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NEUROACTIVE STEROIDS - PHYSIOLOGY AND PATHOPHYSIOLOGY

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ABBREVIATIONS USED

3ß-HSD	– 3ß hydroxysteroid dehydrogenase
A2	–androstenedione
A3α5α	– androsterone
A3α5β	– etiocholanone
A3β5α	– epiandrosterone
A3β5β	– epietiocholanone
ACTH	– adrenocorticotropin
AED	– antiepileptic drug(s)
Allo	– allopregnanolone, P3α5α
AS	- androstane steroids
CIL	- chemimmunoluminescence
CNS	– central nervous system
CRH	– corticotropin releasing hormone
CSF	– cerebrospinal fluid
DHEA	– dehydroepiandrosterone
DHEAS	– dehydroepiandrosterone sulfate
E_2	– estradiol
ELISA	– enzyme-linked immunosorbent assay
GABA	– gamma-aminobutyric acid
GS-MS	– gas chromatography-mass spectrometry
HPLC	– high pressure liquid chromatography
HSOR	– hydroxy steroid oxidoreductase
LH	– luteinizing hormone
MOX-TMS	– TMS, but oxo groups modified to methoxylamine
NAS	– neuroactive steroid(s)
NMDA	– N-methyl-D-aspartate
NS	– neurosteroid(s)
P3α5β	– pregnanolone
P3β5α	– isopregnanolone

P3β5β – epipregnanolone
P – progesterone
PI – pregnanolone isomers
PICs – pregnanolone polar conjugates
PNAS –Proceeding of the National Academy of Sciences of the USA
PS – pregnane steroid(s)
PregS – pregnenolone sulfate
RIA – radioimmunoassay
S – steroid(s)
SHBG – sex hormone binding globulin
SSRIs – selective serotonin reuptake inhibitors
T – testosterone
THDOC3α5α – tetrahydrodeoxycorticosterone
TLC – thin layer chromatography
TMS – steroid hydroxyl groups modified to trimethylsilyl derivatives but intact oxo groups

1. INTRODUCTION

Steroids, which are synthesized in the central nervous system (CNS) are called neurosteroids (NS). Steroids, which act on the nervous system, regardless of their site of synthesis, are known as neuroactive steroids (NAS). Some NS are able to easily pass through the blood-brain barrier (Bixo et al. 1997). Disorders in their biosynthesis or malfunctions in their interactions with target sites can be the cause of many disorders. In 1980, the term neurosteroid appeared for the first time in the Czech literature (Schreiber 1980).

Neuroactive steroids act mainly as modulators of ionotropic receptors in nerve cell membranes responsible for the permeability of relevant ions (Pisu and Serra 2004). Among the most well known receptors affected by NS are the γ-aminobutyric acid type A receptors ($GABA_A$-r), responsible for the influx of chloride ions to nerve cells. They are positively modulated by pregnane and androstane steroids with hydroxylation at the 3α- position. Pregnane steroids with hydroxyl group at the 3β- position, however, reduce the increased capture of chloride ions (Wang et al. 2002, Lundgren et al. 2003). While 3α-PS are therefore extremely effective endogenous neuroinhibitors, 3β-PS and polar conjugates of all pregnane steroids act as their antagonists. 3α-pregnane steroids shorten the period of so-called paradox sleep, lower the liberation of acetylcholine in the neocortex and hippocampus, suppress neurogenesis and worsen spatial memory. Another well-known type of receptor influenced primarily by polar conjugates of NS is N-methyl-D-aspartate receptors (NMDA-r). Conjugates of 5α-PS act as activators of NMDA-r, as do sulfate 3β-hydroxy-5-en steroids, while conjugates of 5β-PS have the opposite effect (Park-Chung et

al. 1994). These receptors are responsible for the influx of chloride ions to nerve cells and subsequent inhibition of nerve function.

An important landmark in the research of the biochemistry of neuroactive steroids was the discovery of their biosynthesis directly in the CNS (Corpechot et al. 1985, Le Goascogne et al. 1987, Schumacher et al. 2003). In the CNS, neurosteroids are synthesized in the mitochondria of glial cells and neurons. Enzymes that are essential for the synthesis of pregnane neurosteroids, such as 3β-HSD and 5α-reductase type 1 (5α-R1) and 2 (5α-R2), are present in the spinal cord and in the brain.

