The effect of propolis produced by *Apis mellifera* as a nutritional supplement on the expression level of NF-κB and LC3 anti-inflammatory immune genes in Iraqi Hamdani sheep

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Abstract

This research aimed to measure the expression levels of the genes LC3 and NF-κB in Hamdani sheep blood that were fed with Apis mellifera propolis. Propolis, a bee product, has been utilized for centuries due to its numerous biological advantages. It was incorporated into their daily diet for six weeks. Propolis is well-known for its antimicrobial, anti-inflammatory, and immune-modulating effects in both animals and humans. The propolis was introduced to the diet every two weeks in quantities of 2, 3, and 4 grams. Following this, blood samples were collected from these 10 sheep biweeklies, and on the same day, RNA was extracted in the lab and converted into cDNA. The expression levels of the genes LC3 and NF-κB were assessed using a Real-Time PCR machine, and the ΔΔCt values were calculated based on reference gene expression. The gathered data were analyzed using the GraphPad software, revealing that the expression of the target genes increased when compared to the control data, with the optimal amount of bee propolis additive being 4 grams per day.

Keywords: LC3, NF-κB, propolis, cDNA and Real-Time PCR

Introduction

Propolis exhibits antimicrobial, antitumor, antioxidant, anti-inflammatory, and immunomodulatory characteristics, showing potential for use in the pharmaceutical sector [1]. Regarding its immunomodulatory effects, propolis increased the expression of TLR-2 and TLR-4 in macrophages,[2]. the co-stimulatory protein CD80 and pro/anti-inflammatory cytokines produced by monocytes,[3] stimulated the NF-κB pathway in dendritic cells and enhanced the production of antibodies [4]. LC3 was first recognized as a protein associated with microtubules, and its main role lies in autophagy, a mechanism that entails the comprehensive breakdown of cytoplasmic components [5]. LC3-associated phagocytosis (LAP) represents a newly identified type of non-canonical autophagy, in which LC3 (microtubule-associated protein 1A/1B–light chain 3) binds to the membranes of phagosomes by utilizing some components of the traditional autophagy machinery.[6] It is recognized to have two variants [7]. LC3-I, located in the

cytoplasm, and LC3-II, which is associated with membranes and is derived from LC3-I to trigger the formation and extension of the autophagosome [8]. It varies from LC3-I solely in that it undergoes covalent modifications with lipid extensions (lipidation). As a result, LC3 has become extensively utilized to track the number of autophagosomes and the level of autophagic activity [9].

Additionally, recent findings indicate that in the process of selective autophagy, LC3 acts as an adaptor protein to attract specific cargo to the autophagosome by interacting with cargo receptors [10]. Nuclear factor kappa B (NF-κB) is a time-honored protein transcription factor and is viewed as a key regulator of the innate immune response [11]. The NF-κB signaling pathway connects signals from pathogens and cellular danger cues, thereby coordinating the cell's defense against invading microorganisms. The mammalian NF-κB transcription factor family is made up of five proteins: p65 (RelA), RelB, c-Rel, p105/p50 (NF-κB1), and p100/52 (NF-κB2), which can interact with one another to create various transcriptionally active homo- and heterodimeric complexes [12]. It is essential in numerous biological functions, such as immune response, inflammation, cell growth and survival, and development [13,14]. Nuclear factor-κB (NF-κB) serves as a transcription factor that is vital for many biological processes, including immune response, inflammation, cell growth and survival, and development [15,16]. NF-κB gets activated by different inflammatory triggers, including growth factors and infectious microbes [17,18]. NF-κB regulates the expression of several genes that influence immune responses, cell growth and proliferation, survival and apoptosis, stress responses, as well as embryogenesis and development in response to various stimuli [19]. NF-κB is essential for human health, and its abnormal activation is linked to the onset of various autoimmune, inflammatory, and cancerous diseases, such as rheumatoid arthritis, atherosclerosis, inflammatory bowel diseases, multiple sclerosis, and tumors [20]. Propolis influenced the functions of monocytes when exposed to retinoic acid (RA), human melanoma-associated antigen-1 (MAGE-1), and lipopolysaccharide (LPS)[21]. Propolis reduced the expression of LC3 that was induced by LPS and also led to lower levels of NF-kB expression compared to the control cells; its combination with RA, however, resulted in a higher expression than the stimulus on its own [22].

