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Influence of Maternal Smoking during Pregnancy on Oxidant Status in Amniotic Fluid

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Abstract. *Background: Approximately 20% of women in Germany are smokers, and some of them are unable to stop smoking during pregnancy. As cigarette smoke generates free radicals, it has been suggested that it may be one of the major sources of oxidant stress in pregnant women and unborn fetuses. On the other hand, the human placenta is known to be a major source of pro-oxidant agents, antioxidant enzyme systems, and hormones, and is able to keep lipid peroxidation under control in normal pregnancy. The aim of the present study was to determine whether it is possible to detect antioxidants in amniotic fluid using the Esterbauer method and to analyze whether there are any differences in the oxidant status of the amniotic fluid between smoking and non-smoking mothers. The results were confirmed by two assays measuring the total antioxidant capacity (TAC), as well as the malon dialdehyde concentration (MDA) in the amniotic fluid of smoking and non-smoking mothers. Materials and Methods: Differences in low-density lipoprotein (LDL) susceptibility to oxidation were measured using the Esterbauer method in the amniotic fluid of smoking and non-smoking mothers. Results: The results showed that there was a significant difference in the duration of susceptibility of LDL to oxidation between smokers and non-smokers (49.47±24.78 min, n=20 and 31.94±14.26 min, n=67; p=0.006). Arithmetic average of MDA was higher in smokers than in non-smokers (11 pmol/mg and 6 pmol/mg); for TAC it was vice versa 840 mM vs. 1054 mM. Conclusion: Measuring the lag phase of LDL oxidation makes it possible to study antioxidative effects. As the lag phase was significantly longer in smokers than in non-smokers, it can be assumed that there must be a substance in the amniotic fluid of smokers which has antioxidative power, inhibits LDL oxidation, and intercepts radicals. It can be assumed that the fetoplacental unit has mechanisms to react against tobacco smoke inhaled by the mother.*

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According to surveys, 20% of women in Germany were smokers in 2009. Many women do not succeed in stopping even during pregnancy: 13% are still smoking at the beginning of pregnancy and only a quarter have stopped by the end of pregnancy (1). The effects of smoking on the fetus have been extensively investigated. Strong associations of smoking with small-for-gestational-age children, premature birth rates, and early rupture of the placenta have been found by several studies. These pregnancy complications may be caused by nicotine, cotinine and carbon monoxide, which are among the most pharmacologically active components of tobacco, crossing the placental barrier and passing into breast milk (2).

As cigarette smoke generates free radicals, it has also been suggested that it may be one of the major sources of oxidative stress in pregnant women and in neonates, following exposure *in utero* (3). Antioxidant systems, which provide protection against the damage caused by free radicals to proteins, DNA and lipid molecules (4), are complex and include both enzymatic and non-enzymatic components (5). It has been shown in several human studies that smokers have greater requirements for antioxidants such as vitamins C and E (6). During normal pregnancy, higher levels of lipid peroxides are accompanied by higher maternal blood levels of vitamin E (7). Maternal smoking has been associated with depletion of serum vitamin C and thiol concentrations (8), but data suggest that selenium plays an active role in the maternal defence system against the toxicity of environmental pollutants such as cigarette smoke (9).

The human placenta, a major source of pro-oxidant agents, antioxidant enzyme systems and hormones, is able to keep lipid peroxidation under control in normal pregnancies (10). The lower incidence of pre-eclampsia in smokers may be explained (11) by the up-regulation of antioxidant systems such as superoxide dismutase, catalase and glutathione peroxidase, with an increase in placental basal plate heme oxygenase activity (12).

It is a matter of controversy whether low-density lipoprotein (LDL) in smokers is more susceptible to oxidation than LDL in non-smokers (13). Several studies have reported that smoking increases the oxidation of LDL cholesterol (14), but it has also been demonstrated that cigarette smoking raises plasma levels of auto-antibodies against oxidized LDL (6).

Several effects that the antioxidant parameters have on maternal and fetal serum and on the placenta have so far been identified, but little is known about the effects of smoking on the amniotic fluid. A study of Uchida *et al*. in 2010 suggested that amniotic fluid protects the fetal organs from oxidative stress (15).

