# Role of Dietary Starches in Induction of Immune Cells Apoptosis in Wistar Rats

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We compared the effects of maize and potato starch on Wistar rats. The level of oligonucleosomal DNA fragmentation increased in thymic and splenic cells of animals receiving potato starch. The expression of Fas/CD95/Apo-1 apoptosis receptor in lymphocytes and their response to exogenous ceramide was also higher in rats receiving potato starch compared to the reference group. This led to intensification of spontaneous and induced apoptosis and decreased cell content in immune organs. The authors conclude that higher resistance of potato starch to digestive enzymes leads to modification of cell metabolism and induction of ceramide-dependent apoptosis in immune cells.

**Key Words:** *starch; lymphocytes; apoptosis; CD95; ceramide*

The diet can modulate gene expression in procaryotes and eucaryotes, including the expression of apoptosis genes. Structural organization of nutrients is essential for their availability for digestive enzymes. This modulates metabolism of intestinal ecosystem components, including the intestinal wall cells, associated lymphocytes, and probiotics, whose interactions are essential for normal gastrointestinal function [7,9,11]. There are no reports about the effects of dietary carbohydrates, *e. g.* starches, on molecular processes of cell death. We studied the effects of different starches on apoptosis induction in immune cells of experimental animals.

Starch granules consisting of two glucose polymers amylopectin crystals (80%) and amylose granules (20%) distributed among them are characterized by different degradation rates [14]. Maize starch is almost completely (99%) absorbed in the small intestine, while potato starch is absorbed only by 28% in the end of the small intestine. If the ration contains 55% of resistant starches, their enzymatic cleavage starts only after 6-7 h [2,7]. Starches accumulating in the large intestine, serve as substrates for bacterial flora and modulate the local immune response [2].

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In rats potato starch induces body weight loss due to the increase in neurotensine concentration in the paraventricular and dorsomedial hypothalamic nuclei leading to inhibition of absorption of dietary lipids [3]. Another possible cause of cell loss is intensive cell death. The aim of this study was to evaluate spontaneous apoptosis in the thymus and spleen of rats receiving rations with different types of starch and to detect the relationship between starch composition and ceramide-dependent induction of lymphocyte apoptosis.

## MATERIALS AND METHODS

Lymphocytes were cultured in RPMI-1640 (Flow) with 10 mM Hepes buffer (Sigma), 2 mM L-glutamine (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin (Sigma), 50 μM 2-mercaptoethanol (Flow), and 10% fetal calf serum (FCS, Flow). Before staining the lymphocytes were washed in phosphate buffer saline (PBS, Sigma). Monoclonal CD95 antibodies (mAb) to rat Fas/Apo-1 receptor (Cedarline) were used. FITC-conjugated $F(ab')_2$ fragments of goat anti-rabbit immunoglobulins (Medbiospectr) served as second antibodies.

The experiment was carried out on 10-week-old Wistar rats (130±5 g) in accordance with Principles of Laboratory Animal Care (1985, National Institute of Health, USA, No. 88-23). The rats (5 animals per group)

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**TABLE 1.** Effect of Starch Type on Body Weight and Weights of Immune Organs in Rats (*M*±*m*)

| Starch type | Mean body weight, g | | Mean weight of spleen, mg | Spleen/body weight ratio | Mean weight of thymus, mg | Thymus/body weight ratio |
|---|---|---|---|---|---|---|
| | day 1 | day 21 | | | | |
| Maize (group 1) | 130±5 | 199±7 | 1389±93 | 0.6986±0.0396 | 441.6±44.1 | 0.220±0.016 |
| Potato (group 2) | 130±5 | 224±15 | 1325±158 | 0.5867±0.0408 | 402.4±83.7 | 0.1733±0.0250 |

received fodder and drinking water *ad libitum* for 21 days. Semisynthetic fodder consisted of 24% casein, 6% yeast, 2% cellulose, 4% salt mixture, and 1% vitamin mixture. The sources of carbohydrates were maize starch (56%) in group 1 and potato starch (59%) in group 2. The animals were weighed after the experiment. Rats were decapitated under narcosis. Peripheral blood was collected, the viscera were removed and weighed.

