Examensarbete

Salivary cortisol and post traumatic stress symptoms
-a ten year follow-up of Swedish UN soldiers
after a 6 months mission
in Bosnia

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Abstract

This is to my knowledge the first time a ten-year follow-up study of salivary cortisol concentrations measured by immunoassays in relation to posttraumatic symptoms according to the Impact of Event Scale (IES) is made. The study was performed on 78 Swedish UN soldiers after a 6-months mission in the former republic of Yugoslavia. Follow-up investigations were performed six months, twelve months and ten years after their return to Sweden. Morning and evening salivary cortisol concentrations were determined by radioimmunoassay (RIA) and enzyme-linked immunoassay (EIA) and subjective posttraumatic avoidance and intrusion symptoms were measured with the IES (see Appendix I).

This study concerns the methodological description of the EIA for determination of salivary cortisol and the comparison of the results from all three follow-up investigations. Post-traumatic stress symptoms according to IES (intrusion subscale and total score) increased significantly over ten years of time. There was an significant interrelationship between the change in both morning and evening salivary cortisol concentrations, measured with immunoassays, and changes in self-rated posttraumatic intrusive symptoms, according to IES, during ten years follow-up, after a six months mission in Bosnia in the way that salivary cortisol concentrations showed a tendency to decrease over ten years of time in subjects with a higher IES score. The rise in morning salivary cortisol, from awakening until 30 minutes later, was significantly correlated with the ratings of posttraumatic stress symptoms according to the IES ten years after the mission.

Nyckelord

Salivary cortisol concentration, post traumatic stress symptoms, ten year follow-up study, UN soldiers.
Preface

I am grateful for having had the opportunity to write this report. It was of big interest and I have been exited from the very beginning. I surely want to thank my tutor Elisabeth Aardal-Eriksson for introducing me in this complex field of research and for all her support and kindness during this assignment. I also want to thank laboratory engineer Agneta Berg for her guidance during my laboratory work and associated professor Lars-Håkan Thorell for invaluable help with the statistical calculations. We complemented each other in a good way.

Stress is a natural part of our daily lives helping us learn and grow. We are exposed to stressful situations throughout all ages in life, at home as well as at work and during leisure. On the other hand, stress can if prolonged, continuous, unexpected or unmanageable lead to health problems by threatening the balance of the organism (1). People of today intend to want very much, and everything ought to be done all at once even if it is unmanageable. This is an example of how our daily life can be unhealthful by prolonged low degree of stress. In addition to our daily life stress, traumatic stress situations (events outside the range of usual human experience) like for instance combat stress can lead to a more defined disorder, Post Traumatic Stress Disorder (PTSD); an anxiety disorder associated with serious traumatic events and characterized by such symptoms as survivor guilt, numbness and lack of involvement with reality (avoidance), or reliving the trauma in dreams, recurrent thoughts and images (intrusion) (2).

During this work I have learned the similarities and differences between immunoassays for determination of cortisol in saliva. I have also come to a better understanding of the possibility to use the results of these determinations and self-rating questionnaires for investigation of the interrelationship between salivary cortisol levels and subjective posttraumatic distress, i.e. how to understand the connection between biology and psychology, between body and soul. I have understood the importance of a careful planning of a long-term follow-up study and the significance of methodological accuracy, so that the sources of error are minimized.
Abstract

This is to my knowledge the first time a ten-year follow-up study of salivary cortisol concentrations measured by immunoassays in relation to posttraumatic symptoms according to the Impact of Event Scale (IES) is made. The study was performed on 78 Swedish UN soldiers after a 6-months mission in the former republic of Yugoslavia. Follow-up investigations were performed six months, twelve months and ten years after their return to Sweden. Morning and evening salivary cortisol concentrations were determined by radioimmunoassay (RIA) and enzyme-linked immunoassay (EIA) and subjective posttraumatic avoidance and intrusion symptoms were measured with the IES (see Appendix I).

This study concerns the methodological description of the EIA for determination of salivary cortisol and the comparison of the results from all three follow-up investigations. Post-traumatic stress symptoms according to IES (intrusion subscale and total score) increased significantly over ten years of time. There was a significant interrelationship between the change in both morning and evening salivary cortisol concentrations, measured with immunoassays, and changes in self-rated posttraumatic intrusive symptoms, according to IES, during ten years follow-up, after a six months mission in Bosnia in the way that salivary cortisol concentrations showed a tendency to decrease over ten years of time in subjects with a higher IES score. The rise in morning salivary cortisol, from awakening until 30 minutes later, was significantly correlated with the ratings of posttraumatic stress symptoms according to the IES ten years after the mission.
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Introduction

Mental effects of severe psychological distress and psychological reactions to combat experiences have been studied in many aspects during the past century. Although psychological approach in measuring stressors provides to some extent the mechanisms in which stress affects health or illness, a better measure would be a physical or biological measure since psychological approach can be difficult. Psychological measures such as questionnaires are shown not to be very reliable, as the individuals interviewed may not report the stressors due to a number of reasons, such as adaptability and self-consciousness (3). Human biologists have shown increasing interest in the use of cortisol as an objective marker of stress in recent years. Cortisol is the predominant glucocorticoid in humans synthesized from cholesterol in the inner layers of the adrenal cortex, zona fasciculata, and to some extent also in zona reticularis, constituting 80% of the 17-hydroxycorticoids in plasma (Figure 1).

