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EVALUTION OF IMMUNOLOGICAL AND MUTAGENIC EFFECTS OF GENETICALLY MODIFIED SOYBEAN 40-3-2 USING AS A RAW MATERIAL FOR FEED

Gerilovych I.O.
National Scientific Center "Institute of experimental and clinical veterinary medicine", Kharkiv, Ukraine, e-mail: toxi-lab@vet.kharkov.ua

The article presents the results of experimental studies of genetically modified soybean line 40-3-2 effects on animals.

Objective was to evaluate the mutagenic effects and potentialimpact on the immune system of animals for genetically engineering modified feed raw materials.

There were used molecular, immunological, clinical, pathological, serological, cytological and statistical methods in our researches.

In the experiment on white rats treated with soy food during 60 days, found that the level of chromosomal damage in bone marrow cells in control and experimental animals were within the physiological norm and didn't differ significantly.

In the experiment on white mice to study the effects of GM soybeans in humoral immunity, was shown that 14 days after the introduction of sheep erythrocytes levels of antibodies in animals of the experimental group was significantly higher than the control at 15.85 %. In the test to assess the validity of genetically modified raw feed on resistance of mice to Salmonella enterica ser. Typhimurium was demonstrated the positive impact of GM soybean line 40-3-2: experimental group of animals that were infected with a dose of 10^6 CFU / mouse, mortality was lower than the control; and the level of antibodies to the pathogen 21 days after infection in animals of experimental groups was significantly higher than the control at 30 %. However, it was experimentally proved that GM soybean line 40-3-2 hasn't got effects on cellular immunity and doesn't act as a sensitizing factor.

Thus it was found that genetically modified soy line 40-3-2 using as raw feed doesn't mutagenic action, but has immunomodulatory properties, that needed to be studied in more detail in long-term experiments.

Keywords: GM plants, soybean line 40-3-2, feed, animals, immune system, mutagenic activity.

Today soybeans are a major biotech crops. The first commercial line of GM soy was launched in 1996. It was Roundup Ready soybeans from the company Monsanto. The genome of this line includes gene 5-enolpyruvylshikimate-3-phosphate (EPSP) from the CP4 strain of *Agrobacterium spp*. This gene causes tolerance to the herbicide glyphosate (Roundup) [1, 2].

In the two years under 8 % of the soybeans in the United States employed biotech crops, and in 2010 this figure was already 93%. In addition, outside the US, transgenic soybean plant started in ten countries, a total area of over 60 million hectares. [3].

Recently, the main soybean production is located in countries such as USA, Brazil, Argentina, China and India, which together produce 91 % of world soybean. Thus the US (85 %) and Argentina (98 %) produce almost exclusively GM soy. In these countries, genetic engineering-modified soybeans are grown without restrictions and enter the market with traditional counterparts. Soybean imports from these countries usually contain a large number of GM grains [2, 3].

In the EU cultivation of GM soya is prohibited, but it could be used for fodder. These GM varieties must be register and included in the State Register of feed produced from GMOs [4, 5].

Ukraine also banned the cultivation of GM soy. [6] And the only one included in the register of genetically modified feed sources are soybean meal MON 40-3-2 (by order of The State Veterinary and Phyto Service [Derzhvetfitosluzhba] from 23.07.2013№ 1752).

From the literature it is known that it was conducted the study of compositional equivalence glyphosate-tolerant and parents soybeans. No significant differences were noted. The concentrations of important nutrients not significantly differ in grain genetically modified and conventional soybeans (Hammond et al, 1996). But there not been evaluated by standard toxicological characteristics [7].

In 1999 Tutelyan V.A. with co-authors conducted a research using laboratory rats which were fed for 5 months protein concentrate derived from genetically modified soybean lines 40-3-2 (Monsanto Co, USA) at a daily dose of 1.25 g/rat. This examined the biochemical

parameters of blood serum (total protein, albumin, globulin, glucose, alkaline phosphatase activity (ALP), alanine and aspartate aminotransferase (ALT and AST), liver (ALP activity, ALT, AST) and urine (pH, relative density, serum creatinine). There have been studied the activity of enzymes of the 1st and 2nd phase of metabolism of xenobiotics. It was found that the addition of GM soy in the diet of rats, changes the functions of hepatocyte membranes and disorders of enzymatic activity compared to physiological norms. At the same time harmful effects on adaptive systems of the body was found [8].

