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Histochemical And Immunohistochemical Studies on The Effect Of Melatonin On Experimental Atherosclerosis In The Aorta of Rabbit

Mattar FE*; Ismael AA*; Kasem MA and Ibrahim KI****
From the Department of Histology, Al-Azhar Faculty of Medicine in Cairo and in Assiut***

Abstract

Treatment of atherosclerosis or its prevention still represents one of the difficult challenges to modern medicine. Free radical peroxidation of LDL has been proposed to have a primary effect on the onset of diet-induced atherosclerosis. Melatonin has been shown to be potentially effective in prevention of some disorders in which free radical processes are involved. The purpose of this study was to investigate the possible protective effects of melatonin on high cholesterol fed animal arteries. Assessment of the atherosclerosis associated foam cells, intercellular adhesion molecule-1(ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) was performed by histochemical and immuno-histochemical methods.

Thirty adult male New Zealand rabbits were divided into three groups, ten animals each. Group-A served as control, group B&C were fed 1% cholesterol-rich diet for 6 weeks. Animals of group-C were drenched, at night, 510 ml saline solution containing 4.2 mg/kg/day melatonin. Frozen sections from the thoracic aorta were prepared for histochemical demonstration of peroxidase & acid phosphatase enzyme activities, and immuno histochemical display of ICAM-1 & VCAM-1. The enzyme activity and adhesion molecule expression were measured by computerized image analysis.

Accumulation of peroxidase & acid phosphatase-rich foam cells was microscopically evident in the sub-endothelium of group-B arteries only. Enzyme activity and expression of ICAM-1 were significantly ($p < 0.0001$) higher in group-B than group-C which was also higher than control, while VCAM-1 expression was significantly ($p \leq 0.013$) higher in group-B than control which was higher ($p < 0.00001$) than group-C.

In conclusion, the results indicate that melatonin could potentially be beneficial in prevention of the food induced atherosclerotic lesions.

INTRODUCTION

Excluding the genetic factors, hyperlipidemia along with enhanced oxidation of lipoprotein lipids are separate but interacting factors giving rise to the formation of atherogenic lesions (Steinberg etal., 1989). Evidence in support of the lipid hypothesis is shown by the dramatic reduction in coronary artery disease arising particularly from cholesterol lowering drugs(Sevanian and Hodis, 1997). On the other hand, Oxidants have been shown to produce athero - genic responses independent of lipid levels and the mechanisms of injury include many of the biological events described for atherogenesis. Support for the role of oxidants in the development of atherosclerosis also comes from clinical trials demonstrating antioxidant

protection beyond that afforded by lipid lowering therapies (Hodis etal., 1995; Rapola etal., 1996). Sevanian and Hodis, (1997) reported the oxidant-mediated processes thought to be of importance in early atherogenesis which included the following:

1) Increased VCAM-1 and ICAM-1 expression and monocyte adhesion protein.

2) The uptake of LDL via scavenger receptors produces dysregulation of intracellular cholesterol synthesis and cholesteryl ester accumulation leading to rapid lipid loading and foam cell formation (Berliner and Heinecke, 1996).These steps apply to the oxidation of serum lipoproteins as well as to vascular tissues. The role of lipop - rotein lipid peroxidation appears to be central to this series of events since inhibition of peroxidation, or peroxide accumulation, tends to suppress, if not eliminate, the progression of atheros - clerosis. Suppression of the atherogenic reaction to oxidants is mediated by several endogenous factors such as metal binding and antioxidant enzymes as well as by a number of antioxidant compounds.

