

Fall 2017


Identifying the inflammatory pathways of genes upregulated in LADMAC cells exposed to BCM7

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Biology, Chemistry, and Environmental Studies Department

The official copy of the thesis

**Identifying the inflammatory genes and pathways upregulated in LADMAC cells exposed
to BCM7**

A Thesis Presented

by

Oumlissa Persaud

To The Undergraduate School in Partial Fulfillment of the Requirements for the Degree of

Bachelors of Science

Major in Biology

at

Molloy College

12/18/2017

Molloy College

Biology, Chemistry and Environmental Studies Department

Signature page

I certify that I have read the thesis and that, in my humble opinion, I feel it successfully meets the requirements outlined by the thesis board in both scope and quality for the completion of the research track in biology at Molloy College.

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Title of Professor: Doctorate

Professor's department: Biology, Chemistry and Environmental Studies Department

Signature _____ Oumlissa Persaud _____ Date _____ 12/18/2017 _____

Abstract of the dissertation:

Identifying the inflammatory pathway of genes upregulated in LADMAC cells exposed to BCM7

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Bachelors in Biology

Molloy College (December)

Biology, Chemistry and Environmental Studies department

Casein is the main protein present in milk and other dairy products. Beta-casein is one of the 3 major proteins in milk. Beta-casomorphin-9 (BCM9) was the original beta-casein protein found in milk, specifically cow's milk. However, a genetic mutation occurred and the 67th amino acid in the BCM9, proline, mutated to histidine (P67H) creating Beta-casomorphin-7 (BCM7). The protein BCM7 gets cleaved in the small intestine, making it readily absorbed in the body. In previous unpublished studies they found that murine leukocytes (LADMAC cells) showed an increase in inflammation when exposed to BCM7, as compared to BCM9. Therefore, the goal of this experiment is to find the inflammatory pathway of genes upregulated in LADMAC cells when exposed to BCM7. The 5 genes we tested were: PTGES, PTGS1, PTGS2, TNF α and NF- κ B. Quantitative polymerase chain reaction (qPCR) was used to identify upregulated inflammatory genes in the LADMAC cells exposed to BCM7, BCM9, lipopolysaccharide (LPS, positive control) and phosphate-buffered saline (PBS, negative control). Our results showed that none of the genes were upregulated when exposed to BCM7 or BCM9. These genes are also a part of the cyclooxygenase pathway, which implies that an inhibitor of this pathway will not stop

inflammation caused by BCM7 or BCM9. Although we did not identify the inflammatory pathway upregulated by BCM7, we were still able to do the opposite. We identified a pathway which is definitely not turned on.

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Abbreviations:

BCM9 - beta-casomorphin-9

BCM7- beta-casomorphin-7

LPS - lipopolysaccharide

PBS - phosphate-buffered saline

qPCR - quantitative polymerase chain reaction

Acknowledgements

I would like to express sincere appreciation to my research mentor Dr. Mary Kusenda, my parents and Jaclyn Kirshbaum. I am very grateful for Dr. Kusenda's guidance throughout my research experience. Dr. Kusenda's thorough and careful explanations are very much appreciated. This project would not be possible without the help of my research colleague, Jaclyn Kirshbaum. We equally worked on this research together and I appreciate all her help. I am very thankful for my parents' tremendous support at all times, especially throughout my academics endeavors.

Furthermore, I would like to thank the Molloy College Biology, Chemistry, and Environmental Studies (BCES) Department for supporting our research. Thanks to Kristen Deutsch a CSTEP research assistant, Eizel Bianca Salonga a summer research volunteer and Senya Huda and Sana Iqbal who worked as Molloy College high school summer research interns and contributed their technical expertise to the project. This research was sponsored in part by the A2 Milk Company. PO BOX 163, Shortland St. Auckland, 1010 New Zealand. Finally, I am grateful for all the experimental advice and helpful discussions given by Dr. Malav Trivedi from Nova Southeastern University.

