

## Determination of the Daily Rhythm of Cortisol in the Saliva of Women and Men

Ljubijankić, N.<sup>a,\*</sup>, Kučukalić, E.<sup>b</sup>, Ljubijankić, S.<sup>c</sup>

<sup>a</sup> Faculty of Science, Department of Chemistry, Zmaja od Bosne 33-35, Sarajevo, Bosnia and Herzegovina

<sup>b</sup> Clinical Center University of Sarajevo, Institute of Clinical Biochemistry, Bosnia and Herzegovina

<sup>c</sup> School of Health Studies, Irfana Ljubijankica, Bihac

### Article info

Received: 08/06/2016  
Accepted: 13/06/2016

### Keywords:

Cortisol  
Saliva  
Daily rhythm  
Woman  
Men

### Corresponding author:

E-mail: [nevzetalj@gmail.com](mailto:nevzetalj@gmail.com)  
Phone: 033 279 956

**Abstract:** Saliva provides a useful and noninvasive alternative to blood for many biomedical diagnostic assays. Recently, saliva has been used as a biological sample of choice for the monitoring of hormones and other clinically important biomolecules. The assessment of cortisol in saliva has proven to be a valid and reliable reflection of the respective unbound hormone in blood and is widely accepted and a frequently employed method. Due to several advantages over blood cortisol analysis (e.g., stress-free sampling, laboratory independence, lower costs) saliva cortisol assessment can be the method of choice in basic research and clinical environments. Synthesis and secretion of cortisol has the most obvious circadian rhythm in nature. The highest concentration of cortisol in extracellular fluids is in the morning hours and the lowest one in the evening. The object of this study was to determine and compare the daily fluctuations of cortisol in saliva by measuring cortisol levels in the saliva of healthy individuals daily in certain periods. Next step was to compare the values of the concentrations of cortisol in test subjects of different sexes and determine the benefits of analysis of cortisol in saliva.

## INTRODUCTION

Cortisol is the main glucocorticoid hormone which is secreted by the cortex of the adrenal gland whose release is controlled by the adrenocorticotropic hormone (ACTH), synthesized from cholesterol (Koraćević et al., 2003). ACTH is controlled by the hypothalamic peptide, corticotrophin releasing factor (CRF). High plasma concentrations of cortisol inhibit the release of CRF and ACTH through the negative feedback mechanism (Kreiger, 1975).

According to its chemical structure cortisol is a steroid hormone (Guyton and Hall, 2006). Cortisol binds to specific intracellular receptors and affects many physiological functions, including the immune system, glucose level, blood vessels and bone metabolism. Cortisol affects metabolism of carbohydrates, fats and proteins (Guyton, 1996). Cortisol is involved in the response to stress and is necessary for proper functioning of organism. Secretion of cortisol in plasma occurs

periodically and starts 5-10 minutes after the secretion of ACTH. The concentration of cortisol in plasma raises gradually and reaches its highest values shortly after waking up, and then during next few hours gradually decreases and reaches the lowest value late in the afternoon and early in the evening (Gafni, et al., 2000). Synthesis and secretion of cortisol has the most obvious circadian rhythm in nature (Kirschbaum and Hellhammer, 1999). The disappearance of this circadian rhythm is present in adrenal pituitary gland, Cushing's syndrome, and Addison's disease. Neuroendocrine mechanism increases the release of cortisol up to 20 times in mental stress conditions (Ardal and Holm, 1995).

Daily rhythm, stimulation of ACTH and cortisol is affected by stress, hard injuries, burns and psychological traumas, what is trouble for diagnosis of mentioned illnesses (Kirschbaum *et al.*, 1995b). Cortisol in plasma circulates in connection with proteins and individually. Most plasma cortisol is bound to corticosteroid binding

globulin (CBG, also known as transcortin) due to its high affinity. About 65% of cortisol binds for CBG, 30% for albumins and 3-5% is free (Kršljak and Gošić, 2008).

Concentration of cortisol in extracellular fluids is a reliable marker of the hypothalamic-pituitary-adrenal feedback system. Stress intensity is related to the cortisol level released in such situations because stress is a generalized body reaction aimed at protecting the organism and preventing injury (Ruzić, 2005).

Cortisol is usually determined in blood and urine, and lately, saliva has been tested for validity of cortisol assay (Nicolson *et al.*, 1997).

Saliva provides a useful and noninvasive alternative to blood for many biomedical diagnostic assays.

Salivary cortisol concentration represents 70% of unbound blood cortisol. Due to its low molecular weight and liposolubility, unbound cortisol penetrates the cellular membrane by simple diffusion which enables the level of free cortisol to be determined in all body fluids (Gafni *et al.*, 2000). There is a high correlation between salivary cortisol levels and unbound free cortisol levels in plasma and serum (Hellhammer *et al.*, 2009).

