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## **Serum Biochemical Response of Growing Rabbits Fed Graded Levels of Genetically Modified Cottonseeds Meal**

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### **Abstract;**

*A 90-day biosafety study was conducted to evaluate the potential unintended effects of GM cottonseeds consumption on rabbits' serum biochemical profile. A total of Forty-eight New Zealand white male and female (24 pairs) rabbits averagely weighing  $570.50 \pm 0.50$  g were assigned to dietary treatments containing different levels of GM cotton seeds (i.e. 20% w/w, 30% w/w and 40% w/w respectively) mixed with standard diet for 90 days. During the course of the study, the levels of various serum enzymes, electrolytes, glucose and serum total cholesterol were measured. The results revealed non-significant ( $P > 0.05$ ) dose-dependent effects in almost all the evaluated parameters except for the serum total cholesterol in which significant differences ( $P < 0.05$ ) were observed. However, such differences were not biologically significant, as all the values were within the reported normal physiological range. The Outcomes from this feeding study demonstrated that the consumption of GM cottonseeds based diets could adversely affect the biochemical profile of rabbits. It is therefore concluded that, the GM cottonseed meal is as safe and nutritious as conventional feed ingredients used in rabbits feeding.*

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**Key words;** Serum Biochemical Profile; Growing Rabbits; GM Cottonseeds: Feed Safety

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### **Introduction**

New varieties of crop plants with improved characteristics such as fortification against common insect pests, tolerance to herbicides, and better quality traits have recently been produced through modern agricultural biotechnology. Notable examples are the genetically modified crops (GM) with the 'Bt' gene that expresses an insecticidal protein (Yang *et al.*, 2014). However, public concerns vis-à-vis the food safety of these transgenic crops have been expressed in respect to human consumption and production of animal products (e.g. meat, milk and eggs) from farm animals fed on transgenic crops (Yang *et al.*, 2013). Several reports on this topic have been published in the past proclaiming no detrimental effects on animal and human health (Reichert *et al.*, 2012; Ibrahim Bala Salisu *et al.*, 2018; Shahid *et al.*, 2016; Yuan *et al.*, 2017), nevertheless, conflicting opinions also do exist and are

experimentally documented (Abdo *et al.*, 2013; Kulikov, 2005; Séralini *et al.*, 2007; Tudisco *et al.*, 2010; Tyshko *et al.*, 2007).

Rabbits are used frequently as a suitable animal model for manipulation in delicate procedure particularly in a scientific study where variations in haematological and biochemical parameters occur, as they have pronounced surface veins which make sampling process easier (Poljičak-Milas *et al.*, 2009). Serum biochemical profile reveals nutritional, physiological and pathological status of an individual (Al-Fartosi *et al.*, 2010; Panda *et al.*, 2017; Sreejaya *et al.*, 2017). Biochemical indices are inter-related with a number of body functions and modification. Any impairment of serum biochemical parameter can ultimately result in structural and physiological anomalies (Mamun *et al.*, 2014). Through the analyses of

serum biochemical profile, the function of vital organs like liver, kidney, heart, and pancreas can be determined easily. Serum biochemical examination, in general, allows for clinical investigation of several constituents of animal bodies. This is especially significant in rabbits, as they demonstrate complex clinical signs that often mask the disease conditions (Molina *et al.*, 2017). Keeping in view the significance of biochemical components in animal, dietary ingredients are found to impart a substantial effect on serology (Ibrahim B. Salisu *et al.*, 2018). While studying and evaluating animal physiology and nutrition, laboratory tests can be used to examine the model animals. The information concerning the potential unintended effects of feeding GM-cottonseed meal on the serum biochemical profile is less, especially in case of rabbits. Thus, this study was conducted to evaluate the effect of dietary incorporation of transgenic cottonseed expressing *Cry* and *Cp4epsps* proteins on the serum biochemical profile of growing rabbits.

## Materials and Methods

### Study area

The study was conducted at National Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan. The site is located between latitude 31° 34' 55.3620"N and longitudes 74° 19' 45.7536" E, at an altitude of 213 m above sea level.

