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1-5 **Short-term effect of the modified diet therapy based on Resting Metabolic Rate (RMR) in obese patients with Type 2 Diabetes Mellitus**.... .....*Nawata, Motoi Sohmiya, Yuzuru Kato (Izumo, Japan)*

6-8 **A radiographic findings in new bone hyperplasia area incident to human teeth movement**..... *Kshitsugu Kawata, Masato Kaku, Tadashi Fujita and Kazuo Tanne (Hiroshima, Japan)*

9-12 **Effect of lead acetate on the tensile strength of incisional wounds in Wistar rats**.....*Salah Eldin O. Elsayed (Riyadh, Saudi Arabia)*

13-18 **Effect of short-term treatment with recombinant Human Growth Hormone (GH) on size eterogeneity of Insulin-Like Growth Factor-I (IGF-I) and IGF Binding Protein 3 (IGFBP-3) in the plasma and urine of patients with chronic renal failure** .....*Motoi Sohmiya, Yuzuru Kato (Izumo, Japan)*

19-25 **Angioarchitecture of the duck pectin**.....*Gaetano Scala, Mario Corona, Alessandra Perrella (Napoli, Italy)*

26-30 **Evaluation of analgesic and anti-inflammatory activity of beta-lactam monocyclic *compounds*** .....*C. Saturnino, M. Buonerba, G. De Martino, JC Lancelot, A. Capasso (Salerno/ Cedex, Italy, France)*

31-35 **Prevalence of modifiable traditional coronary heart disease risk factors among young adult survivors of acute MI: A comparative study between Saudis and Non- Saudi patients**..... *Abdullah Saleh Shatoor (Abha, Saudi Arabia)*

36-39 **Major congenital anomalies in Southwestern Saudi Arabia**..... .....*Mohammed Abdullah Alshehri (Abha, Saudi Arabia)*

40-43 **The involvement of nitric oxide in morphine-induced straub tail in mice**..... *Morteza Samini, Leila Moezi, Shiva Javadi, Hamed Shafaroodi, Shahram Ejtemaei Mehr, Ahmad R. Dehpour (Tehran, Iran)*

44-47 **Intermittent sequential pneumatic lower limb compression and its relationship to prophylaxis of deep vein thrombosis**... .....*G.C. Okoye (Enugu, Nigeria)*

48-51 **Activities of Adenosine Triphosphatases (ATPases in sperm cells: Implications for narrowing down treatment for Asthenospermia**.....*S.O. Ogbodo, E.N. Shu, I.N. Okonkwo J.K. Emeh (Enugu, Nigeria)*

52-57 **Rhegmatogenous retinal detachment: Protein in the subretinal fluid** .....*S. Nasir Askari, Devina Gogi, Shakeer Pullambalavan, M. Saleemuddin, Nafees Ahmed (Aligarh, India)*

58-64 **Genetic Molecular Marker Identification from DNA Polymorphism bands and Virulent genes in *Streptococcus pyogens* and characterization of isolates**.....*S. Mariana, V. Neela, S. Zamberi, S. Mimah (Selangor, Malaysia)*

65-68 **Iron accumulation in hepatocellular carcinoma: A histochemical study of 135 cases**.....*A.A. Ngokere, T.C. Ngokere (Enugu, Nigeria)*

69-72 **Light microscopical details of perineuronal nets in the hippocampus of the mouse revealed by means of methylene blue labelling**.....*Thomas Müller (Mainz, Germany)*

73-75 **Antinociceptive Effects of *Coscinium fenestratum* (Gaertn) on mouse formalin test**.....*K. Chitra, K. Sujatha, S.H. Dhanuskha, et al* (Chennai, India)

76-79 **Antibody response to *M.tb* $H_{37}Ra$ excretory secretory ES-43 and ES-31 antigens at different stages of pulmonary tuberculosis**.....*S Gupta, N Shende, S Kumar, B C Harinath (Sevagram, India)*

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Short-term effect of the modified diet therapy based on Resting Metabolic Rate (RMR) in obese Patients with Type 2 Diabetes Mellitus

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Key words: Type 2 diabetes mellitus, obesity, resting metabolic rate, diet

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Abstract

Twenty-four patients with type 2 diabetes (11 males and 13 females) were randomly divided into two groups: fixed diet group (control) and modified diet group. Control group was maintained on the diet of 105 kJ/kg ideal body weight through the experimental period of four weeks. The modified diet group was started on the diet of 105 kJ/kg ideal body weight, followed by decreased intake based on resting metabolic rate (RMR) measurement when the weight loss rate was decreased. There was no difference in age, sex, therapy, body weight, body mass index and HbA1c levels between two groups.

Body mass index (BMI) was decreased from 31.5±4.2 kg/m^2 at 0 week to 29.8±3.8 kg/m^2 at 4 weeks in modified diet group. BMI was decreased every week for four weeks. BMI was decreased from 29.9±4.6 kg/m^2 at 0 week to 29.1±4.7 kg/m^2 at one week to 28.3±4.5 kg/m^2 at 4 weeks in control group. BMI was significantly decreased every week, however, there was no difference between 3 and 4 weeks in control group. RMR was decreased from 163.0±28.8 kJ/hr/m^2 to 145.5±30.1 kJ/hr/m^2 at 4 weeks ($p<0.005$) in modified diet group. However, LBM was not changed during 4 weeks. RMR at 0 week was positively correlated with LBM ($y=1.39x + 90.78$, $r=0.680$, $p<0.005$) and 4 weeks ($y=1.55x + 65.50$, $r=0.743$, $p<0.005$). The regression curve showed a parallel translation downwards at 4 weeks. HbA1c levels decreased from 7.2±1.5% to 6.0±0.9% ($p<0.05$) in the modified diet group.

In conclusion, we found that the modified diet based on RMR measurement was effectively decreased BMI without change of LBM in obese patients with type 2 diabetes mellitus.

Introduction

It has been recommended to restrict dietary intake to 84 - 105 kJ/kg of ideal body weight in obese patients with diabetes mellitus [1]. However, weight loss is not obtained by the diet therapy in some patients with obesity. Very low calorie diet (VLCD) was more effective for severe obesity [5,6], but considerable rebound weight gain after VLCD was noted after the therapy. Although individual difference of the diet effect may be partly explained by altered beta 3 adrenergic receptors [2-4], the detailed mechanism remains to be fully elucidated.

Resting metabolic rate (RMR), the rate of energy expenditure measured at rest after an overnight fast, accounts for approximately 70% of daily energy expenditure in man [7]. RMR is changed by body temperature [8], age [9], thyroid function [10], sympathetic nerve activity [11] and body composition [12]. It has been reported that RMR is higher in patients with type 2 diabetes than in weight-matched healthy subjects, of which difference is related with hepatic glucose production rate [7,13]. RMR could be evaluated in patients with diabetes mellitus before the start of diet and reevaluated when the weight control was not consistently obtained.

In the present study, we investigated the possible effect of modified diet based on RMR on the control of weight and glucose metabolism in obese patients with type 2 diabetes,

Materials and Methods

Subjects and study protocol

Twenty-four patients with type 2 diabetes, 11 males and 13 females, aged from 19 to 77 yrs were investigated. In all the patients, body mass index (BMI) was more than 26.4 kg/m^2. All of them had been treated for type 2 diabetes mellitus for more than five years. They included 11 patients treated with diet only, 7 pat-

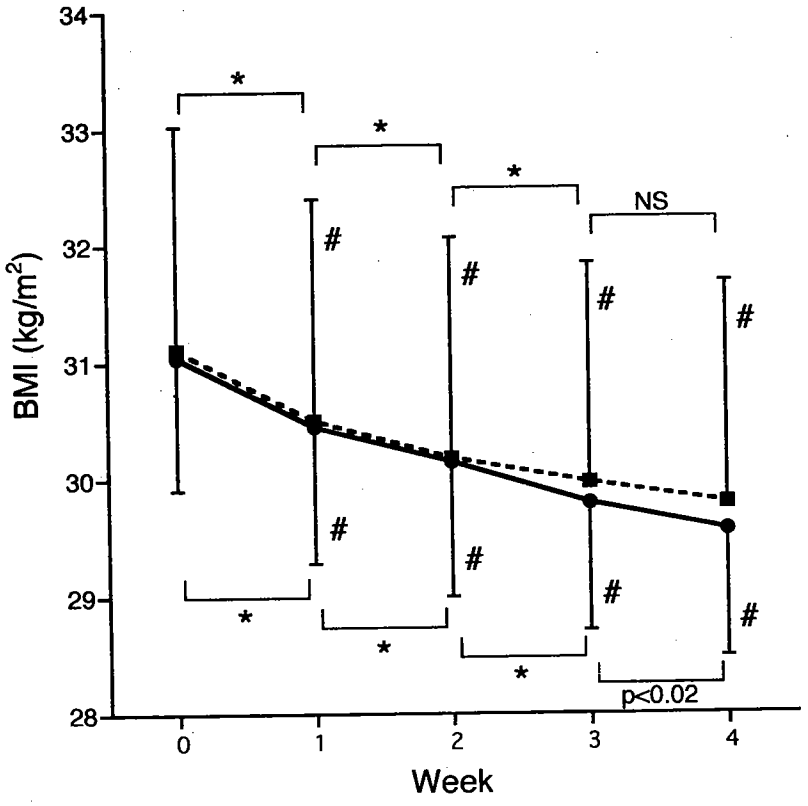

*Fig. 1: Effect of the control diet (105 kJ/kg of ideal body weight) and the modified diet based on RMR measurement on BMI in obese patients with type 2 diabetes mellitus. Closed square and closed circle indicate the control diet group and the modified diet group, respectively. The vertical bars show mean±SE. #: p<0.0001 vs 0 week, *: p<0.001.*

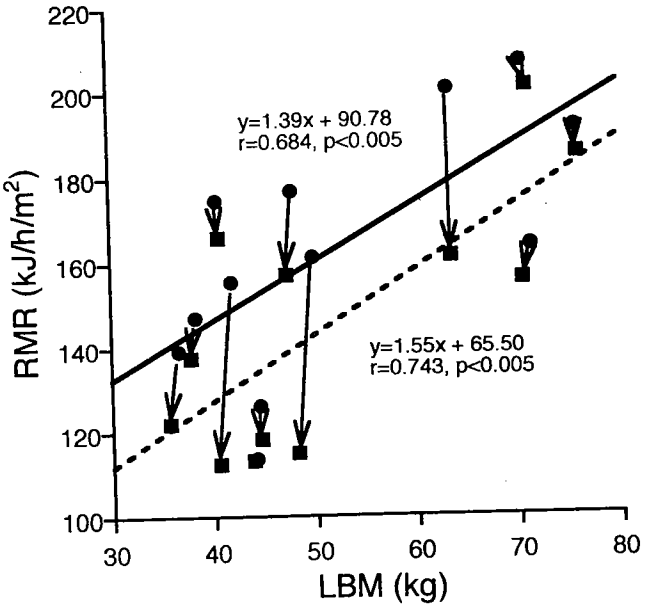

Fig. 2: Relationship between RMR and LBM at 0 week (closed circle) and 4 weeks (closed square) after the modified diet therapy in obese patients with type 2 diabetes mellitus. The linear regression analysis revealed close correlation between RMR and LBM at 0 week (solid line) (y=1.39x + 90.78, r=0.68, p<0.005) and 4 weeks (dashed line) (y=1.55x + 65.50, r=0.743, p<0.005), respectively. The linear regression curve moved downwards at 4 weeks paralleled to that at 0 week. The arrows indicate the changes of individual cases.

Table 1: Clinical characteristics of the control diet and modified diet in obese patients with type 2 diabetes mellitus

		Control diet	Modified diet
n		12	12
Age	(years)	56.5±15.5	44.0±20±4
Sex	(M/F)	7/5	4/8
Therapy	(Diet/OHA/Insulin)	6/2/4	5/5/2
Body weight	(Kg)	78.8±16.5	80.8±16.4
Body mass index	Kg/m^2	31.1±6.7	31.0±3.9
HbA1C	(%)	8.8±2.9	7.2±1.5

Data are expressed by mean ± SD
There was no significant difference between the groups

lents with oral anti-hyperglycemic agents (OHA) and 6 patients with insulin therapy (Table 1). They were admitted to our hospital and maintained on the diet of 105 kJ/kg of ideal body weight for two weeks. They were randomly divided into two groups. Control diet group was maintained on the diet of 105 kJ/kg ideal body weight throughout the experimental period. Modified diet group was started by 105 kJ/kg ideal body weight. The rate of weight loss was assessed every week after the start of restrict diet. If the rate of weight loss was decreased compared with previous weight loss, energy intake was reduced based on measurement of RMR in modified diet group. Energy intake in modified diet group was determined according to following formula: Energy intake (kJ) = [RMR (kJ/day) + energy expenditure by exercise (kJ)] x 10/9 - 15,06 (kJ)/ 7 days. Energy expenditure by exercise was determined by calorie computer (Select 2, Suzuken Co., Nagoya, Japan), and was fixed to 1,255 kJ/day in all the patients. The patients had no diabetic nephropathy except two cases with microalbuminuria. There were no differences in age, sex, therapy, body weight, body mass index and HbA1c levels between the two groups. The written informed consents were obtained from all the subjects.