By determination of concentration of cortisol in serum or plasma, intensity of secretion and its effect on tissue can be defined. In this determination it is necessary to know that analysis of one sample of serum or plasma shows

## EXPERIMENTAL

### Materials and methods

Samples of saliva are taken from 21 healthy individuals, 11 men and 10 women, two times, 8:00-9:00 a.m. and 5:00-6:00 p.m.. Samples of saliva are centrifuged in order to remove cellular debris. Samples of saliva were kept at temperature of  $-24^{\circ}\text{C}$ .

For determination of cortisol on autoanalyzer (Vitros ECI immunodiagnosics system) it was used biochemical laboratory kit: Vitros Immunodiagnosics Products Cortisol Reagent Pack, Ortho-Clinical Diagnostics, Johnson-Johnson company.

### Immunochemical method for determination of cortisol on autoanalyzer

Measuring of cortisol concentrations in serum and saliva is done on autoanalyzer, by immunochemical method, which is based on luminescent immunochemical reaction.

Determination is based on competitive binding of cortisol in sample and cortisol marked with peroxidase, on binding places with special antibodies (sheep polyclonal anti-cortisol). Antibody-antigen complexes which are formed are trapped with streptavidin at barriers of vessel. Materials that are not bound are taken out with washing. The activity of bound peroxidase is measured by luminescence reaction. Bound peroxidase catalyses oxidation of luminol forming derivatives that produce the light measured by analyser. The amount of bound peroxidase is inversely proportional to concentration of present cortisol.

Statistical analysis of the results is done using by Student's *t* test.

concentration in that particular moment. By determination of concentration of cortisol in 24 - hours urine volume, the amount of cortisol secreted during the day is defined, by which the function of the cortex of adrenal gland can be defined (Reid *et al.*, 1992).

Given that, the salivary sample collection is less invasive than blood sampling, many authors agree that determination of salivary cortisol would be more appropriate than blood cortisol (Kirschbaum *et al.*, 1995a).

The cortisol values in plasma and saliva vary during the day. The reference values for cortisol concentration are classified according to the time of sampling. These values are affected by several factors which can vary, so it is recommended that every laboratory should define its own reference values. The reference values of cortisol in saliva, determined in Laboratory of Clinical Biochemistry of Institute for Clinical Chemistry and Biochemistry of Clinical Centre at University of Sarajevo, are following: 3,5 – 49,0 nmol/L (in the morning), and 1,3 – 37,0 nmol/L (in the afternoon).

Since the daily routine (stress, physical effort, nutrition) can significantly affect cortisol concentrations, we determined cortisol concentrations in the late evening hours and compared to those determined in the morning.

## RESULTS AND DISCUSSION

### The concentration of cortisol in saliva of female subjects

The concentrations of cortisol in the saliva of women in the morning and in the afternoon are shown in Table 1, and graphical results are shown in Figure 1. The results showed that in any case, cortisol concentration was lower in the afternoon. The cortisol levels in some individuals were higher than the reference values both in the morning and in the afternoon, which can be attributed to the effect of stress. The mean concentration of cortisol in the morning was 35,8 nmol/L, and in the evening was 23,14 nmol/L.

The results showed that concentrations of cortisol in saliva of female subjects in the morning were statistically significantly higher in comparison to those in the afternoon ( $p^{**}<0.01$ ). There are daily fluctuations in cortisol concentrations in saliva taken from female subjects.

**Table 1.** Concentrations of cortisol in saliva of women in the morning and in the afternoon

Number of sample	Cortisol (nmol/L)	
	In the morning	In the afternoon
1	52,1	40,8
2	37,1	26,6
3	49,1	37,4
4	67,4	57,1
5	19,1	4,9
6	24,5	11,3
7	19,5	9,2
8	32,4	12,1
9	18,9	10,1
10	31,7	21,9

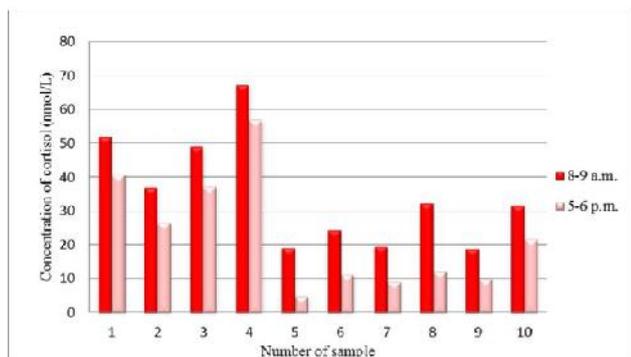


Figure 1. Concentrations of cortisol in saliva of female subjects in the morning and in the afternoon

**The concentration of cortisol in saliva of male subjects**

The concentrations of cortisol in the saliva of male subjects in the morning and in the afternoon are shown in Table 2, and graphical results are shown in Figure 2. The results showed that in any case, the concentration of cortisol was lower in the afternoon. Among males, there are evident individual differences in the concentration of cortisol in saliva. The mean concentration of cortisol in the morning was 32,23 nmol/L, and in the afternoon or evening 20,30 nmol/L. The results showed that concentrations of cortisol in saliva of males were statistically significantly higher in the morning in comparison to those in the afternoon/evening ( $p^{**} < 0.01$ ). Also, the results showed that there are daily fluctuations in cortisol concentrations in saliva of males.