The aim of the present study was to show that it is possible to detect antioxidants in the amniotic fluid using the Esterbauer method, and to analyze whether there are any differences in oxidant status between smoking and non-smoking mothers. Results were confirmed by analyzing total antioxidant capacity (TAC) and malon dialdehyde concentration (MDA) in the amniotic fluid of smoking and non-smoking mothers.

Materials and Methods

Amniotic fluid samples were collected from volunteers who were giving birth at the Department of Gynecology at the University of Erlangen. Twenty smoking and 67 non-smoking mothers donated amniotic fluid, which was collected during cesarean section. According to smoking-status, they were divided into two groups: smokers and non-smokers; all of the smokers had been smoking at least five cigarettes per day during pregnancy. The amniotic fluid samples were centrifuged at 600 ×*g* for 6 min and were frozen at –80˚C until further use.

The assay control group was formed by fasting, healthy, non-smoking non-pregnant women. Equal assay conditions for every spectrophotometric measurement were assured by the use of a single donor with constant lag-time. Blood samples were collected using a 3.2 ml Monovette KE (Sarstedt Inc., Nümbrecht, Germany). The plasma was separated using low-speed centrifugation at 600 ×*g* for 10 min.

After separation of fresh plasma, LDL samples were isolated using a single-step ultracentrifugation. The plasma (2 ml) was adjusted to a density of approximately 1.21 g/ml by adding 0.2 g potassium chloride/ml and layered under 10 ml saline (density 1.006 g/ml) containing 0.01% ethylenediamine tetraacetic acid (EDTA) in 12-ml quick-seal tubes (Beckman Instruments, Munich, Germany). The tubes were centrifuged at 377884 ×*g* for 6 h at 4˚C using a Ti 75 fixed-angle rotor in a Beckman L5-75 ultracentrifuge (Beckman Instruments) (16). The yellow LDL band was removed through the side of the tube with a needle and a syringe. Immediately before the incubation, LDL was separated from EDTA using gel filtration, with Econo-Pac 10 DG columns (BioRAD Ltd., Munich, Germany) (17). The concentration of LDL was measured by the enzymatic colour test (Olympus AU 2700, NY, USA).

Before each measurement, the amount of eluate was calculated in relation to the LDL concentration of the removed band. A volume of 5 μl of amniotic fluid was added to the eluate at a dilution of 1:10. The reaction volume of 1 ml per flask was diluted with oxygen-saturated phosphate-buffered saline at pH 7.4. LDL oxidation was initiated by adding a freshly prepared aqueous $CuSO_4$ solution (18).

Spectrophotometric monitoring was carried out in duplicate, by measuring changes in absorbance at 234 nm in 1-ml quartz cuvettes in a Perkin Elmer Lambda 2 ultraviolet/vis spectrophotometer (PerkinElmer, Inc., Ueberlingen, Germany).

LDL susceptibility to oxidation was measured by continuous monitoring of conjugated diene formation; absorbance readings were made every 5 min at 37˚C until there was no further increase in formation. LDL susceptibility is characterized by lag time, which appears to be a marker for resistance to oxygenation (19, 20).

The duration of the lag phase was measured as shown in Figure 1, following the protocol described by Esterbauer in 1989. Measuring the lag phase of LDL oxidation makes it possible to study the antioxidative effects of test substances (21).

The collected amniotic fluids of smoking and non-smoking mothers were used as samples in the Total Antioxidant Capacity (TAC) Assay Kit and the Malon dialdehyde (MDA) Adduct ELISA Kit.

The TAC Assay Kit (Cell Biolabs OxiSelect™ , San Diego, CA, USA) measures the total antioxidant capacity within a sample. Samples were compared to a known concentration of uric acid standard, within a 96-well microtiter plate format. Samples and standards were diluted with a reaction reagent and, upon the addition of copper, the reaction proceeded for a few minutes. The reaction was stopped and the absorbance was read with a standard 96-well spectrophotometric microplate reader, at 490 nm. Antioxidant capacity was determined by comparison with the uric acid standards.

In the MDA Adduct ELISA Kit (Cell Biolabs OxiSelect), (BSA-) standards or samples were incubated onto a 96-well plate for 2 h, at 37˚C. The MDA -protein adducts present in the sample and standards were probed with an anti-MDA antibody, followed by a (HRP)-conjugated secondary antibody. The MDA-protein adducts content in each sample was determined by comparison with a standard curve that prepared from pre-determined MDA-BSA standards.

