



# Assessment of the impact of genetically modified LibertyLink<sup>®</sup> maize on reproductive function and progeny development of Wistar rats in three generations

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## ABSTRACT

This publication presents the assessment of the impact of genetically modified (GM) LibertyLink<sup>®</sup> maize on reproductive function, prenatal and postnatal progeny development of Wistar rats over three generations. The animals were divided into two groups, which were fed with rodent diet with inclusion of GM LibertyLink<sup>®</sup> ('test' group) and non-GM near-isogenic counterpart ('control' group) maize varieties. The maize was included into the diet at maximum possible level (between 32 and 33%) not causing nutritional imbalance or metabolic disturbance for the experimental animals.

Data analysis showed no impact of LibertyLink<sup>®</sup> maize on the animals' fertility: the observed mating efficiency in both groups was within the normal expected range values under the given experiment conditions. The comparison of progeny prenatal development in the generations F0–F2 has not shown any differences between the groups. Analysis of the physical development of the F0–F2 progeny or pups body weight and length progress did not show any abnormalities. The average number of pups per litter in the control and test groups was within the expected range of variations. Therefore, the results should be considered as direct evidence of the lack of any reproductive toxicity of LibertyLink<sup>®</sup> maize (a.k.a. T25 maize).

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## 1. Introduction

During the past 17 years, GM crops have been broadly adopted. For the period from 1996 to 2013 the global acreage of GM crops increased more than 100-fold, having reached 175.2 million hectares. According to leading experts, the 50% of World agricultural production will have been reached with the excessive use of biotechnology by 2030 [1].

Analysis of publications on GMO safety research varied by design, duration of study, and parameters set leads us to the conclusion that the studies carried out under the standard requirements in terms of the selection of appropriate samples, selection of animals, diet, living conditions, etc., has shown no negative impact of GMOs on the studied parameters [2–10]. In this case the 'standard requirements' mean that the experimental design meets the FAO/WHO/OECD recommendations [11–13] on the safety assessment of GMO.

On the contrary, the majority of experiments conducted with deviations from the standard requirements on the safety assessment of GMO [11–13], has shown exactly the opposite results. Well-known examples of such

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publications are articles of [14–20] and so on. Nevertheless, the debate of this issue is still on, so the completion of science-based evidence of GMO safety has not lost its relevance, particularly, in the field of the next generations' health.

The system of state registration of genetically modified (GM) plants in the Russian Federation includes several toxicological studies, which can be completed by genotoxicological, allergenicity and/or reprotoxicity testing on ad hoc basis. According to established researcher practice, the GMO reprotoxicity study was optional [21,22], but since 2011 the safety assessment of new GM events within their state registration procedure in Russia has also involved the reproductive toxicity studies in experimental animals generations.

The laborious and time-consuming reproductive toxicity methods are a serious restriction of their application. However, their high sensitivity and predictability, focusing on detailed assessment of the health status of the next generations, can be an important argument in favor of conducting investigations of this kind [23–25].

The purpose of the current work was to study the impact of GM LibertyLink® maize on reproductive function and progeny development of Wistar rats over three generations. The animals have been fed with rodent diet containing GM LibertyLink® maize.

GM LibertyLink® maize by Bayer CropScience company came out on the market in the year 1995. This line was developed through chemically mediated transformation of cultured protoplasts obtained from a yellow dent corn (*Zea mays* ssp. *Linnaeus*) with purified DNA containing the *pat* gene isolated from *S. viridochromogenes* utilizing pUC/Ac vector. Vector pUC/Ac contains a *pat* gene expression cassette that encodes synthesis of Phosphinotricin N-acetyltransferase enzyme and a *bla* gene expression cassette included as a selectable marker. The presence of *pat* gene determines this maize line's resistance to glufosinate ammonium (the active ingredient in phosphinothricin herbicides – Basta, Rely, Finale, and Liberty), the *bla* gene is not functional in the modified maize line, as its promoter is only active in bacteria. Protein Pat content in GM LibertyLink® maize tissues is ~0.003% of the total protein [26].

Genetic and phenotypic stabilities of the trait were sustained in several-generations studies of LibertyLink® maize. The maize progeny resistance to glufosinate ammonium is inherited as a dominant trait according to Mendelian principles [26].

GM LibertyLink® maize risk assessment was carried out in accordance with the recommendations of the WHO, the Commission “Codex Alimentarius”, and the Office of the Food and Drug Administration, U.S. Government (Food and Drug Administration) and others [11,12,27–29]. The assessment included the GM maize substantial equivalence evaluation compared to its conventional counterpart [30]; the toxic and allergenic properties estimation of the protein responsible for the resistance to glufosinate ammonium manifestation; the toxic and allergenic properties estimation of LibertyLink® maize grain.

The received data indicate compositional equivalence of GM maize compared to its traditional counterpart (based

on analysis of more than 30 indicators), the absence of toxic and allergenic properties of the protein Pat (based on the number of tests, which included the in vivo study of acute toxicity by oral administration, the in vitro determination of resistance to proteolytic degradation in simulated mammalian gastrointestinal fluids, etc.) and the absence of toxic and allergenic properties of the GM LibertyLink® maize (based on both in vitro and in vivo experiments) [22,26]. The results of these studies have led to entering the food market in the Argentina, Australia, Brazil, Canada, China, Colombia, the European Union, Japan, Malaysia, Mexico, New Zealand, the Philippines, Russian Federation, South Africa, South Korea, Taiwan, USA, with GM LibertyLink® maize [26].

