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Original Article	4500	250 (Structured)	No limit	6	7 or total of 15 images
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Review Article	5500	250	No limit	5	6
Letter to the Editor	500	No abstract	5	No tables	No media

Images should not be labeled (a, b, c, etc.) to indicate figure subunits. Thick and thin arrows, arrowheads, stars, asterisks, and similar marks can be used on the images to support figure legends. Like the rest of the submission, the figures too should be blind. Any information within the images that may indicate an individual or institution should be blinded. The minimum resolution of each submitted figure should be 300 DPI. To prevent delays in the evaluation process, all submitted figures should be clear in resolution and large in size (minimum dimensions: 100 × 100 mm). Figure legends should be listed at the end of the main document.

All acronyms and abbreviations used in the manuscript should be defined at first use, both in the abstract and in the main text. The abbreviation should be provided in parentheses following the definition.

When a drug, chemical, product, hardware, or software program is mentioned within the main text, product information, including the name of the product, the producer of the product, and city and the country of the company (including the state if in USA), should be provided in parentheses in the following format: "Discovery St PET/CT scanner (General Electric, Milwaukee, WI, USA)"

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Books with a Single Author: Sweetman SC. Martindale the Complete Drug Reference. 34th ed. London: Pharmaceutical Press; 2005.

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Scientific or Technical Report: Cusick M, Chew EY, Hoogwerf B, Agrón E, Wu L, Lindley A, et al. Early Treatment Diabetic Retinopathy Study Research Group. Risk factors for renal replacement therapy in the Early Treatment Diabetic Retinopathy Study (ETDRS), Kidney Int: 2004. Report No: 26.

Thesis: Yılmaz B. Ankara Üniversitesindeki Öğrencilerin Beslenme Durumları, Fiziksel Aktiviteleri ve Beden Kitle İndeksleri Kan Lipidleri Arasındaki Ilişkiler. H.Ü. Sağlık Bilimleri Enstitüsü, Doktora Tezi. 2007.

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Manuscripts Published in Electronic Format: Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis (serial online) 1995 Jan-Mar (cited 1996 June 5): 1(1): (24 screens). Available from: URL: http:/ www.cdc.gov/ncidodlElD/cid.htm.

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Contents

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RESEARCH ARTICLE

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The Relationship between Obstructive Sleep Apnea and Gln223Arg Polymorphism in Human Leptin Receptor Gene

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ABSTRACT

Leptin is derived from an adipocyte and acts through the leptin receptor (LEPR). Gln223Arg polymorphism of the LEPR gene is thought to be associated with impaired signaling capacity of *LEPR* and with higher mean circulating levels of leptin and obesity; therefore, it may contribute to the pathogenesis of obstructive sleep apnea syndrome (OSAS). The aim of this study was to investigate the frequency of distribution of LEPR gene polymorphism (Gln223Arg) in OSAS patients in relation to polysomnographic traits and phenotype.

In total, 230 men (152 OSAS patients and 78 controls) were included in the study. All participants were evaluated by polysomnography in addition to anthropometric, metabolic, and hemodynamic variables. The relationship between these phenotypes and polymorphism of the LEPR gene was investigated by PCR-RFLP.

There was no difference between the genotype frequencies of the Gln223Arg polymorphism in the OSAS and control groups. However, OSAS patients carrying the R allele (RR and QR genotypes) had significantly lower Body Mass Index (BMI) than those carrying the Q allele (QQ). OSAS patients with the RR genotype had a significantly lower diastolic blood pressure value than those with the QQ and QR genotypes. When all participants were grouped by blood pressure, the genotype frequency of RR individuals was more prevalent among normotensive men compared to hypertensive men.

Gln223Arg polymorphism of *LEPR* does not seem to be associated with OSAS. This polymorphism may, however, predispose the carrier to reduced BMI and blood pressure. Further studies are needed to unveil the genetic basis of OSAS pathophysiology.

Keywords: Blood pressure, gln223arg, leptin receptor polymorphism, leptin, obstructive sleep apnea

INTRODUCTION

Obstructive sleep apnea (OSA) is characterized by recurrent episodes of upper airway collapse during sleep, which in turn trigger apneas and hypopneas associated with oxyhemoglobin desaturation and arousal from sleep (1). Elevated Apnea-Hypopnea Index (AHI) indicating the severity of the disease in conjunction with hyper somnolence, or related problems in daytime functioning is called OSA syndrome (OSAS) (2). Cardiovascular and metabolic abnormalities are frequent in patients with OSAS (dyslipidemia, hypertension, and diabetes mellitus type 2, along with their cardiovascular consequences) (3,4). Indeed, OSAS is strongly correlated with obesity and obesity-related variables such as body mass index (BMI), neck circumference, and visceral fat deposition (5,6). Recently, it has been speculated that changes in serum leptin levels and leptin-receptor insensitivity may be involved in the pathogenesis of progressive obesity and metabolic abnormalities in patients with OSAS (7,8).

Given the prominence of obesity in the OSAS phenotype, the genetic underpinning of obesity is highly relevant to the study of the genetic basis of OSAS. Furthermore, a number of genes implicated in obesity

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have also been associated with physiological systems relevant to OSAS. For example, leptin, adenosine deaminase, and melanocortin-4 receptor are expressed in a variety of tissues and brain sites important in the regulation of breathing (9). There is growing evidence that leptin, in particular, may possess pleiotropic effects. In addition to its role in appetite regulation and energy expenditure, studies on mouse models suggest that leptin influences lung growth and respiratory control. Leptin treatment also influences sleep architecture in rats. If confirmed humans, these data strongly support the idea that a single gene is implicated in several aspects of the OSAS phenotype (obesity, ventilator control, sleep architecture) (10).

Leptin acts via the leptin receptor (LEPR), which is involved the class I cytokine receptor family. Several single-nucleotide polymorphisms (SNPs) have been described in the human LEPR (e.g., Gln223Arg, Ser343Ser, Ser492Thr, Lys656Asn) of which Gln223Arg (Q223R) is the most common type (11,12). The extracellular region of the LEPR within the first cytokine domain (C domain) containing a leptin-binding site contains the Q223R substitution in exon 6. Consequently, the amino acid change affects all forms of the receptor (13). Therefore this polymorphism of the leptin receptor has been associated with higher level of circulating leptin obesity (as defined by elevated BMI), insulin resistance, and dyslipidemia (14-19). Ogawa et al. (14) reported that the serum soluble leptin receptor (sOB-R) level was negatively correlated with homeostasis model assessment-estimated insulin resistance and serum leptin level and positively correlated with high-density lipoprotein (HDL)-cholesterol and serum adiponectin levels, independent of age, sex, and BMI, in healthy Japanese subjects . Ragin et al. (15) reported higher mean circulating leptin levels of *LEPR* R223R genotype in post-menopausal Caucasian women. Yiannakouris et al. (19) showed that Q223R polymorphism was associated with obesity and predicts approximately 5% of body weight and body composition variability in the Greek population. Hastuti et al. (20) reported that Q223R polymorphism and phenotype of obesity was associated in the Yogyakarta population. Lv et al. (21) analyzed that there was an association between Gln233Arg polymorphism and OSA risk in European but not for Asian populations (22).

The purpose of this study is to investigate the genotype distribution and the allele frequency of *LEPR* Q223R polymorphism in OSAS patients and to estimate the effect of this polymorphism on metabolic and anthropometric traits.

MATERIALS AND METHODS

Study Population

Out of 857 admittants who had complaints of unintentional sleep episodes during wakefulness, daytime sleepiness, unrefreshing sleep, fatigue, insomnia; or loud snoring, breathing interruptions, gasping or choking during sleep; 230 males that were between the ages of 18-70 were included. These patients admitted in the sleep center with their own will and didn't carry the exclusion criteria. Only male subjects were included to eliminate gender-specific genetic and metabolic factors that may be involved in OSAS. Subjects were collected between the years of 2008 and 2011.

After giving a written, informed consent individuals were evaluated on the basis of a detailed physical examination and history for inclusion and exclusion criteria. All subjects had their height, weight, neck circumference and blood pressure measured, and were asked to fill out a standard sleep questionnaire (including an Epworth Sleepiness Scale sheet; ESS) (23). Neck circumference was measured at the level of the cricoid and waist circumference at the level of the umbilicus. BMI was calculated as the weight in kilograms divided by the square of the height in meters (kg/m^2). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured twice with the subjects in the sitting position after resting for at least 5 minutes. To define hypertension, the SBP cut offs in all subjects were set to ≥140 mm Hg, and the DBP cut off was set to ≥90 for hypertension and <140/90 mm Hg for normal blood pressure (24).

Patients with any of the following characteristics were excluded from the study: history of cerebrovascular or cardiovascular disease, diabetes (previous diagnosis or fasting blood glucose >126mg/dL), endocrine disease, significant renal or hepatic disease; age <18 or >70 years old; a diagnosis of central sleep apnea or Cheyne-Stokes respiration; clinical manifestation of severe chronic obstructive pulmonary disease or asthma (post-bronchodilator FEV1<70% predicted); and hypertensives on medication for hypertension.

To confirm the diagnosis, all study groups underwent full-night standard polysomnography. The subject with AHI ≥ 5 were diagnosed as patients (n=152); those with AHI<5 defined as controls (n=78). The patient group consisted of overweight or obese (BMI≥25 kg/m^2, n=86) and non-obese (BMI<25 kg/m^2, n=66) individuals (25), while our control group was composed of non-obese (BMI<25 kg/m^2) individuals with no sleep or respiratory problems. The patients were further classified into three subgroups according to the AHI of each OSAS patient: mild OSAS (5≤AHI<15), moderate OSAS (15≤AHI<30) and severe OSAS (≥30) (26).

To assay for the presence of comorbid disorders, we performed complete blood count and tests for liver, renal, and thyroid function. Hemogram, total cholesterol, triglycerides (TG), HDL-cholesterol, and low-density lipoprotein (LDL)-cholesterol levels were determined, in addition to ECG, chest X ray, and spirometry. The local Ethics Committee approved this study and written consent was obtained from every participant.

Sleep Study

An overnight polysomnography was performed on each subject using the digital polysomnographic system (VIASYS Healthcare GmbH, Hoechberg, Germany). For each subject, we obtained electroencephalograms (3 channels: C3A2, C4A1, and O2A1), left and right electrooculograms, and a chin electromyogram from surface leads. We used nasal cannula and a thermistor to measure airflow, thoracic and abdominal belts to quanti-

fy respiratory effort, a tracheal microphone to record snoring, a pulse oximeter to measure nocturnal oxygen saturation (NOS) and heart beat speed, and a sensor to collect information on body position during sleep. Sleep staging and respiratory event scoring were performed manually according to the AASM criteria (26): Apnea was defined as cessation of airflow for ≥10s; hypopnea as ≥50% reduction of baseline airflow for at least 10s and ≥3% desaturation from present baseline or in case the event is associated with arousal; obstructive respiratory event was scored if it meets apnea criteria and is associated with continued or increased inspiratory effort throughout the entire period of absent airflow respiratory events the which continued thorax and abdominal movements during apnea were defined as obstructive.

Genetic Experiments

Peripheral blood samples were obtained from all patients. DNA was isolated from leukocytes by using phenol chloroform isolation methods (27) and maintained in 500 µl of 10 mM Tris buffer solution. Qualitative and quantitative analysis of DNA was undertaken spectrophotometrically. The *LEPR* Q223R (Gln223Arg or 668A/G-rs1137101 or Glutamine (Q) 223Arginine (R)) polymorphic region was amplified by polymerase chain reaction (PCR). For each sample, genomic DNA of 50–100 ng/µl was used in a 20-µl PCR reaction. Previously reported (28) 5′-ACC CTT TAA GCT GGG TGT CCC AAA TAG-3′ forward and 5′-AGC TAG CAA ATA TTT TTG TAA GCA ATT-3′ reverse primer pair was used for 20 µl reaction mixture in concentrations 1X buffer, 0,025 U/µl Taq polymerase, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, and 2 mM each primer supplied by SibEnzyme (Novosibirsk, Russia). The PCR protocol used was as follows: onset of denaturation at 94°C for 8 min, [denaturation at 94°C for 45 s; annealing at 65°C for 45 s; extension at 72°C for 90 s] 30 cycles and final extension at 72°C for 10 min.

Polymerase chain reaction products of a length of 416 bp were incubated at 37ºC overnight with 10 U/µl MspI restriction enzyme (SibEnzyme, Novosibirsk, Russia) with a C^CGG (cutting site) identification point appropriate for the *LEPR* Q223R polymorphism. To visualize the DNA fragments, a current of 120 V was applied for 20 min.

Statistical Analysis

Descriptive statistics were obtained for clinical and biochemical traits. The Mann-Whitney U test was employed for initial comparison of patient and control groups. Chi-square goodness of fit test was carried out to evaluate for Hardy-Weinberg equilibrium (HWE) in the patient and control groups. Genotype frequencies were compared between groups of hypertensive and normotensive individuals. Analyses were performed using co-dominant, dominant, and recessive models for evaluating the potential effect of R223 (Arg) allele. Dominant or recessive models were defined in terms of R allele effects. In dominant R allele models, homozygous individuals for Q allele were compared with carriers of R allele. In recessive R allele models, homozygous individuals for R allele were compared with carriers of Q allele. ANOVA was performed in the co-dominant model and an independent t test was used in the other genetic models to perform a comparative evaluation of the variables LDL and cholesterol. The results of the variables LDL and cholesterol were expressed as mean±SD (standard deviation). To evaluate the other clinical and biochemical variables, the Kruskal Wallis test was performed in the co-dominant model and Mann-Whitney U test was performed in the dominant and recessive models. Logarithmic transformation (Log 10) was applied to triglyceride measurements, as they were not normally distributed. Log transformation and nonparametric test results were expressed as a back transformed mean±95% CI and median (quartile 1-3), respectively. Odds ratios (ORs) between hypertensive and normotensive groups were obtained to establish the relationship between the SNP allele and hypertension. Pearson correlation was applied between AHI, BMI, waist and neck circumferences. With the current sample size set up, an obstructive difference between the genotypes of 8 unit would be detected with a 70% chance at α=0.05 level. Analyses were carried out using R 2.11.1 (R Foundation for Statistical Computing, Vienna, Austria) (29). In all analyses, a *P* value of <0.05 was considered to be statistically significant.

RESULTS

Demographics and other parameters for both groups were shown in Table 1. BMI (kg/m^2), neck and waist circumference (cm), systolic and diastolic blood pressure (mm Hg), ESS were found to be higher in OSAS patients. Nocturnal mean oxygen saturation [NOS (%)] was significantly lower in the OSAS patient group (Table 1). 52 of the participants were hypertensive. In patients with OSAS compared to the control group was higher the proportion of hypertensive. There was no significant difference between patients and controls for genotype distribution and allele frequencies of *LEPR* Q223R polymorphism (Table 2).

Since genotype frequencies were similar in both OSAS patient and control groups, OSAS patients were subgrouped according to AHI and BMI indices to discern more distinctly the potential impact of the alleles on health. In the subgroup classified according to BMI, a significant difference in allele frequency and genotype distribution was observed under the dominant and co-dominant models. However, no such differences were observed with respect to AHI severity (Table 3).

In OSAS patients, AHI was positively correlated with BMI and both waist and neck circumferences (r=0.233, r=0.244 and r=0.232, respectively all p<0.05).

In OSAS patients, BMI value of men who were carrying the R allele was significantly lower than those who did not have the R allele under the dominant model (p=0.03). Furthermore, we observed lower diastolic blood pressure in men with the RR genotype under the recessive model (p=0.01). Lower AHI values were also noted in men with the RR genotype under the recessive model (p=0.04) and higher NOS values were noted in men with the RR genotype under the co-dominant model (p=0.001) and recessive model (p=0.002) (Table 4). However, no associations were observed between genotypes and values of

Table 1. Demographics and other parameters in OSAS patient and control groups

	Patients (n=152)	Controls (n=78)	p
Age (y)	47.00 (41-56)	46.00 (43-50)	0.500
Body Mass Index (kg/m^2)	30.31 (23.7-33.8)	23.00 (23.1-24)	<0.001
Neck Circumference (cm)	43 (40-45)	40 (38-41)	<0.001
Waist Circumference (cm)	106 (99-116)	98 (94-100)	<0.001
Apnea-Hypopnea Index (events/h)	33.60 (19-58.1)	2.40 (1.1-4.2)	<0.001
NOS (%)	91 (89-93)	93 (92-95)	<0.001
Epworth Sleepiness Scale	10 (6-15)	7 (3-10)	<0.001
Systolic Blood Pressure (mmHg)	130 (120-140)	120 (110-130)	<0.001
Diastolic Blood Pressure (mmHg)	80 (70-90)	70 (70-80)	0.004
Cholesterol (mg/dL)*	213.6±43.23	190.8±28.94	>0.05
Triglyceride (mg/dL) *	190.57±98.30	152.06±92.80	>0.05
Low Density Lipoprotein (mg/dL) *	135.47±32.4	120.60±24.97	>0.05
High Density Lipoprotein (mg/dL) *	38.58±7.90	40.63±6.02	>0.05
Hypertensive	44 (28.94%)	8 (10.25%)	<0.05

Values were expressed as median (quartile 1-3)
*Values were expressed as mean±standard deviation
NOS: nocturnal oxygen saturation; OSAS: OSA syndrome

waist circumference, neck circumference, lipid profiles, SBP, and ESS scores (p>0.05) (Table 4).

When all subjects were classified according to blood pressure (Table 5), the RR genotype appeared to be absent among individuals with hypertension. The odds ratios and 95% confidence intervals (CI) determined suggested that Q allele carriers were at higher risk for hypertension than R allele carriers. The odds ratio results were as follows: OR=2.29 ([%95 CI 1.36-3.83], p=0.002) for R vs. Q alleles; OR = 1.77 ([%95 CI 0.9-3.32], p=0.83) for QR vs. QQ, all under a co-dominant model; OR=2.26 ([%95 CI 1.21-4.19], p=0.012) for QR+RR vs. QQ under the dominant model.

An analysis of the systolic and diastolic blood pressures for all groups led us to understand that both were lower in subjects with the RR genotype. Genotypic differences in SBP were statistically insignificant under the co-dominant and dominant models, while they were significant under the recessive model (p=0.046) (Table 6). There was no significant difference in diastolic blood pressure between the genotypes under any of the genetic models.

DISCUSSION

There was no deviation from Hardy-Weinberg equilibrium in the patient and control groups. In addition, no difference was found in genetic distribution for OSAS patients with different AHI. Thus, present results do not support our initial hypothesis that the polymorphism is associated with OSAS. There is limited data on the occurrence of *LEPR* Q223R polymorphism in OSAS patients. To our knowledge, three other studies on this issue reported results consistent with ours. Hanaoka et al. (30) and Huang et al. (31) have demonstrated that genotype distribution and allele frequencies did not differ in OSAS patients and controls in Japanese populations. Hanaoka et al. (30) suggested that there was a barely significant relationship between the severity of OSAS and the dominant model including groups with or without the R allele. Ultimately, these authors have concluded, as we have, that the *LEPR* Q223R polymorphism was not directly associated with polysomnographic traits and OSAS phenotype. Also , Li et al. (32) reported that association between LEPR and OSAS was not been in Chinese Han population. In contrast, Popko et al. (33) suggested that presence of an R allele in this polymorphism was associated with obesity, thereby constituting a risk factor for OSAS. OSAS is a multi-systemic disorder related to multiple genetic, environmental, and developmental factors and is therefore unlikely to be determined by a single gene mutation. Maxillofacial morphology, respiratory chemo sensitivity, sympathetic nervous system activity, muscular fatigue, hormone levels, and obesity are other parameters that may contribute to the pathogenesis of OSAS (10). Gln223Arg gene polymorphism or LEPR polymorphisms affect obesity, which is a risk factor in OSAS. Also, the studies of population differences between OSAS and Q223R gene polymorphism are insufficient in the literature and its role in the Turkish population is unknown.

