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Mussel RL, De Sa Silva E, Costa AM, Mandarim-De-Lacerda CA (2003). Mast cells in tissue response to dentistry materials: an adhesive resin, a calcium hydroxide and a glass ionomer cement. *J. Cell. Mol. Med.* 7:171-178.

Booth M, Bundy DA, Albonico P, Chwaya M, Alawi K (1998). Associations among multiple geohelminth infections in school children from Pemba Island. *Parasitol.* 116: 85-93.0.

Fransiscus RG, Long JC, (1991). Variation in human nasal height and breath, *Am. J. Phys. Anthropol.* 85(4):419-427.

Stanislawski L, Lefeuvre M, Bourd K, Soheili-Majd E, Goldberg M, Perianin A (2003). TEGDMA-induced toxicity in human fibroblasts is associated with early and drastic glutathione depletion with subsequent production of oxygen reactive species. *J. Biomed. Res.* 66:476-82.

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Full Length Research Paper

Oxidative stress in myocardial infarction: Advanced glycation end-products causes oxidative stress in older myocardial infarction patients

Anjuman Gul^{1*}, M. Aatur Rahman² and Sadaf Hamid³

¹Department of Biochemistry, College of Medicine, Kingdom of Saudi Arabia Ministry Of Higher Education Qassim University, P. O. Box 6666, Buraidah 51452, Kingdom of Saudi Arabia.

²HEJ Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan.

³Department of Anatomy, Dow University, Karachi, Pakistan.

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Older patients with type 2 diabetes mellitus have a two- to four-fold increased risk of myocardial infarction. This study aims to determine the association between oxidative stress and advanced glycation end product (AGE). Human serum samples from normal older subjects ($n = 31$), older diabetic patients without myocardial infarction ($n = 33$), older diabetic patients with myocardial infarction ($n = 32$), older non-diabetic with myocardial infarction ($n = 30$) and normal young subjects ($n = 31$) were investigated. Positive significant correlation was observed between serum AGE and malondialdehyde in older diabetic and non-diabetic patients with myocardial infarction. Negative significant correlation was observed between AGE and vitamin-E in older diabetic and non-diabetic patients with myocardial infarction. However, malondialdehyde and serum AGE were found to be significantly ($P < 0.001$) higher in older diabetic and non-diabetic patients with and without myocardial infarction as compared with older control subjects. In contrast to all four older groups, the serum AGE was significantly ($P < 0.001$) lower in young control subjects. This study revealed that AGE was positively associated with markers of oxidative stress in the older groups.

Key words: Advanced glycation end products, myocardial infarction, diabetes.

INTRODUCTION

Type 2 (non-insulin dependent) diabetes mellitus, the most prevalent form of the disease, is associated with chronic macrovascular and microvascular complications. In the developed world, the risk of myocardial infarction is increased two to four-fold among diabetic patients compared with non-diabetic persons (Aronson, 2008; Saleheen and Frossard, 2004). Recently, it has been documented that, among various factors, advanced glycation end products (AGEs), a heterogeneous group of irreversibly modified products formed in excess during aging and diabetes mellitus, play a crucial role in this

process (Vlassara and Palace, 2003). Hyperglycemia can stimulate non-enzymatic glycation and oxidation of proteins and lipids, leading to enhanced formation of AGEs, which may be involved in the pathogenesis of diabetic vascular diseases (Soldatos and Cooper, 2006).

The presence of AGEs has also been reported in atherosclerotic plaques, and the cross-linking abilities of AGEs may contribute to the increased stiffening of collagen and possibly to vascular hypertrophy (Price and Knight, 2007). Accumulation of AGEs with structural alterations results in altered tissue properties that contribute to the reduced susceptibility of catabolism (Baynes and Thorpe, 2000). Another possible mechanism by which AGEs may contribute to development of atherosclerosis is by activating the transcription nuclear factor κ B (NF- κ B) through RAGE

*Corresponding author. E-mail: anjummurtazagul@yahoo.com.
Tel: 92-21-5862939. Fax: 9221-5862940.

binding, resulting in induction of cellular adhesion molecule expression and cytokine activation or through glycooxidation of lipoproteins and increased foam cell formation (Bierhaus et al., 1997).

Reactive oxygen species (ROS) generated in oxidative stress from a variety of sources can in turn accelerate AGE formation. Oxidative stress originates due to an imbalance between the generation of ROS and the antioxidant defense system. Production of ROS depletes antioxidants and antioxidant enzymes, leading to additional ROS accumulation. AGE formation is dependent on oxidative processes and can create ROS through the Millard reaction (Yamagishi and Imaizumi, 2005). One of the most frequently used biomarkers providing an indication of the overall lipid peroxidation level is the plasma concentration of malondialdehyde (P-MDA), one of several byproducts of lipid peroxidation processes. An increased production of ROS and an enhanced concentration of thiobarbituric acid-reactive substances (TBARS) resulting in oxidative stress, have been observed in diabetes (Surekha et al., 2007). The level of serum AGE could be considered as a marker for the developments of myocardial infarction in older diabetic, as well as in non-diabetic patients.

MATERIALS AND METHODS

Subjects and sample collection

The study included one hundred fifty seven subjects. Out of these, 31 were normal older subjects, 33 were older diabetic patients without myocardial infarction, 32 were older diabetic patients with myocardial infarction, 30 were older non-diabetic with myocardial infarction and 31 were normal young subjects. The blood samples were collected from the subjects during the period of March, 2004 to December, 2007. The ethical committee of Ziauddin University approved the protocol, and consent of the patients was obtained after the nature of the study was fully explained. The older subjects were selected who were over sixty years of age, and young, apparently healthy (age ranging from 20 to 25 years) were selected as control subjects. Sex, weight, duration of diabetes, duration of complication in diabetic and non-diabetic patients, type of diabetes and type of treatments received were also recorded. Physical examination, including measurement of blood pressure was recorded.

Individuals were classified as having diabetes mellitus if any of the following criteria were met (Gabir et al., 2000); fasting serum glucose levels of 7.0 mmol/L or more, random glucose levels of more than 11.1 mmol/L, current use of medications prescribed to treat diabetes (for example, insulin or drugs). Older patients, those with more than one complication, and type 1 diabetics, were excluded from the study. Diagnosed cases of myocardial infarction were included in the study on the basis of chest pain, Electrocardiography (ECG) changes that is, ST elevation and Q wave inversion and biochemical markers that is, raised levels of troponin T, creatine kinase MB (CKMB), aspartate aminotransferase (AST) and Lactate dehydrogenase (LDH). The patients were selected on clinical grounds from National Institute of Cardiovascular Disease, Karachi and Jinnah Postgraduate Medical Centre, Karachi, Pakistan.

Blood was collected in fasting state after a 10 h overnight fast. Samples were withdrawn by venous puncture and distributed

equally into three tubes containing ethylenediaminetetraacetic acid (EDTA) (for HbA_{1c}), heparin (for glucose estimation) and tube with no anti-coagulant (for serum collection). The samples were then immediately stored on ice until processed. Clotted blood was centrifuged at 1,500 rpm for 30 min and the serum was separated and frozen at -70°C until analysis. Blood glucose was determined by glucose oxidase method, glycosylated hemoglobin (HbA_{1c}) was determined calorimetrically using HbA_{1c} kit (Bio Systems Reagents and Instruments, Spain). The serum fructosamine was determined calorimetrically using fructosamine kit (Randox, UK). Vitamin-E was measured on the basis of the reduction of ferric ions to ferrous ions by α -tocopherol and subsequent formation of a pink colored complex with bathophenanthroline which was measured colorimetrically at 536 nm (Baker et al., 1980). Malondialdehyde of the serum sample was reacted with thiobarbituric acid to form a pink coloured pigment, the absorbance of which was measured at 535 nm (Valenzuela, 1991).

Determination of AGEs

Pretreatment of the serum samples for AGEs measurement

To 100 μ l of serum diluted with 100 mM phosphate-buffered saline (PBS), pH 7.2 (PBS), 100 μ l of 0.6% Sodium dodecyl sulfate (SDS)/10 mM Tris-HCl saline, pH 7.4 and 5 μ l of 2 M NaBH₄/50 mM NaOH was added. The mixture was immediately heated at 100°C for 10 min. After cooling in ice water, a further 800 μ l of PBS was added and the samples were then used for AGE assay.

