapeutic standard of practise.

In accordance with the provisions of the Medicines and Related Substance Control Act (Act No. 101 of 1965), a practitioner registered as a homoeopath may prescribe and dispense any homoeopathic substance as well as any unscheduled substance including vitamins, minerals and herbs (Allied Health Professions Act, 1982).

Homoeopathy was founded in the 18th century by Samuel Hahnemann (1755-1843). Homoeopathy is a system of medicine based on the principle that “like cures like”. That means that a substance that is capable of producing certain symptoms when taken by a healthy person is capable of curing any illness that displays similar effects (De Schepper, 2006).

Samuel Hahnemann acknowledged (§125) that various influences including diet, drink and herbal substances may play a stimulating or even medicinal role (Hahnemann, 1843).

2.4.1 FoodState AdrenoState®

2.4.1.1 FoodState®

FoodState® nutrients was established as a company in South Africa in 1999. It sources ingredients from various international sources and all of its products are manufactured and packaged in South Africa. All of the products are manufactured by pharmaceutically licensed manufacturers and hence they comply with the appropriate Good Manufacturing Procedures (GMP) (Pringle, 2008).

2.4.1.2 AdrenoState®

AdrenoState® is a supplement indicated for use in individuals who have adrenal exhaustion and who suffer from the typical symptoms of a stressful lifestyle. It is recommended that 2 tablets be taken every morning as a standard treatment regimen (Foodstate, n.d.). Each tablet of AdrenoState® is comprised of the following ingredients (APPENDIX A):

- Vitamin B₁ (1.25mg)
- Vitamin B₂ (1.5mg)
- Vitamin B₆ (1.5mg)
- Vitamin B₁₂ (75µg)
- Vitamin C (12.5mg)
- Folic acid (50µg)
- Niacinamide (5mg)
- Pantothenic acid (5mg)
- Licorice root (125mg)
- Siberian ginseng (100mg)
- Tyrosine (100mg)
- Avena sativa (50mg)
- Rhodiola rosea (50mg)
- Cordyceps extract (25mg)
- Gotu kola (25mg)
- Reishi mushroom (25mg)

2.5 Stress and cortisol in the literature

Izawa et al., 2007 investigated episodic stress associated with writing a graduation thesis and free cortisol secretion after awakening. This study proved that salivary cortisol levels were higher the day before submission of the thesis as compared to a month before submission and that, therefore, salivary cortisol levels increased as stress increased. This was determined by the DRG® Salivary Cortisol ELISA (SLV-2930) kit. This study did not use a control but rather investigated the intra-individual changes in post-awakening cortisol levels. This study also compared different types of stressors and their effects on cortisol, specifically comparing physical stress such as military training (Gaab et al., 2006) to psychological stress as in the case of writing a graduation thesis. The study found that psychological stress had a far greater effect on cortisol than physical stress. Rozen et al., 2007, examined whether salivary cortisol was a good measure of stress in football players. The results were surprising in that only 7.2 % of the samples had detectable cortisol levels. The reason could be due to an inadequate volume of saliva, incorrect storage and procedure of the assay as well as poor participant compliance in sampling.

Pelser et al., 2002, investigated the effect of Gelsemium sempervirens 200 CH on levels of urinary cortisol and perceived levels of anxiety. In this study the perceived anxiety increased in the control group more than the experimental group but was, however, not statistically significant. The levels of urinary cortisol showed a statistically significant decrease in the experimental group. The researcher’s recommendation for future studies is to take into account
the participants’ socioeconomic backgrounds and to use a much larger sample size. Thomson et al., 2002, investigated the effect of a homoeopathic complex on perceived levels of anxiety and cortisol levels in students. In this study the complex did not significantly alter perceived levels of anxiety but the control group had a more drastic increase in cortisol than the experimental group. In this study it was also stated that due to the uncomfortable process of collection, urinary cortisol measures lead to poor patient compliance. Snyman et al., 2005, investigated the effect of Argentum nitricum 200 CH on pulse rate, blood pressure and perceived levels of anxiety in students undergoing a test. This study proved that the remedy did not reduce test-related stress in a general population of tertiary students. The remedy did, however, have a distinct effect on pulse rate in the experimental group which remained more constant and increased to a lesser extent than in the control group.

In the United Kingdom a randomised double-blind trial was done to test the effect of tea on psychophysiological stress responsivity and post-stress recovery. It was found that the treatment group had lower post-stress cortisol, greater subjective relaxation and reduced platelet activation as compared to the placebo (Steptoe et al., 2006). In Japan, a study was carried out to determine the effect of Lavendula officinalis aroma on salivary endocrinological stress markers. In the aroma group, levels of chromagranin A significantly dropped after the induced stress. There were, however, no statistically significant changes in levels of cortisol in either of the groups (Toda and Morimoto, 2008).

This study is aimed at determining whether AdrenoState® will have an effect on heart rate, blood pressure, perceived levels of stress and salivary cortisol in men.
CHAPTER THREE
METHODOLOGY

3.1 Study Design
This was a randomised, double blind placebo-controlled study conducted over six weeks. Forty participants were recruited and matched, according to levels of exercise as well as their age, into either group A or B. The groups were randomly assigned as either the control (group A) or experimental (group B) group. The experimental group received AdrenoState® and the control group received the placebo. The study was conducted to determine the effect of AdrenoState® on salivary cortisol levels and perceived levels of stress in males.

3.2 Recruitment of participants
Thirty eight male participants were recruited by means of advertising pamphlets (APPENDIX B) at gyms, homoeopathic practises and in and around the University of Johannesburg as well as through word of mouth. Eighteen participants formed part of the experimental group and the other twenty formed part of the control group.

3.2.1 Inclusion criteria
In order to be included in the study, the participant was required to conform to the following criteria. The participant needed to:
• be male;
• be between the ages of 18 and 40 years old;
• have a freezer to store the saliva samples; and
• have obtained a score above 4 in the Stress Status questionnaire (APPENDIX C).

3.2.2 Exclusion criteria
Volunteers were not included if they were on any medication as well as if they were previously diagnosed with any known:
• cardiovascular diseases including hypertension;
• psychological diseases;
• autoimmune diseases;
• liver disorders and
• kidney disorders.
3.3 Research procedure
The study ran over a six-week period where the participants met with the researcher on three occasions.

