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Immunomodulative effect of cimetidine on porcine T lymphocytes in vitro

Immunomodulujące działanie *in vitro* cymetydyny na limfocyty T otrzymane z krwi obwodowej świń

SUMMARY

To assess the influence of cimetidine on porcine lymphocyte activity *in vitro*, the phytohemagglutinin-induced proliferation and viability of lymphocytes T were examined. The cells were incubated with the different concentrations of cimetidine for 72 h in a 5% CO_2 humidified incubator at 37°C. Under the lowest concentration of cimetidine (0.002 μg/ml) proliferative response was not statistically significant. In the concentration range from 0.02 to 20 μg/ml proliferation was significant, and the highest proliferation was at concentration of 2 μg/ml. Viability of cells was above 94% following all experiment. Results of our study confirm that cimetidine has the immunostimulatory effect, and also that it is able to influence on porcine lymphocyte proliferation *in vitro*.

Key words: immunity, porcine T lymphocytes, cimetidine

INTRODUCTION

Cimetidine has been shown to augment cell-mediated immunity *in vitro*, through its blockade of H_2 receptors on T lymphocytes. These receptors are typically stimulated by histamine which exerts diverse suppresive effects on lymphocyte functions, such as suppresive effect on cytotoxic T lymphocytes, mitogen-induced lymphocyte [^{3}H]-thymidine incorporation, and down-regulation of some cytokines [Hahm *et al.* 1995]. Cimetidine has been used in human at high dosages (30--40 mg/kg) in the management of hypersecretory conditions and at these doses it has been seen to have immunomodulatory properties. Soon after introduction of histamine-2 receptor antagonists in peptic ulcer treatment, physicians came to notice that patients treated with these antagonists occasionally experienced regression of their cancers. There are some reports about partial remission of lung cancer, remarkable regression of malignant melanoma, rapid improvement of mycoses fun-

goides and improved survival in patients with gastric cancer. These findings have led to the use of cimetidine in the treatment of various systemic and cutaneous disorders including hypogammaglobulinaemia, papillomatosis, and psoriasis [Harcout *et al.* 1999]. These encouraging results have led to consider use of cimetidine as immunomodulator. In patients receiving cimetidine, the proportion of lymphocytes responding to mitogen stimulation was significantly increased [Rocklin *et al.* 1978, Smith *et al.* 1979, Ershler *et al.* 1983, Feldman *et al.* Burton 1990, Lin 1991, Hahm *et al.* 1995, Bourinbaiar and Fruhstorfer 1996, Harcout *et al.* 1999].

Lymphocyte proliferation is a useful tool for measuring immune function. Proliferative responses of lymphocytes have been used to evaluate the immunological status of the host following onset of disease or vaccination with the specific immunogen. Dorn *et al.* [2002] have documented the proliferative response of porcine T lymphocytes to PHA by a flow-cytometry and [^{3}H]-thymidine incorporation [Dorn *et al.* 2002]. Several authors have reported the use of different substances for nonspecific immunostimulation in pigs [Krakowski *et al.* 2002, Markowska-Daniel *et al.* 2002a, 2002b]. However, any report about using of cimetidine on porcine lymphocyte culture has not been published to date. The aim of the study was to investigate the immunomodulative effect of cimetidine on proliferation of porcine lymphocytes T *in vitro* and possible application of this immunomodulator in nonspecific immunostimulation in pigs.

MATERIALS AND METHODS

Eight crossbred pigs were used for the study. Blood was collected into the tubes with heparin. Lymphocytes were isolated from heparinized blood by density gradient centrifugation. Briefly, blood, diluted with equal volume of PBS (Wytwórnia Surowic i Szczepionek „Biomed" Lublin) was carefully layered on Histopaque-1077 (Sigma) [Tuchscherer *et al.* 2002]. After centrifugation (30 min at 300 × g), lymphocytes were collected from interface and washed twice in PBS (15 min at 400 × g). Isolated lymphocytes were adjusted to a final concentration of 2×10^6 in RPMI 1640 medium (Wytwórnia Surowic i Szczepionek „Biomed" Lublin) supplemented with gentamicin solution (20 µl/ml Sigma) and 10% calf serum (Wytwórnia Surowic i Szczepionek Biomed Lublin).

