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# CAFFEIC ACID FEEDING OF PREGNANT AND LACTATING MICE INFLUENCES THE IMMUNE RESPONSE OF THEIR PROGENY

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We have previously shown that some commonly consumed substances, present in the average human diet, might cross over the placental barrier and affect the development of embryo immune system. The aim of the present study was to examine the cellular and humoral immune response in the group of offsprings from mothers treated with caffeic acid (1 or 6 mg/day) during pregnancy and lactation. We observed that the mean spleen relative weight was reduced in 4-week old progeny of mice treated with 6 mg/day of caffeic acid. The percentage of spleen $CD3^+$ lymphocytes was decreased in both examined doses, while the number of $CD19^+$ lymphocytes was not affected. Local G-v-H reaction induced by splenocytes from 4-week old progeny of caffeic acid-treated mothers was significantly suppressed in both dosage groups. Caffeic acid feeding (6 mg) resulted in up-regulation of the peripheral blood granulocytes chemiluminescent activity as well as in the enhancement of anti-SRBC antibody production in the 6-week old progeny of the treated mothers. We concluded that caffeic acid-enriched mothers' diet may affect progeny immune system function in the postnatal period.

## INTRODUCTION

The everyday diet is rich in the plant-derived polyphenolic substances. They are also present in the commonly consumed beverages like tea, coffee, chocolate and cocoa. Average human diet contains *ca.* 1 g of phenolic acids. Our previous studies have proven that some substances, normally present in the nutritional products, when administered to pregnant mice might disturb the embryonic development of their progeny by influencing the angiogenesis process. Accordingly, the reduction of limbs and thigh bones relative length was observed in the progeny of theobromine- and chocolate-treated mothers [Skopińska-Różewska *et al.*, 2003; Skopiński *et al.*, 2003; Chorostowska-Wynimko *et al.*, 2004]. Plant products might also modulate immune response. We have reported on the influence of plant-derived phenolic acids on the cellular and humoral response generated by the mouse immune system [Sokolnicka *et al.*, 1994; Glinkowska *et al.*, 1997; Barcz *et al.*, 1998].

The aim of the present study was to evaluate the influence of caffeic acid, a polyphenol, administered to pregnant and afterwards lactating Balb/c mice on the immune response of their progeny.

## MATERIAL AND METHODS

Animals were handled according to the Polish law on the protection of animals and NIH standards. All experiments were accepted and supervised by the local Ethical Committee.

The study was performed on 2-month old inbred Balb/c mice, weighing 20 g, fed during pregnancy and lactation 1 or 6 mg/day of caffeic acid (Sigma Aldrich) served with wheat flakes. These doses, after calculation taking into account differences in body size to body weight ratios between man and mouse, corresponded to 0.5 or 3 g administered to a woman weighing about 70 kg. Control mice were fed wheat flakes only.

A group of progeny was sacrificed at the 4[th] week after birth. The peripheral blood from retro-orbital plexus was collected to define chemiluminescence activity of granulocytes, afterwards the total body and spleen weight were measured and results expressed as a relative weight (*i.e.* spleen weight/total body weight). Next, the splenocytes were isolated, the percentage of $CD3^+$ and $CD19^+$ was evaluated by means of immunocytochemistry, while the activity of spleen lymphocytes was estimated in the local graft-versus-host (GvH) reaction.

**Granulocyte chemiluminescence assay.** The granulocyte chemiluminescence test was performed with blood samples taken from control and caffeic acid-fed mothers progeny. Briefly, 0.05 mL of heparinized peripheral blood diluted four times with phosphate buffered saline (PBS, Biomed, Poland) supplemented with 0.1% bovine serum albumin (BSA, Sigma-Aldrich Co. USA) and 0.1% glucose (Polfa,

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Poland) was added to 0.2 mL of luminol ($10^{-5}$ mol/L solution in PBS), (Sigma-Aldrich Co. USA). The background chemiluminescence (CL) was measured at the scintillation counter (RackBeta 1218, Sweden) in the "out of coincidence" mode. Next, cells were activated with 0.02 mL of zymosan (10 mg/mL solution in PBS) and CL activity was measured for the next 15min. The results were expressed as a cpm (count per minute) per $10^3$ granulocytes.