Introduction

Casein is the most predominant fraction (80%) of milk's protein. There are three forms of casein, and beta-casein (β -casein) is one of them. β -casein has 209 amino acids and at least 12 genetic variants (Haq et al, 2014). The two major variants are A1 and A2 which differ at their 67th amino acid position due to a mutation which happened in dairy cattle about 10,000- 5000 years ago. The original A2 milk coded for beta-casomorphin-9 (Bcm9) with proline at the 67th amino acid position (Haq et al, 2014). After the mutation took place, proline was replaced with histidine thus producing A1 milk which coded for beta-casomorphin-7 (BCM7).

β -Casomorphins are opioid like bioactive peptides which are released upon digestion of β -casein (Haq et al, 2014). BCM9 is a larger protein which is not readily absorbed by the digestive system, this is the opposite for a smaller protein such as BCM7. The proline in BCM9 forms a tight bond with amino acids on either side of it, but histidine does not. Due to the weakness of the peptide bonds with histidine, a peptide consisting of 7 amino acids breaks off in our digestive track (Cross, 2015). This peptide is BCM7 which can easily cross the blood brain barrier, bind to opiate receptors and cause inflammation.

It has been previously proven through several experiments that BCM7 causes/increases inflammation (Cross, 2015). In a study conducted by Haq et al, BCM7 was evaluated on murine's gut immune response. Mice were administered the peptides through oral intubation. The researchers found that the BCM7 peptide increased phenotypic expression of inflammation associated molecules (Haq et al. 2014). In unpublished research, LADMAC cells, which are murine monocytes, also showed increased inflammation when exposed to BCM7 as compared to BCM9 by a chemotaxis assay (Amaya et al, 2015).

Inflammation has symptoms of redness, swelling, heat and pain (Scott et al, 2004). It can be classified as a cascade of events and plays a major role in the body's immune response to injury and infection. Of course having an acute inflammatory response is necessary at times: for example when stubbing one's toe. However, chronic inflammatory responses become problematic when they are turned on for unnecessary reasons like, inflammation due to milk.

Therefore, the goal of this experiment is to find the inflammatory pathway of genes upregulated in LADMAC cells when exposed to BCM7. The 5 genes we tested were: PTGES, PTGS1, PTGS2, TNF α and NF- κ B. These genes belong to the cyclooxygenase pathway. This pathway catalyzes the immediate substrate for a series of cell specific prostaglandin and thromboxane synthases (Williams et al, 1999). Prostaglandins are involved in various biologic processes, like the regulation of immune function, inflammation, kidney development etc. (Williams et al, 1999). Therefore, if these genes are upregulated when exposed to BCM7 and BCM9, then an inhibitor of this pathway may prevent inflammation caused by BCM7 or BCM9.

To identify whether this pathway is upregulated or not, the cDNA obtained from inflamed LADMAC cells exposed to BCM7, BCM9, lipopolysaccharide (LPS) as a positive control, and phosphate-buffered saline (PBS) as a negative control will be used. The expression of the genes will be measured by using quantitative polymerase chain reaction (qPCR). Real time qPCR is the most reliable method for detection and quantification of DNA, cDNA and RNA levels (Thornton, 2011).

Materials and Methods

Genes Used

A previous research group selected 12 primer pairs to test for their 12 chosen genes (IL12A, IL12B, PTGES, PTGS1, PTGS2, MPO, NF- κ B_B, NF- κ B_A, MAP3K11, CXCL13, IL1A and TNF α). The genes were chosen based on previous knowledge; they are known to be involved in inflammation. The gene list was later narrowed down to 5 genes after bioinformatics analysis and initial testing of primers by qPCR.

Gathered Bio-informatics data (Genome Browser, Bio GPS & GeneCards)

The BLAT feature of the genome browser mapped the 12 primer pairs, identified their genome coordinates and inferred length of the qPCR product. The genome browser provided other information such as, chromosome number, nucleotide number and melting temperature.

