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Abstract

Background: Acute or chronic stress can lead to physical and mental disorders. Measuring cortisol can objectify the degree of stress. Cortisol is traditionally measured in serum, but recently the relevant fraction of free cortisol can be reliably measured in saliva, using the very sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method. The use of saliva is non-invasive and allows easy serial testing around stressful events. The main objective of this study is to investigate whether serial saliva cortisol determinations using the LC-MS/MS method can be used to assess the stress response that first responders may experience during moments of acute professional deployment in their daily work.

Methods: Healthy first responders (police officers, firefighters, rapid response team, ambulance personnel, first aid and emergency medical personnel) were recruited to participate in a Euregional high-reliability simulation training ("Be aware"-scenario training, April, 19th 2018). At three time points, simultaneous venous blood samples and saliva samples were obtained. These time points were one hour before, immediately after and 10 hours after the simulation training. The correlation between changes in saliva cortisol measured by LC-MS/MS and serum cortisol at all 3 time points was determined. Results were compared with spectators not directly participating in the simulation.

Results: 70 subjects participated in the simulation. There was a strong correlation between the changes in saliva and blood cortisol at the three time points. A significant increase in blood and saliva cortisol was shown 1 hour after the experienced stress-moments. The levels had almost completely returned to baseline in all healthy volunteers 10 hours later. Cortisol in spectators was unaffected.

Conclusion: Serial saliva cortisol-measurements using LC-MS/MS is a reliable and fast non-invasive functional stress-assay, which can be easily collected in daily practice and used for investigation and monitoring of stress response in front line responders.

Key terms: first responder, care giver, saliva, stress, cortisol, validation, simulation

Running title:

Serial salivary cortisol measurements in acute stress moment

What this paper adds

What is already known on this subject:

- Acute or chronic stress can lead to physical and mental disorders
- Investigating the physiological stress response in first responders usually requires blood draw for serum cortisol, hindering evaluation of daily stress as well as research into how to minimise the stress response.
- Although cortisol is also released in saliva, its use has been limited by low levels of excretion and cross-reactivity with other endogenous and exogenous compounds.

What this study adds:

- In this prospective study, first responders participated in a simulation of a stressful situation, and had serum and salivary cortisol collected at three time points. Serum cortisol and salivary cortisol level using innovative LC/LC-MS method were highly correlated with each other. Both rose in the first hour after the stress and returned to baseline at 10 hours.
- The use of saliva as a matrix facilitates using this method to investigate the stress response of first responders.

Introduction

First responders are often exposed to moments of excessive acute stress; repeated episodes of can ultimately lead to physical and mental disorders. Moments of stress elicit a physiological as well as psychological response.[1-4] Cortisol is considered an objective biochemical marker of the physiological stress response.

It is known that the cortisol level increases as part of the systemic nervous system response to psychological and physical stressors, such as life events, exposure to extreme temperatures, a negative energy balance and physical exercise.[5] Cortisol has many specific functions supporting the body to adjust to and deal with the stress, including the mobilization of free fatty acids (FFA) from adipose tissue, protein catabolism, stimulation of liver gluconeogenesis, increased uptake of skeletal muscle glucose and blocking pancreatic insulin production.[6 7] These processes result in a rise in glucose level, which increase the exercise capacity and aid in recovery and adaptation.[8]

At a more detailed level, the stress response is characterised by two major temporal physiological responses, preparing the body to respond adaptively when confronted with a threat. The *first phase* of the response, the so-called fast response, is driven by the autonomic sympatho-adrenal-medullary (SAM) system, causing release of catecholamines (e.g. adrenalin and noradrenalin), increasing the blood pressure, heart- and respiratory rate. The *second phase*, the so-called slower response, is triggered by the hypothalamus-pituitary-adrenal (HPA) axis activation. When stressed, the hypothalamus secretes corticotrophin-releasing hormone (CRH), which activates the anterior pituitary and stimulates the release of the adrenocorticotropin hormone (ACTH). The presence of ACTH stimulates the adrenal cortex to release cortisol.[9] Cortisol secretion is controlled through a negative feedback process, where high levels inhibit the secretion of ACTH from the anterior pituitary. Additionally, high levels of ACTH and cortisol can signal the hypothalamus to reduce the secretion of CRH. This interconnected process is referred to as the hypothalamic-pituitary-adrenocortical (HPA) axis.[9]