The biosynthesis of neuroactive steroids varies depending on age and gender. In women, the largest proportion of pregnane type neuroactive steroids produced from progesterone metabolites are synthesized in the *corpus luteum* (Ottander et al. 2005). The dominant pregnane-type metabolite is allopregnanolone. Changes in the concentration of progesterone that are also reflected in changes in the levels of its reduced metabolites are, together with the abstinence effect, an evident cause of premenstrual syndrome in women. In males, it is clear that the main source of gonadal $GABA_A$-r modulation is testosterone.

The adrenal cortex produces most of the precursors of NS. In the *zona glomerulosa* and *zona fasciculata,* deoxycorticosterone is produced, with subsequent products being the neuroactive isomers of 3α,5α-tetrahydrodeoxycorticosterone (THDOC3α5α). In the 5-en pathway, pregnenolone sulfate is produced in the adrenal *zona fasciculata* (40-800 nmol/l), correlating with the level of

cortisol (de Peretti et al. 1986). Considering the quantity, pregnenolone sulfate is the most significant precursor of progesterone and deoxycorticosterone of adrenal origin. Progesterone is also a substrate for the production of 5α- and 5β-dihydroprogesterone, and further neuroactive pregnanolone isomers. These mechanisms can easily explain the higher levels of brain NS documented in patients having disturbances connected to stress (Higashi et al. 2005).

NS are important modulators of the affects of alcohol on $GABA_A$-r (Morrow et al. 2001). While both activators of $GABA_A$-r and their precursors following relevant conversions intensify these affects, negative modulators of $GABA_A$-r and activators of NMDA-r mitigate them (Barbosa and Morato 2001, Mitchell et al. 2005). Another effect of alcohol, which can be influenced by NAS is appetence for alcohol. Rats given allopregnanolone showed increased appetence for alcohol, while giving 5α-reductases, i.e. one of the enzymes responsible for the synthesis of neuroactive 5α-pregnane steroids, had the opposite effect (Ford et al. 2005).

For both abstinence symptoms and the protective effects of allopregnanolone exist evident gender differences (Devaud et al. 1995), and in females there is also a difference in susceptibility to alcohol addiction with phases of the menstrual cycle.

Alcohol can also influence the concentration of neuroactive steroids in the brain. The acute use of alcohol leads to increased levels of allopregnanolone and progesterone in women during both menstrual cycle phases (Torres and Ortega 2003) as well as in men (Torres and Ortega 2004). The

levels of allopregnanolone present correlates with the intensity of the hypnotic effect of alcohol.

Chronic alcoholism, however, has a completely opposite effect on the concentration of steroid activators of $GABA_A$-r. Considerably lower levels of $GABA_A$-r steroid activators have been described in both human genders (Romeo et al. 1996, Morrow et al. 2001, Hill et al. 2005).

It is known that in addition to 3α-pregnane steroids, benzodiazepine and alcohol also have $GABA_A$-r modulating activity. All of these substances have anxiolytic, ataxic, anticonvulsive, and hypnotic effects.

Stress leads to an increased production of corticosteroids in plasma and in brain. In addition to cortisol, the production of deoxycorticosterone is also increased. 3α-Metabolites of deoxycorticosterone have anxiolytic and anticonvulsive effects through $GABA_A$-r. This could be important for the explanation of the mechanisms of some pathophysiologies related to stress situations, such as epilepsy, panic disorders, post-traumatic syndrome, and depression (Reddy 2006).

It is evident that epilepsy in women is related to reproductive endocrine disorders. In the relationship of progesterone to its reduced metabolites, there is a well-known connection between increased ratios of serum estradiol to progesterone and the frequency of tonic-clonic epileptic seizures in preovulatory and premenstrual phases of the menstrual cycle. In anovulatory cycles, this frequency depends of the levels of estradiol regardless of menstrual cycle phase (Backstrom 1976). Women with catamenial epilepsy showed up to 50-60 % reductions in estrogens and significantly lower

levels of 5α- and 5β pregnandiols (Buntner and Rosciszewska 1985).

