MIGHT CARDIAC CATHETERIZATION INFLUENCE DIURNAL RHYTHM OF THE
STEROID STRESS HORMONES SECRETION?

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Short title: Circadian variation in glucocorticoid levels in young sows

Summary

Glucocorticoids (GCs) are steroid hormones produced by the adrenal cortex in reaction to stress stimuli. GCs production is not stable over a 24-hour period; the plasma concentration peaks in the morning (approximately upon awakening) and then the plasma levels decrease, reaching the nadir in the evening. In our experiments, the levels of cortisol, cortisone, DHEA and DHEAS were tested in young female pigs (n = 23) during heart catheterization at two different day times (in the morning and in the afternoon). The non-parametric Mann-Whitney test for statistical analysis was used. We found only minimal statistical differences in studied markers between the morning and afternoon group (p > 0.05). The absence of circadian variation in GCs levels could originate either at an early age of our experimental pigs, or in stressful conditions on the experiment day, or most likely the day before (e.g. social isolation, fasting, transport, and catheterization), respectively. We can conclude there is no difference in the stress load between morning and afternoon experiments, and therefore we can assume the stress load is not a limiting factor for the timing when catheterization should be preferably performed.

Key words: cortisol, stress, heart catheterization, circadian rhythm

Introduction

Stress has been intensively discussed for many years, not only in human medicine, but also in veterinary medicine and in zoological branches. The term "stress" was used for the first time by Selye (1936), who had described general adaptation syndrome. Stimuli affecting the homeostasis of an organism are called stressors. Stressors could be of many different origins, e.g. ecological (acute environmental changes, nutrition or shelters absence, temperature variations), sociobiological (unstable social hierarchy), health (fever, infection, injury, surgery), transport and many others. Stressors trigger stress response – a complex of physiological, endocrine, metabolic and behaviour reactions protecting organism prior to the injurious effect of stressors (emergency life-history) (Schreiber 1985, Greenberg *et al.* 2002, Möstl and Palme 2002). These reactions are conservative and proceed in most animal species and human in the same way (Breuner and Orchinik 2002, Greenberg *et al.* 2002, Romero 2004).

Glucocorticoids

GCs (main GC in mammals is cortisol, except some rodent taxons, where it is replaced by corticosterone) are steroid stress hormones with 21 carbons, products of adrenal cortex. GCs have an important role in metabolism, especially glucose, and its control.

GCs are small lipophilic molecules; nevertheless only those with a hydroxy group on C-11 (cortisol) are biologically active. he lipophilic character of the unbound GCs allows free entry to the target cells through cell membranes to cytoplasm where they are bound to specific receptors. The GC-receptor complex then enters the nucleus and identifies glucocorticoid response elements (GREs), special palindromic DNA sequences, binds to them and acts as a transcription modulator (Seckl 1997).

Cortisone is a steroid hormone produced by the adrenal cortex. The hormone is characterised by the keto-group on C-11 in its chemical structure. In contrast to cortisol, it cannot be bound

to cytoplasmatic receptors due to its different chemical structure. Cortisone could be considered to be an inactive form of cortisol, but, on the other hand, it represents cortisol's reserve pool in the time when more GCs are needed (e.g. in stress response). Isoenzyme 11β-hydroxysteoriddehydrogenase (11β-HSD) is then an important regulation factor. Cortisone conversion to cortisol is catalyzed by 11β-HSD1 and, conversely, cortisol-to-cortisone conversion is catalyzed by 11β-HSD2.

GC rhythm

GCs production depends on circadian and circannual biorhythms. The peak of GC levels is just before the beginning of activity (in nocturnal animals at a sunset, in diurnal animals at a sunrise) while the nadir of GC levels is before sleeping (e.g. Barnett *et al.* 1981, Becker *et al.* 1985a, b, Bradbury *et al.* 1994, Kramer and Sothern 2001). Pigs are diurnal animals so their GCs peak production is in the morning.

Circadian rhythm develops in ontogeny; young animals have no detectable rhythm as the real onset of the rhythm comes in puberty, in pigs at the age of their 15th week (von Borell and Ladewig 1992, de Jong *et al.* 2000), adult animals already have fully stable rhythm (since the age of 20 weeks) (Ruis *et al.* 1997).