Statistics. The data for amniotic fluid samples from 20 smoking and 67 non-smoking mothers were analyzed using the Statistical Package for the Social Sciences (SPSS, version 18.0 for Windows; SPSS, Inc., Chicago, Il, USA). After the data had been tested for normal distribution using the Kolmogorov–Smirnov test, correlations for continuous variables were assessed using either the Pearson or the Spearman test, depending on normal distribution, and correlations for categorical variables were assessed using the chi-squared test. Correlations between continuous and categorical variables were calculated using the eta test, and the significance of the influence of smoking on the variables was assessed using regression analysis.

All absorbance measurements and determinations of the lag phase were performed in duplicate. The tables produced by the photometer were converted into diagrams using the Excel Software (Microsoft). The duration of the lag phase was determined using the method described by Esterbauer. Measurements were taken from the graph, where $y=OD_{234}$ and x=time and were described by the time point at which the linear extrapolation of the propagation phase intersects $y=y(t_o)$.

The tables produced by the photometer analyzing TAC and MDA data were converted to Excel (Microsoft). Calculation of results were performed as instructed by the assay product manual. Sum, arithmetic average and coefficient of correlation of data were calculated.

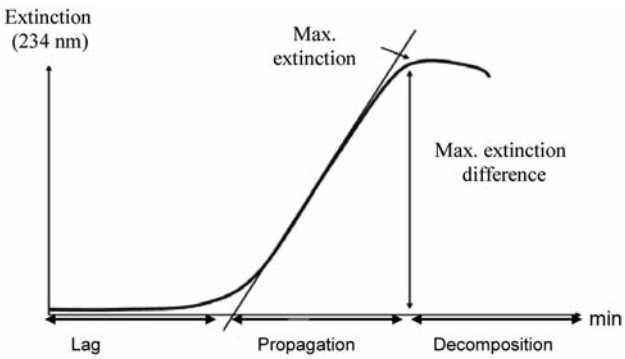

Figure 1. *The principle of continuous monitoring of oxidation of low-density lipoprotein: changes in absorbance at 234 nm are measured using the Esterbauer method. The kinetics of diene formation are clearly divided into three phases: an initial phase with a slow increase in diene formation, a second phase with rapid diene production and a final phase characteristic phase of diene decomposition.*

Table I. *Means with standard deviations and frequencies (%) of the data collected for smokers and non-smokers.*

	Smokers	Non-smokers	*p*-Value
Age of mother (years)	28.81±6.9	33.24±4.8	0.01
Children	2.19±1.0	1.85±0.8	0.29
Duration of pregnancy (days)	268.62±12.7	268.66±15.0	0.85
Weight of child (g)	3024.05±533.7	3265.29±625.7	0.04
Height of child (cm)	49.02±3.3	50.43±3.1	0.16
pH in child	7.31±0.0	7.33±0.0	0.09
BE in child (mmol/l)	–2.25±2.4	–2.01±2.7	0.74
Labor (%)			
Yes	41	22.5	0.08
No	59	77.5	
Cesarean section (%)			
Primary	57.2	67.9	0.24
Secondary	42.8	32.1	
Amniotic fluid (%)			
Clear	95.3	92.2	0.64
Not clear	4.7	7.8	
Gender of child (%)			
Male	66.2	48.4	0.08
Female	33.8	51.6	

BE, Base excess.

Table II. *Duration of the lag phase (minutes) in control individuals, non-smokers and smokers (p-values in the text).*

	Controls	Non-smokers	Smokers
Sum	699.69	2140.44	989.49
Arithmetic mean	22.57	31.95	49.47
Variance	150.12	203.40	614.43
Standard deviation	12.25	14.26	24.79

Results

Arithmetic means and frequencies in the collected data were calculated in order to compare the groups of smokers and non-smokers. The results showed that children born to smoking mothers tended to be smaller (p=0.16) and had significantly lower birth weights. Women who smoked during pregnancy gave birth at a younger age and had more children overall. The mean duration of pregnancy was similar in the two groups. No differences were found in the pH and the base excess (BE) of children's umbilical cord blood. Due to the larger number of non-smokers, frequencies were converted into percentages for comparison. Mothers who were smokers tended to give birth to boys, and non-smokers gave birth mainly to girls (p=0.08). The majority of patients in both groups had clear amniotic fluid and underwent primary cesarean sections without labor (Table I).

