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Research article

PREDICTING THE RISK OF UTERINE FIBROIDS IN FEMALE COPPER SMELTER WORKERS BY CYTOGENETIC ABNORMALITIES OF BUCCAL EPITHELIUM

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Exposure to adverse occupational factors increases the risk of diseases of the reproductive system. Long-term studies have shown a high prevalence of uterine fibroids among all diseases of the reproductive system in women exposed to occupational hazards in industry. The occurrence of fibroids, having such complications as menorrhagia, pain in the pelvic area, and infertility, hampers performance and diminishes the quality of life. The risk of genetic mutations in cells playing a key role in the development of fibroids is raised by inhalation of industrial aerosols containing metals possessing mutagenic and carcinogenic potencies. Cytology of the buccal epithelium can be used as a method objectively indicating the genetic risk.

The purpose of the study was to develop a predictive model for assessing the risk of uterine fibroids in female workers exposed to aerosols containing metal particles at a copper smelter using buccal cytograms.

We examined 47 female workers of a copper smelter, of which 39 formed the main (exposed) group. Uterine fibroids were more frequent in the main group than in the controls (25.6 % versus 0.0 %, $p \leq 0.05$). Genotoxic indicators, such as protrusions and micronuclei, and cytotoxicity indicators were also significantly increased in this group (nuclear vacuolization and cytoplasmic vacuolation were 1.6 times and karyolysis 1.8 times higher compared with the controls, $p \leq 0.05$). We have developed two mathematical models for assessing the risk of uterine fibroids. Model 1 includes variables without restrictions of characteristics, i.e. micronuclei, perinuclear vacuoles, and cytoplasmic vacuolation (AUROC – 0.940), while Model 2 necessarily includes such genotoxic parameters as micronuclei and protrusions (AUROC – 0.883). The 4-member model (M1+M2), which includes both genotoxic (micronuclei and protrusions) and cytotoxic (binucleated cells and cytoplasmic vacuolation) indicators, has the highest significance (AUROC – 0.998).

The results indicate involvement of both genetic and cytotoxic mechanisms in the development of uterine fibroids in female workers exposed to toxic industrial aerosols. The models including both two indicators of genotoxicity (micronuclei and protrusion) and one and/or two cytotoxicity indicators have the greatest predictive value.

Keywords: *buccal epithelium, cytology, uterine fibroids, micronucleus test, cytogenetic index, genotoxicity, cytotoxicity, protrusions.*

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Reproductive health maintenance in working women has always been a priority. Exposure to industrial toxicants is considered an exogenous risk factor for diseases of the reproductive system, especially in cases of hormonal imbalance, obesity, pelvic inflammatory diseases, and low parity [1, 2]. Uterine fibroids are common growths of the womb accounting for 60–70 % of all uterine disorders. Working-age women have the highest incidence rate and the highest frequency of hysterectomies [3]. According to literature data, uterine fibroids tend to "rejuvenate" [4]. The epidemiology of the disease is greatly underestimated due to its asymptomatic stage in most patients. When studying the risks to the reproductive system, much attention is paid to the mechanisms of copper and lead reprotoxicity [5]. The pathogenetic role of industrial mutagens and carcinogens in the development of neoplasms of the reproductive system is still examined since chromosomal and gene mutations are detected in 90 % of cases [6, 7]. Chromosomal abnormalities correlate with tumor phenotypes. At present, informative non-invasive short-term tests are used for screening in both clinical practice and research. Cytological characteristics of the buccal epithelium are a biological indicator of the state of the body, which changes under adverse effects of chemical and biological factors in the industrial setting [8–14]. Functional pathological changes in the buccal epithelium correlate with indicators of impaired homeostasis and can also act as markers of precancer. In 2007, the international project known as the human MN assay in exfoliated cells (HUMN(xL)) was launched [15].

Exfoliative cytology of buccal mucosa is an *in vivo* technique of histological examination that can serve as a source of important diagnostic and prognostic information on health status, exposure to stress and adverse occupational factors; it can also be applied in oncology, dentistry, and pharmacology [16, 17]. Recent publications show that buccal cytograms are widely used to assess micronuclei in those exposed to potentially carcinogenic agents at workplaces [18–21]. According to the latest data on the leading role of the genetic mechanism in the development of uterine fibroids, substantiation of diagnostic criteria based on cytogenetic changes can be useful for early diagnosis of the disease in women exposed to industrial toxicants.