Antioxidants have been shown to protect the cells from the free radical damage *(Mattar and Moustafa* 1995). An anti-atherosclerotic potential has been demonstrated primarily in animals with some antioxidants, these being probucol (Daugherty *et al*., 1989; Hodis *et al*,1992), vitamin E (Wojcicki *et al*. 1991 ; Kleinveld *et al*.,1995 ; Verlangieri *et al*., 1992), vitamin E and C (Bocan *et al*., 1992) and vitamin C (Beetens etal.1984). Some epidemiolo - gical (Bellizi *et al*.,1994 ; Stampfer and rimm *et al*.,1995 ; Gey *et al*., 1987; Rimm *et al*.,1996) and clinical (Rapola *et al*.,1996) studies tended to confirm the results of animal experiments. However, recent, very large, carefully conducted clinical intervention trials using adequate doses of vitamin E demonstrated no effect on a composite endpoint of non-fatal infarction, stroke or death from cardiovascular causes (Steinberg, 2000). One of the compo - unds that are considered as having antioxidant action is the pineal secretory product, melatonin (Goodman & Gilman's, 1990). Melatonin is a horm - one that has beside its antioxidant acti - vity, several other biological effects. Melatonin was reported to regulate the expression of cell adhesion molecules which is one of atherosclerosis –associ ated features. Impaired nocturnal secretion of melatonin was reported to be associated with coronary heart disease (Brugger, *et al.*, 1995). Sakotnik, *et al.*, (1999) postulated that melatonin production may be impaired, due to a lack of synthesizing enzyme, in patients with coronary artery disease. So, decreased melatonin level may be a predisposing factor for coronary artery disease, or the occurrence of coronary artery disease decreases melatonin synthesis.

The aim of this study is, therefore, to reveal the possible protective effect of melatonin on diet-induced atheroscl - erosis in rabbits. Quantitative immuno - histochemical and histochemical method were used to evaluate the expressivity of VCAM-1 and ICAM-1 as well as the accumulation of foam cells as parameters for atherosclerotic manifestation. Such data may add more insight for understanding the cellular and molecular mechanisms of atherosc - lerosis, and possibly its prevention and treatment.

MATERIAL AND METHODS

Material

Adult male New Zealand rabbit was the animal of choice because it is the most sensitive species for induction of

atherosclerosis. The female rabbits were not used to avoid the effect of the cyclic physiologic hormonal changes. The rabbits were about 12 week old. The animals were divided into three groups each of 10 animals.

Group-A: (Control group)

Animals were raised on ordinary, cholesterol-free, diet for 6 weeks.

Group-B (Atherosclerotic group)

Animals were raised on the ordinary diet mixed with 1% cholesterol powder for a period of 6 weeks *(Fennessy, etal., 1994)*. The cholesterol powder used was produced by Merck Company in Germany. Each bottle contained 25 grams yellow cholesterol powder of good quality.

Group-C (Melatonin treated athero - sclerotic group)

Animals were raised on the ordinary diet mixed with 1% cholesterol powder *(Fennessy, etal., 1994)* and were drenched, by naso-gastric tube, 10 ml solution containing 4.2mg Purified pharmaceutical grade melatonin (Memphis Co. For Pharm. & Chem. Ind. Cairo), dissolved in saline every night for 6 weeks. The total daily dose was calculated according to Paget & Barnes(1964).

Monoclonal antibodies of (VCAM-1) and (ICAM-1) were a generous gift from Dr. M. Cybulsky (Department of Laboratory Medicine and Pathobiology, University of Toronto, Canada).

Methods

Frozen sections of 10µm thickness were prepared. Sections of the control and other groups were mounted on the same slide. Sections were stained by the following techniques:

I-Histochemical Techniques for demonstration of foam cells:

(1)Diaminobenzidine method; for peroxidase in peroxysomes *(Carleton, 1980).*

(2)Lead phosphate technique; for demonstration of acid phosphatase enzyme activity in lysosomes *(Pearce, 1972).*

II- Immuno-peroxidase Technique:

ICAM-1&VCAM-1 were demonstrated by the immunoperoxidase method described by Dako manufactural instru - ctions, catalogue number 680. Frozen sections were fixed in cold acetone at -20ºC for 5mins, and then the slides were air dried. Endogenous peroxidase was inactivated by adding 3% H_2O_2 for 15 min. and then washed twice by phosphate buffered saline (PBS). Blocking of non-specific antigens was done for 30 min, and then washed in PBS.

Sections were incubated with the primary antibody at room temperature for 1 hour (including +ve & -ve control), then washed 3 times with Tris-saline pH 7.4, containing 2% fetal bovine serum (FBS) for 5 min./each.

