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# DAYTIME-RELATED RHYTHMICITY OF GAS DISCHARGE VISUALIZATION (GDV) PARAMETERS: DETECTION AND COMPARISON TO BIOCHEMICAL PARAMETERS MEASURED IN SALIVA

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## 1. Introduction and Aims of Study

Chronobiology is a relatively new field of science investigating periodic phenomena in the organism. It is well established that the flow rate and composition of saliva vary rhythmically depending on daytime [1-3]. A number of parameters contained in saliva have been suggested to be reliable measures for detecting stress and related bodily answers on external noxae. In the context of stress and immune answers, alpha-amylase, immunoglobulin A (IgA), cortisol and the regulatory peptide substance P deserve special attention. Relatively fast changes of the levels of those biochemical parameters in saliva have been reported, and their significance to show response on stressing noxae appears to be high [4-6].

As a number of biochemical parameters related to stress are known to vary during daytime, it became obvious to us that mean GDV glow image area values should also follow diurnal changes. Apparently, circadian rhythms of GDV parameters had not been reported yet in literature.

## 2. Test Persons and Methods

Nine persons participated at this experiment (mean age: 38,9 a; median 35 a; SD 12,11; range 24 to 53 years). Five of them were women (mean 41,2 a; range 24 to 53 a), four were men (mean 36 a; range 24 to 43 a). Only (nearly) non-invasive techniques were carried out. The guidelines of the enlarged Helsinki declaration were carefully followed, also including informed consent letters and taking care of data security. Persons with severe acute infections or diseases, or those taking certain kinds of medication (cortisone, psychotropics (psychotropic drugs), beta blockers etc.) were excluded. All test persons were advised not to eat an exuberant dinner or breakfast at the evening and morning before the experiments, not to drink alcohol and only very small amounts of light coffee, black or green tea 20 hours before the experiments. During the tests, no food intake was allowed, only to drink tap water. Mobile phones had to be switched off at least one hour before the tests.

### 2.1. Experimental Timing

Test persons during the experiments were sitting relaxed on a comfortable chair. After one hour of anamnestic and psychological questioning and getting used to the laboratory conditions, 4 experimental phases started: 10, 25 and 45 minutes after the start of each phase, saliva samples were taken. After the second saliva sampling, i.e. 30 minutes after the start of each phase, GDV measurements were carried out (i.e., in between the 2nd and the 3rd sampling of saliva in each phase. At the 50 minutes time point of each phase, 5 minutes break were included. During that period, test candidates were asked to stand up, to go to the bathroom, to drink tap water (thereby flushing their oral cavity) and to carefully wash their hands, as a prerequisite for reliable GDV measurements. No hand cream was allowed. The GDV camera was switched on at 7:30 a.m. at each of the experimental days.

### 2.2. Laboratory Shielding

The experimental room was carefully shielded from high frequency electromagnetic fields using “YShield” shielding paint, a grounded electro-conductive coating attached at the

walls, the ceiling and the floor (type HSF53; www.yshield.com; YShield, Pocking, Germany), as well as "Swiss Shield naturell" shielding curtains (www.esag.at; Esag, Vienna, Austria).

Any electric, magnetic and electromagnetic radiation present, as well as possibly influencing acoustic or vibration-related noxae were examined and documented by an accredited company (ANBUS Co., Fürth, Bavaria; Dr. Ing. Martin Virnich). During the experiments, concomitant high frequency (HF) measurements were carried out continuously, using a Rohde & Schwarz "FSH 3" spectrum analyzer (100 kHz to 3 GHz), and a mobile HD dosimeter (type "ESM-140"; Maschek systems, Bad Wörishofen, Germany; www.maschek.de). Whilst carrying out the experiments documented here, the field strength was at 70 mV/m at mean, corresponding to a mean power density of about 13 $\mu W/m^2$ at the GSM 900 MHz band as the predominating HF field. For other types of HF electromagnetism, the field strengths detected were much below. Static magnetic influences were at 45,7 µT (mean) only, and LF electric fields were below 1 V/m. LF magnetic fields were at 42 nT (mean). None of the equipment used, including the GDV system, did change the electric, magnetic or electromagnetic field strengths significantly when powered.

### 2.3. Saliva Analysis

#### *2.3.1. Saliva Sample Collection*

At each of the experimental time points shown in Fig. 1, during 5 minutes, saliva samples were taken using *Salivette* saliva collection devices (Sarstedt, Nuernbrecht, Germany). Immediately thereafter, the salivettes were centrifuged for 5 min at 1000 x *g*, and the saliva specimen spun into 100 µl of 100 mM HEPES pH 7.0 containing 1 mg/ml bovine aprotinin to prevent proteolytic degradation of one of the markers measured (i.e., substance P). The individual saliva samples were aliquoted and frozen at -20°C until analysis. All biochemical assays used were carried out in triplicate for each of the samples and parameter. Total protein content of saliva samples was measured by the method of Bradford (39) using bovine serum albumin as standard.