Impact of GM and traditional soybeans on immune system of laboratory rats and mice V10A was investigated by Teshima et al. (2000). The study was planned as a comparison of the two groups of animals treated with glyphosate-tolerant and traditional soybeans, respectively. The experiment lasted for 15 weeks. For external features, quality wool, behavior and animal growth rate of both groups did not differ. Histological study of immune organs did not reveal significant changes relative to control. The production of the prizes-specific Ig E was not recorded in any of the experimental group and increase the prizes to content-specific Ig G was identical in animals treated with GM soy and animal control group. Thus, we can conclude no action immunotoxic glyphosate tolerant soybean-provided feeding her laboratory rats and mice [9].

Zhu et al. (2004) did not determine the negative impact of glyphosate-tolerant soybean on the body of laboratory rats by feeding it in terms of volume of more than 90% of the diet [10].

Thus, a large number of authors indicate that transgenic Roundup Ready soybeans in their composition are equivalent to parental varieties and is completely safe for consumption [11, 12, 13]. However, taking into account that this crop is used to produce soy milk and other foods recommended for a healthy diet, many scientists insist on conducting more research on the impact of GM soybeans in the endocrine, immune system, the study of mutagenic and allergenic properties.

Based on the above, our objective was to evaluate the mutagenic effects and potentialimpact on the immune system of animals for genetically engineering modified feed raw materials.

Materials and methods. The identification of GM and traditional soybeans was performed using PCR [14]. Amplification was performed using primer sets and business systems to identify target areas of the standard: CamV35S-promoter (5'-CCACGTCTTCAAAGCAAGTGG-3'; 5'-TCCTCTCCAAATGAAATGAAC TTCC-3') and NOS-terminator (5'-GCATGACGTTATTTATGAGATGGG-3'; 5'-GACACCGCGCGCGATAATTTATCC-3') [15].

Positive and negative controls were used for an objective evaluation of the reaction. As a positive control DNA was obtained from standard samples of certified ERM® – BF410d (Roundup Redy Soya). As negative – DNA was isolated from traditional soybean varieties.

Detection of amplification products was performed in gel electrophoretic system using standard method [16].

The positive samples of soybean grain were further investigated by PCR using a commercial kit for identification of genetically modified soybean 40-3-2 line DNA in food and animal feed. Soybeans, identified as traditional and GM soybean line 40-3-2, used in our further studies.

Study of the impact of GM feed raw materials on immune system, was conducted the experiment using white mice.

Two groups of white mice were formed on the principle of analogues: males, 2.5–3 months old, with an initial weight of 18-20 g.:

- the first animals (I) control group received basic diet, 40 % of which were traditional soybean after crushing and heat treatment (120 ˚C, 30 min.);
- the second animals (II) the experimental group received basic diet, 40 % of which were GM soybean line 40-3-2 after crushing and heat treatment (120 ˚C, 30 min.).

Access to water for all mice was unlimited.

To evaluate the effect of GM material in feed on humoral immunity was conducted to determine the level of hemagglutininin white mice's serum to sheep's red blood cells. For this 21 day experiment and control mice were intraperitoneally injected experimental group 0.5 cm^3 washed sheep red blood cells (20 million. cells/cm^3). Slaughter of animals (by decapitation) and a blood sample for the study was performed at 7, 14 and 21 days after administration of sheep's red blood cells. The resulting white mice serum was used for the reaction of hemagglutination of erythrocytes of sheep by conventional methods [17].

The effect of GM material in feed link cellular immune animals was assessed by delayed-type hypersensitivity reaction to sheep's erythrocytes [18]. For this purpose, experimental mice of both groups on day 21 of the experiment subcutaneously injected in the blade area of 0.5 cm^3 washed erythrocytes (2 million. cells / cm^3). Five days later, all animals in the pad of the left pelvic limb suspension was administered at a dose of sheep red blood cells – 0.02 cm^3 (1 billion. cells / head); in the contralateral limb – 0.02 cm^3 0.95 % sodium chloride solution. Local inflammation assessed after 18–20 hours by determining and comparing the experimental and control limbs.