### Diet Formulation

Diets were formulated according to the CEMB optimized procedure. The test substance is a mature seed of cotton (*Gossypium hirsutum*) variety (VH-289) genetically modified with binary insect resistant genes (*Cry 1Ac* & *Cry 2A*) and glyphosate tolerant gene (*Cp4 epsps*). Dried seeds were crushed with the help of mechanical grinder following the removal of lint from the cotton boll. The seeds were crushed further to remove any remaining lints or strands of tiny cotton fibre. The seeds were then hulled and polished for releasing soft and

highly edible protein. The hulled seeds were then mixed thoroughly with other feed ingredients using a mechanical mixer to produce uniform diet suitable for the animals. Four different experimental diets were formulated to contain varying levels of cottonseed meal at 0 (control), 20, 30, and 40% dietary inclusion levels (Table 1).

### Composition of the experimental diets

Nutritional components of both control and experimental diets were analyzed according to the standard procedure of AOAC (2013). Major components such as dry matter, moisture (DM), crude fibre (CF), ether extract (EE), neutral detergent fibre (NDF), acid detergent fibre (ADF) and hemicellulose were estimated. The total energy content of each diet was also measured (Table 1). All dietary ingredients were thoroughly mixed before subjecting to compositional analyses. Dry matter determination was achieved through drying the samples at 70°C for 24 h with the help of Memmert convection oven. Moisture was calculated using the formula: % Moisture = (100 – %DM). The macro-Kjeldahl method was used to determine the total nitrogen content. The determined nitrogen content was used to calculate crude protein by using a formula (Nitrogen content × 6.25). Crude fibre was estimated as a fraction remaining following digestion with a standard solution of sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) and sodium hydroxide (NaOH) in a controlled condition. Ash determination was achieved by ashing the samples at a high temperature of about 550°C in an oven for about four hours. Soxhlet extraction method was used for ether extract determination. A formula: %NFE = %DM – (%CP + %CF + %EE + %Ash), was applied for the calculation of Nitrogen free extract. The automatic fibre detection system (FIBRE TECH M System; Tecator, Sweden) was employed to determine both Acid detergent fibre (ADF) and Neutral detergent fibre (NDF) as described by Van Soest *et al.*, (1991). Hemicellulose was

calculated by applying formula; (%NDF – %ADF). An adiabatic bomb calorimeter was used for determining the energy content.

**Table 1. Ingredients Compositions and Compositional analysis of the Experimental Diets fed to Rabbits**

Ingredients (%)	Treatments			
	G1	G2	G3	G4
Transgenic Cottonseeds	0	20	30	40
Wheat Offal	40	20	10	0
Corn	28	28	28	28
Dehydrated Lucerne Meal	11	11	11	11
Soybean meal	16	16	16	16
Soybean Oil	1.5	1.5	1.5	1.5
Calcium Phosphate	2.3	2.3	2.3	2.3
Sodium Chloride	0.7	0.7	0.7	0.7
Vitamins and mineral premix	0.5	0.5	0.5	0.5
<b>Total</b>	<b>100</b>	<b>100</b>	<b>100</b>	<b>100</b>
<b>Analysed Composition (%)</b>				
Dry Matter	96.7	95.9	96.3	95.7
Moisture	3.32	4.14	3.73	4.34
Crude Protein	15.7	15.9	16.5	17.6
Crude Fibre	13.5	10.5	14	16
Ether Extract	9.5	11.5	13	14
Ash	9	7.4	7	7.2
Nitrogen Free Extract	47.33	46.3	44.51	47.44
Neutral Detergent Fibre	60.31	59.75	61.22	60.17
Acid Detergent Fibre	37.05	37.59	38.13	38.32
Hemicellulose	23.28	22.18	23.15	21.93
<b>Energy (Kcal/Kg)</b>	<b>2592</b>	<b>2557</b>	<b>2588</b>	<b>2651</b>

G1: Control diet, G2, G3 and G4 represent 20%, 30% and 40% transgenic cottonseeds inclusion levels respectively.