Obesity, central obesity in particular (5,6), is commonly identified as a major OSAS risk factor (10). However, several studies on the association between *LEPR* polymorphisms and BMI have revealed mixed results. For example, Stefan et al. (34) and Gotoda et al. (35) have claimed that Q223R polymorphism has no effect on obesity (11) and only a weak association was established between BMI and Q223R polymorphism in the obese Turkish population in another study (36). Yiannakouris et al. (19) have investigated Q223R, K109R, and K656N polymorphisms in the Mediterranean population, and suggested that not only Q223R was not associated with obesity, but it was also a weak

Table 2. Frequency distribution of genotype and alleles in patient and control groups

	Frequency of Genotype							Frequency of Alleles	
	Co-Dominant Model			Dominant Model		Recessive Model			
Total	**QQ**	**QR**	**RR**	**QQ**	**QR/RR**	**QQ/QR**	**RR**	**Q**	**R**
Patients n=152	71 (46.7)	70 (46.1)	11 (7.2)	71 (46.7)	81 (53.3)	141 (92.8)	11 (7.2)	212 (69.3)	92 (30.7)
Control n=78	30 (38.5)	36 (46.2)	12 (15.4)	30 (43.8)	48 (56.2)	66 (84.6)	12 (15.4)	96 (62.5)	60 (37.5)
$\chi 2$ / p	4.22 / 0.12			1.42 / 0.26		3.80 / 0.06		2.77 / 0.09	

Values were expressed as number of individual and (%)

Table 3. Genotype frequencies with regard to Apnea-Hypopnea Index and Body Mass Index in the patient group

	OSAS group (n=152)	Apnea-Hypopnea Index				Body Mass Index		
	Genotypes	**Mild**	**Moderate**	**Severe**	**$\chi 2$ / p**	**Obese OSAS**	**Non-obese OSAS**	**$\chi 2$ / p**
Co-Dominant Model	QQ	10 (14.1)	20 (28.2)	41 (57.7)		47 (66.2)	24 (33.8)	
	QR	15 (21.4)	15 (21.4)	40 (57.1)	1.72/ 0.42	31 (44.3)	39 (55.7)	8.15/ 0.02
	RR	3 (27.3)	5 (45.5)	3 (27.3)		8 (72,7)	3 (27.3)	
Dominant Model	QQ	10 (14.1)	20 (28.2)	41 (57.7)	1.68/ 0.43	47 (66.2)	24 (33.8)	5.01/ 0.03
	QR+RR	18 (22.2)	20 (24.7)	43 (53.1)		39 (48.1)	42 (51.9)	
Recessive Model	QQ+QR	25 (17.7)	35 (24.8)	81 (57.4)	3.83/ 0.15	78 (55.3)	63 (44.7)	1.26/ 0.35
	RR	3 (27.3)	5 (45.5)	3 (27.3)		8 (72.7)	3 (27.3)	

Values were expressed as number of individuals and (%)

determinant of body fat composition. In our study, in OSAS group, AHI was positively correlated with BMI, neck and waist circumference. Patients with OSAS were divided according to BMI and were found to differ in genotype frequency under the co-dominant and dominant models. We demonstrated that the BMI values of R allele carriers were lower compared to the QQ genotype under the dominant model. The severity of OSAS was decreased in R allele carriers having lower BMI. Lower AHI value in RR genotype under recessive model was distinctive. In agreement with our results, Ben Ali et al. (37) reported that in obese Tunisian men, QR and RR genotypes were associated with lower BMI compared to the QQ genotype . Guizar-Mendoza (28) reported that the RR genotype was more associated with lower body fat percentage compared to QQ and QR genotypes.

The importance of the polymorphism in LEPR on the human body composition has also been reported in a few studies (38,39). Wauters et al. (39) reported that in postmenopausal Caucasian women, the total abdominal fat level, as measured by CT, was higher in Q223 homozygotes. Also, negative results have been reported for the polymorphism in other populations. Heo et al. (40) analyzed entries collected from 9 studies on the LEPR for the association between 3 alleles (Lys109Arg, Gln223Arg and Lys656Asn) with BMI and waist circumference. They reported that there was no association between common LEPR polymorphisms (Lys109Arg, Gln223Arg and Lys656Asn) and BMI or waist circumference in a meta-analysis. In this study, we similarly concluded that the Q223R polymorphism was not associated with neck or waist circumference in patients with OSAS.

We found some differences in the AHI values between the genotypes studied under the recessive model. Differences in the NOS values were also significant across different genotypes under both co-dominant and recessive models. Lower AHI values and higher NOS levels in subjects with a RR genotype led us to hypothesize that the RR genotype can also have an impact on the BMI. Additionally, diastolic blood pressure was associated with certain genotypes under the recessive model, while systolic blood pressure was not significantly different in the various genetic models in OSAS patients. Interestingly, patients with the RR genotype were found to have lower blood pressure levels compared to those carrying a QQ or QR allele. Previously, Rosmond et al. (41) revealed that the disruption of *LEPR* signal might affect sympathetic activation in obese subjects. Similarly, we have examined this condition in all individuals in this study. We evaluated the frequencies of genotypes and allelic distribution after

Table 4. Demographics and other parameters compared with genetic models of OSAS patients

OSAS Group (n=152)	Genotype						
	Co-Dominant Model			Dominant Model		Recessive Model	
	QQ	QR	RR	QQ	QR/RR	QQ/QR	RR
	71	70	11	71	81	141	11
Age†	47.76±9.1	49.10±10.7	43.18±8.2	47.76±9.1	48.30±10.6	48.43±10.0	43.18±8.2
Body Mass Index	31.22¥ (24.0-35.4)	24.10 (23.7-32.5)	30.45 (23-32.7)	31.22 (24.0-35.4)	24.0* (23.1-32.7)	30.30 (23.7-34.1)	30.45 (23-32.7)
Neck Circumference	43 (40.5-45)	42 (40-44.8)	43 (42-44.5)	43 (40.5-45)	42 (40-45)	43 (40-45)	43 (42-44.5)
Waist Circumference	109 (99-117.5)	105.5 (99-114.5)	105 (102-110.5)	109 (99-117.5)	105 (99-115)	107 (99-116)	105 (102-110.5)
Apnea - Hypopnea Index	37 (21.2-60.7)	33.6 (17.9-59.8)	20.8 (14.7-28.4)	37 (21.2-60.7)	32 (17.5-52.5)	35.9 (19.3-61.3)	20.8* (14.7-28.4)
NOS	91 (89-93)	91 (88-92)	93 (92.5-94)*	91 (89-93)	91 (89-93)	91 (88-93)	93 (92.5-94)*
ESS	11 (6-16)	10 (6.3-13)	10 (8.5-12)	11 (6-16)	10 (7-13)	10 (6-15)	10 (8.5-12)
SBP	130 (120-140)	130 (120-140)	120 (115-130)	130 (120-140)	130 (120-140)	130 (120-140)	120 (115-130)
DBP	80 (70-90)	80 (70-90)	70 (65-70)	80 (70-90)	80 (70-90)	80 (70-90)	70 (65-70)*

(*: p<0.05; BMI: ¥QQ>QR; Values were expressed as †: Mean±SD / Other parameters: median (quartile 1-3)
NOS: nocturnal oxygen saturation; ESS: epworth sleepiness scale; SBP: systolic blood pressure; DBP: diastolic blood pressure

Table 5. Genotype frequencies of subjects with hypertensive and normotensive values

	Co-Dominant Model			Dominant Model		Recessive Model		Frequency of Alleles	
	QQ	QR	RR	QQ	QR/RR	QQ/QR	RR	Q	R
Hypertensive (n=54)	32 (59.3)	22 (40.7)	-	32 (59.3)	22 (40.7)	54 (100)	-	86 (79.6)	22 (20.4)
Normotensive (n=176)	69 (39.2)	84 (47.7)	23 (13.1)	69 (39.2)	107 (60.8)	153 (86.9)	23 (13.1)	222 (63.1)	130 (36.9)
χ2 / p	11.279 / 0.004			6.748 / 0.012		7.841 / 0.03		9.51 / 0.001	

Values were expressed as number of individuals and (%)

Table 6. Systolic and diastolic blood pressure values according to genotype distributions

	Co-Dominant Model			Dominant Model		Recessive Model	
All subjects	QQ n=101	QR n=106	RR n=23	QQ n=101	QR/RR n=129	QQ/QR n=207	RR n=23
SBP (n=230)	130 (110-140)	120 (110-130)	120 (110-130)	130 (110-140)	120 (110-130)	125 (110-140)	120 (110-130)*
DBP (n=230)	70 (70-80)	80 (70-80)	70 (70-80)	70 (70-80)	70 (70-80)	70 (70-80)	70 (70-80)

*: p<0.05; Values were expressed as Median (quartile 1-3)
SBP: systolic blood pressure; DBP: diastolic blood pressure

subgrouping according to blood pressure (hypertensive and normotensive). We found no individuals with RR genotype in the hypertensive group. Systolic blood pressure values were also lower in all the RR subjects, a difference that was only statistically significant under the recessive model, while in other models, the difference was only marginal. Odds ratios showed increased risk of hypertension for the Q allele compared to R alelle: R vs. Q (OR=2.29 ([%95 CI 1.36-3.83], p=0.002)). Similarly, this ratio was high in the dominant model (QR+RR vs. QQ: (OR=2.26 ([%95 CI 1.21-4.19], p=0.012). Olivera et al. (42) reported that Gln223Arg polymorphism was associated independently with SBP. Ping Gu et al. (43) reported similar results, showing that the risk of hypertension is higher in Q allele carriers compared to R allele carriers. This outcome confirms the potential protective role of the RR genotype against hypertension (Table 6).

The lack of an obese control group in our study has limited us to show the effect of Q223R polymorphism on OSAS. Inclusion of only male population to the study group and relatively small size of the study group which led to under representation of the population have been the other limitations of the study.

According to our results, the *LEPR* Q223R polymorphism does not appear to be associated with OSAS in men. Our results show that the polymorphism is associated with lower BMI. Lower AHI and higher NOS levels can be attributed to this decrease in BMI. Subjects carrying a RR genotype were found to have lower blood pressure, supporting the idea that the RR genotype may provide protection against hypertension. OSAS is a multifactorial pathology, and therefore, a single gene mutation cannot form the sole basis for explaining the pathophysiology of the disease. Further studies in larger sample populations encompassing other polymorphisms (Ser343Ser, Ser492Thr, and Lys656Asn) in the LEPR are necessary to further clarify the relationship between LEPR polymorphisms and OSAS.

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RESEARCH ARTICLE

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The Effectiveness of the Antimic® Biocide against Nosocomial Bacteria Specified by Different Standard Methods

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ABSTRACT

The effectiveness of Antimic® (3-(trimethoxysilyl)-propyl, cocodimethylammonium chloride) against different nosocomial pathogens was evaluated. Despite the fact that Antimic® biocide is a recommended compound for disinfecting areas, there is no published data about the antibacterial activity of this formulation against nosocomial pathogens (*Acinetobacter baumanii*, methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*). The minimum inhibitory/minimum bactericidal effective concentrations for test bacteria were determined. The bactericidal activity of determined dosages was evaluated using the culture-based quantitative suspension test (British Standards BS EN 1276) for 1 and 5-minute contact period, under clean and dirty conditions. Antibacterial activity was also confirmed by fluorescence staining. The biocide was found to be effective at 25 and 50 mg/L concentrations at contact times of 1 and 5 minute, with ≥ 5 log reduction in all bacteria. According to fluorescent microscopic examinations similar bacteria reduction was determined as ≥ 5 log reduction. The results indicated that Antimic® compound meets the requirements of EN 1276 against the tested bacteria. Moreover, Antimic® biocide provide an advantage by not promoting the viable but non culturable state in the tested bacteria and removing the tested bacteria successfully. The results showed that the execution of different microbiological growth and/or antibacterial activity monitoring tests, simultaneously, provide information about the optimum concentration and contact time of a biocide.

Keywords: Nosocomial pathogens, Antimic®, antibacterial activity, MIC, MBC

INTRODUCTION

In recent years nosocomial infections [hospital associated/acquired infections (HAI)] have been recognised as a serious safety issue for both patients and health care providers. According to the World Health Organization (WHO), nosocomial infection is defined as: "an infection developing in patients during the process of care in a health-care facility which was not present or incubating at the time of admission." This definiton includes infections which can be acquired in the hospital but appear after discharge from hospital, and also occupational-related infections among medical staff (1).

Nosocomial infections occur worldwide as an important healthcare problem both in developed and developing countries. Of every 100 hospitalized patients, 7 in developed and 10 in developing countries will acquire at least one of the health care-associated infections (2,3). At any given time, nosocomial infection prevalence is 5%-12% and 5.7%-19.1% in high-income and in low- and middle-income countries, respectively (3).

Nosocomial infections, cause functional impairment and emotional stress in patients due to increased lengths of stay in hospitals (1,3). It should be noted that HAIs result in increased healthcare costs. The greatest contributor to this cost is increased length of stays for infected patients; also indirect costs due to lost work is also considerable. Most importantly of all, these infections increase morbidity and are a major cause of death (1,3,4).

Patient susceptibility, environmental factors, contaminated environmental sites, microbial agent type and bacterial resistance to antimicrobials can influence the development of nosocomial infections (1). Surveillance studies have proved that many nosocomial infections are caused by antimicrobi-

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al-resistant organisms (4). In general, the agents which are related to nosocomial infections include *Acinetobacter* spp., *Streptococcus* spp., *Staphylococcus aureus* and coagulase-negative staphylococci, enterococci, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Legionella* spp., and Enterobacteriaceae family members including *Proteus mirabilis*, *Escherichia coli*, *Klebsiella pneumonia* and *Serratia marcescens* (5,6). In fact, the methicillin resistant *Staphylococcus aureus* (MRSA) infection is now used as a measure of hygiene in hospitals, since they resist desiccation and can survive in hospital dust for up to a year (1,7-10)

Various measures for controlling and preventing infection, such as the usage of masks and gloves, appropriate hand hygiene and the application of basic precautions are recommended by the authorities (3). However, it has been proven that pathogens such as VRE and MRSA can be transmitted from contaminated surfaces to caregivers' hands (9). Epidemiologic studies have shown that previously colonized places with MRSA, VRE or *Acinetobacter baumanii* are at significant risk of acquiring these organisms (8).

For this reason, HAI prevention/control measures should involve the use of effective biocides (4). Therefore, considering the limited treatment options and the mortality rate, the eradication of the cause of the infection with the correct agent, correct dose and contact time at the infection source points will provide a permanent and effective solution.

There are various reports on cross- and co-resistance to biocidal compounds and antibiotics (4). Therefore, alternative biocidal compounds are being investigated. One of the new durable alternative compounds 3-(trimethoxysilyl)-propyl, cocodimethylammonium chloride (Antimic®), has alkoxy silane functional groups to form covalent bonds at the molecular level and thus, provide antibacterial features to the applied surface. While Antimic®'s hydrophobic long chains approach the lipid membrane, the positively charged quaternary region of the Antimic® breaks down the cell membrane, leading to the death of the bacteria (11).

Four different trimethoxysilyl quaternary ammonium chloride compounds were studied in detailed by the Environmetal Protection Agency (EPA) and were reported as non migrating and non toxic features. (12). Therefore, they prevent antimicrobial resistance and do not cause cross contamination.

Although Antimic® compound is recommended for disinfection, there is no published report on the antibacterial activity of this compound against different nosocomial pathogens. Therefore, in this study the inhibitory characteristics of Antimic® biocide were investigated against *Acinetobacter baumanii*, vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *Staphylococcus aureus* pathogens.

MATERIALS AND METHODS

Test Organisms and Culture Conditions

Acinetobacter baumanii (ATCC 19606), vancomycin-resistant *Enterococcus faecium* (ATCC 51299) and methicillin-resistant *Staphylococcus aureus* (ATCC 33591) organisms were obtained American Type Culture Collection. Test organisms were maintained in phosphate buffer and glycerin suspension separately and stored at - 86°C in cryotubes. Freeze-dried cultures were not subcultured more than 3 times in order to prevent mutations and not affect the resistance of the organisms against antimicrobials (13). For experimental use, freeze-dried cultures of the organisms were grown on tryptone soya agar (Oxoid) at 37°C. After 24 hours, cells were harvested and a suspension was prepared turbidimetrically to a 1.5-5.0 10^8 cfu ml^{-1} concentration in Cl_2-free sterile tap water and used in the experiments.

Biocide

Different dosages of Antimic® biocide (10.000-1 mg/L), were prepared in sterile distilled water. Effective dosages for suspended bacteria were determined by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests.

Determination of Minimum Inhibitory and Minimum Bactericidal Concentration

Broth macrodilution MIC tests were carried out in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (14,15). Briefly, 1 mL of $5x10^5$ cfu mL^{-1} bacterial suspensions were added to tubes containing different concentrations of biocide. After incubation at 37°C for 24 h, the first tube in the biocide/bacteria suspension series without signs of visible growth was considered as the MIC. To determine MBC values, 10 µL samples from each tube showing no visible growth were spread on Mueller Hinton agar plates (Oxoid) and incubated at 37°C for 24 h. MBCs of the compound were determined as the lowest concentration at which no colony formed. The MIC and MBC values were determined in three independent experiments.

Determination of Biocidal Activity with a Quantitative Test Method

The bactericidal activity of the effective dosages (50, 25, 20, 10 mg/L), determined by MIC and MBC tests, was assessed by the culture-based phase 2/step1 quantitative suspension test (BS EN 1276:2009). According to BS EN 1276 (2009) experiments were carried out under clean and dirty conditions for each organism and dosages at contact times of 1 and 5-minute. In this study, tests were undertaken in triplicate.

Briefly, an 8.0 mL biocide sample diluted in standard hard water was added to bovine serum albumin at a final 0.03% (w/v) and 0.3% (w/v) concentrations to represent clean and dirty conditions, respectively. To these tubes 1.0 mL bacterial suspension were added.

After a contact time of 1 and 5 minutes, 1.0 mL of the test blend was pipetted into 8.0 mL neutralizer and 1.0 mL deionized water, After 5 minutes of neutralization. 1.0 mL of test mixture were pour plated, in triplicate with tryptone soya agar. Plates were incubated at 37°C for 48 h prior to counting.

Three control (validation) groups were conducted in parallel for each test:

Validation A. The test was conducted with the addition of 8.0 mL sterile standard hard water in place of the biocide solution to ensure that there was no biocidal activity of the other experimental parameters.

Validation B. The test was conducted with the addition of 8.0 mL neutralizer and 1.0 ml water to the bacterial suspension to ensure that the neutralizer solution did not have biocidal activity.

Validation C. The test was conducted with the addition of 1.0 mL bacterial suspension to the neutralized biocide to ensure that the biocide had been neutralized.

Determination of Antibacterial Activity by Culture Independent (CTC/DAPI Flourescence Staining) Method

At contact times of 1 and 5 minutes, the number of respiring and total cells in biocide exposed and unexposed (control) samples was detected by staining CTC and DAPI, according to a modified technique of Rodriguez et al. (16,17). 900 µL of samples were incubated with CTC redox dye solution (at 5 mM concentration) at 28 °C for 4 hours, in the dark. After that, samples were counterstained with 1.0 µg/mL DAPI for 1 h. Cells were subsequently harvested, after incubation, by vacuum filtration onto black polycarbonate filters (0.2 µm pore size, Millipore, USA). The air-dried filters were mounted with non-fluorescent immersion oil and coverslipped, and stained cells were enumerated microscopically.

Microscope slides were examined using a Nikon 80i epiflouresence microscope. For statistical evaluation, the number of microorganisms was estimated from counts of at least 20 randomly chosen fields (at x 1,000) per sample. The number of microorganisms present in the sample is calculated by applying the following conversion formula:

$$N = \frac{S \times n}{C \times V} \times D$$

where N, microorganism counts per milliliter; S, real filtration area; n, average number of microorganisms per field of vision; C, real microscopic range area; V, filtered sample volume; D, sample dilution.

Following the manufacturer's instructions, respiring cells showing red CTC formazan crystals were considered live cells, while blue cells stained by DAPI were considered dead. Results were expressed as the log number of corresponding bacteria per sample.

Statistical Analysis

The data was analysed by using the Graphpad prism 7. A comparison of biocide exposed and control samples were analyzed using Student's t-test. Differences were considered significant when $p<0.05$.

RESULTS

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory and minimum bactericidal concentrations of Antimic® biocide values against important nosocomial bacteria can be seen in Table 1.

Determination of Biocidal Activity with a Quantitative Test Method

Following MIC and MBC determinations, the bactericidal activity of the effective dosages for each test organisms were further tested by (50-10 mg/L), culture-based standard quantitative suspension test (BS EN 1276:2009). Experiments were performed with the presence of inhibitory substances that simulate the organic load or conditions comparable to the practical use of the product. To simulate dirty and clean conditions, 0.3% or 0.03% (w/v) bovine serum albumin, was used respectively.

According to BS EN 1276 standard (2009), the reduction in culturable bacteria was calculated by subtracting the log of the colony count after biocidal activity (Na) from the log of the initial count in the test chamber ($Nx10^{-1}$). Compounds needed to achieve a five log reduction in culturable colony counts, to pass the test,

The 50 mg/L biocide dosage was effective against all tested bacteria, at each time of contact (1 and 5 minutes), in dirty and clean conditions (Table 2-4).

The lower dosage (25 mg/L) of Antimic also passed against *Acinetobacter baumanii* and vancomycin-resistant *Enterococcus faecium* for both clean and dirty conditions (Table 2, 4). This dosage was also effective against methicillin-resistant *Staphylococcus aureus,* but only after 5 minutes of exposure, in clean conditions (Table 3).

With the 25 mg/L dosage the highest bacterial reduction was achieved against vancomycin-resistant *Enterococcus faecium* bacteria, for this reason, the lower concentrations than this (20 and 10 mg/L) were tested only against *Enterococcus faecium* bacteria.

The 20 and 10 mg/L dosages achieved a 5 log reduction as required by EN 1276, after 5 minutes of exposure, under both clean and dirty conditions (Table 4).

The differences between Antimic® treated and untreated samples were statistically significant ($p<0.05$).

Determination of Antibacterial Activity by Flourescence Staining Method

The log reduction of flourescence stained bacteria counts after Antimic® treatment was found to be similar to the culture based colony counts.

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values in mg/L, exhibited by Antimic® biocide against nosocomial bacteria

Microorganism	MIC mg/L	MBC mg/L
Acinetobacter baumanii (ATCC 19606)	5	50
Methicillin-resistant *Staphylococcus aureus* (ATCC 33591)	20	25
Vancomycin-resistant *Enterococcus faecium* (ATCC 51299)	5	50

*Mean results are expressed as three independent experiments in triplicate. Significant differences between each treatment and microorganism are indicated as $p<0.05$.

