Effects of Glyphosate-resistant Soybeans on Sperm Numbers and Quality in Cyclophosphamide-induced Mice

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Abstract

Roundup Ready soybean harbors a gene encoding 5-enolpyruvylshikimate-3- phosphate synthase (EPSPS), which confers resistance to glyphosate. Cyclophosphamide (CP) is a cytotoxic alkylating agent widely used as anticancer drug and immunosuppressive agent. However, a range of adverse effects including reproductive toxicity could not be ignored. To assess the safety of Glyphosate-resistant soybeans and whether they can impact on the testis, sperm quantity and quality and sperm functions in chemotherapy pathological conditions, which induced by CP. Glyphosate –resistant soybean and non-genetically modified soybeans were fed to the mice for 30 days and 90 days, which injected with CP for 30 days or not. The counts, quality, integrity of sperm membrane, nuclear maturity and acrosomal reaction of sperm were analyzed. The results showed that the sperm count, motility, viability and sperm abnormalities and membrane integrity had a statistically significant difference ($P<0.01$) between the CP group and the CP-untreated group of mice. And sperm nuclear maturity and acrosome reaction also had a significant difference ($P<0.05$). But compared with the non-genetically modified soybeans, Glyphosate –resistant soybean did not cause significant difference ($P>0.05$) on sperm parameters both in CP group and CP-untreated group. The results demonstrated that no matter the mice under normal physiological conditions or chemotherapy pathological conditions, 30 and 90 days of Roundup Ready soybean feeding had non-related potential toxic effects on mice sperm quantity and quality.

Keywords

Glyphosate-resistant; Cyclophosphamide; Reproductive Toxicity; Sperm Count; Sperm Quality; Acrosome Reaction.

1. Introduction

As the most important legume crop on the international market, soybean is cultivated and consumed worldwide, both Amercia, Brazil and Agrentina are the major soybean producing countries [1-2]. To improve the output of soybean, glyohosate was widely used to extripate weed from mid-1990s. Glyphosate, a non selective and broad-spectrum herbicides, works by inhibiting competitively the enzyme 5-enol-pyruvyl shikimate-3-phosphate synthase (EPSPS), which is the essential enzyme of aromatic amino acid biosynthetic pathway and just present in plants and microorganism [3-4]. Although this reaction just occur in plants and microorganism, lacking of selective is still a big challenge of using glyphosate [5]. In order to distinguish the crops from weeds during the use of glyphosate, genetical engineering was applied in agriculture and the glyphosate-resistan soybean, Roundup Ready soybean, has became commercial from 1996 [6]. Since that time, this technology has been applied in many crops, such as maize, cotton and rice. Today, over 90%, 70% and 99% soybean grown in Amercia, Brazil and Argentina are glyphosate resistant.

In 1983, the first glyphosate-resistan tobacco was born in Amercia. According to the report of the International Service for the Acquisition of Agri-biotech Applications (ISAAA) in 2017, after more than 30 years of development, the acreage of genetically modified crops more than 189.8 million hectares, which is 112-fold increase compared with 1996.There are altogether 67 countries and

regions uses genetically modified crops. The reason for widespread use of genetically modified crops is the outstanding contribution they make to improve the socio-economic status of farms and public [7-8]. Through the genetical modification of traditional plants, they can gain many agronomic characteristics, including herbicide resistance, pest resistance, high nutritional quality, high yield and shelf-stable life. To decrease the herbicide dosage in soybean plant, GM soybean event (GTS 40-3-2) with the glyphosate-tolerant trait has been developed and commercialized worldwide.

The tolerance of herbicides in the soybeans was improved by genetically modified. However, the debates on the potential negative environmental and healthy impact of genetically modified soybean has drawn public attention to the safety assessment. A series of processes of safety assessment have been developed by international organizations to evaluate the safety of feeds and foods made from genetically modified plants. The toxicology evaluations could be divided into short-term (14 ~28 days) and long-term (90 days ~ 6 months or 24 months) [9-10]. Furtheremore, multi-generation reproduction test (2-5 generations) experiments were also assessed in Labs [11]. At present, most of the studies at home and abroad focus on organ coefficient, hematology, serum chemistry, neurological deficits on model animals. The results suggested that transgenic crops have no significant adverse effects on any model animals.

As an alkylating agents, Cyclophosphamide (CP) is a mainstay of chemotherapy regimen used in treating cancer and autoimmune diseases [12]. After the hepatic biotransformation by cytochrome P-450 enzymes, CP can be transformed into phosphoramide mustard (PM), which is generally accepted as the toxic metabolite of the treatment. PM damages cells by forming cross-linked DNA adducts to inhibit DNA strand separation during replication [13]. So many studies showed that the vigorously dividing cells, such as ovarian granulose cells and spermatogenic cells are prime target for CP [14-15]. Besides the therapeutic action, a series of side effects can not be ignored. As early as 1972, there was a studies showed that CP could reduce the count of sperms [16]. Since then there were more and more studies have proved that CP could destroy the testicle structure to impact spermatogensis and fertility and with dose-dependent effect [17]. However, the studies on whether Glyphosate-resistant soybeans can impact the destroy caused by CP in the male mice, especially the count and quality of sperm and testicle structure are still relatively rare.

In this study, we established the CP-treatment animal models and fed them with Roundup Ready soybeans for 30 days and 90 days to investigated the effect of genetically modified soybeans on the male reproductive system under the pathological conditions. Moreover, we were proposed to provide more experiment evidences to assess the safety of genetically modified soybeans.

2. Materials and Methods

2.1 Animals feed

The genetically modified soybeans (Roundup Ready Soybeans), GTS40-3-2, were provided by Monsanto and the non- genetically modified soybeans were the parental species. The production of the feed was commissioned to Guangdong Experimental Animal Center, according with the national standard of animal feed ingredients processed requirement. The components and the proportions were same except the soybean of transgenic and non-transgenic.

2.2 Experimental animals

Five-week-old Kunming male mice were obtained from Guangdong Experimental Animal Center. According to the institutional guidelines established by the National Council, the animals room was maintained at a temperature of 22±2℃, relative humidity of 40-70%, 12 h light/dark cycle.