### Experimental Animals and their Management

A total of Forty-eight New Zealand white male and female (24 pairs) rabbits weighing averagely  $570.50 \pm 0.50$  g were purchased from Tollinton Animals Market Lahore, Pakistan. Just after receiving the animals, anti-stress preparations (Symostress-**Product Code:** SSA100 gm) were administered for three days to enable the animals recover from transportation stress (Adeosun and Iyeghe-Erakpotobor, 2014). The animals were allowed to acclimatize to the environment during the first seven days before they were assigned to

their respective treatments. All animals were kept at  $22 \pm 3^\circ\text{C}$  (room temperature), under a period of twelve hours (light / dark cycle) and 45-60% humidity.

### Experimental procedure

Following seven days of the environmental adjustment, rabbits were balanced for weight and randomly assigned to four dietary treatment groups namely: G1, G2, G3, and G4 with 6 pairs per group, (males and females in separate cages) for three-month feeding trial. The rabbits in G1 were raised on a normal diet without transgenic cottonseed. Rabbits in G2, G3, and

G4 were reared on diet containing 20%, 30% and 40% of transgenic cottonseeds, respectively. One hundred and fifty grams (150 g) of the experimental diets were daily served to each animal and allowed for *ad libitum* feeding. Clean water was made available throughout the experimental period.

#### **Blood collection and Serum Biochemistry Determination**

Blood samples were collected from the ear vein of each animal with a sterilized disposable syringe and needle. In order to minimize the standard error in values, the animals were fasted for twelve hours (12 hrs) prior to blood sampling. Blood sample (3 mL) was collected in a labelled sterile serum separator tubes after 90 days of the study. Serum was separated by centrifugation at 3000 rpm at 4°C for 10 minutes and stored immediately at -20°C until use. The biochemical parameters measured include Serum enzymes, Serum electrolytes, Glucose, and total cholesterol. Among serum enzymes, aspartate aminotransferase (AST), alkaline phosphatase (ALP), alanine aminotransferase (ALT), creatinine, and urea (BUN) were studied. For serum electrolytes levels, sodium [Na], potassium [K], calcium [Ca], and chloride [Cl] were measured. All the parameters were estimated in an automated biochemical analyzer (Accurex – Sphera Automated Clinical Chemistry Analyzer, Italy), using commercial kits following manufacturers instruction.

#### **Statistical analysis**

Analysis was done using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, [www.graphpad.com](http://www.graphpad.com)). One-way analysis of variance was used to analyze the data followed by Tukey's Multiple Comparison Test, to determine any significant differences between the treatments. *P* values < 0.05 were considered significant. Results are expressed as mean ± standard error ( $\bar{x} \pm \text{SEM}$ ).

## **Results**

### **Serum biochemical changes and enzymes activities**

Table 2 summarized the changes and activities of serum enzymes such as aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), Creatinine and Urea BUN in growing rabbits in response to the dietary treatments. The biochemical indices in all the four dietary groups of the male and female rabbits after 90 days feeding trial were comparable and no notable changes were detected. Though, there were some variation in the calculated mean values, yet such variations were not statistically significantly ( $p > 0.05$ ).

### **Serum electrolytes**

Table 3 presents the level of various serum electrolytes which includes; Na, K, Ca and Cl of male and female rabbits in response to dietary treatments after 90 days. There were no significant differences ( $P > 0.05$ ) in the level of serum Na in all the dietary groups. Similarly, the level of serum K and Ca were comparable in all the four experimental groups. The response of serum Cl to dietary treatments was also evaluated and all the values remained unaltered in the control as well as in the treated groups of rabbits.

### **Serum total cholesterol and glucose**

The effect of feeding GM cottonseeds on the serum total cholesterol and serum glucose in each group of rabbit was presented in Figure 1. The serum levels of total cholesterol in G1 and G4 males are statistically similar ( $P > 0.05$ ) but were significantly ( $P < 0.05$ ) lower when compared to those of G2 and G3. Correspondingly, significant decrease in the level of serum total cholesterol was also detected in G1 females as compared with the remaining groups. Similarly, significant decrease ( $P < 0.05$ ) in the level of serum total cholesterol was calculated in G2 female as compared to those of G3 and G4 in which

significant increase was recorded. On the other hand, the levels of serum glucose were comparable, and no significant differences ( $P > 0.05$ ) were noticed in all the dietary groups of males and females.