**The local graft-versus-host reaction (GvH).** Local GvH reaction (lymphocyte-induced angiogenesis, LIA test) was performed according to Sidky and Auerbach [1975], with some modifications [Kamiński *et al.*, 1998]. Briefly, splenocytes ($5\times10^5$ cells/injection) were grafted intradermally into anaesthetised F1 crossbreeds Balb/cxC3H mice. After 72 h, animals were sacrificed, new blood vessels were identified and counted in the dissection microscope (6x magnification) in the central 1/3 of the microscopic field.

**Splenocytes phenotyping.** Spleen lymphocytes smears were fixed in acetone. Splenocytes phenotypes ($CD3^+$, $CD19^+$) were determined by immunocytochemistry method with monoclonal antibodies (Serotec) and DAKO APAAP KIT System 40 (DAKO, USA) according to the manufacturer's instructions.

Another group of progeny at the 6th week after birth was intraperitoneally immunized with 0.2 mL of 10% sheep red blood cells (SRBC, Biomed, Poland) suspension. After 7 days the peripheral blood from retro-orbital plexus was collected, sera were isolated and the level of hemagglutinating antibodies was assessed .

**Hemagglutination test.** Hemagglutination test was performed according to Adler [1965] with some modifications [Sokolnicka *et al.*, 1994]. Mice sera were inactivated (56°C, 30 min) and their serial dilutions (from 1:1 to 1:2048) were performed. Afterwards, 0.5% SRBC was added, samples were incubated for 1 h at room temperature, centrifuged (10', 150 × g), and vigorously shacked. Haemagglutination was evaluated in the optical microscope – as the last dilution with at least 3 cells conglomerates present in at least 3 consecutive fields. Results were expressed as logarithms of antibody titer.

**Statistical analysis.** The statistical significance of differences of the experimental groups in comparison to control animals was tested at the p=0.05 level using Student's *t*-test and Mann-Whitney U test.

## RESULTS

The chemiluminescent activity of peripheral blood granulocytes was elevated in progeny group of caffeic acid-treated mothers (dose 6 mg/day) and the difference was close to significance (0.05<p>0.1) (Table 1). Local GvH reaction induced by splenocytes from 4-week old progeny of mice treated with caffeic acid during pregnancy and lactation was significantly suppressed in both caffeic acid groups (p<0.05) (Table 2). The spleen mean relative weight was reduced by the higher dose of caffeic acid (6 mg/day) (p<0.01) (Table 3). The percentage of $CD3^+$ (T lymphocytes) but not the $CD19^+$ (B lymphocytes) spleen cells was significantly decreased by both doses of caffeic acid (p<0.01). Consequently, the mean $CD3^+/CD19^+$ ratio was also lowered (Table 3). On the other hand, the anti-SRBC antibodies titers in the sera from both caffeic acid groups were notably higher than these in the control group (p<0.05) (Figure 1).

TABLE 1. The effect of caffeic acid administration to pregnant and lactating mice on the chemiluminescence activity of their 4 weeks-old progeny peripheral blood granulocytes.

| Caffeic acid (mg/day) | Number of mice | CLmax (cpm±SE) | Statistical significance of difference from control |
|---|---|---|---|
| 0 (control group) | 10 | 20865±3405 | – |
| 1 | 16 | 21343±2716 | n.s. |
| 6 | 23 | 30534±3705 | 0.05<p>0.1 |

TABLE 2. The effect of caffeic acid administration to pregnant and lactating mice on their 4-weeks old progeny splenocytes activity as assessed by the local graft versus host reaction (LIA test).

| Caffeic acid (mg/day) | Number of tests | Mean number of new blood vessels ± SE | Statistical significance of difference from control |
|---|---|---|---|
| 0 (control group) | 66 | 15.9±0.27 | – |
| 1 | 98 | 14.0±0.28 | p<0.05 |
| 6 | 90 | 14.3±0.31 | p<0.05 |

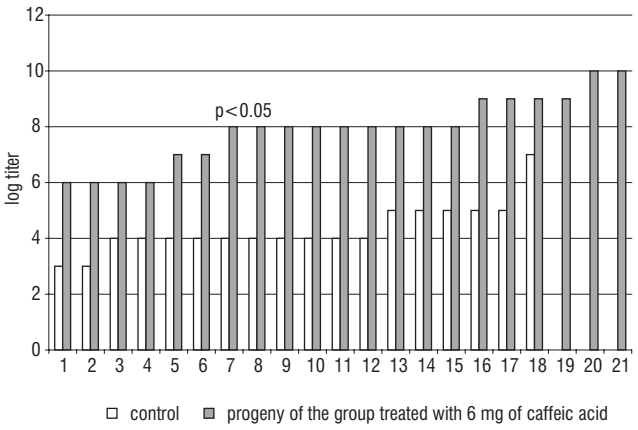


FIGURE 1. The effect of caffeic acid administration to pregnant and lactating mice on anti-SRBC antibody production by their 6-weeks old progeny.