In women with epilepsy, lowered levels of progesterone in the luteal phase of the menstrual cycle have been documented (Herzog 1991). Insufficient progesterone likely leads to an insufficiency of neuroinhibitory 5α/β- reduced 3α-metabolites of progesterone, which could be the cause of an increased frequency of epileptic seizures. Testosterone modulates the propensity for epileptic seizures in both humans and animals. Its aromatization to estradiol increases the likelihood of the onset of a seizure, while its reduction produces neuroinhibitory anticonvulsive $GABA_A$-r positively modulating 3α-hydroxy-androstane metabolites. Indomethacin, as an inhibitor of the 3α-HSOR enzyme that is necessary for the synthesis of 3α-androstane metabolites, tends to increase the pro-convulsive effect of testosterone (Reddy 2004).

2. ***Scope and aims***

Having in mind that many NAS are involved in certain disorders, that there are not strictly male or female hormones, that a limited number of hormones have been sometimes traced in many investigations, hence a limited information might be obtained in this way, we came to the idea that a method suitable for the determination of as many as possible steroids in one assay would be helpful.

Most frequently studied NS such as allopregnanolone, DHEA and pregnanolone, which modulate such events as chronic alcoholism, alcohol detoxication therapy, epilepsy or pregnancy and delivery. Our hypothesis was that also minor

metabolites of progesterone, DHEA or testosterone might be involved in those events.

Considering the above presented facts we have come to the understanding that it may be of interest to point out our attention on several aims, as follows:

AIMS:

1. Methods suitable for determination of some neuroactive steroids to be developed.
2. To investigate the possible linkage between epilepsy in women and the changes in steroidogenesis (especially in steroids sulfates and their unconjugated analogues).
3. To see if the deficiency in progesterone isomers (metabolites) reflects an impaired progesterone biosynthesis in women with severe alcohol addiction.
4. To evaluate the differences in the production of the individual pregnanolone isomers (PI) and their profiles during the reproduction.

3. *Materials and Methods*

Subjects

-Volunteers or outpatients (men – examined for fertility problems of the couple or participating in a study on iodine deficiency; women – regularly cycling, pregnant or during alcohol detoxification – according to the inclusion and exclusion criteria).The CSF was taken from patients operated for either tumorous or non-tumorous lesion.

-After signing written, informed consent, the subjects underwent blood sampling from the cubital vein. All subjects were free of major medical problems or medication known to affect steroid metabolism.

Steroids, chemicals and equipment

The non-radioactive steroids and their conjugates were obtained from Steraloids (Wilton, NH, USA). The solvents for extraction, for HPLC and pyridine were of analytical grade and were purchased from Merck & Co (Darmstadt, Germany), while methoxylamine was from Sigma (St.Louis, MO, USA). The derivatization agent Sylon BFT and TMCS were purchased from Supelco (Bellefonte, PA, USA). When needed the internal standard 3β-hydroxyl(19-H^3)androst-5-en-17-one (trideuterated DHEA) and others were prepared using a method described elsewhere (I. Černý et al., 2004).

The gas chromatography-mass spectrometry (GC-MS) system GCMS-QP2010Plus was supplied by Shimadzu (Kyoto, Japan).

Preparation of the plasma samples for the GC-MS free steroid analysis

The samples (serum, CSF or plasma) were kept frozen at -20^0 C or -80^0 C until analysis. They were thawed and 1 mL of them was spiked with trideuterated DHEA or other steroid (17α-estradiol) as the internal standard to attain a concentration of 1 μg/mL. The spiked sample was extracted with 3mL of diethyl ether. The water phase was kept frozen in a mixture of solid carbon dioxide and ethanol, and the organic extracts were decanted into glass tubes and evaporated to dryness. The dry organic phase residue was used for the determination of free steroids. It was partitioned between 1 mL of 80% methanol with water and 1 mL of petroleum ether (or n-pentane) to eliminate the majority of lipids and sterols. The petroleum ether phase was discarded, while the methanol/water phase containing steroids for analysis was evaporated in a vacuum centrifuge. Sometimes the samples

were prepared twice for further processing using two different derivatization techniques. The first was used for preparation of steroids with hydroxyl-groups modified to trimethylsilyl (TMS) derivatives and with hydroxy-groups modified as in the former case but, in addition, with oxo-groups modified by methoxylamine (MOX-TMS derivatives). The dry organic phase residue was used for the determination of free pregnenolone, DHEA, estradiol and PIs .