The decision to conduct the reproductive toxicity study was to broaden the knowledge base and to confirm that studies, required by the Russian regulations are sufficient to address the safety of GM plants.

In this very feature the research results of rat's reproductive function, prenatal and postnatal progeny development are presented.

## 2. Materials and methods

### 2.1. Experimental design and treatment

The experiment was performed on two animal groups from September 2007 to October 2008. The animals in the 'test' group were exposed to diet containing the LibertyLink® maize, the animals in the 'control' group were exposed to the non-GM near-isogenic counterpart. The animals had been fed with the experimental diets during the entire period of the study.

The experiment was conducted in two stages: the 1st stage was preparatory for generation F<sub>0</sub> rats breeding. Generation F<sub>0</sub> rats had been receiving the experimental diets during the whole ontogenetic development time and were optimally standardized for the purpose of this research. In this case, the animals standardization consisted of females and males selection from litters similar in number of pups and progeny survival skills. The 2nd stage was the direct reproductive function study of generations F<sub>0</sub> and F<sub>1</sub> rats, and progeny F<sub>2</sub> development. A total of 380 adult animals and 1540 of pups were included in the experiment (Table 1).

The basic colony rats (F<sub>b</sub>, males and females age ~30–35 days) fed on conventional rodent diet [31] were randomly divided into two groups and were switched to the above mentioned customized corn diets.

In order to impregnate females, they were housed together with males in 2:1 ratio for the equivalent of one estral cycle (5 days). During the mating period, the rats' age was 100–120 days. Pups were set apart from parental animals on the 26th day of life. The progeny was randomly selected from different females in order to minimize the chance of incest breeding during the course of the experiment.

The study design was adapted from Medico-biologic safety assessment of genetically engineered and modified

**Table 1**  
Composition of the experimental groups and number of animals.

Group		F <sub>b</sub>	F <sub>0</sub>	F <sub>1</sub>	Total
Control group	Adults	35♀ + 20♂	50♀ + 25♂	40♀ + 20♂	190
	Progeny	236	300	242	778
Test group	Adults	35♀ + 20♂	50♀ + 25♂	40♀ + 20♂	190
	Progeny	162	354	246	762

A total of 380 adult animals and 1540 of pups were included in the experiment over three generations.

organisms of plant origin: Methodological instructive regulations MU 2.3.2.2306-07 [31].

## 2.2. Ethics

The experimental protocol was conducted in accordance with the regulations of 'On approval of the Good Laboratory Practice rules' authorized by the Ministry of Healthcare of the Russian Federation (Order No 267 dated July 19, 2003). Federal State Budgetary Institution "Institute of Nutrition" under the Russian Academy of Medical Sciences (FSBI "Institute of Nutrition" RAMS) strictly follows standard procedures of the humane use of laboratory animals as well as in requirements regarding the keeping and breeding of laboratory animals in nurseries and vivariums and their use in scientific, educational and industrial purposes. The research materials were reviewed by the Ethics Committee of the Institute of Nutrition (protocol No. 6 dated December 20, 2010).

## 2.3. Animals

The basic colony rats (F<sub>b</sub>, males and females age ~30–35 days) were received from the animal nursery 'Stolbovaya' of the Russian Academy of Medical Sciences.

Rats had been kept in plastic cages with wood shavings, in heated ( $T \sim 20$  to  $23^\circ\text{C}$ ) and ventilated room with natural light. They had free (ad libitum) access to conventional rodent diet [31] and water. The number of rats in one cage was 3 (2♀:1♂) during mating period, one female with her pups during birthing and lactation periods. During intermediate periods there were 3–5 rats of the same sex in one cage. Animals continued on experimental diets for all period of experiment (from September 2007 to October 2008).

All animals were observed once daily for mortality, moribundity and for overt signs of toxicity. Individual body weights were obtained once weekly for 30–100 days of age. Pups' body weight and length were measured on post-natal days 2, 5, 10, 15, 20 and 25th. Individual food consumption was considered on 30, 45 and 90 days of life.

## 2.4. Maize samples

The samples of LibertyLink® maize and the non-GM near-isogenic counterpart were used. The samples had been grown under identical conditions (i.e. same planting locations in the USA and processing). The material was provided by Bayer CropScience.

All maize grain samples were assessed to confirm that they met the sanitary requirements of the Russian

Federation (Sanitary and Epidemiological rules and regulations, SanPiN 2.3.2.1078-01) [31,32]. The hygienic investigation included the detection of heavy metals, pesticides, mycotoxins and benz(a)pyren content (Table 2). The fumonisins content showed slight variations between the different maize samples. The fumonisin's daily intake was estimated for each experimental group: in the 'test' group's diet the fumonisin contents was  $<0.0128$  mg/kg (0.00085–0.00128 mg/kg of body weight), in the 'control' group's diet the fumonisin content was 0.0173 mg/kg (0.00115–0.00173 mg/kg of body weight) respectively. As described in available sources, kidneys are the most sensitive target organ for fumonisins as far as rats are concerned, with a NOAEL equal to 15 mg/kg of feed (corresponding to 0.25 mg/kg of body weight) [33–35]. In conclusion, the fumonisin levels were shown to be significantly lower than the NOAEL. So the observed fumonisin level variations between the different maize samples would have a minor biological incidence and the safety impact should be negligible.