**Figure 1:** The cortisol synthesis.

\[
\begin{align*}
17^\alpha\text{-Hydroxypregnenolone} & \quad \xrightarrow{3\beta\text{HSD / ISOM}} \\
17^\alpha\text{-Hydroxyprogesterone} & \quad \xrightarrow{P-450c21} \\
11\text{-Deoxycortisol} & \quad \xrightarrow{P-450c11}
\end{align*}
\]

![Cortisol structure](image)
Cortisol is the end product of the hypothalamic-pituitary-adrenal (HPA) axis and is actively involved in the regulation of calcium absorption, blood pressure, metabolism and immune function. During basal conditions, cortisol production has a circadian rhythm regulated by a pulsatile secretion of CRH from the hypothalamus with diurnal rhythmicity stimulating ACTH release from the anterior pituitary lobe. Cortisol secretion also increases in response to food intake, use of tobacco and in connection with physical exercise and psychological stress. Negative feedback of cortisol occurs both at the hypothalamic and the pituitary levels (Figure 2).

**Figure 2:** The cortisol feedback loop.

Cortisol levels peak in the early morning, and drop to the lowest concentration at night (4). Levels rise independently of circadian rhythm in response to stress (5, 6). Across a large group of "normal" subjects 50% show a morning rise in cortisol with a peak at 30-45 minutes after awakening. Moreover, 80% are responders to an awakening challenge (+2.5 nmol/L or more), and 75% show a consistent response over two days. Lack of a morning rise may not be healthy (e.g. morning rise activates appetite and cognitive functioning), so a lack of this rise may indicate a dysfunction of the HPA axis regulation (7). Different patterns of dysfunctions in HPA axis regulations, abnormal cortisol concentrations and diurnal cortisol rhythm have been shown as a consequence of extreme acute, chronic and/or prolonged stress (8-10).

In disaster research there is mostly limited possibility of measuring and following biochemical changes over time due to several practical problems as for example the need for blood sampling, transportation and storage of samples. Nevertheless, several important studies have been performed in particular on Vietnam veterans with PTSD showing low 24-hour urinary cortisol secretion (9-11). While the assessment of cortisol in urine is of declining interest, the development of salivary cortisol assays has become an invaluable tool for both basic scientists and clinicians. A number of significant advantages over the measurement of cortisol in blood have resulted in a steadily increasing interest in salivary cortisol as a marker for stress (12-14).
The level of cortisol in saliva also differs from the cortisol concentration in the general circulation. In the blood only 1-15% of the cortisol is in its unbound or biologically active form. The remaining cortisol is bound to serum proteins, mainly transcortin and albumin. In saliva, cortisol is present in almost exclusively free form i.e. biologically active form and enters the saliva via free diffusion through the acinar cells of the salivary glands (15). The levels are unaffected by salivary flow rate, composition of the saliva concerning serous/mucous content and the presence of salivary enzymes (16, 17). However, a small fraction of the corticosteroid-binding proteins transcortin and albumin is present in saliva (15). Therefore, the salivary cortisol concentration is much lower than in serum, i.e. approximately 3% of the total serum cortisol concentration (16).

**Immuoassay development**

“How could so bulky and large a molecule as an enzyme be attached to an antigen or antibody without sterically hindering the immunochemical reaction between antigen and antibody?” (18). Well, it was proved to work. Solid-phase techniques were used in the development of microtiter plates in which either an antigen or an antibody is non-covalently bound to a solid-phase support. Technical advances led to automated pipetting devices, multichannel pipettes and microtiter plate readers. Today fully automated instruments in the medical laboratories use the immunoassay principle for routine measurements of analytes in patient samples. The number of analytical and clinical investigations relying on diagnostic immunoassays worldwide is exceedingly large (18).