Table 2. Bactericidal activity of Antimic® biocide against *Acinetobacter baumanii* (ATCC 19606) bacteria according to BS EN 1276:2009 standard

			Log Reduction	
Antimic® Dosages	**Initial Count (log_{10})**	**Contact Time**	**In Clean Conditions**	**In Dirty Conditions**
50 mg/L	7.10±0.02	1 minute	> 5.96±0.02	> 5.96±0.01
		5 minutes	> 5.96±0.04	> 5.96±0.03
25 mg/L		1 minute	> 5.96±0.04	> 5.96±0.01
		5 minutes	> 5.96±0.03	> 5.96±0.01

Table 3. Bactericidal activity of Antimic® biocide against methicillin-resistant *Staphylococcus aureus* (ATCC 33591) bacteria according to BS EN 1276:2009 standard

			Log Reduction	
Antimic® Dosages	**Initial Count (log_{10})**	**Contact Time**	**In Clean Conditions**	**In Dirty Conditions**
50 mg/L	7.44±0.06	1 minute	5.49±0.01	5.04±0.04
		5 minutes	> 6.30±0.02	5.39±0.04
25 mg/L		1 minute	4.26±0.04	4.07±0.03
		5 minutes	5.57±0.03	4.00±0.02

Table 4. Bactericidal activity of Antimic® biocide against vancomycin-resistant *Enterococcus faecium* (ATCC 51299) bacteria according to BS EN 1276:2009 standard

			Log Reduction	
Antimic® Dosages	**Initial Count (log_{10})**	**Contact Time**	**In Clean Conditions**	**In Dirty Conditions**
50 mg/L	7.44±0.06	1 minute	> 6.44±0.04	> 6.44±0.01
		5 minutes	> 6.44±0.06	> 6.44±0.04
25 mg/L		1 minute	5.09±0.01	5.05±0.05
		5 minutes	> 6.44±0.01	6.09±0.01
20 mg/L		1 minute	5.02±0.01	4.26±0.04
		5 minutes	5.70±0.01	5.18±0.02
10 mg/L		1 minute	4.69±0.02	4.02±0.02
		5 minutes	5.06±0.04	5.05±0.05

As regards the 50 mg/L biocide dosage a > 5 log reduction was achieved at each time of contact (1 and 5 minutes), in dirty and clean conditions (Figure 1-3).

With the 25 mg/L dosage > 5 log reductions against all bacteria were found at all contact times under dirty and clean conditions; except MRSA bacteria, at 1 minute contact time under dirty and clean conditions, and also at 5 minute contact time in dirty conditions (Figure 1-3) .

20 mg/L and 10 mg/L dosages achieved a 5 log reduction, after 5 minute of exposure, under both clean and dirty conditions (Figure 3), unlike the cultural-based colony counting results.

DISCUSSION

Nosocomial infections can be defined as those occuring in individuals, within 48 hours after entering a health facility (1,18,19). Hospital infections are signs of the service quality of inpatient treatment institutions. Those infections have critical importance, because of the prolongation of treatment duration, loss of work power and productivity, cost increase, and most importantly, increase in morbidity and mortality (1,3,4,9).

According to a survey conducted by the WHO in 55 countries, at any given time 1.4 million people suffer from hospital-acquired infections worldwide (1,9). Annual financial losses due to nos-

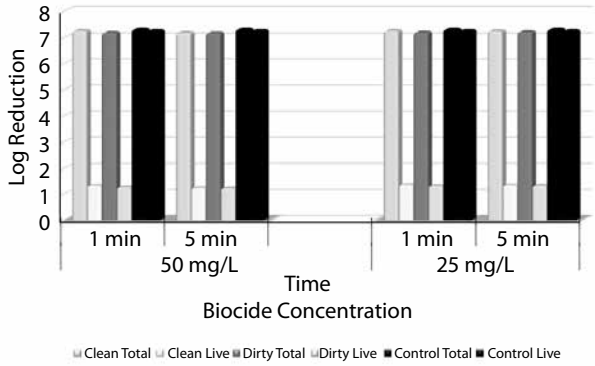

Figure 1. Total cell count (CTC+DAPI positive cells) and live cells (CTC positive cells) of Antimic® treated *Acinetobacter baumanii* (ATCC 19606)

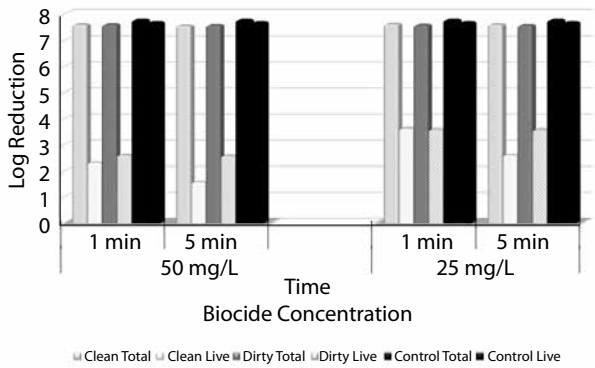

Figure 2. Total cell count (CTC+DAPI positive cells) and live cells (CTC positive cells) of Antimic® treated methicillin-resistant *Staphylococcus aureus* (ATCC 33591)

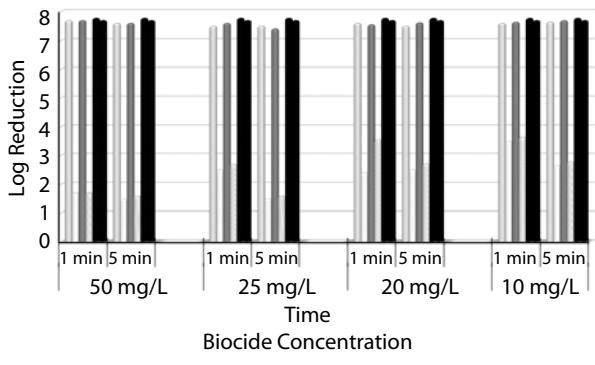

Figure 3. Total cell count (CTC+DAPI positive cells) and live cells (CTC positive cells) of Antimic® treated vancomycin-resistant *Enterococcus faecium* (ATCC 51299)

ocomial infections are estimated at approximately €7 billion in Europe and US$ 6.5 billion in the USA (3). The results of such studies reveal the importance of gaining a better understanding into the prevention of these infections.

The risk factors for the nosocomial infections may be divided into two broad categories: intrinsic and extrinsic factors. Age, immunity, nutrition of the patient and underlying disease conditions constitute intrinsic risk factors. Extrinsic risk factors are composed of factors related to the health care institution such as the architectural structure of the hospital, failure to observe the asepsis/isolation procedures, and lack of attention to hand washing, disinfection and sterilization (1,20-22).

Surveillance studies have shown that many nosocomial infections are caused by antimicrobial-resistant organisms (4). In many scenarios, one of the major reasons for cross-contamination is due to bacterial adhesion of resistant microorganisms from commonly touched places and materials in the hospital (23,24). Since the hospital environment serves as an important reservoir for these pathogens, the eradication of the cause of infection with an effective biocidal agent is a major challenge for the the control of hospital infections (1,5,9,23-25). Thus, in the current study, the inhibitory characteristics of Antimic® biocide were investigated against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, *Acinetobacter baumanii* pathogens.

Moreover, the incorrect and extensive usage of antimicrobials results in the developed resistance (26), thus, effective dosages of biocides should be determined in the laboratory. The MIC and MBC values are helpful parameters to assess the bacteriostatic and bactericidal activity of a biocide, respectively (25). In the current study, MIC and MBC values were between 5-50 mg/L concentration. The highest MIC value was 20 mg/L against MRSA bacteria, which has a particular facility for nosocomial transmission, while the lowest MBC value (25 mg/L) was determined against the same bacteria.

On the other hand, it should be noted that MIC/MBC tests were conducted under nutrient-rich conditions, where high organic material concentrations may interfere with the biocidal action of the substance (4). Therefore, at the second stage of the study (quantitative suspension test, EN1276), effective dosages of biocide against test bacteria were tested with the presence of inhibitory substances that simulate the organic load or conditions comparable to the practical use of the product. The organic material concentration in biocide solutions in EN 1276 tests was lower than in MIC testing. To mimic dirty and clean conditions, 0.3% or 0.03% (w/v) bovine serum albumi, was used, respectively. According to the results, the tested biocide was found to be effective at 25 and 50 mg/L concentrations at contact times of 1 and 5 minutes, with ≥ 5 log reduction in all test bacteria.

Nevertheless, currently, actively used standards are based only on colony count or conventional culture methods. On the other hand, bacteria can enter the viable but non culturable (VBNC) phase as a response to biocidal treatment, which cannot be detected with conventional culture methods and retain sits virulence, posing a public health risk (27,28). In the current study, fluorescent microscopic examinations were carried out to evaluate VBNC state of the bacteria after exposure to Antimic®. Similar bacteria reduction with the culture was

determined. This study indicates the importance of performing *in vitro* biocidal activity tests in relevant simulations. In this regard, the main objective of the current study is to determine the effective biocide dosages, to ensure that those dosages are used in practical conditions. Thus, the emergence of new resistant microorganism strains may be prevented.

The tested biocide is an alternative, ideal disinfectant for hospitals and household facilities, since it is i) safe for the environment and humans, ii) noncytotoxic, iii) stable even at elevated temperatures, iv) readily biodegradable v) has no risk of inducing bacterial resistance vi) non-corrosive vii) does not have harmful effects on materials.

As a conclusion, to prevent nosocomial infections and possible risks associated with resistant microorganisms, specific biocides should be evaluated under simulated conditions. Antimic® biocide provide an advantage by not promoting the VBNC state in the tested bacteria and removing the tested bacteria successfully. Approaches involving producing antimicrobial surfaces and/or furniture may be explored for further investigation into *in vitro* tests for the prediction of the compound's durability of biocidal activity.

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RESEARCH ARTICLE

Eur J Biol 2017; 76(2): 57-60

In vitro Antidiabetic Activities of Two *Sorbus* Species

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ABSTRACT

Fruits of several *Sorbus* species (Rosaceace) are used both in traditional medicine as antidiabetic, antiinflammatory, diuretic, vasoprotective and in foods. In this study, *in vitro* antidiabetic activities of water extracts of *Sorbus aucuparia* L. (rowan tree) and *Sorbus torminalis* L. Crantz (wild sevice tree) fruits were investigated by measuring inhibitory potentials on α-glucosidase and pancreatic α-amylase activities, the most important digestive enzymes. Also, the total phenolic and flavonoid contents of the fruits were determined to evaluate the association between phenolic content and antidiabetic activity. *S. torminalis* and *S. aucuparia* extracts exhibited strong α-glucosidase inhibitory activity, more effective than that of standard drug acarbose. However, *S. torminalis* has shown moderate inhibitory effect against α-amylase while *S. aucuparia* exhibited weak inhibition. The total phenolic and flavonoid contents of the fruits were correlated with andiabetic activities. It has been suggested that antidiabetic effects of the fruits may be due to phenolic compounds present therein. Therefore, *S. aucuparia* and *S. torminalis* fruits might be potential sources of antidiabetic compounds.

Keywords: *Sorbus aucuparia, Sorbus torminalis*, antidiabetic activity, α-amylase, α-glucosidase.

INTRODUCTION

Diabetes mellitus is a group of metabolic diseases (1). Type 2 diabetes mellitus contains β-cell dysfunction and insulin resistance. When the β-cell function decreases over time, fasting blood glucose and postprandial glucose levels begin to rise and remain out of control (2). In the world wide, prevalence of type 2 diabetes is increasing due to lifestyle-related risk factors such as smoking, obesity, poor diabetes and physical inactivity (1). The increased prevalence of type 2 diabetes has led to the development of many new approaches in the treatment of hyperglycemia. The purpose of these treatments is to reduce and maintain glucose concentrations as normal as possible and thus prevent development of complications (3). Sample treatments include α-glucosidase inhibitors (AGIs; acarbose, miglitol and voglibose) to reduce the absorption of carbohydrates in the intestine and control postprandial hyperglycemia. Acarbose inhibits both α-amylase (EC 3.2.1.1) and α-glucosidases (EC 3.2.1.20), thus preventing absorption of starch and other carbohydrates from the intestine also reduces postprandial glycaemia and helps manage diabetes (4). Lately, there has been much interest in the investigations of the natural α-glucosidase inhibitors for diabetes treatment (5).

Nature is a good source of antidiabetic drugs and plants are valuable dietary supplements to improve blood sugar control and prevent long-term complications of type 2 diabetes (2). Polyphenols are naturally occurring compounds found largely in the fruits (especially like grapes, apples, cherries and berries) and vegetables. Several studies revealed that long-time intake of plant polyphenols in diets have a protective effect to development of many diseases such as diabetes (6).

The genus *Sorbus* mostly distributed in Northern Hemisphere, comprises about 250 species of trees and shrubs. Fruits of several *Sorbus* species (berries) included *S. domestica*, *S. aucuparia* and *S. torminalis* from family Rosaceace are consumed as food sources and used as traditional medicine (7). Also, *Sorbus* species are called 'uvez' in Turkish, which have been used as traditional medicinal

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plants for various purposes in Turkish folk medicine (8). *S. domestica* fruits are consumed by the local population in Greece, not only as a nutritious food, but also traditionally as an antidiabetic agent (9). In this study, we investigated *in vitro* inhibitory effects of two *Sorbus* species on α-glucosidase and α-amylase activities. The antidiabetic activities and amount of total phenolics of *S. aucuparia* and *S. torminalis* fruits has been determined, comparatively. Although there are limited number of studies on *S. aucuparia* fruits, there is no study showing the antidiabetic effects of *S. torminalis*.

MATERIAL AND METHODS

Chemicals and Reagents

α-Amylase, α-glucosidase, acarbose, 3,5-dinitrosalicylic acid (DNS), Folin-Ciocalteu reagent, gallic acid, p-nitrophenyl α-D-glucopyranoside (PNPG) and starch purchased from Sigma Chemical Co. (St. Louis, MO, USA). Catechin was purchased from Fluka Chemical Co. (Buchs, Switzerland). All other chemicals or reagents were of analytical grade.

Preparation of Extracts

The fruits of *S. aucuparia* and *S.torminalis* were obtained from Black Sea Region and Istanbul, respectively. Decoctions are one of the most consumed drinkable forms of plants (10). For this reason, the fruit extracts were obtained by using decoction method. After the seeds were removed, fruits were dried in the shade. To prepare the water extracts, 15 g of the fruits were refluxed with distilled water for 3 hours. The extracts were filtered and the solvent was evaporated (Buchi, Switzerland) to dryness under reduced pressure. The fruits extracts were stored in -20°C until needed. For the biochemical assays, the extracts were dissolved in distilled water.

Determination of α-Glucosidase Inhibitory Activity

The α-glucosidase inhibitory effects of the fruit extracts were evaluated using a procedure described by Bothon et al. (11). For the α-glucosidase assay, 25 µL of the fruit extract was mixed with 75 µL of 0.1 M sodium phosphate buffer (pH 6.8) and 50 µL of α-glucosidase solution (1 U/mL) and preincubated at 37°C for 10 minutes. After incubation, 50 µL of substrate solution (5mM PNPG) was added to the reaction mixture and the absorbance change at 405 nm was measured at 37°C for 10 minutes using a microplate reader. Acarbose was used as a standard and replacing the extract with distilled water was used a control. The inhibitory activities of the extracts were identified according to the following formula:

$$\%\,Inhibition = \left(1 - \frac{Reaction\ rate\ of\ sample\ at\ 405\ nm}{Reaction\ rate\ of\ control\ at\ 405\ nm}\right) \times 100$$

Determination of α-Amylase Inhibitory Activity

The inhibition of α-amylase by the *Sorbus* fruits was determined using the DNS method (12). Briefly, 10 µL of each extract were preincubated with 50 µL of α-amylase solution (3 U/mL) and 40 µL of 0.1 M sodium phosphate buffer (pH 6.8) at 25°C for 10 minutes. The reaction was initiated by adding 50 µL starch solution (0.75%). After 5 minutes, the reaction was stopped by adding 75 µL of DNS color reagent (96 mM DNS and 5.31 M potassium sodium tartarate in 2 M NaOH). The mixtures were heated at 85°C for 15 minutes. After cooling, the mixture was diluted 4-fold with distilled water and absorbance was recorded at 540 nm. Acarbose was used as a standard and control was prepared without inhibitor. The inhibitory activities of the extracts were identified according to the following formula:

$$\%\,Inhibition = \left(1 - \frac{Absorbance\ of\ sample\ at\ 540\ nm}{Absorbance\ of\ control\ at\ 540\ nm}\right) \times 100$$

Determination of Total Phenolic and Flavonoid Compounds

Total phenolic and flavonoid contents of the extracts were determined using the Folin-Ciocalteu (13) and the aluminum chloride (14) methods, respectively. For the determination of total phenolics, 5 µL of fruit extract was mixed with 225 µL of distilled water, 5 µL of 2 N Folin-Ciocalteu reagent (previously diluted with distilled water 1:2; v:v) and 15 µL of 2% Na_2CO_3 solution. The mixture was incubated in dark for 2 hours at room temperature. After incubation, absorbance was measured at 760 nm. Total phenolic contents were determined using equation of standard regression curve which obtained by gallic acid solution and were expressed in mg of gallic acid equivalents (GAE).g extract^{-1}.

For the determination total flavonoids, 25 µL of fruit extract was mixed 125 µL of distilled water and 7.5 µL of 5% $NaNO_2$ solution then incubated for 6 minutes. Then, 15 µL of 10% $AlCl_3$ solution was added. After 5 minutes incubation at room temperature, 50 µL of 1 M NaOH solution and 27.5 µL of distilled water was added. The absorbance was recorded at 510 nm. Total flavonoid contents were determined using equation of standard regression curve which obtained by catechin solution and were expressed in mg of catechin equivalents (CE).g extract^{-1}.

Statistical Analysis

The results were evaluated using unpaired t-test with NCSS statistical computer package (NCSS, Kaysville, UT, USA) and the differences were considered significant at $p<0.05$.

RESULTS

In this study, the inhibitory effects of two *Sorbus* species and acarbose on α-glucosidase and α-amylase activities were investigated. It was found that *S. torminalis* and *S. aucuparia* showed strong and dose dependent inhibitory activities against α-glucosidase (Figure 1). The half-maximal inhibitory concentration (IC_{50}) values of the *Sorbus* extracts and acarbose are presented in Table 1. Comparison of the IC_{50} values revealed that the inhibitory effects of both *S. torminalis* and *S. aucuparia* extracts on α-glucosidase were approximately four and two fold higher than that of acarbose, respectively. As shown in Figure 2, *S. torminalis* exhibited 75.32±2.80% α-amylase inhibitory activity at 0.8 mg.mL^{-1} concentration while *S. aucuparia* exhibited only 22.08±1.17 % inhibition at same concentration.

Also, the total phenolic and flavonoid contents of the extracts are shown in Table 2. The results showed that *S. torminalis* water extract had the highest total phenolic and total flavonoid contents. These results demonstrate that there was a high correlation between the antidiabetic activity and the phenolic contents.

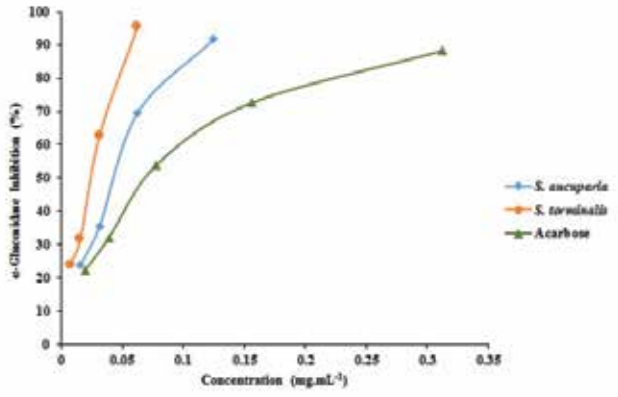

Figure 1. Effects of *Sorbus* extracts and acarbose on α-glucosidase activity

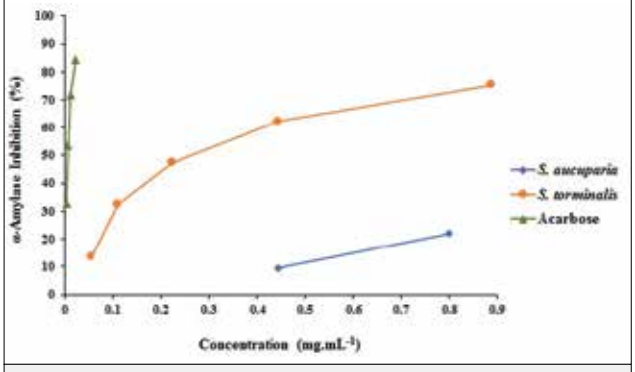

Figure 2. Effects of *Sorbus* extracts and acarbose on α-amylase activity

DISCUSSION

One of the therapeutic approaches in the treatment of diabetes mellitus is reduction of postprandial hyperglycemia (15). The rate of starch digestion is the most important factor affecting of blood glucose level. Since α-glucosidase and α-amylase have a crucial function in carbohydrate hydrolysis, inhibition of these enzymes is one of the most therapeutic strategy for the treatment of diabetes (5). In this study, we evaluated α-glucosidase and α-amylase inhibitory activities of two *Sorbus* species. Our results showed that *S. torminalis* and *S. aucuparia* strongly inhibited α-glucosidase activity indicates tested species have antidiabetic effects. However, *S. torminalis* and *S. aucuparia* showed moderate and weak inhibitory effect on α-amylase, respectively. In literature, there have been limited studies on the antidiabetic effects of *S. aucuparia* fruits while no studies showing antidiabetic activity of the *S. torminalis* fruit were found. In these studies, the antidiabetic effect of *S. aucuparia* fruit extract was reported by measuring α-amylase inhibitory activity (16) and α-glucosidase inhibitory activity (1). These results are consistent with the data obtained from this study. Also, antidiabetic potentials of different *Sorbus* species (S. *decora* and *S. tianschanica*) have been reported in diabetic animal models (17,18).

