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Change in expression of NFκb and MUC5AC in nasal mucosa during pregnancy

Gebelik sırasında nazal mukozada NFκb ve MUC5AC ekspresyon düzeylerinin değişimi

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Abstract

Aim: There is very limited information concerning gestational course of chronic inflammatory diseases of the upper airway. In the current study, we tried to reveal biomolecular changes in the nasal mucosa during pregnancy regarding NFκb and MUC5AC. This way, we may have the opportunity to suggest new treatment strategies for pregnancy induced nasal disorders.

Methods: Thirty adult female rats were separated into pregnant and control groups. Serum estradiole (E2) and progesterone (PG) levels were evaluated by ELISA. Nasal expression of NFκb and MUC5AC were analyzed by polymerase chain reaction testing. Relative expression of NFκb and MUC5AC was compared between two groups. In addition, the influences of PG and E2 levels on NFκb and MUC5AC were determined.

Results: NFκb was significantly low (P=0.015), while MUC5AC was significantly high in pregnant rats (P=0.029). Serum PG had a significantly negative effect on NF-κB expression (P=0.027) and a significantly positive effect on MUC5AC expression (P=0.017). There was a positive correlation between estradiol and MUC5AC (P=0.017).

Conclusions: In the current study, the physiological expression of NFκb and MUC5AC was shown for the first time by PCR in rat nasal mucosa. The effect of PG and E2 on the expression of these biomolecules in the context of pregnancy was also revealed. These findings have the potential to elucidate gestational inflammatory alterations in the upper airway.

Keywords: Pregnancy, Immunity, NFκb, MUC5AC, Nasal mucosa

Öz

Amaç: Üst solunum yolunun kronik enflamatuar hastalıklarının gebelikteki seyri konusunda çok sınırlı bilgi mevcuttur. Bu çalışmada, NFκb ve MUC5AC ile ilgili olarak hamilelik sırasında nazal mukozadaki biyomoleküler değişiklikleri ortaya çıkarmaya çalıştık. Bu sayede, gebeliğe bağlı burun rahatsızlıkları için yeni tedavi stratejileri önerme fırsatımız olabilir.

Yöntemler: Sıçanlar üzerinde yapılan bu çalışma, Deney Hayvanları Araştırma ve Uygulama Merkezinde yapılmıştır. Otuz yetişkin Wister albino dişi sıçan, bir kontrol grubu ve bir hamile grubu olarak ayrıldı. Serum estradiole (E2) ve progesteron (PG) düzeyleri ELISA ile değerlendirildi. NFκb ve MUC5AC'nin nazal ekspresyonu, polimeraz zincir reaksiyon testi ile analiz edildi. İstatistiksel analiz, NFkb ve MUC5AC ekspresyonunun grup bazında karşılaştırılması ile yapıldı. Ayrıca E2 ve PG seviyelerinin bu moleküller üzerindeki etkisi de araştırıldı.

Bulgular: Gebe sıçanlarda NFκb anlamlı olarak düşük (P=0,015) bulunurken MUC5AC anlamlı derecede yüksek bulundu (P=0,029). Serum PG ve NF-κB ekspresyonu arasında istatistiksel olarak anlamlı negatif korelasyon (P=0,027), progesteron ile MUC5AC ekspresyonu arasında ise pozitif korelasyon (P=0,017) bulundu. Ek olarak, estradiol ve MUC5AC arasında pozitif korelasyon tespit ettik (P=0.017).

Sonuçlar: Bu çalışmada, sıçan nazal mukozasında NFκb ve MUC5AC'nin fizyolojik ekspresyonu ilk kez PCR ile gösterilmiştir. E' ve PG'nin bu biyomoleküllerin ekspresyonu üzerindeki etkisi de gebelik bağlamında ortaya konmuştur. Bu bulgular, üst solunum yolunun gestasyonel enflamatuar değişikliklerini açıklama potansiyeline sahiptir.