Generation of bovine serum albumin (AGE-BSA) standard

AGE-BSA was prepared by incubating 5 g BSA with glucose (0.56 M) in PBS under sterile conditions for sixteen weeks at 37°C. Samples were dialysed against PBS and stored at -70°C, protected from light until used. The amount of AGE was determined by non-competitive Enzyme-linked immunosorbent assay (ELISA) using rabbit polyclonal antibodies to AGE (Abcam, UK) (Ono et al., 1998). A 96-wells microplate was coated with 200 μ l of sample and its corresponding control in 50 mM sodium bicarbonate buffer (pH 9.6) and kept at 4°C overnight. After overnight incubation, the wells were washed four times using PBS containing 0.05% Tween-20 (PBST). Each well was blocked for two hours with blocking buffer, washed four times with PBST and incubated with 200 μ l of 1:10⁴ diluted anti-AGE antibody for 2 h. After washing wells four times, 200 μ l of 1:2000 diluted horseradish peroxidase (HRP)-anti-rabbit immunoglobulins (Abcam, UK) were added to each well and incubated for 2 h. Wells were washed five times and reacted with 200 μ l of 3,3',5,5'-tetramethylbenzidine (TMB) solution which was added to each well and incubated for 30 min, and absorbance at 650 nm was measured. Results were expressed as arbitrary AGE units (1 mU of AGE corresponds to 4 μ g of AGE-bovine serum albumin (BSA) standard).

Statistical analysis

Data was analyzed using Statistical Package for Social Sciences (SPSS, v 10.0) (SPSS Inc., Chicago, Illinois). The results were presented as mean \pm standard error of mean (SEM) and standard deviation (SD). The statistical significance of the difference between two mean of various parameters between different groups was evaluated by one-way analysis of variance (ANOVA). The Bonferroni's post hoc test was used to determine which group means differed. With this test, SPSS automatically adjusted the significant level for the multiple comparisons to avoid spurious significant differences being identified (any values below the level of 0.05 was considered as significant) Table 1.

Table 1. Physical features and blood analysis of young healthy subjects, older control subjects, older diabetic patients without myocardial infarction and older diabetic and non-diabetic patients with myocardial infarction.

Parameter	Young Healthy Subjects (31)	Older control subjects (31)	Older diabetic patients without myocardial infarction (33)	Older diabetic patients with myocardial infarction (32)	Older non-diabetic patients with myocardial infarction (30)
Age (years)	21.93±0.28	64.19 ^a ±0.70	64.18± 0.57	66.00±0.78	65.73±0.85
Sex (F/M)	16/15	16/15	16/17	14/18	15/15
Weight (kg)	59.06±1.11	63.61±1.22	65.66±1.53	66.03±1.07	63.33±1.26
Height (m)	1.59±0.01	1.59±0.01	1.59±0.01	1.58±0.01	1.60±0.01
BMI (kg/m ²)	23.39±0.48	25.22±0.53	26.00±0.64	26.20 ^c ±0.51	24.78±0.57
Systolic BP (mmHg)	119.51±1.12	121.54±1.05	119.70±1.19	144.53 ^b ±4.29	139.16 ^b ±4.53
Diastolic BP (mmHg)	79.19±1.01	83.06±1.10	81.66±1.05	91.56 ^b ±1.67	87.33 ^b ±1.80
Fasting Blood Glucose (mmol/l)	4.88±0.09	5.09±0.10	7.46 ^b ±0.24	8.97 ^{bc} ±0.29	5.07±0.12
Glycosylated Hemoglobin (HBA1c %)	4.81±0.07	4.97±0.08	9.02 ^b ±0.28	9.26 ^{bc} ±0.26	5.09±0.10
Serum Fructosamine (mmol/l)	2.10±0.06	2.33±0.06	3.79 ^b ±0.11	3.74 ^{bc} ±0.11	2.08±0.10
Serum-AGEs (mU/ml)	1.71±0.25	04.97 ^a ±0.34	8.10 ^b ±0.49	13.78 ^{bc} ±0.30	9.55 ^b ±0.27
Malondialdehyde (nM/ml)	-	3.56±0.26	7.06 ^b ±0.30	14.33 ^{bc} ±0.53	10.03 ^b ±0.36
Vitamin-E (mg/dl)	-	1.66±0.06	0.89 ^b ±0.05	0.78 ^{bc} ±0.05	1.23 ^b ±0.04

The values are expressed as mean, ± standard error of mean. Units and numbers of cases are shown in parentheses. ^aSignificant as compared with young healthy subjects, ^bsignificant as compared with older control subjects, ^csignificant as compared with non-diabetic older patients with myocardial infarction.

RESULTS

Concentrations of malondialdehyde and serum AGEs were significantly higher ($P < 0.001$) in older diabetic patients with and without myocardial infarction and older non-diabetic patients with myocardial infarction as compared with the older control subjects. When compared with older diabetic patients without myocardial infarction, AGE and malondialdehyde were highest in older diabetic patients with myocardial infarction. Serum vitamin-E was found to be significantly ($P < 0.001$) lower in older diabetic patients with and without myocardial infarction as compared with the older control subjects. The normal older subjects

showed significantly elevated in AGE ($P < 0.001$) as compared with normal young subjects. Fasting blood glucose, HbA_{1c} and serum fructosamine were significantly increased in older diabetic patients with or without myocardial infarction as compared with older non-diabetic patients with myocardial infarction and older control subjects. The increase in the fasting blood glucose level in all older diabetic patients with and without myocardial infarction correlates significantly with glycosylated hemoglobin and serum fructosamine concentrations. Also, the fasting blood glucose, glycosylated hemoglobin and serum fructosamine were not found to be different in older diabetic patients with and without myocardial infarction.

When compared with age matched normal subjects, the older non-diabetic patients with myocardial infarction showed no significant difference in levels of fasting blood glucose, glycosylated hemoglobin and serum fructosamine.

A significantly positive correlation was observed between serum AGEs and malondialdehyde ($r = 0.92$) in diabetic patients with myocardial infarction and in non-diabetic patients with myocardial infarction ($r = 0.98$) (Figures 1 to 4). Significant negative correlations were observed between serum AGEs and vitamin-E ($r = -0.87$) in diabetic patients with myocardial infarction, in non-diabetic patients with myocardial infarction ($r = -0.94$) and between malondialdehyde and

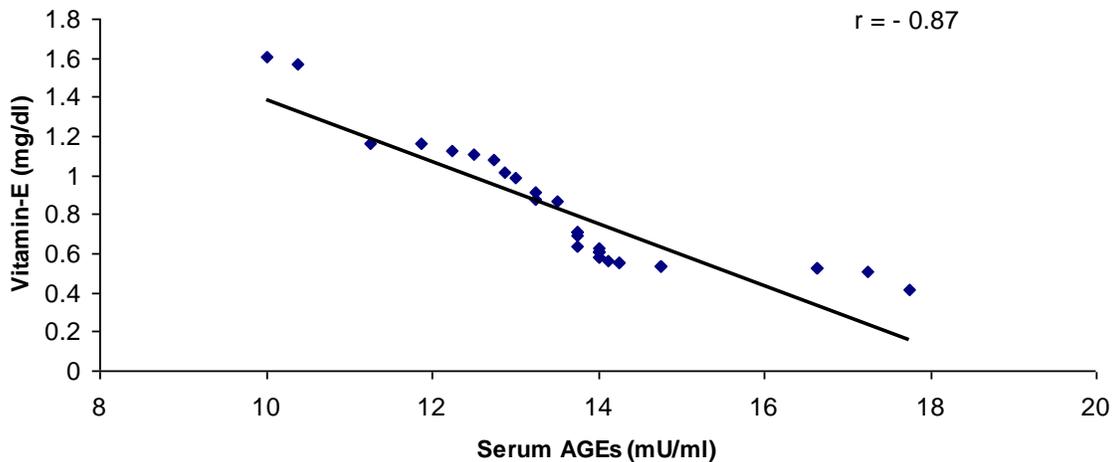


Figure 1. Correlation of serum AGEs versus vitamin-E in diabetic patients with myocardial infarction.