Enzyme labels in immunoassay have been successively used since its invention in the 1960s, by the research group of Peter Perlmann and Eva Engvall (from Sweden, Stockholm University) and the research group of Anton Schuurs and Bauke van Weemen (from The Netherlands, the Research Laboratories of NV Organon). Earlier the use of radioactivity as the reporter label was common, but it was an unhealthy way of analyzing. Special facilities were built in which investigators could work using the amounts of radioactivity (β and γ radiation) required for the labeling of antigens or antibodies. Concerning the safety of laboratory personnel and radioactive waste, this method was gladly altered for Enzyme Immunoassay (EIA). In the early 1980s EIA matched the exquisite sensitivity of existing Radio Immunoassay (RIA) systems for the same analytes (18). From the beginning (in the 1990s) the majority of available immunoassays for saliva cortisol were modifications of protocols developed for the use with serum/plasma. Since the composition of other body fluids is different from serum it is important to use a matrix (for the standards and controls) that matches the contemplated body fluid, and not serum.

**Aim**

The aim of the present study was to investigate possible interrelationships between salivary cortisol concentrations, measured with immunoassays, and changes in self-rated posttraumatic symptoms, according to the self rating scale Impact of Event Scale (IES), in a group of Swedish UN soldiers during ten years follow-up, after a six months mission in Bosnia.
Method and material

Study procedure
The first follow-up investigation occurred in connection with a reunion meeting six months after return to Sweden after the six months mission in Bosnia (BA01). The Senior Medical Officer of the battalion was present giving verbal and written information about the study and administrating the salivary sampling tubes and the IES rating scales. The second follow-up investigation was performed six months later, i.e. one year after the completed BA01 UN-mission. Envelopes were sent out to the home addresses of the participating subjects containing written information about the study, instructions for saliva sampling, two saliva sampling tubes labeled with the employment number and 8 AM and 10 PM respectively and the IES questionnaire labeled with the employment number. The envelopes also contained a paid return envelope to send the samples and the questionnaire to the Laboratory of Clinical Chemistry at Linköping University Hospital, Sweden. The third follow-up investigation occurred in connection with a reunion meeting ten years after returning to Sweden. The Senior Medical Officer of the battalion was present giving verbal and written information about the study and administrating the salivary sampling tubes and the IES rating scales.

Subjects
In October 1993 Sweden sent their first UN-battalion of almost 900 soldiers to Bosnia for sanitarian support, as a part of the United Nation Protection Force (UNPROFOR). The soldiers served for six months, together with Danish forces, providing the people vital things such as food and drinks. They also secured roads and tried to negotiate with the different sides fighting at the time in the former republic of Yugoslavia. During the mission, the UN soldiers were exposed to extreme combat and other severely stressful situations. Some of these situations were the massacre in the village of Stupni Do in October 1993, the battle around and the salvation of captured refugees in the city of Vares in October 1993 and the massacre on the main square in Sarajevo in February 1994 including transport of injured civilians through no man's land. Furthermore, the soldiers were exposed to a mine accident involving a Swedish medical transport vehicle (19). After return to Sweden, members of the company stationed at Vares including escort and medical personnel (in-all 174 subjects) were asked by written information from the Swedish Armed Forces International Command (SWEDINT), the Commanding Officer of the battalion and the Senior Medical Officer to voluntarily participate in a follow-up study. Follow-up investigations were performed on three occasions; six months, twelve months and ten years after return to Sweden. Out of these, 78 subjects (76 men and two women) completed the three follow-up investigations. Medical records from enrolment indicated that the participants were, as far as could be determined; healthy and not taking any pharmacological substances (for example steroids) that directly could affect the cortisol concentrations.
Saliva sample collection
The subjects themselves collected saliva for determination of cortisol in Salivette® tubes (Sarstedt, Rommelsdorf, Nümbrecht, Germany), a plastic tube with an insert containing a cotton-wool swab on which they were instructed to chew lightly until the swab was drained with saliva and then replace it into the insert (Picture 1). Before saliva sampling, the subjects were instructed not to eat, drink, smoke or brush their teeth for 60 minutes. They were told to lie down 30 minutes prior to sampling. These instructions were given in order to minimize possible confounder effects on the salivary cortisol concentrations. On the two first sampling occasions sampling was performed at 8 AM and 10 PM. On the third sampling occasion sampling was performed at wake-up, 30 minutes later and at bedtime.

Saliva sample preparation
All saliva samples were stored at room temperature during transportation to the Laboratory of Clinical Chemistry at Linköping University Hospital, Sweden. On arrival at the laboratory, the saliva samples were centrifuged at 1900 g for 15 minutes and frozen at –20 °C until assayed within six months after sampling. Before assay the samples were thawed completely, vortexed and centrifuged. All samples from each sampling occasion were analyzed in the same assay. Samples from the two first sampling occasions were assayed by duplicate determinations (20). Samples from the third sampling occasion were assayed by single determinations.