## 2.5. Animal diet preparation

The maize grain processing included a gradual whole grain reduction by grinding with micro-mill equipment until the condition of flour substance consisting of 400–500  $\mu\text{m}$  particles. The milled grain was included into the diet to achieve an exposure of 8–10 g/rat/day. Diet ingredient formula was adjusted in order to produce nutritionally balanced diets, in accordance with the principle of isocaloricity and equivalence of chemical composition of diets [31]. The diet compositions are presented in Table 3.

## 2.6. Reproduction and developmental assessment

The assessment of the reproductive function was focused on the fertility of parent animals' as well as on prenatal and postnatal development characteristics of the F<sub>0</sub>, F<sub>1</sub> and F<sub>2</sub> progenies. In the context of this study, the mating efficiency (fertility) was defined as the ability of males to impregnate females, or as the proportion of females to be impregnated. The fertility index was taken as a ratio of fertilizing ability of males over the total number of co-housed males, or as a ratio of pregnant females over the total number of co-housed females. Since the females were put together with males in 2:1 ratio, pregnancy of both, or either female(s) confirmed male's fertilizing ability. In case neither of the females has become pregnant, the male was not considered to be fertile and the females were considered potentially fertile.

**Table 2**  
Sanitary evaluation and chemical composition of maize samples.

Studied indicator	Near-isogenic control	GM maize	Sanitary norms [32]
<i>Toxic elements (mg/kg)</i>			
Lead	n/d	n/d	0.5
Arsenicum	n/d	n/d	0.2
Cadmium	n/d	n/d	0.1
Mercury	n/d	n/d	0.03
<i>Pesticides (mg/kg)</i>			
Hexachlorcyclohexane	n/d	n/d	0.5
DDT and its metabolites	n/d	n/d	0.02
Aldrine	n/d	n/d	–
Hexachloran	n/d	n/d	–
Heptachlor	n/d	n/d	–
Kelthane	n/d	n/d	–
Organic-mercury pesticides	n/d	n/d	Not admitted
2,4-D acid, its salts and ethers	n/d	n/d	Not admitted
Benz(a)pyren	n/d	n/d	0.001
<i>Mycotoxins (mg/kg)</i>			
Aflatoxin B <sub>1</sub>	n/d	n/d	0.005
Desoxynivalenol	n/d	n/d	–
T-2 toxin	0.015 ± 0.005	0.011 ± 0.006	0.1
Zearalenon	n/d	n/d	1.0
Fumonisin B <sub>1</sub>	0.054 ± 0.038	n/d	–
Fumonisin B <sub>2</sub>	n/d	n/d	–
<i>Chemical composition</i>			
Protein (%)	8.54 ± 0.09	9.32 ± 0.06	–
Fat (%)	4.14 ± 0.27	4.45 ± 0.32	–
Carbohydrates (%)	61.40 ± 0.62	60.86 ± 0.32	–
Sum of alimentary fibers (%)	15.64 ± 0.54	15.12 ± 0.32	–
Ash (%)	1.12 ± 0.11	1.05 ± 0.09	–
Moisture (%)	9.07 ± 0.47	9.23 ± 0.48	–
Calories (kCal)	311.8–322.3	316.4–325.2	–

n/d = non defined.

N = 12.

The samples of LibertyLink<sup>®</sup> maize and the non-GM near-isogenic counterpart had been grown under identical conditions (i.e. same planting locations in the USA and processing). The material was provided by Bayer CropScience.

**Table 3**  
Composition of the experimental diets with inclusion of maize.

Diet ingredients	Weight (g)	Protein (g)	Fat (g)	Carbohydrates (g)
<i>Test group (GM maize)</i>				
Casein	19.7	16.7	0.3	0.0
Maize starch	34.6	0.3	0.0	30.0
Sunflower oil	3.7	0.0	3.7	0.0
Lard	5.0	0.0	5.0	0.0
Salt mixture <sup>a</sup>	4.0	0.0	0.0	0.0
Mixture of w/s vitamins <sup>a</sup>	1.0	0.0	0.0	1.0
Mixture of o/s vitamins <sup>a</sup>	0.1	0.0	0.1	0.0
Maize meal	32.0	2.7	1.3	19.6
<i>Total</i>	g 374.8	19.7 78.8	10.4 93.6	50.6 202.4
<i>Control group (near-isogenic maize)</i>				
Casein	19.3	16.3	0.3	0.0
Maize starch	34.0	0.3	0.0	29.4
Sunflower oil	3.5	0.0	3.5	0.0
Lard	5.0	0.0	5.0	0.0
Salt mixture <sup>a</sup>	4.0	0.0	0.0	0.0
Mixture of w/s vitamins <sup>a</sup>	1.0	0.0	0.0	1.0
Mixture of o/s vitamins <sup>a</sup>	0.1	0.0	0.1	0.0
Maize meal	33.1	3.1	1.5	20.1
<i>Total</i>	g 374.8	19.8 79.2	10.4 93.6	50.5 202.0

<sup>a</sup> Diet ingredient formula was adjusted in order to produce nutritionally balanced diets, in accordance with the principle of isocaloricity and equivalence of chemical composition of diets [31].