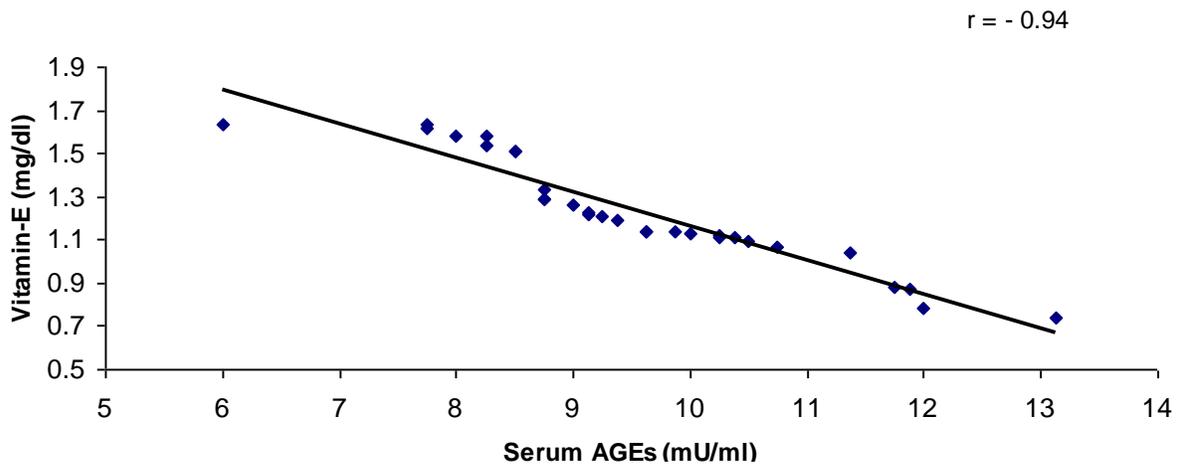


Figure 2. Correlation of serum AGEs versus vitamin-E in non-diabetic patients with myocardial infarction.

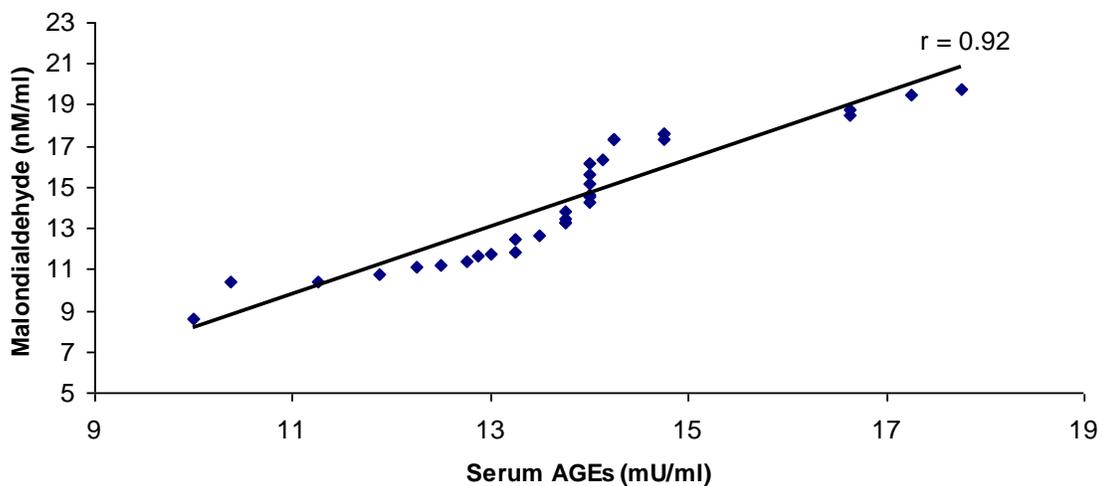


Figure 3. Correlation of serum AGEs versus malondialdehyde in diabetic patients with myocardial infarction.

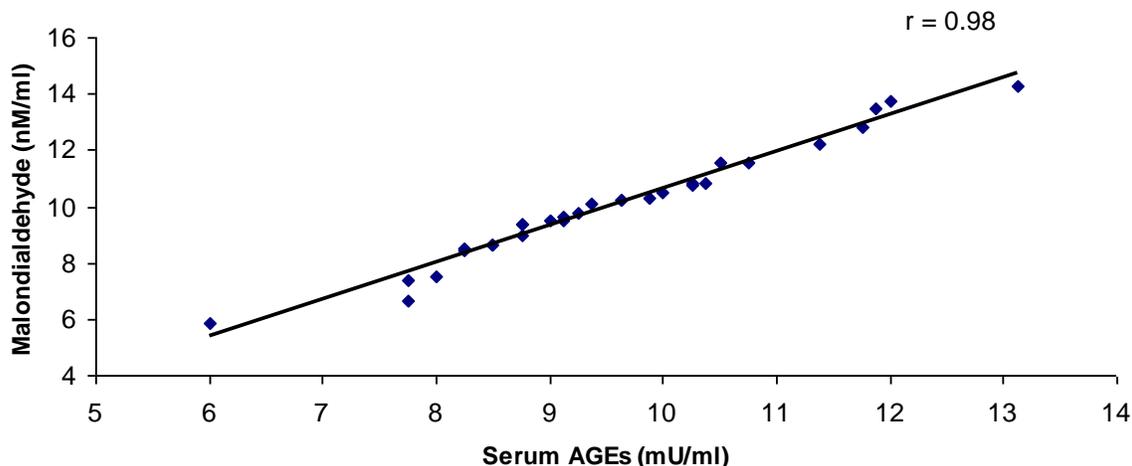


Figure 4. Correlation of serum AGEs versus malondialdehyde in non-diabetic patients with myocardial infarction.

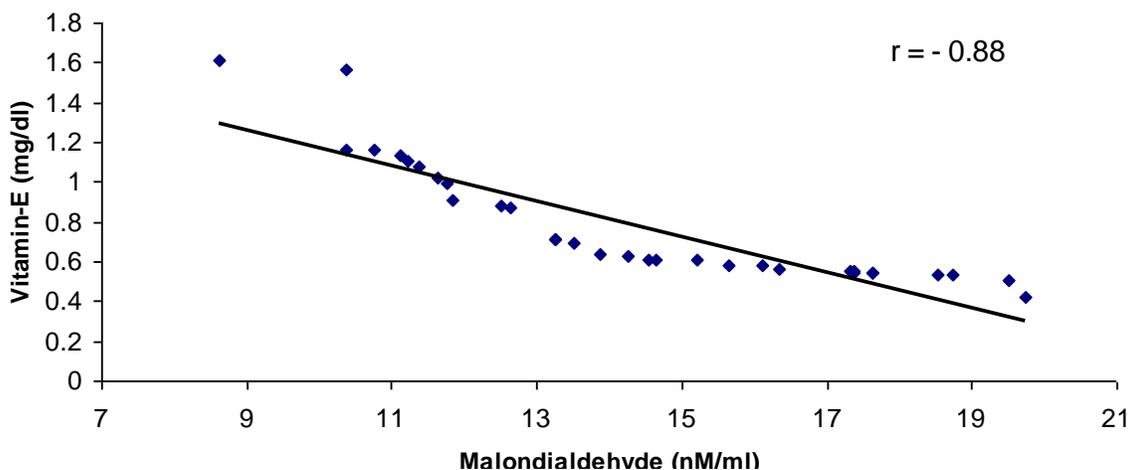


Figure 5. Correlation of malondialdehyde versus vitamin-E in diabetic patients with myocardial infarction.

vitamin-E in diabetic patients with myocardial infarction ($r = -0.88$) (Figure 5).

Significant positive correlations were observed between fasting blood glucose and serum fructosamine ($r = 0.96$), systolic blood pressure ($r = 0.91$), serum AGEs ($r = 0.94$) and between systolic blood pressure and serum AGEs ($r = 0.89$) in diabetic patients with myocardial infarction.

