Phenylalanine Rich Diet (*Vicia faba* L.) Enhances the Expression of Dopamine Receptor D3 (DRD3) Gene in Rabbits

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ABSTRACT

Dopamine is a neurotransmitter hormone for pleasure and reward. It is synthesized only in the brain cells of human and animals and responsible for the regulation of behavior, mood, memory, cognitive, flexible movement, the body weight, and other important functions. The Dopamine Receptor D3 (DRD3) is the most important receptor for dopamine. In this investigation, expression of DRD3 gene was studied in rabbits fed on supplemented diet of dry and fresh faba bean (*Vicia faba* L. Sakha 3). DRD3 gene (≈ 1200 bp) in control and treated rabbits were PCR amplified, sequenced and aligned with reference gene (Acc. No XM_017346708.1). High genetic similarity values were detected among all sequences. DRD3 gene sequences of control, fresh and dry faba bean fed rabbits were deposited in the GenBank with accession numbers MZ714134, MZ714135 and MZ714136 respectively. Direct estimation of blood phenylalanine (Phe) amino acid indicated that feeding rabbits on dry faba bean reflected the highest level of Phe in the rabbit's blood. Quantitative RT-qPCR analysis showed that DRD3 gene was over expressed after feeding rabbits on dry faba bean form compared with feeding on green form and control. Thus, diet rich with phenylalanine like Sakha3 (dry and fresh forms) enhance gene expression of DRD3 gene. However, diet doesn't affect the DRD3 gene sequence and structure. In a conclusion, our findings indicated a direct effect of faba bean supplemented diet on increasing DRD3 expression levels which improve the life quality for human.

Keywords: Dopamine Receptor D3 (DRD3), faba bean, *Oryctolagus cuniculus*, RT-qPCR.

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I. INTRODUCTION

Dopamine is a neurotransmitter hormone for pleasure and reward feeling [1], [2]. It is synthesized only in the brain cells of human and animals [3]. The blood brain barrier (BBB) allows pass of dopamine from brain to the human body, and prevents its entrance to the brain [4]. Dopamine is responsible for the regulation of behavior, mood, memory, cognitive, flexible movement, the body weight, and other important functions [5]-[7]. Decreasing the level of dopamine in the human body, causes serious diseases, for example, Parkinson's disease, Alzheimer [8]-[11], obesity [4], [12], addiction [13], [14], vitiligo [15], [16] and phenylketonuria [17]-[20]. Dopamine also affects the quality of life and health for humans and animals. Low dopamine levels result in increasing the hostility of the animal and its lack of calmness, it also affects its productivity in terms of the amount of milk produced [21], [22].

The dopamine pathway begins with phenylalanine amino acid as the main precursor for dopamine biosynthesis, which

is converted to tyrosine by phenylalanine hydroxylase enzyme. Then, tyrosine is converted to L-Dihydroxyphenylalanine (L-Dopa) by tyrosine hydroxylase enzyme, and L-Dopa is converted to dopamine by L-Dopa decarboxylase enzyme.

The last step in dopamine pathway is binding the dopamine to its receptor in the brain, from this binding dopamine can be spread to all receptors in the body. Alternative pathway is hydroxylation of dopamine to melamine and norepinephrine, which in turn is methylated to produce epinephrine [23]-[25].

Dopamine receptors are divided into two groups; the first one is D1-Like (D1 and D5) receptors and the second group is D2-Like (D2, D3 and D4) receptors [26]-[28]. Dopamine receptors are responsible for receiving dopamine and bound with it after its production inside the brain and it, also responsible for its transmission and distribution to the entire body by transferring it as nervous signals [2], [29]-[31]. The Dopamine receptor D3 (DRD3) gene is located in the nucleus accumbens in the human brain, and any defection in this gene leads to Schizophrenia disease [32], [33]. The cycle of dopamine biosynthesis begins with the phenylalanine

transformation process by phenylalanine hydroxylase enzyme (PAH) and ends at the binding of dopamine to DRD3 in the brain. Then dopamine comes out of the cerebral membrane to the rest of the body [34]. The Dopamine Receptor D3 (DRD3) is the most important receptor for dopamine [9], [31], [35]. Structure of DRD3 receptor is very sensitive and unique, that is made it the perfect target for a lot of drugs as a treatment for vital diseases like Schizophrenia, Depression and Parkinson's disease [36]-[38].

