ORIGINAL ARTICLE

WILEY

Gene expression of adipokines and inflammatory cytokines in peripheral blood mononuclear cells of obese dogs

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Abstract

Background: Peripheral blood mononuclear cells (PBMCs) have been identified as a possible marker of inflammation in obesity. Understanding the expression of pro- and anti-inflammatory cytokines in PBMCs in obese dogs will help control obesity-related inflammatory diseases.

Objectives: The aim of this study was to evaluate the role of PBMCs in obesity-associated chronic inflammation by analyzing the expression of adipokines and inflammatory cytokines.

Methods: Blood samples were obtained from 25 subjects and real-time quantitative polymerase chain reaction determinations were performed to quantify the gene expression levels of adipokines and inflammatory cytokines, including TNF-α, IL-17, leptin, MCP-1, and adiponectin, in the PBMCs.

Results: The results showed that the gene expression levels of TNF-α ($p < 0.001$), IL-17 ($p < 0.0001$), and leptin ($p < 0.0001$) were strongly upregulated in the PBMCs of obese dogs compared to that in non-obese dogs.

Conclusions: The changes in gene expression levels of inflammation-related adipokines and pro-inflammatory cytokines occur in PBMCs, which may contribute to the low-grade chronic inflammation that is present in obesity.

KEYWORDS
adipokine, canine, obesity, peripheral blood mononuclear cell, pro-inflammatory cytokine

1 | INTRODUCTION

Obesity is the state of having an increased adipose tissue mass as a result of an imbalance between energy intake and output. It is also considered a form of chronic low-grade inflammation in humans. Furthermore, human obesity has been proven to be associated with type 2 diabetes mellitus, osteoarthritis, cardiovascular diseases, systemic hypertension and as a risk factor for various kinds of cancer (Enns et al., 2011). To identify how obesity contributes to the development of a chronic inflammatory state, several studies have been conducted to analyse gene expression levels of pro-inflammatory cytokines and adipokines in relation to body mass index and body weight. As in humans, some findings suggest that canine obesity may be related to diseases like osteoarthritis, insulin resistance, pancreatitis and

respiratory disease (Piantedosi et al., 2016). However, limited studies have investigated the difference in gene expression between obese and normal dogs.

Peripheral blood mononuclear cells (PBMCs) are conventional and universal blood samples that are used to analyse biomarkers at a transcriptional level. PBMCs are mainly composed of lymphocytes, monocytes and natural killer cells, which are all essential components of the immune system. They are generally useful in identifying changes in cytokine activity during inflammation since direct measurement of blood concentration level is limited due to the short half-life of cytokines and the presence of many inhibitors in the blood serum (Friberg et al., 1994). In addition, a more suitable amount of sample can be obtained when using PBMCs to confirm gene expression levels, in contrast to other test samples such as fat and muscles (de Mello et al., 2008). Several studies have compared adipokine and cytokine gene expressions in circulating PBMCs to discuss the correlation between obesity and inflammation in humans. However, in veterinary medicine, most studies have evaluated the correlation between obesity and inflammation by comparing cytokine concentration in serum samples, and only one study utilised gene expression (Vendramini et al., 2020). However, this study used whole blood samples instead of PBMCs; hence, there is a need for further research in this area.

In this study, we aimed to evaluate how obesity affects the expression of inflammation-related adipokines in circulating PBMCs. We analyzed the messenger RNA (mRNA) expression of not only adipokines and chemokines such as leptin, adiponectin and monocyte chemoattractant protein-1 (MCP-1), but also pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), and interleukin-17 (IL-17). The haematological effects of obesity were also considered and analysed by comparing the results of general blood tests on obese and non-obese dogs.