DISCUSSION

In the present study, the increased levels of malondialdehyde and decreased vitamin-E clearly show that diabetic patients, irrespective of the gender, were exposed to an increased oxidative stress via lipid peroxidation. This could be due to failure of antioxidant defense system; the ROS accumulates and initiates lipid peroxidation (Kunitomo, 2007). In the present study, antioxidant defense mechanism by vitamin-E and

oxidative stress causing agent by malondialdehyde in older diabetic and non-diabetic patients with and without myocardial infarction, were investigated. Malondialdehyde levels were significantly higher ($P < 0.001$) in older diabetic patients with or without myocardial infarction and older non-diabetic patients with myocardial infarction as compared with older control subjects. In addition, serum vitamin-E was found to be significantly decreased ($P < 0.001$) in older diabetic patients with and without myocardial infarction as compared with older control subjects. Present results are consistent with earlier reports indicating an elevated level of serum lipid peroxide and diminished antioxidant status in diabetic patients (Senthil et al., 2004).

Decreased antioxidant activity and high concentration of lipid peroxidation product may lead to oxidation proteins resulting in myocardial infarction. Our results confirm previous data of enhanced ROS levels in diabetes mellitus (Schleicher and Friess, 2007). Yan et

al. (1994) showed that interaction of AGEs with endothelial cells leads to oxidative stress by a receptor-mediated process. AGE might induce oxidative stress through chemical and cellular mechanisms. In addition to the monosaccharides, the AGEs have also been reported to be produced from dicarbonyl compounds derived from the Millard reaction, autoxidation of sugars and other metabolic pathways for example, glycolysis, and this can account for the increase in the serum AGE in non-diabetic patients with myocardial infarction (Peppia et al., 2004).

Recent studies have brought new insights into broad derangements in non-enzymatic glycation involving not only carbohydrates but also lipids present in diabetes, uremia and atherosclerosis (Chuyen, 2006). Increased level of AGE content in diabetic state was reported earlier (Ahmed, 2005). The results of this study are also being in accordance with these reports. Therefore, it can be speculated that the AGE structures resulting from persisting hyperglycemia are more profusely formed in diabetes and the fact that tissue levels of AGE correlate with prevailing serum concentrations of glucose, fructosamine and glycated hemoglobin, points to a role for hyperglycemia, yet there is good evidence that other carbohydrates such as ascorbate, pentoses may act as potent glycoating agents (Dyer, 1991).

People with diabetes are prone to long-term complications such as myocardial infarction, and development of such complications is a major cause of morbidity and mortality and an ever-increasing burden to healthcare authorities in both developed and developing nations (Veiraiyah, 2005). Epidemiological studies have confirmed that hyperglycemia is the most important factor in the onset and progress of vascular complications in diabetes (Shera, 1998). The formation of AGEs correlates with glycemic control. The AGE hypothesis proposes that accelerated chemical modification of proteins by glucose during hyperglycemia contributes to the pathogenesis of diabetic complications, including atherosclerosis (Yamagishi et al., 2007).

Glycation has both physiological and pathophysiological significance. Under physiological conditions, glycation can be detected in the ageing process, and the reactions are more rapid and more intensive with frequently increased glucose concentrations (Ulrich and Cerami, 2001). The AGE concept proposes that chemical modification and crosslinking of tissue proteins, lipids and DNA affect their structure and function. This in turn contributes to a gradual decline in tissue function and to the pathogenesis of myocardial infarction in diabetic and in non-diabetic patients (Xanthis et al., 2007; Kanauchi et al., 2001). AGEs have previously been shown to accumulate in many tissues with age, independently of diabetes (Lingelbach et al., 2000). Since the body does not contain any single enzyme capable of AGE structure degradation, AGEs accumulate during the biological life

of proteins on which they had been formed (Yan et al., 2006). In addition to the diabetic patients, the serum AGEs was also found to be higher in the older non-diabetic patients with myocardial infarction as compared with older diabetic patients without myocardial infarction, however, this increase was not significant. It states that the role of AGEs in diabetic and non-diabetic patients is potentially important because it induces both the structural and functional implications.

Environmental conditions can result in the formations of various AGEs by a variety of chemical reactions, and the reasons for the formation of such structures in non-diabetic conditions are difficult to explain. Studies have suggested the role of oxidative stress in the formation of AGEs structures, therefore, it might be postulated that reactive oxygen intermediates may accelerate the rate of AGE formation through reactive oxoaldehydes and vice versa; AGEs might induce oxidative stress through chemical and cellular mechanisms (Basta et al., 2005; Miyata et al; 2003). The observations of older groups increased as compared with that of young normal subjects.

Conclusion

Thus, the results of this study support the hypothesis that AGEs may have an important role in myocardial infarction, which in diabetic patients occur much earlier than in those without diabetes. This study also revealed that increased AGEs associated with oxidative stress in the older groups. Taken together the above facts and results, it can be postulated that utilization of antioxidant rich food, along with low AGEs content diet, may be beneficial in delaying myocardial infarction progression, particularly in diabetic subjects.

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Full Length Research Paper

Liquid nitrogen cryotherapy vs. Betamethasone lotion in the management of Alopecia areata

Faghihi Gita^{1*} and Radan Mohammadreza²

¹Department of Dermatology, Skin and Leishmaniasis Research Center, Isfahan University School of Medicine, Isfahan University of Medical Sciences, Iran.

²Supervision Council in Clinical Practice, Isfahan University of Medical Sciences, Iran.

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Alopecia areata (AA) is a common disease of hair follicles. Cryotherapy has been employed to stimulate hair regrowth. We decided to evaluate the efficacy of this method in patchy AA. Forty patients of age >5 years and with 120 recalcitrant patches were enrolled in this study. AA had covered less than 25% of patients' scalps. In each individual, one lesion was treated with nitrogen cryotherapy once weekly for a period of 6 weeks; meanwhile another lesion was treated with topical 0.1% betamethasone lotion. All patients were followed up from weeks 2 to 14. Thirty-nine out of forty patients completed therapy. The overall response rate was 88% for patches treated with cryotherapy and 90% for patches treated with betamethasone lotion. Complete recovery (>90% terminal hair growth) was not obtained in any patient. Chi-square test showed no significant difference between improvements of alopecia in the two groups (reliability 95%). Pearson measurement showed a significant reverse relationship between diameter and duration of patches and the improvement rate in both groups. Liquid nitrogen cryotherapy can be a helpful modality in the treatment of AA.

Key words: Alopecia areata, treatment, liquid nitrogen cryotherapy, betamethasone lotion.

INTRODUCTION

Alopecia areata (AA) is a chronic inflammatory disease of the hair follicle usually manifesting as round or ovoid patchy areas that show hair loss with discrete borders. AA is common but unfortunately has no uniformly successful form of therapy (Schwartz and Janniger, 1997; McElwee, 1999). It is estimated to affect almost 2% of the U.S. population (Safavi et al., 1989). Mild limited involvement of the scalp is the most common presentation; multiple patches may become confluent over time. Regression may occur, with new hair growth taking place; recurrences in different locations occur. More severe forms of the disorder, involving the entire scalp, eyebrows, eyelashes, axillary, pubic areas or the entire body also exist (Madani and Shapiro, 2000; Sharma et al., 1996; Shapiro and Madani, 1999).

Alopecia areata (AA), especially when severe, often profoundly affects the lives of those afflicted. Patients with AA that have a history of atopy may have a less favorable prognosis (Tosti et al., 1994).

Current investigative efforts strongly implicate CD4 and CD8 T-cell lymphocytes in the etiology of this disorder. The CD4⁺ killer T cell is an effector cell which causes hair bulb injury, triggering the AA (Todes-Taylor et al., 1984; McElwee, 1999).

Though autoantibodies are postulated to play an integral role in the disease process, current research implicates a cell-mediated autoimmune mechanism as the underlying pathogenic etiology. CD44v10 is believed to be involved in the activation mechanism of CD4 and CD8 lymphocyte migration into tissue and the initiation of the subsequent defense response against antigenic stimuli (Gilhar et al., 1998). Supporting this theory is the fact that activated CD4 and CD8 T lymphocytes have been found in a characteristic peri- and intra-follicular

*Corresponding author. E-mail: g_faghihi@med.mui.ac.ir.

Table 1. The improvement rate of lesions in the cryotherapy group.

Number of lesions (%)	Terminal hair growth (%)
0	<90
23	75 - 90
33.5	50 - 75
31.5	25 - 50
12	> 25

Table 2. The improvement rate of lesions in the Betamethasone group.