The **purpose** of the study was to develop a prognostic model for assessing the risk of uterine fibroids in female workers exposed to aerosols containing metal particles at a copper smelter using buccal cytograms.

Materials and methods. The study involved 47 female copper smelter workers, of which 39 formed the main group (mean age: 48.7 ± 1.7 years; duration of current employment: 14.8 ± 0.5 years) exposed to adverse occupational factors, the leading one being industrial aerosol of complex chemical composition containing particles of copper, lead, nickel, iron, silicon dioxide, arsenic, etc. The cases worked as crane, hydrometallurgy, and rare metal production equipment operators. According to the results of special assessment of working conditions, crane operators of the anode section of the copper smelter are exposed to the aerosol containing copper, which measured concentration (1.58 mg/m^3) is thrice as high as the maximum allowable concentration (MAC = 0.5 mg/m^3, class 3.2 of working conditions); nickel and its oxides (0.058 mg/m^3), which levels are 1.16 times higher than the MAC (0.05 mg/m^3, class 3.1), and silicon dioxide (< MAC, class 2.0). Hydrometallurgists in the copper sulfate shop of the copper section are exposed to nickel salts in the form of hydrated aerosol (0.006 mg/m^3, class 3.2), copper and sulfuric acid (< MAC, class 2). Operators of the sludge processing section in the production of rare metals of the chemical and metallurgical section are exposed to nickel and its oxides, lead and its inorganic compounds, cadmium and its inorganic compounds (< MAC, class 2), and sulfuric acid, which level (1.8 mg/m^3) is 1.8 times higher than the time-weighted average

concentration (1.0 mg/m^3, class 3.1 of working conditions). The above chemicals have proven carcinogenic potencies (cadmium, lead, nickel, and sulfuric acid) and may have an adverse effect on the reproductive system (copper and lead). The control group consisted of eight female office workers (mean age: 40.1 ± 0.92 years; duration of current employment: 9.25 ± 0.64 years) unexposed to occupational toxicants.

Exfoliated buccal epithelial cells were scraped from the inner cheeks. The material collected with the cytobrush was evenly spread onto a glass slide, dried naturally, and stained according to Pappenheim. The preparations were then analyzed using a Carl Zeiss Primo Star microscope with 16×40 and 16×100 magnifications. Cytogenetic, proliferation, and nuclear destruction indicators were counted per 1,000 cells with the results expressed in ppm (‰) [22]. To assess the risk of cytogenetic abnormalities in buccal epithelial cells in the study groups, we calculated the index of accumulation of cytogenetic damage (*Iac*) using the formula (1) representing the product of the cytogenetic index (*Ic*) and the index of proliferation (*Ip*), divided by the apoptotic index (*Iapop*):

$$Iac = (Ic \cdot Ip / Iapop) \cdot 100, \qquad (1)$$

where *Ic* is the sum of cells with micronuclei and protrusions; *Ip* is the sum of cells with two or more nuclei; *Iapop* is the sum of all cells involved in apoptosis (chromatin condensation, karyopyknosis, karyorrhexis, karyolysis, or apoptotic bodies). Based on the index of accumulation of cytogenetic damage, risk groups were classified as follows: $Iac \leq 2$ – low, $2 < Iac < 4$ – moderate, and $Iac \geq 4$ – high risk [15].

Statistica 6.0 was used for data analysis with the nonparametric Mann – Whitney test applied to assess the significance of differences. We also used ROC analysis to identify cytological features of diagnostic value.

