

# PRENATAL EXPOSURE TO NICOTINE: the effect of dietary supplements on the levels of IL-1a, IL-6a and TNF $\alpha$ expression in the brain, lung and placenta of rats

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## Abstract

**Introduction:** Prenatal exposure to nicotine is a well-known risk factor for adverse consequences during pregnancy, including premature birth. Moreover, some studies have shown that smoking during pregnancy is associated with an increased risk of intrauterine infection and an altered inflammatory profile. The aim of this study was to investigate the effects of dietary supplementation after nicotine exposure measured by cytokine levels in brain, lung and placenta.

**Materials and methods:** The quasi-experimental and cross-sectional study was performed on rats that were subjected to perigestational exposure to nicotine, vitamins and minerals. The rats (n=30) were divided into 3 groups: control group, nicotine exposure group, and nicotine exposure with dietary supplements supplementation group. We collected brain, lung and placenta tissue homogenates from each subject and determined the level of Interleukin-1 alpha (IL-1 alpha), Interleukin-2 (IL-2) and tumor necrosis factor alpha (TNF alpha). Cytokine levels were comparatively analysed to determine differences between them using means comparison and correlation tests.

**Results:** There was an increase in IL-1a, IL-6 and TNF alpha levels in brain, lung and placental homogenates in the nicotine exposure group compared to the control group. The dietary supplements (Se and vitamin E) were associated with a decrease in protein levels of brain homogenates (IL-1a and IL-6), lung (IL-6 and TNF-a) and placenta (IL-1a, IL-6) ( $p < 0,001$ ).

**Discussion:** All study groups exposed to nicotine had increased levels of cytokine. Perinatal administration of dietary supplements was associated with a decrease in the level of IL-6 for brain, lung and placenta homogenates. Further studies are needed to determine the causal interactions between Se, vitamin E and nicotine.

## Rezumat: Expunerea perigestațională la nicotină: rolul suplimentelor nutritive în expresia IL-1a, IL-6 și TNF $\alpha$ în creier, plămân și placenta la șobolani

**Introducere:** Expunerea perinatală la nicotină este o cauză cunoscută a complicațiilor în timpul sarcinii, printre care și nașterea prematură. Mai mult, unele lucrări științifice sugerează că fumatul în timpul sarcinii este asociat cu un risc crescut al infecțiilor intrauterine și cu un profil inflamator alterat. Obiectivul acestui studiu a fost explorarea efectului administrării vitaminelor și mineralelor în cazul expunerii la nicotină, măsurat prin nivelul citokinelor în creier, plămân și placenta.

**Material și metode:** Studiul cvasi-experimental și transversal a fost efectuat pe feteși rezultați de la femele de șobolani expuse la nicotină și suplimente nutritive în perioada perigestațională. Feteși rezultați (30) au fost împărțiți în 3 grupuri de studiu: grupul de control, grupul de expunere la nicotină și grupul de expunere la nicotină cu suplimente nutritive. De la fiecare făt rezultat au fost recoltate probe de țesut din creier, plămân, placenta și din omogenatul acestora au fost realizate determinări pentru Interleukina-1 alfa (IL-1a), Interleukina-2 (IL-2) și factorul de necroză tumorală (TNF-a). Nivelurile citokinelor au fost analizate comparativ pentru identificarea diferențelor, utilizând teste pentru compararea mediilor și pentru corelație.

**Rezultate:** Expunerea la nicotină a înregistrat o creștere a nivelului IL-1a, IL-6 și TNF-alfa la omogenatele de creier, plămân și placenta. ( $p < 0,005$ ) în comparație cu grupul de control. Suplimentele nutritive au fost asociate cu o scădere a nivelurilor proteinelor pentru omogenatele de creier (în cazul IL-1a și IL-6), plămân (IL-6 și TNF-a) și placenta (IL-1a, IL-6) ( $p < 0,001$ ).

**Discuții:** Toate grupurile de studiu au înregistrat creșteri ale nivelurilor citokinelor în cazul expunerii la nicotină. Administrarea perinatală a suplimentelor nutritive a fost asociată în mod consistent cu o scădere a nivelului de IL-6 pentru omogenatul de creier, plămân și placenta. Mai multe studii sunt necesare pentru stabilirea implicațiilor cauzative ale interacțiunii dintre suplimentele nutritive și expunerea la nicotină.