Labeling of the antibody (avidin-biotin ABC technique) was done by adding reagent (1C) for 30 min and washed in Tris-saline, then adding reagent-2 for 30 min. and washed in Tris-saline.

The chromogen substrate was prepared and added for 5-30 min. depending on the control slide. The slides were then washed in distilled water for 5 min. and counterstained with haematoxylin. Slides were washed under tap water then distilled water 3 times, and finally covered with aquamount.

Quantitative Analysis

All slides were examined after staining by the computerized image analyzer using Optimas 6.21 software (Media Cybernetics Inc. 1998).

The statistical analysis was carried out according to the standard statistical procedures and Student (t) test to estimate the significance of results using Microsoft Excel 2000.

RESULTS

Group-A (control group):

There were no foam cells in the intima of the arteries after peroxidase or acid phosphatase techniques. Both peoxidase and acid phosphatase activities were significantly less than the other groups (Table 1, Fig. 1, and Plate 1).

It showed a minimal (basic) expression of both ICAM-1 and VCAM-1 mainly in their endothelial lining (Table 2, Fig. 2 and Plate 2).

Group-B (Atherosclerotic group):

There was accumulation of foam cells in the subintima of the atherosclerotic animal arteries, which have high peroxidase and acid phosphatase activities (plate 1). Both peroxidase and acid phosphatase activities were significantly higher than in other groups (Table 1, Fig.1 Plate1).

The expression of the adhesion molecules (ICAM-1) is represented in plate 2. The intensity of labeling in group-B is higher than group-A (plate 2). The relative amount of ICAM-1 in arterial endothelium (Table 2, Figure 2) was significantly higher (0.74 ± 0.31) than control (0.50 ± 0.15) group.

The expression of the adhesion molecules (VCAM-1) was also higher on the endothelium of atherosclerotic group compared to control (plate 2). The relative amount of VCAM-1 in arterial endothelium (table 2, figure 2) was significantly higher in the atherosclerotic (0.37 ± 0.19) than control (0.33 ± 0.04) group.

Group-C (Melatonin treated atheroscl - erotic group):

In both peroxidase and acid phosphatase stained sections (plate 1) the subendot - helium showed no accumulation of phagocytic cells. Both peoxidase and acid phosphatase activities were signif - icantly less than group-B and higher than group-A (Table1, Fig.1 and Plate1).

Table (1): Mean optical density of acid phosphatase and peroxidase enzymes in sections from all groups (Gr. Comp. = groups compared).

	Group-A		**Group-B**		**Group-C**			
	Acid Ph	**Perox**	**Acid Ph**	**Perox**	**Acid Ph**		**Perox**	
Mean	0.287	0.384	0.385	0.536	0.306		0.426	
STDEV	0.02	0.159	0.032	0.169	0.017		0.189	
Gr.Comp.			A:B	A:B	B:C	A:C	B:C	A:C
P-Value			0.0001	0.0007	0.00025	0.003	0.00012	0.002

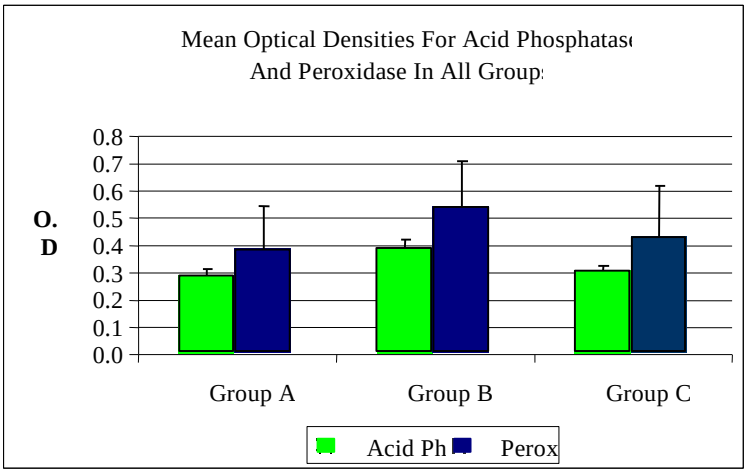

Figure (1): Mean optical density of acid phosphatase and peroxidase enzymes. The error bars represent the standard deviation.