This investigation was aimed to evaluate the influence of direct diet with fresh and dry forms of faba bean (Sakha 3 genotype) on DRD3 levels in rabbits. DRD3 genes of rabbits were PCR amplified, sequenced, and aligned with reference gene in the GenBank.

II. MATERIAL AND METHODS

A. Plant Materials

Faba bean (*Vicia faba* L., Sakha 3 genotype) seeds were obtained from Sakha Agricultural Research Station, Kafr El-Shiekh, Egypt, and planted for a whole season during (September 2018- March 2019) in suitable soil with recommended agriculture conditions. Plants were used as rabbit's food in two forms (dry and fresh) with 150 g feeding dose as shown in Table I.

TABLE I: FABA BEAN FEEDING EXPERIMENT DESIGN

Rabbits	Faba bean feed type	Feeding period	Feeding dose (gm)
1	Control	6 months	0.0
2	Dry		150
3	Fresh		150

B. Animal Samples and Feeding

New Zealand rabbits (*Oryctolagus cuniculus*) were available at the Faculty of Science, Tanta University. They were fed and treated according to the ethical scientific research program, license no. JACUC-SCI-TU-0126 from the Faculty of Science, Tanta University. Male rabbits 6 months aged were fed on fresh and dry beans as shown in Table I, for a period of 6 months. After feeding period, blood samples were collected from the ear vein, after sterilization with 70% ethyl alcohol. Blood samples were drawn using a medical syringe and collected in a separate tube. Rabbits were sacrificed and their brains were collected and stored for further work.

C. Quantification of Phenylalanine Amino Acid

Phenylalanine Assay Kit (abcam, ab83376) was used according to manufacturer protocol for direct quantification of phenylalanine amino acid levels in rabbit blood samples.

D. PCR Amplifications of DRD3 Genes

DRD3 gene fragments of rabbits (*Oryctolagus cuniculus*) were amplified, eluted, sequenced. Nucleotide sequences of treated rabbit samples were aligned and compared with control as well as with reference sequence in the GenBank.

Total genomic DNAs were purified according to manufacturer protocol of GeneJET Genomic DNA Purification Kit (K0721/ Thermo Fisher). Then, Dream Taq PCR Master Mix (2X) (K1071, Thermo Fisher. USA) was

used to amplify DRD3 gene fragments. Primer design and construction was carried out using Primer BLAST Program and rabbit (*Oryctolagus cuniculus*) DRD3, complete cDNA (Accession Number XM_017346708.1) as reference gene. Three specific primer pairs of DRD3 were designed to cover the whole gene sequence as shown in Table II.

TABLE II: SEQUENCE FEATURES OF SPECIFIC DRD3 PRIMERS AND THE EXPECTED SIZE OF AMPLIFIED FRAGMENTS

Primer pair	Sequence	Tm	GC content	Expected size (bp)
Pair no. 1	Forward 5'- GAGTGGCC ACATCAAC TCCA -3'	59.9 °C	55%	211
	Reverse 5'- CAGTAAGT CTGCCACA GCCA -3'	59.9 °C	55%	
Pair no. 2	Forward 5'- TGGCTGTG GCAGACTT ACTG -3'	59.9 °C	55%	581
	Reverse 5'- CAAGGCAG TCTCTTGG CAGA -3'	59.9 °C	55%	
Pair no. 3	Forward 5'- ACGGATCC TTACCCGA CAGA -3'	60.0 °C	55%	363
	Reverse 5'- CTGTTTCAT GTAGCCCA GCCA -3'	60.0 °C	55%	

Thermal cycler (CreaCon, Holland) was used with the following conditions: 40 cycles; each cycle consisted of denaturation at 95 °C for 30 sec followed by annealing at 37 °C for 1 min and extension at 72 °C for 2 min. There was an initial delay for 15 min at 95 °C at the beginning of the first cycle and 10 min delay at 72 °C at the end of the last cycle as a post extension step. Agarose gel (1.5%) was applied to migrate amplicons via MultiSUB Mini Horizontal Electrophoresis System with Power Pro 300 Power supply (Cleaver Scientific, UK) and imaging with Gel documentation system (OmniDOC, Cleaver Scientific, UK). Data analysis was performed using Totallab analysis software (www.totallab.com, Ver.1.0.1).