Anahtar kelimeler: Gebelik, Bağışıklık, NFκb, MUC5AC, Nazal mukoza

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Introduction

Nuclear factor kappa B (NFκb) is a key biomolecule taking role in the transcription of many genes associated with inflammation and immune processes via various pro-inflammatory and anti-inflammatory cytokines. Maintenance of a healthy pregnancy is achieved mainly by the maternal immunologic shift with increasing the ratio of T Helper lymphocytes 2 (Th2) to T helper lymphocytes 1 (Th1) [1]. NFκb plays a key role in this immunologic differentiation in pregnancy. Besides, maternal hormones influence NFκb expression in different tissues [2-5]. Estradiol causes suppression of NFκb expression [3,4]; whereas the effect of Progesterone is conflicting [4,5].

AC subclass of Mucin type 5 (MUC5AC) is the main mucin of the upper respiratory system [6-7]. Overproduction of MUC5AC is related to deterioration of mucociliary clearance which paves the way for rhinitis and nasal obstruction [7]. On the other hand, NFκb takes part in the main pathway regulating the MUC5AC secretion. It has been shown that NFκb has an upregulatory effect on nasal MUC5AC [7]. Recent data showed that Estradiol (E2) also enhances mucin production in bronchial epithelial cells. This finding may be the reason for the higher incidence of chronic inflammatory airway diseases in women [8].

Pregnancy is a unique period which is characterized by varying degrees of immunosuppression and immunologic shift from cell-mediated immunity towards humoral immunity. NFκb is known to play a significant role in this pregnancy induced immune regulation during both implantation window and subsequent trimesters [1]. There is still an ongoing debate concerning the prognosis of chronic inflammatory airway diseases (i.e. asthma, allergic rhinitis) in pregnancy. We aimed to find out the change in the expression of NFκb and MUC5AC in nasal mucosa during pregnancy. This may help to elucidate the pathophysiology of chronic inflammatory conditions of the upper airway in pregnancy. To the best of our knowledge, NFκb and MUC5AC expression in nasal mucosa during pregnancy has not been studied before. In the current study, the effect of maternal hormones on the expression of these biomolecules was also evaluated.

Materials and methods

Animals

The current experimental animal study was approved by the Laboratory Animals Ethics Committee of the institution. The experiment was performed in the Experimental Animals Research and Application Center.

Twenty, 12-week-old Wister albino female rats were enrolled in the study. They were kept at 22 (2) °C on a light-dark cycle of 12 hours. Male rats were kept together with female rats with a M:F ratio of 3:1 for 1 night. Female and male rats were separated from each other in the following morning. Sperm exploration was performed in the vaginal smears of female rats. The day sperm was detected was assumed as Day 0 of gestation, as defined before [9-11]. Group A (control) was constituted by the rats with negative vaginal smear whereas Group B included (pregnant) sperm-positive ones. Pregnancy period of Wister albino rat is around 22 (21-26) days [10-12]. For this reason, we sacrificed the animals at 21st day of gestation with sodium-pentobarbitone (400mg/kg) injection, as reported before [11]. Then, 20 ml of blood sample was obtained by a 23 G needle before the pulse disappeared. Blood samples were sent for detection of serum E2 and PG levels by ELISA. Then we shaved the nasal dorsum. We separated the nasal bones from the maxilla in upward direction and exposed the whole nasal cavity superiorly. Cartilaginous part of the septum (Cartilago septi nasi) with its mucoperichondrium was resected and analyzed by real time PCR.

ELISA

Heparin, EDTA and sodium citrate were mixed into the blood samples. The mixture was centrifugated for 10 minutes at 3000 rpm. The supernatant was kept at -80 °C. General Progesterone (PG) ELISA Kit and Rat E2 (estradiol) ELISA Kit were used for quantitative measurement of serum PG and E2 levels, respectively (MyBioSource, Inc.,CA, USA) [11].