To address the impact of accumulated cytogenetic abnormalities on the risk of uterine fibroids in women exposed to industrial aerosols, we developed a prognostic model of binary classification including logistic regression with the linear relationship with the specified variables (formula 2) or with the linear relationship with cross-terms added (formula 3):

$$y = \frac{\exp\left(b_0 + b_1 x_1 + \cdots + b_n x_n\right)}{1 + \exp\left(b_0 + b_1 x_1 + \cdots + b_n x_n\right)} \qquad (2)$$

$$y = \frac{\exp\left(b_0 + b_1 x_1 + \cdots + b_n x_n + b_{12} x_1 x_2 + b_{13} x_1 x_3 + \cdots b_{n-1n} x_{n-1} x_n\right)}{1 + \exp\left(b_0 + b_1 x_1 + \cdots + b_n x_n + b_{12} x_1 x_2 + b_{13} x_1 x_3 + \cdots b_{n-1n} x_{n-1} x_n\right)}, \qquad (3)$$

where y is the variable showing the probability of the presence ($y = 1$) or absence ($y = 0$) of uterine fibroids; $x_1, x_2, \ldots, x_n$ are independent variables (cytogenetic indicators), and b_0, b_1, … b_n are theoretical coefficients.

We considered the models with three and more and four and more variables since with the same quality of models, preference should be given to that with a smaller number of variables. The McFadden likelihood ratio index (or McFadden's pseudo-R^2 coefficient), hereinafter referred to as pR^2, was chosen as the quality criterion for models (2) or (3). For this coefficient, models with pR^2 values of 0.2 to 0.4 are already considered effective. The mandatory prerequisite for choosing the effective model was the inclusion of cytogenetic indicators (micronuclei and protrusions), without excluding introduction of cytotoxic anomalies into the calculations.

Results and discussion. In the exposed group, uterine fibroids were statistically more frequent than in the controls (25.6 % versus 0, $p \leq 0.05$). Given the facts that myoma is a multifactorial disease and the role of the genetic factor has not been definitely established so far, it is important to select cytometry variables that primarily indicate the genetic risk. To begin with, we compared changes in the cytogenetic status in all workers. Table 1 shows that the exposed women demonstrated

Table 1

Cytogram of buccal epithelium in female copper smelter workers exposed to occupational chemicals and the controls (‰)

Indicators	Controls, $n = 8$	Cases, $n = 39$
Micronuclei	1.88 ± 0.23	4.00 ± 0.12 *
Protrusions	3.13 ± 0.44	5.72 ± 0.20 *
Binucleated cells	2.63 ± 0.26	3.67 ± 0.15 *
Perinuclear vacuole	1.25 ± 0.16	1.56 ± 0.09
Nuclear condensation	3.88 ± 0.30	5.21 ± 0.20 *
Nuclear vacuolization	3.88 ± 0.40	6.59 ± 0.19 *
Cytoplasmic vacuolization	4.50 ± 0.33	7.59 ± 0.20 *
Karyolysis	2.75 ± 0.25	4.97 ± 0.19 *

Note: * $p < 0.05$.

Table 2

Quality indicators of the binary classifier based on logistic regression with three and more variables when choosing the best model based on pR^2 without variable restrictions (M_1 model) and including "micronuclei" and "protrusions" variables (M_2 model)

Quality indicators	Best fit	
	M_1	M_2
	Micronuclei, perinuclear vacuole, cytoplasmic vacuolization	Micronuclei, protrusions, binucleated cells
pR^2	0.570	0.383
AUROC	0.940	0.883
Sensitivity (TPR)	0.900	0.800
Specificity (TNR)	0.828	0.759
Matthews correlation coefficient	0.662	0.501
Diagnostic odds ratio	43.200	12.571
Youden's index	0.728	0.559

an increase in such genotoxic indices as micronuclei and protrusions by 2.2 and 1.8 times, respectively, a 1.3-fold increase in the number of binucleated cells ($p < 0.05$). They also showed changes in early and late destruction of the nucleus and cytoplasm compared to the control group. Their indices of nuclear vacuolization and cytoplasm vacuolization were 1.6 times, karyolysis – 1.8 times, and nuclear condensation – 1.3 times higher than in the controls. The above findings give evidence of a cytotoxic effect.

The index of accumulation of cytogenetic damage in the main group was 3.3 against 1.7 in the controls and corresponded to moderate risk ($2 < Iac < 4$).

To assess the genotoxic risk of uterine fibroids, we chose the prognostic model based on the cytogenetic abnormalities found.