**Cuvinte cheie:** nicotină, suplimente nutritive, IL-1a, IL-6, TNF $\alpha$ , sarcină, șobolani

## **Introduction**

Prenatal exposure to tobacco smoke is a major risk factor for maternal morbidity and mortality (1). Tobacco smoke exposure is associated with premature births, intrauterine growth restriction, low birth weight, sudden infant death syndrome, neurodevelopmental and behavioral disorders, childhood obesity, intrauterine infections, altered inflammatory profile, hypertension, diabetes and asthma (2,3). Although smoking during pregnancy has a major impact in the postnatal development, the negative effects are intensified by smoking during breastfeeding and exposure to second hand smoke (4).

Nicotine is a major component of cigarette smoke that causes addiction, being associated with strong physiopathological characteristics in the human body (5) by stimulating acetylcholine receptors (6). It promotes the presence of free radicals (both in vivo and in vitro) playing an important role in oxidative stress, through the negative impact that it has on the anti-oxidative defenses (7). There is evidence that oxidative stress can be reduced through selenium (Se) and vitamin E supplementation (8). Selenium is present within numerous enzymes that prevent cell damage, by lowering lipid peroxidation and supporting glutathione resynthesis (9). Vitamin E protects cellular structures against free radicals and lipid peroxidation (10).

Cytokines represent a category of small proteins that have the capacity to influence cellular dynamics. Being produced by a wide variety of cells (T cells, B cells, macrophages, endothelial cells, fibroblasts, etc.), these are of particular importance to the immune system, regulating the growth and response of certain cell groups. Some cytokines can amplify or inhibit others effect, acting through complex mechanisms to respond to infections, inflammations, trauma, cancer or reproduction (11). All nucleated cells secrete these proteins but endo/epithelial cells primarily produce interleukin 1 alpha (IL-1a), interleukin 6 (IL-6) and tumor necrosis factor (TNFa) (12).

TNFa is a cell signaling protein involved in the regulation of the immune system, having the ability to inhibit tumor cell genesis and the replication of

viruses, and respond to septicemia via cells that produce interleukins. TNFa disruptions are associated with different disorders among humans, such as Alzheimer (13), cancer (14), depression (15), psoriasis (16) and irritable bowel syndrome (17). IL-1a or hematopoietin-1 is a cytokine of the interleukin 1 family. In general, IL-1a is responsible for inflammation, fever, and septicemia (18). It is located on the activation pathway of alpha necrosis factor, binding itself to the interleukin 1 receptor (19). Being acute phase cytokines, IL-1a and TNF form the strongest synergy, their cumulative effects being uncontestable. These include radioprotection, Schwartzman's reaction, prostaglandin E2 synthesis, body mass loss, insulin resistance and many others (20). IL-6 acts both as a proinflammatory cytokine and an anti-inflammatory myokine. It has a role in the immune system, being secreted by T cells and macrophages during infections and after traumas (especially burns or inflammatory reactions) (21).

The aim of this study was to investigate the effects of dietary supplementation on the levels of IL-1a, IL-6 and TNFa expression, after prenatal exposure to nicotine in a sample of rats.

## **Materials and methods**

### ***Study groups***

We conducted a quasi-experimental, cross-sectional and comparative study. A total of 30 Wistar rat fetuses were included in the sample. The female pregnant rats were primiparous, 16 weeks old, with an average weight of 180-200g. All rats were taken from Iuliu Hatieganu University of Pharmacy and Medicine, Cluj-Napoca, after being kept in proper vivarium conditions, at the Physiology Biobase. The study was reviewed and approved by the Iuliu Hatieganu University of Pharmacy and Medicine Institutional Review Board (IRB 438/24.07.2016). The experiments were conducted in the Experimental Research Laboratory within the Physiology Department, from the same university.

Study groups (10 rats in each study group)

- Group I – Control group;
- Group II – Nicotine exposure for 50 days;

- Group III – Nicotine exposure for 50 days with dietary supplements
- Brain, placenta and lung homogenates.

### **Study technique**

Nicotine was administered by oropharyngeal gavage: 6 mg/kg of nicotine hydrogen tartrate per day (Glentham Life Science, UK), the equivalent of 2-3 packs of cigarettes for an adult weighting 60 kg (22). Dietary supplement Selevit (Pasteur Farmavet, Romania) were administered: 15 mg/kg/day (Recommended Daily Intake) oral veterinary solution with multivitamins, amino acids and trace elements. Female rats were exposed for 50 days: 20 days - the duration of full-term pregnancy, and 30 days - prenatal exposure which replicates chronic exposure (the equivalent of 1 year of pregnancy at humans).