Cimetidine obtained as a gift from „Jelfa" Przedsiębiorstwo Farmaceutyczne Jelenia Góra was in the form of chemically pure powder. For the in vitro assay, cimetidine was initially dissolved in ethanol and then diluted appropriately in complete culture medium in the following final concentrations: 0.002 µg/ ml, 0.02 µg/ml, 0.2 µg/ml, 2 µg/ml, and 20 µg/ml of medium.

Fifty µl of isolated lymphocytes suspended in complete medium together with T cell-specific mitogen phytohemagglutinin (10 µg/ml; PHA, Sigma) were pipetted in duplicate into 96-well microplates (cell culture plates Nunc). To the one part of wells 50 µl of the cimetidine appropriate concentration in complete medium was added and to second part only complete medium (controls) was added.

Cultures were incubated at 37°C and 5.0% CO_2 for 72 h. Proliferation of cells assessment is based on the reduction of tetrazolium salt into a blue formazan by mitochondrial dehydrogenase of viable cells. After 72h of incubation, cultures were pulsed with 15 µl of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide – Sigma) for 3 h at 37°C, and solubilised to dissolve the dark blue crystals overnight [Tuchscherer *et al.* 2002]. Microplate reader (Alab Plate Reader ELISA) measured the optical density (O.D.) of formed blue formazan at a wavelength of 600 nm. The results were expressed as the proliferation index (PI) according to the formula for results of duplicate assays:

$$PI = \frac{\text{OD of stimulated cells}}{\text{OD of non-stimulated cells}}$$

Cell viability: The effect of cimetidine on cell viability was assessed on lymphocytes after 24 h, 48 h, and 72 h of culture with lymphocyte activation with PHA by using trypan blue exclusion test [Lebrec *et al.* 1995].

Statistical analysis: Student's paired t test and analysis of variance were used at $p \leq 0.05$ assert as statistically significant. For calculation of statistical differences 'Statistica' computer program (applications package) was used.

RESULTS

The direct effect of different concentrations of cimetidine was tested on PHA-stimulated peripheral porcine blood lymphocyte cultures (Fig. 1). The mean PI at a concentration of 0.002 μg/ml was 1.037 and differences among this concentration and control was not statistically significant. Those at concentrations of 0.02 μg/ml and 0.2 μg/ml were statistically significant ($p<0.05$ and $p<0.01$ respectively) and averaged PI = 1.062 and 1.082 respectively. The differences between mean PI at a concentration of 2 μg/ml (1.162) and control, and between 20 μg/ml (1.149) and control were also statistically significant ($p<0.01$). The highest proliferative response of lymphocytes observed at concentration of 2 μg/ml was reduced when 20 μg/ml of cimetidine was used. And this is optimally effective in vitro cimetidine concentration.

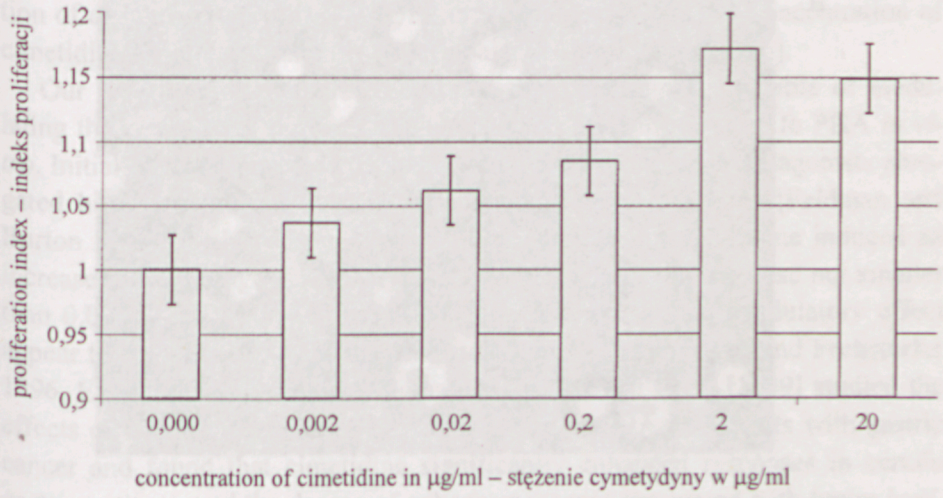
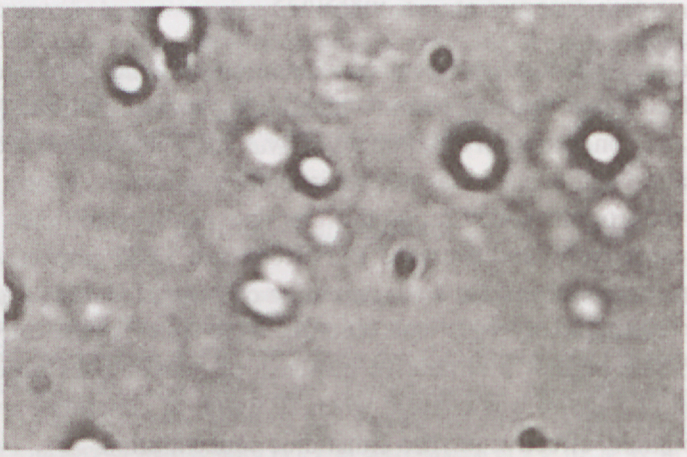