#### *2.3.2. Biochemical Tests*

Salivary alpha-Amylase (1,4 a-D-glucanohydrolase, EC 3.2.1.1) was assayed essentially according to the method of Gillard et al. [7] with 1 mM p-nitrophenyl a-maltoside as substrate with some minor modifications and adaptation to a microplate format. Enzyme activities were calculated as mU/ml.

IgA concentrations in saliva were measured by using a sandwich enzyme-linked immunosorbent assay (ELISA) with a matched pair of mouse monoclonal anti-human-IgA antibodies (G18-1 for capture, and alkaline phosphatase-labeled G20-359 for detection, both from Pharmingen (Becton-Dickinson, Vienna, Austria). IgA concentrations were calculated with respect to appropriate standard concentrations of human IgA run on each plate.

Salivary cortisol levels were examined by a competitive ELISA on microplates coated with goat-anti-rabbit-IgG with rabbit anti-cortisol-antiserum (Fitzgerald, Concord, MA, USA) and a cortisol-3-O-adipic acid dihydrazide-horseradish peroxidase (HRP) conjugate as specific competitor, synthesized essentially as described by Basu et al. [8]. HRP activity was measured with 0.1 mg/ml tetramethyl benzidine and 0.01 % $H_2O_2$ in 0.1 M sodium acetate pH 6.0 at room temperature. Cortisol concentrations were calculated with respect to appropriate standard concentrations of cortisol (hydrocortisone) run on each plate using a 4 parameter fit equation.

Substance P levels contained in saliva were measured by a competitive luminescence immunoassay (LIA) on black microplates coated with goat-anti-rabbit-IgG with a rabbit antiserum directed against substance P and a substance P biotin conjugate, synthesized from substance P with N-hydroxy-succinimidyl-biotin, as specific competitor. Bound substance P-biotin was detected by an Extravidin-HRP conjugate (Sigma). HRP activity was measured by enhanced chemiluminescence (ECL) using a commercial ECL substrate (Roche Diagnostics, Mannheim, Germany). Substance P concentrations were calculated with respect to appropriate standard concentrations of substance P run on each plate using a 4 parameter fit equation.

#### *2.3.3. Data Handling*

Values obtained for the different biochemical parameters of each individual test person were corrected for dilution by the aprotinin-buffer additive and, for some of the diagrams presented here, normalized to the mean values of the first phase to compensate for the variation in individual levels of the parameters tested.

**2.5. Biomedical Statistics**

All experimental data were analyzed using the software packages Sigma-Plot 9.0 (www.systat.com; Systat, San Jose, CA, USA), SPSS 14.0 (www.spss.com; SPSS, Chicago, IL, USA), and Excel 2003 (Microsoft; Redmond, WA, USA). In addition to descriptive data (mean, median, standard deviation etc.), a number of time curse diagrams were calculated.

**3. Results**

Each of the parameters measures showed distinctive values and time curses (***Fig. 1***), with the exception of spatial fractality. In some of the parameters measured, markedly high inter-individual differences were detected. ***Tab. 1*** shows some of the key values obtained as an overview.

For **salivary alpha-amylase**, a mean value of 2,29 mU/ml (ranging from 1,03 to 3,73) was measured for the first test point of the first phase (sample collection in between 9:10 and 9:15 a.m. of each experimental day). The amylase levels went down to a mean minimum of 1,55 mU/ml (ranging from 0,76 to 3.0) measured from the final samples taken in between 12:30 and 12:35 p.m.

**IgA** showed marked inter-individual differences: The mean level measured at the first testing point (at 9:10 to 9:15 a.m.) was 146,23 µg/ml (ranging from 55,64 to 470,76). The highest value measured in one of the test phases was 539,86 µg/ml, the lowest value was at 40,79 µg/ml. At the final test point of each day (at 12:30 to 12:35 p.m.), 157,94 µg/ml were measured as the calculated arithmetic mean (values at that time ranged from 62,40 to 451,73).

For **cortisol** too, high inter-individual variabilities were observed: The mean cortisol levels in saliva measured started at 3,75 ng/ml (ranging from 1,77 to 7,57) obtained from the first sampling time point, and the mean level at the final experimental time point have was at 2,05 ng/ml.

Salivary **substance P** levels detected started at 0,84 ng/ml in the morning at 9:15 a.m. (ranging from 0,10 to 2,16) and ended at a mean of 0,33 ng/ml (ranging from 0,10 to 0,84 ng/ml). Mean maximum levels measured were at 0,97 ng/ml saliva.

