POLISH JOURNAL OF FOOD AND NUTRITION SCIENCES
Pol. J. Food Nutr. Sci. 2004, Vol. 13/54, SI 2, pp. 67–70

THE INFLUENCE OF CHOCOLATE FEEDING OF PREGNANT MICE ON THE IMMUNOLOGICAL RESPONSE OF THEIR PROGENY

Ewa Skopińska-Różewska[1,3], Barbara J. Bałan[1], Ewa Sommer[1], Joanna Chorostowska-Wynimko[1], Janusz Bany[2], Aleksander Wasiutyński[3], Andrzej K. Siwicki[3]

[1]Department of Laboratory Diagnostics and Immunology, National Institute of Tuberculosis and Lung Diseases, Warsaw, Poland; [2]Department of Pharmacology and Toxicology , Military Institute of Hygiene and Epidemiology, Warsaw, Poland; [3]Department of Pathology, Medical University of Warsaw, Warsaw, Poland; [4]Department of Microbiology and Clinical Immunology, Warmia and Mazury University in Olsztyn, Olsztyn, Poland

Key words: mice, pregnancy, chocolate feeding, progeny, immunity

Chocolate which contains flavonoids, a group of compounds which may exhibit biological activity, is made of *Theobroma cacao* beans. It is strongly suggested that certain dietary flavonoids may have the potential to reduce the risk of many diseases. Flavonoids have strong antioxidant potential, so they may help to maintain the cardiovascular health and can influence immune response. Polyphenols from cacao liquor can inhibit mitogen-induced proliferation of T cells and polyclonal Ig production by B cells *in vitro*. They also influence IL-2 secretion by T cells. Chocolate is used by people as a form of self-medication for dietary deficiencies (*e.g.* magnesium) or to balance low levels of neurotransmitters involved in the regulation of mood, food intake and compulsive behaviours. However, our previous research performed in mice showed that chocolate and substances contained in it have many unfavourable effects on organism during its development, *e.g.* they lower embryos mass and their tissues angiogenic activity. Moreover, such treatment was found to cause the shortening of the limbs in offspring of mice fed chocolate during pregnancy, and disturbed the process of bone mineralization. The aim of the present paper is to examine the possible influence of chocolate feeding of pregnant and lactating mice on the cellular and humoral immunological response of their six-week old progeny.

We have found, that a daily dose of 400 mg of chocolate given to pregnant and lactating mice resulted in the enhancement of humoral immunity and suppression of cell-mediated immunity of their 6 week-old progeny.

INTRODUCTION

Chocolate enjoys a very long history. It is thought, that the ancient civilizations, Olmeks, Mayas and Aztecs, living in Central America, first began to grow cacao trees, and produce chocolate drink from their beans. The drink made by them was considered to be magic, because it improved the mood, raised energy, protected against diseases or prevented them. Owing to its magic power it was drunk by rulers, and it lent religious solemnities. The Greek name of the cacao tree – *Theobroma cacao*, directly means the draught of Gods (*Theos* – God, *broma* – the drink). Cocoa beans first appeared in Europe in 1502. Christopher Columbus is attributed to be the discoverer of cocoa beans.

Hernando Cortez, a Spanish conquistador contributed to the spread of cacao trees plantations by bringing cocoa beans to Spain in 1519. Towards the end of the XVIth century chocolate conquered France and Italy and from there reached Holland and the other parts of Europe. The first hard bar of chocolate appeared in the XIXth century [Piotrowska 2004; Poskier, 2004].

Chocolate contains many different substances and chemical compounds essential to the human organism. Among them are proteins, carbohydrates, microelements such as calcium, magnesium, iron, and others as niacin. But first of all chocolate contains numerous alkaloids (theobromine, caffeine, theophylline), and flavonoids. The subclass of flavonoids are flavanols to which belong catechin, epicatechin, quercetin and epigallocatechin, the so-called monomeric flavan-3-ols, and corresponding to them oligomers procyanidins. Besides the chocolate and the cocoa, flavanols appear also in green tea, red wine and many fruits. However, chocolate contains most of all polyphenols that occur in all consumed food-products [Scalbert, 2004].

