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ORIGINAL ARTICLE

Sensitization to inorganic mercury could be a risk factor for infertility

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Abstract

INTRODUCTION: Heavy metals can negatively influence the reproduction due to the fact that they are able to impair the immune reactions including autoantibody production in susceptible individuals. In such a way the infertility could be also caused by altered pathologic immune reaction.

AIM OF THE STUDY: To investigate the *in vitro* lymphocyte reaction after stimulation with metals and production of gamma interferon and antisperm antibodies in supernatants after lymphocyte stimulation in patients with infertility and with proven antisperm antibodies in their serum. The cause of antisperm antibodies presence was not determined.

METHODS: The diagnosis of metal allergy was performed by the lymphocyte proliferation method modified for metals (MELISA®). In supernatants of tissue cultures of lymphocytes without the antigen stimulation and after stimulation with mercury chloride, the *in vitro* production of gamma interferon and antisperm antibodies was studied by ELISA.

RESULTS: More than 50% of patients were reacting to mercury, iron, aluminium and silver as mean by lymphocyte reactivity. When compared the lymphocyte reaction in patients with and without mercury allergy we found that the lymphocytes of patients with mercury intolerance produced less gamma interferon and more antisperm antibodies in supernatants after mercury stimulation of their lymphocytes.

CONCLUSION: In patients with metal intolerance diagnosed by the MELISA® test the release of metal ions from dental materials can be one of the stimulating factors which may adversely affect fertility.

Introduction

Infertility is currently a growing problem. The reasons for infertility may be due to endocrine, genetic, anatomical, immunological or psychogenic problem. It is well known that one of the immunological infertility may be caused by decreased mobility of spermatozoa induced by various physical and biochemical factors. The external sperm membrane consists of soluble antigenic components and in some cases can induce the production of auto–(iso–)antibodies [1].

Heavy metals are biologically active substances and may in susceptible individuals affect the immune system and cause health disturbances. Heavy metals are known to induce so called cellular hypersensitivity (delayed type or type 4 reaction) but humoral responses may be affected as well. An association between undesirable reaction to metals and presence of autoantibodies has been suggested by several authors [2,3,4]. Changes in cytokine production were reported by others [5,6,7,8,9]. Since metals such as mercury, cadmium and lead are toxic in relatively low concentrations [10], undesirable reactions caused by metal antigens may further complicate the health status of the patient. Patients with autoimmune and allergic diseases may be particularly vulnerable [11]. Metal-induced reactions are influenced by genetic background in experimental animals [12] and associated with certain HLA antigens in man [13].

Association between the exposure to organic and inorganic mercury and male infertility has been described by Choy and Hanf with coworkers [14,15]. The exposition to lead and cadmium resulted in the decrease of fertility and in lower quality of sperm as described by several authors [16–20].

Dental amalgam, the most frequently used dental alloy worldwide, is considered as risk factor of pregnancy by Pleva [21]. The aim of this study was to examine the *in vitro* lymphocyte reactivity to wide range of metals in infertile men and women with anti-sperm antibodies. A modified lymphocyte stimulation test, so called MELISA®, was used and the production of interferon γ (IFN-γ) and anti-sperm antibodies in lymphocyte cultures was also determined.

Materials and methods

Patients included in the study were originally referred to The Clinic of Obstetrics and Gynecology, University Hospital, Charles University, Pilsen, Czech Republic. One hundred two subjects were asked to complete a questionnaire regarding metal exposure; among others (occupational exposure, family exposure, residence near sites of pollution, dental restoration, body implants, smoking, tattoos, piercing, vaccination, eye drops use, cosmetics use, diet, chewing) regarding the number and quality of dental metal restorations. Forty patients out of 102 subjects asked to participate, twenty women and twenty men, (mean age 34.6 years, range 26–47 years) agreed to participate in the study. All subjects were treated for primary infertility (unknown patient's inability to have own child) and had anti-sperm antibodies in their blood. Another selection criteria was the presence of only amalgam restorations in the oral cavity. All 40 patients fulfilled this criteria. The study was approved by Ethical Committee of Czech Ministry of Health. Patients were informed about the aim of the study and have given their informed consent. Depending on reactivity to inorganic mercury in MELISA®, patients were divided into two groups (responder and non responder group) before further immunological evaluation.

Control group consisted of eight healthy fertile persons (5 women and 3 men) in the age range 23–54 years (mean 35 years). Control subjects had no anti-sperm antibodies detected in their blood. The control group had similar amalgam restorations as the infertile group.