Radioimmunoassay for determination of salivary cortisol
In connection with the first and second follow-up investigation, salivary cortisol concentrations were determined with a commercial solid-phase radioimmunoassay Coat-a-Count from Diagnostic Products Corporation, Los Angeles, USA designed for determination of cortisol in serum and urine. The radioimmunoassay was modified for determination of cortisol in saliva by diluting of calibrators and controls, increasing the sample volume and extending the incubation time. The method was highly specific with a detection limit of 0.5 nmol/L and a total inter-assay imprecision (CV) of 8.3% for salivary cortisol values < 10 nmol/L and 5.1% for values > 10 nmol/L and intra-assay CV of 4.3% for values < 10 nmol/L and 3.6% for values > 10 nmol/L (21).
**Enzyme immunoassay test principle for salivary cortisol measurement**

In connection with the third follow-up investigation salivary cortisol concentrations were determined with a commercial EIA designed for saliva from Salimetrics, LLC, Pennsylvania, USA. The kit is intended only for research use with saliva (22) and the assay principle is as follows:

A micro titer plate is coated with monoclonal antibodies to cortisol. The cortisol in the samples competes with the cortisol linked to horseradish peroxidase, for the antibody binding sites. The plate is incubated and unbound components are washed off. Bound cortisol peroxidase is measured by the reaction of the peroxidase enzyme on the substrate tetramethylbenzidine (TMB). The reaction is stopped with sulfuric acid and the optical density (OD) is read on a standard plate reader at 450 nm within ten minutes. Correction at 490 to 630 nm is performed. The amount of cortisol peroxidase detected, as measured by the intensity of color, is inversely proportional to the amount of cortisol present.

When pipetting the microtiter plate it is important that the reagent is added following the same sequence as when the samples were pipetted so that every sample has been treated in the same way. Pipetting of samples and reagents must be done without interruption across the plate. Every sample, standard and NSB are duplicated on the plate. Every time running a plate a standard curve should be run too (22). Picture 2 describes the plate layout in the computer software.

**Picture 2: The plate layout.**

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The salivary cortisol EIA was calibrated against liquid chromatography-mass spectrometry (LC-MS) and adapted for automated assay with NexGen Four (ADALTIS, Bologna, Italy). Detection limit was 0.4 nmol/L. Total (inter-assay) imprecision was 11.3% at 3 nmol/L (n=30) and 9.6% at 25 nmol/L (n=30). Picture 3 shows NexGen Four (ADALTIS).
Method comparison

Before analysing the saliva samples from the third follow-up investigation the modified RIA (Coat-a-Count, Diagnostic Products Company) was compared with the new automated EIA (HS-salivary cortisol, Salimetrics with NexGen Four, ADALTIS). Samples were collected at 8 AM and 10 PM from apparently healthy laboratory personnel (n=20; six males and 14 women, age range: 35-60 years). Salivary cortisol concentrations were determined by both methods on the same day. The comparison showed good correlation between the two methods ($r^2 = 0.96$) for concentrations spanning the range 0.4-80 nmol/L. These results agreed with a similar comparison by Raff and co-workers (23).

Impact of Event Scale rating

The IES 15-item version is a self-rating instrument designed for estimating posttraumatic stress reactions comprising intrusive symptoms characterized by unbidden thoughts and images, sleeping disturbances and nightmares, increased startle response and repetitive behavior and avoidance symptoms characterized by total denial of the event, emotional numbness, avoiding situations that remind of the event and loss of interest in activities and other people. The IES is not diagnosing Post Traumatic Stress Disorder (PTSD) but is giving an indication about Post Traumatic Stress Symptoms (PTS) (24). The items are derived from statements frequently used by subjects exposed to recent life events (Appendix I). A cut-off score of $\geq 20$ has been set to identify individuals’ distress with probability for development of PTSD and in the need for follow-up (25). In a review article of the validity of the IES instrument, Joseph offers information of high coefficients of internal consistency and test-retest reliability supporting of the validity of the IES as a measure of trauma-related distress (26). On each sampling occasion, the participating subjects filled out the IES questionnaire in connection with the evening saliva sampling.
Statistics

For estimating central and variation the means and standard deviations were used. The Spearman rank correlation, corrected for ties, was used between rating variables and cortisol values. One factor repeated measures analyses of variance (ANOVA) was applied in studying differences between groups in change of cortisol values over time. All statistical tests were two-sided and the significance level was set to \( \alpha = 0.05 \).

In testing changes between occasions, the Wilcoxon signed rank test for paired observations was used for non-parametric data (27).

The Spearman rank correlation coefficient rho (\( \rho \)), corrected for ties, was used for testing correlations when non-parametric variables were involved.

Results

Material

In the following, the results of morning and evening salivary cortisol and the IES ratings from the 78 subjects completing all three investigation occasions; i.e. six months, one year and ten years after the mission, will be considered.