Steroid analysis

The levels of the 20 unconjugated steroids and 16 steroid polar conjugates were simultaneously measured in the serum using GC-MS. In the GC-MS method some procedures, presented by Stárka et al. (2006) and for initial sample preparation by Havlíková et al. /2006/ were used. Derivatization was done first with methoxylamine-hydrochloride on oxo-groups and then by Sylon B/99% of bis-(trimethylsilyl)-trifluoroacetamide and 1 % of trimethylchlorosilane forming trimethylsilyl derivatives on hydroxyl-groups. A second aliquot of the sample was derivatized by Sylon B only. Steroid conjugates remaining in the polar residue after diethyl-ether extraction were hydrolyzed as described by Dehennin et al. (1996) and further processed in the same way as the free steroids. The samples and polar phases after diethyl-ether extraction used for the quantification of the steroid conjugates were spiked with 17α-estradiol as an internal standard.

The individual samples were processed usually 4 times each time in order best processing of biological materials to be obtained. The temperature and pressure gradients and the effective masses used for the measurement in selective ion

monitoring mode was optimized to obtain maximum sensitivity and sufficient selectivity.

Measurements of steroids by radioimmunoassay

The concentrations of some steroids were evaluated by RIA. Blood progesterone levels were determined by a method originally described by Langer et al. (1978). The amount of pregnanolone sulfate was measured by a specific radioimmunoassay published by Hill et al. (2002). Pregnenolone sulfate, 17α-hydroxy-pregnenolone and progesterone were analyzed also by specific radioimmunoassay of Hill et al. (1999, 2002) and Langer et al. (1978). 17α–Hydroxy-progesterone and dehydroepiandrosterone were measured using RIA kits from Immunotech (Marseilles, France).

Statistical analysis of the data

To evaluate changes in the steroid levels and eventually steroid ratios a one-way (Kruskal-Wallis test) and two-way ANOVA models were used depending on the number of factors under investigations. Multiple testing, when needed, was handle by nonparametric Kruskal-Wallis or Bonferroni multiple comparison to evaluate the differences between individual groups. Statistical computations were performed using NCSS 2007 statistical software (Number Cruncher Statistical Systems, Kaysville, UT, USA). Given the mostly non-Gaussian distribution and non-constant variance in most the steroids studied, the original data underwent a power transformation to attain symmetry and homoscedasticity in the data as well as in the residuals. The group mean values and their 95 % confidence intervals calculated in the transformed data were re-transformed to the original scale for graphical demonstration. For evaluating the difference,

sometimes a robust Wilcoxon,s paired test was also applied. Statistical computations were performed using Statgraphics Plus v5.1 statistical software (Manugistics, Rockville, MA, USA).

4. ***Results***

Neuroactive steroids and epilepsy in women

This study was design to give answers if the steroid metabolism of P and sex S is changed and whether there was an altered balance between steroid sulfates and their unconjugated analogues in women with epilepsy. Considerably lower levels of 17-hydroxy-pregnenolone ($p < 0.0001$, ANOVA; $p < 0.002$, Mann-Whitney test) and 17-hydroxy-progesterone ($p < 0.006$, ANOVA; $p < 0.002$, Mann-Whitney test) were found in patients with epilepsy compared to controls. In the all other S measured, including E2 and P, not significantly differences were observed. Like in the follicular phase, during the luteal phase significant, although not so well pronounced, difference was noticed in 17-hydroxy pregnenolone ($p < 0.008$, ANOVA; $p < 0.05$, Mann-Whitney test). On the other hand, in comparison with follicular phase, even more profound lower levels of 17α-hydroxy-progesterone were detected in the patients in luteal phase ($p < 0.0001$, ANOVA; $p < 0.007$, Mann-Whitney test).