Table 1. α-Glucosidase and α-amylase inhibitory activities of the extracts and acarbose

	Inhibitory activity (IC_{50} mg.mL^{-1})	
	α-amylase	**α-glucosidase**
S. aucuparia	ND	0.050±0.0005[a]
S. torminalis	0.307±0.0158[a]	0.027±0.0006[b]
Acarbose	0.006±0.0002[b]	0.086±0.0027[c]

Data are presented as the mean of three replicates ± standard deviation. Different superscript letters in the same column indicate a significant difference ($p<0.05$). IC50; The inhibitory concentration of the extract or acarbose required to inhibit the activity of the enzyme by 50%. IC50 values were calculated from dose-response curves using Microsoft Excel. All concentrations are the final extract concentrations in the reaction mixture. ND; Not determined.

Table 2. Total phenolics contents (TPC) and total flavonoid contents (TFC) of the extracts

	TPC (GAE.g extract^{-1})	TFC (CE.g extract^{-1})
S. aucuparia	19.13±0.76	9.62±0.27
S. torminalis	24.21 0.61	15.69±0.55

Data are presented as the mean of three replicates±standard deviation.
GAE.g extract^{-1}; mg gallic acid equivalents.g extract^{-1}.
CE.g extract^{-1}; mg catechin equivalents.g extract^{-1}.

In this study, we also determined the total phenolic and flavonoid contents of the fruit extracts. It was found that there was a high correlation between phenolic contents and *in vitro* antidiabetic acitivity. Antidiabetic effects of polyphenolic compounds have been shown in numerous studies (6,19). It has been suggested that hypoglycemic effects of fruits and vegetables may stem from the insulin-like or insulin releasing activities of phenolic compounds present therein (2). Also, α-glucosidase and α-amylase inhibitory potentials of various plant polyphenols such as catechins, diacetylated anthocyanins and alkaloids have been reported in several studies (2,6,20). Phenolic composition of *S. torminalis* and *S. aucuparia* fruits have been shown in previous studies (21,22). Based on the correlation between the results of the assays, we can say that the phenolic compounds in the fruit extracts are responsible for its antidiabetic activity.

In recent work, we demonstrated that the extracts from *Sorbus* fruits especially *S. torminalis*, potently inhibit α-glucosidase and α-amylase *in vitro*. It is reasonable to hypothesize that consumption of *Sorbus* fruits may reduce intestinal absorption of sugars via inhibition of these digestive enzymes. Also, these fruits can be a potential source of natural antidiabetic agents. These findings may scientifically explain some uses of this species in folk medicine as an antidiabetic agent.

Acknowledgement
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RESEARCH ARTICLE

Eur J Biol 2017; 76(2): 61-8

Alterations at the Synthesis and Degradation of E-cadherin in the Human Lungs with Emphysema

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ABSTRACT

Pulmonary emphysema leads to a cascade of events starting with enlarged alveoli, loss of alveoli and, subsequently to the damage and disruption of pulmonary epithelium. The integrity of the pulmonary epithelium, which is constituted by pneumocytes linked to each other through E-cadherin proteins, is important for respiration. The aim of the present study was to detect the content and destruction of E-cadherin protein and to investigate the contribution of E-cadherin to pulmonary emphysema pathogenesis.

The structural changes, reparative capacity of the pulmonary epithelium, amount of E-cadherin protein and, the immunoreactivity of neural precursor cell expressed developmentally down-regulated protein 9 (NEDD9) were evaluated in emphysematous (n=7) and non-emphysematous (n=6) areas of lung samples taken from patients with chronic obstructive pulmonary disease. Emphysematous areas are characterized by enlarged alveoli, disrupted alveolar walls and epithelium, increased type 2 pneumocytes and NEDD9 immunoreactivity, and reduced E-cadherin proteins.

Our data shows that E-cadherin levels are decreased in emphysematous areas due to its degradation by NEDD9. Decreased E-cadherin levels also lead to the disintegration of the pulmonary epithelium by causing the presence of weakness intercellular connections or the absence of intercellular connections. The repair of the pulmonary epithelium could not complete due to the reduced E-cadherin, because type 2 pneumocytes could not differentiate into type 1 pneumocytes. In conclusion, the reduced E-cadherin levels lead to emphysematous alterations in human lungs and contributes to pulmonary emphysema pathogenesis.

Keywords: Pulmonary emphysema, COPD, E-cadherin, pulmonary epithelium

INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is characterized by persistent alveolar loss, abnormal inflammatory response, airflow limitation and tissue remodeling, as well as symptoms such as chronic bronchitis and emphysema (1). Pulmonary emphysema is seen in 20% of COPD patients. It is characterized by inflated alveoli with excessive air, irreversible loss of alveoli, damage of the pulmonary epithelium, and reduced respiratory surface area depending on the extent of injury (2). The destruction of the alveolar structure can occur due to the protease-antiprotease imbalance, degradation of elastic fibers and loss of the tissue elasticity (3,4). Pulmonary epithelial cell loss, failure to achieve pulmonary epithelium integrity, chronic inflammation, and deterioration of repair mechanisms following pulmonary epithelial injury can result in emphysema (3,5,6).

The experimental and clinical studies performed for the pathogenesis of pulmonary emphysema indicate clearly that endogenous repair, following pulmonary epithelial injury, could not occur in the pulmonary emphysema (7). Regeneration of the damaged pulmonary epithelium and ensuring the pulmonary epithelial integrity still remains a challenge in treating the pulmonary emphysema. Therapeutic approaches performed to repair alveolar epithelial and to achieve the integrity of pulmonary epithelium are of a great importance for the renewal and integrity of pulmonary epithelium. Currently there is no therapeutic approach or clinical trial efficient in the repair and keeping the integrity of the pulmonary epithelium for the emphy-

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sema patients. E-cadherin molecule is a cell adhesion protein that is effective on binding of epithelial cells to each other and on the protection of epithelial integrity (8). Oldenburger et al. (9) found that the amount of E-cadherin protein decreased in the lungs of COPD patients compared to control individuals. We suggest that decreases in the levels of E-cadherin protein may result in the failure of binding of pulmonary epithelial cells to each other, and in the disruption of the integrity of pulmonary epithelium. The aims of the present study were to identify the alterations in the levels of E-cadherin protein in the pulmonary emphysematous regions of COPD patients, to explain the possible causes of such alterations, and to determine the relation between E-cadherin and the integrity of pulmonary epithelium.

MATERIALS AND METHODS

Clinical Specimens

The study was ethically reviewed and approved by the Clinical Research Ethics Committee of Istanbul University Cerrahpasa Medical Faculty, Turkey (Diary No a-15/01.10.2013). COPD patients diagnosed with lung cancer were included in the study. COPD diagnosis was made according to the 2016 GOLD criteria. Specimens were obtained from 35 smokers (smoking history: 30 packs/year) and male patients with medium age 48±16. Lung specimens around the tumor areas were removed by thoracoscopic surgery from COPD patients. They were examined microscopically and specimens of emphysematous and non-emphysematous tissue that were free of carcinoma cells were identified in these samples. Two study groups were formed from the samples: Non-emphysematous tissue specimens (n=6, control group)" and "Emphysematous Tissue Specimens (n=7)".

Histology and Immunohistochemistry

The lung specimens were fixed in 10% buffered formalin for 24 h, dehydrated in ascending alcohols and embedded in paraffin. Sections 5 µm thick underwent to hematoxylin-eosin and Verhoeff's elastic stainings (the latter-staining-elastin in blue-black) and were examined under a light microscope.

Sections 4 µm thick were stained using immunoperoxidase-based procedures. After antigen retrieval (10), the sections were incubated in 3% hydrogen peroxide in 1:1 methanol/phosphate-buffered saline mixture, incubated with the indicated antibodies overnight at 4 °C, and then treated with Histostain Plus-peroxidase kit according to the manufacturer's instructions. The indicated primary antibodies are rabbit anti-ki67 (a proliferation marker, diluted to 1:50, Millipore), -prosurfactant C (proSPC, type 2 pneumocyte marker, diluted to 1:500, Millipore) and -neural precursor cell expressed developmentally down-regulated 9 (NEDD9, a digestive protein for E-cadherin, diluted to 1:500, Abcam). The peroxidase activity was revealed by a 3-amino-9-ethylcarbazole substrate kit. Slides were counterstained with Mayer's hematoxylin. For negative controls, phosphate-buffered saline solution was used instead of the primary antibodies.

Five microscopic fields were randomly selected from alveolar areas without bronchioles from sections of each sample. Digital images of these fields were captured at a magnification of 400 and overlaid with transparent grids (1 mm^2). The number of anti-ki67 or anti-proSPC immunoreactive cells was calculated and reported as percentage of the total number of cells.

Western Blotting

Lung samples were snap frozen in liquid nitrogen and stored at -86 °C. Lung samples weighing 200 mg were homogenized in lysis buffer. The lysates were then centrifuged at 13,000*g* for 10 min at 4 °C, and the supernatants were collected and stored at -20 °C. The total protein concentrations were determined using Bradford method (11). Then 80 µg samples were loaded in 10% SDS-PAGE electrophoresis. Next, the protein was transferred onto nitrocellulose membranes and blocked with 5 % non-fat dried milk for one hour. Later on, the protein was incubated overnight at 4 °C with the primary antibodies against E-cadherin and advanced glycosylation end-product specific receptor (AGER, type 1 pneumocyte marker) diluted 1:500 and 1:100 respectively. The membrane was washed, and incubated with the secondary antibody (a goat anti rabbit Ig-G-HRP, diluted 1:500). Finally, the blots were developed with luminol reagent (Santa Cruz, CA, USA). The intensities of the protein bands were quantified using molecular imaging software (Kodak GL 1500, CT, USA) being normalized to β-actin protein bands.

Statistical Analysis

The results were analyzed by *Student T-* test and *Mann Whitney U-2 tail* test to compare differences among groups by using GraphPad Prism software, version 5.00 (San Diego, CA). P values of <0.05 were considered significant.

RESULTS

Histology of Human Lung Specimens

The alveolar structure in the non-emphysematous areas was generally preserved when compared to the alveolar structure of emphysematous areas. Enlarged alveoli were rarely seen in the non-emphysematous areas. In these areas, the integrity of pulmonary epithelium was preserved, and also there were no thinning or breaking of the pulmonary epithelium. The accumulation of macrophages, neutrophils and erythrocytes in the lumens of alveoli were commonly observed in the human lung without emphysema (Figure 1a). It was noteworthy the disrupted lung structure throughout the tissue in the emphysematous areas of human lung. In the emphysematous areas, larger alveoli were observed instead of the small normal-sized alveoli observed in the non-emphysematous areas. There were thinning and breaking in the walls of the enlarged alveoli (Figure 1b). Therefore, the pulmonary epithelial integrity could not be preserved in the human lungs with emphysema. The numbers of accumulated macrophages, neutrophils and erythrocytes in the alveolar lumens were high in the human lung with emphysema.

Elastic fibers were predominantly localized in the alveolar wall, pulmonary interstitial tissue, and the walls of pulmonary arteries and arterioles in the human lung. They were seen as a very curved and long fibers in the lung without emphysema (Figure 2a). There

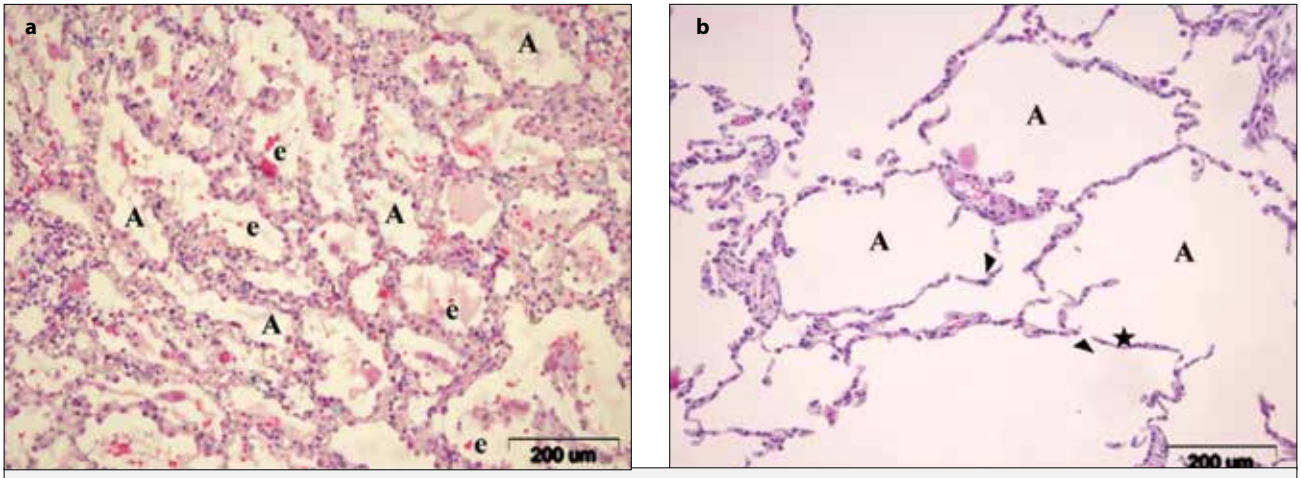

Figure 1. a, b. Histological section of human lung stained with hematoxylin-eosin. a) Lung sample without emphysema; (A) alveoli and (e) erythrocyte in the alveolar lumen; b) Lung sample with emphysema; (A) alveoli, (*) thinning of alveolar wall, and (▲) disruption of alveolar wall. Scale bars = 200 µm.

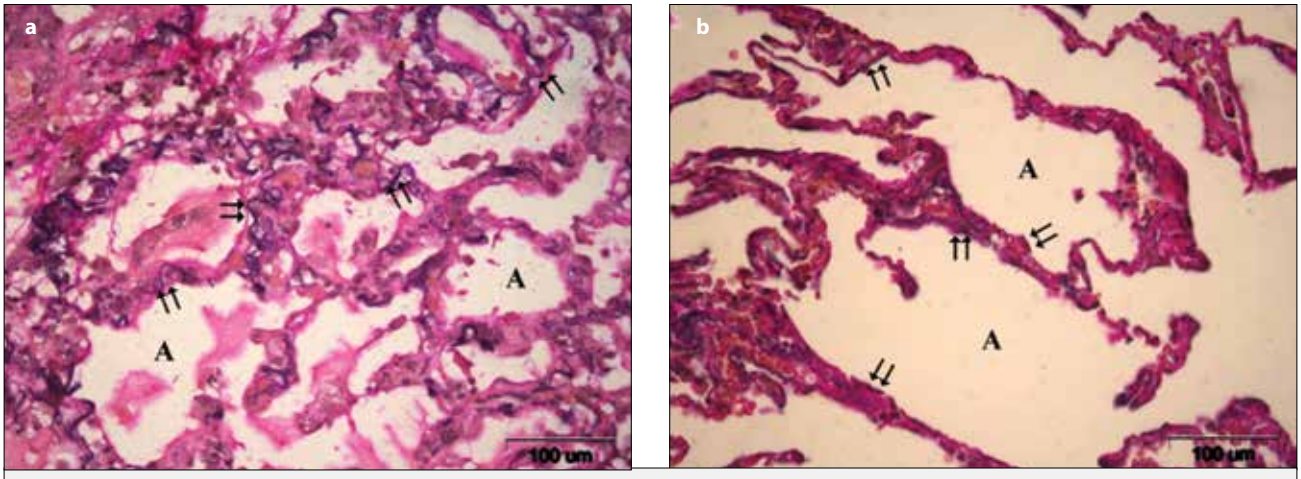

Figure 2. a, b. Elastic fibers of the human lung were seen as blue-black fibers stained with Verhoeff's Elastic Stain. They were more less and discontinuous than that of non-emphysematous areas. a) Lung sample without emphysema; b) Lung sample with emphysema. A: Alveoli, ⇉: Elastic fibers. Scale bars=100 µm.

was a significant decrease in the presence of elastic fibers in the alveolar wall of the emphysematous areas. The elastic fibers in these regions were much less pronounced, more flat and discontinuous compared to those of the non-emphysematous areas (Figure 2b).

Cell Proliferation and Repair of Alveolar Epithelium in Human Lungs with and without Emphysema

Ki67 immunoreactivity was observed in the nuclei of bronchial and pulmonary epithelial cells, and mesenchymal cells in the connective tissue. Ki67 immunoreactive cells were few in number in the non-emphysematous areas of human lung, and also their immunoreactivities were weak (Figure 3). However, there were more Ki67 immunoreactive cells in the emphysematous areas of the human lung. Especially, a lot of these cells in the pulmonary epithelium showed an intense Ki67 immunoreactivity (Figure 3).

ProSPC immunoreactivity was observed in the cytoplasm of some pulmonary epithelial cells in the emphysematous and non-emphysematous areas (Figure 4). A lot of proSPC immunoreactive epithelial cells, strongly expressed proSPC in the emphysematous areas (Figure 4).

The levels of AGER were high in the lungs without emphysema versus the lungs with emphysema [($p<0.01$) (Figure 5)].

Levels of E-Cadherin Protein and Anti-NEDD9 Immunoreactivity in Human Lungs with and Without Emphysema

The levels of E-cadherin were higher in the lungs without emphysema than in the lungs with emphysema [($p < 0.05$) (Figure 6)].

NEDD9 immunoreactivity was also observed in the cytoplasm and nuclei of the bronchial and pulmonary epithelial cells, and in the mesenchymal cells of the pulmonary interstitium. NEDD9 immunoreactive cells were rarely found in the non-emphysematous areas of human lung (Figure 7). However, a large number of NEDD9 immunoreactive cells were identified in the pulmo-

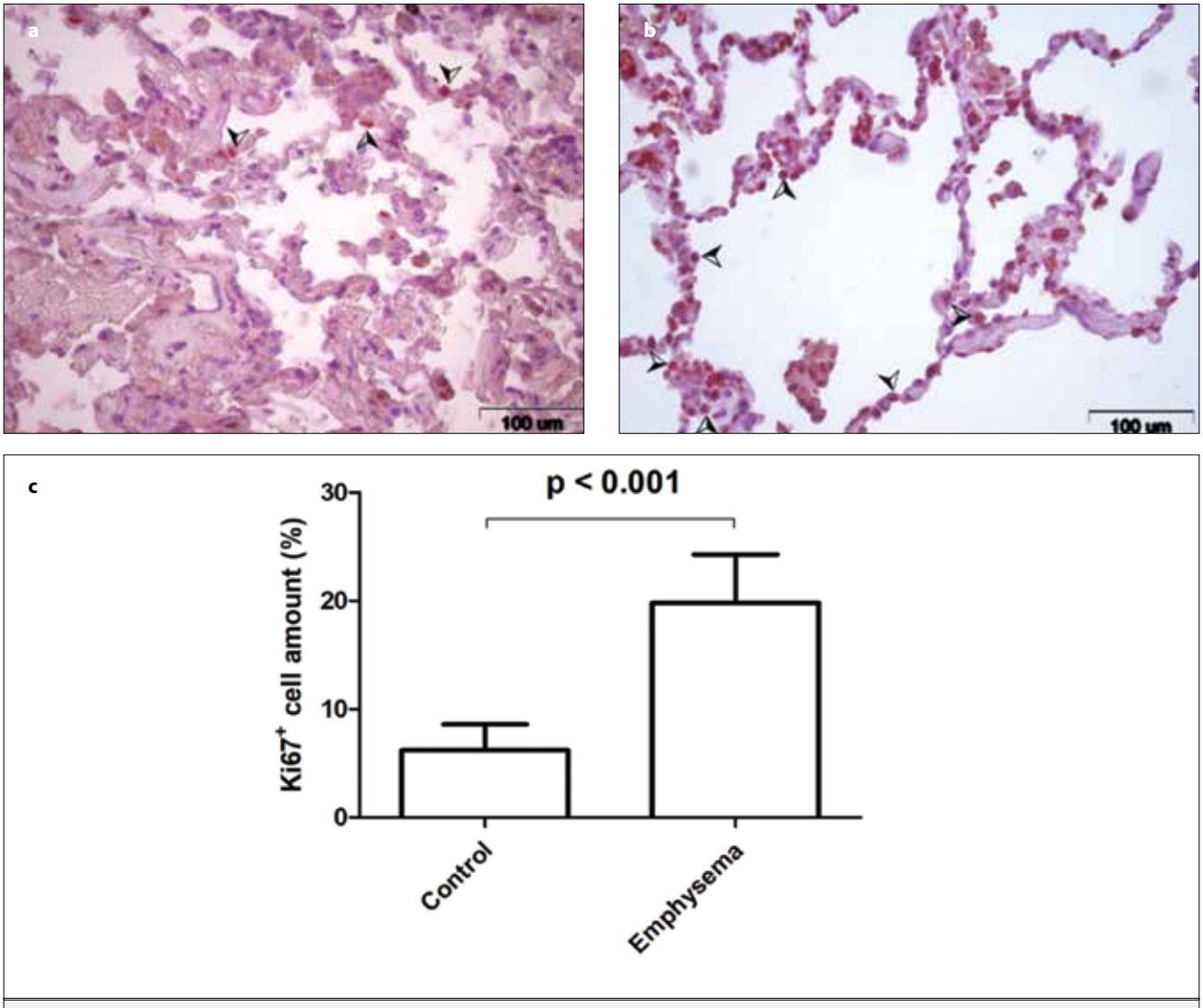

Figure 3. a-c. Ki67 immunoreactivity was marked by arrows in the alveolar epithelium of the non-emphysematous (a) and the emphysematous areas (b). Scale bars=50 µm. Mayer's hematoxylin counterstain. The number of proliferative cells (%) in the alveolar areas the non-emphysematous and the emphysematous regions was shown in "c".

nary epithelium of the emphysematous areas. The number of these cells was much higher than that of the non-emphysematous areas, and these cells were localized into groups at the corners of the alveoli in the pulmonary epithelium (Figure 7).