At the first consultation the participant gave verbal consent and was assessed for suitability for participation in the study, utilising the Stress Status Screening Questionnaire (APPENDIX C). The participant then read and signed the Participant Information and Consent Form (APPENDIX D). The participant subsequently filled in the International Physical Activity Questionnaire (IPAQ) (APPENDIX E), in order to be matched into either group. The participant then filled in the Perceived Stress Questionnaire (PSQ) (APPENDIX F). Approval for the use of the PSQ was provided by the author of the PSQ (APPENDIX G). The researcher took the participant’s blood pressure and heart rate. The participant was provided with a collection tube in order to obtain a saliva sample, first thing in the morning, on three separate occasions during the study. The participant was given clear instructions on how to obtain the sample as well as how to store the sample (APPENDIX H). The participant also received either the AdrenoState® or the placebo (that looked and tasted identical to the active medication) and was required to take it for the full duration of the study, as explained in APPENDIX H.

Two follow-up consultations took place at the end of week three and week six respectively. At these meetings the participant returned their saliva samples. The PSQ (APPENDIX F) was completed by the participant. The researcher took their blood pressure and heart rate and gave the participant a tube in order to obtain their second and third saliva samples. A suitable time was arranged for the collection of the final saliva sample.

3.4 Heart rate and blood pressure
Acute myocardial infarction is more commonly caused by emotional stress than physical exertion. Patients reporting the highest level of stress have the greatest increase in blood pressure. There is a consistent relationship between chronic emotional or psychosocial stress and coronary atherosclerosis (and atherosclerotic risk factors), and these relations persist even after modification in lifestyle (Brotman et al., 2007).

3.4.1 Measurement of blood pressure and heart rate
Three sets of blood pressure and heart rate were taken, the first on day one, the second in week three and the third in week six. Blood pressure was taken with a manual sphygmomanometer on the right arm. Heart rate was taken via the pulsation of the radial artery on the right arm for the duration of one minute. These measurements were a component of the objective part of the study.
3.5 Perceived stress questionnaire (PSQ)

Fliege et al. (2004) recommended the revised version of the Perceived Stress Questionnaire (APPENDIX B) as an economic, reliable, structurally stable and valid instrument that assesses perceived stress in healthy adults. The PSQ emphasizes cognitive perceptions more than emotional states or specific life events and has been shown to be superior to alternative means of predicting healthy outcomes (Bergdahl et al., 2005). It measures three dimensions of a stress reaction (worries, tension and joy) and one stressor dimension (demands). The PSQ is a 20-item questionnaire with scoring from 1-4: 1 being almost never, 2 being sometimes, 3 being often and 4 being usually. Positive leading questions are scored negatively i.e. 5 minus the score given. All other questions are added according to the score that is given. Previous studies have documented that the revised PSQ takes on average, 4.9 minutes to complete and is preferable to the original 30-item questionnaire (Levenstein et al., 1993; Fliege et al., 2004).

3.6 Assessment of salivary cortisol

3.6.1 Salivary cortisol

Cortisol can be measured in saliva, serum and urine (Levine et al., 2006). Jerjes et al., (2005) found that both urinary cortisol metabolite and salivary cortisol assay showed to be effective in accurately measuring changes in the hypothalamic-pituitary-adrenal axis, during urinary collection between 09h00 and 18h00 and salivary sampling at 09h00.

According to Levine et al., (2006) measuring plasma cortisol and salivary cortisol both have advantages and disadvantages but for the purpose of a psychobiological study, salivary cortisol is a reliable reflection and an excellent substitute for plasma cortisol. The normal cortisol reference range for adults is 1.2 – 14.7 ng/mL (Lac, 2001). Salivary cortisol samples may be stored at freezing temperatures over extended periods of time in order to maintain their viability for purposes of analysis (DRG International, 2007).

The DRG® Salivary Cortisol ELISA (SLV-2930) kit (APPENDIX I) available from Biocom Biotech, is an enzyme immunoassay for the quantitative \textit{in vitro} diagnostic measurement of active free cortisol in saliva. Reliable and reproducible results are achieved when the assay procedure is performed with a comprehensive understanding of the package insert instruction and with adherence to good laboratory practice (DRG International, 2007).
3.6.2 Saliva Collection
Participants were required to take a sample first thing in the morning after awakening. They were required to either spit directly into the tube or salivate down a clean plastic drinking straw that was provided with the tubes. The tube then needed to be sealed and labelled with the time that the sample was taken. All three samples were required to be taken at the same time. The sample then needed to be placed in the participant’s home freezer until such time as they were able to deliver it to the researcher.

3.6.3 Assessment of salivary cortisol
The DRG Salivary Cortisol ELISA KIT (slv-2930) is based on the competition principle and the microplate separation. An unknown amount of Cortisol present in the sample and a fixed amount of Cortisol conjugated with horse-radish peroxidase compete for the binding sites of mouse monoclonal Cortisol-antiserum coated onto the wells. After one hour of incubation the microplate is washed to stop the competition reaction. After addition of the substrate solution the concentration of Cortisol is inversely proportional to the optical density measured (DRG International, 2007).

A minimum of 2 ml of saliva was collected in a centrifuge tube with a lid and stored in a -20°C freezer until analysis. The samples were then thawed and centrifuged at 2,200 x g for 5 minutes. The liquid was pipetted into new centrifugal tubes and labelled accordingly. 100µl of each sample was then pipetted into the wells of the plates as per the instructions in the kit (APPENDIX I). The absorbance of each well was determined by the spectrophotometer plate reader at the Laser Research Centre at the University of Johannesburg. All of the samples were run in duplicate to ensure increased reliability of the testing.

