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Original Article

Acyl ghrelin, desacyl ghrelin and their ratio affect hepatic steatosis via PPARγ signaling pathway

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ABSTRACT

Background and study aims: Ghrelin is an appetite hormone-containing 28-amino acid and has 4 different forms in the body. Ghrelin forms have different physiological functions in the body. This study aims to analyze the effect of acyl and desacyl ghrelin hormone on hepatic steatosis and biochemical findings in 36 male Wistar rats.
Materials and methods: Rats were split into 6 equal groups, consisting of control, acyl ghrelin, desacyl ghrelin, acyl/desacyl 3:1, acyl/desacyl 1:1, and acyl/desacyl 1:3 groups, and administered placebo or 200 ng/kg hormone subcutaneous twice a day for 14 days. Oral Glucose Tolerance Test (OGTT) was performed on Day 15, Insulin Tolerance Test (ITT) on Day 16, and scarification procedure on Day 17. Certain biochemical data and liver diacylglycerol (DAG), glycogen, protein kinase C and PPAR-γ levels were detected in the blood. Histological analyses were also conducted on the liver tissues.
Results: The highest plasma total cholesterol and VLDL-K levels were found in the acyl/desacyl 1:3 group, and lower insulin, and HOMA-IR levels were found in groups where acyl and desacyl were administered together ($p < 0.05$). PPAR-γ gene expression level increased in acyl ghrelin and acyl/desacyl 1:3 groups compared to the control group. Protein kinase C gene expression was highest in the acyl/desacyl 1:3 group. The most severe degenerative findings compliant with steatosis in the liver were observed in the acyl ghrelin group ($p < 0.05$).
Conclusion: It was determined that administering rats acyl alone and acyl/desacyl by 1:3 caused the highest PPAR-γ gene expression, serum total cholesterol, HDL-K, and VLDL-K levels in the body. Besides, it is shown that desacyl ghrelin effectively regulates the blood glucose level when administered alone.

Introduction

Ghrelin is an appetite hormone containing 28 amino acids [1–3]. This hormone is secreted by several tissues such as the stomach, brain, kidney, pancreas, liver, adipose tissue, and pituitary [4,5]. The ghrelin hormone has 4 different forms in the body produced by the same ghrelin precursor [nonacylated (desacyl ghrelin), octanoylated (acyl ghrelin), decenoylated and decanoylated]. Acyl and desacyl ghrelin are the forms available in the plasma at the highest level. The desacyl ghrelin is thought to be produced as a result of the des-acylation of acyl ghrelin [6,7].

It is known and available in the literature that ghrelin forms have different physiological functions in the body [8]. Although desacyl ghrelin was considered inactive in the early days, it is now known to have significant endocrine activity in the body [7]. While acyl ghrelin has a characteristic to increase appetite, growth hormone release, body

Abbreviations: NAFLD, Non-alcoholic fatty liver disease; TG, Triglyceride; DAG, Diacylglycerol; PKC, Protein kinase C; AST, Aminotransferase; ALT, Alanine aminotransferase; HDL-C, High density lipoprotein cholesterol; LDL-C, Low density lipoprotein cholesterol; VLDL-C, Very low density lipoprotein cholesterol; OGTT, Oral glucose tolerance test; ITT, Insulin tolerance test; HOMA-IR, Homeostatic model assessment of insulin resistance; RT, Reverse transcriptase; Ct, Cycle threshold; PPAR, Peroxisome proliferator activated receptor; DGAT, Diacylglycerol acyltransferase; AUC, Area under the curve; MTT, Mixed meal tolerans test.

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weight, and insulin resistance, it is indicated that desacyl ghrelin plays a role in decreasing appetite and body weight and increasing insulin sensitivity [4,9].

Non-alcoholic fatty liver disease (NAFLD) is a common public health issue both in developed and developing countries, and it is characterized by a broad liver damage spectrum ranging from simple steatosis to non-alcoholic steatohepatitis. In non-alcoholic fatty liver disease, an increase in de novo synthesis of fatty acids and triglyceride (TG) accumulation in hepatocytes occur [10]. Acyl-CoA, lysophosphatidic acid, diacylglycerol (DAG) lipid metabolites participate in the synthesis of TG in hepatocytes. Diacylglycerol plays an active role in the TG synthesis by regulating protein kinase C (PKC) activation. Therefore, excessive production of DAG leads to the development of NAFLD even in the absence of obesity [10,11].

It is quite important to review acyl and desacyl ghrelins and the ratio of them to each other when investigating the underlying mechanism of hepatic lipogenesis and insulin resistance in NAFLD. In the literature, there are comprehensive studies that examined the effects of acyl ghrelin on appetite and hepatic steatosis; however, no comprehensive study is available on desacyl ghrelin. It is also understood from the recent research that the effects of the ratio of these two hormones to each other on hepatic steatosis, insulin resistance, and biochemical findings are rather important than the amount of acyl and ghrelin. Yet, no data was found in the literature regarding the effective ratios of acyl and desacyl ghrelin. This study was conducted to review the effects of acyl and desacyl forms of ghrelin hormone and the administration of these forms to rats in different doses on hepatic steatosis, insulin resistance, and biochemical parameters.

Materials and methods

Selecting experimental animals, sheltering conditions, and dietary patterns

36 healthy male Wistar rats taken from the Laboratory Animal Breeding and Experimental Research Center, Gazi University according to the principles of "Regulation on the Procedures and Principles Regarding the Operation of Animal Experiment Ethical Committee" [12,13] and weighing 250 ± 30 g were used in the research. The rats were kept in properly ventilated cages with screen covers which were lighted for 12 h and dark for 12 h and kept at room temperature (24 ± 2 °C) and 35–40 % relative humidity. Throughout the research, rats fed ad libitum with standard dry pellet feed and water.