2 | MATERIALS AND METHODS

2.1 | Animals

All animals included in this study were client-owned, young, healthy dogs with no known systemic disease and were brought in for blood donation. Informed consent was obtained from the owners before each procedure. Using a thorough physical examination and the BCS 9 score system, dogs with BCS 7–9 were defined as obese dogs, while dogs with BCS 4–6 were classified as the normal group (Laflamme, 1997). The blood samples taken for analysis were part of their health screening, which is also evaluated and discussed in this study. Furthermore, circulating PBMC samples were taken from whole blood and used to analyse gene expression.

2.2 | Sample collection

All dogs included in this study underwent over 12 hours of fasting. A physical examination was done, after which, blood samples were obtained by venipuncture from either the jugular or cephalic vein.

2.3 | Complete blood count and serum biochemistry

Blood samples treated with EDTA were used for complete blood count (CBC) while samples taken in SST tubes were centrifuged and used for the serum chemistry panel including serum triglycerides (TG) and total cholesterol (T-chol) level measurements.

2.4 | PBMC isolation, RNA extraction and real-time quantitative PCR

PBMCs were isolated from the EDTA-treated blood samples by using density-gradient sedimentation with Ficoll—Paque reagent (GE Healthcare, Little Chalfont, Bucks, UK). Total RNA was extracted from the PBMCs using easy-BLUE reagent (iNtRON, Sungnam, Republic of Korea) after following the manufacturer's instructions. With this RNA, 20 μl of cDNA was synthesized with CellScript cDNA Master Mix (Cell-Safe, Suwon, Korea). Each patient's target gene expression was evaluated in duplicate.

Target adipokines and cytokines included TNF-α, IL-17, MCP-1, leptin, adipokine and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Adipokine and cytokine gene expression was quantified by quantitative polymerase chain reaction (RT-qPCR) using AMPIGENE qPCR Green Mix Hi-ROX with SYBR Green Dye (Enzo Life Sciences, Farmingdale, NY, USA). This was evaluated in duplicate, and GAPDH, being a housekeeping gene, was used as a reference. The sequences of primers used are listed in Table 1.

2.5 | Statistical analysis

All data are presented as mean values and standard error of the mean (SEM). Statistical comparisons between the two groups were performed using an unpaired two-tailed Student's t test. p-values < 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism v 6.01 (GraphPad Software Inc., La Jolla, CA, USA).

3 | RESULTS

3.1 | General characteristics of patients

The general characteristics of each patient are shown in Table 2. A total of 25 dogs participated; five dogs were classified in the obese group (BCS above 7) and the other 20 were classified in the non-obese control group. All patients are large breed dogs, mainly Labradors and Golden Retrievers. The mean BCS of the obese dogs was at 7.2 $\pm$ 0.2, while the

TABLE 1 Primer sequences used to detect gene expression of adipokines, chemokines, cytokines and housekeeping gene

Gene	5'-3' Primer sequence		References
Leptin	Forward	TTCCTGTGGCTTTGGCCCTAT	(Ishioka et al., 2006)
	Reverse	GCCACCACCTCTGTGGAGTA	
Adiponectin	Forward	CCGTGATGGCAGAGATGGC	(Saengsoi, 2018)
	Reverse	AGCCTCGGGGACCTTCAAC	
Monocyte chemoattractant protein 1	Forward	GCTCACCCAGCCAGATGC	(Ryan, 2008)
	Reverse	GCAGTTTGGGTTTGGCTTTT	
Interleukin-17	Forward	CCGATCTACCTCACCTTGGA	(Schmitz et al., 2015)
	Reverse	TCGCAGAACCAGGATCTCTT	
Tumor necrosis factor-alpha	Forward	TCATCTTCTCGAACCCCAAG	(Rai et al., 2008)
	Reverse	ACCCATCTGACGGCACTATC	
Glyceraldehyde-3-phoaphate dehydrogenase	Forward	TATGACATCAAGAAGGTAGTAA	(Kim et al., 2019)
	Reverse	GTAGCCAAATTCATTGTCATACCAG	

TABLE 2 Characteristics including breed, age and sex, of the 25 study dogs, across body condition scores (BCS)