Catechines make one tenth of polyphenols found in the chocolate. The remaining nine tenth are tannins and proanthocyanins – polymers of high molecular weight, which are weakly absorbed from the alimentary tract. Procyanidins extracted from *Theobroma cacao* have antiviral (against HSV and HIV), and antibacterial properties. Flavanols have strong antioxidant potential. Their influence on the production of prostaglandins which are part of inflammatory reactions, modify the synthesis of the nitric oxide, decreases the aggregation of thrombocytes, and it is suggested that chocolate polyphenols may lower the risk of various diseases of the circulatory system, inflammatory diseases and cancer [Bałan, 2002; Natsume *et al.*, 2000; Nelson & Sharpless, 2003; Osakabe *et al.*, 2000; Rein *et al.*, 2000; Richelle *et al.*, 2001; Sanbongi *et al.*, 1998; Wang *et al.*, 2000]. Still, some authors described anti-angiogenic effects of

Author's address for correspondence: Prof. Ewa Skopińska-Różewska, Department of Laboratory Diagnostics and Immunology, National Institute of Tuberculosis and Lung Diseases, Płocka 26, 01-138 Warsaw, Poland; tel.: 0600479495; e-mail: ewaskop@hotmail.com or eskop@wp.pl

green tea catechins, some of these catechins are present also in cocoa [Cao & Cao, 1999; Kim, 2003; Lamy *et al.*, 2002; Ling *et al.*, 1997]. In our previous studies we observed that chocolate feeding of pregnant mice resulted in epigallocatechin-related embryonic angiogenesis suppression, and influenced development of offspring limbs and their bones mineralization [Skopiński *et al.*, 2003, 2004].

The aim of the present paper is to examine the possible influence of chocolate feeding of pregnant and lactating mice on the cellular and humoral immunological response of their six-week old progeny.

MATERIALS AND METHODS

Our experiments were performed on 2–3-month-old female Balb/c mice, fed during pregnancy and lactation with 400 mg of dark chocolate (Wedel) administered with wheat crisp in addition to standard laboratory chow. Control mothers were fed wheat crisp and standard chow. The progeny was sacrificed at the 6th week after birth. In this group, the spleens were collected, weighed, half of each spleen was fixed in 4% buffered formaldehyde and processed for microscopic analysis. From the second part of spleen, the lymphocytes were isolated and their activity was evaluated in the local graft-versus-host (GvH) reaction.

Some of 6-week-old mice were immunized *i.p.* with 0.2 mL of 10% sheep red blood cells (SRBC). Seven days after, mice were anaesthetized with chloral hydrate and the peripheral blood from retro-orbital plexus has been collected. Serum was isolated and frozen at -20°C. Animals were handled according to the Polish law on the protection of animals and NIH standards. All experiments were accepted by the local Ethical Committee.

In the haemagglutination assay the anti-SRBC titre was evaluated. Sera were inactivated at 56°C for 30 min, serially diluted, and supplemented with 0.5% SRBC. After 60-min incubation at room temperature, the samples were centrifuged for 10 min at 150 × g, and vigorously mixed.

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 the mean antibodies titre in the treated group and in the controls.

The local graft-versus-host reaction (GvH) test (lymphocyte-induced angiogenesis, LIA test) was performed according to Sidky and Auerbach [1975] with some modifications [Skopińska-Różewska *et al.*, 2002]. Briefly, splenocytes at a concentration of 1×10^6 cells/injection were grafted intradermally into anaesthetized F_1 (Balb/c × C_3H) crossbreeds mice. After 72 h, the mice were sacrified and the newly-formed blood vessels were identified and counted in the dissection microscope at 6 × magnification, in central 1/3 of microscopic field.

The statistical significance of difference from the control was studied by Student t and Mann-Whitney tests.

RESULTS

Chocolate feeding of mice during pregnancy and lactation period resulted in profound changes in the immunological response of their progeny. Antibody production was highly significantly enhanced, and lymphocyte activity in local graft – versus host response was highly significantly suppressed (Tables 1 and 2).

No differences in relative spleen weight (6.6±0.33 for control spleens and 6.7±0.35 for spleens of progeny of chocolate-fed mice) and in microscopic picture of spleen tissues were seen between experimental and control groups.

TABLE 1. The effect of chocolate feeding of pregnant Balb/c mice on the anti-SRBC antibodies production of their 6-weeks old offspring.

Mean log antibody titre ±SE	Mean log antibody titre ±SE
Control	Chocolate 400 mg daily
4.2±0.11	5.1±0.22***
n=28	n=26

***p<0.001

TABLE 2. The effect of chocolate feeding of pregnant Balb/c mice on the GvH activity of their 6 weeks-old offspring splenic lymphocytes in LIA test.