Knowing that the subjects woke up at the latest 30 minutes before saliva sampling on the first follow-up investigation and were instructed to rest for at least 30 minutes prior to saliva sampling on the second follow-up investigation, the “wake-up+30-minutes” sample on the third follow-up investigation will be used when studying interrelationships between salivary cortisol concentrations measured with immunoassays and changes in self-rated posttraumatic symptoms. On the third follow-up investigation the relationship between the morning cortisol rise, i.e. the difference between the salivary cortisol concentration at “wake-up + 30-minutes” and the concentration at “wake-up”, and self-rated posttraumatic symptoms will be shown.

The subjects

The material comprises 76 men and two women participating in all three follow-up investigations. Their mean age was 27.0 ± 5.7 (\( M \pm SD \)) on the first investigation and 67/78 (86%) had no children. On the first follow-up investigation 50/78 (64%) were single (Figure 3a) and 33/78 (42%) had no more than high-school education (Figure 3b).

Figure 3a: Marital status among the subjects on the first follow-up investigation.
On the ten-year follow-up occasion, 52/78, 67% were married or lived with a partner (Figure 4a) and 27 of the 78 subjects had one child or more; the mean value was 0.6 ± 0.9 (M ± SD). The subjects also had a higher degree of education. On the ten-year follow-up investigation 25/78, 32% had completed university education compared to 14/78, 18% on the first investigation (Figure 4b).
During the ten years since the UN-mission to Bosnia in 1994, 23 of the subjects went on one further UN-mission, 16 subjects went on two or more UN-missions. Thirty-nine subjects did not participate in any further UN-mission.

**Salivary cortisol level**
There was no statistically significant difference between the mean morning cortisol level on the first and second follow-up investigations (11.914 ± 6.673 (M ± SD) nmol/L and 11.112 ± 5.154 (M ± SD) nmol/L respectively). On the first and second follow-up investigation, the morning salivary cortisol sample was taken at 8 AM. On the third follow-up ten years after the mission, morning salivary cortisol sampling was performed at wake-up and again 30 minutes later. The mean cortisol level at wake-up was significantly lower, 8.188 ± 4.871 (M ± SD) nmol/L, than the sample taken 30 minutes after awakening, 13.365 ± 6.856 (M ± SD) nmol/L, p<0.0001. There was also a statistically significant difference between the mean 8 AM cortisol concentration on the first follow-up investigation and the “wake-up” cortisol concentration on the third follow-up investigation. On the other hand, there was no statistically significant difference between the 8 AM cortisol concentration on the first follow-up investigation and the “wake-up + 30-minutes” cortisol concentration on the third follow-up investigation (Figure 5).

**Figure 5:** Mean morning salivary cortisol concentrations on the three different follow-up investigations. 95% confidence intervals are shown in the bar graphs. *** = p<0.0001

The mean evening salivary cortisol levels on the three follow-up investigations were 2.496 ± 1.634 (M ± SD) nmol/L, 3.023 ± 1.872 (M ± SD) nmol/L and 2.782 ± 1.883 (M ± SD) nmol/L respectively (Figure 6). On the first and second follow-up investigation the evening salivary cortisol sample was taken at 10 PM. On the third follow-up, ten years after the mission, evening salivary cortisol sampling was performed just before going to sleep. The mean evening salivary cortisol level on the first follow-up investigation was significantly lower than the second follow-up investigation (p < 0.005). The mean evening salivary cortisol level on the third follow-up investigation did not differ with any statistical significance from either of the two first follow-up investigations.
**Figure 6:** Mean evening salivary cortisol concentrations on the three different follow-up investigations. 95% confidence intervals are shown in the bar graphs.

\[** = p<0.005\]

IES ratings
The mean total IES score increased with statistical significance over time during the ten-year follow-up study. The mean total IES scores on the three different follow-up occasions were 17.8 ± 14.4 \((M \pm SD)\), 20.7 ± 15.2 \((M \pm SD)\) and 26.2 ± 24.1 \((M \pm SD)\) (Figure 7).

**Figure 7:** Mean total IES scores on the three different follow-up investigations. 95% confidence intervals are shown in the error bars.

\[* = p<0.05, ** = p<0.001\]**
The mean IES avoidance subscale ratings on the three different follow-up occasions were 10.4 ± 7.5 ($M \pm SD$), 10.1 ± 8.4 ($M \pm SD$) and 5.9 ± 7.2 ($M \pm SD$). The avoidance subscale ratings (the questions in the IES marked “A” in Appendix I) were significantly lower on the third (ten-year) follow-up occasion than on the first investigation six months after return to Sweden ($F = 20.9$, $df = 2.76$, $p < 0.0001$) (Figure 8).

Figure 8: Mean IES avoidance subscale ratings on the three different follow-up investigations. 95% confidence intervals are shown in the error bars.