3.7 Ethics
Participation in this study was completely voluntary. All participants were well informed of the nature of the study, its intentions and expectations. All information gathered was kept confidential and anonymous. Exclusion criteria were established in order to prevent the admission of any participant to the trial where an established contra-indication exists. There were no anticipated risks by participating in this study and the dosage regimen falls within prescribed safety levels. All Participants had the right to ask questions and the freedom to withdraw at any stage. The results of the study were made available to the participants on their request.
3.8 Data capture and statistical analysis

An analysis of the basic frequencies and descriptives was done by preliminary assumption testing. When the statistics were run it was observed that the data was not as expected and did not form a normal pattern and, therefore, non parametric tests had to be conducted. Within the groups the data was compared using the Friedman test and Wilcoxin’s test. Between the groups the Mann-Whitney test was used. The data analysis and testing was performed by STATKON UJ.
CHAPTER FOUR
RESULTS

4.1 Introduction
A total of fifty two participants were recruited. Five of these were unsuitable for the study as they were on conflicting medication or did not score high enough in the screening questionnaire. Two of the recruited participants’ saliva was contaminated with blood and they were therefore excluded. Of the remaining forty seven, only thirty eight participants completed the study due to dropouts and non compliance. The thirty eight that completed were compliant to the end of the study. All of the participants were male. Group A (control) had a total of twenty participants and Group B (experimental) had a total of eighteen participants. The groups had an equal spread of age and level of exercise according to the IPAQ.

The measurements of blood pressure, heart rate and salivary cortisol at base line, at three weeks and at six weeks as well as the perceived stress questionnaire scores on those days are presented in APPENDIX K.

4.2 Demographics
4.2.1 Age distribution
Ages ranged from 22 to 40 years old. Group A had a minimum age of 23 and maximum age of 40 with a mean age for the group of 28.15 years old. Group B had a minimum age of 22 and a maximum age of 39 with a mean age for the group of 28.39 years old as shown in Figure 4.1 and 4.2.

![Group A Age Distribution](image)

Figure 4.1 Group A participant age distribution showing the majority age between 23 and 28 years old.
4.2.2 IPAQ score distribution

The participants were screened to determine their level of exercise. The scores depicted either low, moderate or high levels of exercise. Group A contained four participants with a low level of exercise, eleven participants with a moderate level of exercise and five participants with a high level of exercise. Five participants in Group B had a low level of exercise, nine participants had a moderate level of exercise and four participants had a high level of exercise as shown in Table 4.1. Both groups therefore had near equal amounts of participants with low, moderate and high levels of exercise.

<table>
<thead>
<tr>
<th>IPAQ Score</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Moderate</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>High</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

4.3 Perceived Stress Questionnaire (PSQ)

Throughout the study the lowest determined PSQ score was 27 and the highest was 72. At baseline the lowest scores were 36 and 37 for Groups A and B respectively. These reduced to 31 and 32 at the end of the third week and further decreased to 27 and 30 by the end of week six. The highest scores at baseline were 72 and 71 for Groups A and B, 64 and 68 at week three and 59 and 63 at week six.

P-values were formulated to determine whether changes were statistically significant. The significance level was 0.05. If $p < 0.05$, a significant difference is indicated and if $p > 0.05$ a non
significant difference is indicated. As seen in Figure 4.3 the average PSQ scores at week three decreased by 2.7 (5.16%) in Group A and 6.17 (11.55%) in Group B. The difference between the three week and six week period was a decrease of 2.55 (5.14%) in Group A and an increase of 1 (2.11%) in Group B. At the end of the six week period, the PSQ scores decreased significantly by 5.25 or 10.03% (p=0.026) in Group A and by 5.17 or 9.68% (p=0.008) in Group B when compared to those scores taken from the baseline reading. However, the comparison of score change between the two groups was not significant at the end of six weeks (p=0.988).

![Average PSQ score between groups](image)

**Figure 4.3** PSQ values over time compared between groups showing a significant decrease in both Group A (p=0.026) and Group B (p=0.008).

**4.4 Heart Rate**

The lowest heart rate recorded was 52 beats per minute (bpm) and the highest was 100bpm. As illustrated in Figure 4.4 the heart rate decreased by 2.1bmp (2.9%) in Group A and increased by 4.51bmp (2.14%) in Group B after the first three weeks. The change between the first three weeks and last three weeks showed an increase of 2.1bmp (3.02%) in Group A and a decrease of 1.67bmp (2.31%) in Group B. Overall, the heart rate was not altered over the six weeks with a 0bmp or 0% change (p=0.124) in Group A and a decrease of 0.16bmp or 0.22% (p=0.282) in Group B, nor was there a difference between the groups (p=0.926).
Figure 4.4 Average heart rate between groups over time showing an initial decrease and then increase in Group A ($p=0.124$) and an initial increase followed by a decrease in Group B ($p=0.282$).

4.5 Blood Pressure

4.5.1 Systolic Readings

The lowest systolic blood pressure recorded was 90 and the highest was 130. As seen in Figure 4.5 the systolic blood pressure decreased in both groups. At three weeks, the systolic blood pressure decreased by 3.5mmHg (3%) in Group A (control) and 1.22mmHg (1.1%) in Group B (experimental). The difference between three weeks and six weeks was a decrease of 9.5mmHg (7.9%) in Group A and 0.56mmHg (0.52%) in Group B. At the end of the six week study there was found to have been a significant decrease in systolic blood pressure of 13mmHg or 11% ($p=0.003$) in Group A, while in Group B the decrease was only 1.78mmHg (1.63%) ($p=0.311$) which was not significant.
Figure 4.5 Average systolic blood pressure comparing the groups showing a decrease in Group A (p=0.003) and no significant change in Group B (p=0.311).

4.5.2 Diastolic Readings

The lowest diastolic blood pressure recorded was 40 and the highest was 85. One participant in group B had a below normal diastolic blood pressure of 50 which remained the same throughout the study. Another participant in group A had an initial diastolic blood pressure of 60, at week three it decreased to 40 and then increased to 60 again at week six. Figure 4.6 illustrates the initial increase and subsequent decrease for Group A over the study period and the consistent decrease of Group B. At three weeks Group A had decreased by an average of 2.5mmHg (3.28%) and Group B by 1.11mmHg (1.56%). The difference between diastolic readings measured at three weeks and six weeks was an increase of 1.25mmHg (1.69%) in Group A and a decrease of 1.39mmHg (1.99%) in Group B. When the six week average results were compared to those taken at the baseline measurement there was found to be no significant decrease in diastolic blood pressure 1.25mmHg or 1.64% (p=0.209) in Group A nor in Group B, which decreased by 2.5mmHg or 3.53% (p=0.150).
Figure 4.6 Average diastolic blood pressure compared between the groups showing an initial increase and then decrease in Group A (p=0.209) and a decrease in Group B (p=0.150).