Extraction of RNA and Analyses of Quantitative Real-Time PCR (qRT-PCR)

TRIzol® Reagent with the PureLink® RNA Mini Kit (Thermo Fisher Scientific, 12183555) was used for total mucosal RNA extraction from the larynx. QuantiFast SYBR Green qRT-PCR Kit (Qiagen, 204154), NF-kB primers and MUC5AC primers were used for qRT-PCR procedure. QuantiFast SYBR Green, NF-kB primers and MUC5AC primers were prepared separately. Next, analyses for the detection of MUC5AC and NF-kB RNA expression levels was performed in Rotor-Gene Q (Qiagen, Hilden, Germany). We have normalized the expression variations of β-microtubulin (B2M) and hypoxanthine phosphoribosyl transferase (HPRT1) by a housekeeping gene. We synthesized reverse and forward primers (Table 1) by Metabion company (Germany). The first step of thermal cycling conditions of the RT-PCR program was the reverse transcription step at 50°C (10 min) which was followed by the PCR step, including an initial activation/denaturation stage at 95°C for 5 minutes. Then we applied 40 cycles of denaturation at 95°C for 15 seconds, accompanying annealing/extension at 60°C for 30 seconds. For calculation of relative variations in gene expression obtained from Real-Time PCR analysis, the $2^{-\Delta\Delta CT}$ method was used [13].

Table 1: Nucleotide sequences used in Quantitative Real-Time PCR analyses

Gene	Primer	Sequence
NF-kB	Forward	5'-ATGTGGTGGAGGACTTGCTG-3'
	Reverse	5'-GCTGCCTTGCTGTTCTTGAG-3'
MUC5AC	Forward	5'-GTTGGCTCTGACTGTACCACC-3'
	Reverse	5'- CCAGTGTGATGATGGTGAGGA-3'
HPRT1	Forward	5'-CGTCTTGCTCGAGATGTGAT-3'
	Reverse	5'- TTCAGTGCTTTGATGTAATCCAG-3'
B2M	Forward	5'-TCTCTCTTTCTGGCCTGGA-3'
	Reverse	5'-TGTCGGATGGATGAAACCC-3'

Statistical analysis

Relative NF-κB and MUC5AC expressions of group A and B were compared. Shapiro-Wilk test was used for evaluation of data distribution. Independent samples t-test or Mann-Whitney U Test were used for comparison of Group A and B according to the results of Shapiro-Wilk test. Pearson correlation test was used for evaluation of the effect of serum E2 and PG levels on TREK-1 and AQP5. Results were presented as mean (SD). Statistical significance was defined as $P<0.05$. We used

Statistical Package for the Social Sciences (SPSS) Version 21.0 (IBM Corp.; Armonk, NY, USA) for statistical calculations.

Results

Twenty Wister albino female rats were enrolled in the study (10 control, 10 pregnant). The mean relative expression of mRNA of NF-κB in groups A and B were 0.10 (0.03) and 0.08 (0.02), respectively. The mean relative expression of mRNA of MUC5AC in groups A and B were 1.06 (0.01) and 1.17 (0.27), respectively. The mean serum E2 levels in groups A and B were 19.15 (5.36) pg/ml and 73.38 (4.26) pg/ml, respectively. The mean PG levels of groups A and B were 14.14 (1.33) ng/ml and 31.99 (4.43) ng/ml, respectively (Table 2). The PCR data of NF-κB and MUC5AC showed non-normal distribution while ELISA of E2 and PG were normally distributed (P>0.05).

Table 2: Expression of NFκb and MUC5AC in nasal mucosa, and serum E2 and PG levels based on groups

Biomolecules & Sex Hormones	Control (Group A)	Pregnant (Group B)	P-value
Biomolecules			
NF-κB (REV)[a]	0.102 (0.027)	0.76 (0.018)	0.015[b]
MUC5AC (REV)[a]	1.063 (0.013)	1.171 (0.272)	0.029[c]
Serum Sex Hormone Levels			
Estradiol (pg/ml)	19.15 (5.36)	73.38 (4.26)	<0.001[b]
Progesterone (ng/ml)	14.14 (1.33)	31.99 (4.43)	<0.001[b]

[a] Denotes Relative Expression Value, [b] P values obtained by Mann-Whitney U Test, [c] p values obtained by Independent Samples t- test

Comparison of NF-κB between the groups revealed significantly lower expression in Group B (P=0.015). On the other hand, MUC5AC was significantly higher in group B (P=0.029) compared to group A (Figures 1, 2).