All patients as well as controls were examined by the dentist and their dental restorations were validated as dental score.

Detection of anti-sperm antibodies in serum was performed by Tray agglutinin test and by mixed reaction anti-globulin test (MAR–test) [22].

The MELISA® test is based on evaluation of proliferation of peripheral blood memory cells *in vitro* after incubation with metal salts [23,24]. Heat-inactivated autologous serum was used for cultivation of lymphocytes [25]. Thirty millilitres of peripheral venous blood was collected and mixed with the equal amount of RPMI 1640 medium containing 10 mM HEPES, gentamycin and glutamine. The blood was layered on Ficoll–Paque gradient (Pharmacia, Sweden) and centrifuged at 600 g for 30 minutes. Mononuclear cells were collected from the interface, washed twice and mixed with 5 ml of RPMI 1640 medium containing 20% of inactivated autologous serum. Plast-adherent cells were partly depleted from leukocyte suspension by incubation on plastic surfaces for 40 minutes at 37°C. After incubation, the lymphocytes were counted and diluted with RPMI 1640 enriched with 10% of autologous serum and glutamine into a final dilution of 1×10^6 cells/ml. Lymphocytes were cultivated for five days with metal salts in an atmosphere of 5% carbon dioxide in humidified air at 37°C. The details such as the metal salts used and their concentrations are described in [23]. Control cultures were incubated under the same conditions in the absence of metal salts. As a positive control, lymphocytes were cultivated with pokeweed mitogen (10 μg per ml, Sigma, USA). After 5 days, lymphocyte proliferation was measured with the help of ^{3}H thymidine incorporation (Lacomed, Czech Republic) as described previously [23] .

The rate of lymphocyte proliferation in metal-treated cultures was compared to the rate in non-stimulated cultures and evaluated by stimulation index (SI). SI= counts per minute in metal-treated cultures divided by counts per minute in non-treated cultures. Stimulation index less than 2 was regarded as negative reaction, SI 2.01–3 as weakly positive reaction, SI 3.01–10 as positive reaction and SI higher than 10 was regarded as strongly positive reaction [23].

Commercial ELISA kit (Serotec, USA) was used for the measurement of cytokine IFN-γ in supernatants of

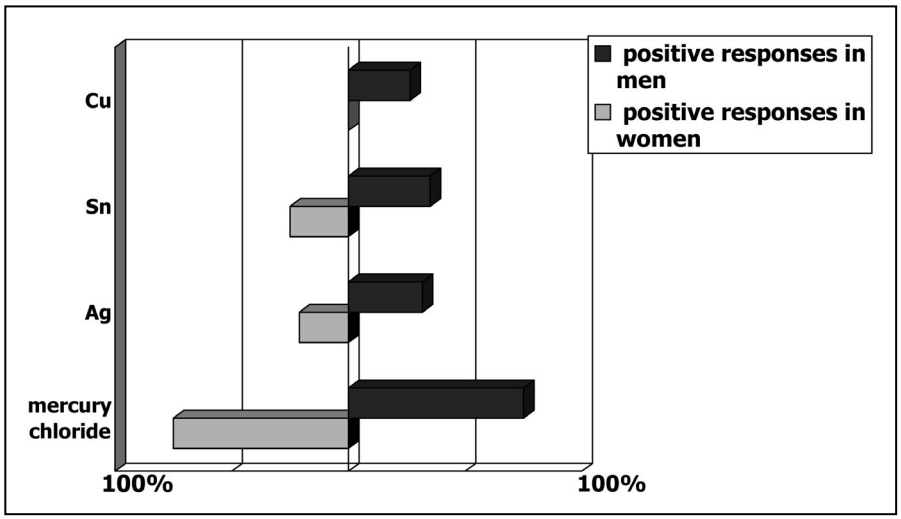

Figure 1: Reaction of infertile patients to metals used in dental amalgam restorations (% of positive reactions in 20 tested women and 20 tested men).

lymphocyte cultures after 5 days cultivation. Results are presented as geometrical means of individual values of IFN-γ content (ng/ml). This assay can detect the levels of interferon from 0 to 10 ng/ml.

The commercial kit Spermatozoa Antibody ELISA (IBL GmbH Hamburg, Germany) was used for the measurement of the anti-sperm antibodies in lymphocyte cultures after 5 days. Results are presented as arithmetic means of the antibody values (mU/100 µl). The detection levels of the kit were from 10 to 284 mU/100 microlitres.