Among the women who gave birth during the study period, the most common reasons for cesarean sections were prior cesarean section in previous pregnancies (24% of cases), fetal risk factors such as positional anomalies (23% of cases); and maternal risk factors, such as gestational diabetes (16% of cases).

The statistical results showed significant difference between the mother's age and parity, between the children's height/weight and the duration of pregnancy, and between the children's weight and height, as expected (p=0.01) (2). There was also a significant difference between the child's height and the mother's age (p=0.05). Regression analysis showed significant associations between smoking and the mother's age (p=0.001), with smokers giving birth at a younger age; between smoking and the number of children a mother had (p=0.040); and between smoking and the child's sex (p=0.026), with mothers who were smokers more often giving birth to boys.

All absorbance measurements and determinations of the lag phase were performed in duplicate. The tables produced by the photometer were converted into diagrams using Excel (Microsoft). The duration of the lag phase was measured as described by Esterbauer (21), and was calculated for each graph. The total and other common arithmetical values for the lag phase were calculated and separated into a control group, and groups of non-smokers and smokers using Excel (Table II).

The mean value for the lag phase was 23±12.2 min in the control group, shorter than that for the groups of non-smokers and smokers. In the group of non-smokers (n=67), the mean value for the lag phase was 32±14.2 min, 40% more, compared with the control group. The mean lag phase in the group of smokers (n=20) was 49±24.7 min, 120%

more, compared with the control group and 53% more than in the group of non-smokers.

The Kolmogorov–Smirnov test was used to compare the duration of the lag phase in all three groups in relation to the normal distribution. A normal distribution was confirmed, and the Student's *t*-test was therefore used to test for significance. There was a significant difference in the duration of the lag phase between smokers and non-smokers (p=0.006).

The analysis of the MDA-assay showed higher concentrations of MDA, as an end-product of lipid peroxidation, in the amniotic fluid of smoking compared to non-smoking mothers. The arithmetic average of MDA amniotic fluid was 11 pmol/mg in smokers and 6 pmol/mg in non-smokers. TAC was lower in the amniotic fluid of smokers than the one of non-smokers, 840 mM and 1054 mM, respectively (Table III). The coefficient of correlation by Pearson was also calculated. No significant correlation was found either in the correlation of LDL/MDA (r=0.24), or in LDL/TAC (r=0.34) in smokers and non-smokers (r=0.44 and 0.34, respectively).

Table III. *Arithmetic average of low-density lipoprotein (LDL), total antioxidant capacity (TAC) and malondialdehyde (MDA) concentration in amniotic fluid of smoking and non-smoking mothers (p-values in the text).*

	Smokers	Non-smokers
LDL (mg/dl)	49	32
TAC (mM)	840	1054
MDA (pmol/mg)	11	6

Discussion

The effects that smoking during pregnancy has on the fetus, have been extensively investigated. Complications during pregnancy may be caused by nicotine, cotinine and carbon monoxide, which are among the most pharmacologically active components of tobacco, which cross the placental barrier and pass into breast milk (2). As cigarette smoke generates free radicals, it has also been suggested that it may be one of the major sources of oxidant stress in pregnant women and in neonates following exposure *in utero* (3). Oxidative stress is characterized by an imbalance in the oxidative burden and in antioxidative capacity (AOC) and has been associated with aging, atherosclerosis and reduced female fertility, among other conditions. Placental and circulating indicators of oxidative stress and AOC in pregnant women have been investigated, however, a role for the amniotic fluid in combating oxidative stress has not been addressed.

Antioxidant systems are complex systems which are triggered by smoking. During pregnancy, active smoking is associated with higher concentrations of placental selenium and zinc (9). These minerals are important components of antioxidant enzymes; selenium acts through the glutathione–peroxidase protein family, and zinc is a constituent of superoxide dismutase. Selenium is conserved in late pregnancy, and the lower maternal blood levels in comparison with higher cord-blood levels indicate active transportation of selenium to the fetus (9). In smokers, the up-regulation of antioxidant systems such as heme oxygenase in the placental basal plate, leading to increasing production of antioxidants such as biliverdin and bilirubin, may explain the decreased incidence of pre-eclampsia (11). Despite the apparent importance of minimizing oxidative stress during pregnancy, the AOC of the amniotic fluid and whether this is affected by other factors, such as smoking, has not been extensively investigated to date.