In both phases of menstrual cycle markedly elevated amounts of pregnenolone sulfate ($p < 0.0001$, ANOVA; were found in women with epilepsy along with almost not changed DHEAS levels.

Profiles of serum neuroactive steroids in women with alcohol addiction

Alcohol abuse is connected with menstrual irregularities, related to the inhibition of progesterone (P) secretion, involved in regulation and organization of the menstrual cycle. During the study it became clear that P biosynthesis was impaired in premenopausal women with history of alcohol intake and subsequently treated for alcohol addiction. As it was expected this steroid rose in the luteal phase during treatment. In the same time allopregnanolone, epipregnanolone and isopregnanolone increased in both phases of the menstrual cycle, but better pronounced in luteal phase, whereas pregnanolone, E_2 and pregnanolone sulfate exhibited significant changes in either phase of the menstrual cycle.

The overall change in the ratio of 3α- to 3β-isomers, expressed as a square root of the ratio of the products of the 3α- and 3β-isomers, did not change during treatment. On the other hand, the overall change in ratio of the 3α- to 5β-isomers showed a gradual increase in the luteal phase. The above cited results and especially the restoration of serum amounts of P, allopregnanolone and probably also epipregnanolone demonstrates the favorable effect of detoxification therapy on reproduction function and psychosomatic stability of premenopausal women treated for alcohol addiction. The other aim of interest concerned altered profiles of serum neuroactive steroids in premenopausal women, treated for alcohol addiction. The most elevated steroid in the patients in comparison to the controls, as it is seen on Fig. 1A is pregnenolone sulfate.

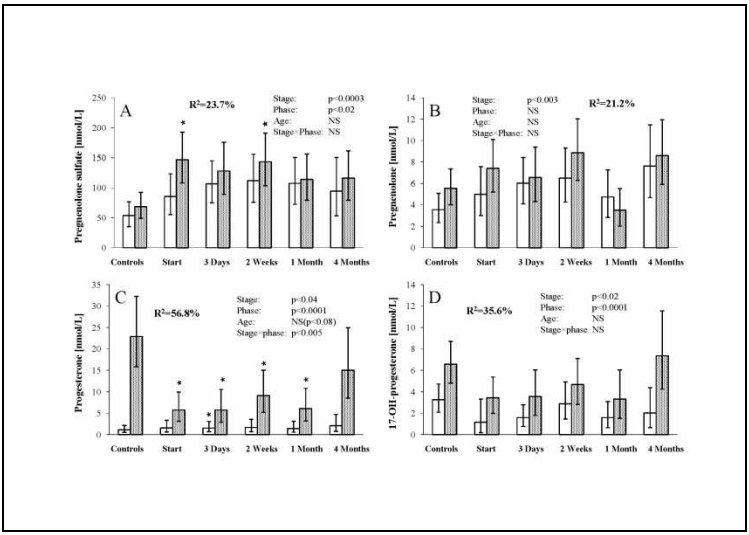

Fig. 1. Serum levels of unsaturated C21-steroids in controls and in women during alcohol detoxication treatment. The empty and dotted bars, with error bars, represent the transformed mean values with their 95% confidence intervals in the follicular and luteal phases respectively; p is the level of statistical significance and R^2 is the squared correlation coefficient expressing percentage of the total progesterone variability explained by an ANCOVA model consisting of subject status (controls or stage of treatment) and phase of menstrual cycle as factors, inter-factor interaction and age as a covariate. The ANCOVA was followed by Bonferroni (controls vs. Stage of the treatment) multiple comparisons and significant differences (p> 0.05) are labeled with an asterisk.

Quite similar situation appeared in unconjugated pregnenolone but was less pronounced – Fig. 1B. The changes in the above mentioned steroids during the treatment were not significant.

No differences between the patients with alcohol abuse and controls and no changes during the treatment were detected in pregnenolone concentrations.