2.3 Animals and treatment

2.3.1 Animals Diet

The male mice were randomly assigned into different group with approximately similar initial mean body weight. The experiment groups were fed on genetically modified soybeans for 30 days and 90 days, which were described as GM_{30} and GM_{90}.The corresponding control groups were fed on non-genetically modified soybeans for 30 days and 90 days, which were described as CK_{30} and CK_{90}.

2.3.2 Actue toxicity test

The mice were randomly divided into two groups(n≥10).CK group (control group) was treated with a single intraperitoneal (ip) injection of saline. CP group (experiment group) received a single ip dose of 200mg/kg Cyclophosphamide (CP, made by Pude Pharma). Then each group was divided into two groups (n≥5) and fed the feed with or without transgenic soybeans for 30 days respectively. After 30 days, all the mice were sacrificed by cervical dislocation for subsequent experiments.

2.3.3 Sub-chronic toxicity test

The mice were randomly divided into two groups(n≥10). CK group (control group) was treated with ip of saline, while CP group (experiment group) received ip dose of 200mg/kg CP at day 1, 30 and 90. Then each group was divided into two groups (n≥5) and fed the feed with or without transgenic soybeans for 90 days respectively. After 90 days, all the mice were sacrificed by cervical dislocation for subsequent experiments.

2.4 Sperm count and motility

The caudal epididymides from mice were washed in sterile saline and then placed in 35mm culture dish with 1ml normal saline. The caudal epididymis was impaled and squeezed with a fine needle to release the sperm into the medium. The sperm suspension was incubated in the 37℃, 5% CO_2 incubator for 5 minutes.

10μl sperm suspension was transferred into a hemocytometer and the sperms(n≥200) were observed with optical electron microscope. According to the laboratory manual of human semen and sperm-cervical mucus interaction of World Health Organization, the sperm motility can be divided into 4 grades: I: strong activity, moving quickly and linear motion; II: normal activity, but the direction of movement is not clear, non-linear or linear motion; III: poor activity, spinning around or wandering, almost without forward motion; IV: no athletic ability. Calculate the sperm motility with the following formula:

$$\text{Sperm motility (\%)} = \frac{\text{I} + \text{II} + \text{III}}{\text{I} + \text{II} + \text{III} + \text{IV}} * 100\%$$

The number of the sperm was counted by hemocytometer and the data were expressed as the number of sperm per ml.

2.5 Sperm quality (Sperm viability rate and Sperm deformity rate)

2.5.1 Sperm viability rate

10μl sperm suspension was mixed with 10μl 0.15% Eosin in 1.5ml EP tube quickly. 10μl mixture was removed on a clean glass slide and pushed it uniformly and quickly. The sperm(n≥200) were observed with optical electron microscope. The live sperm could be stained red or reddish, and the dead sperm could be dark red. Calculate the sperm viability rate with the following formula:

$$\text{sperm viability rate(\%)} = \frac{\text{the number of live sperm}}{\text{the number of total sperm}} * 100\%$$

2.5.2 Sperm deformity rate

80μl sperm suspension was mixed with 20μl 0.15% Eosin in 1.5ml EP tube quickly. After 15 minutes, 50μl mixture was removed on the clean glass slide and pushed it uniformly and air-dried. The stained slide was mounted with neutral resin. The sperm(n≥200) were observed with optical electron microscope. Calculate the sperm viability rate with the following formula:

$$\text{sperm deformity rate(\%)} = \frac{\text{the number of deformed sperm}}{\text{the number of total sperm}} * 100\%$$

2.6 Hypoosmotic swelling test (HOST)

The Hypoosmotic swelling tests were performed as described pervious[13]. The hypoosmotic solution was prepared by mixing 0.375g sodium citrate and 1.351g D-fructose in 100ml of distilled H_2O. 0.1ml sperm suspension was incubated with 1ml hypoosmotic solution for 30 minutes at 37℃. 10μl mixture was removed on the clean glass slide and pushed it uniformly. The sperm(n≥400) were

observed with phase contrast microscope at 400 magnification. The result was showed by the percentage of sperm with typical tail changes of swelling.

2.7 Sperm nuclear maturity analysis

The caudal epididymides from mice were placed in 35mm culture dish with 2ml HTF. HTF should be incubated at 37℃, 5% CO_2 overnight in advance. The caudal epididymis was impaled and squeezed with a fine needle to release the sperm into the medium. The sperm suspension was incubated in the 37℃, 5%CO_2 incubator for 5 minutes. After diluting to 1×10^6/ml, a drop of sperm suspension was removed on the clean glass slide and pushed it uniformly and air-dried. Then the smears were fixed in buffered glutaraldehyde (3%), which prepared by 0.2M phosphate buffer (14 ml NaH_2PO_4 0.2M, 36ml Na_2HPO_4 0.2M, PH 7.2) for 30 minutes. The smears were stained with 5% aqueous aniline blue (Haokai) in 4% acetic acid (PH3.5) for 5 minutes and air-dried. The stained slide was mounted with neutral resin. The sperm($n\geq200$) were observed with microscope at 1000 magnification. The result was showed by the ratio of light blue sperm and blue sperm.

2.8 Acrosome integrity

The sperm suspension was diluting with HTF to 1ml (1×10^6/ml) and was incubated at 37℃, 5% CO_2 incubator for 1h to capacitate. Then 10μl progesterone (1mg/ml) was mixtured with the capacitated sperm suspension and co-incubated 30 minutes to induce acrosome reaction. The sperm suspension was washed with HTF by centrifugation at 2000rpm for 10 minutes, and resuspended it with HTF. A drop of sperm suspension was removed on the clean glass slide and pushed it uniformly and air-dried. Then the smears were fixed in ethanol (90%) for 30 minutes and air-dried.

The smears were stained with PSA-FIFC for 4 hours at 4℃ and then washed by distilled water three times. The acrosome integrity of sperm ($n\geq500$) was observed with fluorescence microscopy. The result was showed by the percentage of sperm with intergrated acrosome reaction .

2.9 Statistical Analysis

The data obtained were analyzed statistically. The student t-test, coefficient of correlation, logistic regression receiver operating characteristic (ROC) analysis were analyzed by SPSS(Version 16.0). The differences between groups were assessed by one-way analysis of variance (ANOVA). Values were expressed as mean±SD. Differences were considered significant at $p<0.05$.