For prenatal development assessment of 9–11 females from  $F_b$ ,  $F_0$  and  $F_1$  of each group were euthanized on the 20th day of pregnancy (one day prior to the expected day of delivery). The uteruses were removed by cesarean section and the uteruses and the fetuses were examined. The females were examined macroscopically for any structural abnormalities or pathological changes.

The number of ovarian corpora lutea, resorptions, implantation sites, the number of alive and dead fetuses, the pre-implantation loss (i.e. difference between the number of ovarian corpora lutea in ovaries and the number of implantation sites in uterus) as well as the post-implantation loss (i.e. difference between the number of implantation sites in uterus and the number of alive fetuses) were determined.

Postnatal  $F_0$ ,  $F_1$  and  $F_2$  progeny development was being assessed during the first month of life by counting the number of alive and dead pups, dynamic of body weight and length, physical developmental landmarks (i.e. ear unfolding, first coat, incisor eruption, eye opening, testicle lowering, vagina opening). Pups' body weight and length were measured on post-natal days 2, 5, 10, 15, 20 and 25th. The average litter size, the male-to-female ratio and the postnatal survival index were calculated from 1st to the 5th day of life (i.e. the ratio between the number of pups being alive on the 5th day to the total number of pups born alive) and from the 6th to the 25th day of life (i.e. ratio between the number of pups being alive on the 25th day to the total number of pups being alive on the 5th day). This assessment was performed in accordance with the Russian Guidelines [21,31] and was also considering the US FDA and OECD methodical guidelines [13,36].

## 2.7. Statistical analysis

According to the experiment structure the comparison between control and GM-fed groups has been performed. Data were presented as  $M \pm SE$  and min–max, where M was

the mean, SE was the standard error, and min–max were the minimal and maximal values, as well as percentage or absolute figures.

Statistical analysis was executed with the use of SPSS 17.0 software package (IBM, USA). The homogeneity of variance and normal distribution between groups was determined by chi-square test, while the variance equality was measured by Levene's test. Reliability assessment of the mean figures differences of data normally distributed was analyzed by one-way ANOVA. For comparison of quantitative parameters not being normally distributed, non-parametric test was applied (U-criterion of Mann–Whitney). Statistical significance was assigned at the  $p < 0.05$  level [37,38].

## 3. Results and discussion

### 3.1. Clinical signs and mortality

Throughout the study, no adverse effects on  $F_b$ – $F_2$  animals' behavior were observed. The animals were examined once daily for well-being. The food and water consumption, appearance and growth of test group rats illustrate normal and similar health condition patterns within and between the two groups. No mortality was recorded during the experiment period.

### 3.2. Body weight progress and food consumption

Weekly body weight progress in all groups of rats at the age of 30–93 days followed the Wistar rats' normal body weight progress (Figs. 1–3) that corresponds with data of reliable sources [39–42] as well as the results of the similar experiment with conventional maize varieties conducted by FSBI "Institute of Nutrition" RAMS. Food consumption was generally similar between test and control groups, and fell within 13.3–29.4 g/rat/day for males and 12.4–25.7 g/rat/day for females in the  $F_b$ – $F_1$  generations.

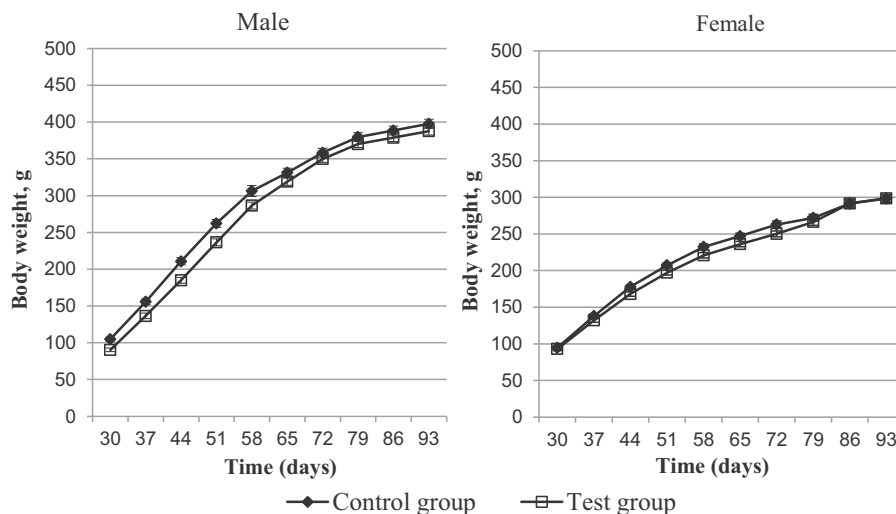


Fig. 1.  $F_0$  body weight progress.