DISCUSSION

Age, gender, various respiratory diseases, smoking, various environmental factors and occupational exposures are found among the causes of pulmonary emphysema (12). In COPD patients, who are aged 45 to 60 and smokers, emphysematous areas are defined as the expansion of the airways and alveoli in areas ranging from distal airways to bronchioles and alveoli (13). In human and animal lungs exposed to cigarettes, emphysematous alterations, such as the enlarged alveoli, the thinned and occasionally disrupted alveolar epithelium, the inflammation in the alveolar spaces and pulmonary parenchyma, and the destruction of the connective tissue have been determined (14,15). In the present study, most of the individuals were male COPD patients, who were with medium age 48±16, smokers and diagnosed with lung adenocarcinoma. Emphysematous areas in lung biopsies taken from these patients were distinguished by the characteristic properties of emphysema mentioned above, such as dilated alveoli, the thinning and occlusion of the alveolar epithelium and infiltration of inflammatory cells, the decreases in amount of elastic fibers, and disorganization of their order.

Responsible mechanisms for the formation of enlarged alveoli in the pulmonary emphysema include the disintegration and disruption of the alveolar epithelium following the injury of alveolar epithelium. The inflammation resulting from cigarette smoke exposure, subsequently, the released agents from the inflammatory cells, and the protease-antiprotease imbalance in

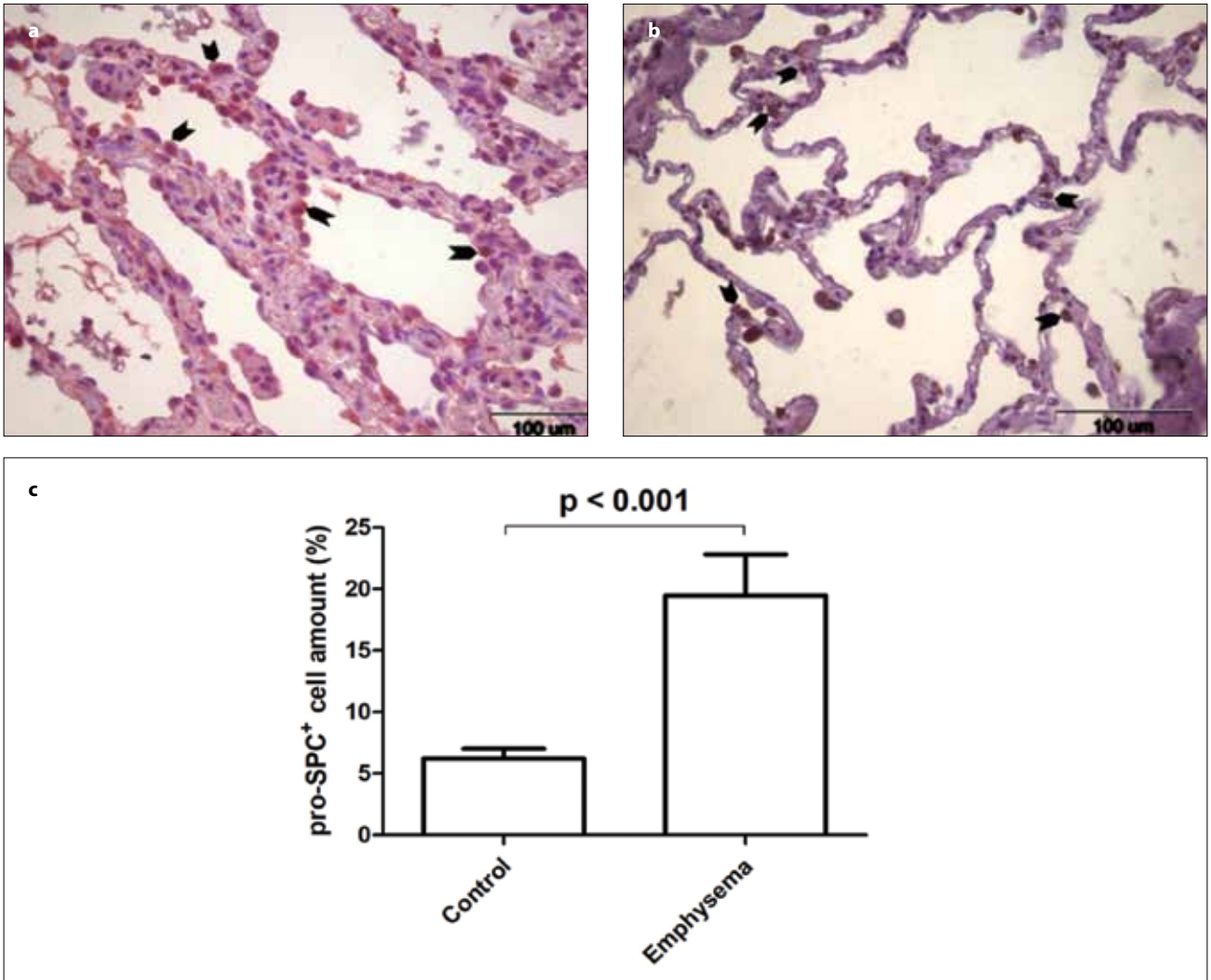

Figure 4. a-c. ProSPC immunoreactivity was marked by arrows in the alveolar epithelium of the non-emphysematous (a) and emphysematous areas (b). Scale bars = 50 µm. Mayer's hematoxylin counterstain. The number of proSPC immunoreactive cells (%) in the alveolar areas the non-emphysematous and the emphysematous regions was shown in "c".

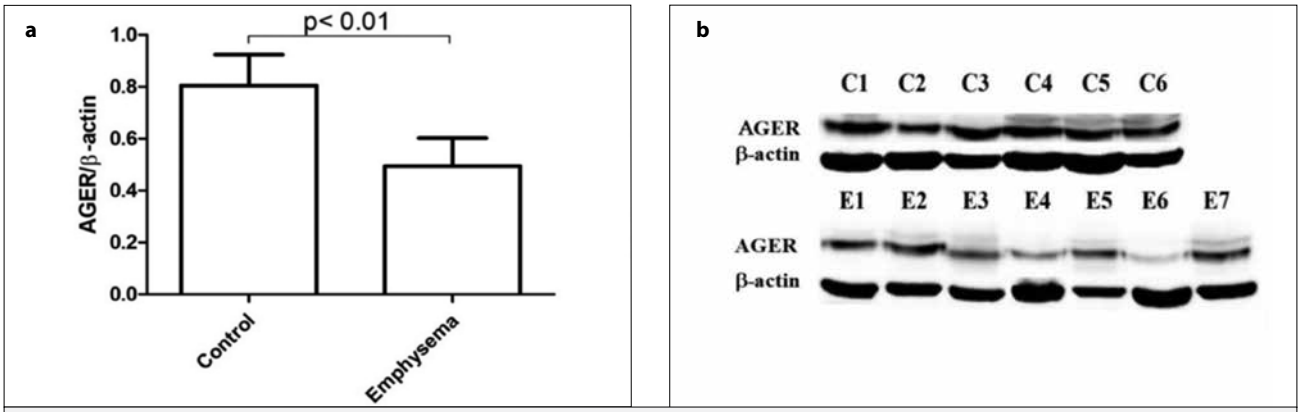

Figure 5. a, b. Alterations of AGER protein levels in non-emphysematous and emphysematous regions areas of lung of COPD patients. a) intensity analysis of the protein bands and b) AGER protein levels in the emphysematous lung samples (E) compared to the non-emphysematous areas (control group, C).

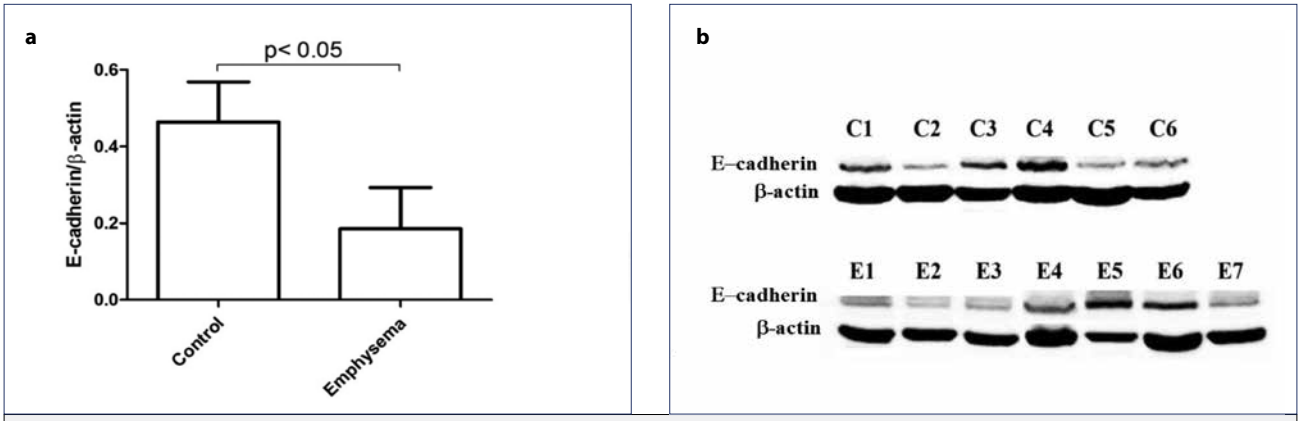

Figure 6. a, b. Alterations of E-cadherin protein levels in non-emphysematous and emphysematous regions areas of lung of COPD patients. a) intensity analysis of the protein bands and b) E-cadherin protein levels in the emphysematous areas (E) compared to the non-emphysematous areas (control group, C).

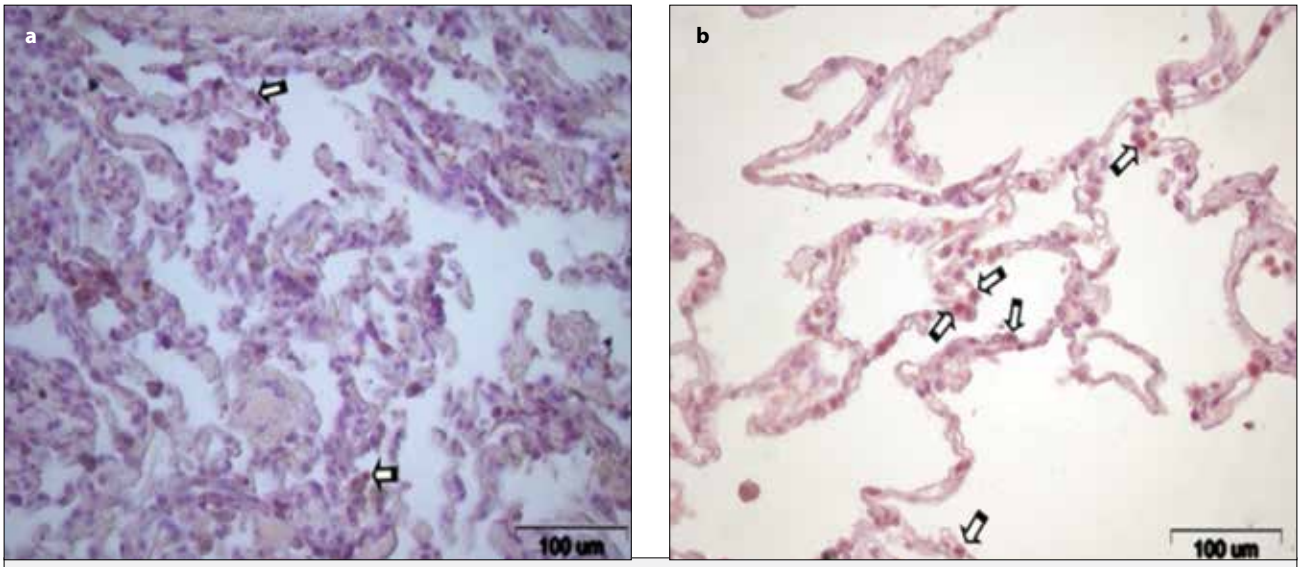

Figure 7. a, b. NEDD9 immunoreactivity was marked by arrows in the alveolar epithelium of the non-emphysematous (a) and emphysematous areas (b). Scale bars = 50 µm. Mayer's hematoxylin counterstain.

the lungs lead to the damage of the alveolar epithelium (2,6). The cell death in the alveolar epithelium cells has been shown in the experimental studies where pulmonary emphysema was induced by several substances such as cigarette smoke, elastase, and papain (7,14,16). It has been suggested that alveolar epithelial damage occurs irreversibly and that the repair of alveolar epithelium cannot be done in the pulmonary emphysema (7). In the present study, we found the disruption of alveolar epithelial integrity and the break of alveolar walls in the pulmonary emphysematous areas of COPD patients. The epithelial cells, which are firmly attached to each other by the E-cadherin molecules, provide the integrity and continuity of the epithelial layer. Type 1 and 2 pneumocytes cover the alveolar surface along the alveolar epithelium in the healthy human lung. Pneumocytes are attached to each other by a variety of tight and adherent cell connections. In a healthy human lung, these cell-cell connections are effective on the preservation and integration of the alveolar epithelium, and in the construction of epithelial barrier function between the alveolar lumen and lung parenchyma (3). It has been reported that cigarettes reduce the expression of genes that encode cell-cell junction proteins, including zonula occludens-1 and E-cadherin and induces structural changes that disrupt the epithelial barriers in the mouse lungs (17). Oldenburger and coworkers (9) found that the amount of E-cadherin protein decreased in the lungs of COPD patients compared to control individuals. The study has also shown that reduction in the amount of E-cadherin protein resulted in the decrease of epithelial barrier functions in human bronchial epithelial cells exposed to cigarette extracts. The increased airway and alveolar epithelial permeability, and the stimulated inflammatory response are accepted as an important risk factors for COPD. The increases in the epithelial permeability and neutrophil infiltrations following cigarette smoke exposure were detected in A549 cells (18). Neutrophil numbers and elastase levels were also increased

in bronchoalveolar lavage fluids collected from COPD patients and residues of digested E-cadherin proteins were found in these fluids (19). Existing reports in the literature suggest that neutrophil-derived elastase may cause the destruction of E-cadherin in human lungs. In the present study, we also found that the levels of E-cadherin protein were reduced and that a large number of neutrophil cells were present in emphysematous areas. Thus, elastases released from these cells may have resulted in the destruction of the E-cadherin. On the other hand, one of the responsible molecules for the intracellular destruction of the E-cadherin protein is NEDD9 protein. Weak expression of NEDD9 in non-neoplastic lung specimens and increased expression of NEDD9 in lung adenocarcinoma cases have been previously reported (20). NEDD9 promotes metastasis in cancer cells by the inhibition of cell adhesion (21). Changes in the protein expressions of NEDD9 family reduced the expression of α-catenin, β-catenin, p120 catenin, and the localization and stabilization of E-cadherin in the cell membrane (22). NEDD9 is an effective protein in transporting E-cadherin to lysosomes and it regulates the lysosomal digestion of the E-cadherin (22). In the present study, NEDD9 immunoreactivity was observed rarely in the alveolar epithelium in non-emphysematous areas, whereas a significant increase in the number of NEDD9 immunoreactive cells was observed in the alveolar epithelium of the emphysematous areas. Therefore, intracellular digestion of E-cadherin induced by NEDD9 may be effective in reducing the amount of E-cadherin proteins in the emphysematous areas.

Our data suggest that the E-cadherin protein contributes to the pathogenesis of pulmonary emphysema via several ways. The first and second ways are the reduced E-cadherin-mediated inflammatory response, which appears due to the increase in the epithelial permeability and the disintegration of the epithelium, which appears due to inability to establish cell-cell connections, respectively. The third way is the successfully uncompleted epithelial repair due to a decreased E-cadherin protein levels. In a damaged tissue, if there are reserve cells or if the cells are capable of proliferation, they first proliferate, and then contribute to the complementation of the tissue repair by differentiating into many specialized types of *cells*. The cumulative data show a relationship between E-cadherin and regulation of cell proliferation. The cytoplasmic portion of the E-cadherin molecule binds to the cytoplasmic β-catenin via mediating proteins. These connection allows the stabilization and localization of E-cadherin molecule in the cell membrane. The disappearing of E-cadherin-β-catenin connection causes the release and transfer of β-catenin from cytoplasm to nucleus, with the subsequent transcription of genes involved in cell proliferation (23). Our findings demonstrate that the alveolar epithelium is damaged, and the alveolar wall is disrupted somewhere. The microscopic data of the present study show that a repair mechanism engages in fact primarily in the alveolar epithelium of the emphysematous areas. We detected the increased number of type 2 pneumocytes and proliferative cells (Ki67 immunoreactive cells) in response to alveolar epithelial damage in the emphysematous areas characterized by reduced E-cadherin levels. The proliferative type 2 pneumocytes differentiate into type 1 and type 2 pneumocytes in the correct repair of the alveolar epithelium (24). Thus, alveolar epithelial repair is completed. However, in the present study, the amounts of AGER and E-cadherin proteins were decreased in the emphysematous areas while the number of type 2 cells was increased. The data suggests that type 2 pneumocytes could not differentiate into type 1 pneumocytes, although the reduced E-cadherin-induced cell proliferation leads to an increased number of type 2 pneumocytes. That might suggest that the epithelial repair has not been completed. Nagaoka and coworkers (25) identified the decrease of E-cadherin levels by Western Blotting in an epithelial cell line transfected with anti-miR-200a, a gene involved in the epithelial cell differentiation and polarization. They noted that the E-cadherin molecule is effective molecule for the epithelial cell differentiation and polarization. Our data suggest that the reduction at the E-cadherin protein levels in the emphysematous areas of the lungs of COPD patients may contribute to the pathogenesis of pulmonary emphysema by causing delays in the differentiation of alveolar epithelial cells in these regions. This situation explains why pulmonary emphysema is characterized by an irreversible loss of alveolar structure.

Pulmonary emphysema is seen in 20% of COPD patients. The loss of transport of respiratory gases due to damage and loss of pulmonary epithelium is shown among the causes of death in COPD patients. The cumulative data have been suggested that pulmonary epithelial damage occurs irreversibly and that pulmonary epithelial repair cannot be performed in the pulmonary emphysema (7). The resolve of the underlying mechanisms related to both pulmonary epithelial injury and the deterioration of its repair in pulmonary emphysema is required for the elucidation of the pathogenesis of pulmonary emphysema. The achievement of proliferation and differentiation of pulmonary epithelial cells in addition to the ensure of the repair and integrity of pulmonary epithelium have been suggested to be an effective approach in improving the pathogenesis of emphysema (26). The data of the present study demonstrate that reductions in protein level of E-cadherin can contribute to the pathogenesis of pulmonary emphysema, leading to emphysematous changes in the alveolar areas of the lung. Approaches to alleviate the increase of E-cadherin protein synthesis or the decrease of its digestion might be utilized in order to reduce the epithelial permeability, preservation of the epithelial integrity, and completion of the epithelial repair in the alveolar areas for the prevention of pulmonary emphysema pathogenesis.

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RESEARCH ARTICLE

Eur J Biol 2017; 76(2): 69-73

Determination of the Distribution of the rs2069514 and rs762551 Alleles of the Cyp1a2 Gene Related to Caffeine Metabolism in Professional Athletes

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ABSTRACT

The objective of this study is to investigate allele distributions of the rs2069514 and rs762551 polymorphisms of the CYP1A2 gene which affect caffeine metabolism in long-distance and short-distance runners in the Turkish population. 20 professional athletes in total were included in the study within the age range of 18-24, 10 of whom were long-distance (female, n=5; Male, n=5) and 10 of whom were short-distance (Female, n=5; Male, n=5) runners. DNA isolation from the athletes was performed from peripheral blood samples and genotyping was determined using the technique of Real Time-PCR. According to the obtained data, the ratio of individuals having AA (n=6), GG (n=9) and AG (n=5) genotype for the region of rs2069514 was found as 30%, 45% and 25% respectively. On the other hand, the ratio for AA (n=4), CC (n=9) and AC (n=7) genotypes in the region of rs762551 allele was determined as 20%, 45% and 35%. According to the results, no significant variance regarding gender was found for either of the different polymorphisms under study in long-distance and short-distance runners while the genotype distributions varied between short and long-distance runners only in the rs2069514 polymorphism. The GG genotype was observed in 9 short-distance runners while it was observed in none of the long-distance runners. As a result, the fact that the GG genotype in case of the Rs2069514 polymorphism was observed in nine of the ten short-distance runners suggests that the athletes in this group metabolize caffeine slowly and this should be considered in the caffeine intake.

