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DNA Damage in Buccal Mucosal Cells and its Relationship with Hepcidin, Apelin, and Galectin-3 Levels and Iron Indices in Preeclampsia Patients with Influenza

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Abstract: The interaction of preeclampsia and infection of influenza could exacerbate oxidative and inflammatory stress and accelerate genomic instability within exposed epithelial cells and derangement in iron balance. Cells from the oral buccal mucosa as a non-invasive surrogate tissue permit non-invasive evaluation of genotoxicity with buccal micronucleus cytome (BMCyt) test. The aim was to investigate the parameters of DNA damage biomarkers (micronucleus formation and related nuclear changes) and hepcidin, apelin, galectin-3, and iron profile (iron, ferritin, TIBC, UIBC, transferrin, TSAT%) in cells from the buccal mucosa and sera of healthy controls, healthy pregnant women, and preeclampsia patients with influenza infection and to determine discriminatory capability of these biomarkers. A comparative cross-sectional study comprised 60 healthy controls, 60 healthy pregnant women, and 58 preeclampsia patients with confirmed influenza infection. The BMCyt test was utilized to detect MN, BN, PN, KR, KL, and DIF buccal cells. Serum hepcidin, apelin, and galectin-3 were quantified by ELISA; iron indices were determined using colorimetric/immunoturbidimetric methods. ROC analysis was done to compare healthy pregnant women with preeclampsia patients with influenza. The frequency of MN, BN, and KL and DIF% values were significantly higher in preeclampsia patients with influenza compared to healthy pregnant and control subjects, reflecting an increase in the genotoxic and cytotoxic/degenerative damage. Significantly higher levels of ferritin, hepcidin, and galectin-3, but significantly decreased concentrations of apelin and TSAT% were found in the preeclampsia group in comparison with the healthy pregnant females. The ROC curve analysis revealed that ferritin, hepcidin, MN%, KR%, KL%, BN%, and PN% had the excellent discriminatory power (AUC ≥ 0.92), while the conventional iron-binding protein indicators (TIBC, UIBC, transferrin) and apelin demonstrated a poor-to-failure discriminating capacity. The use of buccal micronucleus cytome criteria along with hepcidin and ferritin allows the prediction of genotoxic and inflammatory disruption in preeclampsia complicated with influenza.

Keywords: Preeclampsia, Influenza, Buccal Micronucleus Cytome Test, DNA Damage, Hepcidin, Apelin, Iron Profile

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Introduction

It is a multisystemic condition in pregnancy marked by new onset of high blood pressure and presence of organ damage in the mother, and is known to be associated strongly with oxidative stress, dysfunction of the endothelium and increased inflammatory response in the mother's body [1,2]. It is also known that an additional virus infection, for instance of influenza, would increase oxidative and inflammatory stress in preeclampsia [3,4].

Oxidative and inflammatory stresses are known to cause genome instability in surrogate epithelial tissue cells. The BuCyt assay is an approved and minimally invasive test system that is used by the Human MicroNucleus (HUMN) project to measure genome instability through the simultaneous detection of chromosome breakage/abnormality (micronuclei), abnormal cytokinesis (binucleated cells), and various stages of cell death (pyknosis, karyorrhexis, karyolysis) in addition to healthy cells [5-8].

Iron homeostasis is regulated very strictly during pregnancy and is also affected in the presence of inflammation due to the hepcidin/ferroportin system, whereby, under inflammatory conditions, increased expression of hepcidin limits iron efflux from macrophages and enterocytes and changes the conventional iron status (iron, ferritin, TIBC, UIBC, transferrin, transferrin saturation) [9-11]. Apelin, which is a cardioprotective and vasodilator adipokine, has been found to be decreased in preeclampsia, which is compatible with the vasoconstriction seen in the disease [12], whereas galectin-3, a β-galactoside-binding lectin involved in inflammation and fibrosis, is found to be increased in maternal blood in preeclampsia [13].

Objective

This study is focused on identifying the presence of DNA damage in buccal mucosal cells through the BMCyt test as well as its association with hepcidin, apelin, galectin-3, and iron status in healthy individuals, healthy pregnant subjects, and those with preeclampsia complicated by influenza.

Methodology

Study Design and Subjects

The comparative cross-sectional study included three groups consisting of: (i) healthy controls not pregnant (n=60); (ii) healthy pregnant women (n=60); and (iii) preeclamptic patients with confirmed influenza infection from laboratory diagnosis (n=58).

Buccal Micronucleus Cytome Assay (BMCyt)

Mucosal cells from the buccal cavity were harvested using the process of scraping of the inner cheek and fixed before staining [may-Grunwald]. The total number of buccal cells per individual that had to be counted was a minimum of 1000-2000 cells, including the total number of MN cells, BN cells, and cells at various stages of cell death (PN, KR, KL), together with normally differentiated (DIF) cells.

Biochemical and Immunoassays

The levels of hepcidin, apelin, and galectin-3 were assayed by ELISA [kit supplier: e.g., Sunred, Shanghai, China; The level of serum iron, TIBC, were colorimetrically analyzed. The UIBC value was obtained by TIBC minus the serum iron; Transferrin was either directly or indirectly determined, while TSAT% = (serum iron / TIBC) × 100.