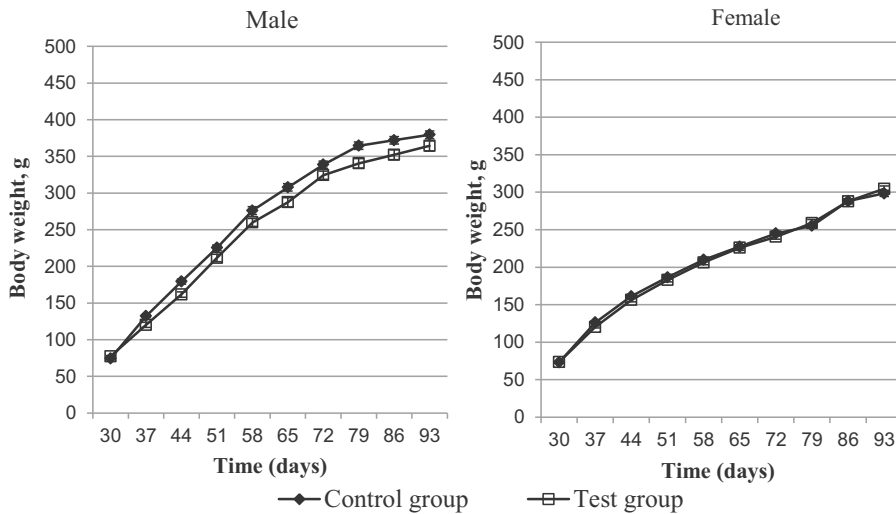


Fig. 2. F<sub>1</sub> body weight progress.

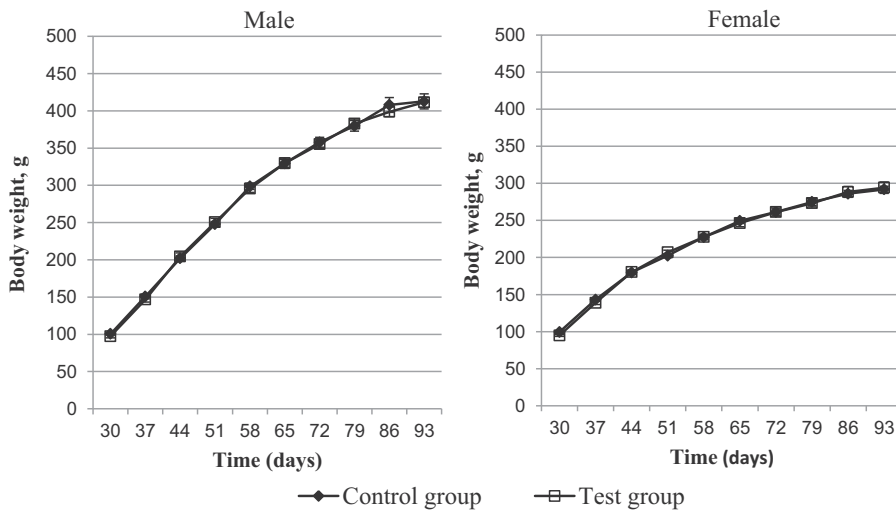


Fig. 3. F<sub>2</sub> body weight progress.

Weekly body weight progress of pregnant females was in whole comparable for both groups. There were no statistically significant differences observed between the test and control groups.

### 3.3. Reproductive function

Key indicator of reproductive function is the mating efficiency, proving experimental animals' fertility. As presented in Table 4, the females' mating efficiency was 71–92%, 79–80% and 77–87% in the F<sub>b</sub>, F<sub>0</sub> and F<sub>1</sub> generations. The males' mating efficiency was 100%, 89–95% and 100% accordingly. The maximum fluctuations range of this parameter, for both females and males, was revealed in the basic colony of rats just received from the external animal nursery. The minimum fluctuations range was revealed in

the F<sub>1</sub> generation, grown under the standardized vivarium conditions.

The observed mating efficiency in both groups was in the normal expected range values under the given experiment conditions [21,43], that matches with the results of the comparable experiment with conventional maize varieties.

The duration of co-housing period (5 days) was equal to the mean rat's estral cycle duration [21]; however, in accordance with Mandl [43], 11–28% of rats may have a longer estral cycle duration (5.5–10 days). Therefore, it is very likely that some of co-housed females never reached estrus phase during the given time period for this particular reason. The number of males considered non-fertile, fell in the range of physiological variations [44] and varied within 0–13%. Several fluctuations of mating efficiency in control



**Table 4**  
Mating efficiency.

Generation	Group	Gender	Number of co-housed rats	Number of impregnate ♀/number of fertile ♂	Mating efficiency <sup>a</sup> , %
F <sub>b</sub>	Control group	♀	24	22	92
		♂	12	12	100
	Test group	♀	24	17	71
		♂	12	12	100
F <sub>0</sub>	Control group	♀	38	26	79
		♂	19	17	89
	Test group	♀	40	30	80
		♂	20	19	95
F <sub>1</sub>	Control group	♀	30	26	87
		♂	15	15	100
	Test group	♀	30	23	77
		♂	15	15	100

<sup>a</sup> The fertility index was taken as a ratio of fertilizing ability of males over the total number of co-housed males, or as a ratio of pregnant females over the total number of co-housed females. Since the females were put together with males in 2:1 ratio, pregnancy of both, or either female(s) confirmed male's fertilizing ability. In case neither of the females has become pregnant, the male was not considered to be fertile and the females were considered potentially fertile.

and test groups had no definite tendency and were thus considered as incidental and not connected with external factors.