Measurement of RMR and lean body mass (LBM)

RMR was determined by indirect calorimetery using closed-circuit spirometry (Chestac 55V, Chest Co., Tokyo, Japan) in early morning after resting in a spine position for more than 8 hours. All the patients were instructed not to leave their bed after midnight and to lie quietly until RMR measurement was completed. Mean value of three consecutive measurement was used for statistical evaluation of RMR. Coefficiency of variation of this measurement was 4.1%. LBM was calculated from body weight by reducing body fat determined by bioelectrical impedance analysis (IBF-300, Omron Co., Tokyo Japan). Coefficiency of variation of this measurement was 0.5%.

Statistical Analysis

Data were analyzed by ANOVA in combination with paired *t*-test. Correlation between two parameters was evaluated by linear regression analysis. $P<0.05$ was considered significant.

Results

As shown in Fig. 1, BMI of modified diet group was decreased every week in the modified diet group; from 31.5±4.2 kg/m^2 at 0 week to 30.8±4.3 kg/m^2 at 1 week, 30.4±4.1 kg/m^2 at 2 weeks, 30.1±4.0 kg/m^2 at 3 weeks and 29.8±3.8 kg/m^2 at 4 weeks. BMI was significantly decreased every week for 4 weeks. On the other hand, BMI of control group was decreased from 29.9±4.6 Kg/m^2 at 1 week, 28.8±4.6 kg/m^2 at 2 weeks, 28.5±4.6 kg/m^2 at 3 weeks and 28.3±4.5 kg/m^2 at 4 weeks. BMI was significantly decreased every week, however, there was no difference between 3 and 4 weeks. There was no difference of BMI between control and modified diet groups.

RMR was decreased in the modified diet group from 163.0±28.8 $kJ/hr/m^2$ at 0 week to 145.5±30.1 $kJ/hr/m^2$ at 4 weeks ($p<0.005$). LBM was not changed during the period (52.1±14.4 kg at 0 week and 51.7±14.5 kg at 4 weeks). As shown in Fig. 2, RMR was correlated with LBM at 0 week ($y=1.39x+90.78$, $r=0.680$, $p<0.005$) and at 4 weeks ($y=1.55x+65.50$, $r=0.743$, $p<0.005$), respectively, making a parallel translation of a line downward. HbA1c levels were decreased from 7.2±1.5% to 6.0±0.9% ($p<0.05$) in the modified diet group, whereas HbA1c levels were not significantly changed in the control diet group (8.8±2.9% vs. 7.1±1.7%, $P>0.05$).

Discussion

We found in the present study that the modified diet based on RMR measurement was useful for constant weight loss in obese patients with type 2 diabetes mellitus. The reduction of RMR was not accompanied by a decrease of LBM.

Many factors are known to regulate RMR in obese patients with type 2 diabetes mellitus. RMR is higher in diabetic patients than in non-diabetic subjects [14,15], possibly reflecting increased production of hepatic glucose. Hepatic glucose production and lipid oxidation are positively correlated with RMR, both of which are correlated in patients with type 2 diabetes [13]. Furthermore, it was reported that glucose excretion into urine and increased RMR were contributed to weight loss during poor control of blood glucose [16]. In the present study, HbA1c levels were decresed

after the diet was modified based on RMR. These findings suggest that an improvement of plasma glucose control could decrease RMR.

RMR was increased in patients with obesity [17,18]. RMR was decreased after reducing body weight in obesity [19-21], but reduced RMR resulted in weight gain [22]. Therefore, monitoring of RMR during the diet was noted in obese patients. Reduced energy intake and decreased physical activity caused a decrease in RMR [23]. On the other hand, some investigators reported that exercise during the diet did not affect a decrease of RMR [19]. In the present study, physical activity was kept constant at 1,260 kJ/day during the experimental period, which was confirmed by calorie meter. Thyroid hormone plays an important role in regulating RMR. Thyroid function was decreased after weight loss in patients with obesity [20,24]. In the present study, thyroid function was not changed in all the subjects.

RMR is correlated with LBM [25]. LBM is the main site of physiological metabolism. In obese patients, both RMR and LBM were decreased by hypocaloric diet, and RMR corrected by LBM was not considerably changed [23]. Therefore, reduced RMR was mainly accounted for reduced LBM in obese patients during the diet. In the present study, RMR was correlated with LBM at 0 and 4 weeks after the start of diet. However, LBM was not changed during the diet in spite of a decrease of BMI. Furthermore, the regression curve was moved downward at 4 weeks in parallel to that of 0 weeks, suggesting that a decreased RMR was independent of LBM in the present study.

In conclusion, we found that the modified diet based on RMR measurement was effective for steady reducing BMI without change in LBM in obese patients with type 2 diabetes mellitus. Long term effect of the modified diet therapy should be further investigated.

References

1. National research council: recommended dietary allowance. 10th ed. Washington DC, National Academy Press, 1989, p29.
2. Groop L. Genetics of the metabolic syndrome. Br J Nutr (Suppl 1) 2000; 83: S39-48.
3. Sakane N, Yoshida T, Umekawa T, Kogure A, Takakura Y, Kondo M. Effects of Trp64Arg mutation in the beta 3-adrenergic receptor gene on weight loss, body fat distribution, glycemic control, and insulin resistance in obese type 2 diabetic patients. Diabetes Care 1997; 20: 1887-1890.
4. Silver K, Mitchell BD, Walston J, Sorkin JD, Stern MP, Roth J, Shuldiner AR. TRP64ARG beta 3-adrenergic receptor and obesity in Mexican Americans. Hum Genet 1997; 101: 306-311.
5. Amatruda JM, Richeson JF, Welle SL, Brodows RG, Lockwood DH. The safety and efficacy of a controlled low-energy ('very-low-calorie') diet in the treatment of non-insulin-dependent diabetes and obesity. Arch Intern Med 1988; 148: 873-877.
6. Burgess NS. Effect of very-low-calorie diet on body composition and resting metabolic rate in obese men and women. J Am Diet Assc 1991; 91: 430-434.
7. Ravussin E, Lillioja S, Anderson TE, Christin L, Bogardus C. Determinants of 24-hour energy expenditure in man. J Clin invest 1986; 78: 1568-1578.
8. Rising R, Keys A, Ravussin E, Bogardus C. Concomitant interindividual variation in body temperature and metabolic rate. Am J Physiol 1992; 263: E730-E734.
9. Vaughan L, Zurlo F, Ravussin E. Aging and energy expenditure. Am J Clin Nutr 1991; 53: 821-825.
10. Bray GA. Effects of caloric restriction on energy expenditure in obese patients. Lancet 1969; 2: 397-398.
11. Saad MF, Alger SA, Zurlo F, Young JB, Bogardus C, Ravussin E. Ethnic differences in sympathetic nervous system-mediated energy expenditure. Am J Physiol 1991; 261: E789-E794.
12. Nelson KM, Weinsier RL, Long CL, Schutz Y. Prediction of resting energy expenditure from fat-free mass and fat mass. Am J Clin Nutr 1992; 56: 848-855.
13. Franssila-Kallunki A, Groop L. Factors associated with basal metabolic rate in patients with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia 1992; 35: 962-966.
14. Fontvieille AM, Lillioja S, Ferraro RT, Schulz LO, Rising R, Ravussin E. Twenty-four-hour energy expenditure in Pima Indians with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia 1992; 35: 753-759.
15. Bogardus C, Taskinen MR, Zawadzki J, Lillioja S, Mott D, Howard BV. : Increased resting metabolic rates in obese subjects with non-insulin-dependent diabetes mellitus and the effect of sulfonylurea therapy. Diabetes 1986; 35: 1-5.
16. Fontvieille AM, Lillioja S, Ferraro RT, Schulz LO, Rinsing R, Ravussin E. Twenty-four-hour energy expenditure in Pima Indians with type 2 (non-insulin-dependent) diabetes mellitus. Diabetologia 1992; 357: 53-759.
17. Chong PK, Jung RT, Rennie MJ, Scrimgeour CM. Energy expenditure in lean and obese diabetic patients using the doubly labelled water method. Diabet Med 1993; 10: 729-735.
18. Vansant G, Van Gaal L, De Leeuw I. Impact of obesity on resting metabolic rate and glucose-induced thermogenesis in non-insulin dependent diabetes mellitus. Int J Obes Relat Disord 1992; 16: 817-823.

19. Geliebter A, Maher MM, Gerance L, Gutin B, Heymsfield SB, Hashim SA. Effects of strength or aerobic training on body composition, resting metabolic rate, and peak oxygen consumption in obese dieting subjects. Am J Clin Nutr 1997; 66: 557-563.

20. Welle SL, Amatruda JM, Forbes GB, Lockwood DH. Resting metabolic rates of obese women after rapid weight loss. J Clin Endocrinol Metab 1984; 59: 41-44.

21. Bray GA. Effects of caloric restriction on energy expenditure in obese patients. Lancet 1969; 2: 397-398.

22. Ravussin E, Lillioja S, Knowler WC, Christin L, Freymond D, Abbott WG, Boyce V, Howard BV, Bogardus C. Reduced rate of energy expenditure as a risk factor for body-weight gain. N Engl J Med 1988; 318: 467-472.

23. Ravussin E, Burnand B, Schutz Y, Jequier E. Energy expenditure before and during energy restriction in obese patients. Am J Clin Nutr 1986; 41: 753-759.

24. Visser TJ, Lamberts SWJ, Wilson JHP, Docter R, Hennemann G. Serum thyroid hormone concentrations during prolonged reduction of dietary intake. Metabolism 1978; 27: 405-409.

25. Cunningham JJ. Body composition as a determinant of energy expenditure: A synthetic review and a proposed general prediction equation. Am J Clin Nutr 1991; 54: 963-969.

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A radiographic findings in new bone hyperplasia area incident to human teeth movement

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Key words: new bone hyperplasia, orthodontic force

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Abstract

This study describes new bone hyperplasia in alveolar buccal site and demonstrates various clinical process in the field of clinical orthodontics using case reports. Extraction of 1-premolars is commonly considered necessary for the proper management of some malocclusion in this case. The force required for space closure were 50-100g delivered by chain elastic. Larger rectangular elgiloy wires, although more rigid, restricted free sliding. In these mechanism, we hypothesized rotation control mainly involves in the teeth adjacent to the extraction sites which tend to roll in ward, if the space is closed too rapidly. Accordingly, buccal alveolar bone showed tension. Our recommended space closure mechanics are straightforward and allow the orthodontist to focus on tooth positioning.

Introduction

New bone hyperplasia is created in the alveolar buccal site after orthodontic extraction. Generally, orthodontic tooth movement is induced by mechanical stimuli and facilitated by remodeling of the periodontal ligament and alveolar bone. In acase requiring extraction during orthodontic treatment, the tooth root was moved to the site of the site of the extracted tooth. It is established that collagen fibers in the periodontal ligament (PDL) increase with the distance between the cementum and alveolar bone as an initial change in periodontal fibers during tooth movement [1, 2]. Also, orthodontic tooth movement is induced by mechanical stimuli and facilitated by remodelling of the periodontal ligament and alveolar bone. In the site of the case of extraction during orthodontic treatment, the tooth root was moved to the extracted tooth. When force is applied to a tooth, periodontal fibers in the PDL are stretched on the side of tension, the osteoblasts and osteocytes transform to an active type and new bone is formed along the stretched area [3]. This article describes new bone hyperplasia in alveolar buccal site and demonstrates various clinical process in the field of clinical orthodontics using case reports.

Mechanics and Space Closure

Extraction of 1-premolars is commonly considered necessary for the proper management of some malocclusion. The 7mm space gained in each quadrant is used in one or more of three ways: relief of crowding, retraction of incisors, mesial movement of molars and premolars. Anchorage control is used to describe the maneuvers performed by the orthodontist, according to the treatment plan, to ensure the correct use of space in an extraction case. In this case, we used an .018×.025 working archwire mosl effectively in an .018-slot standard edgewise system. Larger rectangular elgiloy wires, although more rigid, restricted free sliding. The force required for space closure was 50-100g delivered by chain elastic (Ringlet: Rocky Mountain Morita, Tokyo, Japan) (Fig. 1).

Results and Discussion

A 23-year-old Japanese female presented with anterior crowding malocclusion. Anamnesis was not significant. Panoramic radiograph before orthodontic treatment is shown in Fig. 2. Fig.3 shows an oral photograph. The extraction space was closed using chain elastic. Then, this patient complained of pain when brushing the teeth in this area. Generally, soft tissue hyperplasia can prevent full space closure or allow spaces to reopen after orthodontic treatment (Fig. 3). The bone level of this site is usually low [4-6]. In this case, new bone hyperplasia was completed at the buccal alveolar bone site (Fig. 4B). This new bone was distinguished from the alveolar crest on X-ray (Fig. 4B). We noted a slight me-

sioversion before orthodontic treatment in the lower 1-premolar (Fig. 4A). Moreover, the X-ray before extraction of that the tooth did not show any significant problems (Fig. 4A). In these results, we hypothesized rotation control mainly involves in the teeth adjacent to the extraction sites which tend to roll in ward, if the space is closed too rapidly (Fig. 5). Accordingly, buccal alveolar bone showed tension. Thus, this area has the potential for new bone formation.

