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*This paper is dedicated to the memory of Robert Morfin*

## The Quantitation of 7 $\beta$ -Hydroxy-Epiandrosterone in the Plasma and Seminal Plasma of Men With Different Degrees of Fertility

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### Summary

7 $\beta$ -hydroxy-epiandrosterone (7 $\beta$ -OH-EpiA) is an endogenous androgen metabolite that has been shown to exert neuroprotective, anti-inflammatory and anti-estrogenic effects. However, to the best of our knowledge no information is available about this androgen steroid in relation to sperm quality. We analyzed 7 $\beta$ -OH-EpiA in plasma and seminal plasma using a newly developed isotope dilution ultra-high performance liquid chromatography – mass spectrometry method. Validation met the requirements of FDA guidelines. Levels of 7 $\beta$ -OH-EpiA were measured in 191 men with different degrees of infertility. One-way analysis of variance followed by multiple comparison and correlation analysis adjusted for age, BMI and abstinence time were performed to evaluate the relationships between this steroid and sperm quality. Concentrations of 7 $\beta$ -OH-EpiA in seminal plasma were significantly higher in severely infertile men in comparison with healthy men and slightly infertile men. The same trend was found when blood plasma was evaluated. Furthermore, plasma 7 $\beta$ -OH-EpiA negatively correlated with sperm concentration (-0.215;  $p < 0.01$ ) and total count (-0.15;  $p < 0.05$ ). Seminal 7 $\beta$ -OH-EpiA was negatively associated with motility (-0.26;  $p < 0.01$ ), progressively motile spermatozoa (-0.233;  $p < 0.01$ ) and nonprogressively motile spermatozoa (-0.188;  $p < 0.05$ ). 7 $\beta$ -OH-EpiA is associated with lower sperm quality and deserves more research in that respect.

### Key words

7 $\beta$ -hydroxy-epiandrosterone • Sperm quality • Steroidogenesis • Steroid • Seminal plasma • Reproduction

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### Introduction

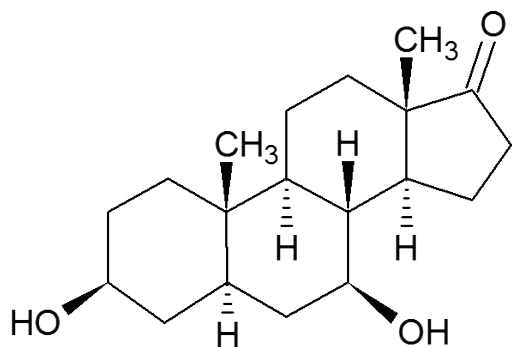
Epiandrosterone (EpiA; 5 $\alpha$ -Androstan-3 $\beta$ -ol-17-one), in the fitness world known as “prohormone”, became very popular in the 1990s. It is commonly available on the black market and advertised to be a DHT booster with no estrogenic side effects such as gynecomastia or water retention. However, increased DHT levels after EpiA administration were not demonstrated in a recent experiment (Piper *et al.* 2017). 7 $\beta$ -hydroxylated EpiA (7 $\beta$ -OH-EpiA; 5 $\alpha$ -Androstane-3 $\beta$ ,7 $\beta$ -diol-17-one; Fig. 1) is an endogenous androgen metabolite of EpiA and dehydroepiandrosterone (DHEA), respectively, and was reported to even have anti-estrogen effects *in vitro* (Niro *et al.* 2012).

In addition to anti-estrogenic properties, neuroprotective (Pringle *et al.* 2003), cytoprotective and immunomodulatory (Davidson *et al.* 2008, Le Mee *et al.*

2008) effects have also been reported.  $7\beta$ -OH-EpiA reduced ischemia-induced neuronal damage (both *in vivo* and *in vitro*) and was suggested as a novel neuroprotective compound. It was concluded that the neuroprotective efficacy lies in the 7-hydroxylation, not in epimerization, because both 7-hydroxy epimers possess neuroprotective properties. However,  $7\beta$ -OH-EpiA was protective at even lower concentrations (10 nM) than the  $7\alpha$ -epimer (100nM)(Pringle *et al.* 2003).  $7\beta$ -OH-EpiA also reduced neurodegeneration in an Alzheimer's disease model (Dudas *et al.* 2004), suggesting that it can have neuroprotective effects in acute as well as chronic diseases. With its anti-inflammatory properties and ability to modulate arachidonic acid metabolism, it was also suggested as an alternative approach in the treatment of inflammatory bowel disease (Hennebert *et al.* 2008).