The effect of GM feed material as sensitizing agent determined by a test of sensitivity to histamine. For this 21 day experiment, mice control and experimental groups were injected intraperitoneally histamine hydrochloride (2.5 mg / mouse in 0.5 cm^3 saline) and observed for clinical signs and severity of anaphylaxis [19].

Impact of GM material feed on resistance of mice to *Salmonella enterica* ser. Typhimurium infection was determined by artificial animals. For this purpose, formed six groups of animals (n = 5): I, II and III - control (treated with conventional soybeans) and I, II and III – research (received GM soybean line 40-3-2). 21 day experiment, mice were intraperitoneally injected with 0.5 cm^3 suspension of *Salmonella* ser. Typhimurium * in saline at doses of 10^4, 10^5 and 10^6 CFU per animal, respectively. Infected mice were observed during 21 days. After that was done euthanasia of animals from blood sampling to determine the level of available antibodies to the pathogen in the agglutination reaction [17, 18]. (* A suspension of *Salmonella* ser. Typhimurium in saline obtained from sector study mycoplasmosis and salmonellosis NSC «IECVM»).

Research potential mutagenic activity of GM material in feed an experiment using laboratory rats. For this purpose, male rats weighing 160-200 g were selected. The animals were divided into two groupson the basis of analogues:

- Rats first (I) control group was received basic diet, 50 % of which were traditional soybean, thermally processed (100 ˚C, 40 min.);
- Rats second (II), research group was received basic, 50 % of which were GM corn soybean 40-3-2 line, thermally processed (100 ˚C, 40 min.).

Access to water for all rats was unlimited. The term of the experiment was 60 days. Slaughter of animals was carried out on 30 and 60 by cervical dislocation. Femur bones were collected, of which received a suspension of bone marrow cells. Smears of bone marrow cells were fixed in methanol (3 min.) and stained with hematoxylin and Karachi dye [20, 21].

Mutagenic activity was assessed by the presence of micronuclei and chromosomal aberrations in metaphase cells from bone marrow that are detected by light microscopy using immersion lens calculation method [20, 22].

Statistical analysis of the results was performed using Student's t-test ($p < 0.05$), as described by Lakin G.F. [23]. Experimental studies on laboratory animals have been conducted in compliance with the fundamental principles of bioethics [24]. Maintenance, care for animals and their feeding were carried out according to the rules and diets recommended for laboratory mice and rats of this age.

Results. During the assessment of humoral immunity it was found that the dynamics of antibody formation against sheep erythrocytes in control and experimental groups were different. Thus, in 7 days after administration of sheep erythrocytes levels of antibodies in the serum of mice of the control and experimental groups was almost identical, whereas in 14 day antibody levels in the animals of the experimental group was significantly higher than the control by 15.85 %. This trend was as well observed in the next week of experiment. Levels of antibodies against erythrocytes of sheep in mice in the experimental group were 12.90 % higher than in the control group of animals (Table 1).

Table 1 – The levels of antibodies in mice's serum to erythrocytes of sheep conditions for introduction in the diet of traditional and GM soybean lines 40-3-2 (log^2, M ± m, n = 4)

Number of days after administration of sheep'serythrocytes	Control group	Experimental group
7	3.38 ± 0.16	3.75 ± 0.17
14	6.00 ± 0.27	7.13 ± 0.08*
21	6.75 ± 0.32	7.75 ± 0.17*
Note. * P ≤ 0.05 relative to control		

The study cellular immunity was assessed by delayed-type hypersensitivity response to sheep's erythrocytes. It was found that after 18 hours in both groups of animals at the point of injection suspension of produced moderate swelling and reddening. The left limb with injection was warmer then right one. Weight of metatarsal of the left limbs in mice treated with GM soy was (0.135 ± 0.031) g, right – (0.123 ± 0.020) g. In the control group, – (0.134 ± 0.036) g and (0.120 ± 0.021) g., respectively. For each animal the response index (DI) was calculated with the following statistical analysis (Table 2).