GeneCards was used to identify the function of each gene and BioGPS looked at the expression of the genes. BioGPS identified the genes expression in all cells, specific cells and macrophages. If the genes were expressed in macrophages then there was a good chance that they would be expressed in our LADMAC cell line. CXCL13 and MPO were eliminated because they were not expressed in macrophages. Although NF- κ B was neither expressed in macrophages, we continued to use it in our study because of its importance in immunological pathways. We were left with 10 genes.

LADMAC cell culture:

LADMAC cells (ATCC CRL-2420) were cultured in Dulbecco's Modified Eagles Medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals, Flowery Branch, GA) 1% Penicillin-Streptomycin (Invitrogen, Carlsbad, CA), 1% Non-Essential amino acids (MEM (Invitrogen, Carlsbad, CA), and 1% Glutamax (Invitrogen, Carlsbad,

CA). Cells were passaged 8 times and the media was changed every 2-3 days and were maintained at 1×10^5 - 1×10^6 cells/ml and incubated at 37°C in 5% CO₂.

Exposing cells to additives:

Cells were cultured at a concentration of 1.44×10^6 cells per flask in 4ml of media and exposed to additives BCM9 (10^6 molar) (A2 Milk Company Auckland NZ), BCM7 (10^6 molar) (A2 Milk Company, Auckland NZ), lipopolysaccharide (LPS) as the positive control and phosphate-buffered saline (PBS) as the negative control. 4µL of each additive was added. All experiments were done as biological triplicates. Cells were incubated in the additives for 48 hours.

RNA extraction:

RNA was extracted from the LADMAC cells following the manufacturer's protocol "Purification of Total RNA from Animal Cells using Spin Technology" (QIAGEN with the exception of the following changes: an extra drying step was added after adding RLT buffer the second time and an extra minute was added prior to final RNA elution.

Reverse transcription

Reverse transcription converted the LADMAC's mRNA to cDNA. The BIORAD protocol "iscript reverse transcriptase supermix for RT-qPCR" (Hercules, CA) was used. We followed this protocol exactly to obtain cDNA as the product.

Nano-Dropping

The samples used in this experiment and the products obtained were all checked for quality and quantity by Nano-drop (Wilmington, DE). For example, Nano-drop confirmed that the reverse transcriptase products were cDNA. The numbers; 1.9 and 1.8 identified RNA and DNA respectively.

Quantitative Polymerase Chain Reaction (qPCR)

We stringently tested the 10 genes using qPCR. qPCR was performed by preheating at 95°C for 3 minutes, followed 39 rounds at 95°C for 10s, 55°C for 10s, 72°C for 30s, with a final step of 95°C for 10s, 65°C for 5s 95°C for 5s. The protocol for 2X SYBR Green qPCR Master Mix (BiMake,Houston,TX.) was followed and the CFX96 Touch Real time PCR detection system (BIO-RAD Hercules, CA) was used. The total reaction was 25ul with a concentration of 10ng/ul cDNA.

We removed MPO, NF κ -B_A, IL12A, IL12B and IL1A from the final analysis because their Cq scores were higher than 30 and an ideal Cq score is usually below 30. A second qPCR test was performed only on the 5 remaining genes: PTGES, PTGS1, PTGS2, TNF α and NF- κ B_B. The results were normalized against two housekeeping genes: GAPDH (F-5' AGGTCGGTGTGAACGGATTTG, R-5'TGTAGACCATGTAGTTGAGGTCA) and ACTIN (F-5' GGCTGTATTCCCCTCCATCG and R-5' CCAGTTGGTAACAATGCCATGT).

Results

Bio-informatics data

Genome browser confirmed that there was a 100% match between the forward and reverse strands of all 12 primers, it also confirmed that the primers identified the same gene. Genome browser provided additional information like number of nucleotides, chromosome number start and end strands and melting temperature (**Table 1**). Gene cards provided the functions of each gene (**Table 1**). BioGPS identified whether the genes were expressed in “all cells”, “specific cells” or macrophages. (**Table 1**).