The thymus and spleen were weighed, washed in PBS, transferred into RPMI-1640, and homogenized. Cell suspension was filtered through a cotton and centrifuged in RPMI-1640 at 200*g* and 4°C. The supernatant was removed, the pellet was suspended, and erythrocytes and dead cells were removed by hypotonic shock. The pellet was washed twice in RPMI-1640 by centrifugation. The cells were counted in a Goryaev chamber. Cell viability was 98-100% (staining with 1% Trypan blue) [10].

The isolated thymic and splenic cells ($10\times10^6$ cells/ml complete RPMI-1640 with 10% FCS) were incubated with specific apoptosis inductor C-2-ceramide (working concentrations 5-50 nmol, Sigma) for 2.5 h at 37°C in a humid atmosphere with 5% $CO_2$, washed 3 times with the medium, then with PBS, and stained with CD95 mAb. Parallel cultures were washed, fixed, and stained with propidium iodide.

Monoclonal antibodies (20 µl) were added to lymphocyte suspension ($1\times10^6$ cells in 50 µl) and incubated for 40 min at 4°C. The cells were washed in PBS, the precipitate was incubated with 50 µl PBS and 20 µl second antibodies for 40 min at 4°C; the cells were then washed in PBS and fixed in 1% paraformaldehyde. In order to evaluate the degree of oligonucleosomal DNA fragmentation, 500 µl propidium iodide (PI; 0.05% PI in hypotonic citrate buffer with 0.5% Triton X-100) was added to $1\times10^6$ cells in 150 µl 1% paraformaldehyde and incubated for 40 min at 4°C in the dark.

The percentage of cells expressing the studied antigen and the fluorescence intensity were determined for each sample in a FACSCaliber flow cytometer

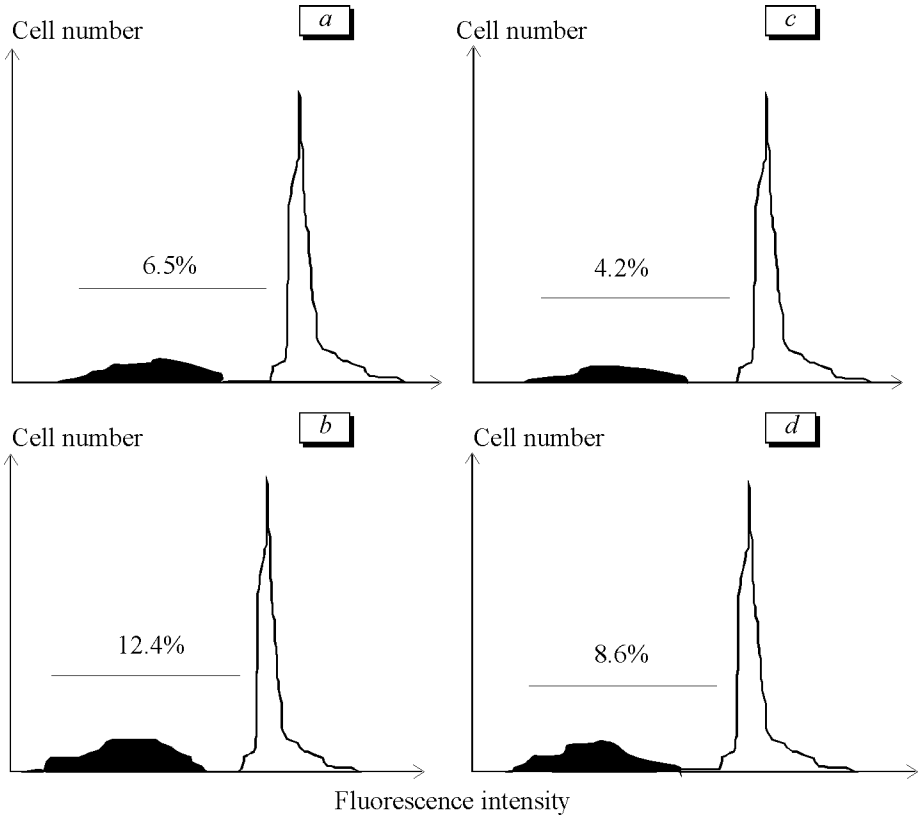


**Fig. 1.** Spontaneous apoptosis in the thymus (*a*, *b*) and spleen (*c*, *d*) of Wistar rats *in vivo*. *a*, *c*) rats fed maize starch; *b*, *g*) potato starch. Dark zone: percentage of cells in apoptosis; light zone: peak of diploid cells.