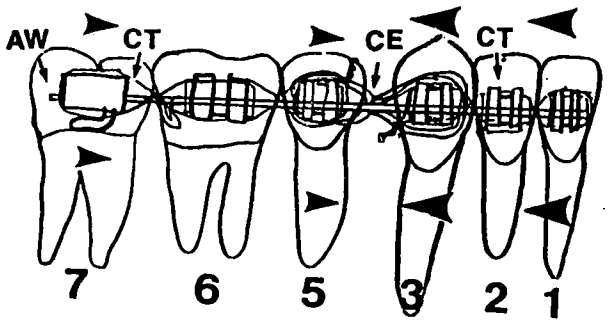

Fig. 1: *Diagram of rectangular .016×.025 elgiloy archwire. A chain elastic was placed between the canine and second-premolar, to initiate sliding mechanics with low-friction. Each teeth were tied individually by wire. AW=archwire, CT= continuous tie (C. Incisor to Canine, second-Premolar to second-Molar), CE= chain elastic*

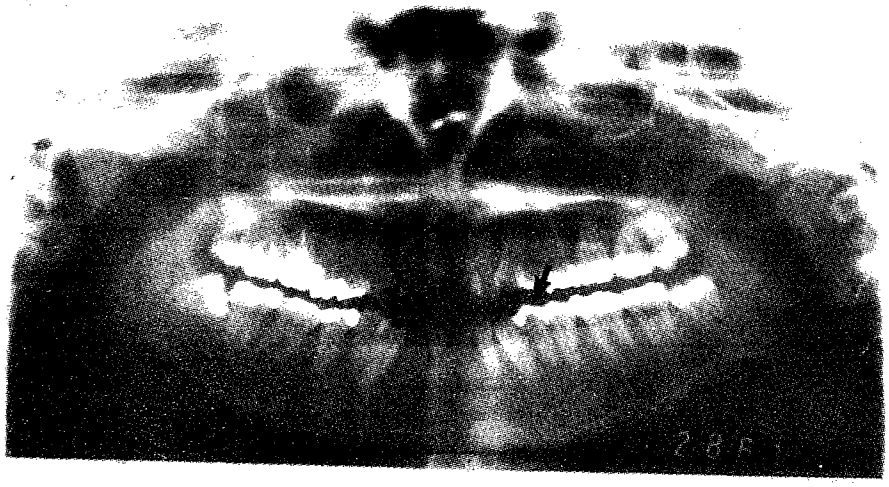

Fig. 2: *Initial panoramic radiograph. Arrow indicate extracted tooth.*

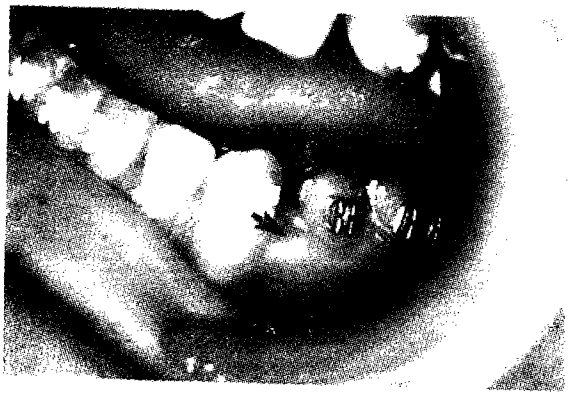

Fig. 3: *Orthodontic treatment intraoral photograph. Arrrow heads indicate frenulum of bucca. Arrow indicate new bone formation in alveolar buccally place.*

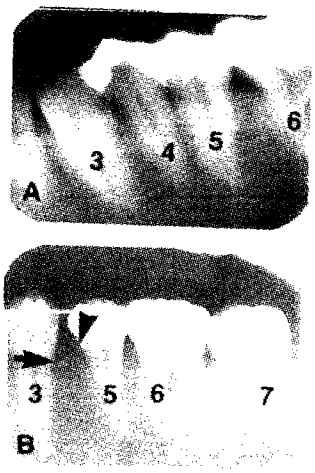

Fig. 4: *A: Initial dental radiograph. B: Orthodontic treatment dental radiograph. 3=Canine, 4=1-Premolar, 5=2-Premolar, 6=1st Molar, 7=2nd Molar, Arrow head indicate new bone formation in alveolar buccally place. Arrow indicate original alveolar crest.*

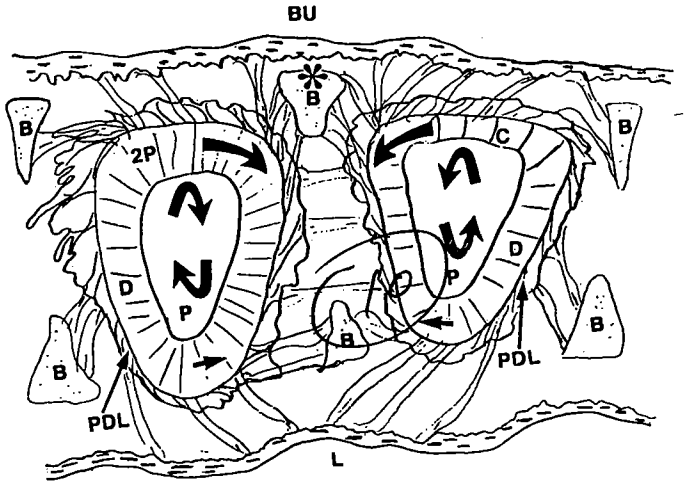

Fig. 5: Diagram of transverse section under the neck of teeth. Teeth adjacent to extraction sites tend to "roll in".
*BU=Buccle, L=lingual, B=bone, P=dental pulp, D=Dentin, PDL= periodontal ligament, C= Canine, 2P=2-Premolar, *=tension side.*

This problem was severe in extraction cases, because the amount of rotation and tipping was usually restricted by the limited extraction space in the arch wire. Moreover, the premature application of elastic tension caused the cuspid and second-premolar to rotate and tip into the extraction sites. Our recommended space closure mechanics are straightforward and allow the orthodontist to focus on tooth positioning.

Conclusions

In the extraction case, most unwanted rotation can be controlled with rubber rotation wedges, Steiner rotation wedges, or longual chain elastics. In conclusion, space closure mechanics can be straightforward and allow the orthodontist to focus on tooth positioning.

References

1. Azuma M. Study of histologic changes of periodontal membrane incident to experimentak tooth movement. Bull. Tokyo Med Dent Univ 1970; 17: 149-178.

2. Kurihara, S. Anelectron microscopic observation on cells found in bone redsorption area incident to experimental tooth movement. Bull Tokyo Med Dent Univ 1977; 24: 103-123.

3. Reitan, K. Tissue reaction as related to the age factor. Dent Record 1954; 74: 271.

4. McLaughlin RP. Bennett JC. The transition from standard edgewise to preadjusted appliance systems. J Clin Orthod 1989; 23: 142-153

5. Bennett JC. McLaughlin RP. Controlled space closure with a preadjusted appliance system. J Clin Orthod 1990; 24: 251-260.

6. Rabie AB. Chay SH. Clinical applications of composite intramembranous bone grafts. Am J Orthod 2000; 117: 375-383.

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Effect of lead acetate on the tensile strength of incisional wounds in Wistar rats

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Key words: Wistar rats, Lead acetate, Tensile strength, wound-healing.

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Abstract

The aim of this study was to investigate the effect of lead acetate toxicity on the tensile strength of incisional wounds in male Wistar rats.

A total of 60 male Wistar rats were used. The experimental /test group (30-Male rats) were allowed to drink aqueous lead acetate at a concentration of 125 mg% for six months. The control group (30-Male rats) were allowed to drink aqueous sodium acetate at the same concentration for the same period. At the end of the 6 months period, serum lead concentration was measured in both groups. A single 5-cm long interscapular incision penetrating the panniculus carnosus was made in the dorsal midline of the rats in both groups. At day-7 and day-14 postoperatively half of the rats from each group were sacrificed by ether inhalation and the entire incision including the surrounding intact, non-wounded, skin was excised. The panniculus carnosus was removed and the tissue immediately placed in ice-cooled containers until tested for tensile strength. The results were analyzed statistically using the student's *t-test*.

At the end of the 6-month period the experimental group of rats showed a statistically significant loss of weight ($P<0.5$). The serum lead level was significantly higher in the experimental group ($P<0.5$) compared to the control group. The tensile strength of the wounds was markedly lower ($P<0.5$) in the test group at day-7 and day-14 than in the control group.

Histologically, there was more collagen, more active fibroblasts and less ground substance in the control wounds compared to the lead-treated wounds.

Lead exposure in rats can lead to loss of weight, a decrease in the tensile strength of the wound and hence delays wound healing.

Introduction

Widespread contamination of the environment by lead (Pb^{++}) may have an impact on human health through the constant and persistent exposure to small doses over long periods of time [1]. Lead toxicity may take various forms depending on the degree of exposure. The present study was undertaken to determine the effect of lead toxicity on wound healing. There appear to be no published reports on the relationship between lead toxicity and wound healing. Lead is known to be one of the commonest environmental pollutants. It is found in soil, grown food produce [2] and in drinking water in some areas [3]. Environmental exposure to toxic levels of lead occurs in a number of industries [4]. In Saudi Arabia, it has been shown that the primary source of lead pollution is motor vehicle emissions [5]. Experimental studies in rats have shown that lead can suppress spermatogenesis [6] and if given simultaneously with N-nitrosodiethylamine it can cause cancer of the kidney [7]. In humans, lead overdose can lead to neuropathies [8].

Healing of skin incisions is a complex, dynamic process, which is characterized by the integrated actions of different cells. The process of wound healing has been described as consisting of broad general phases of inflammation, cellular proliferation, and collagen synthesis, followed by collagen remodeling and maturation. Tensile strength is an essential component of this process. Its measurement is used as a method of assessing wound healing [9]. The strength of the wound is an important factor since it

reflects the subdermal organization of the fibres in the newly deposited collagen. The collagen fibres reorganize in such a way as to secure the interface between the intact dermis and the new epidermis [10].

In general, wound healing in accidental or iatrogenic wounds has an important impact on morbidity in the working population. Factors that adversely affect wound healing can impose a burden on the economies of poor countries by lowering the output of the individuals through absence from work. The present work was undertaken to study the effects of lead in wound healing in the rat.

Materials and Methods

The effect of lead on tensile strength was investigated in 60 male Wistar rats weighing between 180-350 grams. The animals were housed in two groups (Test and control groups, 30 rats each) at a constant temperature of 23 degrees centigrade. Test animals were fed No.1 maintenance diet B.P and tap water containing 125 mg/ 100 ml lead acetate *ad libitum*. The 30 control rats were given No.1 maintenance diet B.P and tap water containing 125mg/ 100ml sodium acetate *ad libitum*. This regime was followed daily for six months.*Weighing of the rats*

Both test and control rats were weighed at the start of the experiment and before making the wounds (i.e after 6 months).

Measurements of lead concenteration in rat venous blood

Venous blood samples were collected from the tail veins of both test and control rats after six months and before making the wounds. The concentration of lead was measured by atomic absorption spectrophotometry [11].

Surgical procedure and Tensile strength measurements

Before the surgical procedure general anaesthesia was induced in the rats by inhalation of ether. Hair was electrically clipped off from the skin of the surgical site. The shaved area was then cleaned with an alcohol- based bactericide (Hibitane). A single linear 5 cm interscapular incision penetrating the panniculus carnosus was made in the dorsal midline. The incisions were then closed with five evenly placed interrupted horizontal mattress sutures of 5-0 Ethilon (Ethicon, Inc., Somerville, NJ). Postoperatively, control and test rats were housed in cages with unrestricted food and water. 7 days after surgery 15 test rats and 15 control rats were sacrificed. The rest of the rats were sacrificed on day-14 postoperatively. The entire incisions, including the surrounding, non-wounded, intact skin were excised and the panniculus carnosus removed. The tissue was immediately placed on ice-cooled containers until tested for tensile strength. From each incision, three test strips approximately 5mm wide were cut perpendicular to the original incision, one from the middle and one from each end. The tensile strength was measured with a Unite-0-Matic FM-20 tensometer (United calibration group, Garden Grove, CA) at a velocity of 20 mm/minute. Tensile strength was expressed as Kg/cm^2 wound tear strength. Mean and standard deviations were calculated for both test and control animals. The data were analysed using the Student's t-test.

Histology

Samples from both test and control incisions were fixed in 10% neutral buffered formalin. They were embedded in paraffin. Sections were stained with hematoxylin and eosin for histological study.