ICAM-1 expression (table 2, figure 2, plate2) in group-C (0.54 ± 0.04) was relatively higher than control (0.50 ± 0.15). It was, however, significantly less than atherosclerotic (0.74 ± 0.31) group.
VCAM-1 expression (Table2, Fig 2 and plate 2) on the endothelium in group-C (0.25 ± 0.08) was less than group-A and also significantly less than group-B.

Table (2): Mean optical density of ICAM-1 and VCAM-1 expression on the endothelial surface of thoracic aorta of all groups (Gr. Comp. = groups compared)

	Group-A		**Group-B**		**Group-C**			
	ICAM.1	**VCAM.1**	**ICAM.1**	**VCAM.1**	**ICAM.1**		**VCAM.1**	
Mean	0.506	0.33	0.747	0.375	0.549		0.254	
STDEV	0.154	0.048	0.313	0.196	0.045		0.089	
Gr.Comp.			A:B	A:B	B:C	A:C	B:C	A:C
P-Value			<0.00001	0.013	<0.00001	0.001	<0.00001	<0.00001

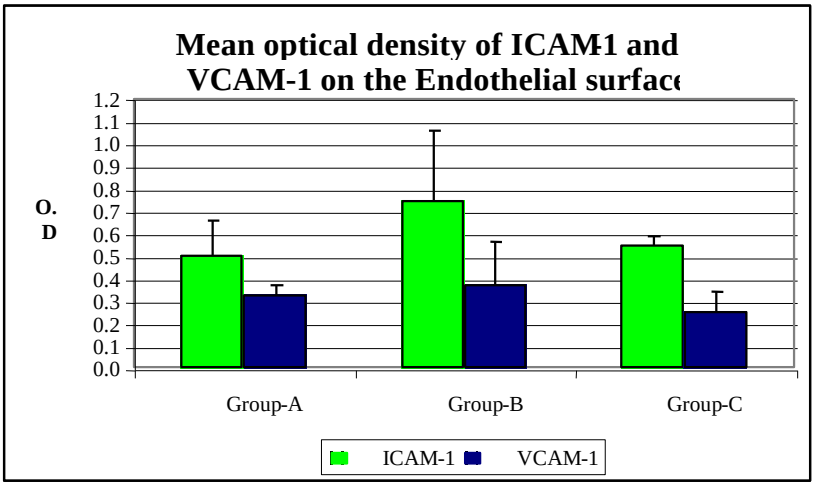

Figure (2): Mean optical density of ICAM-1 and VCAM-1 expression on the endothelial surface of thoracic aorta of different groups. The error bars represent the standard deviation.