Gourp	Breed	BCS (/9)	Age	Sex
Obese	Labrador retriever	7	5 yrs	MC
	Labrador retriever	7	6 yrs	MC
	Golden retriever	7	2 yrs	IF
	Labrador retriever	7	7 yrs	FS
	Labrador retriever	8	9 yrs	MC
Non-obese	Labrador retriever	5	4 yrs	MC
	Labrador retriever	6	3 yrs	MC
	Poongsan Dog	6	5 yrs	MC
	Labrador retriever	4	4 yrs	MC
	Labrador retriever	6	3 yrs	MC
	Labrador retriever	5	5 yrs	IF
	Labrador retriever	6	5 yrs	MC
	Golden retriever	5	2 yrs	MC
	Labrador retriever	6	8 yrs	FS
	Labrador retriever	5	7 yrs	MC
	Golden retriever	5	6 yrs	MC
	Labrador retriever	5	3 yrs	FS
	Labrador retriever	5	3 yrs	MC
	Great Pyrenees	4	6 yrs	M
	Golden retriever	5	6 yrs	FS
	Golden retriever	6	5 yrs	MC
	Old English sheepdog	6	6 yrs	IM
	Labrador retriever	5	4 yrs	MC
	Labrador retriever	6	7 yrs	MC
	English sheepdog	6	6 yrs	MC

non-obese dogs had a mean BCS of 5.333 ± 0.1436; the mean body weight of the obese dogs was 36.16 ± 2.347 kg, while the non-obese dogs have a mean body weight of 32.04 ± 0.9925 kg; and the mean age of the obese dogs was 5.8 ± 1.158 years, while the non-obese dogs had a mean age of 4.9 ± 0.362 years (Table 3).

3.2 | Hematochemical parameters

The results of the blood analysis are shown in Table 4. Among the hematologic tests and chemistry panels performed, only the blood TG concentration level was significantly different between the obese and non-obese dogs ($p < 0.0001$).

3.3 | Gene expression of pro-inflammatory cytokines and adipokines

In comparing the gene expression levels between the obese and non-obese dogs, TNF-α, IL-17 and leptin showed a statistically significant increase in obese dogs ($p < 0.001$) while MCP-1 ($p = 0.7068$) and adiponectin ($p = 0.3800$) showed a tendency to increase and decrease in obese dogs, respectively. Diagrams comparing the expression of mRNAs are given in Figure 1.

4 | DISCUSSION

The aim of this study was to compare the expression levels of pro-inflammatory cytokines and adipokines in the PBMCs of obese and non-obese dogs. Previous studies have confirmed that the gene expression in PBMCs is related to the amount of visceral fat (Yamaoka et al., 2012) and varies depending on the presence of systemic inflammation

TABLE 3 Mean BCS, body weight and age of obese and non-obese dogs

	Non-obese		Obese	
	Mean ± SEM	Median (range)	Mean ± SEM	Median (range)
BCS	5.333 ± 0.1436	5 (4-6)	7.2 ± 0.2	7 (7-8)
Body weight (Kg)	32.04 ± 0.9925	31.9 (24.7-38.2)	36.16 ± 2.347	35 (31.5-44.4)
Years	4.9 ± 0.362	5 (3-8)	5.8 ± 1.158	6 (2-9)