Results

Venous blood lead levels

These measurements confirmed that lead levels in the venous blood of the test group were higher ($P<0.5$) compared to those of the control group. Mean venous blood lead levels in the test and control rats were (1.24 $\pm$ 0.23) micromol/L and (0.47$\pm$ 0.12) micromol/L respectively.

Weight of rats

At the end of the six month of oral lead dosing the test rats looked cachexic and lethargic.There was a statistically significant loss of weight. Small reddish pinhead size swellings were noted in the skin of test rats but not in the skin of control rats. Specimens of these lesions were taken for further histological investigation.

Tensile strength of wounds

The tensile strength was markedly lower in the wounds of the test group than in the control group (Table 1 and Fig 1) at both day 7 and day 14. The difference was statistically signicant ($P<0.5$).

Table 1: Tensile strength of lead-treated and control groups at day-7 and day-14. Values are the means $\pm$ standard deviations

Groups	Day 7	Day 14
Control group	2.42 $\pm$ 0.21 (15)	4.87 $\pm$ 0.36 (15)
Lead-treated group	1.43 $\pm$ 0.16 (15)	2.35 $\pm$ 0.13 (15)

Expressed as kg/cm^2; n for each group is expressed in parentheses.

Histology

Microscopically, the tract of the incisional wounds at day-7 and day-14 of the control group was very narrow with closely apposed edges. Large numbers of active fibroblasts were seen and there

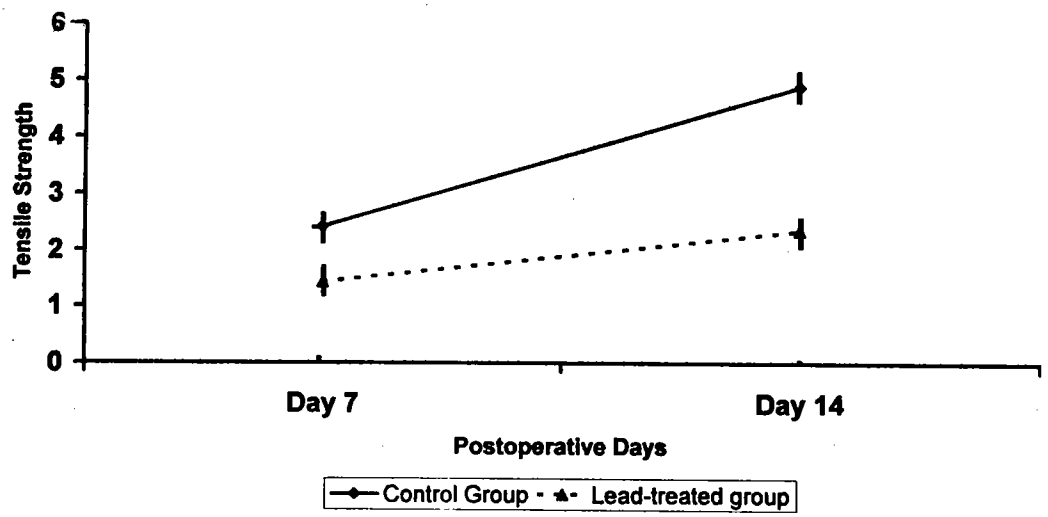

***Fig 1.**: Graph showing the effect of lead acetate on tensile strength (Kg/ cm²) in incisional wounds in Wistar rats. The bars are the standard deviations*

was abundant collagen and sparse ground substance. The wounds of the test group on the other hand were filled by a wide plug of epidermal cells extending the full length of the incision. Fewer active fibroblasts were present. In addition, there was less collagen and more ground substance.

Discussion

Lead compounds are mostly insoluble in water and therefore may pollute the environment in the absence of preventive measures. About 90% of lead in the atmosphere is produced by combustion of lead containing gasoline [5]. Lead enters the body by inhalation or ingestion of contaminated material. In pregnant women, it crosses the placental barrier and poses a hazard to the developing fetus. Evidence suggests that lead intoxication is associated with stillbirths and abortions [4]. The effect of lead poisoning on wound healing appears to have received no attention. The present study is the first report on this subject. It is reported here that oral ingestion of lead acetate in toxic concentrations lowered the tensile strength of incisional wounds in experimental rats. Higher venous blood levels of lead in the test animals confirmed adequate intestinal absorption of lead following oral administration of aqueous lead acetate. Clearly, toxic levels of lead in the blood stream is associated with statistically significant delay in wound healing.

The incisional wound healing model provides an *in vivo* approach for studying the healing of surgical wounds in patients. The rate of healing of incisional skin wounds has historically been assessed using several parameters including tensile strength, histology and immunochemistry [12]. Healing of wounds is a complex process involving epidermal regeneration, fibroblast regeneration, neovascularization, synthesis and remodeling of extracellular matrix components [13]. The mechanism by which lead delays wound healing is not fully understood but it is known that the main target of lead (Pb^{++}) toxicity is the red blood cell [14]. It is also established that lead is a strong enzyme inhibitor [15]. Enzymes can be inactivated or denatured by a variety of chemical means, several of which have clinical importance in wound healing. Many enzymes depend on essential sulfhydryl groups, which form tight covalent bonds with various heavy metals such as lead. For this reason lead can be extremely toxic [15]. Although individual clinical cases of lead poisoning can be treated, preventive measures to minimize pollution must be undertaken to protect the population at large. Further research is required to elucidate the exact mechanism of action operative at the molecular level, that is responsible for the delayed wound healing caused by lead exposure.

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References

1. Needleman HL, Bellinger D, Leviton E and Allred EN.The long-term effects of exposure to low doses of lead in childhood. The New England J Medicine 1990. 322: 83-88.

2. Counter SA, Buchanan LH, Ortega F, Amarasiriwardena C and Hu H. Environmental lead contamination and paediatric lead intoxication in an Andean Ecuadorian village. Int J Occup Environ Health 2000. 6: 169-176.

3. Kessler M, Durand PY, Hestin D, Cao Huu D, Renoult E, Prenat E, Chanliau J, Kaminski M and Duc M. Elevated body lead burden from drinking water in end-stage chronic renal failure. Nephrol Dial Transplant 1995; 10: 1648-1653.

4. Albert N and Moore K. Lead poisoning. New England Journal of Medicine 1990; 325: 1533-1537.

5. Al Saleh I. Sources of lead in Saudi Arabia (King Faisal Specialist Hospital Research Centre). R C Bull 1992; 4: 4-12.

6. Sokol RZ. Hormonal effects of lead acetate in the male rat. Biol Reprod 1987; 37: 1135-1138.

7. Nogueira E. Rat renal carcinogenesis after chronic simultaneous exposure to lead acetate and N-nitrosodiethylamine. Virchows Arch B cell Pathol Incl Mol Pathol 1987; 53: 365-374.

8. Kajiyama K, Doi R, Sawada J, Hashimoto K, Hazama T, Nakata S, Hirata M, Yoshida T and Miyajima K. Significance of subclinical entrapment of nerves in lead neuropathy. Environ Res 1993; 60: 248-253.

9. Nedelec B, Shen YJ, Ghahary A, Scott PG and Tredget EE. The effect of interferon alpha 2b on the expression of cytoskeletal proteins in an *in vitro* model of wound contraction. J Lab Clin Med 1995; 126: 474-484.

10. Kirsner RS, and Eaglsteing WH. The wound healing process. Dermatol Clins 1993; 11: 4629-4635.

11. Lubran M. Determination of Lead by Atomic Absorption Spectrophotometry. In: Laboratory diagnosis of diseases caused by toxic agents. Sunderman FW and Sunderman FW Jr, eds. London: Adam Hilger; 1970; 360-365.

12. Jimenez PA and Rampy MA. Keratinocyte Growth Factor-2 accelerates wound healing in incisional wounds. Journal of Surgical Research 1999; 81: 238-242.

13. Pessa ME, Bland KI and Copeland EM. Growth factors and determinants of wound repair. J Surg Res 1987; 42: 207-217.

14. 14. Taupeau C, Poupon J, Nome F, Lefevre B. Lead accumulation in the mouse ovary after treatment-induced follicular atresia. Reprod Toxicol 2001; 15: 385-91.

15. Singh SP. Enzymes and Isoenzymes. In: A textbook of Biochemistry, 1st ed. Delhi (India): Satish Kumar Jain for CBS Publishers & Distributors 1993; 127-144.

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Effect of short-term treatment with recombinant Human Growth Hormone (GH) on size heterogeneity of Insulin-Like Growth Factor-I (IGF-I) and IGF Binding Protein 3 (IGFBP-3) in the plasma and urine of patients with chronic renal failure

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Key words: Recombinant human GH, IGFBP-3, Radioimmunoassay, Chronic renal failure

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Abstract

We investigated the effect of short term administration of recombinant human GH (rhGH) on plasma and urinary IGF-I and IGFBP-3 levels in five patients with chronic renal failure (CRF). rhGH was subcutaneously infused at a rate of 2 μg/kg/h for 72h. IGF-I and IGFBP-3 levels were measured by specific radioimmunoassay (RIA), which plasma and urine samples were further divided into two components of larger (>100,000) and smaller (<100,000) molecular weight by the ultrafiltration method. Mean (±SD) plasma IGF-I levels increased from 169.2±45.4 μg/L to 404.2±120.7 μg/L after rhGH administration (P<0.05), but urinary IGF-I levels were not significantly changed. Plasma IGFBP-3 levels increased from 2.9±1.8 mg/L to 4.0±1.6 mg/L after rhGH administration (P<0.05), of which larger molecular weight fraction was mainly increased. Urinary IGFBP-3 levels increased from 0.13±0.15 mg/g creatinine to 1.35±0.27 mg/g creatinine after rhGH administration (P<0.05), of which smaller molecular weight fraction increased. Percent increase of urinary IGFBP-3 levels was much more greater than that of plasma IGFBP-3 in these patients (1757.2±1483.6% vs 172.9±39.1%, P<0.05). On the other hand, there was no statistical difference between percent increases of plasma and urinary IGF-I levels (249.3±71.8% vs 368.6±200.9%, P>0.05). These findings suggest that urinary IGFBP-3 levels, especially larger molecular weight of IGFBP-3, are increased in patients with CRF after rhGH administration, which might reflect increased urinary IGFBP-3 protease activity and increased renal clearance rate of smaller molecular weight component of IGFBP-3 after GH treatment.

Introduction

Insulin-like growth factor binding protein 3 (IGFBP-3) is an acid-stable, 45 kilodalton (KD) glycosylated protein with a core molecular mass of 29 KD, which exists in the circulation as a part of 150 KD complex [1,2]. IGFBP-3 is the major regulatory component of insulin-like growth factor I (IGF-I), and IGFBP-3 potentiates and inhibits IGF-I action [3]. Measurement of plasma IGFBP-3 levels by radioimmunoassay (RIA) is a useful assessment of GH secretion in child GH deficiency [4,5,6,7].

In patients with chronic renal failure (CRF), plasma and urinary IGFBP-3 levels were remarkably increased, and the increased plasma IGFBP-3 was accompanied by an increase in the small molecular weight fraction [8,9,10]. It has been also reported that there was discrepancy between IGFBP-3 levels measured by RIA and those of Western ligand blot in patients with renal dysfunction [11,12].

When recombinant human GH (rhGH) was administered to normal subjects, plasma and urinary IGFBP-3 levels increased, and the major fraction of urinary IGFBP-3 was eluted at a smaller molecular weight portion than 150 KD [13]. Urinary IGFBP-3 levels increased in proportion to plasma IGFBP-3 increment during rhGH administration in normal subjects [13]. There has been no report on the effect of rhGH administration on plasma and urinary IGFBP-3 levels measured by RIA in patients with chronic renal failure (CRF).

In the present study, we investigated the effect of short term treatment with GH on plasma and urinary IGF-I and IGFBP-3 levels and their size heterogeneity in patients with CRF.

Materials and Methods

Subjects

ive patients with chronic renal failure were studied. They consisted of 2 males and 3 females, with the mean (±SD) age of 71.0±8.7 yr. Body mass index was 22.1±3.4 kg/m^2. All the patients were diagnosed as CRF due to diabetes mellitus. Glycosylated hemoglobin A1c level was 6.0±0.7%. Urinary albumin excretion was 2.04±1.62 g/day. Plasma creatinine level was 2.6±1.4 mg/dl, and creatinine clearance was 42.1±35.7 ml/min.

Study protocol

All subjects were admitted to our hospital during the experimental period. The diet was restricted to a total calorie intake of 30 kcal/kg of ideal body weight, protein of 1.0 g/kg of ideal body weight and salt of 5 g/day before and during the experimental period. The dosage of other drugs required was not changed during the period. Recombinant human GH (rhGH) (Genotropin, Sumitomo Co., Tokyo, Japan) was subcutaneously infused for 72 h at a flow rate of 2 μg/kg/h using a portable syringe pump (SP-3HQ. Nipro Co., Osaka, Japan). Plasma samples were obtained before and 72 h after the start of rhGH infusion. The samples were immediately centrifuged and stored at -20C until assayed. Urine samples were collected for 24-h before the experiment and on the third day. The urine samples were centrifuged and the supernatants were stored at -20C until assayed. Informed consent was obtained from all subjects.