3. RESULT

3.1 The model of pathological mice

In the actue toxicity test, the treatment of male mice of CP caused a significant decrease ($P<0.01$) in sperm count(47.5%), from 540.00±18.71(10^4/ml) to 28.33±43.65(10^4/ml) and sperm viability (43.3%), from 74.83±2.72(%)to 43.17±3.30(%)in 30 days(Table 1, Fig.1).

Table 1. Effects on mice sperm count and viability by single injection of yclophosphamide

Days	Sperm count(10^4/mL)	Sperm viability(%)
0	540.00±18.71	74.83±2.72
10	451.67±20.95 [a]	62.50±3.46 [a]
20	400.00±25.50 [b]	54.50±2.65 [b]
30	283.33±43.65 [b]	43.17±3.30 [b]
40	388.33±17.00 [b]	47.67±2.95 [b]
50	445.00±14.14 [a]	61.17±3.06 [a]
60	516.67±13.12	71.67±2.01
70	525.00±17.80	73.00±2.48
80	530.00±18.00	72.00±2.48
90	545.00±14.72	76.67±2.78

Data were the mean ±SD. Values with different superscript letters within the same column differ significantly ($P<0.05$) or statistically significantly. Statistical significance was represented as follows: a: $P<0.05$, means significant differences with the 0 day; b: $P<0.01$, means statistically significant differences with the 0 day.

In addition, we observed that the count and viability of sperm have a slowly increase after 30 days, and over time, there was no significant different between 60 days with 0 day (Table 1). Therefore, the dosage regimens of CP was 30 days in order to ensure the mice can keep pathological.

According to the dosage regimens of CP, the mice in Sub-chronic toxicity test was received ip dose of 200mg/kg CP at day 1, 30 and 90. From table 2, we observed that the count and viability of sperm have a permanent decrease, totally 48.9% and 44.5%. This phenomenon can be observed more intuitively in figure 2.

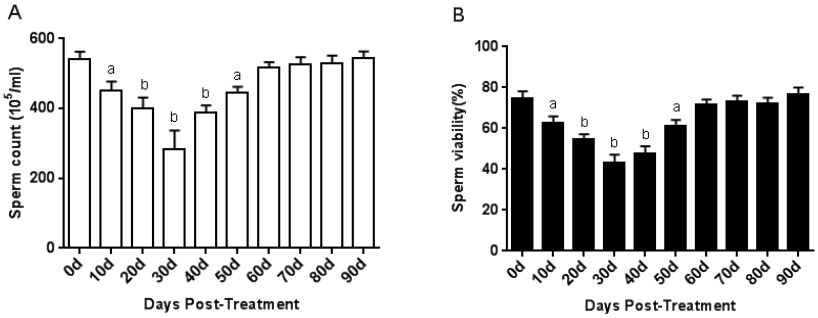

Figure 1. A single dose of CP caused a significant decrease in sperm count and sperm viability in 30 days, but have a slowly increase after 30 days. (A)Sperm count; (B) Sperm viability. Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: P *<0.05*, means significant differences with the 0 day; b: P *<0.01*, means statistically significant differences with the 0 day.

Table 2. Effects on mice sperm count and viability by injection of cyclophosphamide in 30-days cycles

Days	Sperm count (ten thousand/mL)	Sperm viability (%)
0	558.33±20.55	75.80±2.83
10	463.33±19.29 [a]	64.50±3.00 [a]
20	413.33±27.18 [b]	58.83±3.51 [b]
30	288.33±47.14 [b]	43.88±3.42 [b]
40	338.33±30.55 [b]	46.57±3.02 [b]
50	325.30±30.00 [b]	52.17±2.87 [b]
60	285.67±43.20 [b]	46.33±2.59 [b]
70	310.00±38.94 [b]	43.00±2.52 [b]
80	285.00±41.43 [b]	42.00±2.48 [b]
90	285.00±53.07 [b]	42.07±2.55 [b]

Data were the mean ± SD. Values with different superscript letters within the same column differ significantly (P*<0.05*) or statistically significantly. Statistical significance was represented as follows: a: P *<0.05*, means significant differences with the 0 day; b: P *<0.01*, means statistically significant differences with the 0 day.

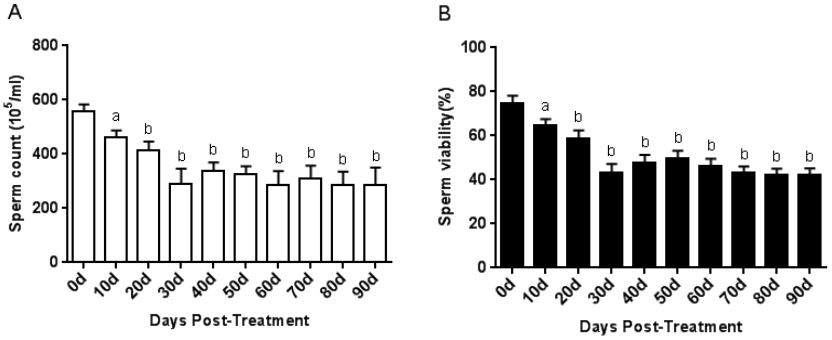

Figure 2. The 30days-cycle dose of CP caused a significant decrease in sperm count and sperm viability in 90 days. (A)Sperm count; (B) Sperm viability. Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: P *<0.05*, means significant differences with the 0 day; b: P *<0.01*, means statistically significant differences with the 0 day.

3.2 Effects on mice sperm count by feeding genetically modified Soybeans.

Under the normal pathological circumstances, the mice was fed on soybeans, which with or without genetically modified for 30 days and 90 days. The count of sperm(10^4/ml) of the GM group were 532.78±25.631 with 30 days diet and 534.44±25.05 with 90 days diet, which had no significant difference with CK group(P>0.05). After the treatment with CP , the different diet, with or without genetically modified soybeans, didn't cause significant difference(P>0.05) in the sperm count between CK group and GM group(Table 3). This result showed that both in the normal pathological circumstances or pathological circumstances, genetically modified soybeans did not affect sperm count of mice (Figure 3).