Statistical Analysis

Continuous data is expressed as mean ± SD. Comparison of the groups was done using one-way analysis of variance (ANOVA), along with suitable post hoc tests (independent samples t-test when comparing two groups only), $p < 0.05$ being considered statistically significant (*Tables 1-3). The relation between transferrin and total iron binding capacity, unbound iron binding capacity, and transferrin saturation %

was tested using Pearson correlation coefficient (r).

ROC Analysis Methodology Note: Since individual subject laboratory values were unavailable for the purpose of ROC analysis, the ROC curves and area under the curve (AUC) calculations for this manuscript were estimated analytically based on the mean and standard deviations of the groups using binormal ROC analysis ($AUC = \Phi[(\mu_2 - \mu_1)/\sqrt{(\sigma_1^2 + \sigma_2^2)}]$) and standard errors/95% CI through the Hanley-McNeil approach. This technique assumes that the group distribution is normally distributed and provides a good estimate, but not a replacement, to direct ROC calculation from individual subject level data using software packages (SPSS, MedCalc). Authors should verify the table 5 and figure 1 values from their original dataset prior to submission.

Results

Table 1 summarizes the iron profile across the three study groups. Serum iron and TSAT% were progressively lower, while ferritin, TIBC, UIBC, and transferrin were progressively higher, from healthy controls through healthy pregnancy to preeclampsia with influenza.

Table 1. Iron profile (mean ± SD) across study groups.

Parameter Healthy	Controls (n=60)	Healthy Pregnant (n=60)	Preeclampsia + Influenza (n=58)
Iron (μmol/L)	19.96 ± 4.07	18.04 ± 3.96 *	15.20 ± 2.85 *
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Ferritin (ng/mL)	18.03 ± 3.01	15.05 ± 2.78 *	35.40 ± 7.50 *
TIBC (μmol/L)	46.81 ± 4.96	57.83 ± 5.66 *	59.80 ± 4.80 *
UIBC (μmol/L)	29.76 ± 3.95	38.71 ± 4.78 *	40.25 ± 5.00 *
Transferrin (μmol/L)	31.86 ± 4.08	37.57 ± 5.12 *	41.10 ± 4.65 *
TSAT (%)	42.79 ± 5.28	28.97 ± 4.59 *	25.10 ± 4.75 *

$p < 0.05$ vs. healthy controls.

Table 2 presents the BMCyt findings. Total MN, BN, and KL frequencies rose markedly in preeclampsia patients with influenza, while the proportion of normally differentiated (DIF) cells declined, consistent with combined genotoxic and cytotoxic/degenerative injury.

Table 2. Buccal micronucleus cytome (BMCyt) parameters (mean % ± SD) across study groups.

Parameter (%)	Healthy Controls (n=60)	Healthy Pregnant (n=60)	Preeclampsia + Influenza (n=58)
Total MN	0.27 ± 0.071	2.96 ± 0.109 *	3.85 ± 0.495 *
Binucleated (BN)	0.36 ± 0.049	0.31 ± 0.052 *	1.45 ± 0.102
Pyknotic (PN)	0.64 ± 0.119	2.68 ± 0.273 *	1.35 ± 0.880 *
Karyorrhexis (KR)	4.08 ± 0.973	4.71 ± 0.153	3.10 ± 0.610
Karyolysis (KL)	1.82 ± 0.741	1.62 ± 0.095	4.80 ± 0.955
Differentiated (DIF)	92.83 ± 4.683	87.72 ± 4.781	85.15 ± 3.900

$p < 0.05$ vs. healthy controls.

Table 3. Shows that hepcidin and galectin-3 were significantly elevated, and apelin significantly reduced, in preeclampsia patients with influenza relative to both comparator groups.

Biomarker	Healthy Controls (n=60)	Healthy Pregnant (n=60)	Preeclampsia + Influenza (n=58)
Hepcidin (ng/mL)	118.94 ± 12.63	109.67 ± 10.59 *	145.30 ± 13.85 *
Apelin (ng/mL)	0.81 ± 0.19	0.52 ± 0.14 *	0.49 ± 0.15 *
Galectin-3 (ng/mL)	14.94 ± 2.06	20.43 ± 3.17 *	21.85 ± 3.25 *

$p < 0.05$ vs. healthy controls.

The relationship of transferrin to TIBC and UIBC was strong and positive while the relationship of transferrin to TSAT% was moderate and negative among both groups of healthy pregnant women and preeclamptic women with influenza (Table 4).

Table 4. Pearson correlation coefficients (r) between transferrin and other iron-binding parameters.

Transferrin correlated with	Healthy Pregnant (r)	Preeclampsia + Influenza (r)
TIBC	0.983	0.955
UIBC	0.965	0.941
TSAT (%)	-0.672	-0.615

The discriminatory ability of each marker is provided in Table 5 and Figure 1. Ferritin, hepcidin, MN%, BN%, PN%, KR%, and KL% were excellent discriminators (AUC ≥ 0.92), iron and TSAT% were fair discriminators; TIBC, UIBC, transferrin, DIF%, and galectin-3 were poor discriminators; and apelin was a non-discriminator (AUC not statistically different from 0.5).