#### 3.4. Prenatal development

The comparison of progeny prenatal development in the test and the control groups of generations F<sub>0</sub>–F<sub>2</sub> (Table 5) has not shown any significant differences between the groups. The number of ovarian corpora-lutea, resorption and implantation sites, the number of alive and dead fetuses, the pre-implantation loss fell within the typical range for Wistar rats physiological variations (Table 6). Spontaneous post-implantation loss in the F<sub>0</sub>–F<sub>2</sub> control and test groups embryos was slightly lower than the normal range of 3.6–9.2% (Table 5). In the generation F<sub>0</sub> post-implantation loss was 0%, in generation F<sub>1</sub> it was 0% in the control group and 0.9 ± 0.9% in the

test group, in generation F<sub>2</sub> it was 0.8 ± 0.8% and 3.7 ± 3.0% in the control and test groups accordingly (Table 5). We should point out that similar-in-design studies have shown very close values of post-implantation loss over three generations of Wistar rats (1.2–9.9%), which confirms the absence of treatment-related finding and illustrates a rather wide variation range of this parameter.

#### 3.5. Postnatal development

Postnatal progeny development in the test and control groups, during first stage of the experiment, is characterized by a transient decrease of F<sub>0</sub> progeny survival rate compared to generations F<sub>1</sub> and F<sub>2</sub> (Table 7). From 1st to the 5th days of life the F<sub>0</sub> control group's progeny survival rate was 96%, during the period from the 6th to the 25th days it was 81%. In the test group of generation F<sub>0</sub> these indicators were 94% and 90% accordingly. The analysis of the causes

**Table 5**  
Prenatal development of progeny F<sub>0</sub>, F<sub>1</sub> and F<sub>2</sub>.

Recorded indicators	Generation F <sub>0</sub>		Generation F <sub>1</sub>		Generation F <sub>2</sub>		
	Control group	Test group	Control group	Test group	Control group	Test group	
Number of pregnant females	10	10	10	9	11	11	
Number of ovarian corpora-lutea	Total	117	104	127	117	124	142
	M ± SE	11.70 ± 0.72	10.40 ± 0.58	12.70 ± 0.54	13.00 ± 0.53	11.18 ± 0.55	12.91 ± 0.55
	Min–max	9–15	8–14	10–15	11–15	8–14	10–16
Number of implantation sites	Total	109	94	120	107	114	132
	M ± SE	10.90 ± 0.67	9.40 ± 0.70	12.00 ± 0.54	11.89 ± 0.77	10.27 ± 0.81	12.00 ± 1.04
	Min–max	7–14	6–13	9–14	8–14	5–14	3–16
Number of alive fetuses	Total	109	94	120	106	113	131
	M ± SE	10.90 ± 0.67	9.40 ± 0.70	12.00 ± 0.54	11.78 ± 0.76	10.27 ± 0.81	11.91 ± 1.12
	Min–max	7–14	6–13	9–14	8–14	5–14	2–16
Number of dead fetuses	0	0	0	1	0	0	
Number of resorptions	Total	0	0	0	0	1 <sup>a</sup>	1
	M ± SE	–	–	–	–	0.09 ± 0.09	0.09 ± 0.09
	Min–max	–	–	–	–	0–1	0–1
Pre-implantation loss (%)	6.7 ± 2.5	10.0 ± 3.6	5.5 ± 1.3	9.1 ± 3.4	8.7 ± 4.7	8.0 ± 6.5	
Post-implantation loss (%)	0	0	0	0.9 ± 0.9	0.8 ± 0.8	3.7 ± 3.0	

<sup>a</sup> Out of uterus pregnancy.

**Table 6**  
Prenatal development of progeny: literature data.

Recorded indicators	Literature references					
	[52]	[45]	[53]	[46]	[50]	[48]
Number of pregnant females	26	150	15	11	47	10
Number of ovarian corpora-lutea	12.3 ± 0.2	–	14.0 ± 0.4	14.5 ± 0.3	13.5 ± 0.2	15.8 ± 1.3
Number of implantation sites	11.2 ± 0.4	–	13.2 ± 0.3	13.5 ± 0.39	12.6 ± 0.2	15.4 ± 1.4
Number of alive fetuses	10.8 ± 0.4	10.6 ± 0.3	10.73	12.3 ± 0.3	11.5 ± 0.3	14.6 ± 1.9
Number of resorptions	0.42 ± 0.2	7.2 ± 1.2	–	0	–	0
Pre-implantation loss (%)	8.5 ± 3.0	14.1 ± 1.5	5.14	6.2	7.1 ± 1.4	–
Post-implantation loss (%)	3.6 ± 1.4	7.4 ± 1.2	5.77	9.2	8.6 ± 1.7	4.69

**Table 7**  
Postnatal development of progeny F<sub>0</sub>, F<sub>1</sub> and F<sub>2</sub>.

Recorded indicators	Generation F <sub>0</sub>		Generation F <sub>1</sub>		Generation F <sub>2</sub>	
	Control group	Test group	Control group	Test group	Control group	Test group
Total number of pups	237	164	300	354	244	246
Of those – still born	1	2	0	0	2	0
Mean litter size (M ± SE)	10.73 ± 0.59	9.53 ± 0.59	11.54 ± 0.60	11.80 ± 0.27	9.68 ± 0.77	10.70 ± 0.50
Ratio ♂/♀ in the litter (%)	47/53	52/48	52/48	49/51	51/49	49/51
Survival from the 1st to the 5th day of life (%)	96	94	98	99	98	99
Number of alive (initial)/number of died	236/10	162/10	300/7	354/4	242/4	246/1
Survival from the 6th to the 25th day of life (%)	81	90	96	95	98	98
Number of alive (initial)/number of died	226/44	152/15	293/12	350/19	238/4	245/5

of this transient higher mortality has revealed that 55% of this index value (24 from 44 dead pups) in control group was mainly due to tree litters with the total number of 32 pups born alive. The most probable hypothesis is that the parental F<sub>0</sub> females, just received from the external animal nursery, did not have enough breast milk. This hypothesis is supported by the fact that the progeny death had occurred in the period from 6th to the 15th days of life, before the pups switched from breast feeding to mixed feeding, and by the pups' relatively low body weight progress: on the 2nd day of life the mean body weight was 6.2 ± 0.1 g (within the normal range), on the 5th day of life it was 7.6 ± 0.2 g (~30% below the normal range), on the 15th day of life it was 21.5 ± 2.7 g (~25% below the normal range).