Materials and Methods

Experimental Design

The study was performed in the Department of Animal Science of the Agriculture Engineering Sciences of Salahaddin University-Erbil on 10 Hamdani sheep aged 2.5 - 3.5 years, divided into the three types of propolis dietary supplement doses at different time intervals were fed a concentrated mixture according to their body weight requirements and the basal diet plus propolis for six weeks in the first two weeks (2 g/day diet), the next two weeks (3g/day diet), and at last two weeks (4 g/day diet).

Blood sampling

In this study, blood samples were obtained from all 10 sheep before initiating the propolis feeding as a control group. Following the diet, blood was sampled from each

sheep every two weeks during three different dosage experiments. The blood samples were collected from the jugular vein of both the control and treated sheep. An anticoagulant (EDTA) was used for the blood collection, and the samples were stored for several hours at −80 °C to facilitate RNA extraction.

Total RNA extraction and cDNA synthesis

Whole blood was utilized to extract total RNA using the Total RNA Extraction Mini Kit (FAVORGENE, Taiwan) and was treated with DNase solution during the on-column extraction, following the guidelines provided by the manufacturer. The RNA samples were preserved at −80 °C until the synthesis of cDNA.The amount and quality of RNA were evaluated using a NanoDrop ND-100 Spectrophotometer (Thermo Fisher Scientific, MA, USA). The A260:280 ratio varied between 2.0 and 2.2 for all samples. The RNA was re-dissolved to achieve a final concentration of 200 ng/µl. Total RNA from each sample was reverse-transcribed and treated with RNAase using the SuperScript™ III first-strand synthesis system with oligo dT20 primers (AddBio, South Korea) following the manufacturer's protocol, which included 4 µl of 5x reaction buffer, 2 µl of a 10 mM dNTP mixture, 2 µl of 10x oligo dT20, 1 µl of 20X addscript enzyme solution, 3 µl of RNA template, and sufficient nuclease-free water to bring the total volume to 20 µl. The temperature cycling involved: priming at 25 °C for 10 minutes, reverse transcription at 50 °C for 60 minutes, RT inactivation at 80 °C for 5 minutes, and cooling to 12 °C for 1 minute. The resulting cDNA was preserved at -20 °C.

Quantitative real-time PCR assays

Real-time quantitative PCR (qPCR) reactions were performed in a total volume of 25 µL using RealQ Plus 2x Master Mix Green (Amplicon, Denmark) with optimized primer concentrations as detailed in Table 2. Each reaction tube contained 12.5 µL of qPCR Mastermix, 1 µL of each primer, 3 µL of cDNA sample, and 9.5 µL of PCR-grade water. A negative control, which included all reagents except the target DNA, was included in each qPCR assay to ensure there was no contamination. The quantity of gene transcripts was determined through absolute qPCR utilizing the 7300 Real-time PCR System (Applied Biosystems, Id.), which included an initial denaturation step lasting 3.5 minutes at 95 °C followed by 40 cycles consisting of 25 seconds at 95 °C, 30 seconds at 58–60 °C (58 °C for NF-κB, 59 °C for LC3, and 60 °C for ACTB), and 40 seconds at 72 °C during which fluorescence was measured. The melting temperature (Tm) of the amplicons was assessed over a range from 65 to 95 °C with increments of 0.5 °C every 5 seconds. No template controls (NTC), which did not contain cDNA template, were processed simultaneously. The qPCR analyses for samples and controls were conducted in triplicate. The analysis for relative gene expression employed the comparative Ct (ΔΔCt) approach, and the overall fold change for the targeted genes compared to untreated controls was calculated as 2^–ΔΔCt [23], with efficiency values ranging from 1.8 to 2.2. Relative fold changes were illustrated graphically using GraphPad Prism software Inc. (Version 5.0, California, USA).