4.6 Cortisol

The lowest cortisol level recorded was 6ng/ml and the highest was 53ng/ml. At baseline 31.6% of the participants were in the normal range of cortisol of 1.2-14.7ng/mL. At three weeks 36.8% of participants were in normal range and at six weeks 39.5% of participants were within normal range. As seen in Figure 4.7, at three weeks there was a decrease in average cortisol levels of 1.37ng/ml (7%) from 19.57ng/ml to 18.2ng/ml in Group A (control) and a decrease of 1.72ng/ml (8.36%) in Group B (experimental) from 20.55ng/ml to 18.83ng/ml. The difference between three weeks and six weeks was an increase of 2.02ng/ml (11.09%) from 18.2ng/ml to 20.22ng/ml in Group A and an increase of 0.52ng/ml (2.76%) from 18.83ng/ml to 19.35ng/ml in Group B. Over the six weeks there was an insignificant total increase of 0.65ng/ml or 3.32% in Group A (p=0.816). The cortisol in Group B decreased by 1.2ng/ml or 5.84% (p=0.513), which was also insignificant.

If the average cortisol values are analysed for each group, it is evident that Group A started off with lower levels of cortisol in general. 45% of Group A increased, 45% decreased and 1% remained the same over time. In Group B 34% increased, 56% decreased and 1% remained the same over time.
Figure 4.7 Average salivary cortisol levels compared between the groups showing an initial decrease in both groups followed by an increase in both groups with an overall non significant change in Group A (p=0.816) and Group B (p=0.513).
CHAPTER FIVE
DISCUSSION OF RESULTS

5.1 Introduction
Four assessment parameters were used in this study. The first parameter was the Perceived Stress Questionnaire (PSQ), the second was heart rate, the third blood pressure split into systolic blood pressure and diastolic blood pressure and the fourth measurement was salivary cortisol. The results of the study are discussed in this chapter with reference to previously mentioned tables and figures where relevant.

5.2 Demographics
The age of the subjects ranged from 22 to 40 years old. As seen in Figures 4.1 and 4.2 the majority of participants in both groups were between the ages of 23 and 28. The remainder of the participants were equally spread out amongst the other age groupings. Although cortisol declines relatively with age (Ferrari and Magri, 2008), this had no significant impact on the study, due to the even distribution of age between the groups. Other factors, however, affecting cortisol levels, such as sleep patterns, physical, mental and emotional stressors, socioeconomic status and individual perception of stress (Kupper et al., 2005), were not taken into account in this study. These factors could explain the vast range distribution of the salivary cortisol levels.

Taking into account the level of physical activity of each participant and grouping them accordingly eliminated the positive effect that physical activity has on stress levels (Schafer, 1998).

5.3 Perceived Stress Questionnaire
Both Groups A (control) and B (experimental) showed significant improvement in the perceived stress scores after the six week period (10.03% (p=0.026) in Group A and 9.68% (p=0.008) in Group B) as seen in Figure 4.3. The results showed an initial decrease after three weeks and a further decrease after six weeks. The change, however, between the groups was not significant. These results could be owing to various causes.

Firstly, the awareness of the participant of their own stress levels and their conscious effort to improve these stress levels can lead to an improvement in itself whether in the treatment or the placebo group (Eller et al., 2006).

Secondly, the mere interaction with the researcher can lend to the idea of psychosocial support supplied by the doctor-patient relationship (Bergdahl and Bergdahl, 2002). The initial
consultation involved an extensive explanation of the study as well as stress in general. The follow-ups became a further platform for more discussion and some participants used these sessions for their benefit. The researcher’s positive expectations of the study may have also lead to the participant’s desire for a positive outcome, however, they did know whether they were on the active treatment or the placebo.

5.4 Heart Rate
The average heart rate among participants of each group did not change significantly over six weeks, 0% (p=0.124) in Group A and 0.22% (p=0.282) in Group B. When readings were compared between the groups at the end of the study, there was also no significant change (p=0.926) (Figure 4.4). This finding was expected as the average recorded heart rate for both groups is regarded as being within the normal range of between 60 bpm to 100 bpm (Martini, 2001). No adverse effects with respect to heart rate were found in six weeks.

5.5 Blood Pressure
5.5.1 Systolic Blood Pressure
The results showed that there was a significant decrease in systolic blood pressure in Group A (control) however there was no significant change in Group B (experimental). The average systolic blood pressure of Group B stayed relatively constant as compared to Group A (Figure 4.5). With reference to Group B Glycyrrhiza glabra, an ingredient present in Adrenostate® has an ACTH-like action on the adrenal cortex, increasing the production of glucocorticoids and mineralocorticoids (Mills and Bone, 2005). Glycyrrhiza glabra can cause an increase in blood pressure (Al-Qarawi et al., 2002). Both groups may have benefited from the psychosocial support but because Group B had Glycyrrhiza glabra, there was no visible change in their systolic blood pressure. No adverse effects were thus noted on the systolic pressure as a result of the treatment.

5.5.2 Diastolic Blood Pressure
No significant change in either of the groups was noted for average diastolic blood pressure readings. This can be an indication that renders Adrenostate® safe for use up to six weeks.

5.6 Salivary Cortisol
There was an initial decrease in both groups after the first three weeks. Between week three and week six, however, Group A (control) increased by 2.02 (11.09%) whereas Group B (experimental) increased by only 0.52 (2.76%). The initial decrease in both groups can be explained by the psychosocial support as well as the awareness of their stress and conscience effort to improve it. After the first three weeks, however, the placebo group increased far more
than the experimental group. This could show that in fact Adrenostate® did have an effect on salivary cortisol levels, which may warrant further research.

The overall finding for average cortisol levels in both groups was not statistically significant. If, however, the average cortisol values are analysed for each group, Group B did in fact have a higher percentage of decreased cortisol levels (56%) over the six week period (45% for Group A) but this was not statistically significant.

Environmental factors such as sleep quality, diet and physical exercise as well as genetic factors and individual temperaments play a role in the extent of cortisol released and it is, therefore, difficult to measure cortisol in isolation of these other important factors (Kupper et al., 2005).