Taken together, in normal pregnancy the AOC is increased in maternal blood, in the basal plate of the placenta and in the umbilical cord. With maternal smoking, the AOC decreases. We wondered whether the amniotic fluid takes part in the antioxidative defence system, as little is known regarding the antioxidative capacity of the amniotic fluid itself. There is some evidence that the amniotic fluid is able to protect the fetal organs from oxidative stress, therefore we investigated whether amniotic fluid or substances in the fluid are able to lengthen the lag time of LDL-oxidation as a marker for anti -oxidative activity (AOA). We used this assay, because LDL particles transport the antioxidants in the body and therefore this test is a true reflection of the real antioxidative potency.

It has been shown that both smokers and pregnant women have greater requirements for antioxidants (22) in order to normalize levels of free radicals in circulating plasma. α-Tocopherol is the most abundant lipid-soluble antioxidant in biological systems and can effectively inhibit lipid peroxidation in membrane systems (23). In contrast, vitamin C is the most effective water-soluble antioxidant in human blood plasma (24) and it is also able to regenerate the lipid-soluble antioxidant vitamin E (25). Uric acid is another potent antioxidant (26). Plasma concentrations of α-tocopherol and uric acid were significantly higher in non-smokers than in smokers, but higher levels of plasma anti-oxidized-LDL immunoglobulin G (IgG) were observed in the group of smokers. Significant negative correlations between plasma anti-oxidized-LDL IgG and α-tocopherol suggest that plasma α-tocopherol may be effective in protecting LDL from oxidative damage (6).

During pregnancy, smokers have been found to have significantly lower serum and amniotic fluid concentrations of vitamin C than non-smokers. These women also had a greater decrease in vitamin C concentration in the amniotic fluid in comparison with non-smokers. Smoking may therefore

increase the fetal utilization of ascorbic acid (27). Studies on vitamin C have shown that amniotic fluid vitamin C levels are significantly higher than those in maternal or fetal plasma at term (28). Although studies in guinea pigs have reported levels of amniotic fluid vitamin C to decrease with advancing gestational age (29), increasing levels of vitamin C during human gestation have also been described (30). A direct correlation has been reported for AOC and gestational age (31). It has been hypothesized that the TAOC of mid-trimester amniotic fluid correlates with vitamin C and α-fetoprotein levels, as well as pregnancy outcomes (30). If α-fetoprotein has antioxidant properties, elevated levels in amniotic fluid might provide protection against oxidative damage. The full effects and role of α-fetoprotein and vitamin C during pregnancy are still being explored.

The aim of the present study was to examine if it was possible to detect the activity of antioxidants in amniotic fluid using the Esterbauer method, and to analyze whether there are any differences in oxidative status between smoking and non-smoking mothers. A significant difference was found in the duration of the LDL oxidation between smokers and non-smokers at delivery.

Measuring the lag phase of LDL oxidation makes it possible to study antioxidative effects for example estriol is an antioxidant that increases the lag phase of LDL oxidation *in vitro*, and its serum concentration rises enormously during late pregnancy. The high levels of estriol observed during pregnancy may be a biological self-protective mechanism for limiting oxidative damage (10).

In our study amniotic fluid lengthened the lag phase of LDL oxidation significantly in smokers. This indicates that there must be substances with antioxidative power in the amniotic fluid in smokers that inhibit LDL oxidation and serve as an interceptor of free radicals. These results were confirmed by the MDA-assay which demonstrates a higher amount of oxidation products in the amniotic fluid of smokers. The TAC-assay showed a better antioxidative status for non-smokers, but this result obviously does not reflect the protecting effect of amniotic fluid on LDL. Pressman et al. (30) showed that the AOC correlated well with vitamin C in amniotic fluid but not with pregnancy outcome, in contrast to alpha-fetoprotein. Recently, oxidized–LDL and malondialdehyde were found to be similar between mothers with hypertensive disorders in pregnancy and healthy pregnant controls, but there was a diffenrence in the amount of free fatty acids in mothers with hypertensive disorders, so an oxidation/reduction imbalance with increase in oxidative stress, coupled with a decreased capacity of antioxidant systems was assumed (32). It can be thought that the fetoplacental unit has mechanisms to react to tobacco smoke inhaled by the mother. These results of course, need to be confirmed in larger group of patients.

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