Fig. 1. The influence of cinetidine on proliferatory activity of porcine lymphocytes T *in vitro*
Rys. 1. Wpływ cymetydyny na aktywność proliferacyjną limfocytów T świń *in vitro*

Tab. 1. Effect of different doses of cimetidine on lymphocyte viability *in vitro*
Wpływ różnych dawek cymetydyny na żywotność limfocytów *in vitro*

Viability of lymphocytes in % – Żywotność limfocytów w %						
Dose (µg/ml) Dawka(µg/ml) / Time (hours) Czas (godziny)	0.000	0.002	0.02	0.2	2	20
0	97	97	97	97	97	97
24	97	96.7	96.9	96.8	97.1	97
48	96.1	95.9	96.2	97.1	96.2	95
72	96.1	95	96.1	96.3	96.3	94.5

A



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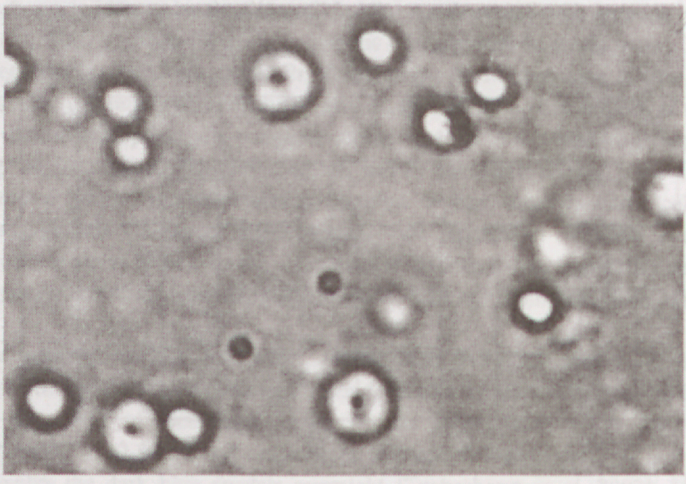

Phot. 1. Blast transformation of lymphocytes, A – cell culture of lymphocytes – control; B – blast transformation – changes in nucleus
Fot. 1. Transformacja blastyczna limfocytów, A – hodowla limfocytów *in vitro* – kontrola; B – transformacja blastyczna – zmiany w jądrze

Viability of examined cells was between 94.5 and 97.1% following all experiment (Tab. 1). This viability slightly decreased in all groups of experiment including control within 72 h of incubation.

DISCUSSION

There are some reports about effects of cimetidine on lymphocyte proliferation *in vitro*. Elizondo *et al.* showed that cimetidine induces an increase in the replication and mitotic indexes, in a dose-response way. The most effective concentrations of cimetidine were from 10^{-3} M to 10^{-8} M [Elizondo and Ostrosky-Wegman 1996]. From the results of Gifford it is clear that the most effective concentrations of cimetidine ranged from 10^{-4} M to 10^{-5} M [Gifford and Tilberg 1987, Gifford *et al.* 1988]. On the other hand Ogden established that the most effective concentration of cimetidine in vitro was 10^{-3} M [Ogden and Hill 1980]. According to Gifford the maximal proliferative response was 22.3% for the 10^{-6} M and 21.6% for the 10^{-5} M cimetidine concentrations. These two concentrations of cimetidine increased lymphocyte proliferation over control to a statistical significance of $p<0.05$ [Gifford *et al.* 1980].