**TABLE 4** Complete blood count, serum chemistry, electrolytes of obese and non-obese dogs

Parameter	Unit	Reference range	Non obese group (Mean ± SEM; n = 20)	Obese group (Mean ± SEM, n = 5)	p value
WBC	/uL	5200-17000	7719 ± 325.2	7914 ± 525.4	0.7877
N/L ratio			2.19 ± 0.1967	2.969 ± 0.4829	0.0943
PCV	%	37.1-57.0	47.26 ± 0.6217	44.68 ± 1.7	0.0977
PLT	10000/uL	14.3-40.0	47.26 ± 0.6217	25 ± 2.901	0.4686
ALT	U/L	5.8-83.3	45.7 ± 2.861	50.2 ± 6.733	0.5027
AST	U/L	11.7-42.5	29.75 ± 1.048	29.2 ± 4.283	0.8523
ALP	U/L	0-97.9	34.25 ± 3.39	25 ± 3.435	0.2039
GGT	U/L	0-14	3.4 ± 0.613	3.2 ± 1.158	0.8842
T-bil	mg/dL	0-0.2	0.096 ± 0.01032	0.064 ± 0.01503	0.1614
BUN	mg/dL	9.6-31.4	15.85 ± 0.8328	12.02 ± 1.373	0.0456
CREA	mg/dL	0.4-1.3	1.03 ± 0.03112	1.072 ± 0.08315	0.5768
Calcium	mg/dL	9.0-11.9	10.06 ± 0.1025	10.26 ± 0.2227	0.3874
IP	mg/dL	1.3-6.3	3.895 ± 0.2188	3.22 ± 0.4176	0.1777
Total protein	g/dL	5.7-7.5	6.712 ± 0.09902	6.928 ± 0.22	0.3489
Albumin	g/dL	2.6-4.4	3.85 ± 0.06835	3.832 ± 0.05826	0.9027
T-Chol	mg/dL	112-312	252.2 ± 14.34	191.8 ± 16.19	0.0661
TG	mg/dL	21-133	52.87 ± 3.348	111.5 ± 17.04	< 0.0001
Glu	mg/dL	74.5-120	104.5 ± 2.472	106 ± 4.29	0.7770
Na	mmol/L	145.1-152.6	146.4 ± 0.2532	146.5 ± 0.405	0.7941
K	mmol/L	3.6-5.5	4.323 ± 0.05241	4.5 ± 0.1118	0.1434
Cl	mmol/L	113.2-122.9	116.6 ± 0.3952	115.7 ± 0.9958	0.3702

(Ghanim et al., 2004). Since PBMCs significantly reflect the expression of obesity-related systemic cytokines and adipokines as well as represent a more convenient sample extraction process in contrast to the use of adipose, liver and muscle tissues, many studies on human obesity and inflammation have been conducted on PBMCs. In this study, PBMCs were used as a novel sample to identify in vivo cytokine gene expression to characterize the effects of dog obesity on adipokine expression and systemic inflammation.

As pro-inflammatory cytokines TNF-α and IL-17 significantly increased in the circulating PBMCs of obese dogs, it can be established that obese dogs are in a low-grade chronic inflammatory status. Studies done in the early 1990s in obese mice showed an increase in the expression of TNF-α. TNF-α is expressed and secreted in adipose tissue; therefore, its expression increases as the degree of obesity increases, thereby causing clinical symptoms such as insulin

resistance (Hotamisligil et al., 1993). Additionally, TNF-α also has tumour-promoting activity; hence, the correlation between obesity and cancer in humans, which is also a major factor discussed in recent studies. IL-17, a cytokine derived mainly from T cells and secreted by adipose tissues, plays a major role in the initiation of inflammation. It is also known to be involved in glucose homeostasis and adipogenesis (Zúñiga et al., 2010). Studies also reported that IL-17 promotes inflammatory response in obese mice (Lee et al., 2017) and is associated with autoimmune diseases and chronic inflammation in humans with obesity (Chehimi et al., 2017).

