Long Term Ingestion of Hormonal Contraceptive Agents – The Exogenous Factor for the Increased Generation of Reactive Oxygen Species (ROS)?

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Abstract: In three different in-vivo studies the formation of reactive oxygen species (ROS) was investigated utilizing the blood of healthy women who use hormonal contraception (HC) and a control group of non-HC users. ROS were quantified by a validated, commercially available photometric kit. In the first study ROS were measured in blood of women from various regions of the Austrian territory. The findings revealed that women using HC had significantly higher ROS levels in the blood (mean 572 ± 136 FORT; one FORT unit represents 0,26 mg hydrogen peroxide per litre) than the non-HC group, HC (347 ± 80 FORT). In the second study, ROS from female students of pharmacy were measured. The results were similar to the first investigation: 519 + 92 FORT in the group using HC and 331 ± 68 FORT in the controlled non-HC group. These outcomes are statistically significant ($p<0.001$). In the third analysis the ROS levels of women using HC, either a progestational agent alone or in combination with estrogen, were measured. ROS values in the progestational agent alone group were 342 + 70 FORT. ROS values in the estrogen combination group had significantly higher ROS values (520 ± 112 FORT, $p < 0.0001$). These results indicate that estrogen may play a role for increased ROS concentrations in the blood of women performing HC.

Keywords: Female, volunteers, hormone, contraception, reactive oxygen species, gestagen, progestational agent, and estrogen, metabolism.

INTRODUCTION

The human organism needs oxygen for the production of energy in order to maintain intracellular biochemical reactions that are necessary for cell living. This energy is required for the oxidation of nutrients or biologicals and for many metabolic reactions including the metabolism of drugs. During such chemical reactions, small amounts of partially reduced and reactive oxygen species (ROS) are formed. Normally, the human organism has sufficient capacity to detoxify these reactive intermediates rapidly.

Oxidative stress (OS) can be defined as an imbalance between the production of ROS and the capacity of the organism to neutralize these reactive oxygene structures. If such an imbalance persists for a longer time period, these oxidative disturbances can cause damage of various cellular structures including lipids, proteins and nucleic acids and can hence lead to lipid- or protein radicals. As a consequence OS is believed to be involved in development of many diseases, such as diabetes, http://en.wikipedia.org/wiki/Atherosclerosisatherosclerosis, Parkinson's and Alzheimer's disease and it may also be important in cell ageing [1]. Various factors have been identified to promote the genesis of ROS, among them the most important are an unhealthy way of life, poor diet, cigarette smoking, alcohol, electro smog, excessive use of computers and mobile phones, travelling by plane, solarium etc. But our organism possesses a large armamentarium (the so called antioxidative capacity) for the detoxification of ROS: first some enzymes like superoxid dismutase, glutathion-S-transferases or catalase destroy ROS leading to the formation of simple water molecules. Another group of compounds represents vitamins (especially ascorbic acid and vitamin E), that protect endogenous structures from radical formation. The third group comprises redox active compounds like glutathion or coenzyme Q. Lastly, trace elements as selenium, copper and others have been found to contribute to the neutralization of ROS.

It is known that drugs can be responsible for the genesis of ROS because either they are metabolized by oxidative mechanisms (CYP450) in the liver (e.g. hormones, antibiotics) or are capable to induce ROS

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formation by electron transfer (e.g. anthracyclines, bleomycin) [2-4].

Objectives

The aim of this study was evaluate the extent of ROS formation in healthy female volunteers who use hormonal contraception (HC) and to compare with ROS values in non-HC women.

EXPERIMENTAL

Volunteers

Three separate investigations were carried out during 2008 to 2010:

Women in the whole federal territory of Austria: city of Vienna, Graz, St. Pölten, Wiener Neustadt, Linz, Salzburg, Innsbruck. The age ranged from 19 - 64 years, N = 165. Women were grouped by HC users or non-HC users.

Female pharmacy student from the University of Vienna: Age range 22 - 26 years, N = 187. Students were grouped by HC users or non-HC users.

Female pharmacy student, as well as other female healthy volunteers (age range from 18 - 44 years, N = 185), were studied for the progestational agent alone group and gestagen /estrogen combination group.

Before ROS measurements, each person entering one of these investigations had to fill out a confidential questionnaire covering their lifestyle, personal status and detailed information regarding the use of HC.