The important statement is given by Hahm *et al.* [1995] who established that the dose of cimetidine in final concentration of 1.0 μg/ml correspond to the serum concentration of the drug necessary to inhibit the secretion of gastric acid by 50%, and the doses are adequate for lymphoblastogenesis. Bournabair estimated that the dose of 200 mg of cimetidine, which corresponds to concentration of drug in serum of 0.5–1 μg/ml is the clinically effective concentration of cimetidine for antiviral therapy [Bourinbaiar and Fruhstorfer 1996].

Our investigations have demonstrated that cimetidine is capable of modulating the response of porcine lymphocytes to mitogenic activity to PHA *in vitro*. Initial observations showed that histamine type-2 receptor antagonists abrogated histamine-induced immunosuppresion generated *in vitro* [Feldman and Burton 1990]. As have been showed in this experiment cimetidine induced an increase in the mitogenic PHA lymphocyte response from the dose not smaller than 0.002 μg/ml. Some authors point out that this immunomodulatory effect appear to be correlated with the imidazole moiety [Bourinbaiar and Fruhstorfer 1996, Elizondo and Ostrosky-Wegman 1996]. Kurosu *et al.* [1989] studied the effects of cimetidine on lymphocyte responses to PHA in patients with gastric cancer and found that cimetidine significantly enhanced responses in certain gastric patients, and the degree of enhancement was associated with tumor load. According to Gifford *et al.* [1980] cimetidine-induced augmentation of human lymphocyte blastogenesis is similar to levamisole in mitogen-induced prolifera-

tive augmentation of normal human lymphocytes. Brockmeyer *et al.* [1989] were investigated the effect of cimetidine on the immune system in men. Cimetidine was administered orally in daily doses of 800 mg for a period of 7 days. Observation of blastogenic response over the entire period of drug administration showed that a significantly enhanced mitogen stimulation response was observed after 7 days of oral cimetidine administration.

In respect of cimetidine dose used in our experiment the differences between our results and other may derived from specific responses of pigs and from another method of lymphocyte proliferation estimation. Nevertheless, the highest and high proliferation induced by 1 μg/ml and 10^{-4} M to 10^{-7} M of cimetidine respectively were confirmed by other authors [Gifford and Tilberg 1987, 1988, Hahm *et al.* 1995, Elizondo and Ostrosky-Wegman 1996]. These values were close to concentration range of cimetidine in medium (0.02 to 20 μg/ml) used in our study.

Viability of cells was above 94% following all experiment, which is in accordance with the results of Lebrec [Lebrec *et al.* 1995].

REFERENCES

Bourinbaiar A.S., Fruhstorfer E.C., 1996: The effect of histamine type 2 receptor antagonists on human immunodeficiency virus replication. Pharmacology Letters 59, 365.

Brockmeyer N.H., Kreutzfelder E., Bluhm C., Shen G., Scheiermann E., *et al.*, 1989: Immunomodulation of cimetidine in healthly volunteers. Klin Wochenschr 67, 26.

Dorn A.D., Waters W.R., Byers V.M., Pesh B.A. Wannemuehler M.J., 2002: Characterization of mitogen-stimulated porcine lymphocytes using a stable fluorescent dye and multicolor flow cytometry. Vet. Immunol. Immunopathol. 87, 1.

Elizondo G., Ostrosky-Wegman P., 1996: Effects of metronidazole and its metabolites on histamine immunosuppression activity. Life Sciences 59, 285.

Ershler W., Hacker M., Burroughs B., Moore A., Myers C., 1983: Cimetidine and the immune response. Clin Immunol and Immunopathol. 26, 10.

Feldman M., Burton M., 1990: Histamine$_2$-receptor antagonists. Drug Therapy 13, 1672.