Animals were prepared for gestation using the Ladosi method, adapted for rodents: pregnant mare's serum gonadotropin (PMSG) – 600 UI for follicle stimulation, and chorionic gonadotropin (hCG) - 1500 IU to induce ovulation. The latter was administered 48 hours after PMSG administration. The fertilization occurred 24 hours after hCG administration. On day 20 of gestation, female rats were anesthetized with ketamine/xylazine (2:1) 0,1 ml/100g/body, and performed caesarean section to deliver the foetuses. After experimental procedures, the animals were euthanatized by an overdose of ketamine 10%.

### **Quantitative analysis of cytokines**

IL-1a, IL-6 and TNFa were assayed from brain, placenta and lung homogenates, using Omnikine Rat Elisa kit (Assay bioTech). The kits contain specific monoclonal rat antibodies precoated onto 96 well plates. After collecting the tissues, the samples were pipetted into wells. As a result, every molecule of IL-1a, IL-6, and TNFa was bound and immobilized by the antibody. The cells were then washed to remove any unbound molecules that could interfere with the binding of reactants. An enzyme-linked polyclonal antibody was added to each well. The cells were then washed again to remove any unbound material. After that, a substrate solution was added to each well so that the product obtained could turn

from blue to yellow upon addition of stop solution. The color intensity is proportional to the quantity of IL-1a, IL-6 and TNFa bound in the initial step. Finally, the resulting samples were analyzed using colorimetry at a wavelength of 450 nm, resulting in IL-1 alpha, IL—6 alpha, and TNF alpha, expressed in  $\mu\text{g} / \text{ml}$ .

### **Data analysis**

We calculated elements of descriptive statistics and the data were presented using indicators of centrality, location and distribution. The Shapiro-Wilk test was used to assess the normal distribution of the data. Student's t-test was used for normally distributed variables, and the Mann-Whitney (U) test for non-parametric data, to compare differences between two independent samples. ANOVA was used to compare three or more samples for normally distributed data, and Kruskal-Wallis for non-parametric data.

We set the level of significance at  $\alpha = 0,05$  (5%),  $\alpha = 0,01$  (1%) or  $\alpha = 0,001$ , as follows:

- $0,01 < p < 0,05$  – statistically significant difference;
- $0,001 < p < 0,01$ – statistically very significant;
- $p < 0,001$ – statistically highly significant;
- $p > 0,05$  – statistical difference not significant.

Pearson's correlation coefficient was used to assess relationship between continuous variables with normal distribution, and Spearman ( $\hat{r}$ ) for non-normal distribution. The analysis of correlation between coefficients was performed using Colton's rule. Correlation coefficients can range from -1.00 to +1.00, the intensity of the association being stronger if the coefficient is close to 1. The following rules, given by Colton (1974), were used to interpret the data:

- little or no correlation  $r \in [-0,25, +0,25]^*$
- fair correlation  $r \in (+0,25, +0,5] \cup [-0,5, -0,25]^{**}$
- moderate to good correlation  $r \in (+0,5, +0,75] \cup [-0,75, -0,5]^{***}$
- very good to excellent correlation  $r \in (+0,75, +1] \cup [-1, -0,75]^{****}$

**Table 1** - Comparative analysis of IL-1a, IL-6 and TNFα values in brain homogenates (µg/ml) within study groups

Indicator	Group	Mean	SE	Median	SD	Min.	Max.	P value (p)		
IL-1α	I	4,05	0,2975	4,02	0,9409	2,94	5,88	I, II, III	I-II	< 0,0001
	II	43,80	2,0286	40,92	6,4148	38,43	59,10	< 0,0001	I-III	6,52 x 10 <sup>-6</sup>
	III	16,14	1,3822	15,00	4,3709	11,12	23,44		II-III	< 0,0001
IL-6	I	11,88	0,2541	12,12	0,8034	10,25	12,94	I, II, III	I-II	2,68 x 10 <sup>-9</sup>
	II	27,33	0,8614	28,16	2,7240	22,15	30,43	1,45 x 10 <sup>-6</sup>	I-III	0,0053
	III	21,71	2,6734	20,21	8,4540	11,60	38,15		II-III	0,0706
TNFα	I	1,40	0,0915	1,45	0,2892	0,95	1,83	I, II, III	I-II	2,8 x 10 <sup>-9</sup>
	II	17,05	0,6785	16,96	2,1456	13,15	20,14	5,23 x 10 <sup>-26</sup>	I-III	9,19 x 10 <sup>-17</sup>
	III	28,73	0,3147	28,95	0,9952	26,49	29,91		II-III	8,34 x 10 <sup>-10</sup>