Table 2. Concentrations of cortisol in saliva of males in the morning and in the afternoon

Number of sample	Cortisol (nmol/L)	
	In the morning	In the afternoon
1	24,7	15,9
2	65,5	40,8
3	49,9	37,4
4	67,4	47,1
5	15,5	9,6
6	41,6	17,4
7	16,9	11,6
8	13,1	5,2
9	16,5	8,5
10	21,2	9,5

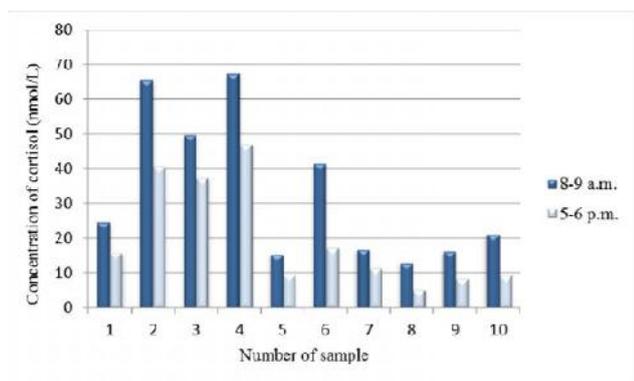


Figure 2. Concentrations of cortisol in saliva males in the morning and in the afternoon

**Comparison of cortisol concentrations in saliva of female and male subjects**

Results presented in Table 3., and graphically presented in Figure 3, show that there are daily fluctuations of cortisol concentrations in saliva of male as well as female subjects. The levels of cortisol in saliva in some participants were higher than the reference values which may be caused by other factors (illness, stress). The cortisol concentrations in saliva of both sexes in the morning were statistically much higher than those in the afternoon/evening (Ljubijankić *et al.*, 2008). Regarding the female subjects, the higher mean cortisol concentrations in saliva were found in both time of sampling (in the morning and in the afternoon/evening) in comparison to males. Women seemed to have higher cortisol levels than men probably due to the fact that women are more sensitive to stress. However, these differences in cortisol concentrations between subjects of different sexes were not statistically significant.

Table 3. The middle value cortisol concentrations (and standard deviations) in saliva of female and male subjects in the morning and in the afternoon

Time of sampling	Middle value of cortisol concentration ± S.D. (nmol/L)	
	Females	Males
8-9 a. m.	35,8 ± 16,42	33,23 ± 21,30
5-6 p. m.	23,14 ± 17,13	20,30 ± 15,39

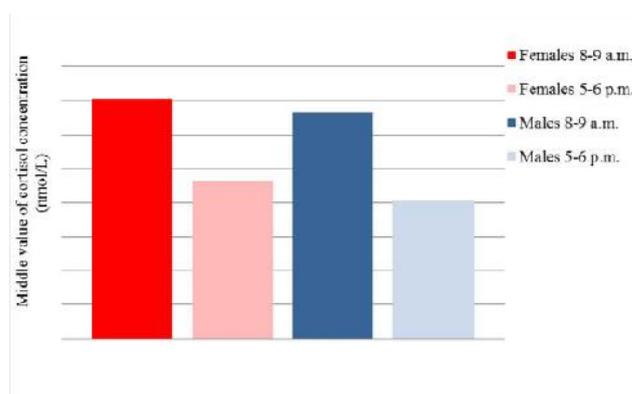


Figure 3. Comparison of cortisol concentrations in saliva of female and male subjects in the morning and in the afternoon

**CONCLUSION**

There are very complicated daily fluctuations of cortisol concentrations in saliva of healthy people of both sexes, because mean cortisol concentrations in the morning were statistically significantly higher in comparison to those in the afternoon/evening. Higher cortisol concentrations in saliva of female subjects were found when compared to male subjects, but these differences were not statistically important. Individual variability of cortisol concentration is evident during the day. The cortisol concentrations in saliva are really low, so it is recommended that in cases of metabolic disorders, sampling time of saliva should be taken in consideration.

Measurement of saliva cortisol, especially in early morning hours could be completely substitute the measurements of cortisol in serum or plasma.

The non-invasive sampling procedure allows saliva to be used for cortisol level determination in situations where blood sampling is difficult to perform.