Table 3. Effects on mice sperm count by feeding genetically modified soybeans.

Days	Groups	Sperm count(10^4/ml)
30 d	CK_{30}	539.44±18.615
	GM_{30}	532.78±25.631
	$CP+CK_{30}$	268.89±36.12 [a]
	$CP+GM_{30}$	270.56±42.58 [b]
90 d	CK_{90}	525.00±25.249
	GM_{90}	534.44±25.05
	$CP+CK_{90}$	262.78±29.486 [c]
	$CP+GM_{90}$	266.11±39.352 [d]

Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: *P <0.01*, means statistically significant differences with CK_{30}; b: *P <0.01*, means statistically significant differences with the GM_{30}; c: *P <0.01*, means statistically significant differences with CK_{90}; d: *P <0.01*, means statistically significant differences with GM_{90}.

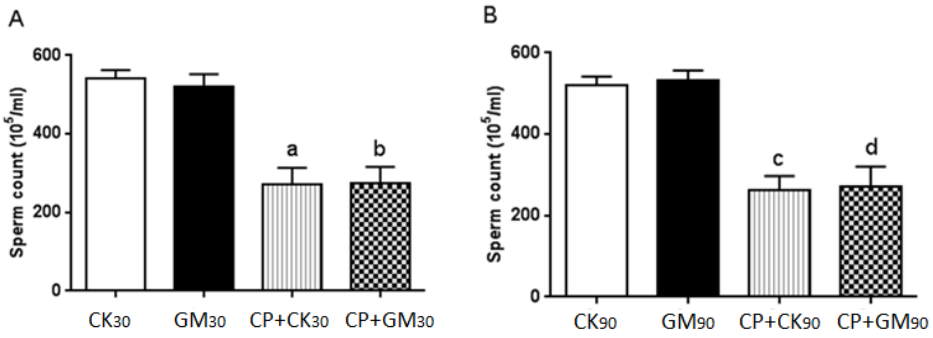

Figure3. CP caused statistical significance difference in the count of sperm, while the diet with or without genetically modified soybeans did not caused significance difference. Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: *P <0.01*, means statistically significant differences with CK_{30}; b: *P <0.01*, means statistically significant differences with GM_30; c: *P <0.01*, means statistically significant differences with CK_{90}; d: *P <0.01*, means statistically significant differences with GM_{90}.

3.3 Effects on mice sperm motility by feeding genetically modified soybeans.

After feeding on genetically modified Soybeans for 30 days and 90 days, the sperm motility (%) of mice were 83.48±3.09 and 84.52±2.44, which showed no significant difference with CK group (P>0.05). With the treatment of CP, it showed the same situation. The sperm motility (%) of mice in GM group were 54.29±2.69 with 30 days diet and 52.89±2.93 with 90 days diet, which had no significant difference with CK group. However, the treatment with CP had statistically significant decreased the motility of sperm from 83.48±3.09 to 54.29±2.69 (34.9%, P <0.01) and from 84.52±2.44 to 52.89±2.93 (37.4%, P <0.01). This result showed that CP could significant decrease the motility of sperm while genetically modified soybeans could not.

Table 4. Effects on mice sperm mobility by feeding genetically modified soybeans

Days	Groups	sperm motility(%)
30 d	CK_{30}	85.61±5.73
	GM_{30}	83.48±3.09
	CP +CK_{30}	54.14±2.85 [a]
	CP +GM_{30}	54.29±2.69 [b]
90 d	CK_{90}	83.86±2.52
	GM_{90}	84.52±2.44
	CP +CK_{90}	53.22±2.62 [c]
	CP +GM_{90}	52.89±2.93 [d]

Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: P *<0.01*, means statistically significant differences with CK_{30}; b: P *<0.01*, means statistically significant differences with the GM_{30}; c: P *<0.01*, means statistically significant differences with CK_{90}; d: P *<0.01*, means statistically significant differences with GM_{90}.

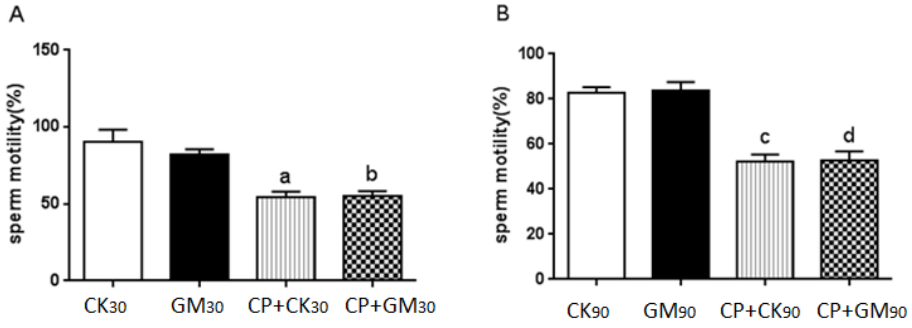

Figure 4. CP caused statistical significance difference in the motility of sperm, while the diet with or without genetically modified soybeans did not caused significance difference. Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: P *<0.01*, means statistically significant differences with CK_{30}; b: P *<0.01*, means statistically significant differences with GM_{30}; c: P *<0.01*, means statistically significant differences with CK_{90}; d: P *<0.01*, means statistically significant differences with GM_{90}.