TABLE 3. The effect of caffeic acid administration to pregnant and lactating mice on their 4-weeks old progeny spleen relative weight and the percentage of T and B spleen lymphocytes.

| Caffeic acid z (mg/day) | Spleens number | Mean spleen relative weight ± SE | Mean % of T lymphocytes $CD3^+$± SE | Mean % of B lymphocytes CD $19^+$± SE | Mean ratio of CD3/CD19±SE |
|---|---|---|---|---|---|
| 0 (control group) | 21 | 6.7±0.33 | 54.1±1.5 | 42.6±1.7 | 1.26±0.02 |
| 1 | 18 | 5.8±0.53 n.s. | 40.6±1 p<0.01 | 40.8±1.3 n.s. | 0.99±0.04 p<0.01 |
| 6 | 15 | 4.8±0.47 p<0.01 | 37.1±1.4 p<0.01 | 43.6±1.9 n.s. | 0.85±0.01 p<0.01 |

## DISCUSSION

Pregnant mouse is a good model to investigate the influence of various substances on the immune system of developing progeny. We previously used this model for evaluating the effect of some drugs (salbutamol, antibiotics, theobromine) on the lymphatic system and various parameters of immune response of adult progeny [Skopińska-Różewska *et al.*, 1985; Kamiński *et al.*, 1998, 2000; Chorostowska-Wynimko *et al.*, 2004].

There is no adequate data in the available literature concerning the influence of phenolic acids on the immune response in the progeny of pregnant and afterwards lactating mothers treated with these substances. Meanwhile, the caffeic acid evaluated in the present study exerted significant modulatory effect on both cellular and humoral response of the mouse immune system.

In the 4-week old offsprings the reduced spleen weight and decreased relative number of the $CD3^+$ spleen lymphocytes was observed. The $CD3^+/CD19^+$ ratio was affected as well due to the $CD3^+$ splenocytes decrease. This phenomenon might reflect the caffeic acid direct impact either on the immune system maturation or the lymphocytes distribution. The lymphocytes reactivity, as assessed by the *graft-versus-host* reaction was down-regulated, proving that both functional as well as quantitative alterations were present in the $CD3^+$ population. Besides, the reduction of alloantigen specific cellular response in the local G-v-H reaction demonstrated for both caffeic acid doses, might mirror the shift in the production of immune mediators by mice spleen cells. The evaluation of selectively induced proliferative activity in different lymphocytes subsets might prove interesting, providing more information concerning the functional activity of these cells.

On the contrary to the observed suppressive effect in the lymphocyte population, the chemiluminescence activity of blood granulocytes was elevated. This observation contradicts the results presented by Limasset *et al.* [1999], who demonstrated the inhibitory effect of some phenolic acids on the polymorphonuclear neutrophils luminol-dependent luminescence. However, this study was performed on the human PMNs and evaluated multiple monocyclic phenolic acids, but not the caffeic acid. Besides, the direct influence of this compound on the CL was examined, while in the present study the indirect effect due to the mothers feeding was evaluated.

As far as humoral response was concerned, the up-regulation of the antibodies production was eminent in caffeic acid group. Likewise, Sokolnicka *et al.* [1994] reported the modulatory effect of phenolic acids including caffeic acid on the antibody production in mice. However, the mechanism responsible for this effect still needs to be examined. Though the relative number of $CD19^+$ cells was not affected in the progeny of the treated mothers but the observed stimulation of antibodies production might reflect some functional shift in this cellular population.

## CONCLUSION

Multiple polyphenols are present in plant products and food included in an everyday diet. As it cannot be excluded that similar effects on the development of the immune system might be observed in humans, obviously some more studies should be conducted to explore this subject.

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