By contrast, 3-oxo-4-ene steroids, P – Fig. 1C and 17α-hydroxy progesterone – Fig. 1D in luteal phase exhibited significant dependence on the subject status, this being more

characteristic for P. Progesterone concentrations in the controls during the luteal phase were significantly higher than in the patients at the beginning of treatment, but no such difference was discovered when comparing the patients themselves after 4 months of treatments. In both luteal and follicular phases E2 demonstrated a similar pattern of differences to the P in luteal phase but with less manifested differences in the follicular phase The DHEA and DHEAS concentrations did not differ significantly between alcoholic subjects and controls and they did not present any changes during the treatment.

The differences in PI did not simply reflect the variation in P levels but were well prominent, particularly in the 5a-PI. This disparity between the controls and the untreated patients was relatively less seen in the pregnanolone. Nevertheless, in contrast to P and similarly as in E_2, the character of the differences in PI did not diverge between follicular and luteal phase.

In order to identify in which steps of NS biosynthesis the alterations occur in women treated for alcohol addiction, the product/precursor ratios were traced in the pathway from pregnenolone and pregnenolone sulfate to PI. As it is apparent, from the ratio of pregnenolone sulfate to unconjugated pregnenolone displayed higher values in the patients, but with a tendency to compensation during treatment. The P/17α-hydroxy-progesterone and pregnenolone/17α-hydroxy-pregnenolone ratios present similar patterns in the luteal phase, with significantly decreased values at the beginning of treatment but with a trend to compensation during therapy.

The overall ratios of 3α– to 3ß-PI and of 5α- to 5ß-PI were also elevated, as were the proportions between neuroinhibiting allopregnanolone and neuroactivating 17α-hydroxy-progesterone. The ratio of the 3α– to 3ß-PI presented no significant differences between the controls and patient during the study. By contrast, the ratio of the 5α- to 5ß-PI showed a tendency to lower values in the patients and again a trend to compensation during their treatment. As far as the ratio of the foremost neuroinhibiting PI allopregnanolone to neuroactivating 17α-hydroxy-progesterone is concerned a markedly depressed values in the patients, but with a trend to incomplete, but significant compensation is observed.

5. ***Discussion***

To obtain a better knowledge of the role of androstane and pregnane metabolites in adult men and of the transformation of these predominantly NA substances to inactive or even reversely active conjugates, a method was developed suitable for the determination of 20 circulating S, including nearly all the C19 S hormones, their precursors and polar conjugates. It is important to notice that some of these hormones are known to act or may possibly operate in the modulation of signal transduction on ligand-gated ion-channels in the central and peripheral nervous system.

The selectivity and sensitivity of the method were sufficient for the entire S measured. As far as sensitivity is concerned it ranged from hundreds of femtograms to tens of picograms, due to the S fragmentation pattern.

Epilepsy in women is usually menstrual cycle-related seizure disorder. It is characterized by an increase in seizures during

particular phases of the menstrual cycle. Many authors suggest that the cyclical changes of ovarian hormones estrogens, mainly E_2, have proconvulsant role, while P and especially its conversion to the NAS allopregnanolone (Kokate et al. 1999; Veliskova, 2007) have anticonvulsant, beneficial neuroprotective properties.

Considerably lower levels of P, 17α-hydroxy-pregnenolone and 17α-hydroxy-progesterone were found in our patients compared to controls. Regarding the significant differences in P concentrations, especially during the luteal phase, the results are in accordance with those of other authors (Rosciszewska et al., 1986;Bonucceli et al. 1990; Herzog, 1991). No difference was observed in P levels between patients and controls in follicular phase. As far as changes in E_2 amounts are concerned, our results are also in agreement of those published by the above mentioned researchers. Galimberti et al. (2009) found that P and E_2 were lower during the luteal phase in women with epilepsy, whereas SHBG levels were higher in the patients than in the controls.

The ratio E_2/P was higher in the investigated patients during the both phases of the menstrual cycle. Recently Shen et al. (2005) claimed that estrogens enhance the effect of P, but the basis of this effect is still not well understood. Evidently the phenomenon suggests that in some physiological situations, as it might be in epilepsy, estrogens enhance the plasticity of $GABA_a$ receptors. At the same time it is well known that the NS can modulate most $GABA_a$ receptor isoforms (Lambert et al. 2003).