Circadian rhythm could be affected by stress situations, e.g. transport (Becker *et al.* 1985b), temperature discomfort (Hillmann *et al.* 2008, Malmkvist *et al.* 2009), moving (Becker *et al.* 1985a), social instability (Ruis *et al.* 1997) or surgery (e.g. Levine *et al.* 1980, McIntosh *et al.* 1981, Becker *et al.* 1985a).

DHEA, DHEAS

Dehydroepiandrosterone (DHEA) and its sulphate (DHEAS) are steroids that mainly originate in the adrenal cortex, to a lesser degree in neuronal brain cells (as neurosteroids), gonads or placenta. DHEA is converted to DHEAS by sulfotransferase in the adrenal cortex, liver or small intestine. DHEAS is the main circulating form; it acts as a pool of DHEA. DHEA has a

wide spectrum of biological influences; it is a weak androgen, but basically it is a precursor of sex steroids, both androgens and estrogens. Both hormones have a positive effect on learning and memory. The lack of DHEA could be a cause of degenerative immune and brain changes in elderly people (Baulieu *et al.* 2000).

DHEA and DHEAS acts via intracellular or membrane receptors, e.g. NMDA, $GABA_A$, $\sigma 1$, etc. These receptors modulate memory processes (Bičíková et al. 2000). *N*-methyl *D*-aspartate (NMDA) receptors are present in many brain and CNS structures, they are important in synaptic plasticity, learning, and memory functions. Positive modulation of NMDA receptors increases inflow Ca^{2+} into cell and consecutive activation calcium dependent enzymes, e.g. phospholipase A_2 (PLA_2) ,Positive modulators could be steroid hormones and their sulphate derivates - DHEA, DHEAS or pregnenolone sulphate (PregS). Positive modulation increases the excitability effect of the target cell and it improves memory functions (Bičíková *et al.* 2000). Another type of receptor modulated by DHEA or DHEAS is the γ-aminobutyric acid ($GABA_A$) receptor. Compared to the NMDA receptor, DHEA and its sulphated metabolite DHEAS are negative modulators (Gartside *et al.* 2010).

Heart catheterization is an invasive cardiac procedure standardly used in human medicine. The aim of our research was to study eventual circadian changes of GCs secretion in young female pigs undergoing this procedure, and to study whether this type of procedure can cause stress and/or affect a stress response due to eventual GC circadian rhythm. The study was designed to correspond to the standard catheterization pattern in human medicine as any positive findings could be then useful for planning these medical interventions in human patients.

Methods

1. Animals:

In our experiment female pigs were used (*Sus scrofa domestica*), crossbred (Landrace x Large white) four months old with body weight from 40 kg to 50 kg. The sows were too young to have a sexual cycle; we could exclude the influence of the oestrus cycle on the stress markers levels. They originated from the AGRO Jesenice, a.s., breeding farm Radějovice (RČH CZ-21045103). The pigs were maintained at the farm under conventional conditions (room temperature about 20ºC, humidity between 40 and 70%, natural lighting and regulated ventilation). They were fed a balanced diet for fattening pigs (Čos II, Velaz, Czech Republic) in accordance with the feeding standard. Drinking water was available *ad libitum*. The end of sleep was determined rather by standardized time of a morning feeding than by the light regime.

Ethical note: The experiment was performed in accordance with Czech law and corresponding EU regulations and was approved by the Institutional Animal Care and Use Committee.

2. Experiment:

23 female pigs were used, divided into two groups: 1) catheterization in the morning (n = 13) and 2) catheterization in the afternoon (n = 10).

2a) Heart catheterization: The heart catheterization was bilateral (sinistral and dextral) performed using a standard catheterization procedure (through arteria and vena femoralis using 7-9F sheet). The catheterizations were carried out as part of electrophysiological projects, in which electrical stimulation and radiofrequency ablation were performed. In all tested animals of both groups four markers were assessed in the blood serum: cortisol, cortisone, DHEA and DHEAS.