The mean **GDV glow image area** at the first test point was at 14.325 pixels (ranging from 11.539 to 17011 pixels at about 9:30 a.m.), and the mean maximum was 20.118 pixels (ranging from 16.080 to 21.388 pixels), reached at the final time point (at about 12.30 p.m.).

**GDV spatial fractality** showed no recognizable daytime dependency.

| | ***First Measurements:*** | | | | ***Final Measurements:*** | | | |
|---|---|---|---|---|---|---|---|---|
| **Factor:** | **Mean** | **SEM** | **Min** | **Max** | **Mean** | **SEM** | **Min** | **Max** |
| alpha-Amylase (mU/ml) | 2,29 | 0,31 | 1,03 | 3,73 | 1,55 | 0,23 | 0,79 | 2,69 |
| IgA (µg/ml) | 146,23 | 45,04 | 55,64 | 470,76 | 157,94 | 40,39 | 62,40 | 451,73 |
| cortisol (ng/ml) | 3,75 | 0,57 | 1,77 | 7,59 | 2,05 | 0,23 | 1,13 | 3,03 |
| substance P (ng/ml) | 0,84 | 0,29 | 0,10 | 2,16 | 0,33 | 0,09 | 0,10 | 0,84 |
| GDV glow image area | 14325 | 798 | 11539 | 17011 | 19121 | 751 | 16081 | 21389 |
| GDV spatial fractality | 1,93 | 0,02 | 1,87 | 2,02 | 1,95 | 0,03 | 1,75 | 2,03 |

**Tab. 1:** Summary of descriptive values obtained from the very first and the very last measurements of salivary and GDV parameters. According to Fig. 1, "first measurements" for the biochemical parameters represents time point 1, i.e. the samples taken in between 9:10 and 9.15 a.m., for the GDV parameters the measurements done between 9:25 and 9:40 a.m. "Final measurements" present data obtained from the saliva sampling in between 12:30 and 12:35 p.m., whereas for GDV, measurements done between 12:15 and 12:30 p.m. were used.

In the following, **diagrams of progression in time** of the different parameters presented in two ways: The first set (***Fig. 1 a-d***) shows individual comparisons of GDV glow image areas and each of the biochemical parameters measured in a smoothened version:

Arithmetic mean values were calculated for each of the four test phases. E.g., the first value contained in the diagram for alpha-amylase represents an arithmetic mean obtained from the three saliva sampling times from the first test phase; the second value in the same diagram is an arithmetic mean of the second test phase, and so on. This calculatory setup has been applied for all diagrams shown in Fig. 1:

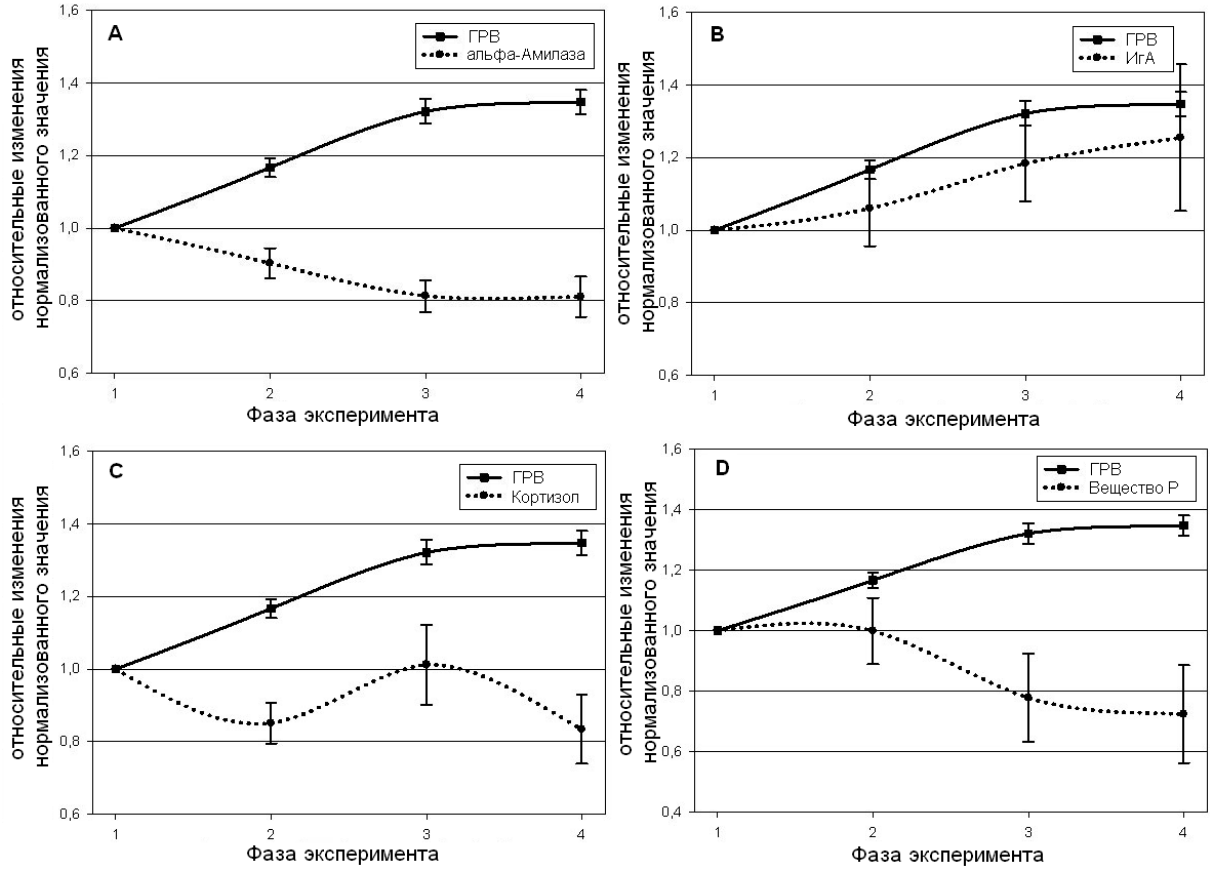


**Fig. 1 a-d:** Diagrammatic representation of the time curses of GDV glow image area in comparison to the salivary parameters measured biochemically. Fig. 1 a shows the comparison between GDV and alpha-amylase, Fig. 1 b compares GDV and IgA, Fig. 1 c is on GDV and the cortisol levels, and Fig. 1 d on GDV and substance P. In every diagram, GDV glow image areas are presented as a time curse line in which the mean values measured during the 4 phases tested are marked as black quadrates. For the associated parameter measured in saliva, dotted time curse lines are used, and slightly bigger black dots each show the time centers of the 4 test phases sampled. The bars show the associated standard errors of the mean (SEM). The y-axis represents the relative change of each parameter, normalized for the starting point (phase 1). The x-axis in each diagram represents a relative time line in which the 4 experimental phases are labeled as 1 to 4. Each experiment started at 9:00 a.m. with phase 1 (see Fig. 1) and ended at approximately 12:35 pm. after phase 4.

## 4. Discussion and Conclusions

The pilot study described here, although done with a comparatively low number of test persons (n=9), can potentially be regarded as kind of a bridge between “school medicine” and complementary medicine. For the first time, the possible existence of a circadian rhythmicity of the mean GDV glow image area was detected. Using standardized clinico-biochemical technology, we could show that there appears to be a characteristic relationship between the diurnal progressions of the mean GDV glow image area and a number of key parameters of stress as detected in human saliva. A closer look comparing the time progressions of the parameters tested shows that each of them appears to follow the expected directions in a remarkably foreseeable way, thus reinforcing that GDV indeed is a scientifically valuable tool to detect stress.

The data presented here undoubtedly justify follow-up studies aimed to test a higher number of persons during longer periods of the whole day. Designing such studies will not be

easy, as no person would agree to sit still or participate at such experiments lasting longer than a few hours. Also, external and intrinsic parameters (hunger, need for sleep etc.) would heavily influence the results. Therefore it might have to be a compromise, in which overlapping groups during several time windows of the day need to be tested, e.g. one group as done here on forenoons, another group at afternoons, but also groups over "lunchtime" and in the evening hours. Such setups would probably also contribute to elucidate possible influences of the experimental setup on the results. Taking salivary samples during the night would be likely to be impossible in the way done during the pilot experiments presented here. Although methods of nighttime sampling of saliva have been described, using automatic blood samples might be another way of approaching. However, taking representative GDV glow image areas in between sleep breaks would cause stress likely to be seen in changes of the GDV glow image area. For such "during night" experiments, hypnosis might be successfully used for allowing GDV tests to be performed during sleep periods.

As a conclusion of the pilot study presented in this manuscript, closer evidence has been presented for the validity of using the mean GDV glow image area as a reliable measure for (at least, certain types of) stress. Comparative analyses of the curve progressions of the parameters tested here in parallel showed the expected connections: Higher GDV glow image area values went in line with increased IgA production in saliva, whilst at the same time, the alpha amylase curve progressed in the opposite way. The meaning of the connections between GDV and cortisol and substance P detected needs to be clarified in subsequent experiments. Overall, such studies will have to include a higher number of test persons, as well as specifically designed experimental setups also needed to elucidate influences of water drinking breaks, of fear and other factors likely to be present.

## 6. Literature


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