Table 2 – The index of delayed-type hypersensitivity response to sheep red blood cells (%)

№	Control group	Experimental group
1	6.25	16.92
2	13.43	5.21
3	7.91	5.04
4	8.33	24.76
5	7.45	5.93
6	10.85	8.63
7	23.42	13.27
8	16.03	13.74
9	7.07	4.08
10	19.63	2.55
M±m	12.04 ± 1.98	10.01 ± 2.35
Note: temp. = 0.7 ($p \leq 0.05$ at $t \geq 2.11$)		

The effect of GM feed material as sensitizing agent determined by a test of sensitivity to histamine. It was found that in mice from the control and experimental groups after intraperitoneal administration of 0.5 % histamine hydrochloride solution (2.5 mg per animal) after 1-2 minutes respiratory and heart rate were increased, mice stooped, most of them grouped, and other kept separately. It was also observed "washing" faces that probably associated with the occurrence of unpleasant metallic taste in the mouth and / or nose mucus hypersecretion that arise when histamine release [19]. In 12-15 minutes after the injection 30 % of the mice in both groups started to itch, due to strong sensation under the action of histamine [19]. At the same time the animals intensified thirst. Finally, evidence of the impact of histamine hydrochloride disappeared within 40-50 minutes after administration. Thus, it was shown that clinical symptoms, duration and severity of the course of anaphylaxis in mice of the control and experimental groups did not differ. Notably sensitizing action of GM material in feed mice was not found.

Table 3 shows the results of the impact of GM row material on resistance of mice to *Salmonella enterica* ser. Typhimurium. The intensity of the symptoms of disease by infection of mice *Salmonella enterica* ser. Typhimurium depended on dose. Thus mice treated with GM soy line 40-3-2, were rather resistant to the action of an infectious agent. In the third experimental group 3 animals died (within 5 days of the experiment), and in third control group - 4 (during the first 3 days). The level of antibodies to salmonellas on 21th day of experiment in mice of the first and second experimental groups was 30 % higher than in animals of the control group. Thus, we considered that GM soybean line 40-3-2 might be as immunomodulator. However, for a more detailed study of its impact on resistance further research is needed on more experimental animals.

Table 3 – Performance of laboratory mice resistance to *Salmonella enterica* ser. Typhimurium under conditions of infection by intraperitoneal administration at various doses.

Dose of 10^4 CFU per mouse		**Dose of 10^5 CFU per mouse**		**Dose of 10^6 CFU per mouse**	
I control gr.	**I exper. gr.**	**II control gr.**	**II exper. gr.**	**III control gr.**	**III exper. gr.**
Clinical signs					
The next day a slight depression of appetite, but in general, the animals were lively, actively moving. Further appetite recovered and symptoms of disease were not observed.		Symptoms noted the first 7 days after infection, depression, lack of appetite, hair disheveled, on the face, especially around the eyes, swelling noted (first 3 days) eyes "narrowed" dull. Over time, appetite and activity resumed animals.		Animals unmovable, always sitting in one place, do not consume food. In the severe swelling of the muzzle, there are purulent discharges from the eyes. Appetite slowly began to recover for 6 days after infection. Even after 14 days the animals were little more active, wool dim, disheveled. 21 day experiment, the mice returned to normal condition.	
Number of the dearth animals					
–	–	–	–	4 (3 on II day, 1 on III day)	3 (2 on II day, 1 onVday)
Pathological changes					
An autopsy was performed after euthanasia on the 21th day of the experiment: liver was red-brown, in one animal from the experimental group and two - the control: gallbladder was full, bile was clear and yellow; intestines, kidneys, lungs and heart were without any changes.		An autopsy was performed after euthanasia on day 21 of the experiment: liver dark cherry color, gallbladder filled with hard, clear bile, yellow; increased spleen; heart and lungs without visible changes.		Liver dark cherry color, gallbladder filled with hard, clear bile, yellow; increased spleen; small and large intestines swollen in the small intestine hemorrhagic inflammation of the mucous membrane; heart and lungs without visible changes. In animals, an autopsy which was performed on day 21 of the experiment, pathological changes in the intestine were not found.	
The intensity of the immune response (RA antibody levels log^2)					
2.0 ± 0.00	2.6 ± 0.24*	2.0 ± 0.00	2.6 ± 0.24*	3.0	2.0 i 3.0
Note: * - P ≤ 0.05 relative to control					

During the experiment, the general condition of the rats in both groups was satisfactory. In appearance, quality wool, animal behavior in both groups did not differ.

According to the cytology average chromosome aberrations and the level of polychromatophil red blood cells with micronuclei in the bone marrow of rats control and experimental groups had no plausible differences (P > 0.05) and were within the physiological norm [20, 21] (Table 4).