Blood Samples

After disinfection, a whole blood sample of 20 µl was drawn from the finger pad of the volunteer, collected in a heparinised glass tube (20 µl volume) and quantification of ROS was performed immediately.

Quantification of ROS

Blood samples in the 20 µl glass tube were transferred into a 1.5 ml eppendorf tube containing 1.0 ml buffer and centrifuged at room temperature for one minute in a microfuge (centrifuge model C6000, Incomat, Germany). By this procedure, red blood cells were hemolysed and separated from plasma. The clear supernatant was put into a “ready for use” cuvette containing chemical reagents and kinetic measurement was performed thereafter at 4 and 8 min. Photometric measurements were carried by use of a photometer (model CR3000, Incomat, Germany) plus commercial available test kit (FORM® test, Callegari, Italy).

ROS quantification based on a modified “Haber – Weiss” reaction: shortly, the radical content of the blood sample (hydroperoxid activity) is transferred by an iron catalyzed electronic shift to a chrome-amine that itself reacts with phenylene-diamine. The resulting chromophor is stable for a few hours and can be quantified by a linear calibration graph [5].

Biometric Calculations

Amount of ROS is expressed as FORT units: one FORT represents the equivalent of 0,26 mg hydrogen peroxide per litre. In healthy people the ROS values range from 160 to 310 FORT; whereby, women show

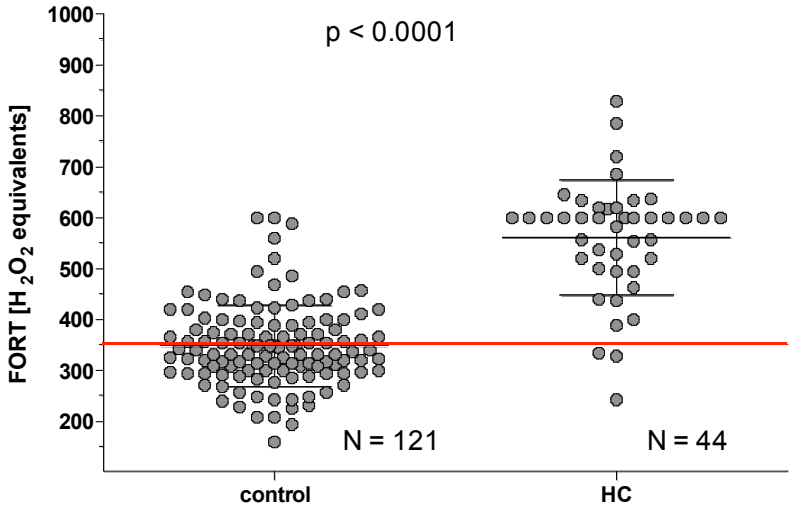

Figure 1: FORT values of healthy women in federal territory Austria: comparison of FORT values in women during HC with a control group without intake of hormones.

generally slightly higher ROS values than men. At 350 FORT a weak oxidative stress can be defined and above 350 FORT strong to very strong oxidative stress (graduated) exists. At the start of our investigations the upper limit of quantitation was 600 FORT. During our investigations we would improve the sensitivity of the assay and were able to quantify up to 1200 FORT.

The scientific softwares InStat 3.0® and GraphPad Prism 5.0® have been used for calculation of descriptive statistics and to evaluate statistical differences of means (unpaired, two-sided Student's t-test, p-level < 0.05).

RESULTS

The results of the first investigation (women in the complete federal territory Austria) are depicted in Figure **1** as a scattergram: for each woman the individual ROS value as well as mean value and standard deviation as lines are given. Mean ROS value in the group without HC was 347 FORT in the control group and 570 FORT in the group with HC, this is an increase of about 60 %. The difference between means of the two groups was statistically highly significant ($p < 0.0001$). Some increased ROS values in the control group can be explained by the fact that ROS formation not only depends on drug intake but also may be influenced by lifestyle. Therefore women had to fill out the questionnaire.

The second investigation comprised female student pharmacists, a HC and non-HC group. The result was similar to the first investigation. In the HC group mean FORT was 519 ± 92 and differed highly significant ($p < 0.0001$) from the control group without HC (331 ± 68 FORT). We have examined the first and second collective, consciously separating from each other, since the life age of the female students of pharmacy was close together: the FORT values in women depend on their age: older women show higher FORT values.