2b) Anaesthesia and medication: Stresnil (azaperone, dose: 5mg/kg), Atropin (atropine sulphate, dose: 0.05 mg/kg) and Narcetan (ketamine hydrochloride, dose: 14 mg/kg) by an intramuscular injection for pre-medication and sedation were used. To obtain intravenous (IV)

access an 18G or 20G IV cannula was inserted into the marginal ear vein. Intravenous anaesthetic introduction started with Propofol (1% propofol: dose 2mg/kg bolus) and Morphine (1% morphini hydrochloridum trihydricum: dose 0.2 mg/kg bolus). Intubation under direct laryngoscopic control was performed with 7 or 7.5 mm orotracheal tubes, depending on the size of the sow. Anaesthesia was maintained with a average propofol (dose: 4 mg/kg/h) IV infusion, and as analgesic morphine (dose; 0.2 mg/kg) IV bolus was administered every hour. Ventilation was sustained at an average volume of 8 to 10ml/kg and respiratory rates of 15 per minute.

During the IV anaesthesia a continuous monitoring of mean arterial pressure (MAP), heartbeat rate (HR), O_2 saturation (SO_2) and exhaled capnometry (PCO_2) was observed on a multiparameter bed-side monitor.

2c) Blood collection: Blood was collected from the jugular vein in four defined periods of the experiment. The first (1) was collected at the farm, in non-stress domestic conditions (control sample, basal levels of the stress markers), other samples were collected 10 minutes after the presumed stress situation: the second sample (2) 10 minutes after intubation and introduction to anesthesia, the third sample (3) 10 minutes after heart stimulation or ablation and the last (4) at the end of the operation, before the animal was sacrificed. Total amount of blood (10 ml) was collected in a 10 ml serum Vacutainer system tube (BD Vacutainer, SSt II Advance), after 30 minutes of incubation in room temperature the tubes were centrifuged (2000 x g) for 15 minutes and then the serum was stored at -20°C till further analyses.

2d) Timing of the experiment: The morning experiments were started at 9 AM, the afternoon ones at 3 PM (by premedication, one hour after sows' arrival to our laboratory). Intubation and introduction to anesthesia were performed usually half an hour after premedication, the heart catheterization itself starts one hour and ends two and half hours after premedication (then the third blood sample was obtained) and experiments ended usually three to four hours

after the premedication. Note: timing could be little bit variable due to complications or unpredictable events.

2e) Laboratory analysis: Cortisol and cortisone was assessed using the method published elsewhere (Šimůnková *et al.* 2008). Briefly, the serum samples were twice extracted and afterwards a high performance liquid chromatography (HPLC) system from Dionex Softron (Germering, Germany) was used for hormone separation. HPLC separation was carried out with reverse phase EC 250/4 NUCLEOSIL® 100-5 C18 column (MACHEREY-NAGEL, Düren, Germany). To avoid possible column contamination the Phenomenex SecurityGuard system with C18 cartridge (Phenomenex, Torrance, CA) was used. The solvents Merck (Darmstadt, Germany) were used as mobile phase for HPLC. Serum cortisol and cortisone concentrations were determined according to a calibration curve using UV/VIS detection. DHEA and DHEAS were measured by commercial radioimmunoassay and immunoradiometric kits (Immunotech; Czech Division, Marseille, France). Intra-assay and inter-assay coefficients of variation (CVs) for DHEA were found below or equal to 7.9 % and 11.9 %. Intra- and inter-assay CVs for DHEAS were below or equal to 7.4 % and 10.6 %, respectively. The analytical parameters corresponded to those stated by the manufacturer.

3. Statistics:

Data were divided into two groups, according to the time of day of the experiment (morning or afternoon hours). For each group there was performed an independent statistical test for evaluating elementary statistical data (median, minimum, maximum) for each marker in each period of experiment.

For testing the difference between morning and afternoon samples, a non-parametric Mann-Whitney test was performed. A non-parametric test was used since our data didn't show standard Gaussian distribution. The Mann-Whitney test was calculated for two independent samples (morning and afternoon) for each marker in each period of the experiment.

Results

Elementary statistical data were calculated for cortisol, cortisone, DHEA and DHEAS for
each period of the experiment (1-4; see section Methods for details) for two different groups
separately (morning and afternoon catheterization). Measured data demonstrated wide
variance, indicating noticeable interindividual variation in these markers' levels (minimum
and maximum values for each marker are shown in tabs 1-4). Measured concentrations in
cortisone, DHEA and DHEAS were in some samples under the detection limit (minimum 0.00
nmol/l).