EpiA can be formed by 2 mechanisms: 1) the classic pathway from androst-4-ene-3,17-dione, or 2) indirectly through a back-door pathway from isopregnanolone ( $3\beta$ -hydroxy- $5\alpha$ -pregnan-20-one) by CYP17A1 (17 $\alpha$ -hydroxylase, 17.20 lyase) (Vankova *et al.* 2016).  $7\alpha$ -hydroxylation of EpiA is catalyzed by CYP7B1 (oxysterol and steroid 7 $\alpha$  hydroxylase) (Kim *et al.* 2004, Starka 2017). NADP(H) dependent 11 $\beta$ -hydroxysteroid dehydrogenase type 1 is then responsible for the conversion of  $7\alpha$ -OH-EpiA to  $7\beta$ -OH-EpiA (Hennebert *et al.* 2007). Oxygenation of EpiA occurs mainly in the intestine, brain and especially liver (reviewed in El Kihel 2012).

The information about  $7\beta$ -OH-EpiA and reproductive health is scarce. 7-hydroxylated derivatives of DHEA have been already found in seminal plasma and their possible immunomodulatory and antioxidative properties in this matrix were discussed (Hampl *et al.* 2003, Pohanka *et al.* 2002). With respect to immunomodulatory effects of  $7\beta$ -OH-EpiA, its presence in seminal plasma is of interest.



**Fig. 1.** Molecular structure of  $7\beta$ -hydroxy-epiandrosterone.

To the best of our knowledge no information is available on plasma or seminal plasma levels of  $7\beta$ -OH-EpiA in humans. Therefore, we aimed to develop a sensitive and accurate method to measure  $7\beta$ -OH-EpiA in both fluids. Furthermore, we determined the relationships between  $7\beta$ -OH-EpiA and sperm quality assessed by spermogram values in men with different degrees of fertility.

## Methods

### Study group

The studied cohort consisted of 191 Czech men (all Caucasian) attending the Pronatal Centre of Assisted Reproduction (Prague, Czech Republic). Our previous study provides a detailed overview of the characteristics of the study population (Vitku *et al.* 2016). All subjects underwent an ejaculate examination according to the World Health Organization (WHO) 2010 criteria, and based on these results they were divided into 4 groups: first group consisted of normospermic men, the second group comprised oligospermic, asthenospermic or oligoasthenospermic men, the third group included teratospermic, oligoasthenoteratospermic and asthenoteratospermic men, while the fourth group consisted of azospermic men. We termed these groups: 1) healthy men, and 2) slightly, 3) moderately and 4) severely infertile men. Table 1 shows the basic characteristics of these groups.

The mean age ( $\pm$  SD) of all participants was  $35.8\pm 5.6$  years and mean BMI value was  $27.2\pm 3.6$  kg/m<sup>2</sup>. 55 % of men in our study were overweight (BMI 25-30) and 20 % were obese (BMI>30). Neither BMI values, nor age, nor abstinence time significantly differed among the groups of men studied (Table 1, Vitku *et al.* 2016).

The study was performed in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The protocol was approved by the Ethical Committee of the Institute of Endocrinology. Informed and written consent with the use of biological materials were obtained from all subjects before participating in the project.