Sample preparation for IGFBP-3 measurement

Plasma and urine samples were diluted to 1:100 and 1:10 with dilution buffer, respectively. The diluted samples were divided into two fractions, one was for IGFBP-3 measurement, and the other was for fractionated IGFBP-3 measurement. The diluted samples were fractionated into large (>100,000) and small molecular weight (<100,000) by using an ultrafiltration membrane (Centricon 100, Amicon Inc., MA, USA). The ultrafiltration membrane retained 95% of bovine IgG fraction II (MW 156,000) and filtered 95% of ovalbumin (MW 67,000). The device with diluted sample was centrifuged at 1000xg for 30 min. The retained fraction was resuspended with dilution buffer to get an equal volume of sample filtrated. The larger molecular weight fraction contained 150 KD fraction of IGFBP-3 and the smaller molecular weight contained 45 KD and 29 KD fractions of IGFBP-3.

Assays

Plasma and urinary IGF-I levels were measured by specific radioimmunoassay after acid ethanol extract as previously described [14]. The detectable quantity was 0.56 μg/L, and the coefficient variance of the inter- and intra-assay was 2.5% and 1.5%, respectively.

Plasma and urinary IGFBP-3 levels were measured by specific radioimmunoassay (Diagnostic Systems Laboratory Inc., TX, USA). IGFBP-3 standard was calibrated to recombinant DNA--derived nonglycosylated human IGFBP-3 (28.75 KD) expressed by *E. coli*. The minimal detectable quantity was 1.0 μg/L. The anti-IGFBP-3 serum crossreacted with 150 KD, 45 KD and 29 KD components of IGFBP-3. There was no considerable crossreactivity of the IGFBP-3 antiserum against purified IGFBP-1, recombinant IGFBP-2, 4, 5, 6, IGF-I and IGF-II.

Urinary creatinine levels were measured by an autoanalyser (TBA50S, Toshiba, Tokyo, Japan). Urinary IGFBP-3 and IGF-I levels were corrected by urinary creatinine concentrations.

Statistical analysis

Statistical analysis of the data was performed by one-way analysis of variance in combination with Student's t-test. $P<0.05$ was considered significant. The data were expressed by means ± SD.

Results

As shown in Fig. 1, mean (±SD) plasma IGF-I levels were increased from 169.2±45.2 μg/L to 404.2±120.7 μg/L inpatients with CRF after rhGH administration ($P<0.05$). However, urinary IGF-I levels did not significantly increase after rhGH administration (2.9±4.1 μg/g creatinine vs 7.9±11.2 μg/g creatinine). As shown in Fig. 2, plasma IGFBP-3 levels increased from 2.9±1.8 mg/L to 4.0±1.6 mg/L ($P<0.05$) after rhGH treatment. The major fraction which increased was the larger molecular weight fraction. The smaller molecular weight fraction

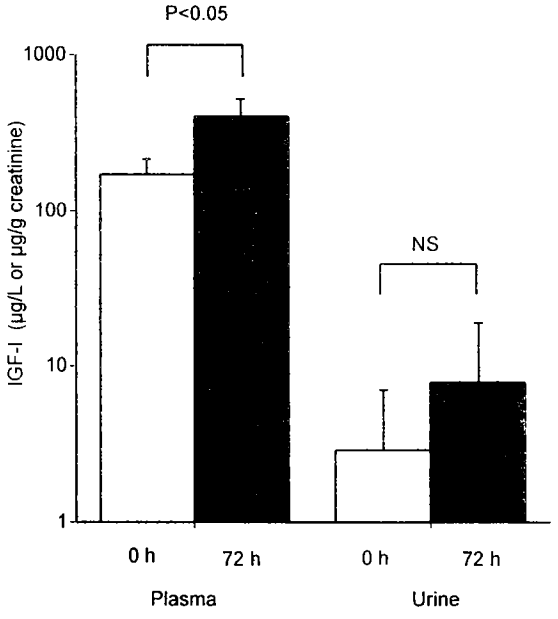

Fig. 1: *Effect of short term treatment with recombinant human GH (rhGH) on plasma and urinary IGF-I levels in 5 patients with CRF. Open column and closed column shows before and after rhGH administration for 72 h, respectively. rhGH was constantly infused subcutaneously at a rate of 2 μg/kg/h for 72 h with a portable infusion pump. Mean±SD values are shown.*

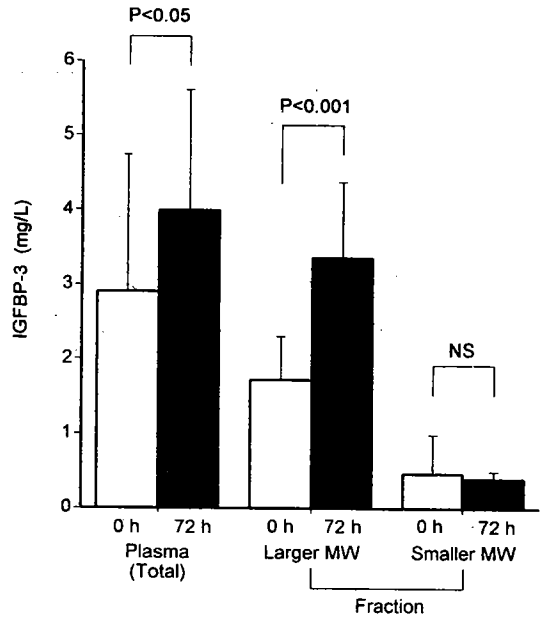

Fig. 2 *Effect of rhGH treatment on total and fractionated serum IGFBP-3 levels measured by specific RIA in 5 patients with CRF. Open column and closed column shows before and after rhGH administration for 72 h, respectively. Serum IGFBP-3 was fractionated into larger molecules (MW >100.000) and smaller molecules (MW <100,000) by means of ultrafiltration method.*

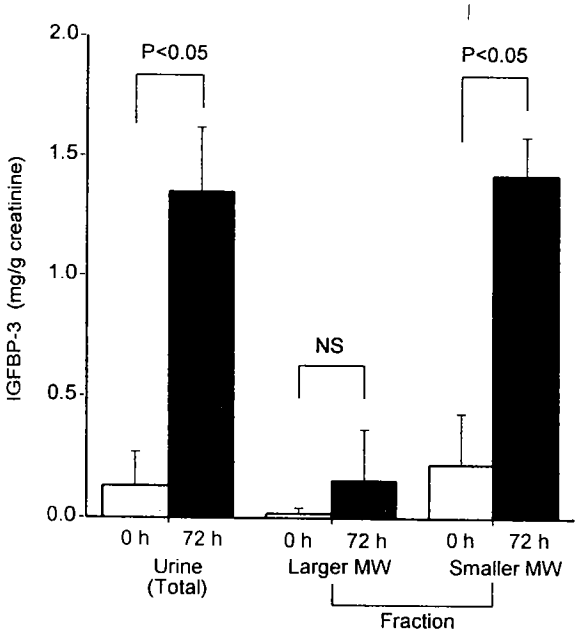

Fig. 3 *Effect of rhGH treatment on total and fractionated urinary IGFBP-3 levels measured by specific RIA in 5 patients with CRF. Open column and closed column shows before and after rhGH administration for 72 h, respectively. Urine IGFBP-3 was fractionated into larger molecules (MW >100.000) and smaller molecules (MW <100,000) by means of ultrafiltration method. Each value was corrected by creatinine concentration in the original urine.*

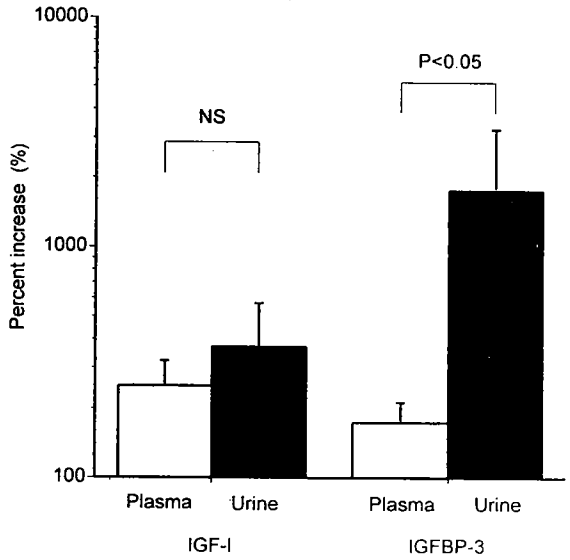

Fig. 4 *Percent changes of IGF-I and IGFBP-3 levels in the plasma and urine in 5 patients with CRF after rhGH administration for 72 h.*

was not changed after rhGH administration. As shown in Fig. 3, urinary IGFBP-3 levels were increased from 0.13±0.15 mg/g creatinine to 1.35±0.27 mg/g creatinine (P<0.05) in patients with CRF after rhGH administration. The major fraction increased was the smaller molecular weight, whereas the large molecular weight fraction was not changed after rhGH administration. As shown in Fig. 4, the percentage increase in plasma and urinary IGFBP-3 was 172.9±39.1% and 1757.2±1483.6%, respectively. The percentage increase in urinary IGFBP-3 was more dominant than that of plasma IGFBP-3 (P<0.05). There was no difference between the percentage increases in plasma and urinary IGF-I (249.3±71.8% and 368.6±200.9%, respectively).

Discussion

In the present study, we found that urinary IGFBP-3 excretion was significantly increased after rhGH administration in patients with CRF. The change in urinary IGFBP-3 was not proportional to the change of plasma IGFBP-3. The major component of IGFBP-3 in the urine during rhGH administration proved to be smaller molecular weight fraction. Circulating IGFBP-3 levels measured by specific RIA included 150 KD, 45 KD and 29 KD component of IGFBP-3. Chromatographic analysis has revealed that IGFBP-3 exists as 150 KD complex in the serum of healthy subjects, whereas smaller molecule components of 45 KD and 29 KD of IGFBP-3 exist in the urine [11]. Some discrepancies between IGFBP-3 levels determined by RIA and those of Western ligand blot analysis have been reported, suggesting the necessity of molecular weight analysis of IGFBP-3 in fluids containing high proteolytic activity [9]. Previous studies of quantitative IGFBP-3 analysis in the serum were performed by gel chromatography followed by specific RIA [8,15]. Western ligand blotting was not sufficient for the quantitative analysis of IGFBP-3. In the present study, we simply divided plasma and urine samples into large (>100,000) and small (<100,000) molecular weight

fractions by means of ultrafiltration which had a cut-off at the molecular weight of 100,000. Our preliminary data showed that more than 95% of 45 KD and 29 KD fractions of IGFBP-3 could pass through the membrane, and more than 95% of 150 KD fraction of IGFBP-3 complex could be retained on the membrane. This enabled the molecular and quantitative analysis of IGFBP-3 in plasma and urine.

Serum IGFBP-3 levels are known to be controlled by GH in healthy subjects and in patients with GH deficiency [5,6]. Urinary IGFBP-3 levels measured by RIA have been shown to be normal or elevated in patients with GH deficiency, indicating that urinary IGFBP-3 is not under the control of GH and unlikely to reflect serum IGFBP-3 filtered through the kidney [11,12]. Serum IGFBP-3 levels were increased in patients with CRF, which consisted of increased smaller fragments of IGFBP-3, suggesting that IGFBP-3 protease activity was rather enhanced in the plasma and urine samples in these patients [11,16].

Serum and urine IGFBP-3 levels are increased in healthy subjects and patients with GH deficiency after rhGH administration [13,17]. However, there has been no report on the effect of rhGH administration on urinary excretion of IGFBP-3 in patients with CRF. Urine IGFBP-3 is considered to come from the circulation and/or the kidney. Northern blot analysis failed to detect IGFBP-3 mRNA in adult human kidney tissue [18,19], indicating IGFBP-3 production was low or absent in the normal human kidney. On the other hand, IGFBP-3 was detected by in situ hybridization method in human kidney [20]. Immunoreactive urinary IGFBP-3 levels were estimated to be approximately 18 μg/g creatinine, which was correlated with urinary IGF-I levels in normal subjects [11]. It was reported that urinary IGFBP-3 levels measured by RIA was normal or elevated in GH deficiency, whereas urinary IGFBP-3 was low or undetectable by Western ligand blot measurement, indicating the presence of high IGFBP-3 protease activity in the urine [11]. Urinary excretion of IGFBP-3 excretion increased in proportion to the change of serum IGFBP-3 in normal subjects after rhGH administration, and urinary IGFBP-3 might reflect mainly renal clearance of smaller fragments of IGFBP-3 from the circulation [13].