Table (1): Description of the investigation's primers

Gene	Primer	Sequence	Size, bp	References
ACTB	Forward	5'-CCAACCGTGAGAAGATGACC-3'	97	[24]
	Reverse	5'-CCAGAGGCGTACAGGGACAG-3'		
LC3	Forward	5'-ATGGTATACGCCTCTCAGG-3'	250	[25]
	Reverse	5'-TTCCCAAAGCTGAATGTGC-3'		
NF-kB	Forward	5'-CAACTGCTCTACCTCCTG-3'	290	[26]
	Reverse	5'-CTTCACTGTTACGGGTTC-3'		

ACTB=Housekeeping gene (beta actin)

Table (2): The sample real-time PCR data for analysis. In this data set, there are two genes (LC3and NF-Kβ); and three concentrations of each combination of gene and sample.

Repli-cate	Samples	Propolis Concen-tration	Ct		2^-(ΔΔCt)	
			LC3	NF-kB	LC3	NF-kB
1	Control	-	31.91	31.99	0.6	1.2
2	Control	-	31.48	32.525	0.8	0.8
3	Control	-	31.135	32.305	0.9	1.0
4	Control	-	31.565	32.32	0.9	1.0
5	Control	-	31.5	32.385	0.8	0.9
6	Control	-	30.495	31.715	2.0	1.4
7	Control	-	31.72	31.58	1.2	1.6
8	Control	-	31.33	32.375	1.4	0.9
9	Control	-	31.315	32.505	1.3	0.8
10	Control	-	31.34	32.775	0.8	0.7
1	T1	2	30.4	30.85	1.83	1.6
2	T1	2	30.41	31.155	1.59	1.1
3	T1	2	30.705	31.55	1.24	0.6
4	T1	2	30.805	30.615	1.49	2.1
5	T1	2	30.39	30.83	1.65	1.7
6	T1	2	31.07	30.6	1.35	2.1
7	T1	2	30.915	31.725	2.10	0.4
8	T1	2	30.405	30.525	2.60	2.3
9	T1	2	30.73	31.25	1.91	1.0
10	T1	2	30.68	31.145	1.29	1.1
1	T2	3	29.435	30.69	3.57	1.9
2	T2	3	29.495	29.92	3.00	4.0
3	T2	3	29.83	31.155	2.27	1.1
4	T2	3	29.195	29.65	4.55	5.1
5	T2	3	29.2	30.115	3.76	3.4
6	T2	3	29.185	29.89	4.98	4.1
7	T2	3	29.485	31.01	5.75	1.4
8	T2	3	29.24	29.67	5.82	5.0
9	T2	3	28.745	30.7	7.55	1.9
10	T2	3	29.025	30.695	3.94	1.9
1	T3	4	28.71	29.41	5.91	6.1
2	T3	4	28.59	29.205	5.63	7.2
3	T3	4	29.18	29.445	3.56	6.0

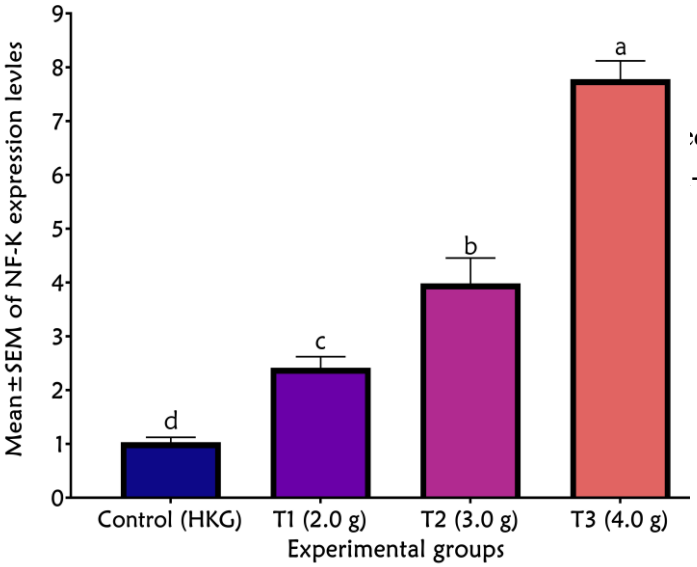
4	T3	4	29.205	29.14	4.52	7.6
5	T3	4	28.855	29.325	4.78	6.6
6	T3	4	28.88	29.11	6.16	7.8
7	T3	4	28.92	29.28	8.35	6.8
8	T3	4	28.585	28.965	9.17	8.7
9	T3	4	28.675	29.545	7.93	5.5
10	T3	4	28.68	29.575	5.00	5.4

Table (3): Impact of propolis produced by *Apis mellifera* as a nutritional supplement on the expression level of NF-κB and LC3 Anti-inflammatory immune genes in Iraqi Hamdani sheep (mean ± SEM, n = 10).