In both phases of menstrual cycle in women with epilepsy elevated levels of 17-hydroxy-pregnenolone sulfate were

detected when compared with controls at almost unchanged DHEAS. Most probably significant higher levels of circulating metabolite with proconvulsant activity might increase frequency of epileptic seizures, but eventually this influence is not connected with the stage of menstrual cycle.

Long-term alcohol abuse among women is a growing thread to their health and reproductive condition (Hugues et al. 1980; Torres and Ortega, 2003). Highly sensitive and selective GC-MS method was used for the analysis of the progesterone isomers, pregnenolone and E_2 in order to evaluate how the NAS reflect impaired P synthesis in women treated for alcohol addiction and whether during the therapy the female reproductive functions could be restored by reinstatement of the steroid biosynthesis. Initially and during the first month of the treatment the levels of P and its reduced metabolites were significantly lower than in controls, which was in accordance with the data of Pettersson et al. (1990) and Sarcola et al. (1999). The beneficial influence of the therapy, as the obtained results pointed out, its compensating effect in P, 17α-hydroxy-progesterone and progesterone isomers. Pregnanolone demonstrated the least pronounced differences, possibly due to its rapid conversation (Parivar et al. 1996). The restoration of P and its isomers to the physiolocical levels during alcohol detoxification therapy could be explain by the regeneration of P synthesis impaired by alcohol abuse or by the recovery of enzyme activities. The restoration of the P metabolism to P isomers is also likely. Third possibility could be the adaptation of the organism to the rising demand for $GABA_a$ receptor activating substances to the cessation of alcohol intake, taking into account the similar mechanism in the effect of alcohol and S activators on it (Morrow, 2001).

It is worth to notice that the ratios of the 5a- to 5ß- isomers showed a gradual increase in the luteal phase, as well as the ratio of neuroactivating pregnanolone sulfate to neuroinhibiting allopregnanolone was expressing a regular decreased tendency during therapy in both phases of the menstrual cycle.

A similar pattern as for the P and its metabolites was found for E_2. This finding suggests that the restoration of ovarian activity during medication is not limited only to P and its metabolites. As already mentioned, estrogens are also neuroprotective. It is quite likely that the reduction of neuroinhibiting pregnanolone isomers in women treated for alcohol addiction is primarily connected with ovarian steroid biosynthesis impairment. The restoration of S concentrations during detoxification therapy for alcohol addiction has a favorable effect on reproductive functions and the psychosomatic stability of premenopausal women.

6. Conclusions

1. A novel and original GC-MS method with sensitivity and selectivity for simultaneous quantification of free and conjugated pregnane and androstane steroids has been developed and evaluated. The sensitivity ranged from hundreds of femtograms to tens of picograms, depending on the steroid fragmentation pattern.

2. Significantly ($p < 0.006$) lower levels of 17α-hydroxy-pregnenolone, 17α –hydroxy-progesterone and higher values of pregnenolone sulfate ($p < 0.0001$) were found in both phases of the menstrual cycle of women with epilepsy when compared with controls. Progesterone concentrations did not

differ in follicular phase, but they were depressed in luteal phase in women with epilepsy.

3. Premenopausal women with alcohol addiction showed depressed progesterone, 17α-hydroxy-progesterone, progesterone isomers and estradiol levels along with elevated pregnenolone sulfate values during the menstrual cycle in comparison with healthy controls. The reinstatement of serum progesterone, estradiol and progesterone isomers during the treatment for alcohol abuse and its favorable effect was demonstrated on both reproductive function and the psychosomatic stability of the patients.

4. The levels of 7-hydroxy-metabolites of DHEA and 3β,17β-androstenediol are age and sex dependent, but independent of the phase of the menstrual cycle. Pregnanolone isomers are of adrenal origin during the follicular phase and of gonadal source during the follicular phase. These isomers and their basic levels are similar in men and women, but increasing in luteal phase (3 times) and escalating (over 38 times) during pregnancy, compared with follicular phase.

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