Cortisol:

For the first period of the experiment (at the farm, non-stress conditions) the median was
145.15 nmol/l for the morning group and 154.90 nmol/l for the afternoon group. At the
second period (after introduction to anesthesia) the median was 272.61 nmol/l in the morning
and 327.75 nmol/l in the afternoon group. After conducting tissue ablation or
cardiostimulation (third period) the median, minimum and maximum were quite close for
both groups. The median was 231.00 nmol/l for the morning and 241.00 nmol/l for the
afternoon group. The minimum concentration was 82.00 nmol/l for the morning and 105.00
nmol/l for the afternoon group. The maximum was 365.00 nmol/l for the morning and 374.68
nmol/l for the afternoon catheterization. For the fourth section of the experiment (at the end of
the catheterization) the median was 143.95 nmol/l in the morning and 224.00 nmol/l in the
afternoon experiment.

We assumed diurnal variation in cortisol, but, surprisingly, we did not find it in any period of
our experiment. The Mann-Whitney test for all stages was statistically non-significant in
significance level 0.05 ($p > 0.05$). Results are shown in tab 1.

Cortisone:

For the first section of the experiment the median was 2.93 nmol/l for the morning group and 8.16 nmol/l for the afternoon group. After introduction to anesthesia the median was 28.36 nmol/l in the morning and 76.50 nmol/l in the afternoon group. For the third section the median was 35.67 nmol/l for the morning and 127.79 nmol/l for the afternoon group. At the end of the operation (fourth period) the median was 19.00 nmol/l in the morning and 124.62 nmol/l in the afternoon experiment.

Results for the Mann-Whitney test were statistically non-significant ($p>0.05$) in all stages of catheterization; there is no diurnal variation in cortisone levels. Results are shown in tab 2.

DHEA:

For the farm sample the median was 0.19 nmol/l for the morning group and 0.12 nmol/l for the afternoon group. At the second section of the catheterization the median was 0.58 nmol/l in the morning and 0.57 nmol/l in the afternoon group. For the third period of the experiment the median was 0.69 nmol/l for the morning and 0.74 nmol/l for the afternoon group. At the end of experiment the median was 0.90 nmol/l in morning and 0.61 nmol/l in the afternoon experiments. Medians for all tested stages were very close in both groups.

The Mann-Whitney test for DHEA was statistically non-significant ($p>0.05$) in all periods of the experiment; there are no diurnal variations in the DHEA levels. Results are shown in tab 3.

DHEAS:

For the first stage of the experiment the median was 0.01 nmol/l for the morning group and 0.11nmol/l for the afternoon group. After introduction to anesthesia the median was 0.83 nmol/l in the morning and 0.10 nmol/l in the afternoon group. At the third stage the median was 0.65 nmol/l for the morning and 0.10 nmol/l for the afternoon group. At the end of catheterization the median was 0.11 nmol/l in the morning and 0.13 nmol/l in the afternoon experiment.

The Mann-Whitney test was non-significant at the significance level of 0.05 in all stages of experiment (p>0.05), there are no diurnal variation in DHEAS levels. Results are shown in tab 4.

Discussion

The aim of the presented study was to find out the circadian rhythm and its eventual changes of cortisol, cortisone, DHEA and DHEAS in young (12 weeks old) female pigs when undergoing standard heart catheterization (stress load). Pigs were chosen because many metabolic and chronobiological similarities with people. In fact, similar experiment could be technically well realized on human patients as well, but the experiment requires to collect several times relatively big volumes of blood (one sample has to contain at least 10 ml of blood), what could be in seriously ill people an unpleasant ethical problem. It is well known that pigs have circadian rhythm of cortisol (e.g Barnett *et al.* 1981, Becker *et al.* 1985a, b, Griffith and Minton 1991). Pigs are animals with diurnal biorhythm, which is why we expected higher levels of GC in the morning than in the afternoon experiments. No circadian rhythm in GCs was found at any period of the experiment; this could result from numerous causes. We can divide the causes into two groups: (1) non-stress and (2) stress reasons, and the second group to subgroups: a) moving to another pen and consecutive stress from social isolation, b) fasting, c) transport from home farm to experimental lab, d) the heart catheterization.