General plan of the research

Rats were divided into 6 groups, each containing 6 rats, weighed one day before the researcher initiated the study, and the weights of the rats in each group were adjusted comparably. The researcher weighed the rats at 8:00 am every day and gave their recorded feed. Control rats received subcutaneous injections of normal saline (200 μl). Both acyl and desacyl treatments were administered at a final dose of 200 ng/kg in 200 μl subcutaneous injections twice a day (8:00 am and 8:00 pm) for 14 days (Table 1). [Acyl ghrelin [AS-24159 Ghrelin, rat 1 mg (Eurogentec)] and desacyl ghrelin [AS-60981 (Des-octanoly)-Ghrelin, rat 1 mg (Eurogentec)]]. As a result of the literature review conducted, it was determined that the long-term tolerable dose for rats without causing any change in body weight or appetite was 200 ng/kg, and this was the dosage used in the study [10,14,15].

Table 1
Hormones administered to rats and their amount (ng/kg).

Groups	Acyl ghrelin	Desacyl ghrelin
Control (n:6)	–	–
Acyl ghrelin (n:6)	200	–
Desacyl ghrelin (n:6)	–	200
Acyl:Desacyl ghrelin 1:1 (n:6)	100	100
Acyl:Desacyl 3:1 ghrelin (n:6)	150	50
Acyl:Desacyl 1:3 ghrelin (n:6)	50	150

Collecting blood and tissue samples

Sacrification and liver tissue of the rats were performed approximately 60 h after the last hormone administration. The reason for this is to perform OGTT and ITT at 1-day intervals to rats and avoid the acute effect of the hormone. On the 17th day of the study, the rats underwent general anesthesia through intramuscular administration of 5 mg/kg Xylazine (Alfazyne 2 %) + 45 mg/kg Ketamine (Alfamine 10 %) by a specialist veterinarian following 12 h of fasting, and they were sacrificed by intracardially collecting their blood respectively starting from the control group. 3–4 mL of the blood collected with 10 mL injectors was taken into EDTA tubes for plasma analysis. To avoid denaturing the ghrelin hormone of protein structure, 20–30 μl DPP IV (Sigma) inhibitor per 1 mL blood was added to the blood in EDTA tubes. The remaining blood was placed into gel vacuum tubes to make serum analysis. The liver tissues collected from the rats were divided into three for liver glycogen, histological analyses, and gene analyses. The liver tissue to be used for glycogen and gene measurement was kept in liquid nitrogen at −80℃, and the tissue to be used for histological analyses was kept in formaldehyde fluid.

Serum parameters

Aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglyceride (TG), total cholesterol, HDL-K, LDL-K, VLDL-K, and glucose in the serum obtained from the blood samples were analyzed with Beckman AU5800 biochemistry autoanalyzer in Gazi University Hospital Biochemistry Laboratory using standard laboratory methods.

Determining plasma insulin, glucagon, fructosamine, hepatic diacylglycerol, acyl and desacyl ghrelin levels

Insulin [rat insulin ELISA kit (SunRedBio)], glucagon [rat Glucagon ELISA kit (SunRedBio)], fructosamine [rat Fructosamine ELISA kit (SunRedBio)], hepatic diacylglycerol [rat Diacylglycerol ELISA kit (SunRedBio)], acyl [rat acyl ghrelin ELISA kit (Reagentgenie)] and deacyl ghrelin levels [rat desacyl ghrelin ELISA kit (Reagentgenie)] in the plasmas obtained from the blood samples collected intracardially while sacrificing the animals were determined using ELISA principle. In the sandwich enzyme immunoassay principle, the substance to be measured in the samples are bound to the reaction plate covered with monoclonal antibodies. An enzyme (HRP) marked second monoclonal antibody is bound to this substance. The intensity of the color resulting from the reaction of the chromatic solution added and the enzyme and the concentration of the substance to be measured is directly proportional.

Oral glucose tolerance test (OGTT) and insulin tolerance test

An oral glucose tolerance test was conducted on Day 15 on unfed rats. Rats were allowed to fast for 12 h; then, they were given glucose solution (2 g/kg) through oral gavage, and the oral glucose test (OGTT) was performed by the researcher and the veterinarian. For OGTT, 1 drop of blood was collected from the rat by pricking the tail end and dripped on On-Call Plus blood glucose strips. The measurements were made at minutes 0, 30, 60, and 120 using the blood glucometer. The tail pricking procedure in rats was performed only once, and 1 drop of blood was collected for other measurements by milking the tail. An insulin tolerance test was conducted on the morning of Day 16. Rats were allowed to fast for 12 h and intraperitoneally injected 0.75 U regular insulin by the researcher and the veterinarian. The blood collection procedure was performed using the same method as OGTT.

Calculating insulin resistance: HOMA-IR (Homeostatic Model Assessment of Insulin Resistance) formula was used to calculate insulin resistance in rats [16].

HOMA-IR = Fasting serum insulin level (μU/mL) x Fasting serum glucose level (mg/dL)/405

Analyzing liver glycogen content

Tissue glycogen levels in liver samples were analyzed in Gazi University Hospital Biochemistry Laboratory according to the method of Seifter et al. [17].

Light microscopic and hematoxylin-eosin staining method

All liver samples were first fixated for 72 h in 10 % formaldehyde solution for light microscope analyses. Following the fixation procedure, the tissues subjected to routine light microscope processes were turned into paraffin blocks. For all groups, hematoxylin-eosin staining was applied to 4–5 μm thick sections obtained from the prepared paraffin blocks. The sections were captured using Leica DCM 4000 (Germany) computer-aided imaging system and analyzed using LAS software.

RNA isolation and real-time QRT-PCR and mRNA expression analyses

Total RNA isolation was performed using the nucleospin method (Macherey-Nagel, Germany). The purity and quantitation of the isolated RNA were determined using a microplate reader (Epoch Biotek, U.S.). 2.5 μg RNA from each isolate was run in 1 % agarose gel electrophoresis and 18 s and 28 s RNA bands were shown. The stable ones of the isolated total RNAs were used in cDNA synthesis.

1 μg RNA was collected from each sample to be used as a template in PCR reaction, and complementary DNA (cDNA) synthesis was performed with reverse transcriptase (RT). Then, 1 μl cDNA from each sample was collected, and the SYBR green master mix and a couple of primers (oligonucleotide) were placed on it. Primers were specific to each transcription analysis, and they were obtained by synthesizing the base sequences previously given in the literature, and approximately 100 ng was used in the reaction. Based on the cycle threshold (Ct) values of amplification curves obtained as a result of RT-PCR, the relative changes of mRNA expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method.