Keywords: CYP1A2, caffeine, short-distance runner, long-distance runner, rs2069514, rs762551

INTRODUCTION

Caffeine ($C_8H_{10}N_4O_2$) is a chemical compound aiding (ergogenic) performance improvement and useful in hastening the response time of athletes and improving their endurance during long workout periods (1,2). It is believed that it acts by blocking adenosine receptors through the central nervous system in the body and increasing neural stimulability in the brain (2). Caffeine increases cyclic AMP (cAMP) due to its inhibiting effect on phosphodiesterase enzyme activity and thus, it increases protein phosphorylation (3). Caffeine inhibits carbohydrate use by encouraging the use of free fat acids, which increase during workouts delaying the depletion of muscle glycogen, as fuel and also improves the fat combustion efficiency of the body (1,4). Excessive caffeine consumption may cause negative effects such as dehydration, vitamin and mineral deficiencies (1).

Having a structure similar to adenosine, caffeine can compete with adenosine in the receptor regions (5). The rate of caffeine metabolism varies between individuals depending on genetic variations (5,6). Caffeine resembles DNA and RNA bases due to its purine structure (1). Caffeine is metabolized by the CYP1A2 gene which encodes the Cytochrome P450 (CYP) enzyme (EC 1.14.14.1), an enzyme from the oxidoreductase class (7,8).

Any polymorphisms were identified on the CYP1A2 gene and it has been reported that certain gene polymorphisms have an effect on caffeine metabolism.

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The presence of the rs2069514 (-3860G>A) polymorphism on the CYP1A2 gene's 1st intron is associated with the gene's low expression rate and is believed to metabolize caffeine more slowly. On the other hand, the rs762551 (-163C> A) polymorphism on the same gene is associated with high enzyme activity (9,10). Thus, the stimulating effects will vary depending on the body's ability to metabolize caffeine. The same amount of caffeine intake causes a stronger stimulating effect on slow metabolizers compared to rapid metabolizers (6,9Pickering, 2017; Sachse, 1999)·

The objective of this study is to investigate the distribution of the polymorphisms (rs2069514 and rs762551) of the CYP1A2 gene metabolizing caffeine, whose ergogenic effect has been shown in athletes, in both long and short distance runners.

MATERIALS AND METHODS

Subjects

20 professional athletes in total, 10 of whom were long-distance (female, n=5; Male, n=5) and 10 of whom were short-distance (Female, n=5; Male, n=5) runners, were included in the study within the age range of 18-24. The group under study consisted of the licensed athletes studying at the Physical Education and Sports Academy at Gelisim University and Istanbul University's Sports Sciences Faculty and signed forms stating they were voluntarily participants in the study. The study protocol was prepared according to the Human Rights Helsinki-II declaration and the study started after the issuance of the ethical committee approval from the Presidency of Istanbul Medipol University non-Entrepreneurial Studies Ethical Committee (Date: 10.03.2017; Number: 10840098-604.1.01-E.6793).

Genotype Analysis

DNA isolation was performed from peripheral blood samples collected from the athletes (Thermofisher Invitrogen (USA) ref no: K1820-02). The Real Time PCR technique was used in Rs2069514 genotyping (Thermofisher TaqMan, ref no: C-15859191-30) and rs762551 (Thermofisher TaqMan, ref no: C-8881221-40).

A 20 µl mixture of 2µL distilled water, 14 µL Taqman Universal Master Mix kit and 4 µL template DNA, in an 1.5 mL Eppendorf tube was used in reproducing the CYP1A2 rs762551 and rs2069514 allele regions. 35 cycles were performed during Real Time-PCR and the pre-denaturation process was performed for 5 minutes at 95 °C. It was followed by the denaturation process at 95 °C for 10 seconds to ensure that the DNA strands separated from each other. The reading was performed in 60 seconds by bringing the temperature to 60 °C. FAM and HEX probes in a Termofisher TaqMan Universal Master Mix were used to determine genotype distributions of the athletes. For the rs2069514 allele region, FAM luminescence identified the 'G' genotype while HEX luminescence identified the 'A' genotype. On the other hand, for the 762551 allele region, FAM luminescence identified the 'C' genotype and HEX luminescence identified the 'A' genotype.

Statistical Analysis

The Statistical Package for the Social Sciences version 20.00 (SPSS Inc.; Chicago, IL, USA) program was used in the statistical analyses conducted in our study. The Chi-square (χ^2), Fisher's Exact test was used in comparing genotypes of the short distance and long distance runners. The significance value was assumed as $p<0.005$ in the conducted analyses.

RESULTS

Table 1 summarizes the rs2069514 and rs762551 polymorphism results and their effects on caffeine metabolism. In our study cohort, rs2069514 polymorphism AA, AG, GG genotypes were found as 6, 5, 9 respectively. Allele frequencies in this polymorphism were determined as A allele number 17 (42.5%) and G allele number 23 (57.5%). For the rs762551 polymorphism, the number of individuals with AA, AC, CC genotypes were found to be 4, 7 and 9 respectively. For Allele numbers, the A allele number was 15 (37.5%) while the C Allele number was 25 (62.5%) (Table 2).

Table 3 shows the genotype distributions of the rs2069514 and rs762551 polymorphisms among short and long- distance runners. In the rs2069514 polymorphism, 1 individual was an A-allele carrier while 9 individuals showed the GG genotype. In the case of the rs762551 polymorphism, 3 individuals had the AA genotype while the remaining 7 individuals were C-allele carriers (CC and AC). In the case of long-distance runners, for the rs2069514 polymorphism, all 10 of the individuals were A-allele carriers. For the rs762551 polymorphism, 1 individual had the AA genotype while the remaining 9 individuals, were found to be C- allele carriers.

According to the obtained results, significant variances were observed only in the rs2069514 polymorphism among short and long-distance runners with respect to the genotype distributions of the CYP1A2 gene's rs2069514 and rs762551 polymorphisms in the athletes ($p<0.005$) while no significant variance was observed in terms of gender in either of the polymorphisms.

DISCUSSION

CYP1A2 enzyme activity, which is associated with performance enhancing effects in athletes, may vary between individuals. Thus, in our study, CYP1A2 rs2069514 and rs762551 polymorphisms, which have an effect on caffeine metabolism were investigated. In the present study, it was observed that 7 of the short-distance runners carried alleles metabolizing caffeine slowly while the remaining 3 individuals had the genotype related to the fast metabolism of caffeine. On the other hand, among the long-distance runners, the slow metabolism genotype was determined in 9 individuals while the compound genotype was observed in 1 individual.

It was shown that people with the AA genotype in the rs762551 polymorphism on the CYP1A2 gene region have higher CYP1A2 enzyme activity and may experience faster caffeine metabolites' accumulation (11,12). To make a comparison with the literature easier and to obtain statistically accurate results, the athletes' genotypes were combined as C-allele carriers in the

Table 1. Athletes' CYP1A2 genotype, branch and caffeine metabolism rates

Athletes	rs2069514	rs762551	Branch	Caffeine metabolism rate (rs2069514)	Caffeine metabolism rate (rs762551)
1	GG	AA	Short distance	Normal	Rapid
2	GG	AC	Short distance	Normal	Slow
3	GG	AC	Short distance	Normal	Slow
4	GG	AA	Short distance	Normal	Rapid
5	AG	AC	Short distance	Slow	Normal
6	GG	AC	Short distance	Normal	Slow
7	GG	AC	Short distance	Normal	Slow
8	GG	AC	Short distance	Normal	Slow
9	GG	AC	Short distance	Normal	Slow
10	GG	AA	Short distance	Normal	Rapid
11	AA	AA	Long distance	Slow	Rapid
12	AG	CC	Long distance	Slow	Slow
13	AG	CC	Long distance	Slow	Slow
14	AG	CC	Long distance	Slow	Slow
15	AA	CC	Long distance	Slow	Slow
16	AA	CC	Long distance	Slow	Slow
17	AG	CC	Long distance	Slow	Slow
18	AA	CC	Long distance	Slow	Slow
19	AA	CC	Long distance	Slow	Slow
20	AA	CC	Long distance	Slow	Slow

Table 2. Distribution of the CYP1A2 gene's rs2069514 and rs762551 polymorphisms among the athletes

		Genotype			Allele Frequency	
		AA	**AG**	**GG**	**A**	**G**
CYP1A2 rs2069514 (n=20)	n	6	5	9	17	23
	%	30	25	45	42.5	57.5
		AA	**AC**	**CC**	**A**	**C**
CYP1A2 rs762551 (n=20)	n	4	7	9	15	25
	%	20	35	45	37.5	62.5

CYP1A2 gene rs762551 polymorphism (AC+CC) and A-allele carriers (AG+AA) in the rs2069514 polymorphism before being analyzed.

As no study related to the rs2069514 polymorphism in distance runners was encountered in the literature, the discussion was focused on the rs762551 polymorphism. More studies should be conducted especially related to the rs2069514 allele region. In one study, the effect of the CYP1A2 gene rs762551 polymorphism on caffeine consumption habits and performance was investigated in college distance runners (13).

Table 3. Short and long-distance runners' genotype distributions in rs2069514 and rs762551 polymorphisms

		Short-distance (n=10)		Long-distance (n=10)		
Polymorphism	**Genotype**	N	%	n	%	**P**
CYP1A2 rs2069514	GG	9	45	-	-	p<0.005
	A allele carriers (AA+AG)	1	5	10	50	
CYP1A2 rs762551	AA	3	15	1	5	0.582
	C allele carriers (CC+AC)	7	35	9	45	

In our study, the rs2069514 polymorphism A-allele carriers was found to be higher in number than the GG genotype expressing normal distribution. In this polymorphism, short-distance runners showed normal distribution while, on the contrary, long-distance runners showed this allele region's variants. Thus, it was concluded that the long-distance runners in our study group may metabolize caffeine more slowly in general.

In the Rs762551 polymorphism, C-allele carriers were found to be higher in short and long-distance runners (Table 3). Similar to our study, in the rs762551 polymorphism, there were more C-allele carriers among professional tennis players (14), recreational trained athletes (5), trained cyclists (15,16) and healthy active individuals (17). In cyclists (18,19) and in a study on soccer players' sprint performance (20), AA genotype and C-allele carriers' number showed an equal distribution.

According to the results from a general literature search, the number of C-allele carriers is higher in the rs762551 polymorphism (14-16). However, although we were not able to conduct a comparison with similar studies in the sports branches under consideration, there are similarities in the literature in general. In other studies, in a comparison of the data related to the rs762551 polymorphism in endurance athletes, the results from the long-distance runners of our study group have strong similarities with the genotype data from the study of Soutward (2016) on athletes and the results of the study of Salinero et al. (17).

In contrast to our study, in certain studies on groups of professional or amateur sportsman, the number of individuals having the AA genotype is higher than that of C-allele carriers in the rs762551 allele region (11,21,22). The reason may be a variation in the ethnic origin of the athletes or a variation in the sports branches.

Although it has been reported in the literature that individuals with the AA genotype showed improved performance with caffeine intake (11,15), they showed that the athletes with the AC genotype obtained a greater improvement in performance compared to those with the AA genotype.

Certain studies showed that caffeine had the most ergogenic effect in endurance athletes and it has a positive effect on muscle endurance (5,23). According to the results produced in this study, the observation of the polymorphisms, which metabolize caffeine slowly, in the long-distance runners, shows that high dose caffeine intake is not meaningful and caffeine may be consumed a much longer time before workouts. Based on this information, it is believed that, especially in endurance sports, a high dose of caffeine support given to athletes may cause a decrease in performance and dehydration due to water loss in the body (5,6).

In the short-distance runners group, although there were fast caffeine metabolizers, more slow caffeine metabolizers were observed in general. In short-distance runners, there is very little acid combustion because most of the energy is produced by creatine phosphate and anaerobic respiration. However, the increase in adrenalin release with caffeine causes performance improvement in this branch in which reaction time is crucial. The International Olympic Committee reported that dehydrated caffeine support at low-medium doses (3-6 $mg.kg^{-1}$) improved sports performance in trained athletes; however, no additional advantages were observed when it was consumed at high doses (≥9 $mg.kg^{-1}$) (24). Accordingly, the rate of caffeine metabolism in the body is crucial. Therefore, more genetic-based studies are needed on the upper dose limits.

Restrictions of our study are the unknown exact running degrees of the athletes, the low number of individuals and the fact that different mutations or polymorphisms on the CYP1A2 gene region, which may be associated with caffeine metabolism, could not be measured. Furthermore, a comparison of genotype statuses could not be carried out because the data on the athletes caffeine consumption habits could not be taken and caffeine could not be introduced to the athletes.

As a result, because there is no data available in literature about the rs2069514 polymorphism, a comparison could not be made in this study with respect to genotype distribution. In the case of the Rs762551 polymorphism, our study has similarities with other studies with respect to genotype distribution. According to our findings, it is preferable to keep caffeine dose support at lower limits in both long and short-distance runners because it increases the number of slow caffeine metabolizing polymorphisms; however, further studies including caffeine introduction should be conducted to find the exact amount. This study is the first study in which the relation between the CYP1A2 gene and caffeine was studied in Turkish athletes. Thus, we believe that our study will contribute to the genetic information pool and will support other studies conducted in this area.

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RESEARCH ARTICLE

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Chemical Analysis and Antimicrobial Effect of Propolis from Hakkari Province of Turkey against Some Pathogenic Microorganisms

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ABSTRACT

This study was performed to investigate the antimicrobial effect of propolis from Hakkari province of Turkey and its chemical content by Gas Chromatography–Mass Spectroscopy. *In vitro* inhibitory activity of propolis was studied by the disc diffusion method against six gram positive, three gram negative bacteria and one yeast like fungi. As a result of chemical analysis, the total flavonoid ratio of the propolis sample was found to be higher than the other compound groups. Pinostrobin chalcone, pinocembrin and chrysin were identified as major flavonoids. Also, all microorganisms tested were susceptible to the propolis extract except for *Klebsiella pneumoniae*. Minimum inhibition concentration (MIC) values were determined by microbroth dilution assay. MIC values against microorganisms ranged from 25 to 200 µg/mL. Antimicrobial susceptibility test results showed that inhibitory effect our propolis sample was somewhat weaker than ampicillin, but it had a broader spectrum.

Keywords: Ampicillin, antimicrobial effect, minimum inhibition concentration (MIC), propolis content

INTRODUCTION

Natural products present a large variety of biological and pharmacological activities and are considered to have beneficial effects in human nutrition (1). In recent years focus on natural products and alternative medicines has renewed interest in bee products such as honey, royal jelly, pollen, and propolis (2).

Propolis is a natural resinous complex collected from different plants by bees. Due to its healing properties, propolis has been used in traditional medicine as an antiseptic, wound healer and therapeutic substance from ancient times to the present (3). Existing studies suggest that propolis, when used as a nutritional supplement, is very important in protecting human health due to its biochemical and biopharmaceutical substances (4). Chemical substances in propolis are generally waxes, resins, balms, aromatic oils, pollen, flavonoids, terpenoids and other organic substances (5). Both the biological activity of propolis as antibacterial, antiviral, antioxidative, antifungal and antiatherogenic, antiproliferative, proapoptotic, antienflamatuar, cytotoxic, and the presence and proportion of bioactive substances in it varies by the phytogeographic properties of the area in which the samples are collected (6-9).

Along with the rising interest in natural products, propolis has been a promising source for discovering new drugs (10). For this reason, propolis has been extensively studied especially due to its chemical structure and biological properties in recent years (11,12). The aim of this study was to investigate the antimicrobial effect of propolis from Hakkari province of Turkey and its chemical composition.

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MATERIALS AND METHODS

Extraction of Propolis

The propolis sample was gathered from Hakkari province of Turkey. The sample was frozen in the freezer (-18 ºC) and then disintegrated with a grinder and 30 g of the powdered propolis sample was dissolved in 90 ml of 96% ethanol. This mixture was incubated for two weeks at 30 °C in a tightly closed dark colored bottle. After two weeks, the supernatant was filtered twice with Whatman No. 4 and No.1 filter papers, respectively. The final solution was diluted in 1:10 ratio (w/v) with ethanol (96%). A portion of this final solution was evaporated to obtain completely dry sample. About 5 mg of dry substance was mixed with 75 µl of dry pyridine and 50 µl bis (trimethylsilyl) trifluoroacetamide heated at 80 °C for 20 min and then supernatant was analyzed by gas chromatography–mass ppectroscopy (GC-MS) (13).

GC-MS Analysis

The gas GC-MS analyses conducted at the Environment and Instrumental Laboratory of Istanbul University using an Agilent brand GC (model 7890A) and MS (model 5975C) equipped with a mass selection detector. The GC was equipped with a (5%-phenyl)-methyl polysiloxane DB-5MS column (30 m length × 0.25 mm i.d. × 0.25 µm df) and an Agilent automatic injection system. The chromatogram was produced by holding the oven temperature at 35 °C for 8 min initially and then increasing the temperature to 60 °C at a rate of 6 °C/min followed by an increase at a rate of 4 °C/min to 160 °C and 20 °C/min to 200°C/min and kept at 200°C for 1 min at which it was held for 1 min (14). Helium was used as the carrier gas at a flow rate of 0.7 mL/min. Split ratio 1:80, injector temperature 280 °C, ionization voltage 70 eV. Identification of components in propolis extract was carried out with the WILEY-NIST MS data library.

Test Microorganisms

In this study, six gram positive bacteria (*Staphylococcus aureus* NCTC 10788, *Staphylococcus epidermidis, Corynebacterium diphtheria, Enterococcus faecalis* NCTC 12697, *Bacillus cereus* ATCC 10876, *Bacillus subtilis)*; three gram negative bacteria (*Escherichia coli* NCTC 9001, *Klebsiella pneumonia, Pseudomonas aeruginosa* NCTC 12924) and one yeast like fungi (*Candida albicans* ATCC 10231) were used for determination of antimicrobial activity of propolis. All microorganisms were provided by the Department of Medical Services and Techniques, Vocational School of Health Services, Bayburt University.

Bacterial strains were cultured overnight at 37 ºC in the trypticase soy broth (Oxoid) and *Candida albicans* ATCC 10231 were cultured overnight at 37 ºC in the Sabouraud liquid medium (SDB, Oxoid). Suspensions were adjusted to 0.5 McFarland standard turbidity and used as inoculum (15).

Screening for Antimicrobial Activity

The *in vitro* inhibitory activity of propolis was investigated by the disc diffusion method. The inhibitory activity of the propolis was detected as a clear zone around the discs. The antimicrobial screening was performed using Mueller-Hinton Agar, (MHA, Oxoid) supplemented with 5% defibrinated sheep blood for bacteria and Sabouraud Dextrose Agar (SDA, Oxoid) for yeast (16). Propolis solutions were impregnated on antimicrobial susceptibility discs of 6 mm diameter (20 µL per disc) and discs were left to dry four hours (15). Clear zones around the discs were measured after 24 h of incubation at 37 ºC for bacteria and 48 h for *Candida albicans* at 25 ºC. The susceptibility of the microorganisms was also tested with commercial discs of ampicillin (10 µg-Oxoid) as a positive control and the %80 ethanol solution as a negative control. All tests were performed in duplicate.

Determination of Minimum Inhibitory Concentrations

Minimum inhibitory concentrations (MIC) have been determined by microbroth dilution method using 96-well microplates. At this stage, the ethanol-free propolis extract was dissolved with 10% dimethylsulfoxide (DMSO) and the concentration was adjusted to 400 µg/µL.

Initially, all wells were filled with 95 µL liquid medium [Mueller-Hinton Broth (Oxoid) for bacteria and Sabouraud Dextrose Broth (Oxoid) for yeast] and 5 µL inoculum. Then, 100 µl DMSO extracted propolis sample (400 µg/mL) was added to the first well. Afterwards, half of the liquid medium-extract mixture in the first well was transferred to the second well and this process was repeated up to 7th well. Thus, the 200 µg/µL starting concentration of propolis sample was diluted in half at each step.

MIC was defined as the lowest concentration which provides complete inhibition on the microbial growth after incubation. MIC values for propolis against the tested strains were determined from 12.5 to 200 µg/mL (17). All tests were performed in duplicates.

RESULTS

Chemical Composition of Propolis

In the present study, the chemical content of a propolis sample obtained from Hakkari province was determined with GC-MS. The ratio of individual compounds varied in the propolis sample (Table 1). Consequently, we found that the sample was rich in hydrocarbons (3.06%), aliphatic acids and their esters (10.49%), cinnamic acids and their esters (0.57%), flavonoids (23.83%), alcohols and terpenes (3.19%), aromatic acids (0.44%) and ketones (1.22%).