We had young (12 weeks old) female pigs in our experiment, which could be the first, stress-independent reason for the absence of circadian variation in cortisol in our experimental animals. Our findings also correspond to some papers (e.g. von Borell and Ladewig 1992, Ruis *et al.* 1997, de Jong *et al.* 2000) that demonstrated the absence of cortisol circadian rhythm in pigs younger than 15 weeks old. According to these studies, a stable circadian

rhythm is aimed only at the age of 20 weeks, with a peak in the morning and a nadir in the evening (Ruis *et al.* 1997, de Jong *et al.* 2000). On the other hand, Ekkel *et al.* (1996) found contradictory findings: circadian rhythm was present in 8-weeks-old piglets.

The second group, stress reasons, is more complex. It was proved that elevated GC levels could affect and disrupt circadian rhythms (references in following text). A stressor could be anything that disturbs welfare or homeostasis and that could activate the HPA axis (Janssens *et al.* 1994, Pol *et al.* 2002). Animals living on a farm are transported to our laboratory one hour before premedication on the day of the experiment; the possible stressors are very numerous: moving to another pen, social stress (social isolation), feeding absence, and the transport itself. On the home farm, the pigs were used to living in natural social groups. The day before the transport to our lab, the experimental pig was separated from its home pen to another pen, where it was alone without social mates. There can be a source of stress from moving and social stress. The pig was suddenly in unknown territory without other members of its social group, without familiar smells, etc. Both moving and exposure to a new territory (Dantzer and Morméde 1983, Becker *et al.* 1985a, Grandin 1997, Désautés *et al.* 1999) and social instability, few or no social contacts (Janssens *et al.* 1994, Ekkel *et al.* 1997, Ruis et al 1997) could elevate GCs levels. On the contrary, Geverink *et al.* (2003) found higher GCs concentrations in females housed in groups than alone, but these group-housed pigs had less circadian amplitude GCs levels. Janssens *et al.* (1995) demonstrated impaired circadian rhythms after chronic social stress, which was not the case in our experiment, but many procedures used could cause acute stress. Moving experiments were carried out by Becker *et al.* (1985a) and they have proved that moving can disrupt diurnal rhythm. Moreover, the same effect can result from social frustration (Barnett *et al.* 1994, Ruis *et al.* 1997).

The day before the experiment, after moving, the pig had to fast (due to anesthesia performed the next day). Food intake can influence circadian rhythm. In some studies two peaks of

corticosteroid levels were found, in the morning and in the afternoon, which was the time when the animals expected to be fed (Geverink *et al.* 2003, Hillmann *et al.* 2008). On the other hand, Barnett *et al.* (1981) didn't find any afternoon peak, but fully confirmed the morning one. Fasting is a stressful event, which can also affect circadian rhythm. Transport from the farm to the laboratory is another stressful event that could cause elevated corticosteroids levels (e.g. Becker *et al.* 1985b, Dalin *et al.* 1993, Averos 2007). Higher cortisol levels were found in females than in males, in winter than in summer and in longer transports (Averos 2007).

Heart catheterization is a minimally invasive intervention, but many studies didn't find differences in elevated GC levels between invasive open surgery and minimally invasive procedures in pigs (Mansour *et al.* 1992, Bessler *et al.* 1994, Burpee *et al.* 2002, Margulis *et al.* 2005, Matsumoto *et al.* 2005, Duchene *et al.* 2008). This means that even minimal intervention can cause a rise in GC levels and consecutive instability in circadian level. But stress response to surgery can be modulated by some other parameters, e.g. type of anaesthesia, hurtfulness, etc. (Marana *et al.* 2008). Particularly numerous anaesthetics (including Ketamine) can influence some diurnal rhythms: melatonin secretion in rabbits (Pang *et al.* 2001), cortisol level and appetite in macaques (Crockett *et al.* 2000) or mesors, amplitudes and acrophases of heart rate, body temperature and locomotor activity in rats (Prudian *et al.* 1997).