In the present study, it was noted that percent increase of urinary IGFBP-3 was much higher than that of plasma IGFBP-3 in patients with CRF after rhGH administration. These findings may be explained by some possible mechanisms. First, small molecular weight fraction of IGFBP-3 generated by IGFBP-3 protease might cause overestimation of IGFBP-3 in RIA [16,21]. Protease activity to proteolyses IGFBP-3 was detected in the serum obtained from pregnant women [22] and rats [23]. The presence of IGFBP-3 protease activity was also demonstrated in the urine of patients with CRF [21]. Furthermore, the IGFBP-3 protease activity was enhanced in nephrotic syndrome [24]. Second, increased smaller molecular weight component of IGFBP-3 might be excreted from serum into urine by increased renal clearance in patients with CRF after GH treatment. Many peptides were filtered in the glomerulus and reabsorbed from the tubules [25]. Therefore, urinary excretion of the peptide was increased due to renal tubular dysfunction [26]. We previously reported that glomerular filtration was increased in patients with CRF after GH treatment [27]. Third, renal production of IGFBP-3 might increase in patients with CRF after rhGH administration although it remains to be elucidated whether local production of IGFBP-3 in the kidney is involved.

Urinary IGF-I levels did not consistently increase in spite of an increase of plasma IGF-I levels after rhGH for 72 h. These findings are similar to other studies in normal subjects [13]. The main origin of urinary IGF-I is not fully clarified. IGF-I was filtered in the glomerulus and reabsorbed in the tubules, and can be produced in the kidney [28]. Therefore, urinary IGF-I levels could be influenced by renal clearance and IGF-I production in the kidney. As IGF-I mRNA was not detected in the kidney under baseline condition in normal subjects, local production of IGF-I in the kidney was not simply determine urinary IGF-I levels [29]. It is possible, however, that specific stimuli with GH might induce IGF-I production in the kidney in patients with CRF. Therefore, urinary excretion of IGF-I might be affected by not only renal clearance of IGF-I and IGF-I production in the kidney after rhGH administration. However, the detailed mechanisms remain to be further elucidated.

We administrated rhGH by means of continuous subcutaneous infusion to obtain a consistent increase of plasma IGF-I and plasma IGFBP-3 levels in a short-term period. Laursen et al [30] reported that continuous subcutaneous infusion of GH induced an increase in serum IGF-I and IGFBP-3 levels more effectively than daily subcutaneous injections. We previously reported that continuous subcutaneous infusion of GH was effective in increasing plasma erythropoietin levels in CRF [27].

In conclusion, we found that there was a greater increase in urinary IGFBP-3 levels than plasma IGFBP-3 levels in patients with CRF after rhGH administration. The major component was the larger molecular weight fraction in the plasma and the smaller molecular weight fraction in the urine. Urinary IGF-I levels were not changed in spite of increased plasma IGF-I levels. These findings suggest that urinary IGFBP-3 might reflect increased urinary IGFBP-3 protease activity and increased renal clearance rate of the smaller molecular weight component of IGFBP-3 from plasma after rhGH administration in patients with CRF.

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References

1. Wilkins JR, D'Ercole AJ. Affinity-labeled plasma somatomedin-C/Insulin-like growth factor 1 binding proteins. J Clin Invest 1985; 75: 1350-1358.

2. Martin JL, Baxter RC. Insulin-like growth factor binding protein-3: biochemistry and physiology. Growth Regulat 1992; 2:88-99.

3. DeMellow JSM, Baxter RC. Growth hormone dependent insulin-like growth factor (IGF) binding protein both inhibits and potentiates IGF-I stimulated DNA synthesis in human skin fibroblasts. Biochem Bioph Res Co 1988; 156: 199-204.

4. Baxter R, Martin J. Radioimmunoassay of growth- hormone dependent insulin like growth factor binding protein in human plasma. J Clin Invest 1986; 78: 1504-1512.

5. Blum WF, Ranke MB, Kietzmann K, Gauggel E, Zeisel HJ, Bierich JR. A specific radioimmunoassay for the growth hormone (GH)-dependent somatomedin-binding protein: its use for diagnosis of GH deficiency. J Clin Endocr Metab 1990; 70: 1292-1298.

6. Hasegawa Y, Hasegawa T, Aso T, Kotoh S, Tsuchiya Y, Nose O, Ohyama Y, Araki K, Tanaka T, Saisyo S. Usefulness and limitation of measurement of insulin-like growth factor binding protein-3 (IGFBP-3) for diagnosis of growth hormone deficiency. Endocrinol Jap 1992; 39: 585-591.

7. Gill MS, Toogood AA, O'Neill PA, Thorner MO, Shalet SM, Clayton PE. Urinary growth hormone (GH), insulin-like growth factor I (IGF-I), and IGF-binding protein-3 measurements in the diagnosis of adult GH deficiency. J Clin Endocr Metab 1998; 83: 2562-2565.

8. Powell DR, Liu F, Baker B, Lee PD, Belsha CW, Brewer ED, Hintz RL. Characterization of insulin-like growth factor binding protein-3 in chronic renal failure serum. Pediatric Res 1993; 33: 136-143.

9. Buyan N, Cinaz P, Hasanoglu E, Gokcora N, Bircan Z. Changes in serum levels of IGF-I and IGFBP-3 in children with chronic renal failure. Endocrine J 1993; 42: 429-433.

10. Hasegawa Y, Cohen P, Yorgin P, Rosenfeld RG. Characterization of urinary insulin-like growth factor binding proteins. J Clin Endocr Metab 1992; 74: 830-835.

11. Gargosky SE, Hasegawa T, Tapanainen P, MacGillivray M, Hasegawa Y, Rosenfeld RG. Urinary insulin-like growth factors (IGF) and IGF-binding proteins in normal subjects, growth hormone deficiency, and renal disease. J Clin Endocr Metab 1993; 76: 1631-1637.

12. Gargorsky SE, Pham HM, Wilson KF, Liu F, Giudice LC, Rosenfeld RG. Measurement and characterization of insulin-like growth factor binding protein-3 in human biological fluids: discrepancies between radioimmunoassay and ligand blotting. Endocrinology 1992; 131: 3051-3060.

13. Tönshoff B, Blum WF, Vickers M, Kurilenko S, Mehls O, Ritz E. Quantification of urinary insulin-like growth factors (IGFs) and IGF binding protein 3 in healthy volunteers before and after stimulation with recombinant human growth hormone. Eur J Endocrinol 1995; 132: 433-437.

14. Yamamoto H, Sohmiya M, Oka N, Kato Y. Effects of aging and sex on plasma insulin-like growth factor I (IGF-I) levels in normal adults. Acta Endocrinol (Copenh) 1991; 124: 497-500.

15. Daughaday WH, Ward AP, Goldberg AC, Trivedi B, Kapadia M. Characterization of somatomedin binding in human serum by ultracentrifugation and gel filtration. J Clin Endocr Metab 1982; 55: 916-921.

16. Lee DY, Park SK, Yorgin PD, Cohen P, Oh Y, Rosenfeld RG. Alteration in insulin-like growth factor-binding proteins (IGFBPs) and IGFBP-3 protease activity in serum and urine from acute and chronic renal failure. J Clin Endocr Metab 1994; 79: 1376-1382.

17. Spagnoli A, Gargosky SE, Spadoni GL, MacGillivray M, Oh Y, Boscherini B, Rosenfeld RG. Characterization of a low molecular mass form of insulin-like growth factor binding protein-3 (17.7 kilodaltons) in urine and serum from healthy children and growth hormone (GH)-deficient patients: relationship with GH therapy. J Clin Endocr Metab 1995; 80: 3668-3676.

18. Naya FJ, Stratheam MD, Spencer EM. Tissue expression and chromosomal localization of the human insulin-like growth factor binding protein 3. In: Spencer EM, editor. Modern concepts of insulin-like growth factors. New York: Elsevier 199: 337-342.

19. Price GJ, Berka JL, Edmondson SR, Werther GA, Bach LA: Localization of mRNAs for insulin-like growth factor binding proteins 1 to 6 in rat kidney. Kidney Int 1995; 48: 402-411.

20. Chin E, Michels K, Bondy CA. Partition of insulin-like growth factor (IGF)-binding sites between the IGF-I and IGF-II receptors and IGF-binding proteins in the human kidney. J Clin Endocr Metab 1994; 78: 156-164.

21. Lee DY, Cohen P, Krensky AM, Rosenfeld RG, Yorgin PD. Insulin-like growth factor binding protein-3 protease activity in the urine of children with chronic renal failure. Pediatr Nephrol 1993; 7: 416-423.

22. Giudice L, Farrell E, Pham H, Lamson G, Rosenfeld R. Insulin-like growth factor binding proteins in maternal serum throughout gestation and in the puerperium: effects of a pregnancy-associated serum protease activity. J Clin Endocr Metab 1990; 71: 806-816.

23. Davenport M, Pucilowska J, Clemmons D, Lundblad R, Spencer J, Underwood L. Tissue-specific expression of Insulin-like growth factor binding protein-3 protease activity during rat pregnancy. Endocrinology 1992; 130: 2505-2512.

24. Hirschberg R, Kaysen GA. Insulin-like growth factor I and its binding proteins in the experimental nephrotic syndrome. Endocrinology 1995; 136: 1565-1571.

25. Maack T, Johnson V, Kau ST, Figueiredo J, Sigulem D. Renal filtration, transport, and metabolism of low- molecular-weight proteins: A review. Kidney Int 1979; 16: 251-270.

26. Hattori N, Kato Y, Murakami Y, Hashida S, Ishikawa E, Mohri Z, Imura H. Urinary growth hormone levels measured by ultrasensitive enzyme immunoassay in patients with renal failure. J Clin Endocrinol Metab 1988; 66: 727- 732.

27. Sohmiya M, Ishikawa K, Kato Y. Stimulation of erythropoietin secretion by continuous subcutaneous infusion of recombinant human GH in anemic patients with chronic renal failure. Eur J Endocrinol 1998; 138: 302-306.

28. Hammerman MR, Ryan G, Miller SB. Expression of insulin-like growth factor in adult and embryonic kidney. Miner Electrol Metab 1992; 18: 253-255.

29. Chin E, Bondy C. Insulin-like growth factor system gene expression in the human kidney. J Clin Endocr Metab 1992; 75: 962-968.

30. Laursen T, Jorgensen JO, Jakobsen G, Hansen BL, Christiansen JS. Continuous infusion versus daily injections of growth hormone (GH) for 4 weeks in GH-deficient patients. J Clin Endocr Metab 1995; 80: 2410-2418.

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Angioarchitecture of the duck pecten

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Abstract

The angioarchitecture of the duck pecten oculi was investigated using light (LM), scanning electron (SEM), and transmission electron (TEM) microscopy techniques. SEM analysis showed that the blood capillaries anastomosed with each other forming a continuous basket-like network that covered both sides of the pecten. Overall, the pecten vascular cast resembled a sandwich-like structure containing large arteries and veins between two layers of capillaries. Small, lymphatic vessels were observed lying on all the blood capillaries of the pecten, and bridging those capillaries on the pleats. These lymphatic vessels ran along the pleats, sometimes connecting to blood capillaries through pores within the internal membrane, and sometimes connecting to lymphatic vessels located within the intercellular spaces between blood capillaries. These intercellular spaces contained fibrillar material, pigmented phagocyte cells, processes derived from these cells and lymphatic precolectors which originated from the bottom of a well structure. Sometimes the intercellular spaces were very deep and completely covered by pigment cells. Near the outskirts of the pecten, some of intercellular spaces were closed off by a covering membrane which permitted the transport of materials and substances from the vitreous body into these spaces. The morphological characteristics of the pecten oculi described in these present study suggest that the pecten plays an important role in the regulation of metabolic exchanges between the ocular blood vessels and the retina, thus preserving the integrity and stability of the intraocular microenvironment.

Introduction

The pecten (*Pecten oculi*) is a characteristic structure of the avian eye. Despite the many detailed investigations that have been carried out on its morphology and biochemistry, the function of the pecten is still not clear [1-11]. It has been hypothesized that the pecten is involved in regulating pH [12], eye-pressure [13], and eye temperature [14]. In addition, pecten has been hypothesized to have a protective role against the reflection of sunshine inside the eye based on the fact that the pecten cones produce shaded areas [15]. The most plausible of the hypotheses proffered to date are those which indicate that pecten plays a role in the nutrition of the retina [16]. Nutrition in the central nervous system involves the selective passage of nutrients through the protective walls formed by the endothelial cells. Gerhardt et al. [17] have proposed pecten as a new in vivo model for the blood-brain barrier since pecten has the four essential characteristics of a barrier: 1) complex tight junctions; 2) the glucose transporter isoform GluT-1; 3) an HT7 molecule; and 4) impermeability to lanthanum-nitrate.

In a recent study of the microvasculature of the duck pecten [18], the presence of lymphatic vessels accompanying the blood capillaries was observed. This suggests that, in addition to a nutritive role, pecten also plays a role in the drainage of waste products from the vitreous body.

In order to better understand the role played by the blood and lymphatic vessels found in the pecten oculi of avian, the authors carried out a morpho-structural study of the angioarchitecture of the pecten in duck using scanning electron (SEM) and transmission electron (TEM) microscopy.

Materials and Methods

Eighteen adult Campbell khaki ducks (*Anas platyrhynchos*) of both sexes were studied. The ducks were anaesthetised by intramuscular injection of Ketamine (25 mg/kg), and sacrificed by exanguination.