From earlier studies it is known that extreme acute, as well as prolonged, stress situations can lead to very high levels of cortisol. This in turn can lead to physiological complaints, damage to tissues and contribute to weight gain, hypertension, sleeping problems, musculoskeletal pain and anxiety.[1] It is known that first responders, such as police officers, firefighters and paramedics, who are the first to arrive and provide assistance at the scene of

an emergency can experience psychological and physical stress, which may lead to, among other consequences, an increase in sick leave and/or post-traumatic stress syndrome.[1 10 11]

Measuring the stress response of first responders can be useful in several ways. First it is important to monitor the cumulative stress on these individuals to prevent negative consequences. Additionally, empirical evidence suggests that continuous professional physical as well as mental training can help to better handle certain possible stressful situations and that such training the increase and peak levels in cortisol are lower.[12-14] Moreover, because of the differences in training level, but also in frequency of training moments between the groups of first responders and caregivers, it is hypothesised that differences in cortisol response to critical situations between subgroups of first responders exist.

A major practical issue limiting the investigation of the stress response in daily practice of first responders or caregivers or evaluating the impact of training or differences in responses by different professions, is that real-time, serial sampling of cortisol levels has not been easy to perform. The most commonly used method requires a blood specimen acquired through venepuncture. In contrast, salivary specimens can be readily collected by the participants themselves, under different conditions and repeatedly throughout the day. However, salivary cortisol levels of low, and tests can be affected by cross reactivity with exogenous glucocorticoids and endogenous cortisol precursors and metabolites in immunoassays. Detection and analysis of salivary cortisol by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) however, provides the specificity required to eliminate cross-reactivity by related steroids.[15] With this relatively new sensitive and specific method, it is possible to investigate the variation in stress response over time by serial salivary cortisol determinations. Although reports of salivary cortisol measurements by LC-MS/MS have been published, studies in the context of daily fieldwork of first responders and caregivers are so far lacking.

It is known that cortisol has a short half-life (in serum 42.1 minutes and in saliva, 28.9 minutes).[16 17] However, non-published data suggest that cortisol is excreted up to 10 hours after an acute stress moment (personal communication, University of Bochum, Germany). It is expected that in healthy persons the cortisol-rise due to the acute stress moment thus returns to base-line levels within 10 hours.

The main objective of this study was to perform serial salivary cortisol measurements before, during and 10 hours after a high-reliability simulation training using LC-MS/MS and using

serum cortisol as the gold standard. This aimed to determine whether the use of salivary cortisol is equivalent to serum cortisol in monitoring the stress response. Additionally, the inclusion of several different groups of first responders allowed us to determine the level of stress-response in different subgroups of first responders.

Methods

Study populations

We recruited a convenience sample of voluntary participants in a Euregional high-reliability simulation training, “Be Aware” April 19, 2018, organised by the Limburgian Society of Intensive Care (LVIZ), Kerkrade, The Netherlands. The volunteers belong to one of five groups of first responders/caregivers: police officers, firefighters, military elite group (rapid response team), ambulance paramedics, emergency department medical personnel. A control group of colleagues of the participants was also recruited as observers. The volunteers had no cardiovascular risk factors (absence of obesity and diabetes mellitus, no smoking), or known heart disease or primary or secondary glucose metabolism disorders.

Simulation scenario

The simulation exercise involved a two-hour simulated terrorist scenario based on the Battaclan terrorist attack of Paris in 2015. The scenario took place outside and made use of a large empty monastery as location were a shooting and hostage took place. A group of actors played the role of terrorists, many hostages and wounded victims. Next to it, a real large fire was made in different parked cars in front of the monastery. Different groups of real first responders were guided to the location of the attack and had to operate with each other for eliminating the terrorists, rescuing the hostages, giving first-aid to the wounded victims at place, and to mobilise them for transport to the nearest emergency department of an hospital, but also to create a safe working space for each other. The control group was watching the scenario by sitting on a stand in the neighbourhood where the action took place.

Consent and data handling

All participants were informed about the nature of the study and signed informed consent. Furthermore, the study was performed according to the Helsinki Declaration and was approved by the Medical Ethical Committee of the Zuyderland Medical Hospital.

Serial samples collection

Simultaneous venous blood samples (serum tubes) and salivary samples were obtained from all participants (including the control group) one hour before, immediately after and 10 hours after the simulation training. Saliva samples were obtained using the salivette tubes (cotton swab with citric acid preparation; Sarsted). For obtaining a saliva sample, patients chewed on the cotton swab for one minute and then placed it back in the plastic container. Samples were processed, including centrifugation, within 2 hours. All volunteers, as well as lab

technicians and investigators, were blinded to the results of the salivary samples of the individual participants as well as the participant groups. Data were analysed anonymously and reported results could not be traced back to individual participants.