In this calculation, $\Delta\Delta Ct = (\Delta Ct$target gene $- \Delta Ct$beta actin) subject group $- (\Delta Ct$target gene $- \Delta Ct$beta actin) control group formula was used; The calculated value was placed in the $2^{-\Delta\Delta Ct}$ formula and the mRNA expression level was determined in the form of a decrease or increase in size. The Beta-actin gene was used as the housekeeping gene, and normalization was applied to the expression levels of other genes based on the beta-actin gene level of each sample. In PCR studies, 5 samples were randomly selected from each group and mean values were taken after 3 repetitions.

In the liver tissue, PPAR-γ and protein kinase C were analyzed using the RT-PCR method. Increased mRNA expression levels of relevant genes in the treatment groups compared to the control group were given as the changes in size.

Statistical analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics 24) package software. Frequency tables and descriptive statistics were used in the interpretation of the findings. All data of the RT PCR study were calculated with REST 2009 V2.0.13 and SPSS 19 package software using the Wise Fixed Reallocation Randomization Test method.

Ethical approval

This study was approved by Gazi University Local Ethics Committee for Animal Experiments (Code Number: G.Ü. ET 19.036) on 7/8/2019.

Result

Rats were divided into 6 groups; control (n:6), acyl ghrelin (n:6), desacyl ghrelin (n:6), acyl/desacyl 1:1 (n:6), acyl/desacyl 3:1 (n:6), and acyl/desacyl 1:3 (n:6) groups. Rats except for those in the control group were administered hormones at different doses for 14 days.

Bodyweight changes

Within the groups, no statistically significant difference was detected between the onset (g) and final body weights of control, acyl, desacyl, acyl/desacyl 1:1, and acyl/desacyl 1:3 groups. The difference between the onset and final body weight in the acyl/desacyl 3:1 group was found statistically significant ($p < 0.05$) (Table 2). There was no significant difference between the onset and final liver weights of the groups ($p > 0.05$) (Not shown in the Table).

Biochemical findings

HOMA-IR values were found higher in the control group at a statistically significant level compared to acyl/desacyl 1:1, acyl/desacyl 3:1, and acyl/desacyl 1:3 groups. Insulin levels were found lower in acyl/desacyl 1:1, acyl/desacyl 3:1, and acyl/desacyl 1:3 groups at a statistically significant level compared to the acyl group. Liver diacylglycerol levels revealed a statistically significant difference between the control group and acyl/desacyl 1:1 group. Liver glycogen (mg/g) was found lower in the acyl group at a statistically significant level in the control group (Table 3).

Total cholesterol level was found higher in the acyl group at a statistically significant level compared to control, desacyl, acyl/desacyl 1:1, and acyl/desacyl 3:1 groups. Total cholesterol level was found higher in the acyl/desacyl 1:3 group at a statistically significant level compared to control, desacyl, acyl/desacyl 1:1, and acyl/desacyl 3:1 groups (Table 3). HDL-C level was detected higher in acyl group at a statistically significant level compared to desacyl, acyl/desacyl 1:1 and acyl/desacyl 3:1 groups, and LDL-C level was detected lower in acyl/desacyl 3:1 group at a statistically significant level compared to acyl and acyl/desacyl 1:3 groups (Table 3). A positive, weak, and statistically significant relation was detected between plasma acyl ghrelin and fructosamine (mmol/L), liver glycogen (mg/g), triglyceride (mg/dL), and VLDL-C (mg/dL) levels (not given in Table).

Table 2
Bodyweight changes of the rats by groups (g).

Groups	Onset $\bar{X} \pm SS$	Final $\bar{X} \pm SS$	p
Control (n:6)	255,00 ± 24,84	265,83 ± 12,94	p = 0,097
Acyl (n:6)	251,50 ± 20,83	252,83 ± 24,34	p = 0,743
Desacyl (n:6)	256,67 ± 19,69	263,00 ± 23,77	p = 0,077
Acyl/descyl 1:1 (n:6)	256,17 ± 14,73	259,50 ± 12,85	p = 0,193
Acyl/desayl 3:1 (n:6)	253,50 ± 14,90	260,83 ± 16,90	**p = 0,027**
Acyl/desacyl 1:3 (n:6)	256,00 ± 27,44	261,17 ± 21,20	p = 0,462
p	p = 0,998	p = 0,740	

"Paired Sample-*t* test (t-table value)" was used in the comparison of measurement values of two dependent groups with normal distribution, and "ANOVA" test (F-table value) statistics were used in the comparison of the measurement values of three or more independent groups. "Wilcoxon" test (Z-table value) was used in the comparison of measurement values of two dependent groups without normal distribution, and "Kruskal-Wallis H" test (χ^2-table value) statistics were used in the comparison Z. measurement values of three or more independent groups.

Table 3
Mean and standard deviation values of fasting biochemical findings of rats by group.