Scanning electron microscopy – vascular corrosion cast technique

After sacrifice, six ducks were each incannulated through the common carotid artery (*A. carotis communis*) and perfused with

physiological solution to wash the vascular bed. They were then injected with a low viscosity, colored methylmethacrylate mixture [19]. After methylmethacrylate polimerization, eye-casts were collected and corroded by immersion in KOH solution (30%) for 1 to 2 weeks. The specimens were then rinsed with tap water, rinsed with bi-distilled water and dried in a desiccator. The pecten was removed from the eye-casts, mounted on stubs (12 mm, diameter) and coated with gold using a sputter coater (BIORAD, SC500, Hemel Hempstead, UK). All gold coated samples were examined and photographed under a scanning electron microscope (SEM-LEO 435 VP, Cambridge, UK) at 10 kV.

Scanning electron microscopy – intact tissue technique

Six ducks were each perfused through the common carotid artery with phosphate buffer 0.1 M, pH 7.3, to wash blood vessels, and then fixed with Karnovsky's solution (4% parafolmaldehyde, 2.5% glutaraldehyde). After 12 h, the pectens were separated from the eyes, immersed in a glucose phosphate buffer for 24-48 h, and dehydrated first in ethyl alcohol, and later using critical point (BALZERS CPD 030, Liechtenstein)). The piece specimens were then mounted on stubs (12.5 mm, diameter), examined under SEM (LEO 435 VP) at 17 kV, and photographed.

Transmission electron microscopy

Six ducks were each perfused through the common carotid artery with a cacodilate buffer 0.1 M, pH 7.2 to wash the vascular bed. A mixture of this buffer and 2% glutaraldehyde was then injected into the artery. Pectens were removed from the eyes and immersed in glutaraldehyde for 1 h, rinsed in cacodilate buffer, postfixed with 2% OsO_4 for 2 h, dehydrated, and embedded in an EM bed 812 resin. All embedded specimens were sliced into ultrathin sections using an ultramicrotome (Ultratome IV-LKB), stained with uranyl acetate and lead citrate (Ultrastain-LKB), examined under a transmission electron microscope (TEM-Philips EM 201, Eindhoven, The Netherlands) at 40 kV, and photographed.

The nomenclature in this paper has been adopted from the Handbook of Avian Anatomy: Nomina Anatomica Avium [20].

RESULTS

SEM Observations

1) General Vascular Organization of the Pecten

SEM examination of the duck pecten showed a very well developed vascular organization. The integral cast of the pecten was a trapezoid (6.5 mm long, 4 mm high, 0.5 mm thick) that had 12 folds, and a closely woven overall texture which tended to loosen towards the apex.

2) Blood Vessel Architecture

Blood was supplied to the pecten by 4 arteries (*Aa. pectinis oculi*) originating from the ophtalmotemporal artery (*A. ophthalmotemporalis*). The *Aa. pectinis oculi* lay at the base of the pecten and within the wall of the choroids, and then branched into several perpendicular arteries that ran within the wall towards the apex of the pleats. The arteries of the pleats gave rise to several arterioles that ran between the sides of the pleats, and terminated in a superficial network of capillaries (Fig.1). These capillaries anastomosed with each other forming a continuous basket-like network that covered both sides of the pecten. Overall, the pecten vascular cast resembled a sandwich-like structure containing large arteries and veins between two layers of capillaries. These capillaries varied in diameter and shape, and formed an uninterrupted covering on the pectin (Figs. 2a, 2b). Contiguous capillaries of the pecten were frequently connected to each other by small, hollow, communicating structures situated at slits in the capillary walls (Fig. 3). In addition, the surfaces of the capillaries were extensively coved by depressions of various sizes and shapes. The capillary network gave rise to the post-capillary venules that converged within the pleats. At the base of the pecten, these venules gave rise to the pecten veins (*Vv.pectinis oculi*) that connect to the ophtalmotemporal vein (*V. ophthalmotemporalis*).

3) Lymphatic Vessel Origin and Architecture

Small, lymphatic vessels (*Vas lymphaticum precolectorium*) 3-4 µm in diameter were observed lying on all the blood capillaries of the pecten, and bridging between capillaries on the pleats. The lymphatic system originated from lymphatic capillaries 0.8-1.2 µm in diameter located at the apex of the pecten, and formed a network (*Rete lymphatica initiale*) composed of links that continued to the precollector lymphatic vessels. These precollectors ran along the pleats, sometimes connecting to blood capillaries through pores (0.5-0.6µm, diameter) in the internal membrane, and sometimes connecting to lymph vessels (*Vas lymphaticum initiale*) located within the intercellular spaces between the blood capillaries (Figs. 4a, 4b). They then followed the pleats to the base of the pecten, where they exited the organ (*Vas lymphaticum collectorium*).

TEM Observations

1) Blood Vessel Structure

The endothelial cells observed in the duck pecten blood capillaries were either smooth, or covered with microfolds. The former cells were thin, with few apical processes, and were not frequent. The latter cells, which often filled the entire capillary lumen, had many microfolds on the luminal and basal surfaces, and in some cases, their microfolds had enlarged extremities. In general, the transition area between the two cell types was neat and clear-cut (Fig. 5). Adjacent cell types were conjoined to each other forming thin strips of desmosomes along the entire length of cell surface contact. The nuclei of the cells were ovoid in shape and protruded towards the lumen. Cytoplasmic organelles (mitochondria, granular endoplasmic reticulum fragments, and ribosomes) were observed in the perinuclear region of the cells. Small, elongated, electron-dense cytoplasmatic granules were observed in the cytoplasm. The smooth endothelial cells frequently showed large clear cytoplasmatic vesicles. In addition, there were blood

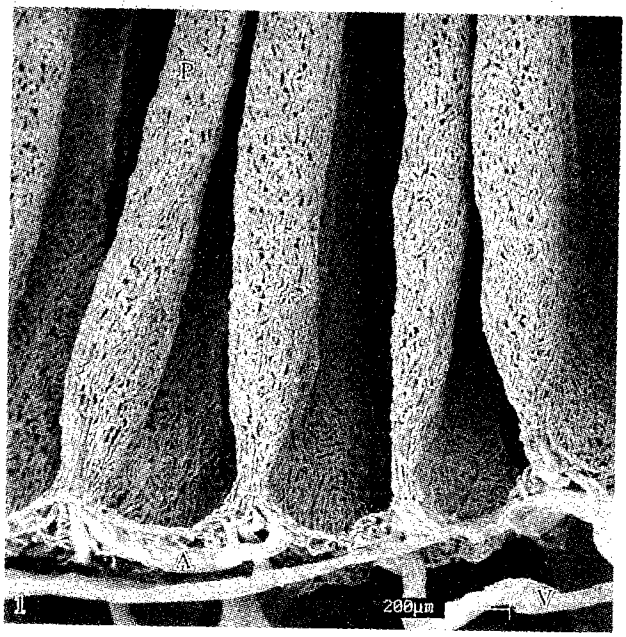

Fig. 1: SEM of microvascular corrosion casts of the duck pecten oculi.
A, pecten artery. P, pleat of the pecten. V, pecten vein.

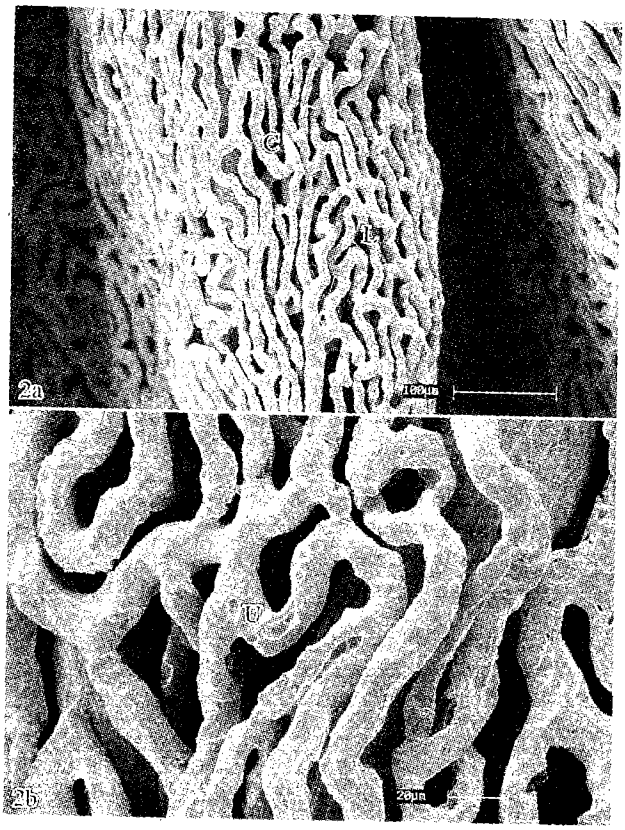

Fig. 2: ***SEM of the superficial blood capillary network of pleats.***
2a: Capillary bed of the pleat vievewed from the side. 2b: Detail of capillaries.
C, blood capillary network. L, lymphatic precolector. U, imprints of the endothelial processes.

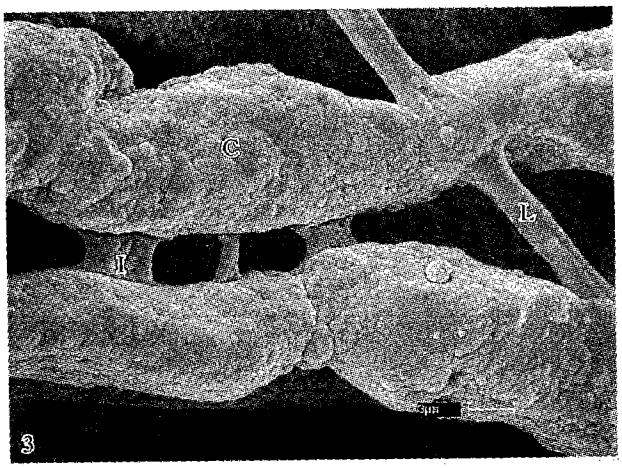

Fig. 3: SEM of high magnification of the superficial blood capillary network of pleats.
C, blood capillary network. I, communicating structures situated at slits in the capillary walls. L, lymphatic precolector.

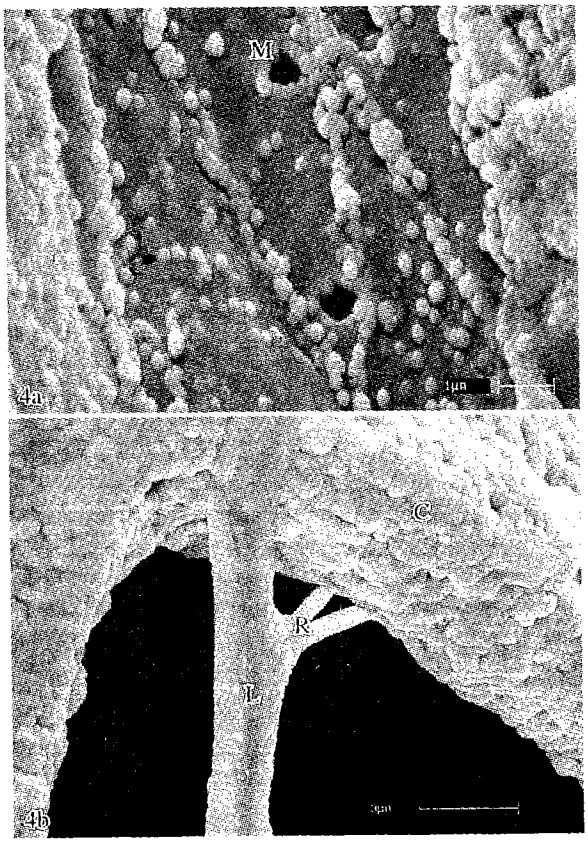

Fig. 4: SEM of the connections between blood capillaries and lymphatic vessels.
4a: Endothelial cell pores of the blood capillaries. 4b: Casts of connections between blood capillaries and lymphatic vessels. C, blood capillary network, L, lymphatic precolector. M, endothelial cell pores of the blood capillaries. R, connections between lymphatic precolectors and blood capillaries.