### Chemicals and reagents

The steroids  $7\beta$ -OH-EpiA and D2- $7\beta$ -OH-EpiA were synthesized in Laboratoire de Chimie moléculaire, Conservatoire National des Arts et Métiers as previously described (Ferroud *et al.* 2012, Ricco *et al.* 2011). 2-hydrazinopyridine, ammonium formate and trifluoroacetic acid were from Sigma-Aldrich (St. Louis,

**Table 1.** Comparison of age, BMI, and semen parameters among men with different degrees of infertility.

	Group 1 (n=89)	Group 2 (n=59)	Group 3 (n=25)	Group 4 (n=18)	p-value	Multiple comparisons
Age (years)	35.9 (34.8; 37.0)	35.7 (34.3; 37.0)	35.8 (33.8; 37.8)	35.2 (32.9; 37.6)	0.9715	
BMI (kg/m <sup>2</sup> )	27.7 (26.7; 28.8)	26.9 (25.8; 28.1)	26.1 (24.9; 27.5)	26.4 (24.8; 28.1)	0.2748	
Abstinence time (days)	4.072 (3.722; 4.481)	4.152 (3.711; 4.687)	3.884 (3.265; 4.725)	3.415 (2.846; 4.201)	0.3953	
Concentration (mil/ml)	61.3 (55.7; 66.8)	25.4 (18.7; 32.2)	18.5 (7.2; 29.8)	0	0.0000	1>2,3>4
Total count (mil)	228.1 (202.7; 253.5)	96.1 (65.1; 127.1)	68.7 (16.7; 120.7)	0	0.0000	1>2,3>4
Motility (%)	58.2 (55.2; 61.0)	42.7 (39.4; 46.0)	23.3 (17.7; 28.8)	0	0.0000	1>2>3>4
- Progressively motile sperms	47.3 (44.7; 49.9)	30.2 (27.0; 33.4)	13.1 (7.8; 18.5)	0	0.0000	1>2>3>4
- Nonprogressively motile sperms	10.9 (9.7; 12.2)	12.5 (10.9; 14.0)	10.2 (7.6; 12.8)	0	0.0000	1,2,3>4
- Immotile sperms	41.8 (39.1; 44.5)	57.3 (54.0; 60.6)	76.7 (71.1; 82.2)	0	0.0000	3>2>1>4
Morphology (%)	13.4 (12.5; 14.3)	7.9 (6.8; 9.0)	2.4 (0.1; 4.8)	0	0.0000	1>2>3,4

Data are shown as means and 95.0 % confidence intervals (in the parentheses) for each group, the levels of significance of the model and multiple comparisons are provided. Group 1 – normospermic men, Group 2 – oligospermic/asthenospermic/oligoasthenospermic men, Group 3 – teratospermic/oligoteratospermic/oligoasthenoteratospermic men, Group 4 – azoospermic men.

**Table 2.** Retention times and MS/MS conditions.

Analyte	Retention time (min)	Precursor ion	Quantification ion	Confirmation ion	DP (V)	EP (V)	CEP (V)	CE (V)*	CXP (V)
7 $\beta$ -OH-EpiA	3.77	398.3	105.1	108.0	61	7	18	75 (41)	4
D2-7 $\beta$ -OH-EpiA	3.76	400.4	108.0	105.2	66	7	20	41 (71)	4

DP – declustering potential, EP – entrance potential, CEP – collision entrance potential, CE – collision energy, CXP – collision cell exit potential, V – volts. \* Values for the confirmation ion are given in the brackets.