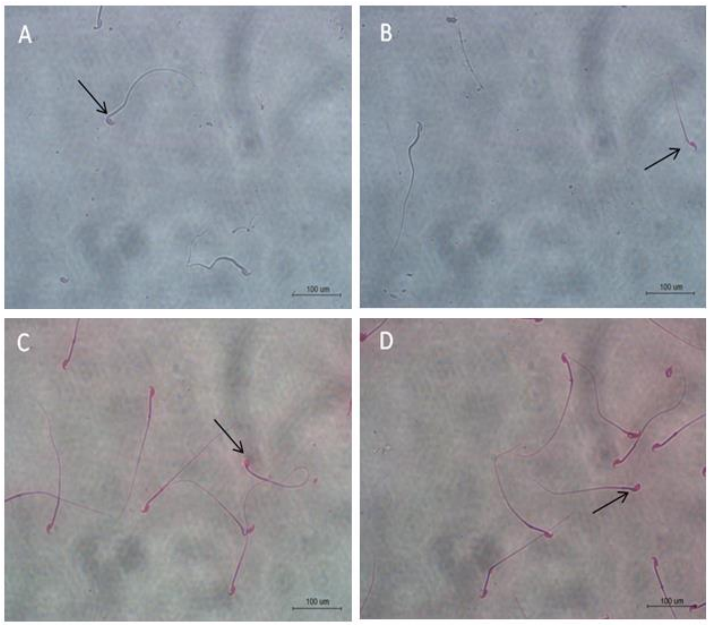

Figure 5. Esion staining of mice sperm. A and B were stained with 0.15% esion to observed sperm viability. The arrow indicated the death sperm. C and D were stained with 1% esion to detect sperm deformity. The arrow was indicated the abnormal sperm morphology The abnormal sperm had head deformity, fat head, banana-like or amorphous head or no hook. Other sperm were normal with the hooked sickle head.

3.4 Effects on mice sperm quality by feeding genetically modified Soybeans.

0.15% eosin staining could stain the death sperm with red head to calculate the viability of sperm (Figure 5A and 5B), while 1% eosin staining could assess the deformity of sperm (Figure 5C and 5D). After feeding on genetically modified diets for 30 days and 90 days, the viability of sperm was 73.94±2.34% and 76.61±3.27%, and the deformity rate was 1.45±0.23% and 1.60±0.27% respectively. This result showed that neither the viability nor deformity could be affected by genetically modified soybeans both in the normal pathological circumstances or pathological circumstances.

After the treatment of CP, the quality of sperm, both in the CK and GM group, had statistically significant decreased (P <0.01). However, the sperm viability (%) of mice in GM group were 45.61±2.91 with 30 days diet and 43.00±2.75 with 90 days diet, and the deformity(%) were 4.46±0.32 with 30 days diet and 4.58±0.38 with 90 days diet, which had no significant difference with CK group (Table 5, P>0.05).In short, this result showed genetically modified soybeans did not affect sperm quality.

Table 5. Effects on mice sperm quality by feeding genetically modified soybeans

Days	Groups	Sperm viability(%)	Sperm deformity(%)
30 d	CK_{30}	74.61±2.79	1.67±0.26
	GM_{30}	76.61±3.27	1.60±0.27
	CP +CK_{30}	43.22±2.83 [a]	4.36±0.33 [a]
	CP +GM_{30}	45.61±2.91 [b]	4.46±0.32 [b]
90 d	CK_{90}	76.22±3.45	1.60±0.27
	GM_{90}	73.94±2.34	1.45±0.23
	CP +CK_{90}	41.78±3.57 [c]	4.56±0.26 [c]
	CP +GM_{90}	43.00±2.75 [d]	4.58±0.38[d]

Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: *P <0.01*, means statistically significant differences with CK_{30}; b: *P <0.01*, means statistically significant differences with the GM_{30}; c: *P <0.01*, means statistically significant differences with CK_{90}; d: *P <0.01*, means statistically significant differences with GM_{90}.

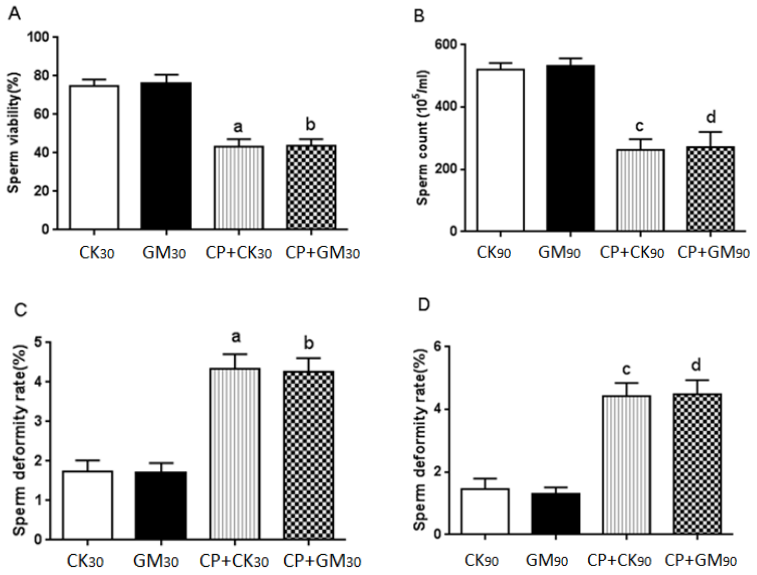

Figure 6. CP caused statistical significance difference in the quality of sperm, while the diet with or without genetically modified soybeans did not caused significance difference. A and B: sperm viability; C and D: sperm deformity. Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: *P <0.01*, means statistically significant differences with CK_{30}; b: *P <0.01*, means statistically significant differences with GM_{30}; c: *P <0.01*, means statistically significant differences with CK_{90}; d: *P <0.01*, means statistically significant differences with GM_{90}.

3.5 Hypoosmotic swelling test (HOST) on mice fed with genetically modified Soybeans

HOST was performed to evaluate the functional integrity of the sperm's plasma membrane. Only the sperm with intact membranes would swell in hypotonic solutions, which showed the expansion of the tail. In the GM30 and GM90 group, the percentage of swollen sperm (%) were 62.83±2.50 and 60.87 ±3.31, respectively, which neither showed significant difference with CK group (P> 0.05, Table 6).

Table 6. Hypoosmotic swelling test (HOST) on mice fed with genetically modified Soybeans

Days	Groups	sperm tail swelling degree(%)
30 d	CK_{30}	62.17±2.50
	GM_{30}	62.83±2.50
	CP +CK_{30}	47.06±3.88 [a]
	CP +GM_{30}	46.50±3.98 [b]
90 d	CK_{90}	61.16±2.87
	GM_{90}	60.87±3.31
	CP +CK_{90}	47.64±3.17 [c]
	CP +GM_{90}	45.44±3.78 [d]

Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: *P <0.01*, means statistically significant differences with CK_{30}; b: *P <0.01*, means statistically significant differences with the GM_{30}; c: *P <0.01*, means statistically significant differences with CK_{90}; d: *P <0.01*, means statistically significant differences with GM_{90}.