5.7 Summary
The aim of the study was to determine whether Adrenostate® had an effect on salivary cortisol and perceived levels of stress in males. The results obtained conclude that Adrenostate® had a lowering effect on average cortisol levels when compared to that of the control group although it was not statistically significant. It can also be established that cortisol levels are dependent on many other factors and therefore lends to the need for further research. Participants in both groups reported a significant improvement in their perceived level of stress, however, the change between the groups was not statistically significant.

There were no significant changes in heart rate as expected because participants’ heart rates were within normal range. Both the average systolic and diastolic readings of participants in both groups were within normal range but a significant decrease was seen in the systolic blood pressure of Group A (control) with no significant change for Group B (experimental). There were no significant changes in diastolic blood pressure.
CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions
The aim of the study was to determine the effect of Adrenostate® on salivary cortisol levels and perceived levels of stress in males. Potential effects on heart rate and blood pressure were also measured. There was found to be no statistically significant change in salivary cortisol levels over six weeks, however, there was an overall decrease in salivary cortisol in the Group B (treatment group) as opposed to Group A (control group) over the six week period and this warrants further research. The Perceived Stress Questionnaire (PSQ) demonstrated an overall statistically significant improvement over time, however, the improvement was observed in both Groups A (control) and B (experimental).

With regards to the heart rate there was no statistically significant change. The systolic blood pressure in Group A decreased significantly and there was no significant change in diastolic blood pressure.

Although most evaluated criteria of recruited participants fell within the normal ranges of measurement, the decrease (although insignificant) of cortisol may warrant further investigation over a longer period of time utilising stricter control measures. The study did not evaluate individuals with measurably severe physiological signs of stress as an average and consequently would not be expected to demonstrate significant changes. Adrenostate® can however be considered a safe product as no aggravation of measured values was noted.

6.2 Recommendations
The following recommendations should be considered in future studies of a similar nature:

• Future studies should consider a larger sample group with greater physiological severity measures.
• In order to lessen the effect of natural cortisol decrease with aging, the age range of the participants should be narrowed to 20-30 years old.
• The design of the study is more suited to the evaluation of chronic stress levels and thus a longer study period should be considered in order to evaluate natural influences on cortisol and bodily rhythms.
• It is recommended that the salivary cortisol sample be taken at the same time by all of the participants. Sampling in the first hour of awakening is ideal but that may mean a different time of day for each participant. Multiple samples should be taken throughout the morning and an average obtained for the morning may provide more accurate results.
• Future studies should only consider participants with a high level of cortisol in order to evaluate its potential to lower cortisol levels.

• Other hormonal measures such as Adrenocorticotropic hormone (ATCH) and Dehydroepiandrosterone (DHEA) could be made in conjunction with cortisol.

• Additional measures of perceived levels of stress, other than the Perceived Stress Questionnaire (PSQ) should be considered.
REFERENCES


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APPENDIX A
Adrenostate® ingredients and therapeutic uses

Vitamin B complex – Thiamine (B₁): 1.25mg, (normal dosage: 50mg/day), Riboflavin (B₂): 1.5mg, (normal dosage: 50mg/day), Pyridoxine (B₆): 1.5mg, (normal dosage: 100-200mg/day), Cobalamin (B₁₂): 75 µg, (normal dosage: 10-100mcg/day), Folic acid (B₉): 50µg, (normal dosage: 400mcg-800mcg/day), Niacinamide (B₃): 5mg, (normal dosage: 100mg/day), Pantothenic acid (B₅): 5mg, (normal dosage: 300mg/day).

The B vitamins help to maintain healthy nerves, skin, eyes, hair, brain function and muscle tone especially in the lining of the gastrointestinal tract. They act as coenzymes and are involved in energy production. The B vitamins should always be taken together (Balch, 2006).

Ascorbic acid (Vitamin C) (12.5 mg) is an antioxidant that is necessary for approximately three hundred metabolic functions in the body. These functions include: growth and repair, adrenal gland function, healthy gums and the production of anti-stress hormones and interferon. Vitamin C has shown to improve asthma, prevent cancer, defend against infection and boost immunity (Balch, 2006). Typical dosage ranges are 500mg - 1g/day (Sullivan, 2002).

Avena sativa (Wild oats) (50mg) is made up of the following constituents: Benzaldehyde, betacarotene, beta-ionone, beta-sitisterol, betaine, caffeic acid, campesterol, caryophyllene, chlorophyll, ferulic acid, lignin, limonene, p-coumaric acid, quercetin, scopoletin, sinapic acid, stigmaterol, vanillic acid, vanillin, calcium, folate, iron, magnesium, manganese, phosphorus, potassium, selenium, zinc, vitamins A, B₁, B₂, B₃, B₅, B₆ and E. Avena sativa is indicated for use in nervous exhaustion, insomnia, depression, stress, debility and skin disorders (Balch, 2006). There are no known contraindications. The typical daily dosages are 3 - 6ml/day (Bone, 2003).

Cordyceps sinesis (25mg) is a rare and exotic medicinal fungus used in Chinese medicine. Cordyceps contains a wide range of compounds, which are considered nutritional. It contains all of the essential amino acids, vitamins E and K, B₁, B₂, and B₁₂. In addition, it contains many sugars, including mono-, di-, and oligosaccharides, and many complex polysaccharides, proteins, sterols, nucleosides, and trace elements (K, Na, Ca, Mg, Fe, Cu, Mn, Zn, Pi, Se, Al, Si, Ni, Sr, Ti, Cr, Ga, V, and Zr). Cordycepin and cordycepic acid are the main biochemical constituents. Of particular mention are various immunosuppressive compounds, including cyclosporin. Research has shown that the polysaccharides, found in cordyceps, are effective in
regulating blood sugar, and have antimetastatic and antitumour effects. In traditional Chinese medicine, Cordyceps has been used to treat conditions including respiration and pulmonary diseases, renal, liver, and cardiovascular diseases, hyposexuality, and hyperlipidemia. It is also used in the treatment of immune disorders and as an accessory to modern cancer therapies. Cordyceps is frequently prescribed for the elderly to alleviate general aches and pains. Practitioners of traditional Chinese medicine also recommend the regular use of Cordyceps to strengthen the body’s resistance to infections, such as colds and flu. Therapeutic applications of Cordyceps are hypothesised to be centred primarily on the key effects of increased oxygen utilisation, increased ATP production, and the stabilisation of blood sugar metabolism therefore combating weakness and fatigue. Typical daily dosages are 3 - 4.5g/day. No human toxicity has been reported (Holliday et al., 2005).

