STUDY OF DNA DAMAGES INDUCED BY SMOKING AND ITS REPAIR IN HUMAN CUMULUS AND GRANULOSA CELLS

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Summary of Ph.D. thesis

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Introduction

The human genome is being subjected to growing numbers of new environmental agents (food additives, herbicides, new chemical agents, exhaust gases etc.). Smoking as a source of huge numbers of harmful chemical agents can cause a lot of modifications in essential biological molecules. Cigarette smoke and cigarette tar contains high concentrations of reactive oxygen species (ROS) and other molecules. These molecules cause loss and change of genetic information, since they chemically modify the DNA bases (oxidation, deamination, methylation etc.) (Halliwell and Gutteridge 1999). ROS can react with other molecules in the cells which generate chain reactions of secondary radical formation. These radicals can cause damage far from the source of ROS. The imbalance between the ROS and protective mechanisms (enzymatic and non enzymatic antioxidants, DNA repair) can cause oxidative stress. Damages develop mutations which result metabolic alterations, tumors and death of the cells. The chemicals in cigarette smoke can cause all range of DNA damage which repaired by different DNA repair mechanisms (DeMarini 2004, Christmann et al. 2003).

Besides the well known health hazardous effect smoking has influence on almost all fields of human reproduction (Schiverick and Kalifa 1999). In female reproduction the production of oocytes with normal developmental potential and creating appropriate hormonal background are very important factors. The somatic cells in the growing follicle take fundamental part in these processes (Kidder 2002, Vanderhyden 2002). In my PhD thesis I summarize our experiments on the *in vitro* and *in vivo* DNA damaging effects of smoking on human cumulus and granulosa cells.

Aims

Numerous data demostrate direct effect of smoking on DNA damage and reproductive functions. The role of oxidative stress in female reproduction has been demonstrated in several processes but little is known about the real molecular mechanisms taking place in the damaging effects.

Human oocytes can be studied during the *in vitro* fertilization treatment (IVF). Ethically however only remaining - not fertilized and pathological - cells could be used for experimental purposes. The wellness of cumulus and granulosa cells sorrounded the oocyte in the growing follicle is very important for the proper development of the oocytes. These cells

can be isolated as „by-product" of *in vitro* fertilization, thus could be used for screening reproductive toxicants.

The aim of our experiments was as follows:

- to measure the generation of reactive radicals with ESR and their DNA damaging effect with comet assay after cigarette smoke and cigarette tar extract treatment in granulosa cell culture,
- to study the *in vitro* repair of oxidative DNA damage caused by cigarette smoke and cigarette tar extract treatments in granulosa cell culture,
- to study the *in vivo* effect of smoking on DNA damage in human cumulus cells, and the effect of smoking on success rate of IVF,
- to measure the activity of alkyltransferase enzyme responsible for the repair of methylated bases caused by smoking.

Results and conclusions

ESR spectroscopy is able to detect ROS directly in the case of radicals with unpaired electron (Halliwell and Gutteridge 1999). DNA damage caused by smoking can be measured with a simple, cheep and quick method, the single cell gel electrophoresis or comet assay (Moller és mtsai 2000). Alkaline comet assay with DNA oxidation specific endonucleases was used in our *in vitro* repair experiments. The Fpg enzyme recognizes oxidized purines while EndoIII detects oxidized pirimidines.

1. The absorption spectrum of DMPO-radicals generated during 2 and 4 hours of CSE treatment of granulosa cells was tipical for hidroxyl radicals. The measured DMPO-OH adduct level increased in time, but less characteristic DMPO-radicals are detectable. We detected significantly elevated single strand DNA break level after CSE treatment with comet assay. There were no significant difference in the level of single strand DNA breaks between 2 and 4 hours treatment of CSE.
2. After 2 and 4 hours treatment of granulosa cells with CTE we detected increasing level of DMPO-OH adducts. The characteristics of the ESR spectra refers to the presence of numerous unidentified reactive radicals. Significantly elevated level of DNA strand breaks was measured as compared to the CSE treatment. 4 hours

treatment with CTE significantly increased the level of single strand breaks compared to the 2 hours CTE treatment.