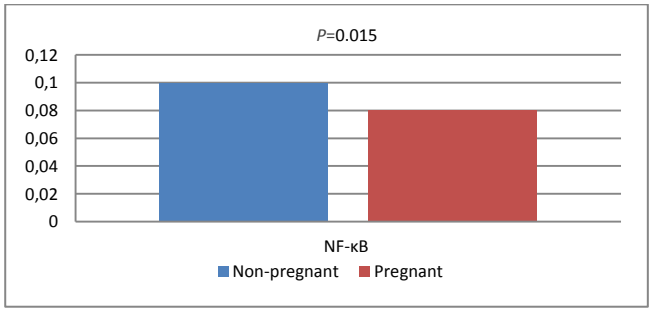

Figure 1: Graphic showing the increase of NF-κB expression in nasal mucosa during pregnancy

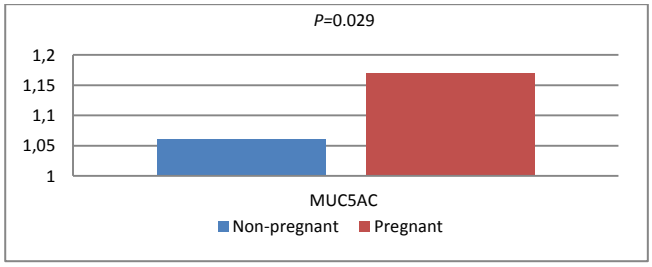

Figure 2: Graphic showing the increment of MUC5AC expression in nasal mucosa during pregnancy

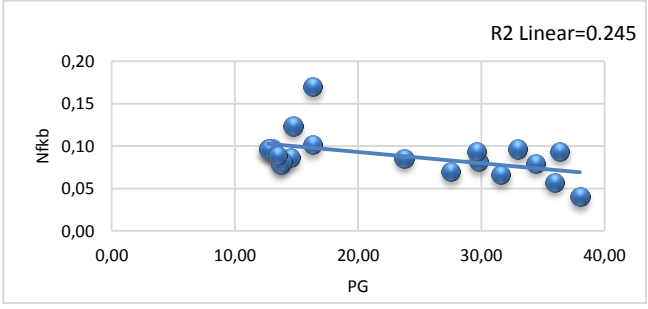

Figure 3: Scatter plot graphic showing the relationship between NF-κB expression and serum PG levels

A statistically significant negative correlation was found between serum PG and NF-κB expression (P=0.027), while a positive correlation was found between PG and MUC5AC expression (P=0.017) (Figure 3, 4). We did not find any correlation between E2 and NF-κB expression (P=0.126); however, E2 and MUC5AC were positively correlated (P=0.017) (Figure 5).

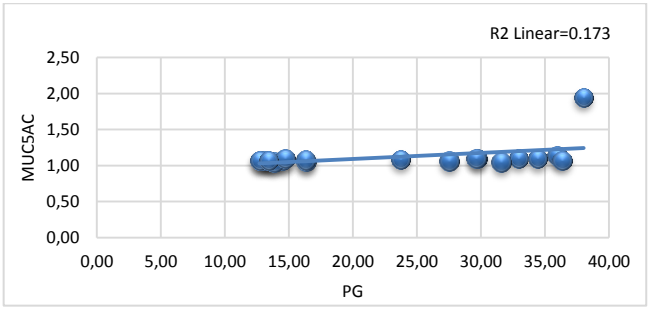

Figure 4: Scatter plot graphic showing the relationship between MUC5AC expression and serum PG levels

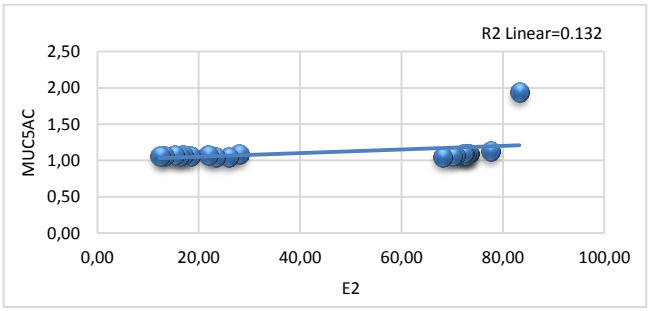

Figure 5: Scatter plot graphic showing the relationship between MUC5AC expression and serum E2 levels