In this investigation our FORM test had a lower sensitivity: the upper cut off was 600 FORT, this cut off can be seen in the data of Figure **2**. Scattering of individual data (and results) was high because several factors favour the generation of ROS and had not been evaluated in this study. But in both investigations we could give evidence that HC induces significantly higher FORT values compared to the control groups.

In the third study we investigated whether the observed high FORT values in women with HC of study 1 and 2 are caused by the presence of estrogen in the preparation. Therefore, we compared a pure gestagen group and a gestagen – estrogen combination group. As illustrated by Figure **3**, pure gestagen did not increase FORT levels in the blood of volunteers. When a gestagen – estrogen combination is used the FORT values are elevated by a statistically significant margin. This may indicate, that of estrogen is responsible for the increased FORT values.

Table **1** compares results and statistical data for all three investigations. FORT values in all three control groups did not show any statistical significant difference between each other.

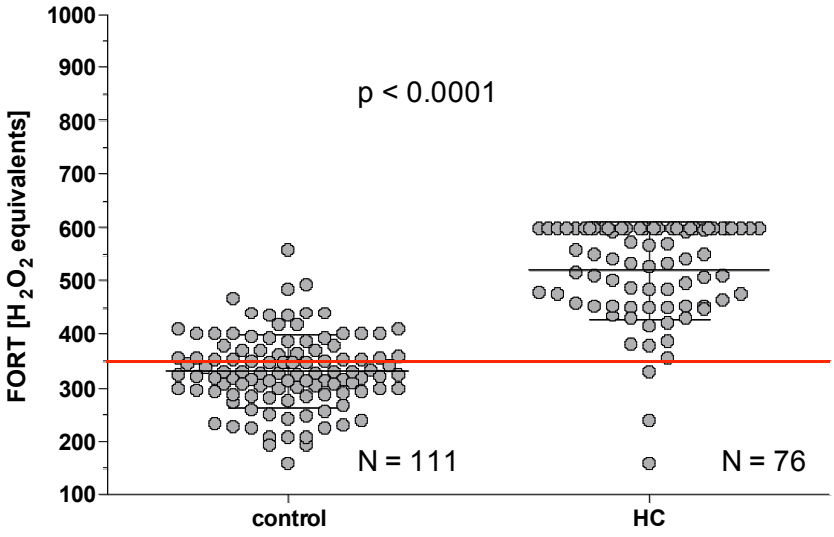

Figure 2: FORT values of female students of pharmacy: comparison of FORT values in female students during HC with a control group without intake of hormones.

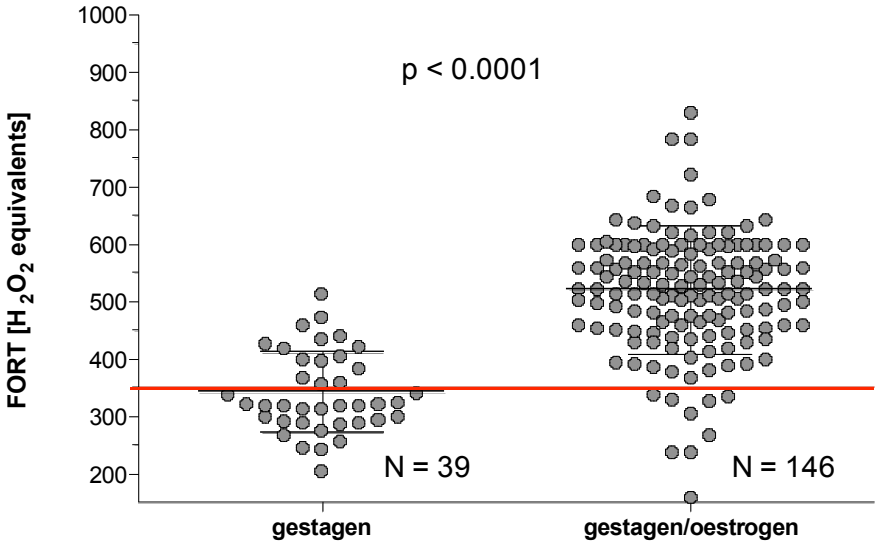

Figure 3: Comparison of FORT values in female healthy volunteers taking pure gestagen preparations or gestagen/estrogen combination preparation.