Number of lesions (%)	Terminal hair growth (%)
0	<90
28	75 - 90
34.5	50 - 75
27.5	25 - 50
10	> 25

inflammatory infiltrate of anagen hair follicles.

MATERIALS AND METHODS

Inclusion criteria

1. Age >5 years.
2. AA coverage less than 25% of scalp area.
3. Presence of at least two symmetrical patches over the scalp.
4. Maximum diameter of each lesion less than 7 cm.
5. Absence of any other severe medical illness.
6. No simultaneous immune-suppressive use and discontinue of any other treatment at least 4 weeks earlier.

Exclusion criteria

1. Pregnancy.
2. Lactation.
3. Any newly onset medical systemic illness.
4. Progression of AA into more than 25% of scalp area.
5. Severe hemorrhagic bulla or any documented hypersensitivity to each of the procedures.

In each individual, one lesion was treated with nitrogen cryotherapy once weekly for a period of 6 weeks. The patients were matched by their ages and sexes. A cryogun was used to spray the liquid nitrogen to the area for 2 to 3 s, until it became slightly frozen. After the frozen area thawed (about 3 to 5 s), a second spray was done in the same manner. Meanwhile another lesion on the same patient was treated by topical 0.1% betamethasone lotion twice daily for 6 weeks. All patients signed the consent and were followed up from weeks 2 to 14, fortnightly.

The side effects and amount of hair regrowth during the study were recorded. The evaluation was performed with clinical examination. According to the extent of terminal hair growth, the lesions were grouped into 4 categories:

1. Good response (regrowth of >75% terminal hair).
2. Moderate response (regrowth of 50 to 75% terminal hair).
3. Poor response (regrowth of 25 to 50% terminal hair).
4. No response (regrowth of less than 25% terminal hair).

RESULTS

Thirty-nine out of forty patients (17 men, 22 women) completed the study. The patients' ages ranged from 9 to 58 years. The mean age was 22 ± 2.5 years. The duration of disease ranged from 3 months to 10 years. The average disease duration was 8 months. The two groups were not significantly different in their demographic characteristics, and in the sizes and patterns of patches at baseline (Tables 1 to 4).

The average diameter of lesions in cryo group was not statistically different from that of the other group, and was 3 to 3.5 cm. The partial hair regrowth was seen in 88% of patches in the cryotherapy group and 90% of patches in the other group. No one attained full hair regrowth in each group.

One patient (2.5%) discontinued the treatment by cryotherapy due to pain during cryo spraying and dissatisfaction.

The results in lesions treated with cryotherapy, were composed of:

1. Good response 23% (regrowth of >75% terminal hairs).
2. Moderate response 33.5% (regrowth of 50 to 75% terminal hairs).
3. Poor response 31.5% (regrowth of 25 to 50% terminal hairs).
4. No response 12% (regrowth of less than 25%).

And in the other group of lesions treated by topical betamethasone the results were as follows:

1. Good response 28% (regrowth of >75% terminal hairs).
2. Moderate response 34.5% (regrowth of 50 to 75% terminal hairs).
3. Poor response 27.5% (regrowth of 25 to 50% terminal hairs).
4. No response 10% (regrowth of less than 25%).

A comparison of improvement rate of the results of the two groups showed no significant difference ($p=0.75$) with Chi-Square test (reliability co-efficiency 95%).

The response of lesions according to the duration of disease was as follows:

Cryotherapy group

Good response

1. The lesions below 6 months: 46.5%

Table 3. The relationship between lesion diameter and response rate in the cryotherapy group.

Percentage of improvement	Lesion diameter		
	<2.5cm	2.5-5 cm	>5 cm
Good response (Growth of more than 75% of terminal aesthetically acceptable hair)	45%	35.5%	14%

Pearson measurement test analysis: (P=0.03); r=- 0.25.

Table 4. The relationship between lesion diameter and hair regrowth rate in the betamethasone group.

Percentage of improvement	Lesion diameter		
	<2.5 cm	2.5-5 cm	>5 cm
Good response (Growth of more than 75% of terminal aesthetically acceptable hair)	46.5%	34%	15%

Pearson measurement test analysis: p=0.025; r= - 0.8.

Table 5. The hair regrowth rate according to lesion duration in the cryotherapy group.

Percentage of improvement	Lesion duration		
	<6 months	6-24 months	>24 months
Good response (Growth of more than 75% of terminal aesthetic acceptable hair)	46.5%	37.5%	16%

Pearson test analysis; (P=0.037); r= - 0.25.

- The lesions between 6 and 24 months duration: 37.5%.
- The lesions above 24 months: 16%.

Betamethasone group

Good response

- The lesions below 6 months: 48.5%.
- The lesions between 6 and 24 months duration: 32%.
- The lesions above 24 months: 18.5%.

Pearson measurement test analyses showed that there was a reverse relationship between lesion duration and the rate of hair regrowth ($r = -0.25$, $p=0.037$), ($r = -0.75$, $p=0.045$), in the two groups sequentially. Besides that, in each group, there was a significant improvement compared to the baseline status (Tables 4 to 7). The response of lesions according to the diameter of patches was as follows:

Group 1 (Cryotherapy)

Good response

- The lesions below 2.5 cm diameter: 45%
- The lesions between 2.5 to 5 cm diameter: 35.5%
- The lesions above 5 cm diameter: 14%.

Topical betamethasone group

Good response

- The lesions below 2.5 cm diameter: 46.5%.
- The lesions between 2.5 to 5 cm diameter: 34%.
- The lesions above 5 cm diameter: 15%.

Pearson measurement test analyses showed that there was a reverse relationship between lesion diameter and rate of hair regrowth ($r= -0.25$, $p=0.03$), ($r= -0.8$, $p=0.025$), in the two groups sequentially (Tables 3 and 4). Of course, in each group there was a significant improvement compared to the baseline status.

The recurrence (by definition)

Loss of more than 50% of hairs or increase of more than 50% in the extent and size of the patches after good terminal hair regrowth (defined previously), occurred in 41% of group A and 68% of group B. On the other hand, betamethasone-treated patches were more easily prone to recurrences than cryotherapy-treated ones {(Chi-Square (reliability coefficient 95%) ($p<0.05\%$, $p=0.02$))}.

The most frequent side effect in the cryotherapy group was short term transient erythema and some mild stinging pain. There were no significant or dangerous side effects during nitrogen application or in the betamethasone treated group. Only in one patient of the

Table 6. The improvement rate according to lesion duration in the betamethasone group.

Percentage of improvement	Lesion duration		
	<6 months	6-24 months	>24 months
Good response (Growth of more than 75% of terminal aesthetic acceptable hair)	48.5%	32%	18.5%

Pearson test analysis: (P=0.045); r= -0.75.

Table 7. Patients demographic and lesions characteristics.

Sex	Population (no.)	Mean age (years)	Duration (months)
Male	17	19.5	14
Female	22	24	9

cryotherapy group was the treatment discontinued due to dissatisfaction and painful sensation. Therefore, the adverse events were mild and except for one patient; they did not warrant discontinuation of therapy. In addition, no dyspigmentation occurred in areas treated by liquid nitrogen cryotherapy at the end of this trial.

DISCUSSION

Various therapeutic agents have been described for the treatment of AA, but none are curative or preventive. Cryotherapy has also been employed to stimulate hair growth in AA (Hong et al., 2006; Hyung et al., 1994).

One study utilizing both children and adults, revealed hair regrowth in greater than 60% of affected areas in 70 of 72 patients. In that study still, after 6 months only 3 out of 66 patients had recurrences. The average size of their studied lesions was 2×3 cm (Lei et al., 1991). In this study, 23% of lesions showed a good response (regrowth of >75% terminal hairs) and 33.5% showed only a moderate response (that is, regrowth of 50 to 75% terminal hairs) in cryotherapy mode of treatment. The average diameters of our studied lesions were 3 to 3.5 cm sequentially in the two groups.

The cause of such significant difference could be racial variation of response in autoimmune diseases, criteria of inclusion for example severity of hair loss at the start of trial, or a technical difference in mode of treatment.