In the generations F<sub>1</sub> and F<sub>2</sub>, the progeny survival rates from 1st to the 5th days of life were 98–99% and 95–96% from 6th to the 25th days of life, respectively (Table 7). Significant differences between control and test groups have not been shown.

The observed progeny survival rates in both groups was in the normal expected range values that corresponds with the results of the similar experiment with conventional maize varieties conducted by FSBI "Institute of Nutrition" RAMS: the progeny survival rates from 1st to the 5th day

of life were 87–99%, from 6th to the 25th day of life were 94–100%.

In accordance with several sources [44], Wistar rats line has a relatively high variability of some reproductive function indicators, including the progeny survival rate during the first month of life (this index may not exceed 68%) (Table 8). In addition, it has been shown that standardized living conditions help reducing the variability to a great degree [45]. In the present study, the maximum survival fluctuations rate was observed during the first stage of the experiment in progeny of F<sub>0</sub> rats, just received from the external animal nursery. The minimum survival fluctuations rates were observed in progenies of F<sub>0</sub> and F<sub>1</sub> rats grown under the Institute of nutrition's vivarium conditions. This fact speaks volumes about general keeping conditions having key influence on the researched indicators.

The average number of pups per litter in the control and test groups were within the expected range of variations, that agrees with the results of the own identical researches with conventional maize varieties (the range was within 9.53–11.80 pups per litter) and values of reliable sources (Table 8). No statistically significant differences were detected between groups (Table 7). The ratio

**Table 8**  
Postnatal development of progeny: reliable sources.

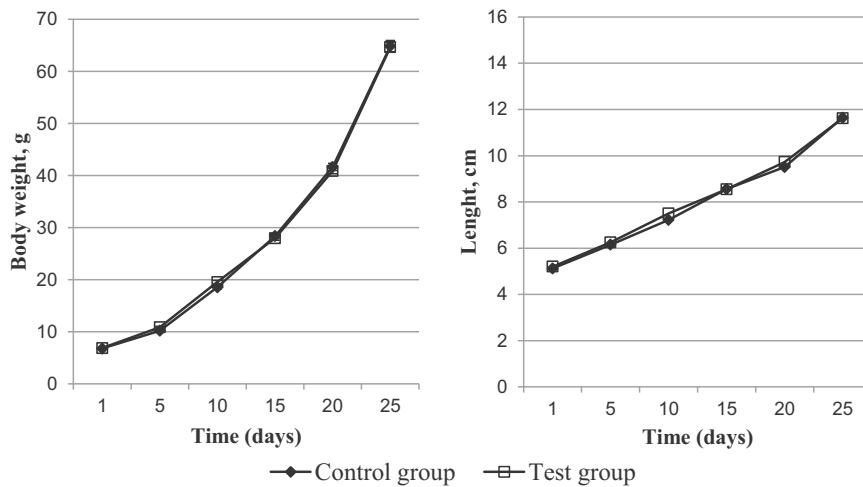
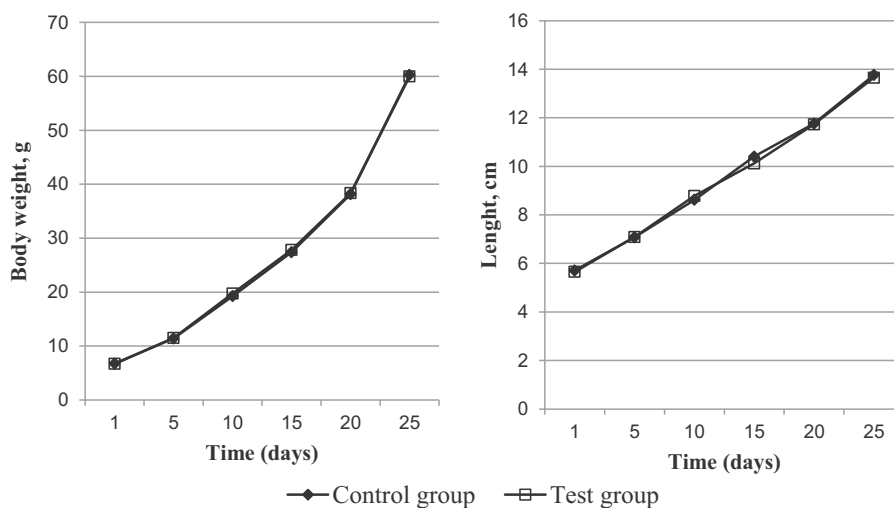
Recorded indicators	References					
	[49]	[51]	[47]	[48]	[46]	[44]
Total number of pups	215	133	28	146	135	>300
Of those – still born	4	5	1	–	1	–
Mean litter size (M ± SE)	11.4 ± 0.2	11.1 ± 2.0	9.5 ± 0.5	14.6 ± 0.6	12.3 ± 0.1	11.67 ± 0.1
Survival from the 1st to the 5th (%)	97.0	96.0	99.3	–	–	96.8
Survival from the 6th to the 25th (%)	99.0	–	98.6	–	–	67.9
Ratio ♂/♀	46/54	45/55	–	48/52	47/53	47/53