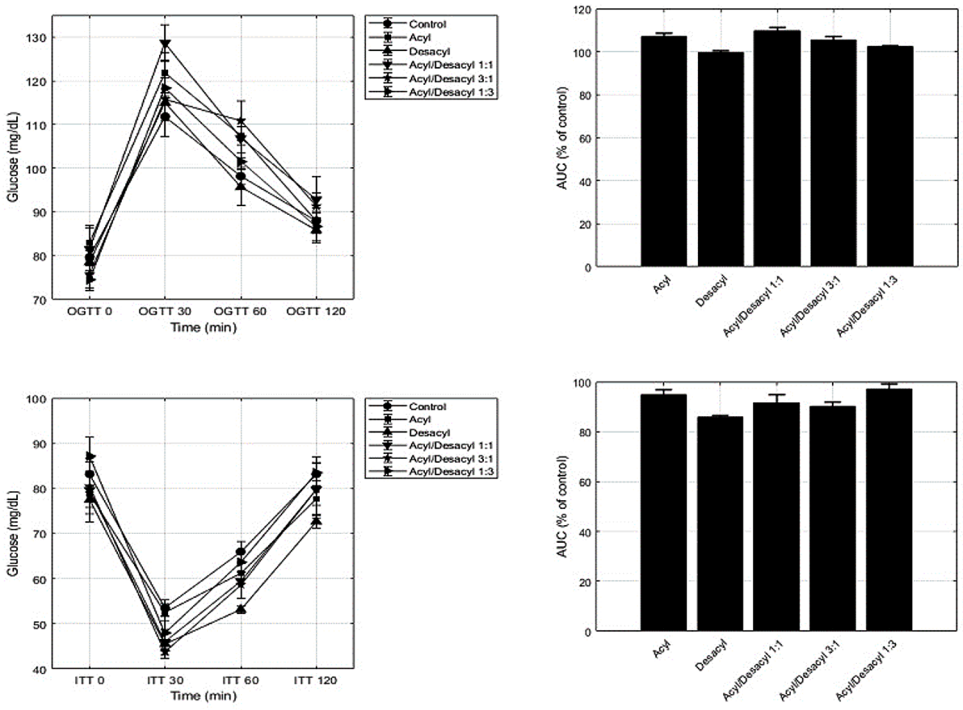
Biochemical parameters	Control (n:6) [1] $\bar{X} \pm SS$	Acyl (n:6) [2] $\bar{X} \pm SS$	Desacyl (n:6) [3] $\bar{X} \pm SS$	Acyl/desacyl 1:1 (n:6) [4] $\bar{X} \pm SS$	Acyl/desacyl 3:1 (n:6) [5] $\bar{X} \pm SS$	Acyl/desacyl 1:3 (n:6) [6] $\bar{X} \pm SS$	p	Difference
Acyl ghrelin (pg/mL)	227,50 ± 87,96	142,83 ± 65,01	168,00 ± 84,35	114,33 ± 57,72	259,83 ± 180,79	211,33 ± 87,09	p = 0,156	
Desacyl ghrelin (pg/mL)	466,00 ± 67,90	435,00 ± 177,54	490,00 ± 103,83	547,33 ± 145,58	372,17 ± 108,15	460,33 ± 121,13	p = 0,266	
Acyl/desacyl ghrelin	0,51 ± 0,23	0,46 ± 0,45	0,37 ± 0,21	0,23 ± 0,16	0,74 ± 0,48	0,50 ± 0,25	p = 0,143	
Glucose (mg/dL)	119,80 ± 7,10	124,81 ± 14,55	118,05 ± 9,73	122,56 ± 18,04	114,04 ± 14,18	112,03 ± 7,29	p = 0,378	
HOMA-IR	2,08 ± 0,27	2,03 ± 0,53	0,98 ± 0,63	0,81 ± 0,16	0,40 ± 0,21	0,26 ± 0,11	**p = 0,000**	**[1–4,5,6]**
Insulin (μU/mL)	7,02 ± 0,75	6,56 ± 1,39	3,68 ± 2,10	2,67 ± 0,42	1,45 ± 0,82	0,93 ± 0,41	**p = 0,000**	**[1–4,5,6] [2–5,6]**
Glucagon (ng/L)	83,45 ± 15,86	83,92 ± 10,41	83,45 ± 9,32	76,53 ± 15,12	65,03 ± 9,54	67,13 ± 7,34	p = 0,084	
Fructosamine (mmol/L)	5,52 ± 0,85	4,56 ± 1,44	5,50 ± 1,65	5,85 ± 1,38	5,04 ± 1,13	5,88 ± 0,81	p = 0,441	
Liver glycogen (mg/g)	7,60 ± 0,60	5,66 ± 1,12	6,05 ± 0,59	6,30 ± 1,26	7,00 ± 1,48	6,45 ± 0,93	**p = 0,003**	**[1,2]**
ALT (U/L)	48,33 ± 5,89	46,50 ± 7,71	45,33 ± 5,68	46,50 ± 10,48	48,17 ± 7,25	41,67 ± 4,76	p = 0,636	
AST (U/L)	145,33 ± 20,72	147,00 ± 13,25	161,17 ± 64,62	167,00 ± 44,71	142,17 ± 36,59	118,00 ± 27,06	p = 0,149	
Liver DG (nmol/g)	23,00 ± 7,01	30,00 ± 12,46	30,50 ± 8,19	40,83 ± 7,41	33,33 ± 9,56	34,50 ± 6,16	**p = 0,037**	**[1–4]**
Triglyceride (mg/dL)	95,87 ± 49,43	103,07 ± 28,30	88,48 ± 12,63	88,57 ± 26,30	126,30 ± 26,10	117,20 ± 28,73	p = 0,201	
Cholesterol (mg/dL)	32,07 ± 5,29	41,15 ± 4,58	29,88 ± 5,49	32,28 ± 1,97	30,88 ± 4,05	42,07 ± 6,92	**p = 0,001**	**[2–1,3,4,5] [6–1,3,4,5]**
HDL-C (mg/dL)	19,57 ± 3,49	24,53 ± 3,11	17,88 ± 3,13	17,95 ± 1,70	17,82 ± 2,52	23,98 ± 4,73	**p = 0,002**	**[2,3,4,5]**
LDL-C (mg/dL)	11,32 ± 2,63	14,08 ± 2,62	10,02 ± 2,52	10,60 ± 2,06	9,18 ± 1,17	14,03 ± 2,43	**p = 0,002**	**[5–2,6]**
VLDL-C (mg/dL)	19,17 ± 9,87	20,60 ± 5,66	17,68 ± 2,51	17,72 ± 5,26	25,25 ± 5,24	23,45 ± 5,76	p = 0,201	

"Paired Sample-*t* test (t-table value)" was used in the comparison of measurement values of two dependent groups with normal distribution, and "ANOVA" test (F-table value) statistics were used in the comparison of the measurement values of three or more independent groups. "Wilcoxon" test (Z-table value) was used in the comparison of measurement values of two dependent groups without normal distribution, and "Kruskal-Wallis H" test (χ2-table value) statistics were used in the comparison Z. measurement values of three or more independent groups.
HOMA-IR: Homeostatic Model Assesment Insulin Resistance, DG: Diglyceride, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, HDL-C: High density lipoprotein, LDL-C: Low density lipoprotein, VLDL-C: Very low density lipoprotein.

Table 4
Mean and standard deviation levels of OGTT and ITT values of rats over time by groups.