Nevertheless, we assume that absent cortisol circadian rhythm in our experimental young pigs was caused particularly by the immaturity of the hypothalamo-pituitary-adrenal (HPA) axis. Moreover, circadian rhythm could be theoretically affected by the procedures held on the day before the experiment (moving, social deprivation, fasting), but no difference was found between GCs levels during the whole catheterization procedure and the basal value of GCs levels from the preceding evening (Skarlandtová *et al.* in press). The heart catheterization

could influence circadian rhythm, but the time of acute stress is relatively too short time to affect the whole rhythm. Cortisol/cortisone ratio indicates that pigs are not in a stress; even during intervention (Skarlandtová *et al.* unpublished data). However, in stress studies it is important to monitor not only GCs levels, but also the circadian rhythm (de Jong *et al.* 2000). The concentration of DHEA and DHEAS is higher in stress events, as cortisol concentration (e.g. Nieschlag et al. 1973). These steroids have antiglucocorticoid effect, they acts as "antistress" steroids and minimize negative glucocorticoid effects (e.g. Kimonides et al. 1998, 1999, Charney 2004, Maninger 2009). As cortisol, DHEA and DHEAS have circadian variability with the highest levels in the morning and the nadir in the evening. In our experiment, there were no differences between morning and afternoon levels of these steroids. Because the process of concentration elevation of DHEA and DHEAS acts in the same way as in cortisol elevation, we could assume, our experimental sows are too young to have stable circadian rhythm in DHEA and DHEAS, because of the immaturity of the HPA axis.

Conclusion

Our experiments surprisingly revealed minimal changes ($p > 0.05$) in tested markers during heart catheterization in the morning when compared to the afternoon hours in each period of the experiment in any marker (cortisol, cortisone, DHEA and DHEAS). This means that morning and afternoon levels are not different; our results have not proved any diurnal rhythm or variation in GCs production in the studied group of experimental animals, but the results have also suggested that there is no difference in the stress load between morning and afternoon catheterization. Consequently, we can assume that there is no diurnal variation in the stress markers levels under the stress load and that timing is not a limiting factor for the elective cardiac interventional procedure.

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Tab 1: Median, minimum, maximum and Mann-Whitney test for two independent samples (morning and afternoon) for cortisol levels.

Period of experiment	Group	Median (nmol/l)	Minimum (nmol/l)	Maximum (nmol/l)	p
1	morning	145.15	58.11	716.71	0.55
	afternoon	154.90	121.00	445.53	
2	morning	272.61	58.00	394.22	0.14
	afternoon	327.75	118.00	435.34	
3	morning	231.00	82.00	365.00	0.58
	afternoon	241.82	105.00	374.68	
4	morning	143.95	50.34	405.69	0.20
	afternoon	224.00	122.11	396.00	

Tab 2: Median, minimum, maximum and Mann-Whitney test for two independent samples (morning and afternoon) for cortisone levels.

Period of experiment	Group	Median (nmol/l)	Minimum (nmol/l)	Maximum (nmol/l)	p
1	morning	2.93	0.00	52.00	0.32
	afternoon	8.16	0.00	55.53	
2	morning	28.36	16.68	94.56	0.52
	afternoon	76.50	13.00	151.31	
3	morning	35.67	15.25	204.00	0.15
	afternoon	127.79	20.93	290.00	
4	morning	19.00	0.00	363.77	0.15
	afternoon	124.62	9.08	231.00	

Tab 3: Median, minimum, maximum and Mann-Whitney test for two independent samples (morning and afternoon) for DHEA levels.

Period of experiment	Group	Median (nmol/l)	Minimum (nmol/l)	Maximum (nmol/l)	p
1	morning	0.19	0.00	0.76	0.59
	afternoon	0.12	0.00	0.53	
2	morning	0.58	0.44	1.40	0.47
	afternoon	0.57	0.33	0.93	
3	morning	0.69	0.52	1.40	0.54
	afternoon	0.74	0.22	2.12	
4	morning	0.90	0.37	1.49	0.31
	afternoon	0.61	0.37	1.04	

Tab 4: Median, minimum, maximum and Mann-Whitney test for two independent samples (morning and afternoon) for DHEAS levels.

Period of experiment	Group	Median (nmol/l)	Minimum (nmol/l)	Maximum (nmol/l)	p
1	morning	0.01	0.00	0.14	0.31
	afternoon	0.11	0.00	0.14	
2	morning	0.83	0.02	0.18	0.38
	afternoon	0.10	0.00	0.26	
3	morning	0.65	0.00	0.23	0.32
	afternoon	0.10	0.07	0.20	
4	morning	0.11	0.07	0.19	0.40
	afternoon	0.13	0.07	0.25	