**Table 9**Physical development of the pups F<sub>0</sub>, F<sub>1</sub> and F<sub>2</sub>.

Recorded indicator	Generation F <sub>0</sub>		Generation F <sub>1</sub>		Generation F <sub>2</sub>	
	Control group	Test group	Control group	Test group	Control group	Test group
Ear unfolding, day	2.66 ± 0.08	2.71 ± 0.06	2.81 ± 0.05	2.95 ± 0.04	2.86 ± 0.08	2.89 ± 0.07
First coat, day	5.48 ± 0.08	5.56 ± 0.13	5.31 ± 0.10	5.74 ± 0.11 <sup>a</sup>	5.98 ± 0.07	5.76 ± 0.09
Incisor eruption, day	9.07 ± 0.11	9.29 ± 0.13	9.25 ± 0.14	9.72 ± 0.12 <sup>a</sup>	9.46 ± 0.04	9.59 ± 0.15
Eye opening, day	15.05 ± 0.09	15.26 ± 0.11	15.44 ± 0.11	15.62 ± 0.14	15.18 ± 0.07	15.26 ± 0.22
Testicle lowering, day	23.86 ± 0.11	24.00 ± 0.16	23.98 ± 0.22	23.90 ± 0.18	23.72 ± 0.22	23.97 ± 0.23
Vagina opening, day	28.75 ± 0.09	28.97 ± 0.10	28.73 ± 0.26	28.29 ± 0.17	28.70 ± 0.12	28.83 ± 0.12

<sup>a</sup> According to the reliable sources [21,50], the normal post-natal indicators of Wistar pups physical development are as follows: ear unfolding from 2nd day, first coat from 5th day, incisor eruption from 6th day, eye opening from 12th day, testicle lowering from 18th day, vagina opening from 28th day.

**Fig. 4.** F<sub>0</sub> pups body weight and length progress.**Fig. 5.** F<sub>1</sub> pups body weight and length progress.

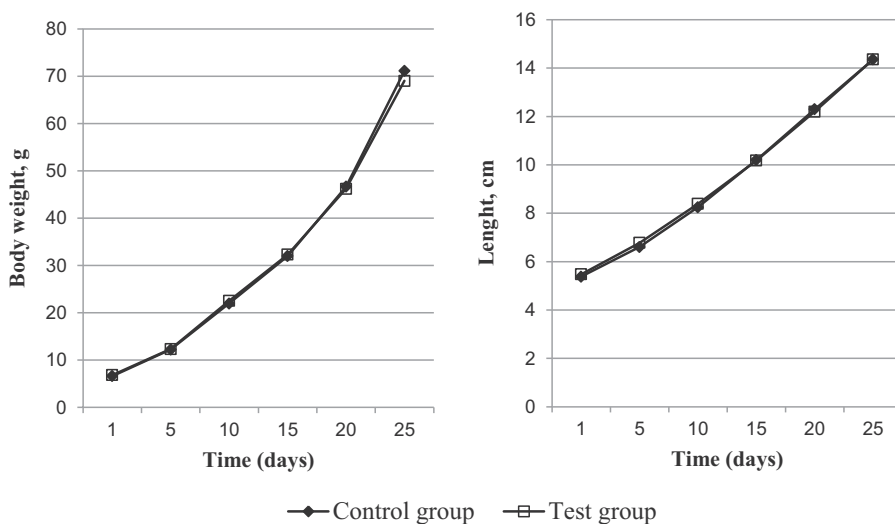


Fig. 6. F<sub>2</sub> pups body weight and length progress.

between males and females in the groups differed slightly in each generation. However, these variations did not have any distinct trends and fell within the typical value limits for Wistar rats [44,46–49]. Analysis of the physical development of the F<sub>0</sub>–F<sub>2</sub> progeny (ear unfolding, first coat, incisor eruption, eye opening and others) (Table 9) or pups body weight and length progress (Figs. 4–6), did not show any abnormalities [48–51].

#### 4. Conclusion

In conclusion, the findings from rat fed diets containing LibertyLink<sup>®</sup> maize were similar to those fed diets containing non-GM near-isogenic counterpart. The evaluation of LibertyLink<sup>®</sup> maize potential reproductive toxicity over three generations of Wistar rats has not revealed any negative impacts on the reproductive function or on the pre- and post-natal rat progeny development.

The obtained data corresponds with the results of the identical reproductive toxicity study performed with conventional maize varieties, as well as a review of published information and demonstrates that the reproduction and development findings observed with LibertyLink<sup>®</sup> maize were within the normal range of variations for the various studied parameters. Therefore, the results of this investigation should be considered as evidence of the lack of any negative impact of GM LibertyLink<sup>®</sup> maize on the reproductive function and Wistar rats' progeny development.

#### Conflict of interest

The authors declare that there are no conflict of interest.

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