Gene name	Function	Expression in all cells	Expressed in specific cells	Expressed in macrophage	Forward 5'	Reverse 5'	Chromosome	Strand	Start	End	# nucleotides	Tm
PTGS1	Increased in inflammation and inhibited by NSAIDs	Most cells	white blood cells, mast cells	YES	GACAACTGGAGGG AGGAGTG	2	(+)	36230432	36230451	438	58.09	
					CCCAGAGCCAG TATCCATGT	2	(-)	36237718	36237737	58.4		
TNF	Pro-inflammatory cytokine, secreted by macrophages, neuroprotective function of cytokine	No	only macrophages 1 hr most, and decreased by half by 7hrs	YES	CCCTCACACTCAG ATCATCTTCT					61	58.27	
					GCTACGACGTG GGCTACAG					57.91		
IL1A	Inflammatory response in macrophages, stimulate the release of	yes	macrophages and bone marrow	YES	GCAACGGGAAGAT TCTGAAG	2	(-)	129306534	129306553	177	58.31	
					TGACAAACTTCT GCCTGACG	2	(+)	129304760	129304779	58.43		
CXCL13	Promotes the migration of B-lymphocytes- chemotaxis	no	Lymph node, spleen	Very little	AGGCCACGGTATT CTGGAAG					250	59.91	
					AGCTTGGGGAG TTGAAGACA					58.3		
MAP3K11	Activates MAPK8/JNK kinase, and activates JNK signalling pathway, PI3K and IKKalpha, and transcription of NFkB, mediates Rho family/ GTPases and cdc42	Most cells	Bone marrow	high	TAGATTCCGAGCC ATCCAGT	19	(+)	5696778	5696797		58.13	
					AGGTACCACGT GGCTTCATC	19	(-)	5697574	5697593	192	58.43	
PTGS2	Prostanoic acid biosynthesis in inflammation	no	Bone marrow macrophage 6hr lps	YES	TGAGCAACTATTC CAAACCAGC	1	(+)	150104078	150104099	74	60.08	
					GCACGTAGTCT TCGATCACTATC	1	(-)	150104296	150104318	1,287	56.62	
IL12A	Subunit of a Cytokine, that acts on T and NK cells, Cytokine may need NO synthase 2A is found to be required for the signalling process of this signalling in innate immunity.	No	Macrophage after 7 hrs LPS	YES	TAGACAAGGGCAT GCTGGT	3	(+)	68695540	68695559		57.67	
					AGATAGCCCAT CACCCCTGT	3	(-)	68698018	68698037		55.37	
MPO	It is a heme protein. Plays a role in neutrophils	No	Bone Marrow CD34 cells	No	CCATGCTTCAGAC CTCCAAT					195	58.53	
					CCGGCTGCTTG AAGTAAAC					58.43		
PTGES	Prostaglandin E synthase, activated by I11B, expressed by TP53. Involved in inflammation	No	Bone marrow 6hr	YES	GGATGCCTGAAA CGTGGAG	2	(-)	30900913	30900932	132	64.06	
					CAGGAATGAGTA CACGAAGCC	2	(+)	30892760	30892780		58.25	
IL12B	A cytokine that acts on T cells and NK cells as well as a broad variety of cellular	YES	Macrophage bone marrow	YES	TGGTTTGCCATCG TTTTGCTG	11	(+)	44404064	44404084	123	65.85	
					ACAGGTGAGGT TCACTGTTTCT	11	(-)	44407848	44407869		56.32	
NFKB_A	Controls cytokine production and cell survival	YES	Lymphoblasts mainly	No	ATGGCAGACGATG ATCCCTAC					111	58.45	
					TGTTGACAGTG					58.63		
NFKB_B	Controls cytokine production and cell survival	YES	Lymphoblasts mainly	No	GACCAAGGAGATG GACCTGA					271	59.76	
					AACTCCGCCAT TTTCTTCT					58.47		