Table 4 – The level of chromosomal damage in bone marrow cells of laboratory rats by feeding them the terms of conventional and GM soybean lines 40-3-2 for 60 days (M ± m,%)

Term research	Group	Chromosomal aberrations, %	Polychromatide erythrocytes, %	Polychromatide erythrocytes with micronucleus, %
30 days	Control	1.86 ± 0.06	46.42 ± 3.05	0.48 ± 0.09
	Experimental	1.86 ± 0.09	46.16 ± 2.97	0.47 ± 0.07
60 days	Control	1.78 ± 0.06	47.47 ± 1.35	0.45 ± 0.05
	Experimental	1.8 ± 0.07	46.11 ± 2.46	0.47 ± 0.07

There was no genotoxic effect of GM soybean lines 40-3-2 using as raw feed for white laboratory rats during 60 days.

Conclusions. 1. It was found that genetically modified soybean line 40-3-2 as raw feed did not cause mutagenic action, but showed immunomodulating properties.

2. It is experimentally proved that GM soybean line 40-3-2 had no effect on cellular immunity and did not affect the induction of sensitization.

3. In the experiment for the effects on humoral immunity it was found that in 14 days after administration of sheep's erythrocytes level of antibodies in animals from the experimental group was significantly higher than the control by 15.85 %.

4. In the test of estimation of GM fodder effect on resistance of mice to *Salmonella enterica* ser. Typhimurium the positive impact of GM soybean 40-3-2 line was demonstrated. The experimental group of animals that were infected with a dose of 10^6 CFU / mouse, mortality was lower than the control; and the level of antibodies to the pathogen hemaglutination to 21 days after infection in animals of experimental groups was significantly higher than the control group by 30 %.

5. The level of chromosomal aberrations in bone marrow cells of laboratory rats which were fed by traditional and GM soybean lines 40-3-2 for 60 days was not different in animal of both groups and was within the limits of the physiological norm.

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ОЦІНКА ІМУНОМОДЕЛЮЮЧОЇ ТА МУТАГЕННОЇ ДІЇ ГЕНЕТИЧНО МОДИФІКОВАНОЇ СОЇ ЛІНІЇ 40-3-2 ЗА УМОВ ВИКОРИСТАННЯ ЇЇ В ЯКОСТІ СИРОВИНИ КОРМІВ

Герілович І.О.
Національний науковий центр «Інститут експериментальної і клінічної ветеринарної медицини», Харків, Україна

У статті наведені результати експериментального вивчення впливу генетично модифікованої сої лінії 40-3-2 на організм тварин.

Метою роботи було оцінити мутагенну дію та встановити ймовірний вплив на імунну систему тварин генно-інженерно-модифікованої сировини кормів рослинного походження.

При проведенні експериментів застосовували молекулярні, імунологічні, клінічні, патологоанатомічні, серологічні, цитологічні та статистичні методи дослідження.

У досліді на білих щурах, які одержували сою з кормом упродовж 60 діб, було встановлено, що рівні хромосомних ушкоджень у клітинах кісткового мозку у контрольних і дослідних тварин були в межах фізіологічної норми і вірогідно не відрізнялись.

В експерименті з вивчення впливу ГМ сої на гуморальну ланку імунітету, визначено, що через 14 діб після введення еритроцитів барана рівень вмісту антитіл у тварин дослідної групи був вірогідно вищим від контролю на 15,85 %. У тесті з оцінки дії генетично модифікованої сировини кормів на резистентність мишей до Salmonella enterica ser. Typhimurium продемонстровано позитивний вплив ГМ сої лінії 40-3-2: у тварин дослідної групи, що були заражені в дозі 10^6 КУО/миша, смертність була нижчою від контролю; а рівень аглютинуючих антитіл до збудника на 21 добу після зараження у тварин дослідних груп був вірогідно вищим від контрольних на 30 %. Водночас експериментально доведено, що ГМ соя лінії 40-3-2 не впливає на клітинну ланку імунітету і не виступає в якості сенсибілізуючого фактору.

Таким чином встановлено, що генетично модифікована соя лінії 40-3-2 при використанні її в якості сировини кормів не чинить мутагенної дії, але має імуномоделюючі властивості, що потребує більш детального вивчення.

Ключові слова: ГМ рослини, соя лінії 40-3-2, корми, тварини, імунна система, мутагенна дія.