The comparison of two models based on logistic regression with three and more variables described in Table 2 showed that the best model without choosing variable restrictions had the McFadden's coefficient of determination of 0.570 while in that with micronuclei and protrusions included as variables pR^2 equaled 0.383 with the area under the ROC curve of 0.940 and 0.883, respectively (Figure 1).

Despite the differences in the coefficients of determination, characteristics of the models with/without genotoxic indicators are quite similar, which can be considered as the aptness of mandatory inclusion of specific indicators of genotoxic effects in the model for determining the risk of uterine fibroids in the study population.

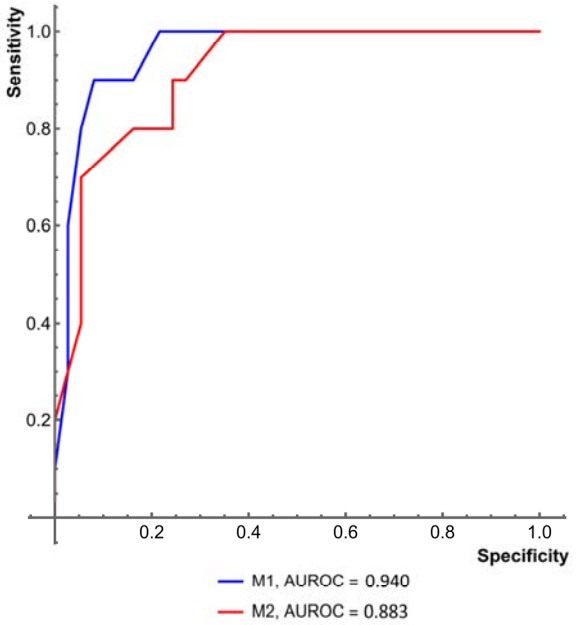

Figure 1. ROC-curves for M_1 and M_2 models from Table 2

When including four variables in the model, the coefficient of determination has little significance (pR^2 = 0.105 for the best model), but when including two genotoxicity and two cytotoxicity indicators, it improves significantly (pR^2 = 0.938 for the best model of four variables selected from all predictors). Thus, this model has the greatest diagnostic value (Figure 2). The model is described in Table 3.

One of the risk factors for female reproductive health in copper smelting is the aerosol of complex chemical composition. Lead, nickel and cadmium, which are part of the aerosol, are known to be both mutagenic and reprotoxic substances inducing chromosomal aberrations in somatic and germ cells [23]. According to the literature data, exposure to high copper concentrations causes an increase in the frequency of mitotic aberrations in cells. High exposures to nickel compounds, which also have a clastogenic effect, lead to the formation of micronuclei. The mechanism of action is based on DNA rupture and generation of DNA-protein crosslinks. An increase in the frequency of sister chromatid exchange and chromosomal aberrations is also noted. Cadmium and its compounds raise the frequency of micronuclei and chromosomal aberrations. Some mechanisms of action include inhibition of DNA repair systems, effects on cell proliferation and functioning of tumor suppressors. When exposed to sulfuric acid, binding to DNA molecules occurs, which leads to disruption of DNA chain division. Since uterine fibroids have multiple etiologies, with the molecular genetic mechanism being one of them, the study of genotoxic effects of the above chemicals on myometrium and endometrium cells remains relevant. According to foreign scientists, copper and lead "…could negatively affect endometrial functionality, compromising the decidualization

Table 3

Quality indicators of the binary classifier based on logistic regression with four variables when choosing the best model based on pR^2 without variable restrictions (M_1 model) and including the variables "micronuclei" and "protrusions" (M_2 model)

Quality indicators	Best fit
	$M_1 = M_2$
	Micronuclei, protrusions, binucleated cells, cytoplasmic vacuolization
pR^2	0.938
AUROC	0.998
Sensitivity (TPR)	1.000
Specificity (TNR)	0.966
Matthews correlation coefficient	0.937
Diagnostic odds ratio	$+\infty$
Youden's index	0.966