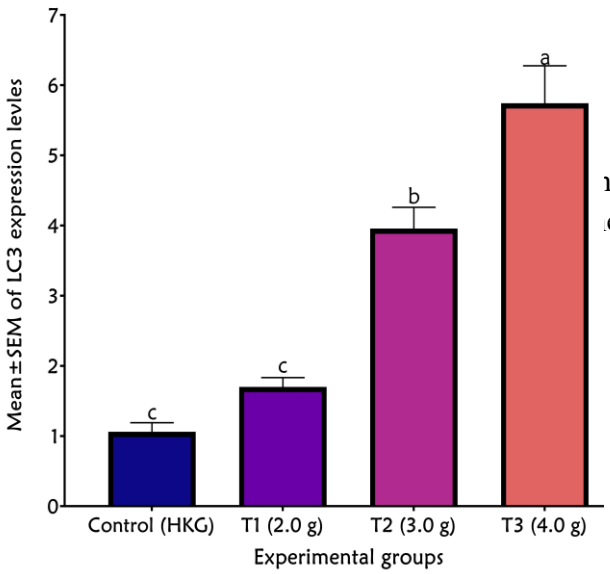
Genes name	Experimental groups				p-value
	Control (HKG)	T1 (2.0 g)	T2 (3.0 g)	T3 (4.0 g)	
LC3	1.06+0.1307c	1.7+0.1341c	3.957+0.3034b	5.742+0.5354a	<0.0000
NF-Kβ	1.032+0.0909d	2.417+0.2054c	3.984+0.473b	7.78+0.3405a	<0.0001

T1 (2.0 g): (2 g/day diet) propolis, T2 (3.0 g): (3 g/day diet) propolis, T3 (4.0 g): (4 g/day diet) propolis. $^{A\text{-}B\text{-}C\text{-}d}$ different superscript letters in the same row represent significant difference.

A



B



C

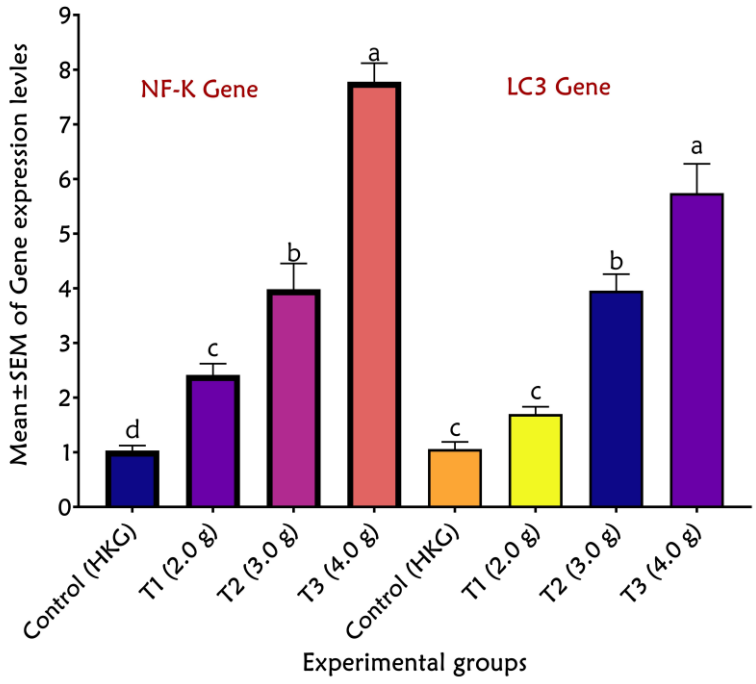


Figure (1): A, B, C Impact of propolis produced by *Apis mellifera* as a nutritional supplement on the expression level of LC3 and NF-κβ Anti-inflammatory immune genes in Iraqi Hamdani sheep. (HKG): Housekeeping gene (ACTB).