In the CP-induced pathological mice, the percentage of swollen sperm (%) in GM group were 46.50±3.98 with 30 days diet and 45.44±3.78 with 90 days diet which had no significant difference with CK group(Table 5, P>0.05). However, it was obvious that CP induced significant decrease of functional intact sperm both in CK and GM group (Figure 7, P<0.01). In short, this result showed genetically modified soybeans did not affect the functional integrity of the sperm's plasma membrane.

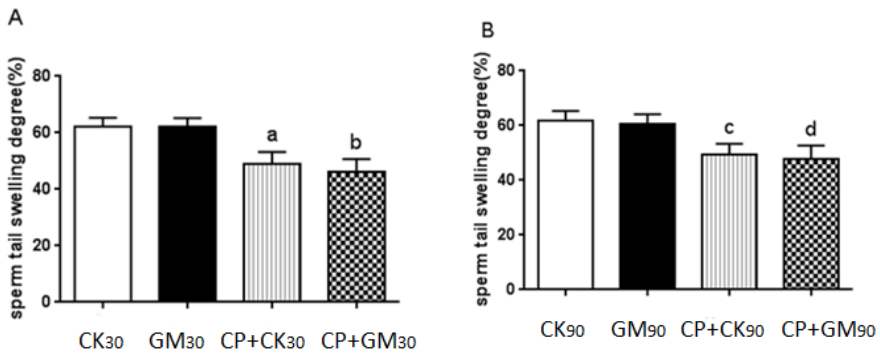

Figure 7. Hypoosmotic swelling test (HOST) on mice fed with genetically modified soybeans in 30 days (A) and 90 days (B). CP caused statistical significance difference in functional integrity of the sperm's plasma membrane, while the diet with or without genetically modified soybeans did not caused significance difference. Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: *P <0.01*, means statistically significant differences with CK_{30}; b: *P <0.01*, means statistically significant differences with GM_{30}; c: *P <0.01*, means statistically significant differences with CK_{90}; d: *P <0.01*, means statistically significant differences with GM_{90}.

3.6 Sperm nuclear maturity analysis on mice fed with genetically modified Soybeans

The maturity of sperm nuclear was assessed by aniline blue staining. Aniline blue can combine with histone specifically to reflect the content of histone in sperm nuclear. There is rare histone in mature sperm, so that they will not be stained or stained weak, while the immature sperm, with more than 15% histone will be stained dark blue (Figure 8).

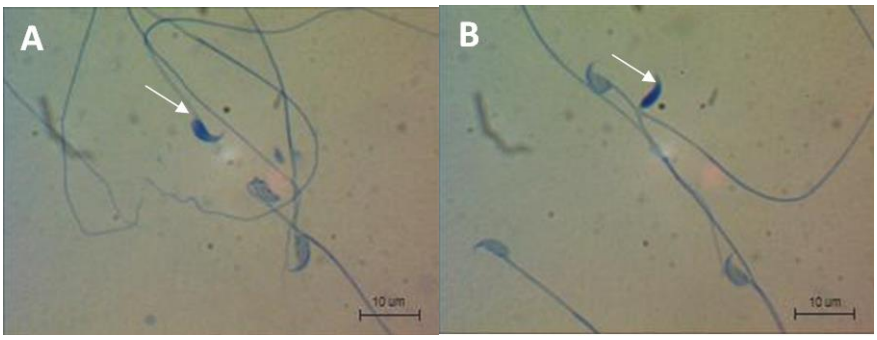

Figure 8. Aniline blue staining of sperm nuclear maturity analysis. A: the arrow was indicated an immature sperm with light blue. B: the arrow was indicated a mature sperm with dark blue.

From the data in Table.7, it showed that nuclear maturity of sperm (%) in GM group was 93.17±2.90 with 30 days diet and 593.33±2.4 with 90 days diet, didn't differ significant (P>0.05) with CK group. After the treatment of CP, the nuclear maturity of sperm had significantly decreases (P<0.01) both in CK and GM group, however, there was still no significantly difference (P>0.05) between CK and GM group, no matter 30 days and 90 days diet.

Table 7. Sperm nuclear maturity analysis on mice fed with genetically modified Soybeans.

Days	Groups	Sperm nuclear maturity(%)
30 d	CK_{30}	92.64±2.27
	GM_{30}	93.17±2.90
	CP +CK_{30}	82.61±2.26 [a]
	CP +GM_{30}	83.22±2.20 [b]
90 d	CK_{90}	91.67±2.29
	GM_{90}	93.33±2.42
	CP +CK_{90}	82.50±2.84 [c]
	CP +GM_{90}	83.22±3.12 [d]

Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: *P <0.01*, means statistically significant differences with CK_{30}; b: *P <0.01*, means statistically significant differences with the GM_{30}; c: *P <0.01*, means statistically significant differences with CK_{90}; d: *P <0.01*, means statistically significant differences with GM_{90}.

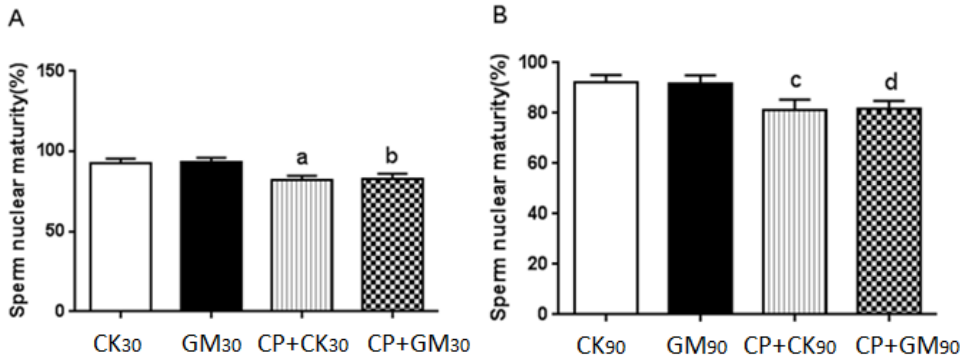

Figure 9. Sperm aniline blue staining on mice fed with genetically modified soybeans in 30 days (A) and 90 days (B). CP caused statistical significance difference in nuclear maturity of sperm , while the diet with or without genetically modified soybeans did not. Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: *P <0.01*, means statistically significant differences with CK_{30}; b: *P <0.01*, means statistically significant differences with GM_{30}; c: *P <0.01*, means statistically significant differences with CK_{90}; d: *P <0.01*, means statistically significant differences with GM_{90}.