Heneicosane, nonadecane, pentacosane, cyclohexadecane, tricosene, docosane, eicosane compounds from hydrocarbons; palmitic acid, ethyl oleate, octadecanoic acid, decanedioic acid compounds from aliphatic acids and their esters; cinnamic acid, ferulic acid from cinnamic acids and their esters; pinostrobin chalcone, pinocembrin, chrysin compounds from flavonoids; β-eudesmol, guaiol, 2-methoxy-4-vinylphenol, l-limonene, α-muurolene, farnesol, γ-terpinene, β-myrcene, α-pinene compounds from alcohols and terpenes; benzoic acid, propanoic acid, 3,4-dimethoxycinnamic acid compounds from aromatic acids; 2-nonadeca-

none, 2-pentadecanone compounds from ketones were determined in the propolis sample by GC-MS. The ratio of these individual compounds varied in propolis sample. Among them, pinostrobin chalcone, pinocembrin, chrysin and ethyl oleate were detected at high concentrations; 8.85%, 9.16%, 5.82%, 8.15%, respectively.

Antimicrobial Activity and Minimum Inhibition Concentration

The antimicrobial activity of propolis were tested against six gram positive (*Bacillus cereus, Bacillus subtilis, Corynebacterium diphtheriae, Enterococcus faecalis, Staphylococcus aureus, Staphylococcus epidermidis*), three gram negative bacterial strains (*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa*) and one yeast like fungi (*Candida albicans*). The disc diffusion method was used to determine the antimicrobial activity. The diameters of inhibition zones and minimum inhibition concentrations (MICs) results are illustrated in Table 2. The minimum inhibitory concentration against microorganisms ranged from 25 to 200 µg/mL.

DISCUSSION

Physical appearance and chemical composition of propolis differs depending on seasonal, geographical and botanical factors but it generally contains 50% resin and vegetable balsam, 30% wax, 10% essential and aromatic oils, 5% pollen and 5% various other substances. So far, more than 300 compounds, such as polyphenols, terpenoids, steroids, sugars and amino acids were detected in raw propolis (18). However, many studies have reported that the flavonoids present in the propolis are responsible for its biological activities (19-23).

Several studies have been carried out to investigate the chemical content of propolis collected from many different regions using different techniques (24-26). Congruent with existing studies (24), our data showed higher rates of flavonoids found in almost all propolis samples from many different geographical regions. Celemli (24) has reported compounds belong to alcohols, aldehydes, aliphatic acids and their esters, carboxylic acids and their esters, cinnamic acids and their esters, ethers, flavo-

Table 1. Chemical content of a propolis sample from Hakkari province of Turkey

Compounds	RT(min)	%TIC	Compounds	RT(min)	%TIC
Hydrocarbons			***Alcohols and Terpenes***		
Heneicosane	18.44	0.38	β-Eudesmol	17.09	1.17
Nonadecane	17.29	0.21	Guaiol	15.59	0.35
Pentacosane	20.35	0.33	2-Methoxy-4-vinylphenol	10.90	0.09
Cyclohexadecane	18.26	0.19	l-Limonene	6.53	0.11
Tricosene	19.30	0.3	α-muurolene	13.74	0.01
Docosane	19.41	0.58	Farnesol	14.62	0.64
Eicosane	17.32	1.07	γ-Terpinene	4.91	0.01
Aliphatic acids and their esters			β-Myrcene	10.25	0.52
Palmitic acid	17.90	1.15	α-Pınene	4.92	0.29
Ethyl Oleate	18.82	8.15	***Aromatic acids***		
Octadecanoic acid	18.93	0.71	Benzoic acid	9.22	0.24
Decanedioic acid	20.65	0.48	Propanoic acid	11.25	0.03
Cinnamic acids and their esters			3,4-Dimethoxycinnamic acid	17.64	0.17
Cinnamic acid	13.29	0.45	***Ketones***		
Ferulic acid	17.57	0.12	2-Nonadecanone	18.51	0.36
Flavonoids			2-Pentadecanone	18.55	0.86
Pinostrobin chalcone	20.24	8.85			
Pinocembrin	21.02	9.16			
Chrysin	22.61	5.82			

TIC: total ion current; RT: retention time

Table 2. Diameters of inhibition zones and minimum inhibition concentrations

	Diameters of inhibition zones (mm)		
Microorganisms	**Propolis**	**Ampicillin**	**MIC (µg/mL)**
Staphylococcus aureus NCTC 10788	18±3	23	25
Staphylococcus epidermidis	16±2	13	50
Corynebacterium diphtheriae	17±1	17	50
Enterococcus faecalis NCTC 12697	14±2	21	100
Bacillus cereus ATCC 10876	19±1	18	25
Bacillus subtilis	20±2	21	25
Escherichia coli NCTC 9001	10±2	-	200
Klebsiella pneumoniae	-	-	-
Pseudomonas aeruginosa NCTC 12924	9±2	9	200
Candida albicans ATCC 10231	13±1	-	100

MIC: minimum inhibition concentration

noids, hydrocarbons, ketones and terpenes in different propolis samples. The same group has also reported that flavonoid content correlates inversely with hydrocarbon and aliphatic acid contents. Likewise, it is stated that the basic structure of the samples collected from Turkey (Bursa) and Bulgaria is similar in flavonoids, pinocembrin and pinobanksin, the main content the samples collected from İzmir was 3,4-dimethoxycinnamic acid (25). On the other hand, it has been reported that the propolis samples collected from Hatay, Adana and Mersin Provinces of Turkey contain aromatic acids, terpenoids, hydrocarbons, fatty acids, alcohols and many other chemical compounds (26). Contrary to our work, Sahinler and Kaftanoğu (26) have not reported any flavonoid group compounds in the propolis samples used in their study.

According to our results in the Table 2; ethanol extracts of the propolis from Hakkari showed antibacterial activity against all target strains, except *Klebsiella pneumoniae*. In addition, it showed antifungal effect against *C. albicans*. The results of *in vitro* antimicrobial assay indicated that our propolis sample inhibits the growth of gram positive bacteria better than gram negative bacteria studied. Our results are in agreement with the findings of Stepanovic et al. (27). Many studies have shown that gram positive bacteria are more susceptible to the antimicrobial effect of propolis than gram negative bacteria (28,29). Silici and Kutluca (16) studied the antimicrobial activity of propolis samples collected by three different races of bees against *S. aureus, E. coli, P. aeruginosa* and *C. albicans* and found that their propolis samples show strong activity against gram positive cocci (*S. aureus*), but had low activity against gram negative bacteria (*E. coli* and *P. aeruginosa*) and yeast (*C. albicans*). Similarly, Daugsch et al. (30) investigated the antimicrobial activity of six propolis samples against *Staphylococcus aureus* ATCC 259232. As a result, they reported that the activity grades of six different samples were related to the propolis chemical content and botanical origin of their samples. In a different study, the antimicrobial activity of Tabora and Iringa propolis against gram positive and gram negative bacteria was compared. Researchers reported that propolis samples contained flavonoids, but the activity of the propolis from Tabora was higher than that of propolis from Iringa (31). Gao et al. (32) reported that the pinocembrin (5,7-dihidroksiflavanon) is a compound that can be found in very high concentrations in propolis and, is responsible for antimicrobial activity of propolis. Accordingly, antimicrobial activity of the propolis sample we investigated can be related to the rate of this compound.

The results of our study show that, Hakkari-Turkey propolis has weaker antimicrobial effect compared to ampicillin, but it has a broader spectrum. These findings are also consistent with a previous study. Kalogeropoulos et al. (18) has determined chemical composition and antimicrobial properties of propolis from Greece and Cyprus and they demonstrated that propolis inhibitory spectrum is broader and its activity stronger even at very low concentrations compared to nisin. The main reason for these effects could be the fact that propolis contains many different components which act synergistically while nisin has only one ingredient.

The present study is the first to investigate antimicrobial effect of a propolis sample collected from Hakkari province of Turkey. Our results have showed that propolis from this region contain flavonoids in higher ratio than the other common bioactive compounds such as hydrocarbons, aliphatic acids and their esters, cinnamic acids and their esters, alcohols and terpenes, aromatic acids and ketones. Therefore, propolis from this region is expected to have different biological activities, besides having antimicrobial effect. Hence, it could be concluded that our results support the present usage of propolis as a therapeutic agent in alternative medicine. However, individual isolation of its bioactive substances is necessary in order to explain the full mechanism of propolis action on pathogenic microorganisms.

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Cell Surface Sialylated N-Glycan Alterations during Development

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ABSTRACT

This brief survey focuses on the comparison of sialylated N-glycans of embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), mesenchymal stem cells (MSCs) and of differentiated cells. In addition, the impact of sialic acid (Sia) deficiency on cell surfaces during development is summarized. The most common Sia is N-acetylneuraminic acid (Neu5Ac). The branched structures of complex- and hybrid- type N-glycans are the carrier for Sia. Transmembrane adhesive proteins, voltage-gated ion channels and many ligand-activated receptors are some examples of heavily sialylated N-glycan bearing membrane proteins. Their oligosaccharide extensions provide an important contribution to glycocalyx glycans. ESCs and iPSCs are characterized with high mannose-type and biantennary complex-type core structures. Two branches terminate with α2,6- linked Sia. MSCs contain high mannose, hybrid- and complex- type N-glycans. Linear poly-N-acetyllactosamine (poly-Galβ1-4GlcNAc, poly-LacNAc) chains are the characteristic structures. Both α2,3- and α2,6- linked Sias are seen in a species-specific manner in MSCs. α2,6- linked Sia is probably a marker associated with the multipotency of human MSCs. Differentiated healthy cells contain the most abundant 2-branched complex structures. The bisecting branch on the core structure appears as a differentiation marker. poly-LacNAc chains are terminated with α2,3- and α2,6- linked Sia, with the former being higher. poly-LacNAc sequences have a high affinity for β-galactoside recognizing lectin and galectin. Galectin forms a lattice structure with the N-glycans of glycoproteins anchored to the plasma membrane. The impact of N-glycan-galectin complexes in cell biology is summarized. Finally, the effect of reduced Sia on clearance of aged cells is explained. Experimental evidence for the masking role of Sia in the regulation of histolysis in aged cells is revealed.

Keywords: Sialic acid, sialylated N-glycan carriers, embryonic stem cell, mesenchymal stem cell, differentiated cell, sia deficiency, galectin lattice

INTRODUCTION

Glycosylation of the proteins starts with transferring the common N-glycan precursor to the growing peptide in the lumen of endoplasmic reticulum. The precursor (2 GlcNAc, 9 Man, 3 Glc) is attached in the amide nitrogen of the asparagine residue in the β-glycosidic linkage (GlcNAc β1-Asn) by oligosaccharyltransferase. Maturation reactions continue in endoplasmic reticulum and Golgi lumens by the successive actions of glycosidases and glycosyltransferases. The structure of the common glycan precursor changes. These enzymes cause the formation of high mannose-, hybrid-, and complex- types of N-linked oligosaccharides (Figure 1) (1-3). The branched structures of complex- and hybrid-type N-glycans are the carrier for Sia.

Many carrier proteins for sialylated N-glycans are present within the structure of the plasma membrane and extracellular matrix. The effects of sialylation on the structure/function of a glycoprotein is dependent on the localization of the Sia on the glycoprotein (4). Sialylated glycans significantly changes during development depending on the manner of cell and tissue type (5). Light and heavily sialylated plasma membrane glycoproteins are responsible for different functions. Transmembrane adhesive proteins (cadherins and integrins), voltage-gated ion channels (for Na^+, K^+ and Ca^{+2}) and many ligand-activated receptors (for EGF and others) are examples of heavily sialylated membrane glycoproteins. Cadherins are responsible for attachment to neighboring cells and provide a strong intercellular adhesion (6). Integrins mediate attachment to the ex-

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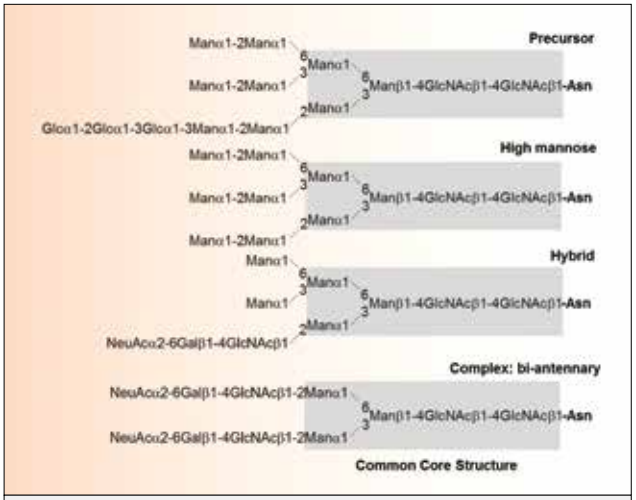

Figure 1. Precursor and basic types of N-glycans. The common core structure is shaded

tracellular matrix and transmit the signals across the plasma membrane in both directions (7). Voltage-gated ion channels are largely distributed in cell membranes and regulate membrane permeability for Na^+, K^+ and Ca^{+2} ions. Excitable cells in the nerve, skeletal and cardiac muscles play a significant role in regulating electrical excitability. In comparison with other membrane glycoproteins voltage-gated ion channels contain larger amount of Sia molecules (8).

Voltage-gated Na^+ channels (Na_v) are responsible for initiation, conduction and termination of the action potential in excitable cells. Sias alter Na_v gating activity in an isoform-specific manner. The number and location of sialylated chains and their attached pore forming or auxiliary subunits change the activity of Na_v (9,10). The comparison of N- and O- linked Sia contributions on channel gating is found to be similar (11) but, Sia and polySia have different effects on gating of the Na_v (12). Slight changes in channel function may cause several cardiac disorders (13).

Voltage-gated potassium channels (K_v) are responsible for repolarization of excitable cells (14). They contain significant amounts of Sias (8,15). Negative charges of Sias on N- or O-glycans cause conformational changes in the voltage-sensing domains but their effects on the channel gating mechanism don't appear the same (16). Cell surface expression of potassium channel $K_{v1.3}$ was investigated in tissue culture conditions on CHO pro 5 cells. Preventing N-glycosylation of $K_{v1.3}$ caused a significant decrease in its surface protein level. Supplementation of GlcNAc, L-fucose and Sia to the culture medium promoted $K_{v1.3}$ surface protein expression. Supplementation of GlcNAc lead to an increase in the degree of branching in the N-glycan core of $K_{v1.3}$. The increase in branching degree caused a decrease in internalization of $K_{v1.3}$ protein, but at the same time an increase in it's half-life on the cell surface (17). It is known that congenitally reduced sialylation causes altered gating of voltage gated Na^+ and K^+ channels, but the impact of reduced sialylation on cardiac electrical signals is not well known (18).

G-protein-coupled and ligand-activated receptors are other examples of sialylated membrane glycoproteins. In G-protein-coupled receptors, N-glycosylation and Sia have a role in the stabilization of the receptor dimers participating with disulfide bonding (19). Among the GFRs, EGFR is the most studied receptor, tyrosine kinase (20). For downstream signaling, the first necessity is the binding of the ligand, EGF, to its receptor. EGF-binding causes conformational changes of EGFR and it forms dimers. Dimerization leads to intracellular tyrosine kinase activation and subsequently auto-phosphorylation (21). EGFR bears twelve N-linked glycosylation sites on the extracellular region (22) and is heavily glycosylated (23). Sialylation diminishes EGFR activity by preventing its dimerization (24). Inhibition of sialylation on N-glycosylation is made by removing the Asn 420 and 579-linked glycans which causes ligand-independent dimerization (25). As well as, sialidase treatment promotes EGFR signaling (23). It is clear that sialylated N-linked glycans can affect the conformational stability of the channel and receptor proteins.

The presence of N-glycans on these heavily sialylated glycoproteins is important in cell physiology. They dynamically change by specific glycosyltransferases and glycosidases. In this way, biological events are regulated by covering or uncovering certain glycan sequences for recognition of specific lectins (galectins and siglecs) (26). Galectins with affinity for β-galactoside (Galβ1-4GlcNAc, LacNAc) form complexes with N-glycans on the cell surface of glycoproteins. They don't require a specific receptor and can bind with any of the suitable oligosaccharides from the cell surface or extracellular matrix (27). Galectin lattice regulates diffusion, selection, activation, arrest of T-cells, receptor kinase signaling and functionality of membrane receptors, glucose and amino acid transporters (28), and cell growth and differentiation (29).

Galectin-glycoprotein lattices control the organization of a plasma membrane domain like lipid rafts (30-33). Galectin lattices regulate lateral mobility of integrins (34), junctional stability of N-cadherins (32), receptor distribution at the cell surface (35), turnover of endocytic receptors (33), and intracellular signaling pathways (31,36-38). Galectins exhibit a remarkable functional diversity that participates in developmental processes, such as cell differentiation and pathophysiology, (39) cell adhesion and motility, regulation of immune homeostasis, and recognition of glycans on pathogens (26,28,40). It has been proposed that Galectin-glycoprotein lattices at the cell surface function as an "on and off switch" that regulates cell proliferation, differentiation, and survival (33). It appears that the cells may change cellular growth, differentiation, function, and probably pathologic transformation by altering the galectin glycoprotein lattice (35).

STRUCTURE, BIOSYNTHESIS AND FUNCTION OF SIALIC ACIDS

Sialic acids (Sias) are a large family of nine carbon monosaccharide sugars. The most common Sia is N-acetylneuraminic acid (Neu5Ac). Neu5Ac is the biosynthetic precursor for all other members of the family. It is usually found in a six-membered

ring configuration and the positions of carbon (C) 7-9 stay outside the ring as a glycerol side chain. C-1 has a carboxyl group, which is ionized at physiological pH to give a negative charge. C-5 determines the type of the four "core" Sia molecules. Presence of the N-acetyl group at the C-5 is known as Neu5Ac. Hydroxylation of the N-acetyl group at the C-5 produces N-glycolylneuraminic acid (Neu5Gc). De-acetylation of the N-acetyl group to an amino group forms neuraminic acid (Neu). The presence of a hydroxyl group at the C-5 produces 2-keto-3-deoxynonic acid (KDN) (41-44). Various substitutions (O-acetyl, O-methyl, O-sulfate and O-lactyl groups) of one or more of the hydroxyl groups at the 4-, 7-, 8- and 9- carbon positions of these "core" molecules form different modifications. Nearly fifty modifications exist in nature (42,45).

The sialylation pattern of a cell is regulated by three group enzymes; Sia Synthase, Sialyltransferase, and Sialydase or Neuraminidase. Sia synthase localized in the nucleus is responsible for the synthesis and availability of the activated Sia substrate, CMP-Sia. Sialyltransferase adds Sia during sialo-oligosaccharide biosynthesis using CMP-Sia as a donor, in trans-Golgi. The sialidase family separates from Sia during degradation of sialoglycoconjugates in lysosomes or endosomes (44). The specific sialylated pattern of a cell is produced by the activities of these three enzyme groups.

The C-2 on a Sia structure can form α glycosidic linkage with another sugar. The most common Neu5Ac can be found in four distinct linkage types to penultimate sugars. There are twenty known genes in mice and humans with encoding sialyltransferases for synthesizing these four major linkages. Some sialyltransferases (ST3GAL subfamily with six members) add Sia in an α2,3-linkage to Galactose (Gal), whereas others (ST6GAL subfamily with two members and ST6GALNAC subfamily with six members) add Sia α2,6-linkage to either Gal or GalNAc (N-acetyl-galactosamine). The fourth type of Sia linkage is directed by the polysialyltransferase family (ST8SIA subfamily with six members) which adds an α2,8 linked Sia to another Sia (4,46,47). Sialidase or neuraminidase enzymes are classified in four different groups, according to their subcellular localization, in mammals. The NEU1, NEU2, NEU3 and NEU4 enzymes are located in lysosomes, cytosol, plasma membranes, and lysosome/mitochondria (48-50). Combinations of different glycosidic linkages with the various substitutions produce structural diversity in hundreds of Sia molecules (51). This wide structural diversity of Sia molecules contributes to the enormous diversity of carbohydrate parts of proteins and lipids in cell membranes and secreted molecules (45).

Bio synthesis of Sia begins with the epimerization and subsequent phosphorylation of UDP-GlcNAc (Uridine Di Phosphate-N-Acetylglucosamine) to ManNAc-6-P (N-acetylmannosamine-six-phosphate), in cytosol. The bi-functional enzyme, UDP-N-acetylglucosamine 2-epimerase /N-acetylmannosamine kinase (GNE) catalyze these reactions. The enzyme Neu5Ac-9-P synthase combines with ManNAc-6-P and phosphoenol pyruvate to form Neu5Ac-9-P. Following dephosphorylation of Neu5Ac-9-P by Neu5Ac-9-P phosphatase, Neu5Ac is formed and transported into the nucleus. In the nucleus, Neu5Ac is changed to CMP-Neu5Ac by CMP-Neu5Ac synthetase and transported to the Golgi apparatus by the CMP-Sia transporter (16,52). Only the activated sugar is transported into the Golgi apparatus. In trans Golgi lumen, activated Sia serves as a substrate for sialyltransferases. Linkage-specific sialyltransferases add the Sia as a terminal unit to a selected glycan chain, depending on the availability of the preferred acceptor chain sequence (53,54).