**Table 3.** LC conditions and parameters of validation for plasma and seminal 7 $\beta$ -OH-EpiA.

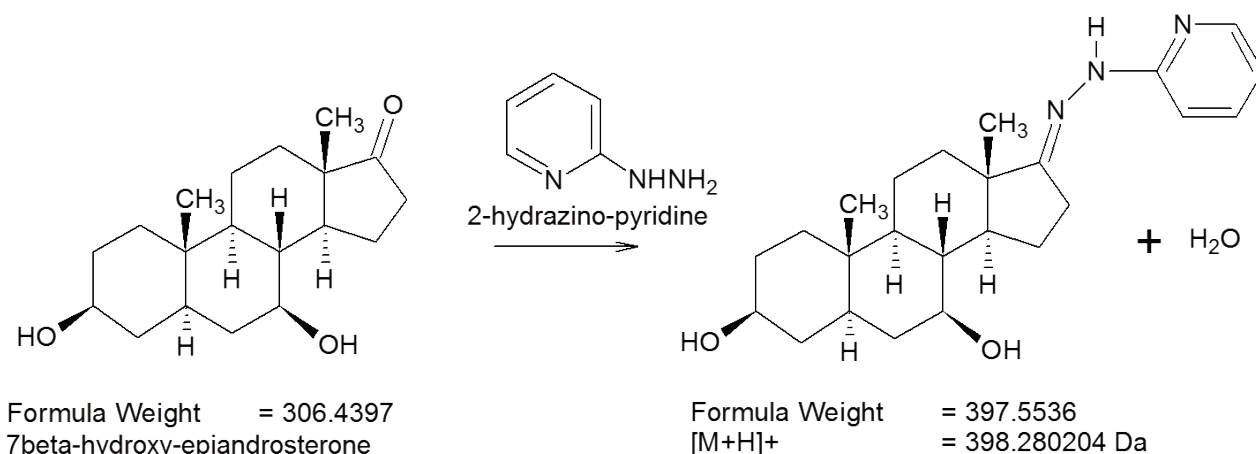
	Calibration range (ng/ml)	Correlation coefficient	LLOQ (pg/ml)	Precision (%)		Recovery (%)		
				Intra-day	Inter-day	Addition 1	Addition 2	Addition 3
<i>Plasma 7<math>\beta</math>-OH-EpiA</i>	0.024-3.0	0.9991	11	6.95-9.18	6.44-15.17	92.22	106.11	105.60
<i>Seminal 7<math>\beta</math>-OH-EpiA</i>	0.024-3.0	0.9991	27	5.06-11.93	1.00-9.49	106.96	89.38	

Standard additions were 0.06, 0.3 and 0.72 ng/ml in plasma and 0.12 and 0.6 ng/ml in seminal plasma. Precision is presented as ranges from all additions (during single analytical run for intra-day precision and during three runs in different days for inter-day precision, respectively). LLOQ – lower limit of quantification.

**Table 4.** Comparison of plasma and seminal levels of 7 $\beta$ -OH-EpiA in ng/ml in groups of men with different degrees of infertility.

	Group 1 (n=89)	Group 2 (n=59)	Group 3 (n=25)	Group 4 (n=18)	p-value	Multiple comparisons
<i>Plasma 7<math>\beta</math>-OH-EpiA</i>	0.027 (0.020; 0.034)	0.030 (0.021; 0.039)	0.039 (0.024; 0.053)	0.040 (0.024; 0.056)	0.234	-
<i>Seminal 7<math>\beta</math>-OH-EpiA</i>	0.071 (0.015; 0.127)	0.086 (0.021; 0.151)	0.183 (0.082; 0.285)	0.316 (0.200; 0.431)	<b>0.022</b>	1,2<4

Data are shown as means and 95.0 % confidence intervals (in parentheses) for each group; the levels of significance of the model and multiple comparisons are provided. p-value<0.05 is highlighted in bold. Group 1 – normospermic men, Group 2 – oligospermic/asthenospermic/oligoasthenospermic men, Group 3 – teratospermic/oligoteratospermic/oligoasthenoteratospermic men, Group 4 – azospermic men.



**Fig. 2.** Derivatization reaction of 7 $\beta$ -OH-EpiA with 2-hydrazinopyridine.

MO, USA). Methanol and water for chromatography were of HPLC grade and were purchased from Merck (Darmstadt, Germany). Diethyl ether was obtained from Lach-Ner, s.r.o. (Neratovice, Czech Republic). The physiological solution (0.9 % sodium chloride) was from B. Braun (Melsungen AG, Germany).