Table 1: Statistical Overview for Studies 1, 2 and 3 (Values in FORT)

Study	Group	N	Mean	SEM	SD	CV	Min	Max	Difference HC - control	p-Level HC <> control
1	1. control	121	347	7	80	22,9	160	600	- 225	0.0001
	2. HC	44	572	21	136	23,8	241	828	---	---
2	1. control	111	331	7	68	20,7	160	559	- 188	0.0001
	2. HC	76	519	11	92	17,8	160	600	---	---
3	1. gestagens HC	39	342	11	70	20,5	206	512	- 178	0.0001
	2. gest + oest HC	146	520	9	112	21,6	160	1074	---	---

HC … hormone contraception; CV … coefficient of variation [%]; p-levels between controls: 1<>2: 0.104 , 1<>3: 0.727, 2<>3: 0.390

DISCUSSION

Metabolism of Steroid Hormones

Steroid hormones show strong lipophilic character and therefore they must be biotransformed into more hydrophilic metabolites to be accessible for the elimination from human body. For these processes there are two principle phases available:

In the so-called phase-I reactions comprises hydroxylation, oxidative desamination, ester or amide splitting or also reduction processes. Phase-I reactions are usually catalysed by CytochromP450 isoenzymes or esterases. The phase-II reactions cover conjugation reactions, the drug (or its metabolite) is coupled to endogenous structures under energy consumption: The most frequent reactions are the ß-D-glucuronidation, the sulphatation and the glutathione conjugation [6].

As depicted in Figure **4**, gestagens are metabolized by reductive pathways. First in a phase-I reaction the keto-groups in ring A and D are reduced into the corresponding secondary alcohol. In a side reaction, a reduction of the double bond occurs in the ring A to the analogous single bond. In a following phase-II reactions two ß-D-O glucuronides are formed by ß-D-glucuronic acid and the hydroxy-group at the rings A and/or D and the resulting conjugates are eliminated with the bile. So the complete metabolism of gestagens occurs without consumption of oxygen, an increased formation of ROS during these biotransformation processes can be excluded.

The metabolism of the estrogens is quite different from that of the gestagens (see Figure **5**). In phase-I reactions the estrogens are predominantly hydroxylated at the rings A and D of the steroid

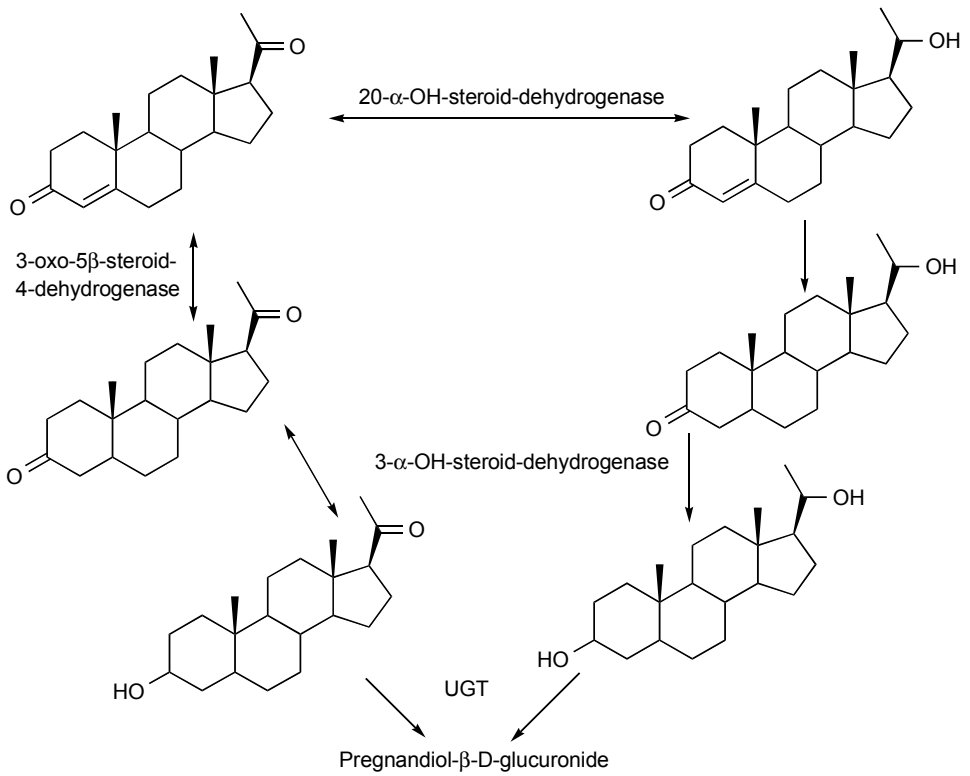

Figure 4: Metabolic conversion of progesteron in man (simplified schedule).