Test	Control (n:6) [1] $\bar{X} \pm SS$	Acyl(n:6) [2] $\bar{X} \pm SS$	Desacyl (n:6) [3] $\bar{X} \pm SS$	Acyl/desacyl 1:1 (n:6) [4] $\bar{X} \pm SS$	Acyl/desacyl 3:1 (n:6) [5] $\bar{X} \pm SS$	Acyl/desacyl 1:3 (n:6) [6] $\bar{X} \pm SS$	p Difference
OGTT Glucose							
0.min	79,67 ± 4,72	83,00 ± 9,67	78,50 ± 6,47	81,50 ± 11,99	75,83 ± 9,43	74,50 ± 4,85	$\chi^2 = 5,317$ p = 0,378
30.min.	111,83 ± 11,34	121,83 ± 10,98	115,00 ± 7,95	128,67 ± 10,13	115,83 ± 12,09	118,83 ± 15,62	F = 1,594 p = 0,192
60.min.	98,17 ± 4,83	107,50 ± 5,09	95,67 ± 10,11	106,83 ± 10,87	106,83 ± 11,21	101,50 ± 4,72	$\chi^2 = 11,485$ **p = 0,043 [2-1,3]**
120.min.	88,00 ± 7,38	87,83 ± 5,74	85,83 ± 7,03	92,83 ± 12,97	91,33 ± 7,20	86,67 ± 7,89	$\chi^2 = 2,789$ p = 0,733
ITT Glucose							
0. min	83,17 ± 6,65	78,83 ± 10,72	77,50 ± 4,42	79,67 ± 17,20	80,50 ± 8,29	87,17 ± 10,32	$\chi^2 = 5,845$ p = 0,322
30. min	53,50 ± 4,32	52,50 ± 4,64	45,50 ± 2,34	46,00 ± 7,07	43,83 ± 3,60	48,00 ± 9,01	F = 2,951 **p = 0,028 [1-5]**
60. min	66,00 ± 5,62	61,17 ± 5,23	53,17 ± 1,94	59,50 ± 9,42	58,67 ± 7,20	63,67 ± 5,61	F = 3,028 **p = 0,025 [1-3]**
120. min	83,17 ± 6,21	77,67 ± 10,01	72,67 ± 3,44	79,83 ± 13,88	79,83 ± 8,70	83,50 ± 8,53	F = 1,167 p = 0,348

"Paired Sample-t test (t-table value)" was used in the comparison of measurement values of two dependent groups with normal distribution, and "ANOVA" test (F-table value) statistics were used in the comparison of the measurement values of three or more independent groups. "Wilcoxon" test (Z-table value) was used in the comparison of measurement values of two dependent groups without normal distribution, and "Kruskal-Wallis H" test (χ2-table value) statistics were used in the comparison Z. measurement values of three or more independent groups.
ITT: Insulin Tolerance Test, OGTT: Oral Glucose Tolerance Test.



AUC: Area Under the Curve, ITT: Insulin Tolerance Test, OGTT: Oral Glucose Tolerance Test

Fig. 1. Glucose and AUC values according to OGTT and ITT. AUC: Area under the curve, ITT: Insulin tolerance test, OGTT: Oral glucose tolerance test.

Table 5
Multiple changes in the mRNA expression levels of the relevant genes in the treatment groups compared to the control group.

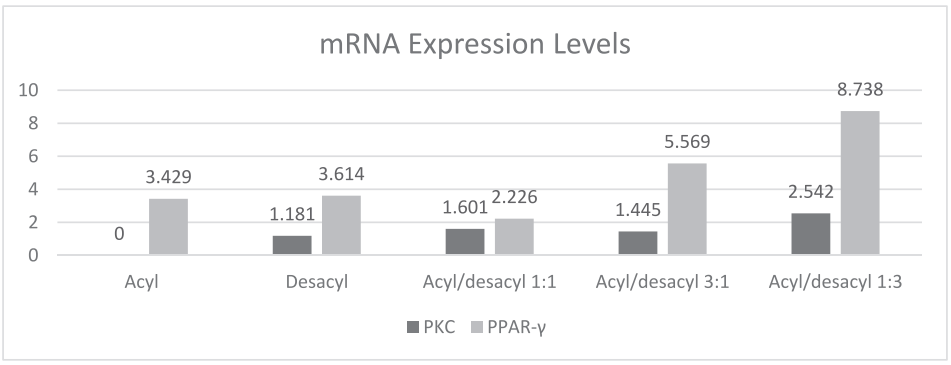
Groups	Gen	Expression Levels	p
Acyl Ghrelin	PKC	0,170	0,117
	PPAR	3,429	**0,028**
Desacyl Ghrelin	PKC	1,181	0,848
	PPAR	3,614	0,096
Acyl/desacyl 1:1	PKC	1,601	0,622
	PPAR	2,226	0,282
Acyl/desacyl 3:1	PKC	1,445	0,492
	PPAR	5,569	0,216
Acyl/desacyl 1:3	PKC	2,542	**0,034**
	PPAR	8,738	**0,001**

PKC: Protein Kinase C, PPAR: Peroxisome proliferator activated receptor.

60-minute OGTT glucose levels of those in the acyl group were higher at a statistically significant level compared to those in the control and desacyl groups. 30-minute ITT glucose levels of those in the acyl/desacyl 3:1 group were higher at a statistically significant level compared to control groups. 60-minute ITT glucose levels of those in the desacyl group were higher at a statistically significant level compared to those in the control group (Table 4). Additionally, as a result of OGTT and ITT, the lowest AUC value was observed in the desacyl group ($p > 0.05$) (Fig. 1).

Results of Real-Time PCR and mRNA expression levels

In the acyl ghrelin group, it was detected that PPAR-γ gene expression was stimulated 3.429 times more than the control group and this up-regulation was statistically significant. Although the PKC gene expression level of the acyl ghrelin group was detected to be moderately increased compared to the control group, this increase was indicated not to be statistically significant.



PKC: Protein Kinase C, PPAR: Peroxisome proliferator activated receptor

Fig. 2. Liver PKC and PPAR-γ mRNA expression levels of rats. PKC: Protein Kinase C, PPAR: Peroxisome proliferator activated receptor.