**Table 2. Changes in the levels of serum enzymes in the four experimental groups**

Parameters	Feeding groups (Males)				P-values
	G1	G2	G3	G4	
ALP (U/L)	42.17±7.87	37.83±3.17	40.50±5.76	42.50±5.30	0.9369
ALT (U/L)	47.67±5.11	47.17±9.79	49.67±4.96	47.83±4.48	0.9932
AST (U/L)	92.83±5.27	95.00±3.38	97.67±4.72	89.67±6.23	0.715
Crea (Mg/dL)	1.34±0.08	1.34±0.08	1.43±0.05	1.28±0.14	0.728
Urea (Mg/dL)	21.83±1.33	22.50±1.23	21.50±1.57	22.17±1.58	0.9646

Parameters	Feeding groups (Females)				P-values
	G1	G2	G3	G4	
ALP (U/L)	41.33±3.68	43.33±2.06	41.00±3.06	41.17±3.85	0.9513
ALT (U/L)	49.50±5.14	48.17±4.30	48.17±5.31	49.50±4.85	0.9947
AST (U/L)	91.17±5.42	93.17±12.19	90.17±11.24	86.33±11.70	0.9729
Crea (Mg/dL)	1.32±0.06	1.38±0.10	1.35±0.10	1.39±0.09	0.9435
Urea (Mg/dL)	24.83±2.93	32.667±3.27	32.50±4.18	29.33±3.19	0.3416

ALP: alkaline phosphatase, ALT: aminotransferase, AST: aspartate transaminase, Crea: Creatinine, Urea: Urea BUN, G1: Control, G2, G2, and G4 represent 20%, 30%, and 40% transgenic cottonseeds fed groups respectively.

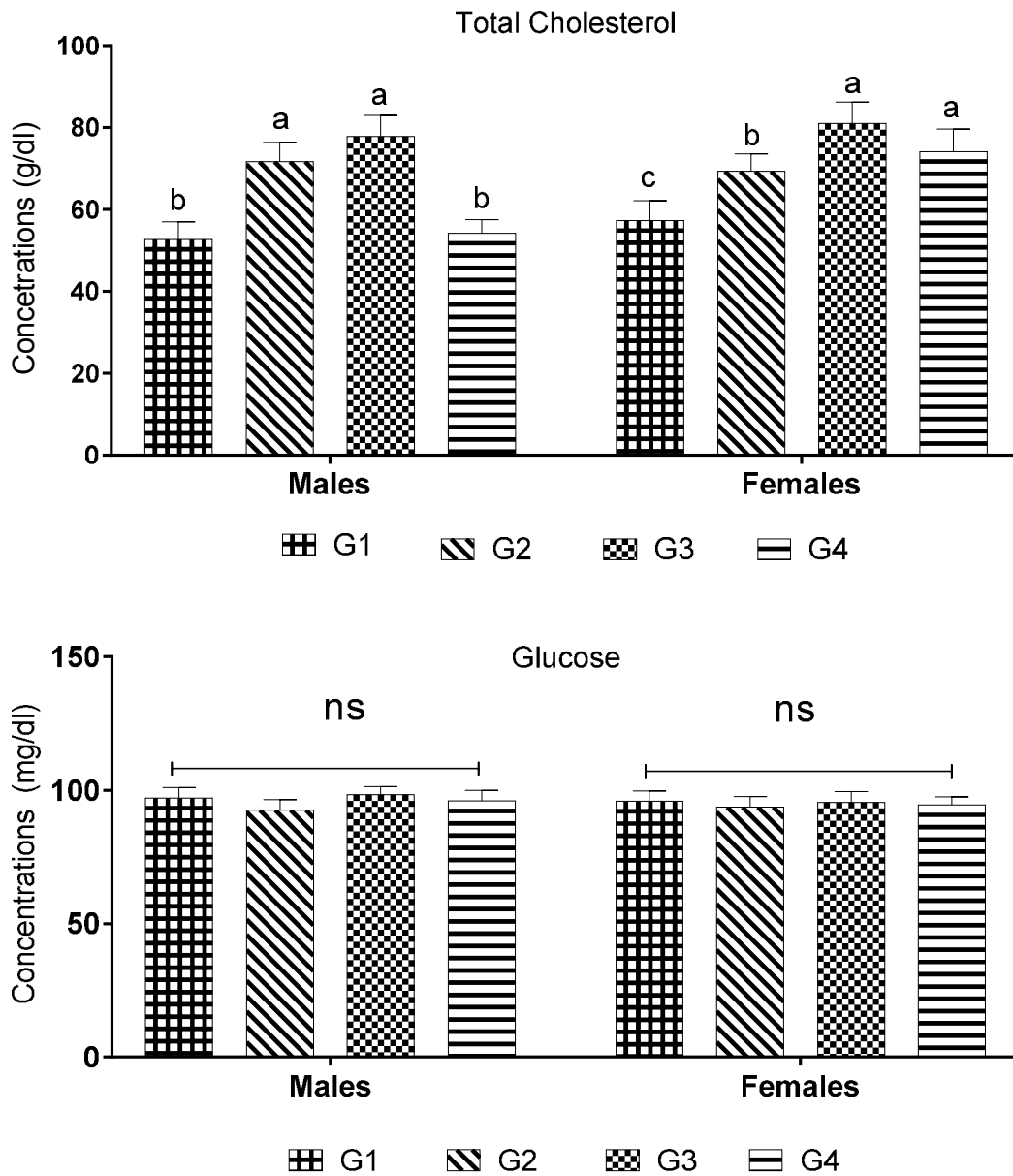
**Table 3. Changes in the levels of serum electrolytes in the four experimental groups**