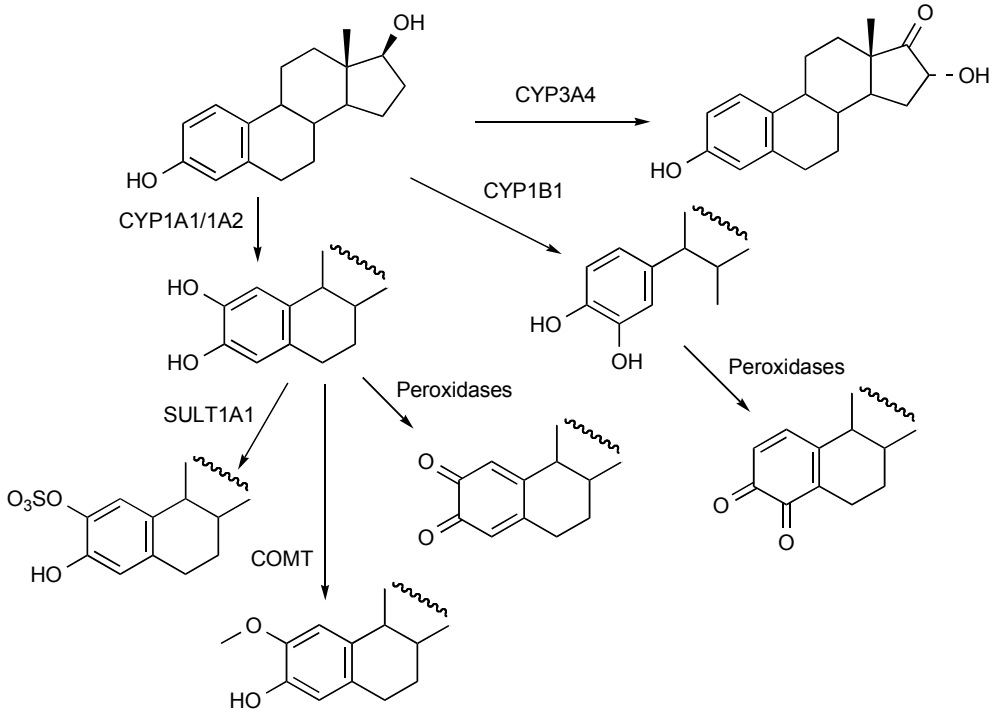

Figure 5: Metabolic conversion of estrogens in man (simplified schedule).

structure by which much more polar hydroxyl metabolites are formed. These metabolites comprise catechol, semiquinon and quinon structures that are conjugated in phase-II reactions with endogenous sulfate or glutathione. This hydroxylation needs activated oxygen as a co-factor and therefore in such side reactions ROS may be formed by different electronic transitional stages.