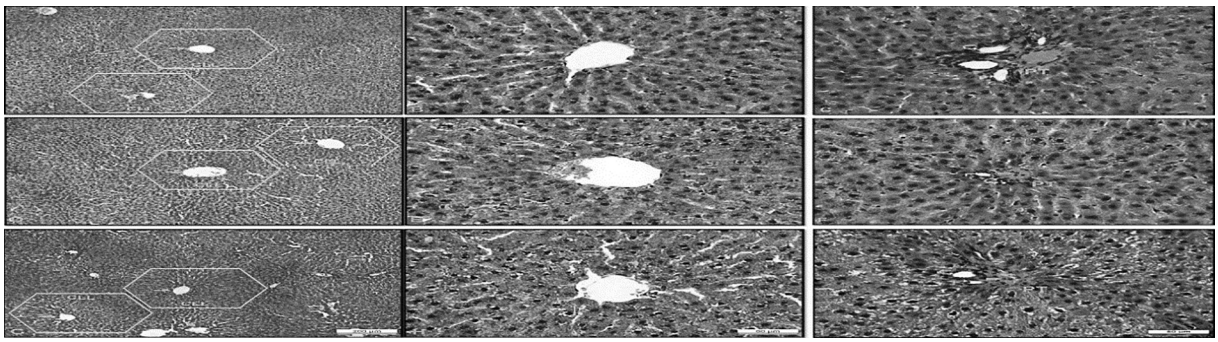


Fig. 3. CLL: Classic liver lobule, →: vena centralis, ▶: hepatocyte, ⇉: sinusoid, ▷: nucleus, PT: portal region, ↯: hepatic artery, ↱: hepatic vein, ↳: bile duct, ↔: congestion, ➔: vacuolar degeneration, ➡: lipid droplets, ■: infiltration, ⇨: hepatocytes with impaired morphology are being observed (Hematoxylin Eosin ×100 [A, D,G], ×400 [B-I]). (A-C: Control Group, D-F: Desacyl group, G-I: Acyl/desacyl 1:3 Group).

When compared to the control group, the highest increase in both PKC and PPAR-γ mRNA expression levels was observed in acyl/desacyl 1:3. In the liver tissues of this group, a 2.542-fold increase was detected in PKC mRNA expression levels, and an 8.738 fold increase was detected in PPAR-γ mRNA expression levels. These increases, which were detected in comparison to the control group, were found statistically significant (Table 5) (Fig. 2).

Histochemical results

Liver section lobules and portal regions of the control group were distinguished from their histological appearances (Fig. 3A-B-C).). Even though the liver section lobules and portal regions of the desacyl group were similar to the control group in general, dilatation and congestion were observed in some sinusoids, vacuolar degeneration in some hepatocytes, and an increase in lipid droplets in hepatocyte cytoplasm (Fig. 3D-E). A slight infiltration was distinguished in the hepatic artery (Fig. 3F). Dilatation and congestion were detected in vena centralis and sinusoids of acyl/desacyl 1:3 group, impairment in the morphologies of some hepatocytes located at the lobule center, and cytoplasmic lipid droplets in some hepatocytes (Fig. 3G-H). Dilatation, congestion, and infiltration in the hepatic artery and intense lipid droplets in peripheral hepatocytes were monitored (Fig. 3I). In the acyl/desacyl 1:1 group, dilatation and severe congestion were distinguished in vena centralis, dilatation in some sinusoids (Fig. 4A), intense lipid droplets in hepatocytes located in the peripheral of the lobule, and sporadic existence of hepatocytes with the pycnotic nucleus at the center of the lobule. Impairment in the morphology of some hepatocytes and vacuolar degeneration was monitored in this region (Fig. 4B). An increase in the ligament amount in the portal region, intense dilatation and congestion in the hepatic artery, slight infiltration, and degenerative change in the epithelium of bile discharging canal towards multiple layers in certain regions instead of single-layered cubic structure of the epithelium were observed (Fig. 4C). In the acyl/desacyl 3:1 group, an increased degeneration in the liver, dilatation in vena centralis and sinusoids, and congestion and infiltration in vena centralis were distinguished parallel to the increase in the acyl ghrelin amount administered. Vacuolar degeneration in hepatocytes and morphological impairment in hepatocytes at the lobule center were monitored. Overwhelming and abnormal appearing basophilia was distinguished in some hepatocytes in this group. While the reason of such hepatocytes might be clarified through advanced immunohistochemical techniques, it was considered to be caused by cell proliferation or lysosomal ribonuclease (Fig. 4D-E). It was observed that lipid droplets compliant with steatosis became quite widespread; the optical arrangement was completely impaired in regions where such hepatocytes were intense; and dilatation and congestion in sinusoids became denser (Fig. 4E-Inset). It was detected that the bonds of portal region elements with hepatocytes were impaired, and there was an intense separation between the cells in this region. An intense dilatation was observed in sinusoids surrounding the portal region, and it was observed that the number of hepatocytes showing basophilia relatively increased in the extreme (Fig. 4F). Histologically, it was distinguished that the acyl ghrelin group was the group which showed the most intense degeneration compared to all other groups. It was detected that the vena centralis was dilated and expanded by extending within the lobule by impairing the arrangements of hepatocytes and sinusoids, and the lobule structures were particularly impaired in these regions. Severe congestion and infiltration in the vena centralis