Polynomial regression was used to determine the mathematical equation to predict the response of a dependent variable based on an independent variable. Data were processed and analyzed using StatsDirect v.2.7.2, OpenEpi 3.03, and Excel (Microsoft Office 2010). The graphical representation of the data was obtained using Excel (Microsoft Office 2010).

## Results

### Brain homogenate

Intense statistically significant differences were recorded for at least two study groups ( $p < 0,001$ ) when comparing interleukin 1 (IL-1Laa) values. The independent samples tests yielded statistically significant differences observed between groups I-II, I-III and II-III ( $p < 0,001$ ).

When comparing interleukin 6 (IL-6) values intense statistically significant differences were also recorded for at least two study groups ( $p < 0,001$ ). The independent samples tests yielded statistically

significant differences observed between groups I-II ( $p < 0,001$ ) and I-III ( $p < 0,01$ ).

For TNFα intense statistically significant differences were also recorded for at least two study groups ( $p < 0,001$ ) when taking into account all study groups. The independent samples tests yielded statistically significant differences observed between groups I-II, I-III și II-III ( $p < 0,001$ ).

### Placenta homogenate

Intense statistically significant differences were recorded for at least two study groups ( $p < 0,001$ ) when comparing interleukin 1 (IL-1Laa) values. The independent samples tests yielded statistically significant differences observed between groups I-II ( $p < 0,001$ ), I-III and II-III ( $p < 0,05$ ).

When comparing interleukin 6 (IL-6) values intense statistically significant differences were also recorded for at least two study groups ( $p < 0,001$ ).

The independent samples tests yielded statistically significant differences observed between groups I-II and II-III ( $p < 0,001$ ).

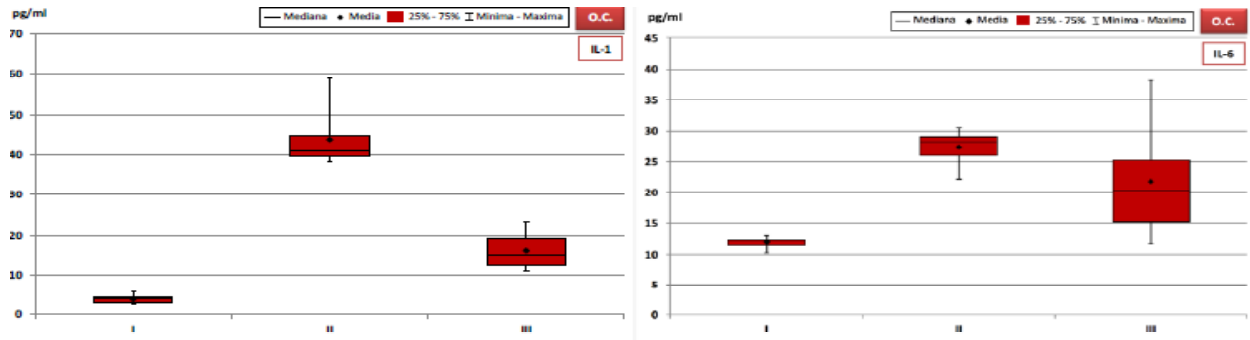


Fig 1 - IL-1a and IL-6 descriptive values in brain homogenate

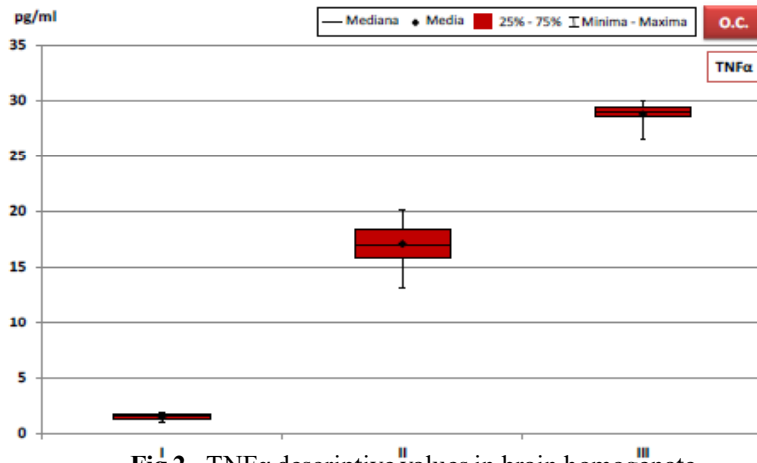


Fig 2 - TNFα descriptive values in brain homogenate

Table 2 - Comparative analysis of IL-1a, IL-6 and TNFα values in placenta homogenates (µg/ml) within study groups