On the other hand, the intrusion subscale ratings (the questions in IES marked “I” in Appendix I) were significantly higher on the ten-year follow-up investigation than on the first investigation six months after the mission ($F = 15.5$, $df = 2.77$, $p < 0.0001$). The mean intrusion ratings on the three different follow-up investigations were 7.4 ± 8.4 ($M \pm SD$), 10.6 ± 8.5 ($M \pm SD$) and 12.6 ± 10.8 ($M \pm SD$) (Figure 9).

Figure 9: Mean IES intrusion subscale ratings on the three different follow-up investigations. 95% confidence intervals are shown in the error bars.
To study the impact of changes in cortisol concentrations over time on posttraumatic avoidance and intrusion symptoms according to the IES subscales, the morning salivary cortisol concentration 30 minutes after awakening on the third follow-up investigation was subtracted from the morning salivary cortisol concentration on the first follow-up investigation. The same procedure was performed with the evening salivary cortisol concentrations. The subjects who had a higher salivary cortisol concentration on the first follow-up investigation than on the third follow-up investigation were classified as “Decreasing” ($n = 33$ for morning cortisol and $n = 37$ for evening cortisol concentrations). The subjects who had a lower level of cortisol on the first follow-up investigation than on the third follow-up investigation were classified as “Increasing” ($n = 45$ for morning cortisol and $n = 41$ for evening cortisol concentrations). The subjects who had a lower level of cortisol on the first follow-up investigation than on the third follow-up investigation were classified as “Decreasing” ($n = 33$ for morning cortisol and $n = 37$ for evening cortisol concentrations). The subjects who had a lower level of cortisol on the first follow-up investigation than on the third follow-up investigation were classified as “Increasing” ($n = 45$ for morning cortisol and $n = 41$ for evening cortisol concentrations). The figures 10 and 11 display the results of the ANOVA regarding the IES avoidance subscale ratings over the three follow-up investigations within the “Decreasing” and “Increasing” groups. There was no significant difference in either morning cortisol concentration or evening cortisol concentration over the three follow-up investigations between the “Increasing” and “Decreasing” groups. There were however significant differences in IES avoidance subscale rating between the “Increasing” and “Decreasing” groups for both morning ($p < 0.0001$) and evening cortisol ($p < 0.0001$). The “Increasing” morning cortisol group showed lower IES avoidance subscale ratings on all three follow-up investigations. On the other hand, the “Increasing” evening cortisol group showed higher IES avoidance ratings on all three follow-up investigations. There was no interaction between the two groups regarding the change in IES avoidance subscale ratings over time.

**Figure 10:** Two-way repeated measures ANOVA of the IES Avoidance ratings over time in subjects classified with “Increasing” and “Decreasing” morning salivary cortisol concentrations. The 95% confidence intervals are shown in the error bars.

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![Graph showing mean IES Avoidance ratings over follow-up investigations for decreasing and increasing morning cortisol groups.](image-url)
Figure 11: Two-way repeated measures ANOVA of the IES Avoidance ratings over time in subjects classified with “Increasing” and “Decreasing” evening salivary cortisol concentrations. The 95% confidence intervals are shown in the error bars.

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Figures 12 and 13 display the results of the ANOVA regarding the IES intrusion subscale ratings over the three follow-up investigations within the “Decreasing” and “Increasing” groups. There was a statistically significant difference in both morning cortisol concentration ($p < 0.05$) and evening cortisol concentration ($p < 0.05$) over the three follow-up investigations between the “Increasing” and “Decreasing” groups. There were also statistically significant differences in IES intrusion subscale rating between the “Increasing” and “Decreasing” groups for both morning ($p <0.0001$) and evening cortisol ($p <0.0001$). There was a significant interaction between the two groups for both morning ($p <0.0001$) and evening cortisol ($p <0.0001$) regarding the change in IES intrusion subscale ratings over time.
**Figure 12:** Two-way repeated measures ANOVA of the IES Intrusion ratings over time in subjects classified with “Increasing” and “Decreasing” *morning* salivary cortisol concentrations. The 95% confidence intervals are shown in the error bars.

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**Figure 13:** Two-way repeated measures ANOVA of the IES Intrusion ratings over time in subjects classified with “Increasing” and “Decreasing” *evening* salivary cortisol concentrations. The 95% confidence intervals are shown in the error bars.

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<td>IES Intrusion * Subjects</td>
<td>152</td>
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On the third follow-up investigation the morning salivary cortisol sampling was performed just after awakening and again 30 minutes later. As stated, at a consensus meeting, lack of a morning rise may not be healthy (7). We therefore correlated the morning salivary cortisol rise to the IES avoidance and intrusion subscale ratings. There was a statistically significant correlation between the rise in morning salivary cortisol from awakening until 30 minutes later and the ratings of both avoidance ($\rho = -0.47$, $p<0.0001$) and intrusion ($\rho = -0.61$, $p<0.0001$) symptoms according to the IES. Regression plots are shown in Figures 14 and 15.