Gifford R.M. Tilberg A., 1987: Histamine type-2 receptor antagonist immune modulation II. Surgery 102, 242.

Gifford R.M. Voss B.V., Schmidtke J.R. Ferguson R.M., 1988: Histamine type-2 receptor antagonist immune modulation I. Surgery 103, 184.

Gifford R.M., Hatfeld S.M., Schmidtke J.R., 1980: Cimetidine-induced augmentation of human lymphocyte blastogenesis by mitogen, bacterial antigen, and alloantigen. Transplantation 29, 143.

Hahm K.B., Kim W.H., Lee S.I., Kang J.K., Park I.S., 1995: Comparision of immunomodulative effects of the histamine-2 receptor antagonist cimetidine, ranit-

dine, and famotidine on perpheral blood mononuclear cells in gastric cancer patients, Scand. J. Gastroenterol. 30, 265.

Harcout J.P., Worley G., Leighton S.E.J., 1999: Cimetidine treatment for reccurent respiratory papillomatosis. Int. J. Pediatr. Otorinolaryngology 51, 109.

Krakowski L., Krzyżanowski J., Wrona Z., Kostro K., Siwicki A., 2002: The influence of nonspecific immunostimulation of pregnant sows on the immunological value of colostrum. Vet. Immunol. Immunopathol. 8, 89.

Kurosu Y., Tanaka N., Furusho Y., Morita K., 1989: Cimetidine-mediated augmentation of lymphocyte responses to phytohemagglutinin in gastric cancer patients. Jpn. J. Clin. Oncol. 19, 56.

Lebrec H., Roger R., Blot C., Burleson G.R., Bohuon C., Pallardy M., 1995: Immunotoxicological investigation using pharmaceutical drugs. Toxicology 96, 147.

Lin J., 1991: Pharmacokinetic and pharmacodynamic properties of histamine H_2-receptor antagonists. Clin Pharmacokinet. 20, 218.

Markowska-Daniel I., Stankiewicz I., Wałachowski M., Pejsak Z., 2002: Wpływ skojarzonego stosowania żelaza i izoprynozyny na zdrowotność prosiąt. Medycyna Wet. 58, 45.

Markowska-Daniel I., Żmudzki J., Pejsak Z., 2002: Wpływ skojarzonego stosowania żelaza i izoprynozyny na wskaźniki immunologiczne prosiąt. Medycyna Wet. 58, 598.

Ogden B.E., Hill H.R., 1980: Histamine regulates lymphocyte mitogenic responses through activation of specific H_1 and H_2 histamine receptors. Immunology 41, 107.

Rocklin RE., Greineder D., Littman B., Melmon K.L., 1978: Modulation of cellular immune function in vitro by histamine receptor-bearing lymphocytes: mechanism of action. Cellular Immunology 37, 162.

Smith M., Couhig E., Miller J., Salman J., 1979: Cimetidine and the immune response. The Lancet 30, 1406.

Tuchscherer M., Kanitz E., Otten W., Tuscherer A., 2002: Effects of prenatal stress on cellular and humoral immune responses in neonatal pigs. Vet. Immunol. Immunopathol. 86, 195.

STRESZCZENIE

W celu oceny działania cymetydyny na aktywność limfocytów T wyizolowanych z krwi obwodowej świń w warunkach *in vitro*, zbadano ich zdolność do proliferacji i żywotność. Wyizolowane limfocyty inkubowano przez 72 godziny w atmosferze 5% CO_2 w temperaturze 37°C pod działaniem różnych stężeń cymetydyny. Przy najniższym stężeniu cymetydyny (0,002 µg/ml) odpowiedź proliferacyjna była zwiększona, ale nie w stopniu statystycznie istotnym. Przy stężeniach pomiędzy 0,02 a 20 µg/ml proliferacja była znacząca, osiągając wartość najwyższą przy stężeniu 2 µg/ml. Żywotność komórek pozostała powyżej 95% podczas trwania eksperymentu. Wyniki naszych badań potwierdzają immunostymulujące działanie cymetydyny i jej zdolność do wpływania na proliferację limfocytów pochodzących z krwi obwodowej świń w warunkach *in vitro*.

Słowa kluczowe: odporność, świnie, limfocyty T, cymetydyna