Unlike blood, saliva contains free cortisol. Bearing in mind that the only biologically active form is free cortisol, the value obtained in saliva could be used as an objective parameter for changes in the value of cortisol in one day (morning, noon and evening).

## REFERENCES

- Ardal, E., Holm, A.C., (1995). Cortisol in Saliva Reference Ranges and Relation to Cortisol in Serum. *European journal of clinical chemistry and clinical biochemistry*, 33, 927-932.
- Gafni, R.I., Papanicolaou, D.A., Nieman, L.K. (2000). Nighttime salivary cortisol measurement as a simple, noninvasive, outpatient screening test for Cushing's syndrome in children and adolescents. *The Journal of Pediatrics*, 137(1), 30-35.
- Guyton A.C, (1996), *Hormones of cortex of adrenal Gland. Medicalphysiology*, Savremena administracija, Beograd, 854-862
- Guyton, A.C., Hall. J. E., (2006). *Endocrinology and reproduction, Medical physiology*. Medical Biochemists, Zagreb, 905-1024.
- Hellhammer, D. H., Wust,S., Kudielka, B. M. (2009). Salivary cortisol as biomarker in stress research. *Psychoneuroendocrinology*, 34, 163-171
- Kirschbaum, C., Hellhammer, D.H., (1999). Noise and stress – salivary cortisol as a non-invasive measure of allostatic load. *Noise Health*, 1, 57-65.
- Kirschbaum, C.,Klauer, T., Filipp, S.H., Hellhammer, D.H., (1995a). Sex-specific effects of social support on cortisol and subjective responses to acute psychological stress. *Psychosomatic Medicine*, 57(1), 23-31.
- Kirschbaum, C., Prussner, J.C., Stone, A.A., Federenko, I., Gaab, J., Lintz, D., Schommer, N., Hellhammer, D.H., (1995b). Persistent high cortisol responses to repeated psychological stress in a subpopulation of healthy men. *Psychosomatic Medicine*, 57(5), 468-474.
- Koračević, D., Bjelaković, G., Đorđević, V., Nikolić, J., Pavlović, D., Kocić, G., (2003). Biochemistry of hormones, Saliva, *Biochemistry, Savremena administracija, Beograd*, 706-815; 1040- 1044.
- Kreiger, D.T., (1975). Rhythms of ACTH and corticosteroid secretion in health and disease, and their experimental modification. *Journal of Steroid Biochemistry*, 6, 785-791.
- Kršljak, E., Gošić, K., Determination of cortisol levels in plasma and saliva in women exposed to chronic stress. (2008). *Serbian Dental Journal*, 55, 163-169.
- Ljubijankić, N., Popović–Javorić, R., Šćeta, S. Šapčanin, A. Tahirović, I., Sofić, E. (2008). Daily fluctuation of the cortisol in the saliva and serum of healthy persons. *Bosnian Journal of Basic Medical Sciences*, 8(2), 110-115.
- Nicolson, N., Storms, C., Ponds, R., Sulon, J.(1997). Salivary Cortisol Levels and Stress Reactivity in Human. Aging, *Journal of Gerontology*, 52A (2), M68-M75.
- Reid, J.D., Intrieri, R.C., Susman, E.J., Beard, J.L., (1992). The Relationship of serum and salivary cortisol in a sample of healthy elderly. *Journal of Gerontology*, 47(3), 176-179.
- Ruzić, B., (2005). Systemic stress responses in patients undergoing surgery for BPH. *British Journal of Urology*. 95, 77-80.

## Summary/Sažetak

Pljuvačka predstavlja korisnu i neinvazivnu alternativu krvi za mnoge biomedicinske dijagnostičke testove. U novije vrijeme se koristi kao dijagnostička tečnost za monitoring mnogih supstanci, pa i hormona. Određivanje kortizola u pljuvački je dokazano valjan i pouzdan odraz kortizola u krvi i široko je prihvaćena i često korištena metoda. Zbog nekoliko prednosti u odnosu na analizu kortizola u krvi (uzorkovanje bez stresa, laboratorijska nezavisnost, niži troškovi) mjerenje pljuvačnog kortizola može biti metoda izbora u osnovnim kliničkim istraživanjima. Sinteza i sekrecija kortizola ima najizrazitiji cirkadijalni ritam u prirodi. Najveća koncentracija kortizola u ekstracelularnim tečnostima je u jutarnjim satima, a najniža u večernjim satima.

Cilj ovog rada je da se utvrde dnevne oscilacije kortizola u pljuvački mjerenjem nivoa kortizola u pljuvački zdravih osoba u određenim dnevnim periodima, zatim da se uporede vrijednosti koncentracije kortizola u osoba različitog spola i utvrde prednosti analize pljuvačnog kortizola.