Indicator	Group	Mean	SE	Median	SD	Min.	Max.	P value (p)					
IL-1α	I	2,51	0,3389	2,37	1,0716	1,24	4,63	I, II, III	I-II	0,0003			
	II	7,15	0,8534	7,03	2,6986	3,47	12,74				0,0001	I-III	0,0282
	III	4,30	0,6484	3,91	2,0505	1,90	8,61						
IL-6	I	4,16	0,3604	4,33	1,1398	2,65	5,51	I, II, III	I-II	< 0,0001			
	II	12,51	1,6135	11,27	5,1022	7,43	24,22				< 0,0001	I-III	0,1038
	III	5,00	0,3290	5,29	1,0404	3,25	6,13						
TNFα	I	3,00	0,4016	2,76	1,2700	1,24	5,36	I, II, III	I-II	0,0003			
	II	1,32	0,1205	1,11	0,3810	0,98	2,11				0,0019	I-III	0,0373
	III	1,92	0,2506	2,07	0,7925	0,99	3,22						

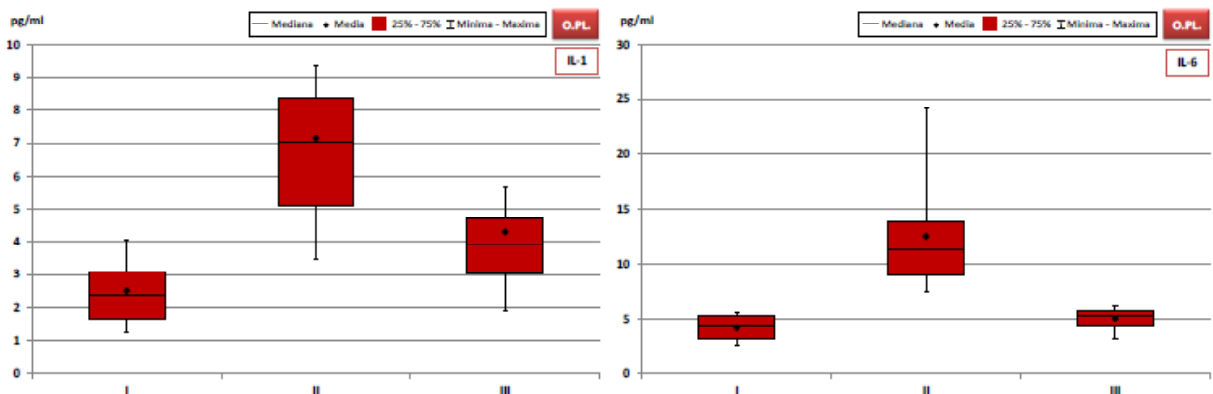


Fig 3 – IL-1a and IL-6 descriptive values in placenta homogenate

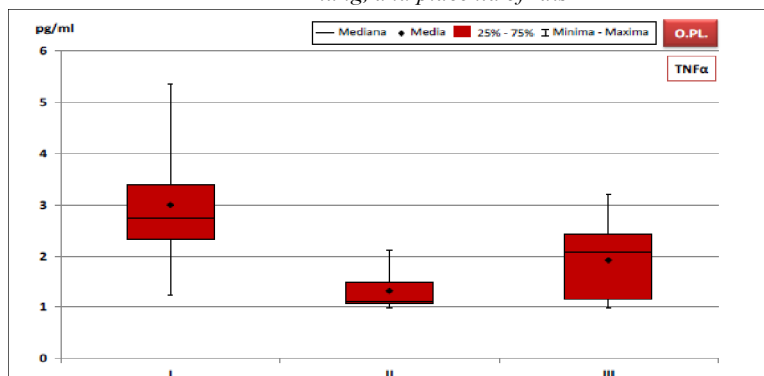


Fig 4 - TNF $\alpha$  descriptive values in placenta homogenate

Table 3 – Comparative analysis of IL-1 $\alpha$ , IL-6 and TNF $\alpha$  values in lung homogenates ( $\mu$ g/ml) within study groups