Laboratory assays

The serum sample was analysed using an automated competitive immunochemoluminescent assay (Siemens Immulite, lower limit of quantitation 30 nmol/L, within-run CV 4.0, 4.4% and between-run CV 3.6, 2.7% at 156 and 1137 nmol/L respectively).

Salivary cortisol was analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Salivette tubes were centrifuged at 800g for 10 minutes and the recovered saliva was stored at -20°C until analysis. 500 µl of sample, calibrator plus internal standard (d2-cortisol) was transferred to dedicated sample tubes. Extract (50 µl) was injected onto a 4 X 2 mm C8 Gemini guard cartridge for on-line solid phase extraction. The compounds were eluted from the guard cartridge onto a Phenomenex 30·3 mm 4 lm Synergy Hydro-RP C18 analytical column. The eluant was then injected directly into a high-performance liquid chromatography system (Shimadzu model LC-MS 8050), coupled to a triple quadruple mass spectrometer. Cortisol and d2-cortisol were monitored using transitions (m/z) 363.2 > 121.1 and 365.1 > 122.2 respectively.

The determination of salivary cortisol using the LC-MS/MS-method was validated internally according to the ISO15189-guidelines, as well as based on the guideline for bioanalytical method validation for the European Medicines Agency.[18] This test has a lower limit of detection of 20 pmol/L, a linear range from 2 to 30 nmol/L, an intra-assay coefficient of variation (CV) of 2.42% and total CV of 4.36%. Method accuracy determined by spike recovery averaged 91%.

Sample stability over time from saliva collection using the salivette tubes was tested prior to the study by storing freshly collected saliva samples of 5 healthy volunteers at room temperature (RT) and preparing them at time points 0 (T= 0) and 5 days (T= 120 hrs). Salivary cortisol was determined by the LC-MS/MS-method described above and the differences between the two measurements were expressed as relative coefficients of variation (RCV; %). The mean concentration at each level should be within ±15% of the concentration at T=0.[18]

Outcomes:

The primary outcome of interest was the correlation of serum and salivary cortisol at the three time points of collection. Secondary outcomes were the pattern of rise and fall

associated with the stages of the simulation, and differences in this correlation between subgroups of first responders.

Statistical analysis

Continuous variables were expressed as mean ± standard deviation for variables with a normal distribution and median (interquartile range) for skewed variables. Normality was tested by the Kolmogorov-Smirnov test. Because the results were normally distributed, the Pearson-r method was used for the correlation between serum total cortisol and salivary cortisol. Statistical significance was assumed at $p<0.05$. Comparisons among the different groups of participants were analyzed by ANOVA.

For the analysis of the sample stability experiment a paired t-test was used. Statistical analysis was performed using Microsoft Excel (Microsoft, Redmond, WA) and GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) software.

Results

Seventy volunteers (42 males and 28 females) from six different professional groups participated in the study. 36 were in the simulation group and 34 were in the control group. Overall mean age was 39. The mean ages of those participating in the simulation ranged from 26-59 depending on profession, with the highest age in emergency department professionals. The mean age of the control group was 48. All participants provided serum and saliva samples at all 3 time points.(Table 1) There were no missing data.

Cortisol measurements during the simulation training

Table 1 shows the results of the serum cortisol as well as the salivary cortisol determinations at the three different time points for the different groups of first responders. To correct for the relatively large inter-individual biological variation the results were also expressed as relative change (expressed in %) using the time point '1 hour before the simulation training' as basal level/output value (100%), see figure 1. No significant differences were found between the different groups of first responders.

Cortisol in blood (the gold standard) showed a significant increase between the measurement before and 1 hour after the training ($p<0.05$) in all subgroups of first responders, with exception of the control group. Cortisol levels ten hours after the training showed a significant decrease when compared to + 1 hour ($p<0.01$), in the total group as well as in *almost all* different groups of first responders (with exception of the personnel of the emergency room). The rapid response team subgroup showed the largest increase, while the emergency room personnel had the smallest increase.

Salivary cortisol also increased in *almost all* subgroups 1 hour after the scenario training. It was most pronounced in the rapid response team subgroup. Finally, it showed a significant decrease in all subgroups ten hours after the training when compared to +1 hour ($p<0.001$). The control group showed no significant differences in serum as well as salivary cortisol between the 3 different time points

Correlation between serum and salivary cortisol

The correlation between the cortisol concentration in serum and saliva of all participants (including control groups) determined at the 3 different time points is presented in Figure 2; a significant linear correlation was found between serum and salivary cortisol ($R= 0,75$, $p<0.0001$), yet the data better fitted an exponential curve ($R^2= 0,78$, $p<0.00001$).