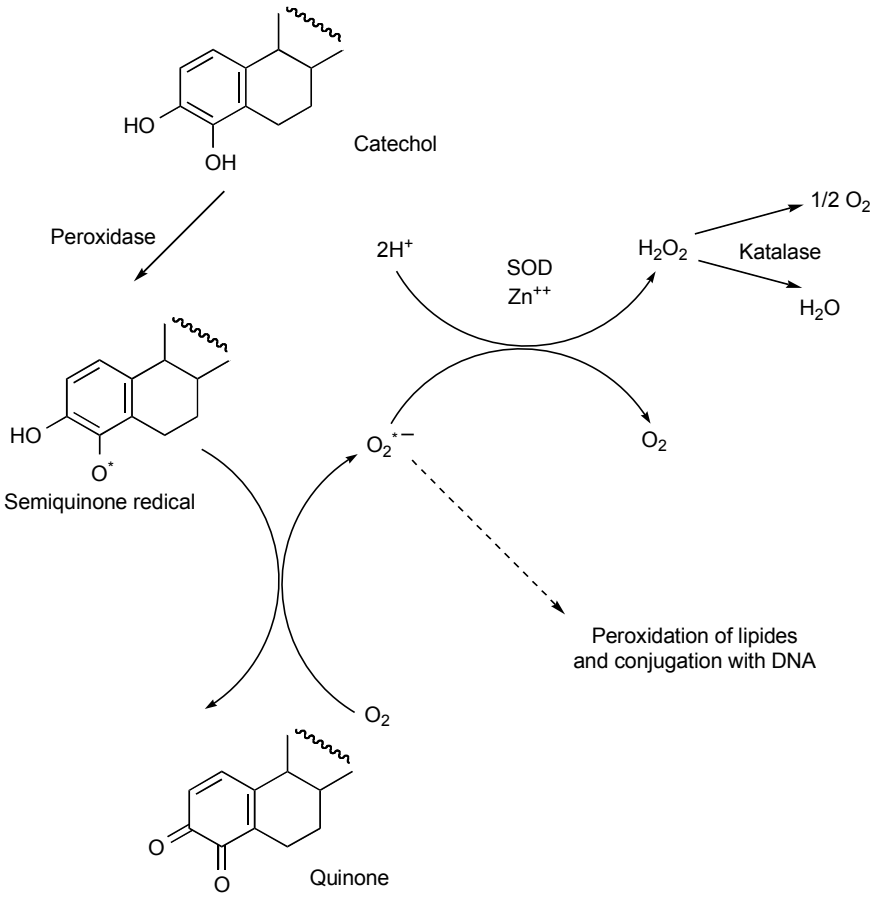

Figure 6: Mechanisms of ROS generation and detoxification during oxidative metabolism of estrogens.

Figure **6** gives an example for the formation of ROS during the oxidation of catechol into 3,4-quinone structure. This reaction proceeds via a transition state in which semiquinone radicals are formed. These semiquinone radicals can now produce under aerobic conditions oxygen radicals, that ultimately generate lipid peroxides and oxygen - DNA adducts [7]. Only the rapid conversion of these oxygen radicals into hydrogen peroxide by superoxide dismutase (SOD) can prevent from further radical chain reactions. The resulting hydrogen peroxides are splitted by the enzyme catalase into oxygen and water. Cardiac muscle cells do not possess these anti-oxidative defense SOD / catalase and are therefore very sensitive for oxidative damage. This mechanism is therefore the reason for the cardiotoxicity of cytostatics from the anthracycline type.

The resulting lipid peroxides (formed by these side reactions) are chemically stable and can be quantitated by the FORM test. As a consequence these lipid peroxides give indirectly evidence for the initially existence of short-living oxygen radicals. Although only small amounts of estrogens are taken (low mg amounts), the resulting hormone concentration in the blood already after continuous intake is sufficient for an increased formation of ROS. ROS generation induces chain reactions of oxygen radicals that form lipid peroxides that cannot be detoxified any more by limited antixodative defense.

Further, increased estrogen concentrations in the organism may lead to a deficit of zinc ions. Since zinc is an important cofactor of SOD, such a zinc deficit may decrease the antioxidative capacity [8]. The data on hand show that the regular taking of estrogens leads to considerably increased ROS concentrations. It is also interesting that after stopping intake of estrogens, ROS concentrations in the blood decrease very slowly (over a period of several weeks) until values below 350 FORT are reached.

CONCLUSION

This is the first time in Austria that ROS concentrations (quantitated as lipid peroxides) have been measured in the blood of women using HC. The data show that continuous intake of drugs containing

estrogens increases ROS concentrations in the blood of female volunteers whereas HC with gestagenes did not increase ROS levels. Comparing the metabolism of gestagens and estrogens in humans, the oxidative metabolism of estrogens might be one reason for the observed higher ROS levels. Estrogens are metabolized by hydroxylation and quinone formation. This reactive transitient status of semiquinones and the resulting semiquinone radicals may increase the formation of ROS. Gestagens are metabolized via reductive metabolism; therefore, ROS are not generated. The question arises whether such high ROS concentrations can be decreased by improving the antioxidative defense. This would be of great interest and importance for women who continuously use HC.

In a further study we will investigate various hormonal contraceptive formulations (implants, nuva ring, plaster) and their impact on ROS formation.

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