Parameters	Feeding groups (Males)				P-values
	G1	G2	G3	G4	
Sodium (mEq/l)	142.50±2.77	142.17±2.55	139.67±4.54	142.17±2.99	0.9233
Potassium (mEq/l)	4.40±0.42	4.43±0.32	4.52±0.40	4.38±0.47	0.9951
Calcium (mEq/l)	10.12±0.68	10.02±0.69	9.68±1.06	10.00±0.95	0.9852
Chloride (mEq/l)	101.83±3.32	101.83±4.56	102.33±3.42	104.33±5.30	0.9706

Parameters	Feeding groups (Females)				P-values
	G1	G2	G3	G4	
Sodium (mEq/l)	141.33±4.99	143.17±5.93	141.67±4.18	143.33±4.01	0.9871
Potassium (mEq/l)	4.50±0.39	4.65±0.56	4.65±0.74	4.20±0.39	0.9256
Calcium (mEq/l)	9.50±0.83	9.92±1.02	9.70±0.41	9.70±0.88	0.9873
Chloride (mEq/l)	103.17±5.88	103.83±5.12	102.17±5.24	100.00±3.24	0.9512

G1: Control, G2, G2, and G4 represent 20%, 30%, and 40% transgenic cottonseeds fed groups respectively.



**Figure 1.** Changes in the levels of serum total cholesterol and Glucose in the four experimental groups of male and female rabbits. G1: Control group, G2, G3 and G4 represent 20%, 30% and 40% transgenic cottonseed fed groups, respectively. ns: means not significantly different. Bars bearing different alphabets are significantly different,

### Discussion

Genetically modified (GM) crops have huge potential in plant production however, their safety as food and feed remains debatable (Ibrahim B. Salisu *et al.*, 2018). Various Conflicting reports and controverting opinions were raised continuously regarding the

potential risks of GM crops on both human and animal health. Nevertheless very little is known about the validity of the various safety assessment tests that were performed for these crops (Ibrahim and Okasha, 2016). Therefore, this study was carried out to evaluate the effect



of feeding GM cottonseed on serum biochemical indices of rabbits.