Table 1: Bio-informatics data (BioGps, Gene cards & Genome Browser)

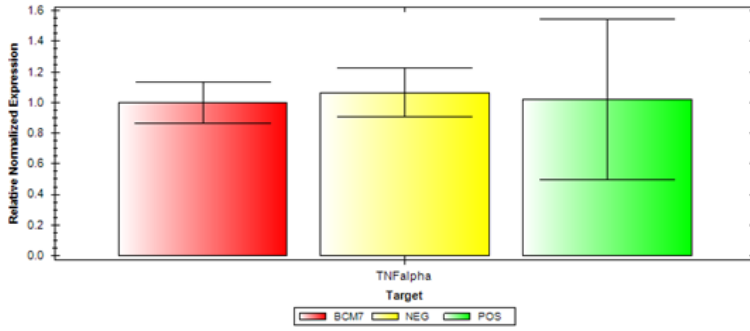
Quantitative Polymerase Chain Reaction (qPCR)

We performed qPCR on 5 genes (PTGES, PTGS1, PTGS2, TNF α and NF- κ B). They are a part of the cyclooxygenase pathway in LADMAC cells exposed to BCM7, BCM9, LPS and PBS.

The qPCR data of the 5 genes were normalized against two housekeeping genes: GAPDH and ACTIN. The results indicated that none of the 5 genes were upregulated by BCM7 or BCM9 (**Figure 1**). Expression of each gene tested didn't surpass the expression of the negative control. For example, expression of PTGS2 when exposed to BCM7 and BCM9 was not significantly higher than the negative control (**Figure 1**).