In another Korean study, those over 50 years of age and with more than 3 weeks of treatment interval seemed to have a relatively poor response rate. Other patient related factors such as sex and age or demographic characters were not significant. There were no significant side effects, except slight pain, swelling and erythema (Hong et al., 2006). The overall improvement rate of the Korean study was significantly higher than that of the present study.

In the opinion of Hyung et al. (1994), the overall respondents were 22 (66.7%). There seemed to be good response rates of 70.0% in females and 71.4% in AA

multiplex. These data showed a better outcome than that of the present study and the difference may be due to shorter intervals of cryo application (Hyung et al., 1994).

The best mechanisms explaining the efficacy of cryotherapy in AA are as follows:

1. Vascular changes
2. Immunomodulation

According to Hong et al. (2006), if cryotherapy is applied to diseases of the hair superficially, one can expect regrowth of the hair. The supposed mechanism is that cryotherapy dilates the vessels around hair follicles, thus improving follicular nutritional status. Their report showed that superficial cryotherapy promotes eyebrow hair growth in several patients with AA.

On the whole, nitrogen cryotherapy in patients with AA can be an effective treatment. It is a simple and convenient method, and has a relatively good therapeutic response with fewer side effects. We have reported our recent experience, which shows that cryotherapy with liquid nitrogen appears to promote hair growth in patients with AA.

Cryotherapy in AA may be regarded as a safe, efficacious and easily available treatment.

Conclusion

We recommend the use of superficial cryotherapy as a helpful and practically effective treatment for patients with a mild, isolated form of AA, especially in children who are vulnerable to side effects of immunosuppressive drugs. Of course in future there is a plan for a longer follow up to see if there is any sustained result from cryotherapy.

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Full Length Research Paper

Revealing the effect of probiotic combination: *Lactobacillus rhamnosus* and *Lactobacillus acidophilus* (Lacidofil®) on acute diarrhea in adult patients

Marcellus Simadibrata^{1*}, Suzanna Ndraha², Robert Tedjasaputra³, Ari Fahrial Syam¹, Achmad Fauzi Aan Santi¹ and Aziz Rani¹

¹Gastroenterology Division, Department of Internal Medicine, Faculty of Medicine, University of Indonesia/Cipto Mangunkusumo Hospital Jakarta, Indonesia.

²Department of Internal Medicine Koja Hospital Jakarta, Indonesia.

³Department of Internal Medicine, Tarakan Hospital Jakarta, Indonesia.

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The concept of taking probiotic supplementation for better health and improving acute episodes of diarrhea has been well-known. Several studies have indicated that probiotic supplementation bring beneficial effects for patients with diarrhea. However, there is a striking lack of evidence on the supplementation of probiotic products in adult patients with acute diarrhea and prescribing probiotics for acute diarrhea has been underestimated. A prospective, double-blind, randomized, controlled clinical trial was conducted in adult patients who visited the study sites at three hospitals (Cipto Mangunkusumo, Koja and Tarakan Hospital) in Jakarta between 2007 and 2010. The subjects were divided into 2 groups, that is, the treatment group receiving *Lactobacillus* spp. product at the dose of 3 × 2 capsules for 7 days and the control group receiving placebo. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software program. The p value of < 0.05 was considered significant. The study demonstrated a significant result of faster recovery from diarrhea 1.02 ± 0.48 days sooner than the placebo group (p = 0.018). Greater improvement on clinical outcomes (frequency of stools, stool consistency, abdominal pain, nausea, vomiting, bloating, headache, fever and tenesmus) were observed in the group receiving *Lactobacillus* spp. supplementation; however, the result was statistically significant on stool consistency only. Combined probiotic supplementation appears to be a promising therapeutic agent for treatment of acute diarrhea in adults. However, further controlled clinical studies as well as good storage, handling and distribution is essential to explore the therapeutic benefit for possible practical clinical application.

Key words: *Lactobacillus rhamnosus*, *Lactobacillus acidophilus*, probiotic supplementation, acute diarrhea, adults.

INTRODUCTION

As one of developing countries with poor hygiene and sanitation, Indonesia has a considerable morbidity and mortality rate for diarrheal diseases (Unicef Indonesia, 2012; World Bank-Agriculture and Rural Development, 2012; USAID Indonesia, 2012). Acute diarrhea is one of the most common diagnoses in daily clinical practice (Hendarwanto, 1996). It is often a primary sign and

symptom of gastrointestinal tract disorder which may cause serious problems both in children and adults. The symptom ranges from mild and self-limiting to severe, which may become a common cause of death in developing countries. Worldwide, acute diarrhea causes over 5 million deaths per year.

In developing countries, it remains the second most common cause of death in children. It also causes a substantial problem in adult patients. With over 600 million international trips being taken annually, the traveler's diarrhea in adults would be a special problem.

*Corresponding author. E-mail: marcellus_sk@yahoo.com.

Therefore, a prompt and effective treatment to manage acute diarrhea is critical in reducing the high morbidity and mortality rate. Furthermore, there are great concerns on the use of antibiotics in managing acute diarrhea, as most of infectious diarrhea cases are viral. Inappropriate use of antibiotics will disturb the gastrointestinal flora, which may cause a longer duration of diarrhea, greater side effect and lead to the development of antibiotic resistance (Manatsathit et al., 2002; Rani, 2002; Field, 2003; Qadri et al., 2005; Oyofe et al., 2002). Thus, oral rehydration therapy, ongoing fluid replacement and nutritional support, including prescribing probiotic supplementation to promote recovery from acute diarrhea are parts of the core foundation treatment for acute diarrhea.

The concept of taking probiotic supplementation for better health and improving acute episodes of diarrhea is not new. Several studies have indicated that probiotic supplementation may normalize the gut microflora, which is an important protection of the host against gastrointestinal (GI) tract diseases (De Roons and Katan, 2000; van Niel et al., 2002; Tiaskal et al., 1995, 2005; Reid et al., 2003; Guandalini et al., 2000; Shornikova et al., 1997; Guerrant et al., 2001).

Other studies have provided evidences that the supplementation of probiotic products containing *Lactobacillus acidophilus* and other *Lactobacillus* spp has significantly shortened the duration of diarrhea and offered faster recovery from diarrhea 1 day sooner in children and infants (Canani et al., 2007; Agustina et al., 2007; Sazawal et al., 2006; Hatta et al., 2011; Putra et al., 2007). Several clinical studies have demonstrated that probiotic combination of *L. rhamnosus* and *L. acidophilus* (Lacidofil®) as an effective supplement for various gastrointestinal diseases in children (Foster et al., 2011). However, many probiotic supplementation studies have yielded conflicting results, and prescribing probiotics for acute diarrhea has been underestimated. Moreover, there is a striking lack of evidence on the supplementation of probiotic products in adult patients with acute diarrhea. To our knowledge, this is the first study in Indonesia that investigated the effect of combined probiotic agents on acute diarrhea in adult patients.

The aim of our study was to reveal the beneficial effect of combined probiotic product of *L. rhamnosus* and *L. acidophilus* (Lacidofil®) on acute diarrhea in adult patients including shorter diarrhea duration, improved stool frequency and consistency, as well as improved abdominal and other symptoms compared to placebo.

MATERIALS AND METHODS

Study subjects and design

A prospective, double-blind, randomized, controlled clinical trial was conducted at three hospitals (Cipto Mangunkusumo, Koja and Tarakan Hospital) in Jakarta between 2007 and 2010. Those

hospitals are public hospitals funded by Indonesian government and Cipto Mangunkusumo Hospital is the top National Referral Center Government Hospital. Subjects enrolled in our study were eligible patients who visited the three hospitals during the study period. The inclusion criteria were: (1) patients with acute diarrhea; (2) aged 13 to 60 years; and (3) did not have severe complications. Acute diarrhea was defined as the passing of three or more loose or watery stools (> 200 mL/h) within a 24 h period, lasting less than 14 days. Patients were excluded from study if: (1) they had a serious complication (renal failure, metabolic acidosis, severe dehydration, hypovolemic shock, heart failure, vomiting, anorexia); (2) had a history of allergy to *Lactobacillus* spp.; (3) had diarrhea caused by amoeba, colorectal cancer or tumor; (4) had exclusion criteria with a proven cause of diarrhea, amoeba, colorectal cancer or tumor; (5) were taking antibiotic therapy.