Sample stability over time

Table 2 shows the results of salivary cortisol stored for 0 hours and for 120 hours in five healthy volunteers. This shows that the differences (RCV) in salivary cortisol levels (nmol/L)

between the two time points (0 hr vs 120 hrs) were smaller than 15% in all samples, illustrating the sample stability assessment of salivary cortisol when using the salivette tubes.

Discussion

This study compared serially collected serum and salivary samples of different groups of first responders at fixed time points during and after simulation training. Consistently lower levels of cortisol were found in saliva when compared with paired blood samples, yet a strong correlation between these two measurements was demonstrated. One hour after a stressful period, a rise in cortisol (serum as well as salivary cortisol) was seen, which was normalized 10 hours later. This rise and fall of cortisol were absent in the control group.

When a person perceives a personal situation as stressful, a series of physiological mechanisms are set in motion, notably activation of the sympathetic- and adrenomedullary system and of the hypothalamic-pituitary-adrenal axis (HPA). Like the sympathetic nervous system, the hypothalamic-pituitary-adrenal axis (HPA) is influenced by physical training of the stress-situation.[19 20] In this study this is reflected by the fast and highest increase of serum as well as salivary cortisol, followed by a complete normalisation of cortisol at time level +10 hours.

The very low mean salivary cortisol concentrations determined with the relatively new, sensitive and specific LC-MS/MS-method show a strong correlation with values obtained with the gold standard assays as performed on peripheral serum, although an exponential relationship was the best fit for the data. A similar finding was reported by Perogramvros et al.[21] who proposed that this relationship supports the concept of corticosteroid binding globulin (CBG) binding capacity saturation. Bound steroids are biologically inactive; the unbound or free fraction is active. Since cortisol is a lipophilic steroid hormone with a low molecular weight, the cortisol that is unbound to CBG can enter the cells through passive diffusion. Since only a small, unbound fraction of the hormone is available to diffuse into the saliva, concentrations are consistently lower than in serum. Salivary cortisol has been traditionally measured by immunoassays, in which cross-reactivity by other steroids, exogenous glucocorticoids and endogenous cortisol precursors and metabolites can be a major limitation.[21] In contrast to previously used antibody-based assays, LC-MS/MS is a high specific method that eliminates cross-reactivity. Perogamvros et al showed in their study, that the method used in the present study, had no interference with other steroids, including prednisolone and cortisone.[21] In addition to the study by Jonsson et al, they stated that this explains the consistently lower salivary cortisol values generated by LC-MS/MS when compared with that by various immunoassays.[15 21]

The noticed rise-and-fall in cortisol after a stressful event was most pronounced in the members of the rapid response team. This elite-group of first responders can be considered as the group with the highest exposure to training and consequently skills, which thus seems

to increase the capability to optimally handle stress moments. However, the exact mechanism behind this finding is unclear and needs further investigation.
The emergency room physicians showed the lowest rise-and-fall of all groups of first responders investigated. This can be explained by the fact that they were not at the location where the attack took place, but in their confident surrounding of their workspace in the hospital. Besides the fact of numerous victims, they were not confronted with other stressful events.
Finally, the control group that had no part in the scenario training, but in which cortisol was determined at the same time, showed no increase and normalisation, indicating that the noticed rise-and-fall was consistent with the stressful situation.

The stability of cortisol during sample storage and transport conditions are critical for the results of cortisol determinations and necessary to facilitate daily fieldwork sample collection. When allowing not more than 15% difference[18] between paired measurements over time, the stability assessment experiment using the salivette tubes revealed that salivary cortisol is stable for a minimum of 5 days. In addition, Nalla and co-workers used the same tubes in their study and showed that it was also stable under six different storage conditions.[22] However, they used an enzyme immunoassay for the determination of salivary cortisol. Our study is supportive of the fact that this also applies to the salivary cortisol determination using the LC/LS-MS method.

Limitations

The study has a number of limitations. It is known that cortisol has a diurnal rhythm and reaches its maximum a few hours before and lasts 1 hour after awakening. Cortisol then declines progressively during the day to reach its nadir in late evening. The simulation training was conducted in the morning. This strategy of serial measurements in time needs further validation for their value in assessing stress moments occurring at other moments of the day.
In this high-reliability simulation training all participants had to deal with events containing physical and mental stressors. However, they all knew that this was a training and not a real-life event. Future studies should be performed in real life-situations of stressful events.
Finally, we chose a 10-hour interval before determining final salivary cortisol levels. This choice was based on unpublished results that found that in patients who experience a heavy acute stressful moment, the excretion of cortisol by the adrenal glands can exist for 10 hours. The presented study was performed in healthy first responders, and in all participants the cortisol levels at time point +10 hrs returned to baseline. Future studies need to be performed to investigate if it is possible to determine first responders in which cortisol does not decline

within 10 hrs after experiencing a stressful moment, and if these persistent elevated levels of cortisol are associated with more sickness, somatic complaints, or psychological impairment.