Serum biochemical profile remained very crucial for clinical investigation of several constituents of animal bodies. It has been reported that some of the serum biochemical markers such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transferase, nucleotidase, ceruloplasmin, alpha fetoprotein, amylase, lipase, creatinine phosphokinase and lactate dehydrogenase are used to evaluate diseases of liver, pancreas, skeletal muscles, bone, etc. Such enzyme test may assist the physician in diagnosis and treatment (Gowda *et al.*, 2009; Pratibha *et al.*, 2004; Ruhl and Everhart, 2005). In this study, various serum enzymes were measure in response to GM feed consumption. No substantial changes were observed in all the tested serum enzymes of both male and female rabbits. Although, there were some differences in the calculated mean values, however such differences were only numerical and remained within the normal reference range reported for rabbits (Ahamefule *et al.*, 2008; Archetti *et al.*, 2008). The results of serum enzyme activities obtained in this study obviously revealed that, exposing the rabbits to dietary GM cottonseeds for 90 days did not adversely affect the function of their vital organs such as the liver, kidneys and heart. These results are consistent with the report of Yonemochi *et al.*, (2003), who found no harmful effect of feeding GM maize on serum lactate dehydrogenase levels in dairy cows. Similar results were also reported by Tudisco *et al.*, (2006), suggesting that GM soya bean did not affect the several specific enzymes in serum, heart, skeletal muscle, liver and kidney of the experimental animals. On the other hand, Singh *et al.*, (2003), reported decreased levels of ALT in serum of buffaloes raised on GM cottonseed. However, the authors observed that there was no perilous effect on the health

buffaloes as confirmed from the result of haematological parameters.

Serum electrolytes such as Na, K, Ca and Cl are minerals found in body tissues and blood in the form of dissolved salts. As electrically charged particles, electrolytes help in transporting of the nutrients into and wastes out of the body's cells, maintain a healthy water balance, and also assist in stabilizing the body's acid/base (pH) balance (Ahmad *et al.*, 2009; Mushtaq and Pasha, 2013; Oviedo-Rondon *et al.*, 2001). The results obtained from this study revealed that the electrolytes balance was not influenced by incorporation of GM cottonseeds in the diet. Similar conclusions have been made by Zhou *et al.*, (2014), when Sprague–Dawley rats were fed high-amylose transgenic rice for up to three generations. The outcomes of this study agreed with that of Prasad, (2008), who fed rabbits with a high-cholesterol diet for six-months.

Cholesterol is a sterol, a type of lipid molecule, and is biosynthesized by all animal cells as an essential structural component of all animal cell membranes (Hanukoglu, 1992). Likewise, glucose is an important energy source not only for erythrocytes but also act as an indispensable short-term energy source for many other tissues e.g. central nervous system (Wasserman, 2009). The level of serum glucose was not influenced by the dietary treatments but that of serum total cholesterol was changed significantly in both experimental males and females. However, the observed changes were still within the normal reference range reported for healthy rabbits (Ahamefule *et al.*, 2008; Archetti *et al.*, 2008). It is therefore speculated that these changes were not due to GM cottonseeds consumption but could linked to other factors such as gender or physiological differences among the experimental animals. These results, were in line with that of Rahman *et al.*, (2015), who found no noticeable changes in the levels of serum total cholesterol and glucose when rabbits were fed with GM cottonseeds and fresh

leaves for 90 days. Consistently, Yuan *et al.*, (2017), reported no observable differences in the levels of serum total cholesterol and glucose when glyphosate-resistant transgenic soybean GTS40-3-2 was fed to rats for 195 days. Furthermore, Yuan *et al.*, (2013), reported no significant differences in the levels of triglyceride and total cholesterol when rats were fed GM and conventional rice for a 90-day study.

### Conclusion

Under the given experimental conditions, there were no detrimental effects or significant biological changes in the control and experimental rabbit following 90-day

consumption of a diet formulated with the graded levels (0%, 20%, 30% and 40%) of transgenic cottonseeds. The outcomes of this study, therefore suggested that the GM cottonseed meal could be safely used as a feed constituent like other conventional feed ingredients in the diet of rabbits.

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