Indicator	Group	Mean	SE	Median	SD	Min.	Max.	P value (p)		
IL-1 $\alpha$	I	2,88	0,2141	2,83	0,6769	2,09	4,03	< 0,0001	I-II	0,0009
	II	10,76	1,6001	11,64	5,0600	2,36	16,99		I-III	< 0,0001
	III	16,58	1,9700	15,37	6,2298	11,22	31,97		II-III	0,0524
IL-6	I	5,30	0,9379	4,38	2,9658	2,22	10,99	0,0002	I-II	0,0003
	II	14,62	1,3852	16,10	4,3804	3,79	17,85		I-III	0,0005
	III	10,90	0,8215	11,16	2,5978	6,85	14,24		II-III	0,0115
TNF $\alpha$	I	2,88	0,4818	2,55	1,5234	1,12	6,16	4,18 x 10 <sup>-11</sup>	I-II	3,26 x 10 <sup>-9</sup>
	II	9,61	0,4063	9,40	1,2848	7,82	11,41		I-III	0,0002
	III	5,70	0,3508	5,66	1,1094	4,21	7,50		II-III	9,05 x 10 <sup>-7</sup>

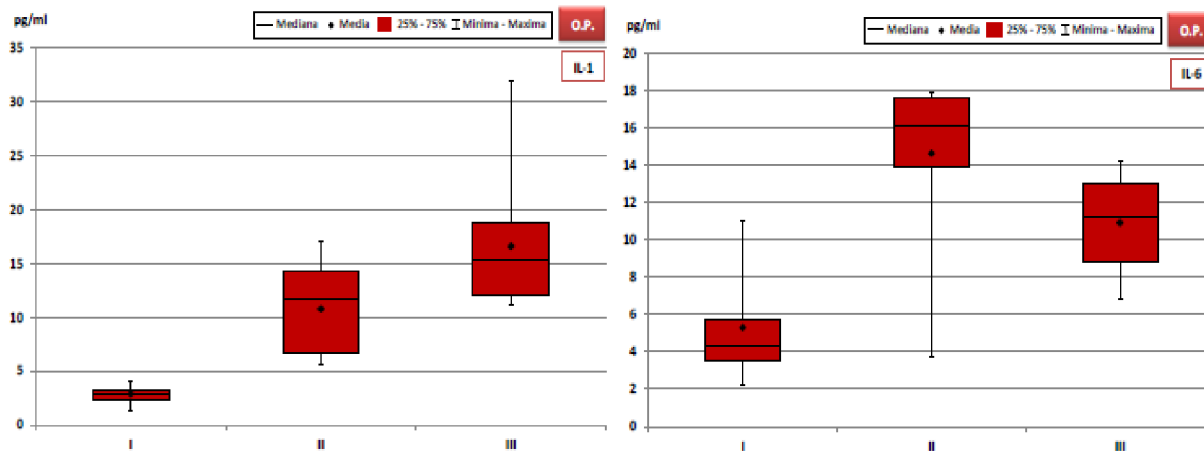


Fig 5 - IL-1 $\alpha$  and IL-6 descriptive values in lung homogenate

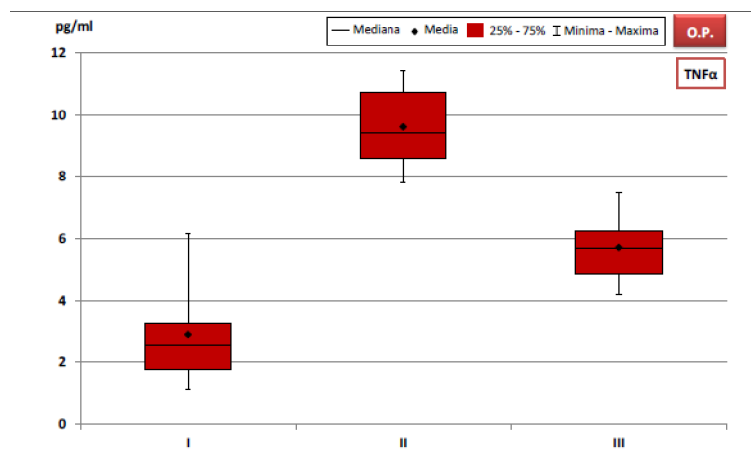


Fig 6 - TNF $\alpha$  descriptive values in lung homogenate

For TNF $\alpha$  intense statistically significant differences were also recorded for at least two study groups ( $p < 0,001$ ) when taking into account all study groups. The independent samples tests yielded statistically significant differences observed between groups I-II ( $p < 0,001$ ) and I-III ( $p < 0,05$ ).