In summary, comparison of the results of the salivary cortisol determination using the salivette tubes reveals a strong correlation with the results of the serum cortisol determination. This serial salivary cortisol testing method is a reliable and fast non-invasive functional assay to gain objective insight in the stress response experienced by first responders during acute events. Due to the sample stability for minimally 5 days, it is a feasible and acceptable collecting method in the daily fieldwork of stress-investigations.

Tables

Table 1 Median values and interquartile ranges for cortisol (serum as well as salivary) at the 3 different time points in the different groups of first responder's versus control group

Parameter		First responders active in the simulation					Control group
		Ambulance	Emergency department	Firefighters	Police officers	Rapid Response Team	
Number of participants	n=	5	5	10	8	8	34
Gender	M/F	4/1	2/3	9/1	7/1	8/0	12/22
Age	Mean	26	59	41	35	26	48
Serum cortisol	-1 hr	0.27 [0.19- 0.36]	0.28 [0.26-0.44]	0.22 [0.19-0.24]	0.28 [0.20-0.34]	0.24 [0.20-0.37]	0.33 [0.27-0.41]
(µmol/L)	+ 1 hr	0.32 [0.19-0.49]	0.35 [0.26-0.46]	0.29 [0.25-0.37]	0.35 [0.27-0.41]	0.44 [0.32-0.50]	0.24 [0.18-0.30]
	+10 hrs	0.14 [0.09-0.18]	0.16 [0.08-0.27]	0.10 [0.07-0.16]	0.11 [0.08-0.23]	0.11 [0.10-0.23]	0.13 [0.09-0.18]
Salivary cortisol	-1 hr	2.35 [0.56-3.86]	1.77 [1.64-4.16]	1.82 [1.48-2.19]	2.24 [1.01-2.81]	1.61 [1.13-5.01]	4.14 [2.77-6.77]
(nmol/L)	+ 1 hr	3.67 [1.56-7.40]	5.44 [2.35-6.82]	2.84 [2.41-4.28]	4.17 [1.30-7.25]	5.79 [1.78-9.80]	1.66 [1.17-2.99]
	+10 hrs	0.75 [0.34-0.91]	1.17 [0.30-2.22]	0.61 [0.34-0.78]	0.55 [0.29-1.24]	0.50 [0.37-1.03]	0.56 [0.31-1.31]

Data are presented as median [25^{th}-75^{th} percentile]

Table 2 Stability of salivary cortisol using Salivette tubes (time frame= 5 days)

Sample nr	Salivary cortisol (nmol/L)		Relative difference (%)
	T= 0 hr	T= 120 hrs	
1	6,34	6,87	8,5
2	0,63	0,60	5,5
3	14,85	14,59	1,7
4	1,05	0,97	7,8
5	0,95	0,98	3,2
Paired t-test			
Mean of differences	-0.070		
95% Confidence interval	-0.45 to 0.31		
Correlation coefficient	0.999		
P-value	<0.0001		

CI= confidence interval

Legends to the figures

Figure 1

Plots of cortisol in blood vs salivary cortisol measured in the different groups of first responders, just before, immediately after (+ 1hr) and 10 hr after the simulation training. The vertical dashed line represents the moment of the stress event.

Figure 2

Curve fitting of serum versus salivary cortisol data (n= 210 paired samples).

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Ethical approval statements

All participants were informed on the nature of the study protocol and signed informed consent. The study was performed according to the Helsinki Declaration and was approved by the Medical Ethical Committee of the Zuyderland Medical Hospital (registration no. METCZ20180039). Data were analysed anonymously and reported results could not be traced back to individual participants.

Clinical Trial Registration

Not applicable.

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Competing interests

None declared.

Authorship contributions

M.S. and M.P.G.L. designed and conceptualised the research. M.S. and M.P.G.L wrote the first concept of the article. M.S. and M.P.G.L. coordinated all aspects of this study. PvdB, WD and WvM commented on the initial draft version of the manuscript and were involved in actively revising the subsequent draft and discussing them in the research until submission of the final version.

Conflict of interest disclosures

No potential conflict of interest was reported by the authors.

Patient and Public Involvement

There were no patients involved in this study. However, from each group of first responders one representative was involved in designing the scenario training. The volunteers in each group of first responders were recruited by their supervisors.

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