#### Sample preparation

Determination of 7 $\beta$ -OH-EpiA in plasma and seminal plasma was added to an existing method that has already been published (Sosvorova *et al.* 2015, Vitku *et al.* 2016). In brief, a sample of plasma (500  $\mu$ l) or seminal plasma (1,000  $\mu$ l) was spiked with 10  $\mu$ l of an internal standard (IS) mixture (deuterated analogues of 7 $\beta$ -OH-EpiA, pregnenolone, 17-OH-pregnenolone, dehydroepiandrosterone -DHEA, 7 $\alpha$ -OH-DHEA, 7-oxo-DHEA, cortisol, cortisone, testosterone, androstendione, dihydrotestosterone) and diluted with 500  $\mu$ l of physiological solution. Samples were shaken, and a liquid-liquid extraction using diethyl ether (3 ml, 1 min) was performed. Dry residues were derivatized by 100  $\mu$ l of 2-hydrazinopyridine (Fig. 2) in methanol with the addition of trifluoroacetic acid (1 mg: 5 ml: 1.63  $\mu$ l) according to Higashi *et al.* (2007). The samples were again shortly shaken and then sonicated for 15 min. After evaporating under a gentle stream of nitrogen, samples were redissolved in 100  $\mu$ l of 5 mM ammonium formate in 60 % methanol, of which 50  $\mu$ l was injected into the liquid chromatograph.

#### Liquid chromatography/mass spectrometry (LC-MS/MS) of steroids

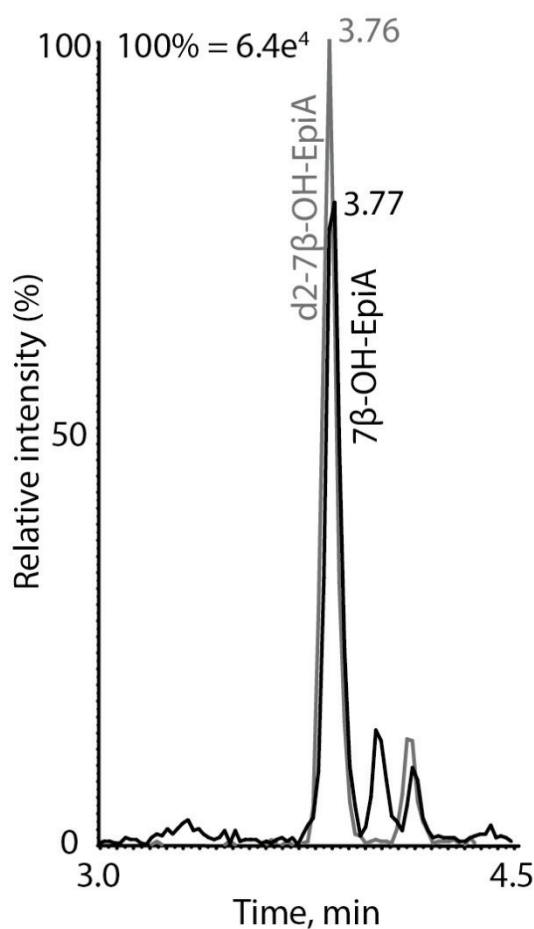
An ultra-high pressure liquid chromatography (UHPLC) Eksigent ultraLC 110 system (Redwood City,

CA, USA) equipped with a Kinetex C18 column (100 x 3.0 mm, 2.6  $\mu$ m; Phenomenex, Torrance, CA, USA) and Security Guard ULTRA cartridge system (UHPLC C18 for 3 mm ID column; Phenomenex, Torrance, CA, USA) was used for analyte separation. Column temperature was maintained at 50  $^{\circ}$ C and separation was carried out at a flow rate of 0.75 ml/min. Detection of the analytes was performed on an API 3200 mass spectrometer (Sciex, Concord, Canada) with an electrospray ionization (ESI) probe operating in positive mode. Retention times and transitions with optimized conditions for MS for 7 $\beta$ -OH-EpiA and D2-7 $\beta$ -OH-EpiA are summarized in Table 2. Example of the chromatogram obtained from the real sample (concentration of 7 $\beta$ -OH-EpiA 2.64 ng/ml) is provided in Figure 3. Analyst 1.6 software was used for system control and data evaluation. More information about the LC-MS/MS conditions can be found in the study of Sosvorova *et al.* (2015). Calibration ranges, correlation coefficients of calibration curves and lower limits of quantifications (LLOQs) are provided in Table 3.