The subjects were divided into 2 groups. The first group of 38 patients received oral rehydration salt (Oralit®) and the probiotic combination product of *Lactobacillus* spp. (*L. rhamnosus* and *L. acidophilus*) at the dose of 3 × 2 capsules for 7 days. Each probiotic capsule contained a combination of two strains, that is *L. rhamnosus* R0011 (1.9×10^9 CFU) and *L. acidophilus* R0052 (0.1×10^9 CFU); while, the other group (n = 37) received oral rehydration salt and placebo capsule at equal dose of 3 × 2 capsules for 7 days. The subjects received capsules of *Lactobacillus* spp. or placebo for 7 days. They were also asked to contact the investigators if diarrhea recurred in follow-up period of 1 week. The treatment was administered as early as possible after the informed consent had been obtained. There was no minimal time interval in patient between the beginning of the symptoms and the administration of the *Lactobacillus* spp. capsules.

Clinical presentation of illness before and after treatment, including symptoms of diarrhea, abdominal complaints and other complication were recorded for all the cases. Laboratory examinations of blood and stool analysis were carried out. Blood specimens were collected for blood analysis (hemoglobin level, leukocyte count, hematocrit, platelet count, ureum and creatinine level) to exclude any complication. Stools were collected from each subject for routine stool analysis to determine whether the diarrhea was caused by infectious agents, such as bacteria, amoeba or fungi. The stool consistency and the severity of diarrheam, as well as other symptoms (abdominal pain, nausea, vomiting, bloating, tenesmus, headache and daily activity disturbance) were monitored throughout the study period and rated on a 4-point scale: no symptom (0), mild symptom (1), moderate symptom (2), and severe symptom (3). The symptoms of fever and thirst were self-reported and recorded as positive and negative results.

Statistical analysis

Data was entered in specific forms (case report forms/CRFs) of data Epi template. Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software program to determine the statistical differences between study groups. Independent t-test was used to determine the mean value and 95% confidence interval for the duration of diarrhea. The p value of < 0.05 was considered significant.

Ethical approval

The study protocol had been approved by the Ethical Review Committee of Medical Faculty, University of Indonesia

RESULTS

A total of 90 patients (n = 45 for each group) were initially

Table 1. Socio-demographic characteristics of patients receiving *Lactobacillus* spp. or placebo.

Characteristics	<i>Lactobacillus</i> Spp (%) (n = 38)	Placebo (%) (n = 38)	P value
Sex			
Male	15	16	1.000
Female	23	22	
Age: Mean ± SD (years)	32.74±12.65	35±13.58	0.869
Ethnicity			
Javanese	18	18	0.665
Sundanese	6	5	
Aceh	1	0	
Batak	4	1	
Padang	2	2	
Bugis/Makassar	1	1	
Timor	1	0	
Ambon	1	0	
Betawi	2	6	
Chinese	1	1	
Others	1	4	
Type of diarrhea:			
Infectious*	8	7	0.914
Non-infectious	36	25	

*Infectious diarrhea was defined based on positive laboratory test results revealing the causative infectious agents, such as bacteria, amoeba or fungi.

enrolled in our study and included in the per-protocol analysis. However, only 76 patients (n = 38 for each group) had completed the study, who were included in the intention-to-treat (ITT) analysis. Socio-demographic characteristics of our subjects are presented in Table 1. Both groups were similar with respect to most characteristics. Most subjects were female, Javanese and had suffered from non-infectious diarrhea.

The per-protocol analysis indicated that the duration of diarrhea in group 1 (*Lactobacillus* spp.) was significantly shorter than group 2 (placebo) with p-value of 0.018 (Table 2). The results demonstrated faster recovery from diarrhea 1.02 ± 0.48 days sooner than the placebo group. The recovery from diarrhea was determined by complete decline of clinical symptoms (stool consistency, number of stool and other symptoms such as stomach pain, vomiting, fever, and lack of appetite). Greater recovery of stool frequency on day 0 to 3 was also noticed in group 1 (*Lactobacillus* spp.) than the placebo group; however, such improvement was not statistically significant (p > 0.05). Better improvement of stool consistency was observed in group 1 compared to placebo group during the study period. Although the difference was statistically significant on day 2 (p = 0.049) and day 5 (p = 0.048), but no significant differences were observed on the other days (Table 2).

In *Lactobacillus* spp. group, there was improvement of other clinical symptoms comparing day 0 and day 7 significantly (p < 0.001) (Table 3). Faster improvement on some clinical outcomes were observed in group 1 (*Lactobacillus* spp.) than the placebo group when comparing the complaints on Day 0 and 7. Only the stool consistency in *Lactobacillus* spp. group was improved faster than in placebo group significantly (p = 0.022) (Table 3). No serious adverse events was found both in *Lactobacillus* spp. group and the placebo group.

DISCUSSION

Most of our study subjects were female, Javanese and had suffered from non-infectious diarrhea. Our findings seem to be consistent with other studies which also found acute non-infectious diarrhea predominantly in women. Conversely, other studies have found inconsistent results which demonstrated that acute diarrhea is also found in men (Supcharassaeng and Suankratay, 2011; Guandalini, 2012). As one of tropical developing countries, we could expect that there would be more infectious cases found. However, in this study, we observed a majority of non-infectious acute diarrhea cases. In contrast, contradictory results were observed by

Table 2. Clinical outcome of patients receiving *Lactobacillus* spp. or placebo.

Parameter	<i>Lactobacillus</i> spp. (Lacidofil®)	Placebo	p value per protocol analysis
Duration of diarrhea			
Mean (days) ± SD	2.45±1.60	3.47±2.08	0.018
Mean (hours) ± SD	58.74±38.54	83.37±49.83	
Frequency of stools (Mean value ± SD)			
Day 0*	2.79±0.96	3.05±0.87	0.215
Day 1	1.53±0.89	1.68±1.04	0.480
Day 2	1.18±0.61	1.26±0.86	0.646
Day 3	1.03±0.64	0.97±0.72	0.736
Day 4	1.08±0.43	1.03±0.49	0.620
Day 5	1.03±0.54	1.00±0.61	0.837
Day 6	1.00±0.61	0.95±0.40	0.659
Day 7	1.00±0.57	0.95±0.40	0.642
Stool consistency (Mean value ± SD)			
Day 0	1.50±0.51	1.68±0.47	0.105
Day 1	0.86±0.65	1.12±0.77	0.133
Day 2	0.47±0.56	0.78±0.71	0.049
Day 3	0.37±0.55	0.37±0.56	0.953
Day 4	0.13±0.35	0.23±0.49	0.332
Day 5	0.06±0.23	0.24±0.50	0.048
Day 6	0.03±0.17	0.15±0.36	0.083
Day 7	0.03±0.16	0.15±0.36	0.071

*Day 0 corresponds to the first 24 h period from the initiation of treatment.

Table 3. Improvement of other clinical outcomes of patients receiving *Lactobacillus* spp. or placebo.

Parameter	<i>Lactobacillus</i> spp. (Mean ± SD)		Placebo (Mean ± SD)		p value
	Day 0	Day 7	Day 0	Day 7	
Frequency of stools	2.79±0.96	1.00±0.57	3.05±0.87	0.95±0.40	1.000
Stool consistency	1.50±0.51	0.03± 0.16	1.68±0.47	0.15±0.36	0.022
Abdominal pain	1.53± 0.86	0.13±0.58	1.82±1.11	0.13±0.41	0.820
Nausea	1.42±0.95	0.08±0.36	1.45±0.95	0.05±0.23	0.570
Vomiting	0.82±1.04	0.03±0.16	1.26±1.11	0.03±0.16	0.921
Bloating	1.32±0.87	0.16±0.55	1.21±1.07	0.16±0.55	0.964
Tenesmus	0.68±0.87	0.08±0.36	0.53±0.95	0.000	0.615
Headache	1.13±1.04	0.11±0.39	1.24±1.02	0.05±0.23	0.740
Fever (%)	52.6	5	36.8	7.9	0.276
Thirsty (%)	65.8	28	65.8	18.4	0.271

other investigators in developing countries (Supcharassaeng and Suankratay, 2011; Wanke, 2011; Okeke et al., 2003). Due to the limited coverage of our study sites, which are the top referral hospitals in our country, it is not possible to extrapolate our findings as a representation of overall condition in the population.