**Figure 14:** Regression plot of morning salivary cortisol rise (cortisol concentration at awakening +30 minutes – cortisol concentration at awakening) and IES Avoidance subscale ratings on the third follow-up investigation.

![Regression plot ofAvoidance](image1.png)

Regression line: $Y = 8.819 - .562 * X; R^2 = .157$

**Figure 15:** Regression plot of morning salivary cortisol rise (cortisol concentration at awakening +30 minutes – cortisol concentration at awakening) and IES Intrusion subscale ratings on the third follow-up investigation.

![Regression plot ofIntrusion](image2.png)

Regression line: $Y = 18.709 - 1.182 * X; R^2 = .306$
Discussion

This study is to my knowledge the first ten-year follow-up of UN soldiers with the aim to investigate the possible interrelationships between salivary cortisol concentrations, measured with immunoassays, and changes in self-rated posttraumatic symptoms, according to the IES self rating scale.

In the study only two of the 78 subjects included were women. This might be a methodological problem comparing the results. However, the salivary cortisol concentrations and the IES ratings from the two women included in the study did not diverge markedly from the results of the rest of the subjects. Therefore, the uneven sex diversion would probably not affect the results of the study.

The study population did not differ much regarding demographic data in comparison to the general population in Sweden during the ten-year follow-up according to the Statistiska Centralbyrån (SCB). When the study started, approximately 55% of all men in Sweden in the age span 20-45 years old were not married compared to 64% in this study. Most of the subjects, 86%, did not have any children when entering the study. This could indicate that one of the reasons the subjects went on the mission was that they did not have a family of their own to see to. Another reason could have been unemployment and the will to get more life experience by carrying out a humanitarian initiative before getting a higher degree of education. However, the number of unemployed at the start of this study did not differ from the number of unemployed in the general population. Nine percent of all men in Sweden in the age span 20-45 years were unemployed compared to 10% in this study. Ten years later 5% of all men in Sweden in the age span 30-55 years were unemployed compared to 3% in this study. Furthermore, 9% of all men in Sweden in the age span 20-45 years had completed a university degree compared to 18% in this study at the start of the study. Ten years later, 16% of all men in Sweden in the age span 30-55 years had completed a university degree compared to 32% in this study. It could be suggested that going away on at least one UN-mission was an inspiration to further education. Only a few subjects had combat experiences from earlier UN-missions.

During the ten years time of this follow-up study, our laboratory had worked on a methodological development of salivary cortisol assays. Concerning the safety of laboratory personnel and radioactive waste, Radio Immunoassay (RIA) was exchanged for Enzyme Immunoassay (EIA). On the two first follow-up investigations, salivary cortisol concentrations were determined with a modified commercial radioimmunoassay from Diagnostic Products Corporation, designed for determination of cortisol in serum and urine. On the third follow-up investigation, salivary cortisol concentrations were determined with a commercial EIA designed for saliva from Salimetrics, LLC. This salivary cortisol EIA was calibrated against liquid chromatography-mass spectrometry (LC-MS) and adapted for automated assay with NexGen Four (ADALTIS). The use of two different assay methods in this study is of course a methodological problem that has to be taken into account. In order to minimize this problem, the modified RIA was compared with the new automated EIA, before analyzing the saliva samples from the third follow-up investigation as earlier mentioned. The comparison showed good correlation and agrees with a similar comparison by Raff and co-workers (23). Therefore, we decided it possible to compare the results from the three follow-up investigations.
One other fact that has to be taken into account is the maybe small, but yet, differences in time points and situations for saliva sampling on the three follow-up investigations. On the first follow-up investigation, the subjects performed the morning saliva sampling at 8 AM after having been woken up at 7 AM. On the second follow-up investigation, the subjects performed the morning saliva sampling at home. However, they were instructed to perform the sampling at 8 AM after lying down for at least 30 minutes and not to eat, drink, smoke or brush their teeth for 60 minutes prior to sampling. The different situations could have affected the results, as the subjects might not have been as accurate when taking the saliva samples at home. On the third follow-up investigation, the saliva sampling was performed at wake-up, 30 minutes later and at bedtime. On this occasion, wake-up was at 7 AM and bedtime at 10 PM. These facts could indicate that the salivary cortisol value at “wake-up + 30-minutes” probably could be compared to the 8 AM values from the two first investigations.