3.7 Assessment of acrosome integrity of mice fed with genetically modified Soybeans

PSA-FITC staining was performed to assess the integrity of acrosome, by combining with pre-acrosome enzyme in acrosome to fluoresce during acrosome reaction (Fugure 10. A C E). The acrosome integrity sperm would show bright green fluorescence in whole sperm head.

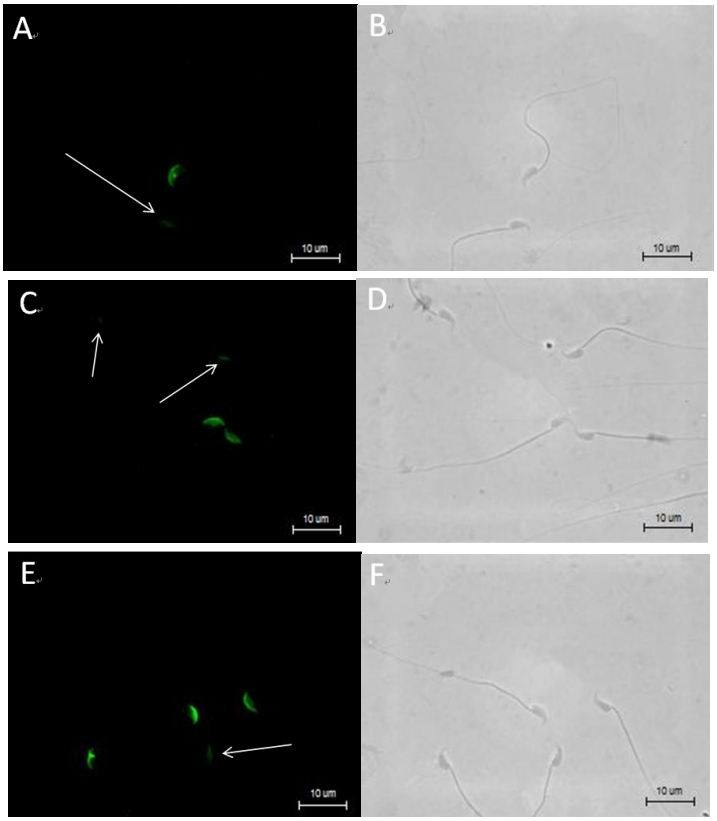

Figure 10. FITC-PSA fluorescence staining for mice sperm to assess acrosome reaction. (1000X) A, C and E: The arrow was indicated the partial absence of fluorescence meant acrosome reaction. B,D and F: corresponding smears with A C and E in phase contrast microscope.

From the data in Table.8, it showed that the rates of sperm acrosome reaction (%) in GM group were 54.89±2.55 with 30 days diet and 55.33±3.10 with 90 days diet, didn't differ significant ($P>0.05$) with CK group. After the treatment of CP, the rate of sperm acrosome reaction had significantly decreases ($P<0.01$) both in CK and GM group, however, there was still no significantly difference ($P>0.05$) between CK and GM group, no matter 30 days and 90 days diet.

Table 8. Rate of acrosome integrity of mice fed with genetically modified Soybeans

Days	Groups	Acrosome reaction(%)
30 d	CK_{30}	53.72±2.68
	GM_{30}	54.89±2.55
	CP +CK_{30}	43.94±2.30 [a]
	CP +GM_{30}	43.19±2.56 [b]
90 d	CK_{90}	55.83±3.23
	GM_{90}	55.33±3.10
	CP +CK_{90}	43.83±2.65 [c]
	CP +GM_{90}	42.83±2.09 [d]

Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: $P<0.01$, means statistically significant differences with CK_{30}; b: $P<0.01$, means statistically significant differences with the GM_{30}; c: $P<0.01$, means statistically significant differences with CK_{90}; d: $P<0.01$, means statistically significant differences with GM_{90}.

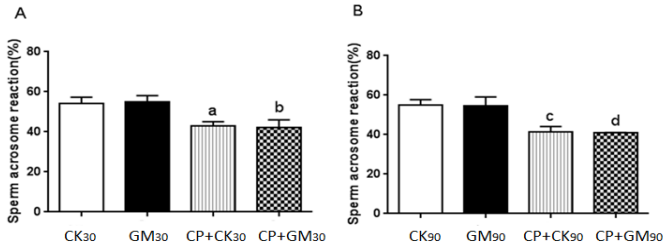

Figure 11. Rate of sperm acrosome reaction on mice fed with genetically modified soybeans in 30 days (A) and 90 days (B). CP caused statistical significance difference in nuclear maturity of sperm, while the diet with or without genetically modified soybeans did not. Data were mean ± S.D. (n = 9 per group). Statistical significance was represented as follows: a: *P <0.01*, means statistically significant differences with CK_{30}; b: *P <0.01*, means statistically significant differences with GM_{30}; c: *P <0.01*, means statistically significant differences with CK_{90}; d: *P <0.01*, means statistically significant differences with GM_{90}.