The results of MELISA® were evaluated by Fisher's exact test and by Student's paired t–test. Student's paired t–test was used to compare the values of IFN-γ and of anti-sperm antibodies in lymphocyte cultures of two groups of patients.

Results

Reactivity of infertile men and women to metals being commonly used in dental amalgam is shown in Fig. 1. Fifteen women out of 20 tested (75%) and 15 men out of 20 tested (75%) responded to inorganic mercury with increased lymphocyte proliferation. Lymphocyte stimulation with other metals often used in dentistry or present in the environment are shown in Fig.2. More than one half of tested patients showed positive reaction to iron and to aluminium. Positive responses to other metals were seen less frequently (in 20–25% tested persons to cadmium and platinum, in 10–20% tested persons to titanium, organic mercury, lead, nickel, chromium, molybdenum, gold and zinc). The results of organic mercury reaction are presented as the mean value for four forms tested – methylmercury, ethylmercury, phenylmercury and thimerosal. Significant difference in the reactivity of lymphocytes to metals between men and women was detected only for cadmium but not for other metals (Fig.2).

Based on the results patients were divided into two groups (responders and non responders) depending on their lymphocyte reactivity to inorganic mercury *in vitro*.

The responder group consisted of 30 patients (15 men and 15 women) and the non responder group consisted of 10 patients (5 men and 5 women). The pro-

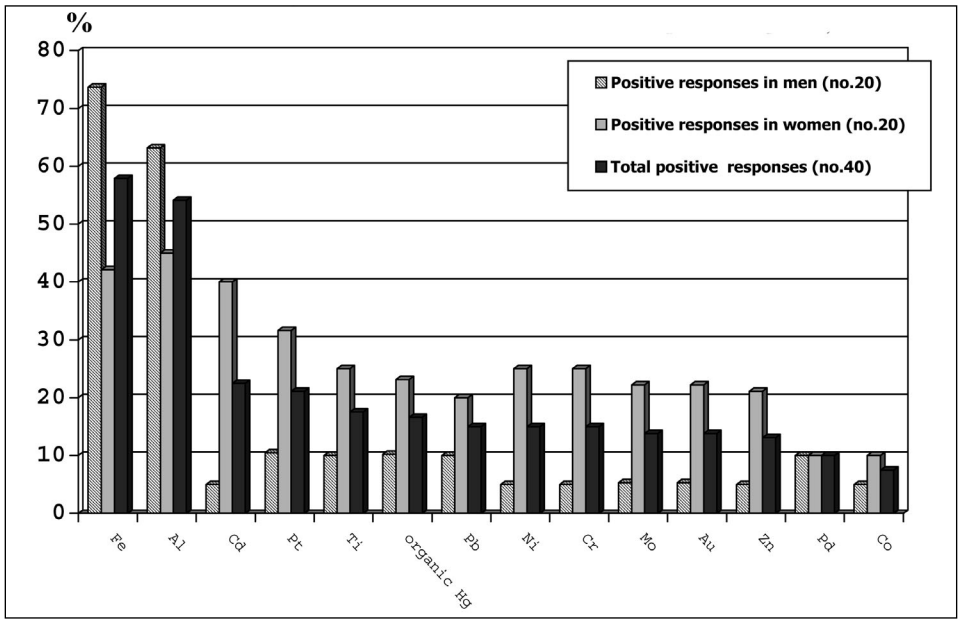

Figure 2: Lymphocyte stimulation to other metals used in dentistry or present in the environment in infertile men and women (% of positive responses).