Discussion

Pregnancy is characterized by unique immunomodulation supporting the healthy development and maintenance of the feto-placental unit. The pregnancy related physiological alterations of the immune system raise questions concerning the course of the allergic and other immunologic disorders which already exist before pregnancy [14]. NFκb is well-known with its key role in certain immunologic processes, especially in pregnancy [4-5]. Previous studies showed that NFκb also modulates MUC5AC levels by taking part in certain pathways [7]. As MUC5AC is the main mucin type seen in upper airways, its alterations cause deterioration in mucociliary clearance and transport which lead up to chronic inflammatory airway diseases. From that point of view, we hypothesized that these biomolecules may also play a role in pregnancy related airway diseases and may lead to exacerbation of preexisting chronic airway diseases during pregnancy. In this context, we evaluated whether expressions of nasal MUC5AC and NFκb changed in pregnancy. We also tried to find out whether E2 and PG affected the levels of both biomolecules.

There is a consensus that all pregnant women have a certain level of airway inflammation. Nonetheless, the effect of pregnancy on preexisting chronic inflammatory airway diseases has not been studied comprehensively. As pregnancy is characterized with some degree of immune suppression, deterioration of preexisting inflammatory conditions might be expected. For example, the course of asthma in pregnancy is variable. Namely, one third of patients experience worsening of the symptoms whereas nearly 20% of patients experience

improvement [15]. Allergic rhinitis is also one of these inflammatory conditions which is known to exacerbate in pregnancy. Although the course of allergic rhinitis in pregnancy is still hazy, there are reports concerning adverse outcomes caused by nasal obstruction. Nasal obstruction may lead to maternal hypertension, intrauterine growth restriction, low Apgar scores and increased admission in neonatal intensive care units [16].

Gestational NFκb suppression is particularly important in maternal immunologic differentiation. McCracken et al. revealed downregulation of NFκb in T cells isolated from pregnant women. They suggested that NFκb suppression leads to reduction of cytokine release from T Helper type 1 cells, which is essential for maintaining a healthy pregnancy [17]. NFκb modulates the expression of the genes related to immunity, especially in antigen presenting cells, lymphocytes, and cytokines [1]. On the other hand, MUC5AC is known to be the main mucin constituting a physical barrier in mucosa of nasopharyngeal lymphoid tissues [18]. Thus, it plays a critical role in nasal immunity [19]. Placental NFκb is suppressed in healthy pregnancies while overexpressed in pathological states like preeclampsia [20]. Similarly, we found significant suppression NFκb expression in the nasal mucosa of pregnant rats (Figure 1).

PG is known for its immunomodulatory effect in pregnancy by induction of immune tolerance in favor of Th2 [21]. In the current study, increased PG was associated with suppressed NFκb levels (Figure 2). From that point of view, we suggest that NFκb may take part in immunomodulatory effect of PG. In contrast to PG, E2 levels had no correlation with NFκb in our study. Data about the relationship between E2 and NFκb is controversial. Stice et al. showed that E2 treatment activates protective response via rapid NFκb stimulation in ischemia and trauma cases [22]. However, prior studies showed that prolonged E2 exposure causes inhibition of NFκb expression [23].

We showed that nasal MUC5AC was upregulated by E2 and PG. In contrast, Lange et al found no effect of E2 and PG on MUC5AC expression in ocular epithelial surface of mice [24]. They concluded that regulation of epithelial mucin genes was tissue-specific because previously, mucin in reproductive tract epithelium was found to be regulated by E2 and PG [24,25].

Conclusions

The physiological alteration of NFκb and MUC5AC in nasal mucosa of pregnant rats was shown for the first time. Using real time PCR, we also determined the association of E2 and PG with these biomolecules in rat nasal mucosa. Our findings may partially reveal the biomolecular background of mucosal changes of upper airway during pregnancy. Furthermore, tissue specific regulation of these biomolecules with E2 and PG in nasal mucosa may also elucidate the course of inflammatory airway diseases during pregnancy. Limitation of the current study is that we did not study mucosal expression of NFκb and MUC5AC with immunohistochemistry. Future nasal immunohistochemical studies concerning these biomolecules will elucidate the structural (laminar) localization. By this means, potential influence of topical agents on these biomolecules can be studied in the context of pregnancy.

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