*Centella asiatica* (Gotu Kola) (25mg) is a plant used in Ayurvedic medicine to alleviate symptoms of anxiety and to promote a deep state of relaxation and mental calmness. The main constituent is triterpene rich fractions found within the plant extracts (Wijeweera et al., 2006). *Gotu Kola* is said to decrease fatigue and depression, increase sex drive, stimulate the central nervous system, stimulate appetite and assist with sleep disorders (Balch, 2006). Gotu Kola should be used with caution in patients with coeliac disease, fat malabsorption, vitamins A, D, E and K deficiencies and upper digestive irritations. There are no reports of overdose with Gotu Kola. Typical dosage ranges are 1.8g/day (Mills and Bone, 2005).

*Glycyrrhiza glabra* (Licorice) (125mg) is made up of the following constituents: Apigenin, benzaldehyde, beta-carotene, beta-sitosterol, betaine, camphor, carvacrol, estriol, eugenol, ferulic acid, formononetin, geraniol, glabrene, glabridin, labrol, glycyrrhetinic acid, glycyrrhizin, isoliquiritigenin, isoliquiritin, isoquercitrin, lignin, mannitol, phenol, quercetin, salicylic acid, sinapic acid, stigmasterol, thymol, umbelliferone, vitexin, calcium, choline, iron, magnesium, manganese, phosphorus, potassium, selenium, silicon, zinc and vitamins B1, B2, B3 and C. *Glycyrrhiza glabra* has a very wide range of functions which include anti-inflammatory, anti-microbial, anti-viral and anti-bacterial. It also helps with muscle spasms and promotes adrenal gland function. *Glycyrrhiza glabra* has shown to be beneficial for allergies, asthma, chronic fatigue, depression, emphysema, prostatic hypertrophy, fever, hypoglycaemia, inflammatory bowel disease, premenstrual syndrome, menopausal symptoms, upper respiratory infections and can protect again liver cancer, cirrhosis and atherosclerosis (Balch, 2006).
*Glycyrrhiza glabra* has an ACTH-like action on the adrenal cortex, increasing the production of glucocorticoids and mineralocorticoids. Typical dosage ranges between 3 - 12g/day. Licorice is contraindicated in patients with liver disorders, hypertension, hypokalaemia, kidney insufficiency and pregnancy. Licorice should not be prescribed concomitantly with Digoxin, diuretics or laxatives (Mills and Bone, 2005).

**Ganoderma lucidum (Reishi mushroom) (25mg)** is a fungi that has been used to treat various diseases such as hepatitis, hypertension, arthritis, bronchitis, tumours and cancer. *G. lucidum* contains Ganoderic acid, lucidenic acid, ganoderma acid, ganodosterone and oleic acid (Chen and Chen, 2004). *G. lucidum* has also been reported to contain polysaccharides and protein bound polysaccharides which have antitumour and antihypertension activities and have also shown to decrease blood glucose levels (Seong-Kug et al., 1999). *G. lucidum* increases monocytes, macrophages and T-lymphocytes, production of cytokine, interleukin, tumour-necrosis-factor and interferon. *G. lucidum* has been shown to increase cardiac contractility, lower blood pressure, and increase resistance to hypoxia in the cardiac muscles. *G. lucidum* has a broad spectrum of antibacterial activity. *G. lucidum* exerts hepatoprotective, antidiabetic, antitussive, expectorant, sedative, analgesic, and antiasthmatic effects (Seong-Kug et al., 1999). Typical dosage ranges are 1.5 - 3g/day. No contraindications have been found (Chen and Chen, 2004).

**Rhodiola Rosea (50mg)** has been used in traditional medical systems in Europe and Asia as an adaptogen to increase an organism’s resistance to physical stress (Schriner et al., 2009). *Rhodiola Rosea* contains 40 chemical constituents, comprising of salidroside, p-tyrosol, rosavins (including rosavin, rosin, and rosarin), rhodioni-side, rhodiolin and rosiridin. The pharmacological effects of *Rhodiola rosea* include adaptogenic and anti-stress effects, antianoxia, anti-fatigue, immunity improvement, protection of the central nervous system and of the cardiovascular system (Chen et al., 2009). Typical dosage ranges are 340 - 680mg/day. No side-effects have been reported (Shevstov et al., 2003).

**Eleutherococcus senticosus (Siberian ginseng) (100mg)** is used for improving stamina, vitality and generally one’s ability to withstand stress. It also helps eradicate debility and depression. Typical dosage ranges are 2 - 3g/day. Siberian ginseng is contraindicated in patients with hypertension. There are incidences of overdose, however, and it is recommended that Siberian ginseng only be taken for a six week period followed by a two week break before recommencement (Mills and Bone, 2005). The main constituents include: phenylpropanoids,
saponins, coumarins, vitamins (e.g. vitamin E) and provitamins (provitamin A, i.e. b-carotene (Davydov and Krikorian, 2000).

**Tyrosine (100mg)** is an amino acid commonly found in foods such as avocados and bananas. Tyrosine is a precursor of adrenaline and the neurotransmitters noradrenalin and dopamine, which regulate mood and stimulate the nervous system (Balch, 2006). These neurotransmitters play a key role in a variety of stress related-behaviours. In addition, noradrenalin is critical for modulating the central stress response (Lieberman, 2003). It helps to combat chronic fatigue, anxiety and depression (Balch, 2006). Administration of tyrosine has also been shown to improve performance on an array of tasks and reduce behavioural deficits associated with stressful conditions (Thomas et al., 1999).
APPENDIX B
Advertising pamphlet

Are you male and between the ages of 18 and 40 years old?

ARE YOU

STRESSED?