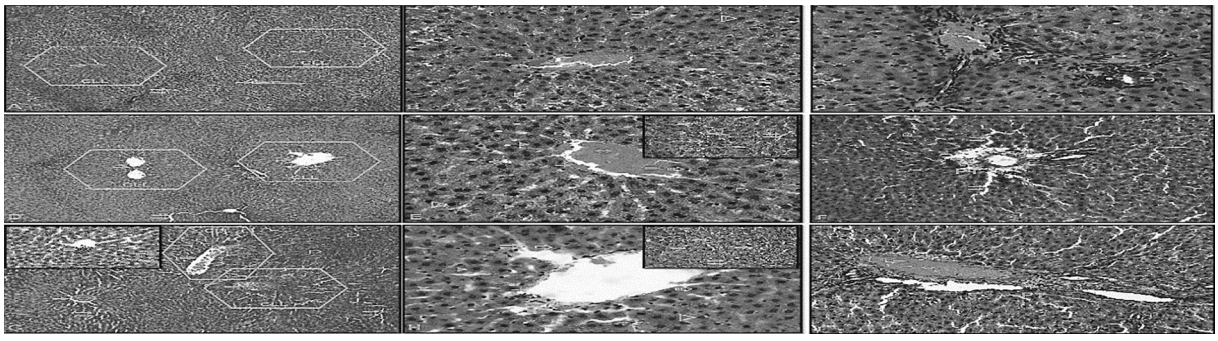


Fig. 4. CLL: Classic liver lobule, →: vena centralis, ▶: hepatocyte, ▷: nucleus, ⇉: sinusoid, ↔: congestion, ➔: vacuolar degeneration, ⇨: hepatocytes with impaired morphology, ⇪: pycnotic nucleus, ➡: lipid droplets, PT: portal region, ↯: hepatic artery, ↱: hepatic vein, ↳: bile duct, *: intensified connective tissue, ■: infiltration, ➧: bile duct epithelium out of the usual arrangement, ⌂: hepatocytes showing abnormal basophilia, ➲: a separation between cells are being observed. (Hematoxylin Eosin ×100 [A,D,G], ×400 [B-I]). (A-C: Acyl/desacyl 1:1 Group, D-F: Acyl/desacyl 3:1 Group, G-I: Acyl Group).

and congestion in dilated sinusoids were distinguished (Fig. 4G and 4G-Inset). It was monitored that hepatocytes lost their optical arrangements almost in every region, and they were organized in form of cell masses. In this region, it was observed that there was a separation in the intercellular bonds and that some hepatocytes with pycnotic nucleus existed. It was distinguished in the studies with other groups that the number of polynuclear hepatocytes generally decreased directly proportional to the increased acyl ghrelin administration; however, it was determined that it relatively decreased in this group in comparison to all groups. The cause of this was considered to be the impairment in the metabolic activities of the hepatocytes. It was determined that there was a morphological impairment in the hepatocytes located at the lobule center (Fig. 4H), there was an increase in the intensity of the hepatocyte containing lipid droplets compliant with steatosis; the hepatocytes in regions where such hepatocytes became intense were organized in form of cell masses; and the incidence of sinusoidal congestion became intense in such regions (Fig. 4H-Inset). The most intense degeneration between all groups was detected in the portal region of this group. In addition to the increase in the ligament amount compliant with fibrosis, congestion was observed in the severely dilated hepatic artery and the hepatic vein, and the bile discharge canal in this region might not be clearly distinguished due to the severe degeneration. Infiltration was also detected in this region (Fig. 3I).

Discussion

Blood glucose regulation

Acyl ghrelin stimulates calcium in the pancreas cell and increases the cAMP level and insulin release. Desacyl ghrelin is known to increase insulin sensitivity independent from this mechanism, but how it does this is unknown [18]. In some studies conducted on humans and rats, it was found that the lowest fasting insulin level was observed in groups taking deacyl [15,19], and there was a negative correlation between plasma insulin, glucose, and HOMA-IR and desacyl ghrelin [2,10,15,20]. Besides, it was detected that desacyl ghrelin decreases hepatic glucose release and insulin secretion in cell cultures in addition to in vivo studies on rats and mice [2,20–24]. In this study, plasma insulin and HOMA-IR levels of the desacyl ghrelin group were found lower than the control group and acyl ghrelin group. The highest insulin levels in this study were observed in the control and acyl ghrelin groups ($p < 0.05$) (Table 3).

Recent research indicates that the ratio of acyl and desacyl ghrelin can have metabolic effects on the body [25–27]. In a study by Dallak, it was demonstrated that the combined administration of acyl and desacyl ghrelin increased insulin levels more than in the group receiving only acyl or desacyl ghrelin [10]. Another study found the lowest insulin levels in the group receiving both acyl and desacyl ghrelin simultaneously [20]. In the present study, insulin and HOMA-IR values were lower in the groups receiving combined hormone treatments compared to those receiving a single type of hormone. Gauna, in his research, categorized subjects into five groups: placebo, acyl ghrelin, desacyl ghrelin, acyl + desacyl ghrelin combination, and acyl ghrelin + GH. Four hours after hormone intervention, the highest total ghrelin level was observed in the acyl ghrelin and desacyl ghrelin combination group. Similarly, in another study, it was demonstrated that administering acyl and desacyl ghrelin together after 4 h increased plasma acyl and desacyl ghrelin levels more than in the groups given only acyl or desacyl ghrelin [10]. The lower insulin levels observed in the combined hormone application group in this study are thought to be attributed to the increased total ghrelin levels, primarily desacyl ghrelin. The absence of a significant difference in ghrelin levels in Table 3 of the study is likely due to blood samples being taken 60 h after the last hormone application and the hormones' half-life. But surprisingly, it was observed that these values were lower in the groups with acyl/desacyl ratios of 3:1 and 1:3 than in the group with a 1:1 ratio. However, since there are no studies similar to this one in the literature, it has not been discussed sufficiently. Therefore, it is recommended to conduct more detailed studies on this subject in the future.

While in a study, it was detected that acyl and desacyl ghrelins did not change the fasting plasma glucose level [28], some studies indicated that acyl ghrelin increased blood glucose level [10] and desacyl ghrelin decreased it [10,15]. In a study by Dallak, the highest fasting blood glucose acyl and desacyl ghrelin ratio was detected in the group administered 1:1 [10]. In our study, no significant difference was found in fasting blood glucose levels among the groups (Table 3). The contradiction between the study results is considered to arise from the differences in hormone forms administered in the studies and the methodologic differences between doses and periods.

Some studies on humans have demonstrated that plasma glucose levels and the area under the curve (AUC) of acyl ghrelin during the Mixed Meal Tolerance Test (MTT) were higher than those in the placebo group but lower in the desacyl group [29,30]. In a study involving rats, the oral glucose tolerance test (OGTT) and the AUC value during the Insulin Tolerance Test (ITT) were found to be lower in the group receiving desacyl ghrelin compared to the group receiving acyl ghrelin [10]. The presence of desacyl ghrelin was confirmed in the desacyl ghrelin group (Fig. 1). Furthermore, while the highest blood glucose value was detected in the acyl group at the 60th minute of the OGTT, the lowest blood glucose levels were observed in the acyl/desacyl 3:1 group at the 30th minute of the ITT, and in the desacyl ghrelin group at the 60th minute (Table 4). This suggests that desacyl ghrelin may act as an antagonist to acyl ghrelin in lowering blood sugar levels. Additionally, the observation of the lowest blood glucose level at the 30th minute of the ITT in the acyl/desacyl 3:1 group contributes new insights to the literature, indicating that the acyl/desacyl ratio of blood may have an impact on glucose levels.