	Mean number of blood vessels ± SE Control	Mean number of blood vessels± SE Chocolate 400 mg daily
First experiment	14.5±0.26	11.9±0.19***
	n=26	n=24
Second experiment	13.3±0.3	11.8±0.24***
	n=28	n=29

*** p<0.001

DISCUSSION

Chocolate contains flavonoids, a group of compounds, which may exhibit biological activity. It is strongly suggested that certain dietary flavonoids may have the potential to reduce the risk of various diseases. Flavonoids have strong antioxidant potential, so they may help to maintain the cardiovascular health and can influence immune response [Osakabe *et al.*, 2000, 2002]. Polyphenols from cacao liquor can inhibit mitogen-induced proliferation of T cells and polyclonal Ig production by B cells *in vitro*. This effect is dose-dependent. They also can inhibit IL-2 secretion by T cells [Sanbongi *et al.*, 1997]. Chocolate appears to support heart health also by positively affecting the way some blood components function, especially platelets [Rein *et al.*, 2000]. Chocolate is used by people as a form of self-medication for dietary deficiencies (*e.g.* magnesium) or to balance low levels of neurotransmitters involved in the regulation of mood, food intake and compulsive behaviours (*e.g.* serotonin and dopamine) [Bruinsma & Taren, 1999]. However, it is known that dopamine at non-toxic levels, strongly and selectively inhibits the vascular permeabilizing and angiogenic activities of VEGF, the important cytokine of embryonic angiogenesis. Dopamine acts through D2 dopamine receptors to induce endocytosis of VEGF receptor 2. This receptor is critical for promoting angiogenesis. The action of dopamine is specific for VEGF and do not affect other mediators of microvascular permeability or endothelial cell proliferation and migration [Basu *et al.*, 2001]. Also such flavonoids as catechin and epicatechin contained in chocolate may exert antiangiogenic activity through inhibition of IL-8 production, or/and because of the inhibition of growth, migration and tube formation of endothelial cells [Tang & Meydani, 2001].

Chocolate contains high levels of monomeric, oligomeric, and polymeric catechins. Plasma concentrations of epicatechin and also theobromine are increased markedly after chocolate consumption. Plasma level of this substances reaches the maximum 2–3 h after consumption of chocolate and is highly correlated with the dose of chocolate [Richelle *et al.*, 1999].

Pregnant mouse is a very good model to investigate the influence of various drugs, xenobiotics and products of natural origin on the immunological response and the immune system of her progeny. This practical model was used by us many times previously [Skopińska-Różewska *et al.*, 1996, 2003; Kamiński *et al.*, 1998, 2000]. Our previous studies have demonstrated the inhibitory action of chocolate and theobromine on tumour and embryonic angiogenesis. We have observed significant inhibition of embryos growth and also the effect of shortening of limbs and bones in 4-week-old offspring of female mice fed chocolate during pregnancy and lactation. In other study we have observed that chocolate given to mice during pregnancy may disturb the processes of bone mineralization in offspring. 400 mg of chocolate given to pregnant mice has significantly suppressed angiogenic activity of 18-days embryos tissues and this effect was correlated with epigallocatechin concentration [Skopiński *et al.*, 2003, 2004].

Except for flavonoids, chocolate contains some methyloxantines (theobromine, caffeine, *etc.*). There is proved with no doubt the strong immunomodulatory and the anti-inflammatory activity of methyloxantines. Their activity is exerted through the activation and function of various haematopoiethic cells.

From our previous research it results also that both the theophylline and the theobromine significantly inhibit the ability of mononuclear blood cells of healthy donors, patients suffering from diabetic retinopathy or rheumatoid arthritis, as well as of the cells isolated from various human cancers, to the induction of neovascularisation in mice cutaneous test. We also observed some effects of theobromine on the fetus development and the postnatal status of the immune system [Chorostowska *et al.*, 1995, 2004; Skopiński *et al.*, 1998; Skopińska-Różewska & Skopiński, 2001]. We suppose that immunological abnormalities observed by us in the present work are partly connected with the influence of theobromine and partly with catechins present in chocolate.

CONCLUSION

As far as eating of chocolate, for its wholesome value is advisable for men and not pregnant women, insomuch pregnant women and children should themselves beware for the possibility of appearing unwanted side effects of eating chocolate.

ACKNOWLEDGEMENTS

The study was supported by grant 3PO5EO372 of the State Committee for Scientific Research.