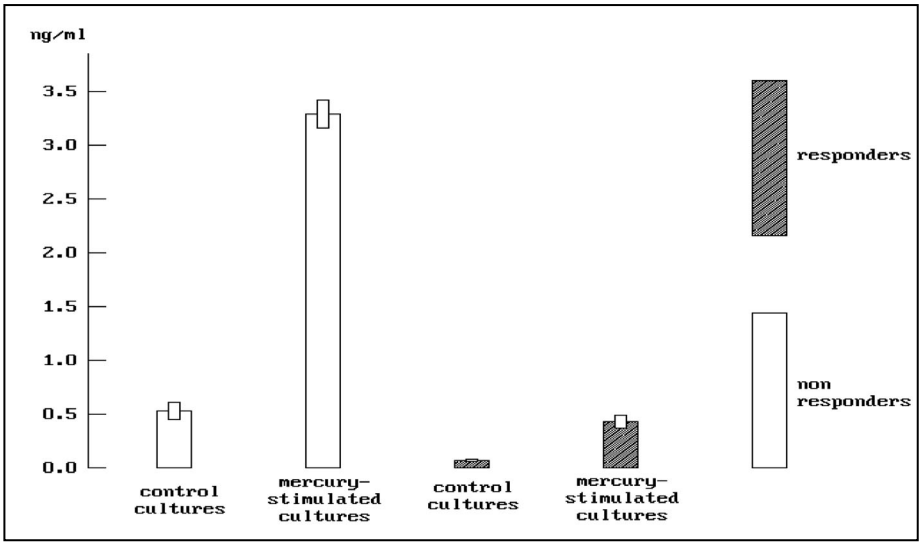

Figure 3: Interferon–γ production by lymphocytes exposed to mercury chloride and in control non exposed cultures. The results are divided according to reactivity to mercury chloride in MELISA® (means in ng/ml).

duction of anti-sperm antibodies and of cytokine IFN-γ was further determined.

The results of the cytokine IFN-γ production in lymphocyte cultures are shown in Fig 3. Significantly higher production of IFN-γ was found in supernatants from responder lymphocyte cultures treated with mercury chloride (3.28 ng/ml) as compared to non responders (0.45 ng/ml, P <0,05). The difference in the concentration of IFN-γ in supernatants from responder lymphocyte control cultures and in supernatants from non responder control cultures was also significant (0.51 ng/ml and 0.08 ng/ml, P<0,05). We didn't find any significant difference between the genders in IFN-γ production.

Titres of anti-sperm antibodies of lymphocyte cultures with and without cultivation with mercury chloride are shown in Fig 4. In mercury-stimulated cultures the amount of anti-sperm antibodies was higher in responders as compared to the non responders (53.4 mU/100µl and 20.4 mU/100µl respectively, P<0,05).

Surprisingly, some anti-sperm antibodies were also found in control cultures. The titre of anti-sperm antibodies in control cultures was also higher in supernatants from responders as in non responders (58.0 mU/100µl and 17.9 mU/100µl respectively, P<0,05). We didn't find any significant difference between the genders in anti-sperm antibodies production.

After evaluation of questionnaires we did not find any dose-response relationship between the number of amalgam restorations and positive responses to mercury *in vitro*. In control fertile group we have found positive reaction in MELISA® test only in one woman to lead and in two women to inorganic mercury. Quite minimum amounts of anti-sperm antibodies were present in lymphocyte cultures from the fertile group (control culture – 2.5 mU/100µl, mercury-treated culture – 2.1 mU/100µl) and titres of IFN-γ in control group (control culture – 0.07 ng/ml, mercury-treated culture – 0.39 ng/ml) were similar to these in non responder group.

Discussion

In this study, infertile patients frequently showed increased lymphocyte stimulation *in vitro* to inorganic mercury and other metals ubiquitous in the environment. Compared to fertile group the results in infertile patients were significantly higher for all metals tested. The highest reactivity was detected to inorganic mer-