Lipid profile

In the literature, it is mentioned that acyl ghrelin has a stimulating effect on triglyceride, cholesterol, and fatty acid levels in serum and fat tissue [31]. Some studies indicate that acyl ghrelin increases serum cholesterol and triglyceride levels [10,15]; desacyl ghrelin does not change [15] or decreases [10] serum cholesterol and decreases triglyceride level [15,10,28]. In this study, total cholesterol and LDL-C levels were found significantly high in acyl and acyl/desacyl 1:3 groups compared to other groups (Table 3). The root cause of this increase is considered to be associated with the increase in PPAR-γ gene expression (Table 5).

Diacylglycerol

Acyl ghrelin activates the diacylglycerol acyltransferase (DGAT) enzyme by causing an increase in PPAR-γ gene expression after stimulating GHSR-1a. Diacylglycerol stimulates TG synthesis by increasing protein kinase C (PKC) activation. Desacyl ghrelin is considered to lower TG synthesis through a receptor specific to itself independent from GHSR-1a [10,11]. It was shown in the studies conducted that DAG might induce both hepatic steatosis and IR even in the absence of peripheric insulin resistance (IR) [32–34]. In research conducted on rats, DAG level was detected highest in the acyl ghrelin group and lowest in the desacyl group [10]. In this study, while DAG levels in acyl and desacyl groups were similar, the highest DAG level was detected in the acyl/desacyl 1:1 group (Table 3). It is considered that this may arise from the rapid metabolization of DAG in the body (30 min) and the removal of the rat livers in the study approximately 60 h after administering the hormone.

Liver glycogen

In the literature, it is shown that acyl ghrelin administration leads to increased gamma coactivator 1-alfa (PGC1α) (glucogenesis activator)

protein expression in the liver and decreased glycogen synthesis kinase [35,36]. In a research, it was found that acyl ghrelin has no effect on glycogen synthesis in short term [37], and desacyl ghrelin was more effective than acyl ghrelin in increasing the liver glycogen level in two weeks [10]. In this study, the lowest liver glycogen level was observed in the acyl ghrelin group as in the literature ($p < 0.05$) (Table 3). This is considered to arise from the decreased glycogen synthesis kinase by acyl ghrelin.

PPAR-γ

Peroxisomes are subcellular organelles performing various functions, including β-oxidation of fatty acids and cholesterol metabolism in most plant and animal cells. PPAR-γ is known to regulate adipocyte differentiation, fatty acid storage, and glucose metabolism regulation [38]. In some studies on rats, acyl ghrelin was shown to increase the gene expression of certain mediators such as SREBP 1c [39], PPAR-γ, and mTOR [38] that stimulate lipogenesis in adipose tissue and decrease PPAR-α mRNA level [10]; and desacyl ghrelin was shown to increase PPAR-α mRNA level [10]. In this study, it was detected that there was an increase in PPAR-γ mRNA expression levels in liver tissues of rats in the acyl ghrelin and acyl/desacyl 1:3 group compared to the control group (Table 5). This is considered to arise from the increase caused in PPAR-γ gene expression by acyl ghrelin after stimulating GHSR-1a.

Protein kinase C

Protein kinase C is included in the serine/threonine kinase family, and PKC isozymes are lipid-dependent kinases. Protein kinase C is activated through DAG and calcium [10,11]. In a study, a 3–3.5 times increase was observed in the protein kinase C membrane fraction of the acyl ghrelin group compared to the rats in the control group, but desacyl ghrelin caused no change in protein kinase C [10]. In this study, PKC mRNA expression levels of the acyl:desacyl 1:3 group increased 8.738 times compared to the control group ($p < 0.05$) (Table 5). Besides, there had been an increase in protein kinase C level independent from the change in DAG level. It is considered that this may arise from the rapid metabolization of DAG in the body and the removal of the rat livers in the study approximately 60 h after administering the hormone. The observation regarding the highest PKC increase in the acyl/desacyl 1:3 group provides new data to the literature as there are no studies where acyl/desacyl was administered by 1:3.

Hepatic steatosis

In the literature, it is reported that acyl ghrelin stimulates and desacyl ghrelin decreases adipogenesis [38,40]. In a study on rats, it was indicated that acyl ghrelin caused the development of hepatic steatosis in rats, but administration of desacyl ghrelin alone and in combination with acyl ghrelin did not lead to any change in the liver [10]. In this study, the intensity of hepatocyte-containing lipid droplets compliant with steatosis was observed in the acyl ghrelin group at the highest and at the lowest in the desacyl ghrelin group. Besides, it was detected that the lipid droplets increased parallel to the amount of acyl ghrelin in combined treatments (Figs. 3 and 4). This is considered to be caused by acyl ghrelin which increases the expression of genes stimulating fatty acid synthesis.

In this study, no difference was observed in the levels of these hormones despite acyl and desacyl administration to rats. This is considered to be caused by blood collection approximately 60 h after the last hormone administration to rats (the half-life of ghrelin is 30 min).

In conclusion, the highest total cholesterol, HDL-C, and VLDL-C levels, and PPAR-γ mRNA expression were observed in acyl and acyl/desacyl 1:3 groups. As a result of the histological analyses in the liver, it was detected that acyl ghrelin increases hepatic steatosis and desacyl ghrelin operates, unlike acyl ghrelin, by mitigating this impact. It was also found that desacyl ghrelin was more effective in regulating blood glucose levels.

Authors' contributions

Emine Elibol and Yasemin Akdevelioğlu designed the experiment. Emine Elibol drafted the manuscript. Canan Yılmaz, Belkıs Narlı, Serkan Şen, Cemile Merve Seymen and Gülnur Take Kaplanoğlu participated in the experiment and helped analyze the data. All authors have read and approved the final manuscript.

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Declaration of competing interests

Authors state no conflict of interest.

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