E. Nucleotide Sequencing of DRD3 Genes

Amplified PCR fragments were purified with E.Z.N.A.®Gel Extraction Kit, (D2500-01, Omega BIO-TEK, USA). Samples were sent to Micron-Corp. Korea for sequencing with ABI PRISM®3100 Genetic Analyzer. Aligned DRD3 sequences were analyzed on NCBI website (<http://www.ncbi.nlm.nih.gov/webcite>) using BLAST to confirm their identity. Genetic distances and Multi Alignments were computed by Pairwise Distance method using Clustal Omegasoftware analysis (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The nucleotide sequences were also compared with highest homology sequences available in the GenBank.

F. Quantitative RT-qPCR analysis of DRD3 genes

Quantitative RT-qPCR was applied to detect DRD3 gene expressions after feeding rabbits on dry and fresh beans.

DRD3 gene expression was determined using Maximas SYBR Green/Fluorescein qPCR Master Mix by Rotor-Gene Q (Qiagen, USA) using two-step cycling protocol and Glyceraldehyde-3-Phosphate as a housekeeping gene. TRIzol's Reagent (15596026, Life Technologies, USA) was applied for total RNA purification from brain samples. Yield and quality of total RNA were determined Spectrophotometrically at 260 and 260/280 nm ratio, respectively.

III. RESULTS

A. Levels of Phenylalanine (Phe) Amino Acid in Blood

For evaluating direct effect of faba bean feeding dietary on phenylalanine (Phe) level in rabbits, Phe content was estimated in blood of rabbit under study. Compared with control sample, feeding on dry faba bean was superior to fresh faba bean for Phe levels. As shown in Table III, rabbits fed on 150 g of dry bean reflected highest increase of Phe level (311 nmol/L), compared to those fed on 150 g of fresh bean (98 nmol/L) Control rabbits indicated a relatively low level of Phe (77 nmol/L).

TABLE III: PHENYLALANINE LEVELS IN BLOOD FOR CONTROL AND TREATED RABBITS

Rabbit samples			
Phenylalanine levels (nmol/L)	Control	Fresh beans (150 g)	Dry beans (150 g)
	71	98	311

B. Amplification and sequences of DRD3 gene fragments

DRD3 gene in control and faba bean fed rabbits were amplified, sequenced, and aligned with reference gene (Accession Number XM_017346708.1). Specific DRD3 amplicons with 581, 363 and 211 bp were detected for the three samples (Fig. 1).

Fig. 1. Amplified PCR products of DRD3 gene with $\approx$ 581, 363 and 211 bp for control and rabbits fed on 150 g of dry and fresh faba bean.

High genetic similarity values were detected in DRD3 gene sequences for both control and faba bean fed rabbits with reference gene sequence as shown in sequence alignment (Fig. 2) and similarity percentage (Table IV). DRD3 gene sequence for control sample reflected the highest genetic similarity with reference sequence (98.14%). For fresh faba bean fed rabbits, the lowest genetic similarity (97.97%) was recorded after feeding with 150 g faba bean. Nucleotide sequences of dry bean fed rabbits indicated (98.06%) similarity with reference gene sequence. DRD3 gene sequences of control, fresh and dry faba bean fed rabbits were deposited in the GenBank with accession numbers MZ714134, MZ714135 and MZ714136 respectively.

TABLE IV: SIMILARITY OF DRD3 GENE SEQUENCES FOR ORYCTOLAGUS CUNICULUS WITH REFERENCE GENE (ACCESSION NUMBER XM_017346708.1) FROM GENBANK

Dopamine D3 Receptor Gene Sequence	Similarity (%)
Rabbit fed on (150 g) of fresh faba bean.	97.97 %
Rabbit fed on (150 g) of dry faba bean.	98.06 %
Rabbit control.	98.14 %

Fig. 2. Nucleotide sequence alignment of amplified DRD3 genes with reference gene (Accession Number XM_017346708.1).