Result and Discussion

The summary of the effects of varying levels of propolis (2 g/day diet for the first two weeks, 3 g/day diet for the next two weeks, and 4 g/day diet for the final two weeks) on the expression levels of NF-κ and LC3 anti-inflammatory immune genes in Iraqi Hamdani sheep are presented in Tables 1 and 2. The expression levels of the NF-κβ gene significantly increased ($p \leq 0.05$), reaching (T1 2.417+0.2054c, T2 3.984+0.473b, and T3 7.78+0.3405a) with the different levels of propolis compared to the internal housekeeping gene reference (ß-actin), which was given a value of 1.0. The various levels of propolis (last two weeks at 2 g/day, the next two weeks at 3 g/day, and last two weeks at 4 g/day) significantly enhanced ($p \leq 0.05$) the expression of the LC3 gene, as shown in Figure 1, indicating that the highest expression level of NF-κβ anti-inflammatory immune genes in Iraqi Hamdani sheep was notably recorded (7.78+0.3405a) in lambs consuming a diet supplemented with propolis T3 (4 g/day) when compared to the internal reference ß-actin, assigned a value of 1.0 (1.032+0.0909d). Furthermore, lambs provided with a diet containing propolis T3 (4 g/day) during the last two weeks showed an increased expression level of LC3 anti-inflammatory immune genes that significantly ($p \leq 0.05$) reached (5.742+0.5354a) in comparison to the internal reference ß-actin, which had a value of 1.0 (1.06+0.1307c).

Based on these results, it can be concluded that propolis supplementation in Hamdani sheep enhances the anti-inflammatory immune genes. Our findings are consistent with those by [29], who noted that propolis extracts can influence the nonspecific immune response by activating macrophages, promoting the release of hydrogen peroxide, and suppressing the production of nitric oxide in a dose-dependent manner. Supporting our findings, [30] demonstrated that propolis suppresses both inducible nitric oxide synthase (i-NOS) expression and its enzymatic activity.

The most widely researched and biologically active component in propolis inhibits the production of cytokines and chemokines, T cell proliferation, and lymphokine production, leading to a reduction in the inflammatory response. This mechanism is believed to be associated with the NF-κB signaling pathway [31]. CAPE is recognized as a strong inhibitor of nuclear factor -κB (NF-κB) activation [33], and the inhibition of NF-κB may lead to decreased expression of COX-2, whose gene is regulated by NF-κB [27], alongside a significant reduction in NO production by inhibiting INOS activation [34]. Conversely, our findings align with those of [28], who observed the production of IL-1ß and IL-12 by macrophages, as well as IL-2, IL-4, IL-10, and transforming growth factor beta (TGF-β) in their study of the impacts of propolis and some of its constituents on the basic functions of mitogen-activated immune cells in human blood, including DNA synthesis and cytokine production in vitro. These results indicate that propolis and many of its components exert a direct regulatory influence on fundamental immune cell functions and can be viewed as a natural alternative to anti-inflammatory agents. Present findings are supported by numerical studies, with [35] reporting that propolis possesses a wide array of additional beneficial biological activities, including anti-inflammatory properties, as well as antioxidant effects [36].

Assessing the impact of propolis derived from Apis mellifera as a dietary supplement on the expression levels of NF-κB and LC3 anti-inflammatory immune genes in Iraqi Hamdani sheep, our findings indicate that administering 4 g/day for six weeks improved the sheep's health by significantly ($p \leq 0.05$) reducing inflammation and oxidative stress, while also notably up-regulating the expression levels of NF-κB and LC3 anti-inflammatory immune genes. Conversely, both lower doses of propolis (2 and 3 g/day) also significantly ($p \leq 0.05$) elevated the expression levels of NF-κB and LC3 anti-inflammatory immune genes when compared to internal (housekeeping) reference genes.

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