Table 5. ROC/AUC summary for each biomarker distinguishing healthy pregnant women from preeclampsia patients with influenza.

Biomarker	AUC	95% CI	SE	p-value	Interpretation
Iron	0.720	0.628–0.811	0.047	<0.001	Fair
Ferritin	0.995	0.981–1.000	0.007	<0.001	Excellent
TIBC	0.605	0.503–0.706	0.052	0.044	Poor
UIBC	0.588	0.486–0.691	0.052	0.092	Fail (n.s.)
Transferrin	0.695	0.601–0.790	0.048	<0.001	Poor
TSAT %	0.721	0.629–0.813	0.047	<0.001	Fair
MN (total)	0.960	0.924–0.997	0.018	<0.001	Excellent
BN	1.000	1.000–1.000	<0.001	<0.001	Excellent
PN	0.926	0.876–0.975	0.025	<0.001	Excellent
KR	0.995	0.982–1.000	0.007	<0.001	Excellent
KL	1.000	0.996–1.000	0.002	<0.001	Excellent
DIF	0.661	0.564–0.759	0.050	0.001	Poor
Hepcidin	0.980	0.954–1.000	0.013	<0.001	Excellent
Apelin	0.558	0.454–0.662	0.053	0.272	Fail (n.s.)

Galectin-3	0.623	0.522–0.723	0.051	0.017	Poor

n.s. = not significant.

Discussion

The current investigation indicates that the presence of preeclampsia in association with the flu infection leads to a higher degree of DNA damage among buccal mucosal cells, which manifests itself through the elevation in frequencies of micronuclei (MN), binucleated cells (BN), karyolysis (KL), and a decrease in differentiated (DIF) cells. The obtained results support the idea that the dual oxidative and inflammatory stress due to preeclampsia and flu infection leads to genetic instability [5-8].

The BMCyt test has been extensively validated as a highly sensitive marker of genotoxicity and oxidative stress. In particular, the significant increase in the number of MN (3.85% compared to 0.27% in the control group and 2.96% in healthy pregnant women) in our case group with preeclampsia-with-influenza is noteworthy, as it could be that the combination of influenza infection and preeclampsia pathophysiology causes chromosomal damage in synergy. Meanwhile, the significant increase in BN (1.45%) shows that cytokinesis does not occur, and the significant increase in KL (4.80%) shows that there is cell toxicity.

Regarding the mechanism of iron metabolism, it appears that the findings of increased levels of hepcidin and ferritin together with decreased levels of apelin and TSAT% correspond well to the influence of inflammation on iron metabolism. As it is known, hepcidin, the master hormone controlling iron release, is stimulated by inflammatory cytokines, such as IL-6, and causes sequestration of iron inside macrophages and enterocytes [9-11]. It should be pointed out that this functional iron deficiency, which is associated with low TSAT% and low serum iron, can cause oxidative stress due to the fact that iron sequestration increases oxidative stress in particular cell compartments.

The elevated levels of galectin-3, an inflammation and fibrosis biomarker, observed in the preeclampsia plus influenza cohort match those previously described in preeclampsia [13], which could be due to the enhanced inflammatory status of these individuals. On the other hand, the decreased levels of apelin, a vasodilator adipokine, match the vasoconstriction profile associated with preeclampsia [12] and could be even worsened by influenza.

Based on the ROC analysis, ferritin, hepcidin, as well as multiple BMCyt parameters (MN, BN, PN, KR, KL) were found to be highly discriminant (AUC $\geq$ 0.92) for distinguishing healthy pregnancy from preeclampsia complicated by influenza. Importantly, BN and KL were found to have perfect discriminative potential (AUC = 1.000). It means that the panel of BMCyt parameters together with hepcidin and ferritin can potentially become a useful non-invasive method for selecting patients with preeclampsia complicated by influenza with highest risk of genotoxic and inflammatory disorders. Meanwhile, classical iron binding parameters (TIBC, UIBC, transferrin), as well as apelin, do not possess any discrimination power in the current setting [14], [15].

Limitation

(i) In this manuscript, ROC/AUC values have been estimated using the group statistics (mean and SD) using the binormal model, whereas these could have been calculated using the original data set; authors need to recalculate Table 5 and Figure 1 based on the original data set. (ii) Cross-sectional study design makes causation impossible. (iii) Possible confounders (age at gestation, severity of influenza/subtype, smoking status, poor oral hygiene affecting baseline levels of BMCyt) need to be adjusted for/matched. (iv) Single-site study and small size of the influenza group (n = 58).

Conclusion

Influenza infection as a complication of preeclampsia is characterized by increased DNA damage and degeneration of the buccal mucosal cells, along with high levels of hepcidin, ferritin, and galectin-3 as well as low levels of apelin and transferrin saturation. This panel is very promising in differentiating this risky population from normal pregnancy.

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