C. Expression of DRD3 gene

Quantitative RT-qPCR was used to evaluate effect of feeding by fresh and dry faba bean on DRD3 gene expression levels. Our findings indicated overexpression of DRD3 gene as a direct influence of feeding rabbits on dry and fresh faba bean. As shown in Table V and Fig. 3, rabbits fed on 150 g

of dry faba bean reflected superior increase of DRD3 gene expression level (almost three folds) compared with those fed

on 150 g of fresh faba bean which DRD3 gene expression level with one and half fold.

TABLE V: EXPRESSION LEVELS OF DRD3 GENE

Samples	Gene being Tested Experimental (TE)	Gene being Tested Control (TC)	Housekeeping Gene Experimental (HE)	Housekeeping Gene Control (HC)	Δ Ct values for the experimental (Δ CTE)	Δ Ct values for the control (Δ CTC)	Δ Ct Value ($\Delta\Delta$ Ct)	$2^{\Delta\Delta$ Ct (Expression fold change)	-fold Expression level in the experimental condition the expression as in the control condition
Control	35.7	21	34	20	1.7	1	0.7	0.6	
Treatments dry beans (150 gm)	32.2	21	32	20	0.2	1	- 0.8	1.7	
fresh beans (150 gm)	34.7	21	33.6	20	1.1	1	0.1	0.9	

Δ Ct values (Experimental) = Ct (Tested Exp) – Ct (Housekeeping Exp).

Δ Ct values (Control) = Ct (Tested Control) – Ct (Housekeeping Control).

$\Delta\Delta$ Ct values = Δ Ct values (Experimental) - Δ Ct values (Control).

Relative gene expression fold change = $2^{\Delta\Delta$ Ct.

Fig. 3. Expression of DRD3 gene for three rabbit samples. 1: control, 2: rabbit fed on 150 gm of dry beans and 3: rabbit fed on 150 gm of fresh beans.

IV. DISCUSSION

In this investigation, rabbits (*Oryctolagus cuniculus*), as a biological model, were used for Dopamine Receptor D3 (DRD3) gene sequence and expression [39], [40] indicated perfect matching dopamine biosynthesis pathway between rabbits and human and high similarity of DRD3 gene sequence between rabbits and human. Faba bean (*Vicia faba* L.) Sakha 3 genotype was chosen as a diet rich in phenylalanine to study the effect of different feeding conditions on DRD3 expression. Previous studies showed that faba bean plants contain high amounts of phenylalanine and phenolic amino acids [41].

Results of the present study indicated an increase of phenylalanine level in rabbit blood after fed on faba bean. This is in accordance with [42] who indicated direct influence of diet rich in phenylalanine as faba bean Sakha 3 genotype on increasing phenylalanine (Phe) amino acid in rabbit blood.

Our findings obtained by quantitative RT-qPCR reflected high increase of DRD3 gene expression level in rabbit fed on fresh and dry faba bean (one and half and three folds, respectively). This indicates a direct correlation between faba bean diet rich in phenylalanine and increasing the level of phenylalanine amino acid in the blood, which enhance DRD3 gene expression.

The cycle of dopamine biosynthesis begins with the phenylalanine transformation process by phenylalanine hydroxylase enzyme (PAH) and ends at the binding of

dopamine to DRD3 in the brain. The DRD3 is the most important receptor for dopamine [31], [34], [35]. The steps of dopamine biosynthesis cycle in rabbits are the same in humans [43]. Diet rich with phenylalanine like Sakha3 (dry and fresh forms) is the main precursor for dopamine [41], [42], [44]-[46]. It is increased the gene expression of DRD3 gene.

In this investigation, DRD3 gene fragments were PCR amplified, sequenced, and aligned with reference gene. The results indicated high sequence similarity with reference gene. Thus, diet rich with phenylalanine like Sakha3 (dry and fresh forms) enhance gene expression of DRD3 gene. However, diet doesn't affect the DRD3 gene sequence and structure [47]-[51].

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