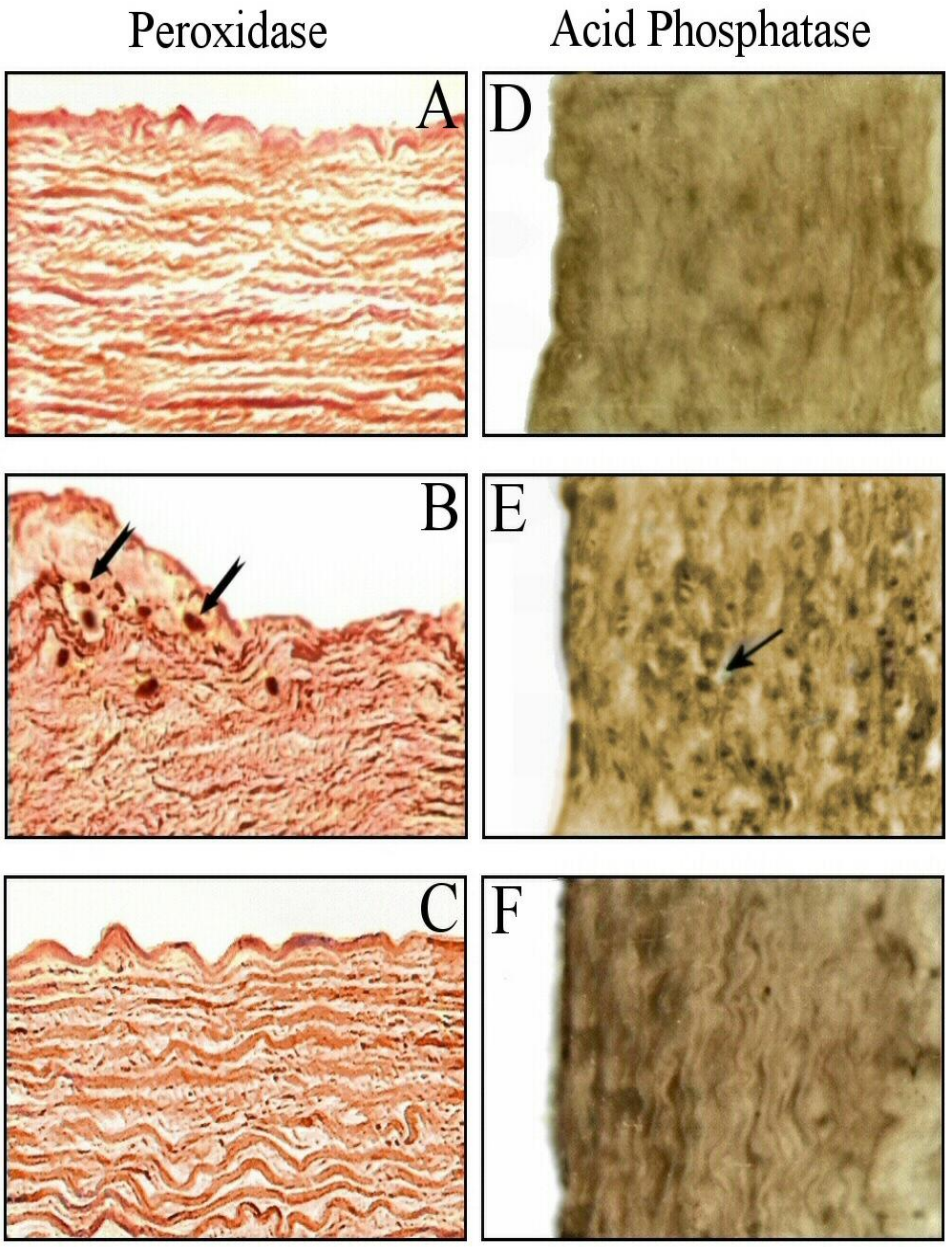

Plate 1: On the left side (Peroxidase reaction x 500), figure-A,B and C represent sections from group-A, B and C respectively. Note the marked accumulation of phagocytic cells which have high enzymatic activity in the subendothelium of group-B only (arrow). On the right side (Acid phosphatase reaction x 500), figure-D,E and F represent sections from group-A, B and C respectively. Note that accumulation of foam cells is only seen in group-B (Figure-E).