REFERENCES

1. Bałan B.J., Działanie przeciw-drobnoustrojowe i przeciwzapalne substancji pochodzenia naturalnego. 2002, *w*: Wpływ substancji naturalnych na układ odpornościowy (ed. E. Skopińska-Różewska). Fundacja Pomocy Zdrowiu-Medycyna Naturalna, Warszawa, pp. 111–129 (in Polish).
2. Basu S., Nagy J.A., Pal S., Vasile E., Eckelhoefer I.A., Bliss V.S., Manseau E.J., Dasgupta P.S., Dvorak H.F., Mukhopadhyay D., The neurotransmitter dopamine inhibits angiogenesis induced by vascular permeability factor vascular endothelial growth factor. Nat. Med., 2001, 7, 569–574.
3. Bruinsma K, Taren D.L., Chocolate: food or drug? J. Am. Diet. Assoc., 1999, 99, 1249–1256.
4. Cao Y., Cao R., Angiogenesis inhibited by drinking tea. Nature, 1999, 398, 381–383.
5. Chorostowska-Wynimko J., Skopińska-Różewska E., Sommer E., Bałan B.J., Skopiński P., Demkow U., Żukowska M., Gil M., Some immunotropic effects of theophylline and theobromine. Eur. Resp. J., 1995, 8, suppl. 19, PO315.
6. Chorostowska-Wynimko J., Skopińska-Różewska E., Sommer E., Rogala E., Skopiński P., Wojtasik E., The multiple effects of theobromine on the fetus development and postnatal status of the immune system. Int. J. Tissue Reactions, 2004, 26, in press.
7. Kamiński P., Skopińska-Różewska E., Wąsik M., Bany J., Barcz E., Sommer E., Marianowski L., Thymus and lymph node cell CD4 and CD8 marker expression and their angiogenic activity of offspring of salbutamol-treated pregnant C3H mice. Pharmacol. Res., 2000, 41, 95–99.
8. Kamiński P., Skopińska-Różewska E., Bany J., Wąsik M., Sokolnicka I., Barcz E., Rogala E., Sommer E., Brajczewska-Fisher W., Filewska M., Bałan B.J., Skopiński P., Marianowski L., The effect of salbutamol treatment on the cellular immunity of the offspring of pregnant mice: spleen cell activity. Drugs Exptl. Clin. Res., 1998, 24, 77–83.
9. Kim M.H., Flavonoids inhibit VEGF/bFGF-induced angiogenesis *in vitro* by inhibiting the matrix-degrading proteases. J. Cell. Biochem., 2003, 89, 529–538.
10. Lamy S., Gingras D., Beliveau R, Green tea catechins inhibit vascular endothelial growth factor receptor phosphorylation. Cancer Res., 2002, 15, 62, 381–385.
11. Ling Y.C., Lin-siau S.Y., Chen C.F., Lin J.K., Suppression of extracellular signals and cell proliferation through EGF receptor binding by (-)-epigallocatechin gallate in human A431 epidermoid carcinoma cells. J. Cell. Biochem., 1997, 67, 55–65.
12. Natsume M., Osakabe N., Yamagishi M., Takizawa T., Nakamura T., Miyatake H., Hatano T., Yoshida T., Analyses of polyphenols in cocoa, and chocolate normal-phase and reversed-phase HPLC. Biosci. Biotechnol. Biochem., 200,64, 2581–2587.
13. Nelson B.C., Sharpless K.E., Quantification of the predominant monomeric catechins in black chocolate standard reference material by LC/APCI-MS. J. Agric. Food Chem,. 2003, 51, 531–537.
14. Osakabe N., Baba S., Yasuda A., Iwamoto T., Kamiyama M., Takizawa T., Itakura H., Kondo K., Daily cocoa intake reduces the susceptibility of low density lipoprotein to oxidation as demonstrated in healthy human volunteers. Free Radical Res., 2000, 34, 93–99.