### ***Lung homogenate***

Intense statistically significant differences were recorded for at least two study groups ( $p < 0,001$ ) when comparing interleukin 1 (IL-1Laa) values. The independent samples tests yielded statistically significant differences observed between groups I-II and I-III ( $p < 0,001$ ).

When comparing interleukin 6 (IL-6) values intense statistically significant differences were also recorded for at least two study groups ( $p < 0,001$ ). The independent samples tests yielded statistically significant differences observed between groups I-II, I-III ( $p < 0,001$ ) and II-III ( $p < 0,05$ ).

For TNF $\alpha$  intense statistically significant differences were also recorded for at least two study groups ( $p < 0,001$ ) when taking into account all study groups. The independent samples tests yielded statistically significant differences observed between groups I-II, I-III and II-III ( $p < 0,001$ ).

## **Discussion**

The aim of this study was to explore the effects of Se and vitamin E supplementation after nicotine exposure before and during pregnancy, by measuring IL-1 $\alpha$ , IL-6 and TNF $\alpha$  in the placenta, brain, and lung homogenates. A strong effect observed in this study was the impact of nicotine on the sample included in the study. Nicotine administration resulted in statistically significant increase in cytokine values in 11 out of 12 cases. This finding supports previous studies that have analyzed the association between nicotine exposure during pregnancy and the negative effects described before (23), stressing the importance of IL-1 $\alpha$ , IL-6 and TNF $\alpha$  influence in pathogenesis (24).

The difference between the nicotine exposure group and the control group, supports the testing of the main hypothesis, focusing on the role

of dietary supplements before pregnancy. A strong result of this study was observed for IL-6:

An important result of the study was observed for IL-6: in all observations ( $n=9$ ), Se and VE supplementation recorded lower protein values in comparison with lot II of exposure. Analyzing this increase in relationship with the control group, we assert that supplementation may be associated with mitigation of nicotine exposure effects.

This result (although limited empirical evidence is presented in this stage) could bring up additional research hypothesis to determine the specific mechanisms through which Se and vitamin E interact with nicotine exposure. This study complements existing literature on the benefits of Se therapy (25,26), by discussing the implications of Se on pregnancy after prenatal exposure to nicotine (7).

Dietary supplementation could be a cost-effective measure to address an important public health issue - smoking during pregnancy, with positive effects that have already been discussed in the literature (8). It would be interesting to analyze the effects of this synergy on cytokines expression during nicotine exposure. However, several studies (9,26,27) recommend caution in what concerns excessive supplementation, and careful monitoring of Se levels not to impact the oxidative processes of the host body. Moreover, longitudinal studies are needed to establish the long-term effect of Se and vitamin E therapy.

Both IL-1 $\alpha$  and TNF $\alpha$  recorded amelioration and amplification values following supplementation. A possible explanation for these increases could be the administration of vitamin E. One study that analyzed the response of vitamin E supplementation on cytokines (28), identified that this response is strictly dependent on the individual's values before supplementation. Therefore, it is possible that vitamin E has influenced IL-1 $\alpha$  and TNF $\alpha$  values in some cases, due to the limited statistical power of the model and the impossibility to discuss cause-effect.

In what concerns the brain, literature discusses the ability of nicotine to inhibit TNF $\alpha$  neuroprotection, but not of IL-1 and IL-6 (29). TNF $\alpha$  recorded increased values after supplementation, but IL-1 $\alpha$  and IL-6 followed the model of amelioration described above.

All study groups recorded increased levels of cytokines after nicotine exposure. Perinatal administration of Se and vitamin E was consistently associated with a low level of IL-6 compared to the control and exposure group, on every analyzed sample (brain, lung, and placenta homogenate). Interactions among cytokines, nicotine, supplements, and the body are highly complex and must be taken into account when interpreting the results. Further studies are necessary to determine causal implication of the interaction among Se, vitamin E and nicotine.

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