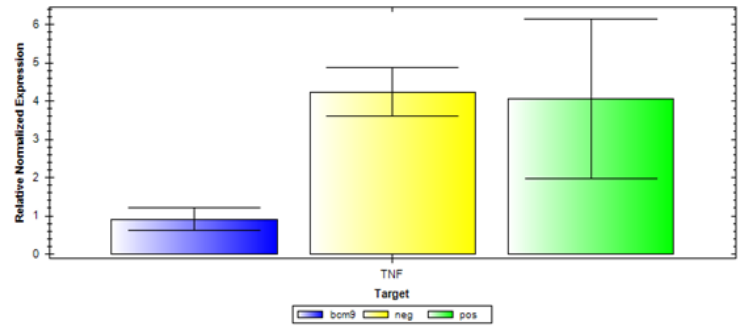
BCM7

TNF α

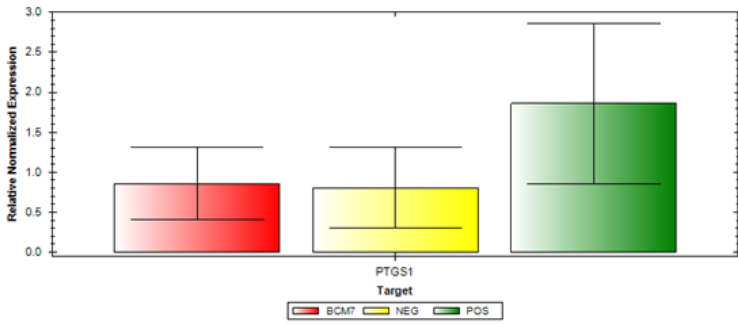


BCM9

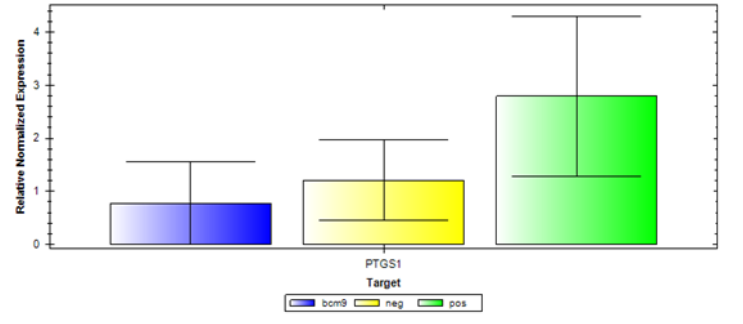
TNF α



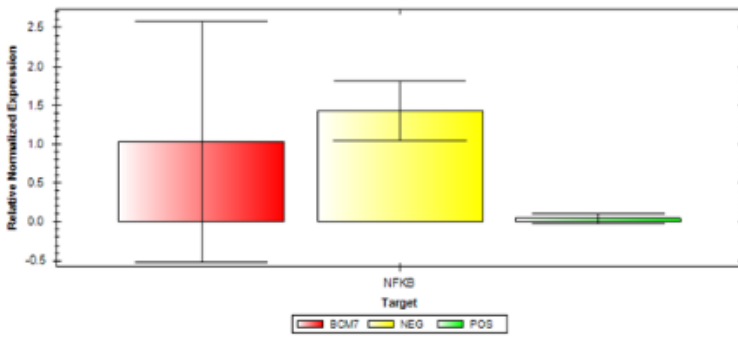
PTGS1



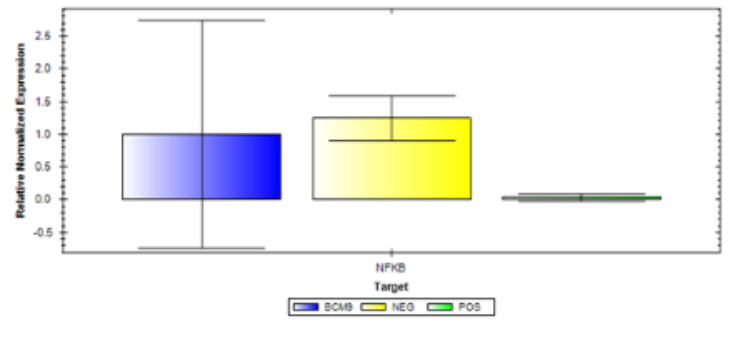
PTGS1



NF- κ B



NF- κ B



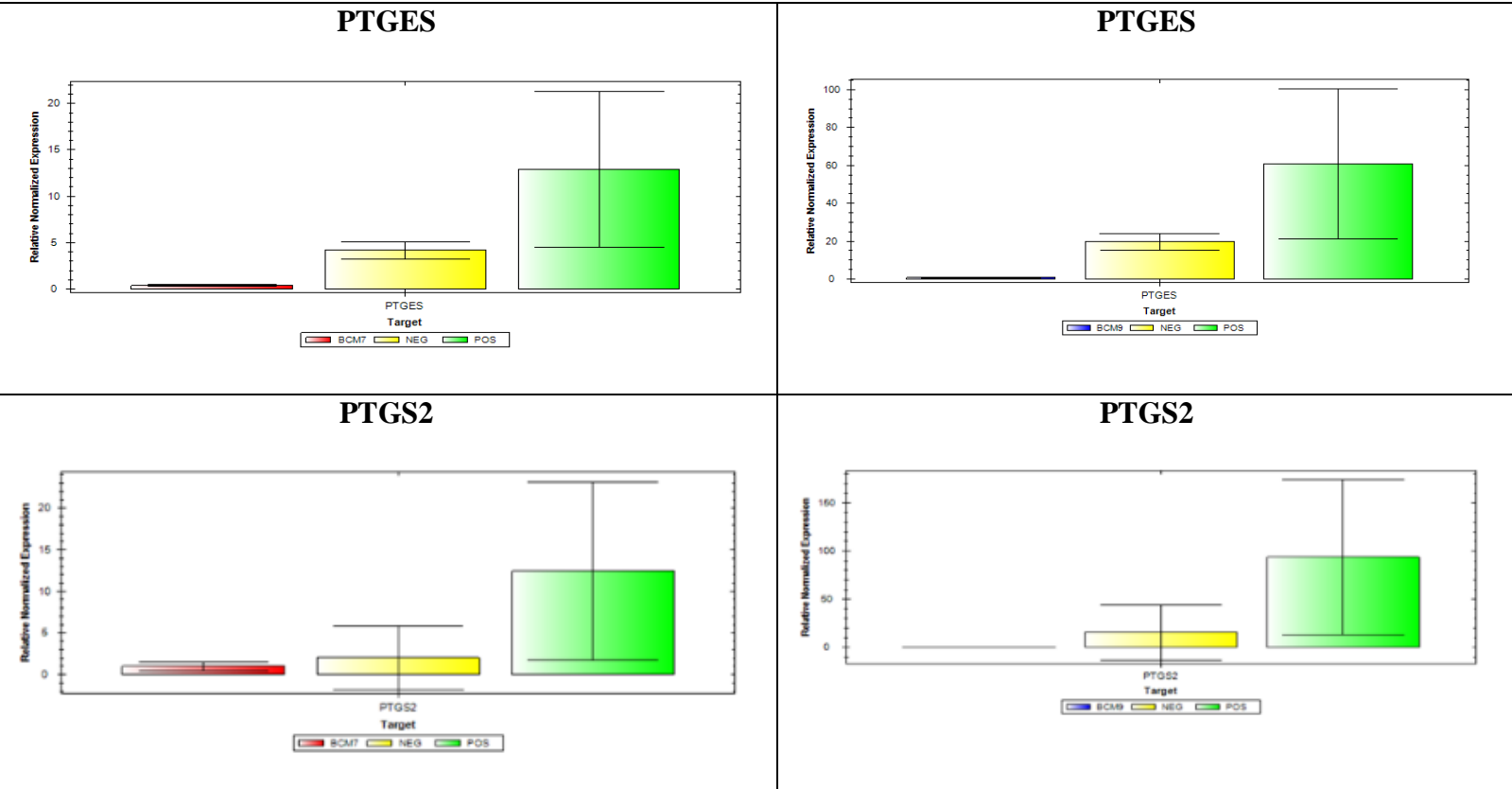


Figure 1: Graphs showing the expression of the genes when exposed to BCM7, BCM9, LPS and PBS. The genes expression when exposed to BCM7 and BCM9 is in red and blue respectively. The negative control and the positive control are represented by yellow and green respectively. The genes expression when exposed to BCM7 and BCM9 were lower than the expression of the negative control. Therefore, the genes when exposed to BCM7 and BCM9 were not upregulated.

Discussion

The qPCR results showed that none of the 5 genes tested were upregulated when exposed to BCM7 and BCM9. Our findings on the 5 genes suggested that, inhibition of the cyclooxygenase pathway will not stop inflammation caused by BCM7 or BCM9. For example, aspirin is an inhibitor of the cyclooxygenase pathway (Ohmori et al, 2006). However, aspirin will not alleviate or stop inflammation caused by BCM7 or BCM9.

A study performed by Pennesi et al, reported that a casein free diet helped autistic patients. The diet alleviated symptoms of inflammation and it improved other ASD symptoms like social behavior (Pennesi et al, 2012). These findings implied that casein probably caused these symptoms or enhanced them. Such complications by casein can be attributed to its BCM7 portion. As such, it is still very important to identify the specific inflammatory pathway turned on by BCM7. If this is done then an inhibitor of the pathway can be developed to help those patients. More research is clearly needed in this area.

Although we did not identify the inflammatory pathway upregulated by BCM7, we were still able to do the opposite. We identified a pathway which is definitely not turned on. For future studies, our findings gave researchers additional background information to work with. Future researchers can now proceed by testing other genes aside from the ones we already tested.

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