15. Osakabe N., Yasuda A., Natsume M., Takizawa T., Terao J., Kondo K., Catechins and their oligomers linked by C4-C8 bonds are major cacao polyphenols and protect low-density lipoprotein from oxidation *in vitro*. Exp. Biol. Med., 2002, 227, 51–56.
16. Piotrowska A., Boska czekolada. Newsweek, 2004 [www.google.pl/search].
17. Poskier E., Czekolada dobra na wszystko, 2004 [www.nsik.com.pl/archiwum].
18. Rein D., Paglieroni T.G., Wun T., Pearson D.A., Schmitz H.H., Gosselin R., Keen C.L., Cocoa inhibits platelet activation and function. Am. J. Clin. Nutr., 2000, 72, 30–35.
19. Richelle M., Tavazzi I., Offord E.A., Comparison of the antioxidant activity of commonly consumed polyphenolic beverages (coffee, cocoa and tea) prepared per cup serving. J. Agric. Food Chem., 2001, 49, 3438–3442.
20. Richelle M., Tavazzi I., Enslen M., Offord E.A., Plasma kinetics in man of epicatechin from black chocolate. Eur. J. Clin. Nutr., 1999, 53, 22–26.
21. Sanbongi C., Osakabe N., Natsume M., Takizawa T., Gomi S., Osawa T., Antioxidative polyphenols isolated from *Theobroma cacao*. J. Agric. Food Chem., 1998, 46, 454–457.
22. Sanbongi C., Suzuki N., Sakane T., Polyphenols in chocolate, which have antioxidant activity modulate immune functions in humans *in vitro*. Cellular Immunology, 1997, 177, 129–136.
23. Scalbert A., Chocolate, health and polyphenols, 2004 [www.inra.fr/PRSSE/nov03/gb/nb1.htm].
24. Skopińska-Różewska E., Janik P., Przybyszewska M., Sommer E., Białas-Chromiec B., Inhibitory effect of theobromine on induction of angiogenesis and VEGF mRNA expression in v-raf transfectants of human urothelial cells HCV-29. Int. J. Mol. Med., 1998,2, 649–652.
25. Sidky Y., Auerbach R., Lymphocyte-induced angiogenesis, a quantitative and sensitive assay of the graft-versus--host reaction. J. Exp. Med., 1975, 141, 1084–1093.
26. Skopińska-Różewska E., Furmanowa M., Guzewska J., Sokolnicka I., Sommer E., Bany J., The effect of *Centella asiatica*, *Echinacea Purpurea* and *Melaleuca alternifolia* on cellular immunity in mice. Centr. Eur. J. Immunol., 2002, 27, 142–148.
27. Skopińska-Różewska E., Bany J., Demkow U., Chorostowska-Wynimko J., Rogala E., Barcz E., Różycka B., Filewska M., Kamiński P., Sokolnicka I., Biological monitoring of the antibiotics toxicity on the model of pregnant mice. 1996. *w*: Biologiczne monitorowanie skażenia środowiska (ed. A.K. Siwicki). Instytutu Rybactwa Śródlądowego, Olsztyn, pp. 47–53 (in Polish).
28. Skopińska-Różewska E., Chorostowska-Wynimko J., Sommer E., Rogala E., Demkow U., Filewska M., Radomska-Leśniewska D., Skurzak H., Bany J., Siwicki A.K., Wpływ diety wzbogaconej w czekoladę na angiogenezę embrionalną i nowotworową u myszy. 2003. *w*: Rola immunomodulatorów pochodzenia naturalnego w zapobieganiu i leczeniu chorób (eds. E. Skopińska-Różewska, A.K. Siwicki). MEDYK, Warszawa, pp. 245–250 (in Polish).
29. Skopińska-Różewska E., Skopiński P., Modulacja procesów angiogenezy – nowy kierunek w immunologii klinicznej. Terapia 2001, 9, 3, 3–6 (in Polish).
30. Skopiński P., Skopińska-Różewska E., Sommer E., Chorostowska-Wynimko J., Rogala E., Cendrowska I., Chrystowska D., Filewska M., Białas-Chromiec B., Bany J., Chocolate feeding of pregnant mice influence length of limbs of their progeny. Pol. J. Vet. Sci., 2003, Suppl., 6, 57–59.
31. Skopiński P., Skopińska-Różewska E., Kamiński A., Dziedzic-Gocławska A., Sommer E., Chorostowska--Wynimko J., Cendrowska I., Chrystowska D., Sadlo J., Chocolate feeding of pregnant mice resulted in epigallocatechin-related embryonic angiogenesis suppression and bone mineralization disorder. Pol. J. Vet. Sci., 2004, 7, 131–134
32. Tang F.Y., Meydani M., Green tea catechins and vitamin E inhibit angiogenesis of human microvascular endothelial cells trough suppression of IL-8 production. Nutr. Cancer, 2001, 41, 119–125.
33. Wang J.F., Schramm D.D., Holt R.R., Ensunsa J.L., Fraga C.G., Schmitz P., Keen C.L., A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage. J. Nutr., 2000, 130 (8S Suppl), 2115S–2119S.