(Becton Dickinson) using SimulSet software. FITC-labeled $F(ab')_2$ fragments served as nonspecific control. FITC fluorescence was measured on an FL1 channel at $\lambda$=530±15 nmol. PI fluorescence was measured on an FL2 channel at $\lambda$=585 nm (10,000 events per lymphocyte gate were analyzed in each sample). The data are presented as a histogram with the abscissa showing the fluorescence intensity and the ordinate showing the percentage of stained cells.

All measurements were repeated at least 3 times for each point; the data were analyzed using Biostat software. The results are presented as $M\pm m$. The groups were compared using Student's $t$ test. The differences were considered significant at $p\leq 0.05$).

## RESULTS

After the end of the experiments body weight in groups 1 and 2 was 199±7 and 224±15 g, respectively. In group 2 the mean weight of the spleen and spleen/body weight ratio were lower, the thymus weight and thymus/body weight ratio tended to decrease. Hence, maize starch decreased the weight of immune organs in comparison with body weight (Table 1).

In order to evaluate the relationship between the type of starch and spontaneous apoptosis in the thymus and spleen, the distribution of PI-stained cells was studied in the lymphocyte gate by flow cytometry (Fig. 1). Since PI binds not only oligonucleosomal DNA fragments, but also single-stranded DNA terminals during cell cycle, the samples always has a diploid peak in the zone of intense fluorescence. The first peak on the histogram corresponds to the number of diploid cells in a sample. The zone to the left from the diploid peak, denoted with M1 cursor, corresponds to fluorescence of apoptotic cells. The number of cells in apoptosis is determined by the total number of events at an area limited by the respective gate, for which the histogram serves as a two-dimensional reflection.

The level of spontaneous apoptosis in thymic and splenic cells was significantly higher in group 2 (12.4±0.7% *vs.* 6.5±0.4% and 8.6±0.6% *vs.* 4.2±0.5%, respectively). Replacement of maize starch with potato starch for 3 weeks intensified death of immune cells, which was confirmed by intensification of oligonucleosomal DNA fragmentation.

$CD95^+$ lymphocytes were isolated directly after the end of the experiment and cultured for 2.5 h with cell-penetrating C-2-ceramide in concentrations of 5, 10, 20, and 50 nmol (doses inducing apoptosis) (Fig. 2). The initial number of $CD95^+$ thymocytes in groups 1 and 2 was 7.28±0.34% *vs.* 12.84±0.26%, respectively ($p<0.05$). Addition of potato starch to the ration for 3 weeks increased the number of cells expressing apoptosis receptors.

Incubation with C-2-ceramide for 2.5 h dose-dependently increased the number of $CD95^+$ cells in the thymus in both groups (Table 2), the effect was more pronounced in group 2.

For splenic cells the initial percentage of CD95 antigen expression was 5.18±0.50% in group 1 and 10.44±0.60% in group 2. Incubation of splenic cells with C-2-ceramide for 2.5 h more significantly increased the number of $CD95^+$ splenocytes in group 2 (Table 2).

**TABLE 2.** Effect of Starch Type on Rat Thymocyte and Splenocyte Sensitivity to Ceramide-Induced Apoptosis: Percentage of CD95 Antigen Expression after Incubation with C-2-Ceramide ($M\pm m$, %)

| C-2-ceramide concentration, % | Starch | |
|---|---|---|
| | maize (group 1) | potato (group 2) |
| Thymus | | |
| control | 7.28±0.34 | 12.84±0.26 |
| 5 nmol | 8.16±0.20 | 13.94±0.07 |
| 10 nmol | 10.22±0.28 | 14.74±0.29 |
| 20 nmol | 15.24±0.25 | 22.74±0.29 |
| 50 nmol | 17.76±0.50 | 28.34±0.26 |
| Spleen | | |
| control | 5.18±0.50 | 10.44±0.60 |
| 5 nmol | 6.0±0.26 | 10.72±0.80 |
| 10 nmol | 6.88±0.20 | 11.84±0.29 |
| 20 nmol | 12.88±0.36 | 17.88±0.54 |
| 50 nmol | 16.54±0.30 | 21.56±0.61 |