According to previous research 50% of healthy subjects show a morning rise in cortisol concentration with a peak at 30-45 minutes and approximately 80% are responders to an awakening challenge of +2.5 nmol/L or more (7). Lack of morning cortisol rise might indicate cognitive dysfunction (7). In this study, the mean morning cortisol rise on the third follow-up investigation, i.e. the difference between the salivary cortisol concentration at “wake-up + 30-minutes” and the concentration at “wake-up”, was approximately 5 nmol/L indicating an overall fairly normal average morning cortisol rise. However, the morning cortisol rise on the third follow-up investigation was significantly correlated to the IES ratings in the way that subjects with lower than a 5 nmol/L morning cortisol rise showed a higher IES rating and in particular in the intrusion subscale. The substantial relationship between the morning cortisol rise and the ratings of intrusion symptoms with IES and the much more moderate relationship between the morning cortisol rise and the ratings of avoidance symptoms might indicate that the IES should not in the first place be evaluated by the total score but by the subscales individually.

Probably, all experience was psychologically processed with time and the subjects could handle their problems in a better way ten years after the mission, when it comes to avoidance, allowing themselves to think back and talk about different situations. Maybe this was hard when just coming home to Sweden and to their families. In Bosnia their lives changed; no beds, no proper food, nothing exclusive, little sleep, it was unsafe and they had to be on guard all the time. It is difficult to understand the situation during a war, even for a psychiatrist. Even though they were offered professional help not all found it helpful, because it was not easy explaining and describing everything, and that was not what they wanted to do. They rather wanted someone who knew what it was like, so that they did not have to tell basic facts such as explaining the geography, military terms etcetera.

When comparing the results of the IES ratings from the three follow-up investigations it should be noticed that the total IES score increased over ten years of time. However, when looking at the two different subscale ratings the intrusion symptom ratings increased while the avoidance symptom ratings decreased, why it is central to evaluate the subscales separately and not only the total score. One possible explanation to these results could be that experiences that have been denied have fallen into oblivion.
Another point to consider is that memories that have been avoided will be denied over time. A third reflection might be that persons who do not have the mental power to avoid or deny the memories may suffer from intrusive memories and/or thoughts. However, a theoretical explanation to this result is lacking today. It is however not quite unlikely that there is a physiological explanation to the relationship between the change in cortisol concentration and especially intrusive symptoms since these are represented by for example flash-backs, nightmares and panic attacks expressed by increased heart frequency and blood pressure, sweating etcetera.

When looking at the ANOVA calculations of the interrelationships between changes in salivary cortisol concentrations and IES ratings over time, it can be stated that neither change in morning nor evening cortisol was related to avoidance symptoms. However, both increasing evening cortisol over time and a flattening of the diurnal cortisol rhythm (i.e. decreasing morning cortisol and increasing evening cortisol over time) was correlated to higher ratings of intrusive symptoms.

**Conclusions**

There was no statistically significant change in either morning or evening cortisol concentrations over ten years of time after a six months UN-mission to Bosnia.

The intrusive post-traumatic stress symptoms according to the IES subscale increased while the avoidance subscale ratings decreased over ten years of time after a six months UN-mission in Bosnia.

There was an interrelationship between the change in both morning and evening salivary cortisol concentrations, measured with immunoassays, and changes in self-rated posttraumatic intrusive symptoms, according to IES, during ten years follow-up, after a six months mission in Bosnia.

There was a statistically significant correlation between the rise in morning salivary cortisol, from awakening until 30 minutes later, and the ratings of both avoidance and intrusion symptoms according to the IES.

This study suggests an interesting relationship between physiology and psyche. Maybe in future research, the concept of this study could be used more often on subjects exposed to traumatic stress for a better understanding of long term changes in somatical and psychological symptoms.
References


Appendix I

Impact of Event Scale – Revised (28).

INSTRUCTIONS: Below is a list of difficulties people sometimes have after stressful life events. Please read each item, and then indicate how distressing each difficulty has been for you DURING THE PAST SEVEN DAYS with respect to ___________. How much were you distressed or bothered by these difficulties?

Response Anchors: 0 = Not at all; 1 = A little bit; 2 = Moderately; 3 = Quite a bit; 4 = Extremely.

1. Any reminder brought back feelings about it. I
2. I had trouble staying asleep. I
3. Other things kept making me think about it. I
4. I felt irritable and angry. A
5. I avoided letting myself get upset when I thought about it or was reminded of it. A
6. I thought about it when I didn’t mean to. I
7. I felt as if it hadn’t happened or wasn’t real. A
8. I stayed away from reminders of it. A
9. Pictures about it popped into my mind. I
10. I was jumpy and easily startled. A
11. I tried not to think about it. A
12. I was aware that I still had a lot of feelings about it, but I didn’t deal with them. A
13. My feelings about it were kind of numb. A
14. I found myself acting or feeling like I was back at that time. I
15. I had trouble falling asleep. A
16. I had waves of strong feelings about it. I
17. I tried to remove it from my memory. I
18. I had trouble concentrating. A
19. Reminders of it caused me to have physical reactions, such as sweating, trouble breathing, nausea, or a pounding heart. I
20. I had dreams about it. I
21. I felt watchful and on guard. A
22. I tried not to talk about it. A