Upon comparison of the expression of adipokines and chemokines such as leptin, adiponectin and MCP-1 in circulating PBMCs, a difference between obese and non-obese dogs was found. Among them, leptin is an adipokine produced in adipocytes and is known to be greatly affected by adipose tissue (Münzberg & Morrison, 2015). Obesity can
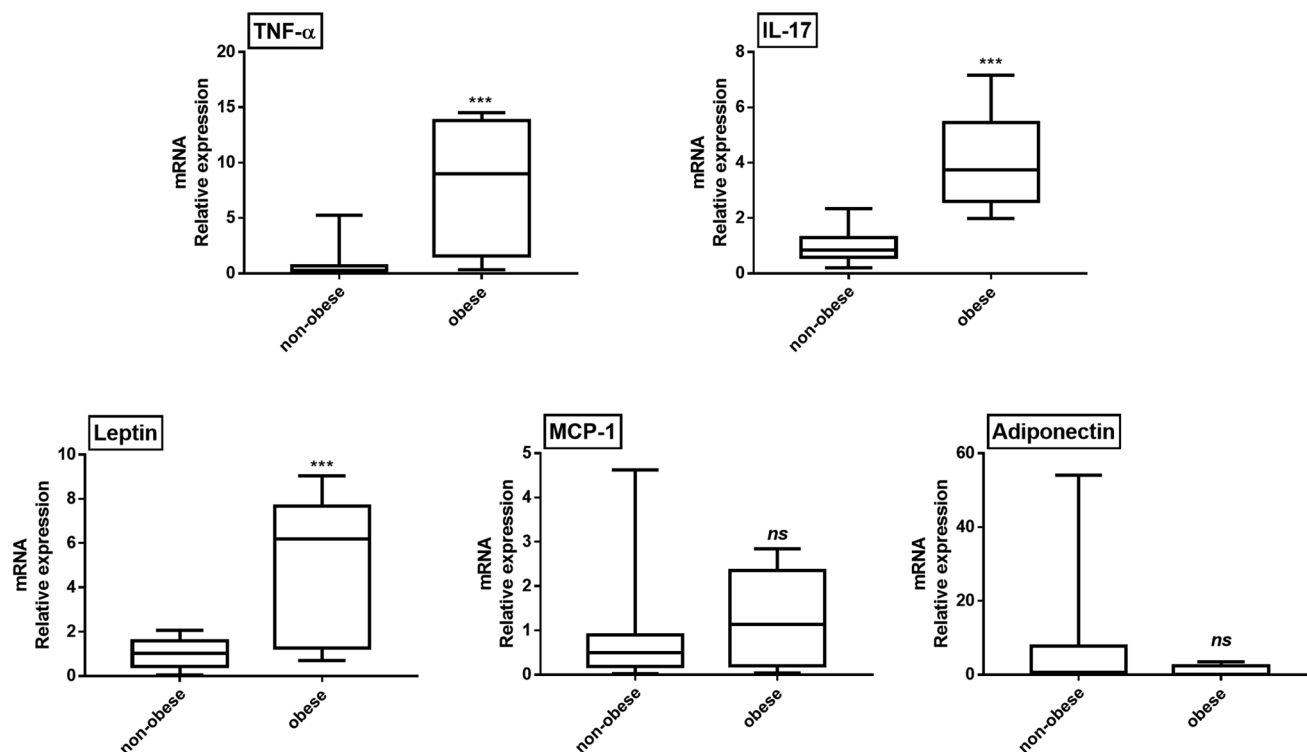
FIGURE 1 mRNA expression of pro-inflammatory cytokines and Adipokines in PBMC of obese dogs. Expression of pro-inflammatory cytokines such as TNF-α and IL-17 and Adipokine such as Leptin, MCP-1, adiponectin were analyzed using RT-qPCR. Results are shown as mean $\pm$ SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ns, not significant

result in an increase in blood leptin levels that can lead to various clinical symptoms due to its effects on glucose and lipid metabolism, angiogenesis, cell-mediated immunity, blood pressure homeostasis, body temperature regulation and appetite regulation (Francisco et al., 2018). However, so far, there has been no study confirming the expression level of leptin in the PBMC of obese dogs. Interestingly, in this study, it was confirmed that the expression of leptin was significantly increased in PBMC of obese dogs.