**Note.** Here and in Table 3: $p<0.05$ between groups 1 and 2.

**TABLE 3.** Apoptosis Induction Evaluated by PI Incorporation into Thymocytes and Splenocytes of Rats Receiving Maize or Potato Starch ($M\pm m$, %)

| C-2-ceramide concentration, % | Starch | |
|---|---|---|
| | maize (group 1) | potato (group 2) |
| Thymus | | |
| control | 6.32±0.19 | 12.64±0.23 |
| 5 nmol | 6.98±0.22 | 12.84±0.68 |
| 10 nmol | 7.62±0.52 | 13.98±0.55 |
| 20 nmol | 11.14±0.45 | 16.56±0.38 |
| 50 nmol | 13.44±0.44 | 18.94±0.62 |
| Spleen | | |
| control | 4.20±0.21 | 8.58±0.28 |
| 5 nmol | 4.74±0.24 | 8.86±0.37 |
| 10 nmol | 6.28±0.25 | 10.06±0.44 |
| 20 nmol | 9.76±0.48 | 13.78±0.43 |
| 50 nmol | 12.46±0.38 | 14.92±0.53 |

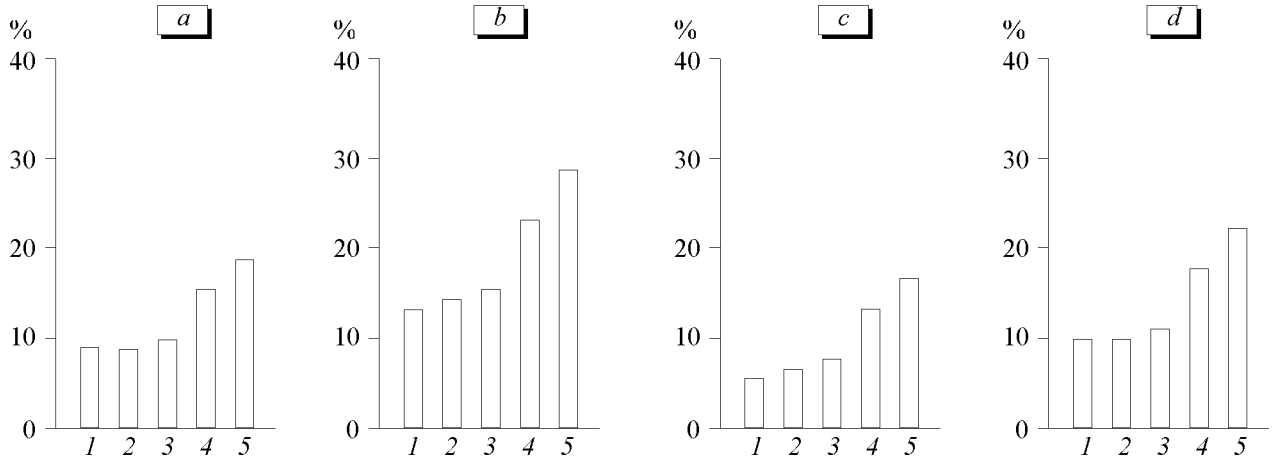


**Fig. 2.** Ceramide-induced expression of CD95 antigen (Fas/Apo-1) in thymic (*a*, *b*) and splenic cells (*c*, *d*) of rats receiving maize (*a*, *c*) or potato starch (*b*, *d*). Ordinate: percentage of $CD95^+$ cells in lymphocyte gate. *1*) initial cell population; *2-5*) incubation with C-2-ceramide: *2*) 5 nmol; *3*) 10 nmol; *4*) 20 nmol; *5*) 50 nmol.

The percentage of cells with DNA fragmentation was evaluated by PI incorporation in parallel with expression of CD95 antigen in cell cultures. Statistically significant induction of ceramide-dependent apoptosis was observed, it was more pronounced in group 2 (Table 3). Addition of potato starch to rat ration increased expression of apoptotic antigen CD95 and stimulated ceramide-induced apoptosis in the thymus and spleen of experimental animals.

Hence, structural organization of food components and their resistance to digestive enzymes modify cell metabolism, which can cause changes in receptor expression on the plasma membrane. The increase in the number of apoptosis receptors on immune cells leads to activation of ceramide-dependent signaling pathways, which determines cell death and decrease of cell content in immune organs.

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