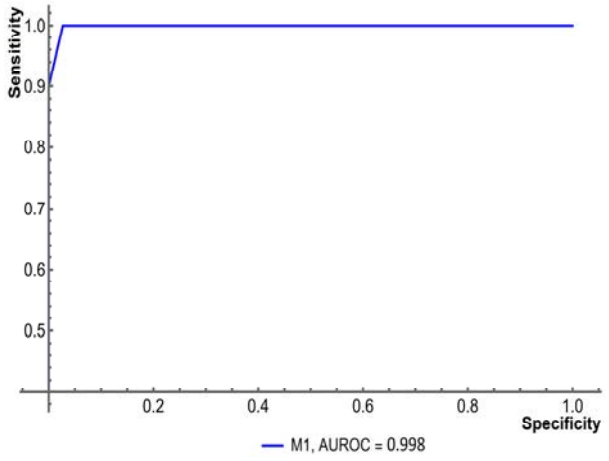

Figure 2. ROC-curve for the $M_1 = M_2$ model from Table 3

process and disrupting endometrial regeneration and embryo adhesion" [24], thus increasing the risk of endometrial cancer. The relationship between blood concentrations of cadmium and its compounds and uterine fibroid volume was also proven [25]. Nickel and its compounds affect endometrial cells and increase the risk of endometriosis. In this regard, it is worth emphasizing that the micronucleus test of buccal epithelium reveals micronuclei in cells that are formed directly as a result of DNA damage following exposure to industrial toxicants. Micronuclei are known to be formed via different pathways: as a result of DNA damage, thus representing a chromosome fragment, or damage to the spindle of division in the form of one or more whole chromosomes, which may indicate both gene and genomic disorders. Our findings showing an increase in the number of cytogenetic disorders, such as micronuclei and protrusions in the female copper smelter workers, are consistent with the results of previous studies and confirm genotoxic properties of industrial toxicants, which may be a risk factor of uterine fibroids. An increased frequency of binucleated cells in the workers is also associated with toxic effects of chemical compounds. It is also known that binucleated cells are formed as a result of nuclear division that takes place without cytoplasmic division, while their ploidy increases. The frequency of binucleated cells is an indicator of proliferative activity [26]. Thus, the variables included in the prognostic model, such as micronuclei and protrusions, can play the leading role in identifying risk factors of uterine fibroids. However, exposure to industrial chemicals can develop acquired cytotoxic effects and promote congenital genotoxic effects. The established increase in the number of cells with early and late destruction also allow us to use these variables to assess the risk of endometrial pathology. Again, since uterine fibroids have multiple potential etiologies, the role of the endocrine system in the mechanisms of their development is among the leading ones. Heavy metals within industrial aerosols have a toxic effect on the hypothalamic-pituitary system of the body. Yet, the mechanisms of genotoxic and cytotoxic effects of heavy metal aerosols have not been fully studied [27]. Our results can therefore be used to identify groups at risk of uterine fibroids in female workers exposed to occupational hazards. The presented methodology for using buccal cytograms to establish groups at risk of uterine fibroids in female workers of a copper smelter and the study results support our hypothesis that geno- and cytotoxicity of industrial toxicants in relation to buccal epithelial cells are similar in mechanism to the effects of developing pathological processes in myometrium, which allows their extrapolation. Inclusion of data on cytogenetic and hormonal status in the developed model for predicting the risk of uterine fibroids is relevant and significant for the tasks of forming groups at risk of health problems in female workers exposed to aerosols containing mutagenic and reprotoxic elements.

Conclusions:

1. Genotoxic and cytotoxic indices in buccal cytograms are significantly more frequent in women exposed to industrial aerosols containing mutagenic and reprotoxic elements compared to unexposed workers.

2. The risk of uterine fibroids can be calculated using the mathematical model that includes genotoxic and cytotoxic indices.

3. Calculation of the parameters of the model that includes genotoxic prognosis indices and, above all, such variables as micronuclei and protrusions play a decisive role in identifying risk factors of uterine fibroids.

4. It is expedient to continue the research by introducing hormonal status parameters into the models.

5. These models are relevant for the tasks of forming groups at risk of health disorders among female workers exposed to aerosols containing mutagenic and reprotoxic elements.

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