You may qualify to participate in a Research study being conducted through the Department of Homoeopathy at the University of Johannesburg on:

The effect of AdrenoState® on salivary cortisol levels and perceived levels of stress

Ethical clearance number: 39/09

Participation is voluntary and strictly confidential

Consultations and treatment are FREE OF CHARGE

For more information please contact

Kelly Joffe

083 287 4409/ stresstrial@gmail.com
APPENDIX C
Stress Status Screening Questionnaire

1. Name: ___________________________________________________________

2. Age: ___________________________________________________________

3. Have been previously diagnosed with:
   3.1 Any cardiovascular diseases including hypertension? Y N
   3.2 Any psychological diseases? Y N
   3.3 Any autoimmune diseases? Y N
   3.4 Any liver disorders? Y N
   3.5 Any kidney disorders? Y N

4. Are you on any chronic medication? Y N
   4.1 If yes, please state what medicine you are on:

5. Do you smoke? Y N

6. How stressed are you? (Richardson, n.d.)
   6.1 Do you worry about the future? Y N
   6.2 Do you sometimes have trouble falling asleep? Y N
   6.3 Do you often reach for a cigarette, a drink, or a tranquilizer in order to reduce tension? Y N
   6.4 Do you become irritated over basically insignificant matters? Y N
   6.5 Do you have less energy than you seem to need or would like to have? Y N
   6.6 Do you have too many things to do and not enough time to do them? Y N
   6.7 Do you have headaches or stomach problems? Y N
   6.8 Do you feel pressure to accomplish or to get things done? Y N
   6.9 Are you very concerned about being either well-liked or successful? Y N
   6.10 Do you perform well enough in life to satisfy yourself? Y N
   6.11 Do you get satisfaction from the small joys or simple pleasures of life? Y N
   6.12 Are you able to really relax and have fun? Y N

SCORE | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12

48
APPENDIX D
Information and consent form

Dear Volunteer

My name is Kelly Joffe, I am a masters student of homoeopathy at the University of Johannesburg. I am undertaking this study in completion of my M.Tech Homoeopathy qualification.

This study uses a supplement called AdrenoState® for the possible treatment of stress. The study will be assessing perceived levels of stress in the form of a questionnaire as well as saliva cortisol levels.

Long term exposure to stress can lead to many harmful effects on the body. Cortisol is the major stress hormone and long term exposure to high cortisol levels can cause harm to the body. The intention of this study is to determine whether AdrenoState® will have an effect on cortisol levels as well as perceived levels of stress. This study may benefit in the result of reduced stress and will contribute to scientific knowledge. You are invited to participate in this research study.

In order for you to participate, will need to be male, between the ages of 18 and 40 years old, have a freezer in order to store the saliva samples and be experiencing symptoms of stress.

Participants may not be included if they have been previously diagnosed with any cardiovascular diseases including hypertension, any psychological diseases, autoimmune diseases, liver disorders, kidney disorders or if they are taking any chronic medication.

The study will run over a six week period where the participants will meet with the researcher on three occasions: day one, week three and week six. At all three meetings the participant will need to fill in the perceived stress questionnaire. The researcher will take their blood pressure and heart rate and give the participant a tube in order to obtain a saliva sample first thing in the morning upon waking. The participant will also receive either the AdrenoState® or the placebo (that will look and taste identical to the active medication) at the first visit and will be required to take four capsules every morning for the full duration of the study. It is recommended that no lifestyle changes should be made during the study.

Participation in this study is completely voluntary. All participants will be well informed of the nature of the study and what it entails. All personal information gathered will be kept
confidential and anonymous. All Participants have the right to be informed and to ask questions. You are free to withdraw at any stage. There are no anticipated risks involved but if you should experience anything that concerns you please contact the researcher. The results of the study will be made available to the participants on their request. The control group may also receive AdrenoState® if the outcome is successful.

In order that you are fully informed about the nature and requirements of the study you are required to carefully read this information form and give consent by signing your signature. I……………………………………., the participant have been completely informed about the procedure of the study. I acknowledge that I may withdraw at any time in the study. I acknowledge that I am free to inquire about the research and ask questions, which will be answered by the researcher and supervisor to the best of their ability.

Name:………………………………… Signature:…………………….. Date:…………………..

I, Kelly Joffe, the researcher, have given a comprehensive explanation of the intended study procedure and treatment. I will provide the best explanations that I can with regards to questions posed by the participants.

Name: Kelly Joffe Signature:…………………….. Date:……………………

Contact details:
Researcher: Kelly Joffe Cell: 083 287 4409
Supervisor: Dr. Neil Gower Cell: 011 5596779

A copy of this Information and Consent form must be handed to the participant. The original will be kept in the participant’s file.
APPENDIX E

International Physical Activity Questionnaire (IPAQ)

Please answer each question even if you do not consider yourself to be an active person. In answering the following questions,

**Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder that normal.

**Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder that normal.

1a. During the last 7 days, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling? Think about *only* those physical activities that you did for at least 10 minutes at a time.

<table>
<thead>
<tr>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
</table>

2a. Again, think *only* about those physical activities that you did for at least 10 minutes at a time. During the last 7 days, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

<table>
<thead>
<tr>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
</table>

3a. During the last 7 days, on how many days did you **walk** for at least 10 minutes at a time? This includes walking at work and at home, walking to travel from place to place, and any other walking that you did solely for recreation, sport, exercise or leisure.

<table>
<thead>
<tr>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
</table>

The last question is about the time you spent sitting on weekdays while at work, at home, while doing course work and during leisure time. This includes time spent sitting at a desk, visiting friends, reading travelling on a bus or sitting or lying down to watch television.

4. During the last 7 days, how many hours per day did you usually spend sitting on a **weekday**?

<table>
<thead>
<tr>
<th>&lt; 1</th>
<th>1-3</th>
<th>3-5</th>
<th>5-7</th>
<th>7-9</th>
<th>9-11</th>
<th>&gt; 11</th>
</tr>
</thead>
</table>

1b. How many minutes did you usually spend on one of those days doing vigorous physical activities?

<table>
<thead>
<tr>
<th>&lt; 10</th>
<th>10-20</th>
<th>20-30</th>
<th>30-40</th>
<th>40-50</th>
<th>50-60</th>
<th>&gt; 60</th>
</tr>
</thead>
</table>

2b. How many minutes did you usually spend on one of those days doing moderate physical activities?