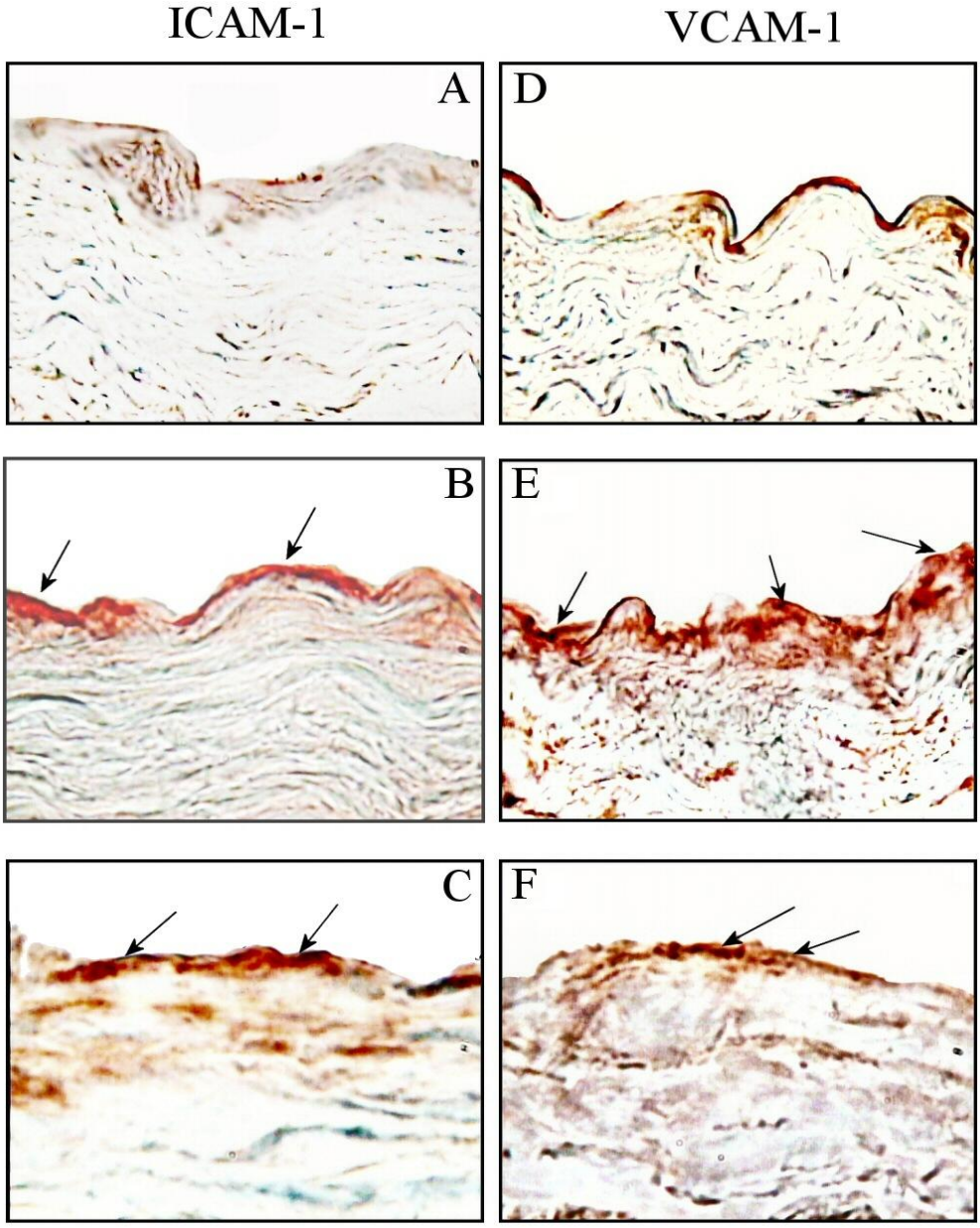

Plate 2: On the left side (ICAM-1 expression by immunoperoxidase x1000), figure-A,B and C represent sections from group-A, B and C respectively. Note the high expression of ICAM-1 in group B (Arrows). On the right side (VCAM-1 expression by immunoperoxidase x1000) figure-D, E and F represent sections from group-A, B and C respectively. Note the high expression of VCAM-1 in group B compared to other group (Arrows).

DISCUSSION

Several theories have been proposed for the pathogenesis of ather – osclerosis. However, all of these theories suggested that lipid peroxida - tion has a primary effect on the onset of atherosclerosis *(Chisolm, 1991; Navab, etal., 1995; Berliner & Heinecke, 1996; Steinberg & Lewis, 1997).* Antioxidants, such as melatonin, were shown to exert a protective effect from atherosclerosis by prevention of lipid peroxidation *(Carr, et al., 2000)*. The first component

of the blood vessel affected by atherosclerosis is the endothelial lining. Abnormalities of the endothelium are induced by certain lipids, peroxidants, and inflammatory cytokines *(Hennig & Toborek, 2001).*

In the present study, induction of atherosclerosis resulted in aggregation of macrophages in the aortic subintimal areas of the atherosclerotic group and increased the expression of both ICAM-1 and VCAM-1 on the endothelial lining. Both features were reported as consequences of lipid peroxidation leading to atherosclerosis (Sevanian and Hodis 1997)