#### Validation

Validation parameters met the criteria of the FDA Guidance for Industry (Food and Drug Administration 2001) in terms of selectivity, accuracy, precision, recovery, calibration curve, and stability of analyte in spiked samples. Acceptable selectivity was defined as the absence of any detectable SRM LC-MS/MS ion currents at the retention time regions of each analyte and its deuterated standards in charcoal treated plasma samples. Samples were tested for further interference during prevalidation phase. Validation experiments included standard additions of 7 $\beta$ -OH-EpiA

in six replicates. Four different concentrations were measured in the plasma matrix (pooled plasma + 3 additions), whereas only 3 different concentrations were measured in seminal plasma (pooled seminal plasma + 2 additions) due to the limited amount of seminal plasma matrix. Accuracy, precision and recovery were then determined and are shown in Table 3. The spiked concentrations were 0.06, 0.3 and 0.72 ng/ml in plasma and 0.12 and 0.6 ng/ml in seminal plasma. Stability tests and matrix effects were evaluated as well, with satisfactory results. More details on performing validation tests can be found in Vitku *et al.* (2016).



**Fig. 3.** Chromatogram of 7 $\beta$ -OH-EpiA in plasma. Measured concentration was 2.64 ng/ml.

#### Statistical analysis

Data that were below the limit of detection were replaced by  $LOD/\sqrt{2}$  (Hornung and Reed 1990). All data were subsequently transformed by Box-Cox transformation due to non-Gaussian distribution and heteroscedasticity of variables. One-way analysis of

variance (ANOVA) followed by least square difference multiple comparisons was used to evaluate differences between groups of variously infertile men. Partial correlations adjusted for age, BMI and abstinence time were used for assessing relationships between levels of 7 $\beta$ -OH-EpiA and parameters of sperm quality. The statistical software Statgraphics Centurion XVI from Statpoint Inc. (Warrenton, VA, USA) was used for data transformations, ANOVA testing and multiple comparisons. Partial correlations analyses were performed using NCSS 2007 (Kaysville, UT, USA).

## Results

We analyzed 191 samples of plasma and seminal plasma. 7 $\beta$ -OH-EpiA was detected in 65 % of plasma samples and 84 % of seminal samples. Differences between plasma and seminal levels of 7 $\beta$ -OH-EpiA in 4 groups of men with various degree of infertility are given in Table 4. Seminal plasma showed a mild accumulation of this steroid in comparison with blood plasma. Furthermore, 7 $\beta$ -OH-EpiA concentrations in seminal plasma increase from healthy men towards severely infertile men. The same trend was also observed for 7 $\beta$ -OH-EpiA levels in plasma.

Partial correlations adjusted for age, BMI and abstinence time between sperm parameters and 7 $\beta$ -OH-EpiA in both body fluids are given in Table 5. Plasma 7 $\beta$ -OH-EpiA negatively correlated with sperm concentration and total count, while 7 $\beta$ -OH-EpiA in seminal plasma was negatively associated with motility including progressively motile spermatozoa and nonprogressively motile spermatozoa. Taken together, these observations indicated negative associations of this steroid with sperm quality.

To be complete, we investigated the relationships between plasma and seminal 7 $\beta$ -OH-EpiA and the following other steroids measured in our previous study (Vitku *et al.* 2016) in the same cohort – pregnenolone, 17-hydroxy-pregnenolone, cortisol, cortisone, DHEA, 7 $\alpha$ -hydroxy-DHEA, 7 $\beta$ -hydroxy-DHEA, 7-oxo-DHEA, testosterone, androstenedione, dihydrotestosterone, 17 $\beta$ -estradiol, estrone and estriol. No correlations except for a positive correlation of 7 $\beta$ -OH-EpiA with DHEA in seminal plasma ( $r=0.227$ ;  $p=0.009$ ) were found.

**Table 5.** Partial correlations adjusted for age, BMI and abstinence time between sperm parameters and 7 $\beta$ -OH-EpiA in both body fluids.