<table>
<thead>
<tr>
<th>&lt; 10</th>
<th>10-20</th>
<th>20-30</th>
<th>30-40</th>
<th>40-50</th>
<th>50-60</th>
<th>&gt; 60</th>
</tr>
</thead>
</table>

3b. How many minutes did you usually spend walking on one of those days?

<table>
<thead>
<tr>
<th>&lt; 10</th>
<th>10-30</th>
<th>30-60</th>
<th>60-90</th>
<th>90-120</th>
<th>120-180</th>
<th>&gt; 180</th>
</tr>
</thead>
</table>
APPENDIX F

Perceived Stress Questionnaire

Participant:                                                        File number:

PSQ20 (Fliege et al., 2004; Levenstein et al. 1993)

For each sentence, mark the number that describes how often it applies to you during the last 4 weeks. There are no right or wrong answers. Please work quickly, without bothering to check your answers, and do not skip any question.

<table>
<thead>
<tr>
<th>Number</th>
<th>Statement</th>
<th>Almost never (1)</th>
<th>Sometimes (2)</th>
<th>Often (3)</th>
<th>Usually (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>You feel rested</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>02</td>
<td>You feel that too many demands are being made on you</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>03</td>
<td>You have too many things to do</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>04</td>
<td>You feel you’re doing things you really like</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>05</td>
<td>You fear you may not manage to attain your goals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>06</td>
<td>You feel calm</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>07</td>
<td>You feel frustrated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>08</td>
<td>You are full of energy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>09</td>
<td>You feel tense</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Your problems seem to be piling up</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>You feel you’re in a hurry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>You feel safe and protected</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>You have many worries</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>You enjoy yourself</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>You are afraid for the future</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>You are lighthearted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>You feel mentally exhausted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>You have trouble relaxing</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>You have enough time for yourself</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>You feel under pressure from deadlines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX G

Approval to use Perceived stress questionnaire (PSQ)

Dear Ms Joffe,

Feel free to use the PSQ. Files attached, long and shorter version. Choose the time-perspective as it suits your research purpose. We currently use 4 weeks. Several time-frame versions are validated (see papers by Susan Levenstein).
I wish you success with your projects!

With kind regards
Herbert Fliege

-----Ursprüngliche Nachricht-----
Von: Kelly Joffe [mailto:kelly@joffefam.co.za]
Gesendet: Montag, 4. August 2008 17:01
An: Fliege, Herbert
Betreff: Perceived stress questionnaire

Dear Dr. Fliege,

My name is Kelly Joffe, I am a student at the University of Johannesburg in South Africa currently doing my masters. I would like to know if it would be possible to please get the rights to use the perceived stress questionnaire for my master’s study.

Thank you

Kind Regards
Kelly Joffe
kelly@joffefam.co.za
APPENDIX H

How to take and store the saliva sample and how to take the medicine

Eating, drinking, chewing gums, smoking or brushing teeth should be avoided for 30 minutes before sampling. Otherwise, it is recommended to rinse mouth thoroughly with cold water 5 minutes prior to sampling. Samples must be taken within 1 hour of waking in the morning. Do not collect samples when oral diseases, inflammation or lesions exist (blood contamination). If there is visible blood contamination in the patient specimen, it should be discarded, rinse the sampling device with water, wait for 10 minutes and take a new sample.

Procedure:

1) Wake up in the morning
2) Spit into the tube provided to fill up to the line demonstrated
3) Close the tube
4) Place in the freezer until collection
5) Saliva is stable at room temperature but should still however be frozen for storage
6) AFTER the sample has been taken you may eat, drink and take the AdrenoState® or identical placebo.

Samples must be taken at the same time on all three occasions.

How to take medicine or placebo

Four capsules must be taken, with water, every morning from the beginning to the end of the study. On the days that you have to take the saliva sample the AdrenoState® or placebo must be taken AFTER the saliva sample.
APPENDIX I

The saliva cortisol assay insert

ASSAY PROCEDURE
Each run must include a standard curve.
1. Secure the desired number of coated strips in the frame holder.
2. Dispense 100 µL of each cortisol Standard and Control into appropriate wells.
3. Dispense 100 µL of each sample into selected wells.
4. Dispense 200 µL of Enzyme Conjugate into each sample and standard well and mix the plate thoroughly for 10 seconds.
5. Incubate for 60 minutes at room temperature.
6. Briskly shake out the contents of the wells and rinse the wells 3 times with diluted Wash Solution (400 µL per well). Strike the inverted wells sharply on absorbent paper towel to remove residual droplets.
7. Add 200 µL of Substrate Solution to each well.
8. Incubate for 30 minutes at room temperature.
9. Stop the reaction by adding 100 µL of Stop Solution to each well.
10. Determine the absorbance of each well at 450 ±10 nm.

It is recommended that the wells be read within 10 minutes.

Calculation of Results
1. Calculate the average absorbance values for each set of standards, controls and patient Samples.
2. Construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical(Y) axis and concentration on the horizontal (X) axis.
3. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.
4. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred calculation method. Other data reduction functions may give slightly different results.
5. The concentration of the samples can be read directly from this standard curve. Samples with concentrations higher than that of the highest standard have to be further diluted. For the calculation of the concentrations this dilution factor has to be taken into account.
APPENDIX J

Chemical reagents of the kit

1. **Microtiterwells**, 12x8 strips, 96 wells, coated with (mouse) anti-Cortisol antiserum.
2. **Standard** (Standard 0-6), 7 vials, 1 ml each, ready to use;
   Concentrations: 0.0-2-5-10-20-40-80 ng/mL contain 0.003% Proclin 300 as a preservative.
3. **Control low/ control high**, 2 vials, 1.0 mL each, ready to use;
   Contains 0.003% Proclin 300 as a preservative.
4. **Enzyme conjugate**, 1 vial, 26 mL, ready to use
   Cortisol conjugated to horseradish peroxidise, contains < 0.019% BND and < 0.017% MIT as preservative.
5. **Substrate Solution**, 1 vial, 25 mL, ready to use; Tetramethylbenzidine (TMB)
6. **Stop Solution**, 1 vial, 14 mL, ready to use; contains 0.5M H$_2$SO$_4$
7. **Wash Solution**, 1 vial, 30 mL (40x concentrated); concentrate for 1200ml
APPENDIX K
Data Collection Tables

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