The absence of macrophage aggregations in the subendothelial layer of the intima in group-C is probably due to the action of melatonin as an antio - xidant, which protects the endothelial cells against injury. Such protection may be through inhibition of ICAM-1 expression which in turn inhibits mono - cyte recruitment, infiltration and transf - ormation into active macrophages in the subendothelial layer of the intima. As reported for other antioxidants *(Gordon, etal., 1989; Berthou, et al., 1997; Fruchart, 1997; Kirchgassler, et al., 1998; Packard, 1998; Hennuyer, et al., 1999; Poulter, 1999; Krempf, et al., 2000),* melatonin may affect peroxi - dation of circulating lipid leading to prevention of its phagocytosis.

Expression of ICAM-1 on the endothelial cells at certain sites may be related to complex hemodynamics in these regions. Hemodynamics may increase local permeability or transport of lipoproteins by endothelium. They promote lipoprotein retention in the intima. Local oxidation of lipoproteins trapped in the intima may generate soluble factors that induce endothelial adhesion molecule expression. Introdu - ction of shear stress can activate various endothelial cell signal transduction pathways *(Ishida, et al., 1997; Bhullar, et al., 1998).* They influence the expression of adhesion molecules *(Nagel, et al., 1994)*.

The localized expression of ICAM-1 in aortic endothelium of normal animals may provide a milieu for atherosclerotic lesion formation. It may result in occasional recruitment of monocytes into the intima. A potential mechanism for this is production of chemokines and inflammatory cytokines during engulf - ment of oxidized lipoproteins and transformation into foam cells *(Malinauskas, et al., 1995)*.

The endothelial expression of ICAM-1 in group-B was significantly increased when compared to group-A. This can be explained by LDL oxidation in atheros - clerosis which enhances the expression of ICAM-1 that influences the adhesion and endothelial transmigration of monocytes *(Cominacini, et al., 1997).*

Oxidized LDL stimulates the expression of the peroxisome proliferator-activated receptor γ (PPAR γ) in macrophages. Immunocytoche - mical localization of PPAR and Ox LDL in macrophages (and probably also in endothelial cells) of human atherosc - lerotic lesions suggested that Ox LDL also promotes expression of this nuclear transcription factor which enhances ICAM-1 expression by end -othelial cells *(Ricote, et al., 1998).*

Treatment of rabbits with high cholesterol diet and melatonin in group-C significantly decreased ICAM-1 endothelial expression in thoracic aorta compared to group-B. Melatonin seems to inhibit monocyte adhesion by increasing the resistance of LDL to oxidation *(Cominacini, et al., 1991; Esterbauer, et al., 1989)*.

The endothelial expression of VCAM-1 in group-B was significantly increased compared to group-A. This can be explained by the fact that some

byproducts of LDL oxidation, such as lysophosphatidyl choline, increases the expression of VCAM-1 by endothelial cells in atherosclerosis *(Khan, et al., 1995; Kume, et al., 1992 ; Marui, et al., 1993)*. The induced expression of VCAM-1 is largely dependent on synthesis of new mRNA and protein. There are apparently no storage forms of VCAM-1 *(Pober, et al., 1986)*.

Treatment of rabbits with high cholesterol diet and melatonin signify - cantly decreased VCAM-1 endothelial expression compared to group-B. Similar results were obtained by *Fruebis, et al., 1999* as due to the antio - xidant effect of vitamin E. Therefore, melatonin may decrease endothelial monocyte adhesion by down-regulating expression of VCAM-1 which was proposed for other antioxidants *(Ferns, et al., 1993; Kaneko, et al., 1996)*. As an antioxidant, melatonin may also inhibit monocyte adhesion by supp - ressing nuclear factor NF-κB mobiliz - ation and the induction of VCAM-1 in endothelial cells stimulated by tumor necrosis factor (TNF) *(Marui, et al., 1993; Weber, et al., 1994)*. NF-κB activation by TNF was found to be controlled by reactive oxygen interm - ediates (ROIs) *(Satriano, et al., 1993)*.