This study is the first to discuss changes in the pro-inflammatory cytokine and adipokine expression in the circulating PBMCs of obese dogs. Confirmation of results at the actual protein level was not performed, nevertheless, the significant results of this study can serve as a major foundation for studying the correlation between obesity and immune cells, in addition to the existing studies on serum cytokine and adipokine concentrations in obese dogs. A follow-up study confirming the change in expression of more cytokines and adipokines in the circulating PBMCs is recommended. Since dogs and humans share a similar environment, this study can also serve as an important reference for mediating research.

Blood tests clearly indicated an increase in the TG levels of obese dogs. Although no significant difference was found in the T-chol levels, some studies have shown that T-chol in obese dogs (José Lahm Cardoso et al., 2016; Piantedosi et al., 2016; Tropf et al., 2017) could be used to evaluate the effects of obesity on hyperlipidemia. Therefore, hyperlipidemia can be regarded as a clinical symptom associated with obesity in dogs, as in humans. The risk of metabolic syndrome, in which

type 2 diabetes mellitus, cardiovascular diseases and high blood pressure appear together, has been very well established. Obesity has also been presented as a problem in dogs, furthering interest in metabolic syndrome. Our results support previous evidence that confirmed the presence of hyperlipidemia in obese dogs, and thus, contribute to the clinical significance of metabolic syndrome in obese dogs.

The neutrophil-lymphocyte ratio (N/L ratio) has been used as a marker of systemic inflammation and as an indicator of some inflammatory diseases in dogs (Benvenuti et al., 2020; Pierini et al., 2019). In this experiment, we were able to confirm if the N/L ratio increased in obese dogs. Several studies conducted on human obesity have reported an increase in the N/L ratio (Furuncuoğlu et al., 2016; Rodríguez-Rodríguez et al., 2020); however, based on the author's knowledge, no previous studies regarding changes in the N/L ratio have been conducted on dogs. Therefore, we recommend further discussion on the use of the N/L ratio as an inflammatory marker in future studies on dog obesity.

Another limitation of this study is the imbalance in the number of dogs included in the obese group (n = 5) and the control group (n = 20). Analysis of small breeds was also limited, and advanced tests such as blood insulin concentration and lymphocyte differentiation were not performed. Nevertheless, the results of this experiment confirmed that the expression of inflammatory cytokines and adipokines was significantly high in obese dogs, it could serve as the basis for further research on larger population sizes, more diverse breeds and more comprehensive laboratory blood test items.

5 | CONCLUSIONS

This is the first study to confirm changes in the expression levels of inflammatory cytokines and adipokines in PBMCs of obese dogs. Our findings could serve as a basis for further research on the association of obesity with chronic inflammation as well as its correlation with the incidence of systemic diseases associated with adipokines in dogs.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICS STATEMENT

All animal experiments in this study were approved by the institutional animal care and use committee of Seoul National University (SNU), Republic of Korea; all protocols were in accordance with the approved guidelines (SNU, protocol no. SNU-200519-6). The clients agreed to provide peripheral blood mononuclear cells of the dog to the laboratory of Veterinary Internal Medicine, Seoul National University and to fully assume the future management. All informed consents are documented.

AUTHOR CONTRIBUTIONS

Conceptualization: An JH, Youn HY; Data curation: Hwang SY, An JH, Kim KB, Lee JH, Park SM, Oh YI, Chae HK, Youn HY; Formal analysis: Hwang SY, An JH, Youn HY; Funding acquisition: Hwang SY, An JH, Youn HY; Investigation: Hwang SY, An JH, Youn HY; Methodology: Hwang SY, An JH, Youn HY; Project administration: Hwang SY, An JH, Youn HY; Resources: Hwang SY, An JH, Youn HY; Software: Youn HY; Supervision: Youn HY; Validation: Youn HY; Visualization: Youn HY; Writing - original draft: Hwang SY, An JH; Writing - review & editing: An JH, Youn HY.

DATA AVAILABILITY STATEMENT

Data openly available in a public repository that issues datasets with DOIs.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1002/vms3.713.

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