The terminal location of Sia on the glycan chain facilitates the cell surface interactions between the cells and the immediate environment. These acidic monosaccharides act directly as a biological target for many pathogenic microorganisms. For example, human parainfluenza virus initiates an infection through Sia recognition and binding in a glycosidic linkage specific manner. α2,3- linked and α2,6- linked Sia residues are receptors for the type-1 and type-2 parainfluenza virus, respectively (55). In addition, Sias have a masking role, covering penultimate sugars. Sia covers penultimate galactose residue that is recognized by an asialoglycoprotein receptor. In a similar way, Sia covers penultimate LacNAc (Galβ1-4GlcNAc) that is recognized by a β galactoside recognition lectin, it is known as galectin. These two main functions of Sia participates in the regulation of many important events during development, such as cell communication, differentiation, aging, adhesion, migration, self/non-self-discrimination and many cell signaling events.

EMBRYONIC STEM CELL SIALYLATED N-GLYCANS

Stem cells are undifferentiated cells with a high capacity for self-renewal (proliferate indefinitely) and pluripotency (differentiated into three germ layers) (56). Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are valuable resources for cell replacement therapies. Determination of specific markers are required for effective utilization of stem cells and elimination of tumor initiating cells (57). For this purpose, cell surface glycan variations have been investigated to detect the most appropriate markers.

A study to determine the characteristic surface of glycosylation patterns was conducted with labeled lectin applications on glycosidase digested or nondigested cells by microscopic methods (58-60). Useful impressions of mainly terminal and some internal monosaccharide residues of oligosaccharide chains were obtained from these studies. Advanced analytical techniques including nuclear magnetic resonance (NMR) and mass spectrometry (61) and newly developed microarray technology (62,63) have provided additional structural information about stem cell glycosylation.

In human stem cells, the major pluripotency-specific N-glycosylation is made up of a high mannose-type and a biantennary complex-type core structure as determined with MALDI-TOF mass spectrometry NMR spectroscopy (61), flow cytometry and immunohistochemistry, (58, 64,65) and fluorescein labeled lectin staining (59). Upon induction of pluripotency, the occurrence of a significant increase in the high mannose-type N-gly-

cans (66,67) indicate that it is in an immature stage of N-glycoproteins (68,69). Biantennary complex-type core structures bear type 2 N-acetyl lactosamine (LacNAc, Galβ1-4GlcNAc) chains in hESCs (61). However, Type 1 LacNAc (Galβ1-3GlcNAc) is a characteristic feature of hiPSCs (62,68). The LacNAc chains are terminated with α2,6- and α2,3 linked Sias, but the α2,6- Sia linked higher in hESCs. In iPSCs, a linkage type of Sia changed from α2,3 to α2,6. However, large branched poly-LacNAc chains present in mouse ESCs suggest that it might participate in cell survival by increasing the interaction among membrane molecules (70). Complex terminal fucosylation is also characteristic of the N-glycosylation structure of hESCs (61,62,68). One terminal bears an α1,2-linked fucose residue (Fucα1-2Galβ1-4GlcNAc) while the other terminal bears an α1,3- or 1,4-linked fucose residue producing a Le^x structure (Gal β1-4 (Fucα1-3) GlcNAc). In addition, the most abundant fucose linkage in hESCs N-glycans is a core α1,6- linked fucose residue which links to the asparagine-linked GlcNAc residue.

MESENCHYMAL STEM CELL SIALYLATED N-GLYCANS

Mesenchymal stem cells (MSCs) are adult multipotent progenitor cells. They differentiate into mesenchymal cell lineages. Adipose tissue, bone marrow, and umbilical cord blood are harvested sources of MSCs (71,72). MSCs are considered very valuable cell sources for stem cell-based therapy because of the probability of teratoma formation in ESCs and iPSCS (73,74). The ability to adhere to plastic surfaces is one of the main characteristics of MSCs and it is also useful for glycoengineering studies (71).

High mannose-type N-glycans are characteristic for undifferentiated bone-marrow derived MSCs from humans (73,75) and equines (76). Biantennary N-glycans are detected in adipose tissue-derived hMSCs (72). Hybrid type N-glycans are found to be the less abundant type of N-glycosylation in undifferentiated and adipogenically differentiated hMSCs (73,75). In mice, an extraordinary branch is demonstrated by expression of GnT-V (the corresponding gene is Mgat5) in neural progenitor cells that have a self-renewal ability and multipotency (77). This is amazing because GnT-V is associated with oncogenic differentiation (78,79). Although a suggested function of GnT-V is related with cell growth and migration by regulating integrins (80) in cancer cells, expressed GnT-V in neural progenitor cells may perform a similar function during neural development and brain injury conditions (77). GnT-V and its reaction products appeared in proliferating cells. In differentiated cells, they were markedly reduced (77).

Linear poly-LacNAc chains terminated with Sia are the characteristic structures in undifferentiated hMSCs (75,73). Using mass spectrometry-based quantitative techniques, different amounts of α2,3-linked Sia obtained from the hMSCs expanded on different surfaces were detected. These results reveal that α2,3- sialylation participates in controlling hMSC multipotency by regulating cell adhesion (81). It is known that MSCs change differentiation potentials depending on the stiffness or chemically modified substrates (82,83). In addition, bone marrow-derived hMSCs, osteogenic precursors, and poly-LacNAc chains are terminated with α2,3- linked Sia (75).

Comparisons of lectin binding intensity of early (with differentiation ability) and late (without the ability to differentiate) passages of adipose derived hMSCs, using high density lectin microarray, demonstrated that early passage cells show stronger binding to specific lectins for α2,6-linked Sia (74). Bone marrow-derived hMSCs and cartilage tissue-derived chondrocytes also showed this similar binding activity, but no binding activity is indicated between α2,6-linked Sia and its specific lectins in human dermal fibroblasts. Using HPLC analysis combined with MS spectrometry, α2,6-linked Sia on sialylated N-glycans is detected at a higher percentage (24-28 %) in early passage cells than late passage cells (13-15 %) in adipose tissue-derived hMSCs (72). These results suggest that α2,6-sialylation is a marker associated with the differentiation potential in stem cells (74,72). The presence of α2,3- and α2,6 linked Sia on bone marrow derived macrophages and osteoclast precursors (MSCs) is demonstrated with lectin labeling in mouse (84). α2,3- linked Sia was detected throughout osteoclastogenesis, but α2,6-linked Sia disappeared at the terminal stage of differentiation. Desialylated cells, particularly α2,6-linked Sia-deficient cells do not develop into multinuclear osteoclasts. This suggests that α2,6-linked Sia may be involved in osteoclast differentiation and regulating the cell fusion process (84).

Fucosylated N-glycans of human bone marrow MSCs bear at least two or more fucose residues, one of which is a core fucose that is α1,6-linked to the asparagine-linked GlcNAc residue of the N-glycans (73). Using a cell microarray procedure, a core fucose addition to α1,3 terminal fucose and complex terminal fucose were detected on N-glycans MSCs of canine and ovine species (76). Finally, according to a study performed on veterinary species in bone marrow derived MSCs, terminal Sia linkage shows variability in a species-specific manner. For example, while the MSCs of canines contain more α2,3- linked Sia, equine MSCs have a higher density of α2,6- linked Sia (76).

DIFFERENTIATED CELL SIALYLATED N-GLYCANS

The dynamics of expression on cell surface glycans vary widely depending on cell types and stages of development. Characteristic changes during healthy or pathologic differentiation processes have been reported (85-87). Some glycan structures on ESCs, iPSCs and MSCs disappear and a new glycan profile occurs on differentiated cells. Alterations appear mainly in core branching, in poly-LacNAc extension and in terminal units, such as in Sia and fucose content.

High mannose-type N-glycans represent immature forms of N-glycans (65). In differentiated cells, the quantity of high mannose-type N-glycans decreases and complex/hybrid types of N-glycans start to appear in abundance (61,66,88). N-acetylglucosaminyltransferases (GlcNAcTs, GnTs) (as productions of Mgat genes) conduct the production of Tri and tetraantennary and more branched, complex N-glycans in Golgi apparatus. Sequential activity rules for the GnTs were established by Brockhausen

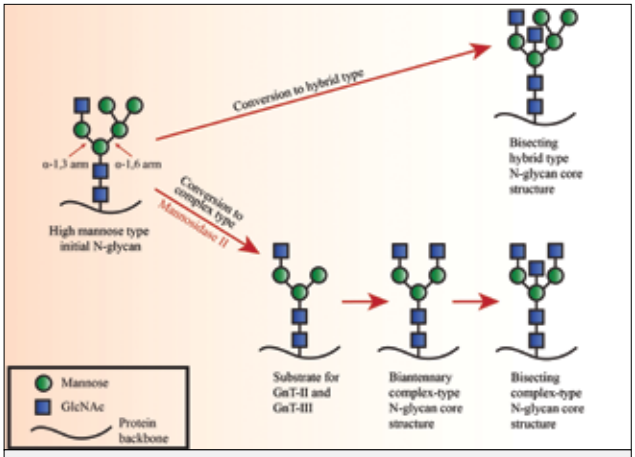

Figure 2. Two- three- and four branched N-glycan core structures, modified from Taniguci and Korekane (2011) with permission

et al. (89). These enzymes are responsible for the branching of the core structure *in vitro* (90-94).

"GnT-1 acts before all other GnTs and is responsible for the conversion from the high mannose-type to the hybrid and complex-types of N-glycans (Figure 2) (91). It catalyzes the formation of β1-2 linkage by transferring GlcNAc sugar to the Man residue on the α1-3 arm of the core structure with 5 mannose (Man). α-mannosidase II removes two Man residues on the α1-6 arm and a substrate for the GnT-II and GnT-III enzymes form. GnT-II controls the conversion of a hybrid type to complex type structures and catalyzes the β1-2 linkage by adding GlcNAc to the α1-6 arm on the core. Activity of GnT-II is a prerequisite for the GnT-IV, GnT-V and GnT-IX enzymes (95) that are responsible for cancer progression. GnT-III catalyzes the formation of β1-4 glycosidic linkage by transferring GlcNAc to the first Man residue on the core (Figure 2). The formation of a bisecting GlcNAc prevents subsequent processing and elongation of N-glycans (90) which inhibits the catalytic activity of the GnT-II, GnT-IV, GnT-V and FUT8 enzymes that are responsible for branching of the core structure *in vitro* (89,91).

Differentiated healthy cells contain the most abundant 2-branched complex structures on their N-glycoproteins. A few 3- and 4- branched glycans are also present (3). The distribution of PHA.E lectin ligands (58,59) and N-glycan signals (61) indicate the presence of bisecting GlcNAc on the N-glycan core of differentiated cells. During neural differentiation of murine iPSCs and ESCs, the bisecting N-glycan is upregulated and suggested as a differentiation marker (96). In caco-2 cell differentiation, the levels of bisecting N-glycan increase (88). Similarly, human induced pluripotent stem cell-derived cardiomyocytes also contain bisecting GlcNAc in relative abundance (97).

During primary human hepatocyte differentiation from hESCs, the first bi and triantennary complex N-glycans were found in hepatocyte-like cells (98). They were asialylated, monosialylated or fully galactosylated N-glycans. When they differentiated to primary human hepatocyte, bisialylated biantennary and trisialylated triantennary N-glycans were dominant. Similar changes observed during monocyte-derived macrophage differentiation show an increase particularly in triantennary glycans (99). One (Y101) of the mesenchymal stromal cell clones showed an abundant amount of complex N-glycan, during differentiation into osteoblasts (100). In addition, another mesenchymal stromal cell clone (Y202), which cannot differentiate into osteoblasts, showed a similar reduction in oligomannose glycan content when incubated in an osteogenic medium. Based on these observations, it suggests that expressed N-glycans, upon induction of differentiation, may be important for self-renewal rather than for cell fate determination (100).

Several N-glycans that have bisecting GlcNAc carry LacNAc repeats and a core fucose sugar in their glycomic profiles (101). The presence of the bisecting GlcNAc on the cell surface glycoproteins affects their interaction with galectins and siglecs, probably altering N-glycan conformation (92).

Characteristic changes occur on terminal sialylation and fucosylation of branched N-glycans during differentiation. Hybrid type N-glycan branches in a transition embryoid body bear both α2,6- and α2,3- linked Sia in humans (61). Differentiated chondrocytes have α2,6- linked Sias. The expression ratio of α2,6- to α2,3- linked Sias determines the differentiation status of chondrocytes (102). α2,6- sialylation decreases in adipogenesis (103) and osteoclastogenesis (84) in mouse but desialylated cells do not differentiate into osteoclasts despite the normal expression of an osteoclast marker.

During brain development, completely differentiated cells bear mostly α2,3- linked Sia as compared with α2,6- linked Sia, in rats (104). This development is explained with a linkage shift from α2,6- linked Sia to α2,3- linked during mouse brain development (105). This linkage shift may affect the biological functions of endogenous lectins, such as galectins and siglecs. The binding activity of galectins is greatly diminished when LacNAc chains are capped with α2,6- linked Sia (106,107).

N-GLYCAN-GALECTIN LATTICES

Galectins are soluble proteins and found within the cell, cell surface, and ECM as well as, in biological fluids. (108). They recognize and bind to β galactoside (Galβ1-4GlcNAc, LacNAc) sequences. LacNAc sequences are seen on poly-LacNAc extensions of tri- and tetraantennary N-glycan chains on cell membrane glycoproteins. In general, affinity of galectins to poly-LacNAc sequences is higher when compared with the affinity of LacNAc alone. However, galectin types (Gal-1, Gal-2, and Gal-3) display some differences in glycan binding properties. For example, only Gal-3 is bound to proximal LacNAc of poly-LacNAc extensions (109).

Galectin binding is influenced by N-glycan branching, LacNAc content and the balance of α2,3- and α2,6- linked terminal Sia (38). While Gal-1 is connected to only α2,3- sialylated poly-LacNAc, Gal-3 is connected to both α2,3- and α2,6- sialylated glycans (109). α2,6- sialylation alters binding of specific galectins, being consistent with biological function differences (35).

A detailed study on oligosaccharide binding specificity of galectins was performed using frontal affinity chromatography (106). Three (OH) groups on LacNAc, i.e. 4-OH and 6-OH of Gal, and 3-OH of GlcNAc are required for binding of galectins. In complex type N-glycans, no galectin could bind if 6-OH of Gal linked to a Sia. However, it did show that modified glycans such as α1-2 Fuc, α1-3- Gal, α1-3- GalNAc and α2,3- Sia have a preference for galectins (106).

Galectins released from cells are concentrated on the cell surface and generate galectin-glycan complexes. Gal-3, existing as a monomer in solution, produces a pentameric structure through self-associated intermolecular interactions and mediates crosslinking of proteins, forming a lattice organization (108, 110,111).

IMPACTS OF SIALIC ACID DEFICIENCY AT CELL SURFACES

Disorders at any stage of Sia biosynthesis, inactivation of the functional enzymes, such as GNE and activity of endogene sialydases cause Sia deficiency on cell surfaces. GNE (UDP-GlcNAc2-epimerase/ManNAc kinase) catalyzes the first two steps of Sia biosynthesis. Inactivation of GNE causes early embryonic lethality in mice (112-114). The GNE-deficient ESCs are not successful in the formation of embryoid bodies in the first day of culture. However, following the addition of a Sia, GNE-deficient ESCs form normal embryoid bodies (115). Proliferation of ESCs is correlated with GNE-expression and the cellular Sia concentration (113).

Comparison of the developmental profiles of wild type and GNE knockout mice indicates that GNE plays an important role in the development of excitable tissues (114). Genetic defects of the GNE cause GNE myopathy, a disease related with progressive muscle atrophy and weakness. Hyposialylation and production of reactive oxygen species, ROS, are correlated with muscle atrophy but an increase in sialylation causes a reduction in ROS. Depending on this relation, it suggests that Sia has a role as a ROS scavenger in skeletal muscles (116). In GNE myopathy, lectin binding and MS analysis show that GNE-deficient cells contain low levels of sialylation and distinct N-glycans, differentiated in branching of core structure and in poly-LacNAc extensions. These N-glycan chains display a binding affinity with galectin 1 (117).

The effect of desialylation was first described during a clearance of serum glycoproteins (118,119). At the same time, a hepatic galactose specific receptor (asialoglycoprotein receptor) from a rabbit liver was characterized (120) and a similar clearance mechanism for the desialylated cells, for erythrocytes in the rabbit was reported for the first time (121,122). According to a postulated hypothesis; a time-dependent loss of Sia residues uncovers the penultimate galactose residues. An asialoglycoprotein receptor, a lectin (123-125), in rat liver and peritoneal macrophages, recognizes and captures these galactose residues.

This receptor, a C-type lectin, is involved in the recognition and binding of terminal galactose on the glycan chains and is responsible for the clearance of asialoglycoproteins, and redetermined as an Ashwell-Morell Receptor (126). In a similar way, Sia residues of membrane glycoconjugates control the lifespan of erythrocyte. Desialylation of glycophorin (127) is responsible for the clearance of aged erythrocytes (128). Enzymatically desialylated erythrocytes in vitro show that the desialylation rate of aged cells is low but sufficient to lead to their capture by macrophages (124). Comparisons of quantified Sia on young and old cells claim that the decrease in Sia with cell aging may act as a senescent cell marker, capable of triggering their selective removal (129). Insufficient sialylation can causes rapid clearance of the cells. On the contrary, a long half-life is related to high level terminal Sia on the cell surfaces. This point is extremely important to the development of therapeutic glycoproteins (130).

In vitro desialylated platelets are also cleared rapidly from circulation similar to erythrocyte clearance (131,132). Platelets that lost the Sias during circulation are cleared by the hepatic endocytic Ashwell-Morell Receptor (133-136).

Sia depletion in aged cells is displayed in the human diploid fibroblastic cell line, TIG3. The young cells grow at a higher rate than aged cells in vitro conditions (137). The results obtained from lectin blot analysis of membrane glycoproteins show that the α2,6- sialylation, but not α2,3- sialylation, of N-glycans decreases markedly in the aged cells when compared to the young cells. The gene expression of the α2,6- sialyltransferase I (ST6Gall), which transfers Sia to the galactose residue of N-glycans, decreases in the aged cells which supports the results mentioned above. Some valuable information was obtained using lectin microarray. Human skin samples that taken from different age groups were tested for the glycan changes on diploid fibroblasts. α2,6- sialylated glycans, in particular, were found to differ between elderly and fetus derived cells at the early passage. In addition, both cell types exhibited sequentially decreasing α2,3- sialylated O-glycan structures (138). Comparisons of early and late passage cells by fluorescence activated cell sorting analysis using lectins, show that a decrease in sialylation and an increase in sialidase NEU1 occurs, in aged cells. Myofibroblast differentiation was inhibited by the reduction of sialylation. Using a sialidase inhibitor, a demonstration of restored myofibroblast differentiation in late passage fibroblasts provides evidence that Sias decrease in aging cells (139).

Additional evidence for the masking role and decreased capacity in aged cells of Sia were obtained from insect tissue, prothoracic glands, in *Galleria mellonella* (Lepidoptera). Prothoracic glands secreting ecdysone hormone in larval instars, are larval structures that disintegrate gradually by hemocytic autolysis in the pupal period and then disappear completely in the adult life of the insect. The presence of Sia on prothoracic glands was determined by electronic ionization mass spectroscopy, electron microscopy, and spectrophotometry (140). For investigation of the role of Sia during the degeneration process of prothoracic glands, neuraminidase digested larval glands (young cells) were incubated within the hemolymph collected from the same age larvae. Light and electron microscopic observations

showed an incomplete capsule formation by the accumulation of hemocytes around desialylated gland cells but not in the control group (141). Desialylated larval cells were recognized as a foreign structure by hemocytes. According to the tested idea, a progressive loss of Sia at distinct developmental stages uncovers the penultimate sugar in glycan chains, allowing for the adhesion of hemocytes during the degeneration period of the glands. The encapsulation and degeneration of experimentally created desialylated larval cells, like the behavior observed in pupal cells, constitute the evidence for the masking role of Sia in the larval period (141). Observation of similar results around experimentally created desialylated larval surfaces in nervous tissue (142), in corpus cardiacum-corpus allatum complex (143) and in testis (144) indicates that Sia acts as a mask for hemocytic receptors during the larval period under normal conditions. These studies provide an explanation for the functionality of Sia in insects, confirming that Sia is a universal molecule.

CONCLUSION

N-glycan patterns of the plasma membrane glycoproteins contribute a large amount to the glycosylation of the glycocalyx. Like a tag, the total N-glycan pattern on the cell surface produces specific markers for the types of embryonic differentiation and aging stages and physiological and pathological state of the cells. The determination of cell surface markers is essential for basic studies and clinical applications. For the effective use of stem cells in cell therapy, it is necessary to know the pluripotency and multipotency associated glycans, for discrimination from other differentiated cells (145-147).

An important function of N-glycan-galectin lattices is to regulate the differentiation mechanism. Lattice structures forming between LacNAc repeats on N-glycan branches, and galectins determine plasma membrane glycoprotein residency time by inhibiting endocytosis of them (31,34). It has been suggested that galectin mediated glycoprotein assemblies are responsible for the signaling, adhesion, migration, and proliferation in many cell types. N-glycan-galectin lattices that affect the activities of membrane glycoprotein can control the decision between cell growth and arrested growth by regulating receptor turnover (26,28,29,39). Since dysregulation of the N-glycan-galectin lattices is responsible for many chronic diseases, it should be targeted for development of new strategies in medical treatment.

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