3. The DNA repair capacity of oxidative damage in granulosa cells after CSE and CTE treatment was compared. Two and 4 hours CSE treatment initially caused significantly increased level of single strand DNA breaks and oxidative base damages compared to the untreated controll cells. The measured comet tail lengths in 1 hour repair time increased 2 times, since the base excision repair glycosylases incise the DNA at the damaged purines and pirimidines which can be detected as new strand breaks in comet assay. The level of DNA damage after 4 hours repair decreased to the level detected immediately after 2 hours of CSE treatment. After 4 hours CTE treatment the level of single strand breaks and oxidative base damages continously increased. We detected higher level of oxidised purines in these experiments.

   Both after 2 and 4 hours of CTE treatment the detected DNA damage increased in time continously with high frequency of oxidized purines. The cells visually contained apoptotic level of fragmented DNA which means that the cells probably were not able to repair the DNA damage caused by the cigarette tar components.

4. Comet assay has been done using shorter CSE treatments (30 min, 1 and 2 hours) and longer repair times (0, 4, 12 and 24 hours) in granulosa cells. The level of DNA damage increased with the duration of the treatment. The repair of single strand breaks and oxidized base damages was most effective after the 30 min treatment, but the remaining level of DNA damage did not decrease to the level of untreated controll cells. Using longer treatments with CSE (1 and 2 hours) the kinetics of repair was the same, the correction of damaged bases was slow and the remaining damage level stays at relatively high level.

   We detected the highest level of the oxidized purines in these experiments. The speed of repair of purines and pirimidines is the same after short treatment time (30 min). The repair of oxidized purins was slowest in the case of longer treatments (1 and 2 hours).

5. The detected reduced repair activity in granulosa cells is in aggreement with the differentiation-associated decreased DNA repair processes in terminally differentiated cells (Nouspikel and Hanawalt 2002), since granulosa cells during the hormonal changes in ovulation differentiate into progesterone-producing cells of the corpus luteum. The remained numerous unrepaired DNA damage sites could cause diminished progesterone production. This is in aggreement with the literature, where alkaloids in

cigarette smoke has been shown to inhibit progesterone synthesis (Gocze és Freeman 2000). These effects partly explain the high rate of early abortions in smokers, since the efficient progesterone production of the corpus luteum is critical for preparing the uterus for the implantation of the embryo.

6. We studied the *in vivo* effect of smoking on cumulus cells of smokers compared to non-smokers with the comet assay. The cumulus cells of smokers contain significantly elevated levels of single strand DNA breaks. The difference was also significant after induced oxidative stress (100µM H_2O_2 treatment) between smokers and non-smokers. The cells of smokers did not seem to phisically more sensitive to the induced oxidative stress, the elevated level of DNA damage in cumulus cells of smokers originates from the smoking itself.
7. Despite the higher number of percentage of transferred morula stage and best quality preembrios in the smoker group, the pregnancy rate in the smoker females was significantly lower. Regarding the basic parameters of assisted reproduction we found significant difference only in the FSH level between smokers and non-smokers.
8. We detected significant difference in the activity of alkyltransferase enzyme between cumulus and granulosa cells, which can indicate the different repair activity of these cells in the developing follicle. We measured significantly increased activity of alkyltransferase enzyme in the granulosa cells of smokers as reported in the literature previously (Drin és mtsai 1994, Slupphaug és mtsai 1992). This can indicate the presence of inducible enzyme activity initiated by the DNA damages produced by smoking. This has also been shown in other tissues.

In our experiments the detected high level of remaining DNA damage with increasing treatment time can explain the high frequency of cancers developed in smokers. The reduced repair activity of granulosa and cumulus cells could explain the significantly lower pregnancy rate and the high level of early abortions in smokers, as observed in clinical practice.

In conclusion, our study provides new information about the role of smoking and ROS on female reproduction.