4. Discussion

With the fast development of Genetic engineering techniques, genetically modified (GM) crops maybe the best solution to growing problem of food shortages. However, the public is more concerned about the safety of GM crops and focused on the potential risks such as allergic, food toxicity, resulting in antibiotic resistance and the impact on the environment [15]. Chukwudebe et al.(2012) [16] and He et al.(2016) [17] performed a subchronic research(90 days) on rats, to observed their growth performance, food consumption, hematology, metabolism and histopathology, which aimed at assessing the safety and nutritional properties of CV127 soybeans(at levels of 11% and 33%). However, they found a few significant differences with its near isogenic conventional variety in growth and performance response variables, which were considered isolated and no biologically meaning. Simliar results were reported by Qi et al. (2012) [18], they found that some significant differences between in rats, which dietary exposured to 3Ø5423 X 40-3-2 and non-GM soybeans, the authors also thought that these differences were within normal ranges, and concluded that the GM soybeans 3Ø5423 X 40-3-2 was as safe as non-GM soybeans. Date from Wang's (2016) 90-day subchronic toxicological study of the dicambatolerant soybean (MON87708) showed that there were no indication that GM soybeans would caused adverse effects. [19]

People could be less resistant to external stimulation and potential toxicity, therefore, it is meaningful to evaluate the toxicity in pathological conditions. Though there were many research on GM crops safety evaluation at domestic and foreign, they more focused on normal physiological conditions and studies in animal models of pathological states were almost blank. So it is necessary to build a stable pathological damage model within toxic substances to assess genetically modified soybeans effects on sperm. Cyclophosphamide (CP) is an alkylating agent which widely used as a chemotherapy drug to resist cancer. However, acts as a cell-cycle non-specific drug, CP can form DNA croos-linking to inhibit DNA synthesis to damages the reproduction system in the treatment of cancer, which used to build a stable reproductive pathology model during the experimental study. [20] In our studies , we focused on whether GM soybeans could induce adverse effect of sperms, including quantity, quality, structure and function on pathological mice model, which was induced by CP. Our previous studies showed that a single injection of different dose (50 mg / kg, 100 mg / kg, 200 mg / kg, 250 mg / kg) could decrease sperm count in varying degrees and with a positive dose-dependent. LD50 dose of CP in mice is 250mg/kg, which induces the symptoms of weight loss, less sperm and even half of the deaths. However, the dose of 200mg/kg could significant impact the quantity, quality and vitality of mice sperms but deaths. Therefore, we chose 200 mg / kg dose as a standard dose of CP to establish the reproductive injury animal model.

Sperm is germ cells of men or other male mammals. The semen quality has a very close relationship with male reproduction, which reflects reproductive capacity of male. The sperm parameters to assess semen quality contain semen volume, sperm count, sperm viability and morphology, sperm motility

and sperm concentration [21]. To date, a great number of factors might take an negative part in male sperm quality such as overweight, social stress, special disease and chemotherapy [22]. Active sperm has accounted for more than 60% of the total, and abnormal sperm should be 10% or less. Sperm viability, measured as the proportion of live sperm [23]. Sperm morphology is controlled by a large number of genes traits, any external substances, which can affect these genes expression, are likely to induce sperm mutations, and sperm deformity can reflect genetic damage of sperm in some extent [24]. Sperm motility is perhaps the most obvious measure of sperm function which may be showed in vitro and this has been compared with actual fertilizing ability in some studies [25]. So we had chosen sperm count, sperm viability, sperm motility and sperm morphology to assess sperm quality of the mice which fed on GM and non-GM soybeans in normal physical conditions and CP-induced chemotherapy model for 30 days and 90days. The results displayed that in normal conditions, there had no significance between GM group and CK group both in 30 days and 90 days. In the administrated CP groups, sperm parameters, including sperm count, sperm viability, sperm motility and sperm morphology both have extremely decreased compared to normal groups. However, when we compared between GM group and CK group in CP-induced model, we also had concluded no significance between them no matter in 30 days groups or 90 days groups. At all, our study demonstrated that GM had no worse effects on mice sperm in normal conditions or pathological state.

Sperm membrane is essential to maintaining the biochemical and structural integrity of sperm, and membrane integrity is crucially importance in assessing the fertilizing capacity, therefore, plasma membrane is seen as one of the most considerable prerequisites of sperm fertilization in assessing semen quality [26-28]. In our research, we had chosen mice sperm membrane integrity as the sperm parameters to assess the quality of sperm. We found that fed on GM soybeans in 30 days and 90 days could not significantly affect the membrane integrity of mice sperm both in normal model and CP-induced model compared to the CK groups. At the same time, there were no significant difference between GM groups and CK groups in CP-induced model could be observed in hypotonic swelling of mice sperm. At all, no matter in normal or CP- pathological conditions, GM soybeans had no adversely effects on sperm membrane integrity.

In routine semen analysis, the maturity of sperm chromatin nuclear is regards as a main assessment to evaluation of male infertility [29]. Maturity of sperm chromatin occurs during spermiogenesis, epididymal maturation and sperm formation dues to histone-protamine replacement [30]. Nuclear histone contain numerous lysine ,while protamine generally do not , so aniline blue staining, which can bind to lysine-rich histones for detecting sperm nucleoprotein transform could reflect sperm maturation. The progress of sperm chromatin nuclear maturity is essential for fertilization. We studied on mice sperm chromatin nuclear maturity in feeding on GM soybeans and non-GM soybeans under normal or CP-induced pathological state. The research had found that sperm chromatin nuclear maturity of the GM groups and CK groups had no significant difference both in 30 days and 90 days, the same results could be observed in CP-induced groups. In other words, both in normal physiological and pathological conditions, 30 days and 90 days GM soybeans feeding had no adverse effects on nuclear maturity of mice sperm.

One of most used fluorochrome combinations for assess of acrosome integrity is fluorescein isotiocyanate-conjugated pea (Pisum sativum) allutinin (FITC-PSA) [31-32]. We can observe that sperm with intact acrosome fluorescence in the whole head, but sperm cells with a reacted show green fluorescence in sperm equatorial region. We assessed acrosome integrity on sperm of mice which fed on GM soybeans and non-GM soybeans in 30 days or 90 days. The dates showed that under normal conditions and pathological state, there were no significant difference with each other. It was also suggested that GM soybeans had no adversely effects on sperm acrosome integrity in normal physical conditions and pathological state.

5. Conclusion

Our research had comprehensive analyzed sperm parameters including sperm count, sperm quality, sperm morphology, sperm motility and acrosome reaction. The experimental results showed that 30

days and 90 days Roundup Ready soybean feeding would not affect the count and quality of mice sperm, such as the sperm membrane integrity, nuclear maturation and development, and acrosome reaction. In short, Roundup Ready soybeans could not produce toxic effects in mice sperm numbers and quality.

Acknowlegments

These author's contributed equally to this work.The authors acknowledgment support from the National Major Special Projects for Genetically Modified Organisms.

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