Our study demonstrated that supplementation of combined probiotic product (*L. rhamnosus* and *L.*

acidophilus (Lacidofil®) has shortened the duration of diarrhea 1.02 ± 0.48 days sooner than the placebo group. Similar result was also observed in other studies and has been discussed in a meta-analysis on acute diarrhea in children (De Roons and Katan, 2000; Van Niel et al., 2002; Tlaskal et al., 1995, 2005; Reid et al., 2003; Guandalini et al., 2000; Shornikova et al., 1997; Sazawal et al., 2006). Furthermore, several studies have also

reported that probiotic supplementation of *Lactobacillus* spp. may accelerate healing process leading to the recovery of acute diarrhea in children and adults (Van Niel et al., 2002; Tlaskal et al., 2005; Guandalini et al., 2000; Shornikova et al., 1997; Canani et al., 2007).

Some investigators found that children with acute gastroenteritis treated orally with the probiotic combination of *L. acidophilus* and *L. rhamnosus* experienced significantly faster improvement of stool consistency compared to the control group ($p < 0.003$). They demonstrated that improved stool consistency was observed in 4 ± 2.02 h for children treated orally with probiotic products and the improvement was observed in 5.45 ± 2.33 h for those without probiotic treatment (Tlaskal et al., 2005).

Several studies have indicated that Lactobacilli may bring remarkable recovery of acute diarrhea and improved clinical outcomes as a result from some mechanisms, such as by (1) binding the intestinal mucosa receptors and preventing pathogen adhesion and invasion to the intestinal epithelium; (2) stimulating immune response and also (3) producing protective immunomodulatory effects by increasing Ig A secretion (Tlaskal et al., 2005; Reid et al., 2003). Moreover, a systematic review and meta-analysis suggests that probiotics are associated with a reduction in antibiotic-associated diarrhea (Hempel et al., 2012).

In another study, however, the beneficial effect of probiotic supplementation on the recovery of acute diarrhea were not observed compared to placebo (Ritchie et al., 2010). Greater improvement on clinical outcomes (frequency of stools, stool consistency, abdominal pain, nausea, vomiting, bloating, headache, fever and tenesmus) were observed in the group receiving *Lactobacillus* spp. supplementation; however, the result was statistically significant on stool consistency only. Similar results were found in other studies (De Roons and Katan, 2000; Van Niel et al., 2002; Tlaskal et al., 1995, 2005; Reid et al., 2003; Guandalini et al., 2000; Shornikova et al., 1997).

In contrast, significant improvements were observed in children with gastrointestinal disease treated with probiotic supplementation including stomach pain, vomiting, fever and loss of appetite. Children who received probiotic supplementation of *L. acidophilus* had significant faster recovery than those without the probiotic (Tlaskal et al., 1995, 2005). No serious adverse event was found in our study, since acute diarrhea is usually mild and self-limiting. Moreover, placebo treatment may bring therapeutic-like effect in 40 to 50% cases. Other randomized-control trials (RCTs) have also shown similar results (Guandalini, 2012; Hatchette and Farina, 2011; Patro et al., 2010).

The limitation of our study includes inadequate laboratory examination such as stool culture and viral test to reveal the infectious causative agents of acute diarrhea, which could not be performed due to limited budget and facilities; therefore, infections was defined based on

history, physical examination and positive laboratory test results of blood test and routine stool analysis.

Conclusion

Our study has demonstrated the beneficial effect of combined probiotic supplementation of *L. rhamnosus* and *L. acidophilus* on improving the clinical outcomes of acute diarrhea and shortening the duration of diarrhea in adult patients. The results are consistent with the well-known, reproducible capability of *Lactobacillus* spp. to shorten the duration of diarrhea by approximately 1 day.

Combined probiotic supplementation appears to be a promising therapeutic agent for treatment of acute diarrhea in adults. However, there is concern that although probiotics are considered nonpathogenic, it may be infective when the patient is severely ill or immunosuppressed. Moreover, some factors should be considered, including the safety, strain selection, product stability and formulation. It should always be kept in mind that probiotic must stay viable to bring the beneficial effect and its viability is affected by temperature, humidity and oxygen concentration. The product should also be delivered in right dose. Lower dose of probiotic in the market may not provide the beneficial effects as reported in clinical studies. Therefore, further controlled clinical studies, as well as good storage, handling and distribution is essential to explore the therapeutic benefit for possible practical clinical application.

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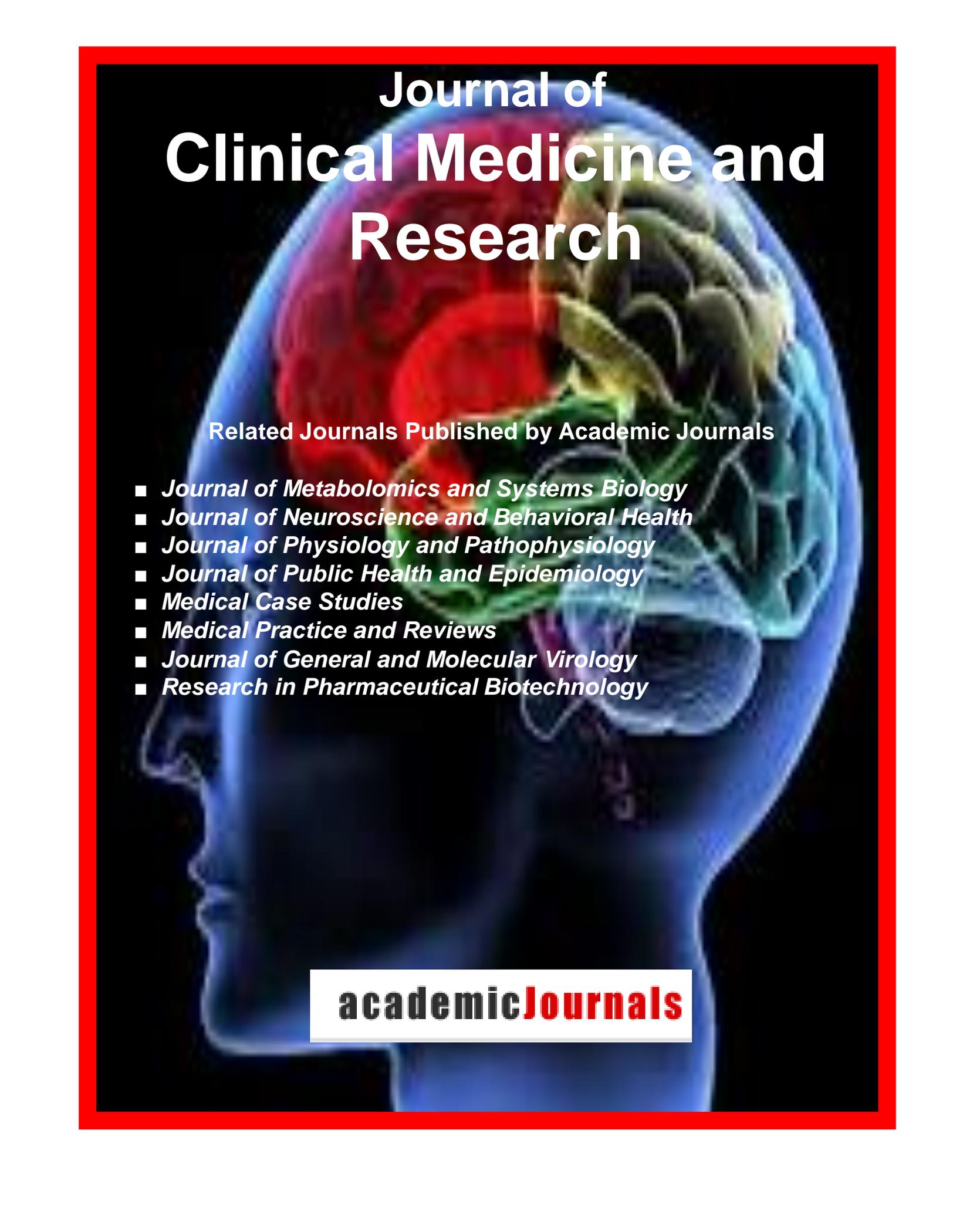
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