In conclusion, the present study suggests that melatonin administration ameliorates signs of atherosclerosis in high cholesterol-fed rabbits. Its effect may be due to its antioxidant action. However, it may also act as hormone supplement, since decreased night-time level of melatonin were reported in patients with coronary artery disease *(Sakotnik, etal., 1999)*. Further studies are needed, however, to evaluate the effect of melatonin administration in human atherosclerosis clinically.

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دراسات هستوكيميائية وهستوكيميائية مناعية لتأثير الميلاتونين على الشريان الأبهر فى حالة تصلب الشرايين التجريبي فى الأرنب

فتحي السيد مطر*- عبد الموجود أنس اسماعيل*-
محمد أحمد قاسم –إبراهيم كمال ابراهيم ****
من أقسام الهستولوجيا بكليات طب بنين القاهرة *وأسيوط**- جامعة الأزهر

يعتبر علاج أو منع حدوث تصلب الشرايين من التحديات التى تقابل الطب الحديث. وقد أظهرت الدراسات أن عملية فوق أكسدة الليبيدات منخفضة الكثافة نتيجة وجود الشقاق الحرة قد تكون المسبب الأ ولي في الإصابة بتصلب الشرايين. ولقد أثبتت التجارب أن للميلاتونين تأثير وقائي في الحالات المرضية الأخرى التى تسببها الشقاق الحرة. وعلى ذلك فقد صمم هذا البحث لدراسة التأثير الوقائي المحتمل للميلاتونين من الإصابة بمظاهر التصلب فى شرايين ألأرانب المغذاة بطعام عالى الكولسترول. وقد تم تقييم مدى الوقاية بتتبع الخلايا الرغوية التى تصاحب تصلب الشرايين وكذلك جزيئات الإلتصاق الخلوية-1 (ICAM-1) وجزيئات الإلتصاق الوعائية-1 (VCAM-1) وذلك باستخدام الطرق الهستوكيميائية و الهستوكيميائية المناعية الكمية.

استخدم في هذه الدراسة ثلاثون من ذكور الأرانب النيوزيلاندي البالغة حيث قسمت إلى ثلاث مجموعات بكل مجموعة عشر أرانب. وقد غذيت المجموعة الضابطة بطعام خال من الكولسترول. أما المجموعتين الثانية والثالثة فقد قدم لهما غذاء يحتوي على 1% كولسترول لمدة ستة أسابيع وعلاوة على ذلك جرعت المجموعة الثالثة مساء كل يوم بمحلول مائي يحتوي على 4.2 مجم/كجم من الميلاتونين. وبعد استخلاص الشريان الأبهر الصدرى تم إعداد قطاعات مثلجة لاستخدامها فى الدراسات الهستوكيميائية لتبيان إنزيمى البيرأوكسيديز والفوسفاتيز الحمضى الموجودين فى الخلايا الرغوية، وكذلك لتبيان الجزيئات اللاصقة الخلوية والوعائية بالطرق الهستوكيميائية المناعية. وقد تم قياس نشاط الإنزيمين ومدى ظهور الجزيئات اللاصقة باستخدام جهاز تحليل الصور بالحاسب الآلي.

وقد أظهرالفحص المجهري تراكم الخلايا الرغوية ذات النشاط العالي لإنزيمي الفوسفاتيز الحمضى والبيرأوكسيديز فى طبقة ماتحت بطانة الشريان فى المجموعة الثانية بينما كان نشاط هذه الإنزيمات وكمية كلا النوعين من جزيئات الإلتصاق عاليا فى هذه المجموعة. أما فى المجموعة الثالثة المعاملة بالميلاتونين فقد كانت القيم التى قيست للعوامل الثلاثة أقل بمقدار ذو دلالة احصائية عالية عنها فى المجموعة الثانية وإن كانت أعلى مما فى المجموعة الضابطة.

ويستخلص من البحث أنه يمكن للميلاتونين أن يقوم بدور فى الوقاية من المظاهر التى تصاحب تصلب الشرايين.