Sperm parameters	Plasma 7 $\beta$ -OH-EpiA		Seminal 7 $\beta$ -OH-EpiA	
	r	p-value	r	p-value
Concentration (mil/ml)	-0.215	<b>0.004</b>	-0.045	0.611
Total count	-0.150	<b>0.046</b>	-0.112	0.205
Motility	-0.035	0.643	-0.260	<b>0.003</b>
- Progressively motile spermatozoa	-0.024	0.754	-0.233	<b>0.008</b>
- Nonprogressively motile spermatozoa	-0.144	0.055	-0.188	<b>0.032</b>
- Immotile spermatozoa	-0.149	<b>0.048</b>	-0.022	0.805
Morphology	-0.145	0.067	-0.105	0.262

The correlation coefficient of partial correlation *r* is a measure of the strength between variables and the *p*-value shows statistical significance. *p*-value<0.05 are highlighted in bold.

## Discussion

To the best of our knowledge, this is the first study to determine 7 $\beta$ -OH-EpiA concentrations in human body fluids. Derivatization by 2-hydrazinopyridine in the method enhanced the sensitivity and enabled us to analyze this androgen metabolite in the pg/ml order of magnitude, which are the concentrations occurring in plasma and seminal plasma. 2-hydrazinopyridine has also been previously used for derivatization of oxosteroids with satisfactory results (Higashi *et al.* 2007, Lionetto *et al.* 2017).

The concentrations of 7 $\beta$ -OH-EpiA were found to range from LLOQ-0.167 ng/ml (0.549 nmol/l) in normospermic male plasma and LLOQ-0.358 ng/ml (1.176 nmol/l) in seminal plasma. In comparison with other 7-hydroxylated C19 steroids, the plasma concentrations were within the same order of magnitude or slightly lower (Hampl *et al.* 2003, Macova *et al.* 2014, Starka *et al.* 2006, Vitku *et al.* 2017). Concentrations of 7 $\beta$ -OH-DHEA have been found to be lower in seminal plasma than in plasma (Vitku *et al.* 2016), in contrast to 7 $\beta$ -OH-EpiA where an accumulation in seminal plasma was observed.

The most striking differences were found between seminal concentrations of 7 $\beta$ -OH-EpiA in healthy men and severely infertile men; however, the whole steroid hormonal balance seems to be disrupted in men with impaired spermatogenesis. Seminal T (Zalata *et al.* 2014, Zhang *et al.* 2010), DHT (Vitku *et al.* 2016, Zalata *et al.* 1995) and androstenedione (Zalata *et al.* 2014) levels have been found to decrease towards infertile men. On the other hand, seminal concentrations of 17 $\alpha$ -hydroxy-progesterone (Zalata *et al.* 2014), progesterone (Zalata *et al.* 2014), DHEA (Vitku *et al.*

2016), 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (Zalata *et al.* 2014), E2 (Bujan *et al.* 1993, Luboshitzky *et al.* 2002, Vitku *et al.* 2016, Vitku *et al.* 2015, Zalata *et al.* 2014, Zhang *et al.* 2010), other estrogens (Vitku *et al.* 2016), as well as 7 $\beta$ -OH-EpiA have been found to increase.

These are preliminary data and the molecular mechanism of 7 $\beta$ -OH-EpiA action in semen is unknown so far. We could hypothesize that the steroidogenic pathway towards DHT is suppressed on behalf of the production of E2 and 7 $\beta$ -OH-EpiA. The elevated levels of the cytoprotective 7 $\beta$ -OH-EpiA in infertile men could be a compensatory mechanism towards an improvement of sperm quality.

## Conclusion

Many steroids circulate in the human body, some of which have biological activity, while others are precursors or metabolites (without biological activity). The functions of many of these other steroids including 7 $\beta$ -OH-EpiA are frequently insufficiently explored. Here we contribute information on the concentration ranges of this steroid in healthy men compared to men with different degrees of fertility. The data revealed a negative relationship between 7 $\beta$ -OH-EpiA and sperm parameters, and may help to better understand changes in the steroid pathway in relation to partial or complete infertility.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

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