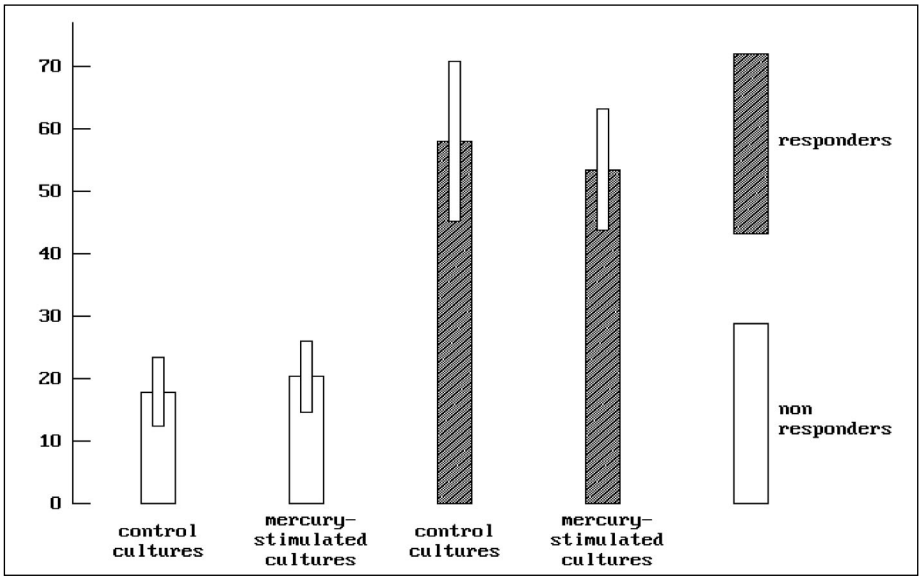

Figure 4: Anti-sperm antibody production by lymphocytes exposed to mercury chloride and in control cultures. The results are divided according to reactivity to mercury in MELISA®(arithmetic means in mU/100µl).

cury, followed by iron and aluminium. Mercury is main component of dental amalgam, a frequently used dental material in restorative dentistry.

Increased *in vitro* lymphocyte stimulation triggered by mercury may indicate the possibility of mercury-induced inflammation *in vivo*. It is generally accepted that some metals, such as nickel, chromium, cobalt, mercury and gold are highly allergenic as demonstrated by standard patch testing and often cause oral lichen planus [26,27,28], contact allergy [29,30,31,32] or fatigue and autoimmunity [33]. During the last years there has been an increase in diseases characterized by hyporeactivity or hyperreactivity of the immune system. In young generation, infertility is now affecting one couple in six [34,35]. This may be due to the decrease in sperm quality as described previously [36,37,38]. Various other factors such as exposure to heavy metals [15,16,18,20,21,39], diet [14], smoking [17,19,35,40] and diverse environmental factors [37,38,41] were implicated to explain infertility in men and women. The most frequent factors implicated in male infertility are occupational exposure to heavy metals, such as Cd, Pb, Mn and Hg, to pesticides and solvents, smoking (exposure to Cd, Pb, Hg, Ni, Ar, Mn) and diet rich in sea food (exposure to methylmercury). In women, diet rich in sea food (exposure to methylmercury), delayed childbearing, changes in sexual behavior, smoking and long-term use of contraceptives are most often implicated. Contraceptives such as vaginal gels may contain mercury preservatives.

In the context of autoimmune etiology we postulate that metals might participate in autoantibodies formation and inflammation reactions that may have negative impact on the outcome of pregnancy. Infertile men have high levels of anti-sperm antibodies in the blood and the ejaculate, which implies clinical relevance of anti-sperm antibodies [1]. In genetically susceptible animals, mercury and gold induce local and systemic autoimmunity [42–46]. Since our patients were exposed to amalgam, the negative effect of inorganic mercury leaking from amalgam restorations and affecting the outcome of pregnancy cannot be excluded.

The group from Heidelberg (Germany) reported that removal of mercury and other metals by chelation with DMPS (2,3–dimercaptopropan–1–sulfonic acid, sodium salt) improved the spontaneous conception chances of infertile women [39].

The study of cytokine pattern in responders and non responders showed that IFN-γ production in mercury-treated cultures in both groups increased as compared to IFN-γ production in control cultures. Increase in IFN-γ production was higher in non responder group as in responders. This could possible be due to different kinetics in the production of cytokine where responder cultures produced IFN-γ during 24 to 48 hours. Since cytokine measurement was performed at the end of the culture, the cytokine synthesis could have been down-regulated in responder cultures.

Regarding the titers of anti-sperm antibodies, the responder group had at least three times higher levels of anti-sperm antibodies in the culture supernatant as did the non responder group. The presence of mercury in the cultures did not affect the levels of anti-sperm antibodies. The low presence of anti-sperm antibodies in control group might be due to its low content in patient's sera used for lymphocyte culture cultivation. We can speculate that mercury-sensitive individuals (responders) may have activated B lymphoblasts in the blood which may secrete anti-sperm antibodies during the cultivation *in vitro*. The clinical relevance of anti-sperm antibodies for infertility has been described in workers occupationally exposed to mercury [47].

Some authors reviewed that existing scientific evidence does not demonstrate that mercury from dental amalgam poses a public health hazard [48]. Although there exists a controversy regarding the effects of dental amalgam on health [48,49], there is accumulating evidence of pathological effect of mercury on susceptible individuals [7,11,12,13,21,33,50] and on susceptible groups such as fetuses and young children [51–4].

In conclusion, results of this study indicate that mercury from amalgam fillings could be a risk factor which could negatively influence fertility in metal-sensitive patients.

Acknowledgements

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