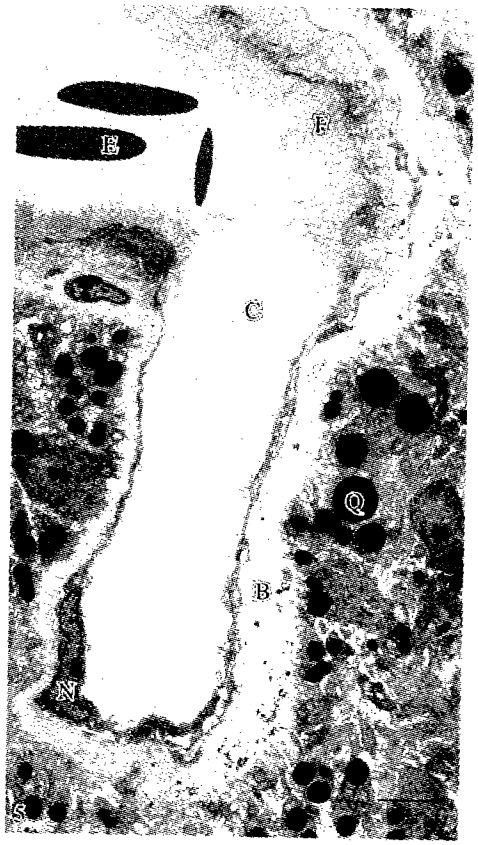

Fig. 5: TEM of the blood capillary network (Photomontage).
B, basal lamina of the blood capillary pecten. C, blood capillary pecten. E, erythrocyte. F, processes. N, nucleus of endothelial cell. Q, pigmented phagocyte cell. Bar=5μm

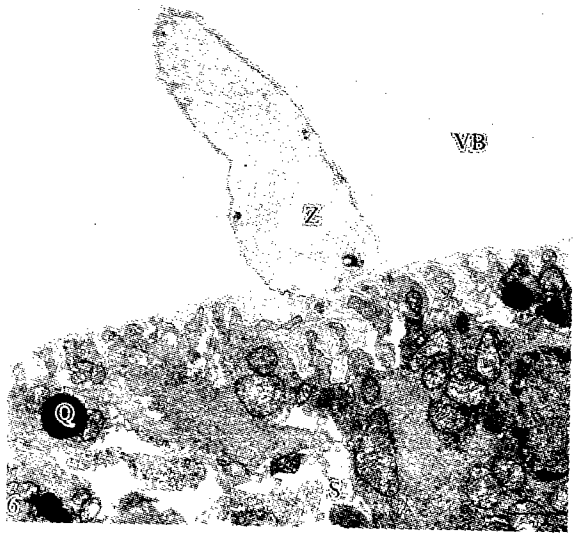

Fig. 6: TEM of intercellular space of the duck pecten.
Q, pigmented phagocyte cell. S, intercellular space. VB, vitreous body. Z, discoid detachment. Bar=1.5μm.

vessels that lay along the well-defined basal membrane whose surface was very irregular at points where the plasmalemma of the endothelial cells had basal processes. The basal lamina (*Membrana basalis*) of the pecten capillaries was thick (1-3μm), and consisted of concentric layers of loosely compacted fibrillar materials which very often contained macromolecules moving in both directions between the intercellular spaces and the blood vessels.

2) Intercellular Spaces

The intercellular spaces observed between blood vessels contained fibrillar material, pigmented phagocyte cells, processes derived from these cells and lymphatic precolectors (3-4 μm, diameter) which originated from the bottom of a well structure. The intercellular spaces were sometimes very deep and completely covered by pigment cells. Near the outskirts of the pecten, some of the intercellular spaces were closed off by a covering membrane which permitted the transport of materials and substances from the vitreous body into the spaces (Fig. 6).

Discussion

The present study provides evidence for the hypothesis that the pecten of the duck plays a role in the preservation and maintenance of retina homeostasis. In contrast to the mammalian eye, the avian retina is avascular, and its nourishment must come from adjacent regions. As regards general angioarchitecture, the duck pecten is organized to facilitate the exchange of materials and substances between the blood vessels and vitreous body via diffusion. That functional aspect of the pigeon pecten was convincingly demonstrated by experiments in which blood flow to it was surgically interrupted [21]. In the pecten, the blood vessels were packaged together forming an extensive capillary network composed of irregular links along the pleated surface, there by forming a large surface contact area. The blood capillaries were often in an anastomotic relationship with each other. In addition, there were sometimes bridge structures at apertures in the capillaries which served to slow blood flow, and to regulate intravascular blood pressure. The anastomoses and bridge structures presumably facilitate the exchange of materials and substances between the blood vessels and vitreous body.

As regards vessel organization, the pecten microvasculature has been taken as evidence that the duck pecten is involved in the transportation of materials and substances to and from the avascular retina. Braekevelt [7] concluded as much in his study of the pecten oculi of the red-tailed hawk. As regards the vessel structure, the capillaries of the duck pecten, as in other avian species such as the night hawk, loon and pigeon, are characterized by large increases in the endothelial surface at points where the vasal wall is very thin [5, 6]. This too facilitates the exchange of materials and substances. The presence of numerous processes on both the luminal and basal borders of the endothelial cell plasmalemma, considered together with the presence of cell organelles in paranuclear clusters, indicate that these cells play a role in the absorption and transportion of substances. This is consistent with Raviola and Raviola [22], who hold that a role in active transport still remains the only reasonable inference to be drawn from the special morphological structures of the endothelial cells in the blood vessels of the bird pecten oculi. Additional support for the above hypothesis is provided by the high alkaline phosphatase content in the pecten, since alkaline phosphatase is necessary for the active transport of materials across endothelial cell membranes [23]. Finally, several other aspects of the endothelium that comprise the pecten blood vessels, namely, its lack of vascular pores, its impermeability to horseradish peroxidase throughout ontogeny [24], and its similarity to a blood-brain barrier endothelium [17], indicate that there are many morphostructural components of the pecten involved in retinal homeostasis which are essential for retinal trophism.

The presence of a lymphatic capillary network beneath the vitreous membrane in the pecten suggests that the pecten also plays a role in the drainage of the retinal catabolites from the vitreous body. During vision activity, the cones and rods lose portions of the apical cytoplasmatic due to light stimuli. These portions take the form of discoid detachments [25], and loosened and worn protein [26] which are subsequently emptied into the vitreous body, and later drained into the pecten where they are demolished by the pigment cells. The pigment cells are tightly packed in the bridge of pecten, and loosely packed into groups lying between the wall and superficial membrane of the pecten. After demolition, the cones, rods and waste material is directed towards the lymphatic system, and the pigment cells are absorbed into the endothelium of the blood capillaries. However, the tremendous variation in its size and complexity indicates to many that the organs must have a function in addition to that of nutrition. The hypothesis that the diurnal visual capacity of birds depends on pecten pleat development was advanced by Braekevelt [7]. Indeed, in the highly visually oriented red-tailed hawk, the pecten forms a highly vascular, heavily pigmented and extremely pleated, fan-like structure situated over the oval optic nerve head. In contrast, nocturnal birds, such as owls and goatsuckers, have relatively small pectens with few folds. In fact, the pecten of the owl is incomplete in that it does not have a bridge joining the vane-like structures. Agreeing with Pezzolo [27], we assert that the trophic system formed by the pecten in birds is morphologically the simplest, structurally the most elaborate, and functionally the best among all the Verterbrates.

In conclusion, in the duck, the pecten seems to be primarily involved in the maintenance of eye homeostasis. The morphological characteristics of the pecten described in the present study suggest that the pecten plays an important role in the regulation of metabolic exchanges between ocular blood vessels and the retina, thus preserving the integrity and stability of the intraocular microenvironment.

References

1. Jasinski A. Fine structure of capillaries in the pecten oculi of the sparrow, Passer domesticus. Z Zellforsch 1973; 146: 281-292.

2. Bawa SR, YashRoy Rakesh C. Structure and function of vulture pecten. Acta Anat 1974; 89: 473-480.

3. Tucker R. The surface of the pecten oculi of the pigeon. Cell Tissue Res 1975; 157: 457-465.

4. Yew DT. The origin and initial development of the pecten oculi. Anat Anz 1978; 143: 383-387.

5. Braekevelt CR. Electron microscopic observations on the pecten of the nighthawk (Chordeiles minor). Ophthalmologica 1984; 189: 211-220.

6. Braekevelt CR. Fine structure of the pecten of the pigeon (Columba livia). Ophthalmologica 1988; 196: 151-159.

7. Braekevelt CR. Fine structure of the pecten oculi of the red-tailed hawk (Buteo jamaicensis). Anat Histol Embryol 1991; 20: 354-362.

8. Braekevelt CR. Fine structure of the pecten oculi in the great horned owl (Bubo virginianus). Histolol Histopathol 1993; 8: 9-15.

9. Braekevelt CR. Fine structure of the pecten oculi in the American crow (Corvus brachyrhynchos). Anat Histol Embryol 1994; 23: 357-366.

10. Braekevelt CR. Fine structure of the pecten oculi in the Australian galah (Eolophus roseicapillus). Histolol Histopathol 1996; 11: 565-571.

11. Braekevelt CR. Fine structure of the pecten oculi of the emu (Dromaius novaehollandiae). Tissue Cell 1998; 30: 157-165.

12. Dieterich CE, Dieterich HJ, Spycher MA, Pfautsch M. Fine structural observations of the pecten oculi capillaries of the chicken. Z Zellforsch 1973; 146: 473-489.

13. eaman AR, Storm H. A correlated light and electron microscope study of the pecten oculi of the domestic fowl. (*Gallus domesticus*). Exp Eye Res 1975; 2: 163-172.

14. Bachsich P, Gellèrt A. Beiträge zur Kenntnis der Struktur und Funktion des Pecten oculi im Vogelauge.Graefe's Arch Ophthlmol 1935; 133: 448-460.

15. Barlow HB, Ostwald TJ. Pecten of the pigeon's eye as an inter-ocular eye shade. Nature 1972; 236: 88-90.

16. Pettigrew JD, Wallman J, Wildsoet CF. Saccadic oscillation facilitate ocular perfusion from the avian pecten. Nature 1990; 343: 362-363.

17. Gerhardt H, Liebner S, Wolburg.The pecten oculi of the chicken as a new in vivo model of the blood-brain barrier. Cell Tissue Res 1996; 285: 91-100

18. Scala G, Corona M, Mirabella N, Perrella A, Pelagalli GV. Microvasculature of the pecten oculi in *Anas plathyrhynchos*. It J Anat Embryol 2002; 107: 65-75.

19. Gannon BJ. Preparation of microvascular corrosin casting media: procedure for partial polymerisation of methyl methacrylate using ultraviolet light. Biomed Res 1981; 2: 227-233.

20. Baumel JJ. King AS, Breazile JE, Evans HE, Vanden Berge JC. Handbook of avian anatomy: NOMINA ANATOMICA AVIUM. Second edition. Published by the Club, Cambridge, Massachusetts, 1993.

21. Wingstrand KG, Munk O. The pecten oculi of the pigeon with particular regard to its function. Biol Skr Dan Vid Selsk 1965; 14: 1-64.

22. Raviola E, Raviola G. A light and electron microscopic study of the pecten of the pigeon eye. Amer J Anat 1967; 120: 427-462.

23. 23.Welsch U. Enzyme histochemical and structural observation on the pecten oculi of the birds. Z Zellforsch 1972; 132: 231-244.

24. Latker CH, Beebe DC. Developmental changes in the blood-ocular barriers in the chicken embryos. Exp Eye Res 1984; 39: 401-414.

25. Anderson DH, Fischer S. The photoreceptors of diurnal squirrels: outer segment structure, disc shedding and protein renewal. J Ultrastruct Res 1976; 55: 119-141.

26. Young RW. Biogenesis and renewal of visual cell outer segment membrane. Exp Eye Res 1974; 18: 215-223.

27. Pezzolo D 1994. Adattamenti morfologici all'ambiente dell'occhio dei Vertebrati. Atti della Società Italiana di Anatomia. XLVII Convegno Nazionale. It J Anat Embryol 1990; 99: 17-100.

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Evaluation of analgesic and anti-inflammatory profile of beta-lactam monocyclic compounds

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Abstract

In this short paper we report the pharmacological activity of some azetidinone derivatives. In fact, we have examined the *in vivo* anti-inflammatory and analgesic profile of a series of monocyclic β-lactam (azetidinones) which structurally are very similar to some inhibitors of human leukocyte elastase (HLE), an enzyme involved in degradation processes of connective tissue. Our new compounds were administered orally (15 mg/Kg) to albino rats 30 minutes before injecting carrageenin in the plantar aponeurosis. Tested compounds demonstrated a good activity; in particular two of them markedly reduced paw edema formation, with activity very similar to indomethacin (reference compound, 5 mg/Kg). To evaluate the analgesic activity we carried out the acetic acid writhing test, pretreating rats orally with our compounds 30 minutes before injecting the acid solution i.p. The same two compounds which showed the anti-inflammatory activity also, demonstrated a very good analgesic activity. These results indicate that these new azetidinone derivatives possess a good analgesic and anti-inflammatory activity. Considering that these compounds are structurally very similar to HLE inhibitors, we cannot exclude the possibility that their pharmacological activity may be related to HLE inhibition.

Introduction

Several laboratories, including our own, have reported the synthesis and activity of certain low molecular weight inhibitors of mammalian serine proteases, especially human leukocyte elastase [1]. Human leukocyte elastase (HLE, EC 3.4.21.37) which is involved in the pathogenesis of chronic inflammatory diseases such as pulmonary emphysema, which is present in the azurophil granules of human polymorphonuclear leukocytes (PMN), was chosen as the target enzyme. Normally, these granules fuse with phagosomes containing engulfed foreign material (such as bacteria), and HLE, in combination with other lysosomal enzymes, catabolizes the particles [2]. Under certain pathological conditions, however, PMN become attached to host protein (elastin fibres, basement membrane, connective tissue, immune complexes), and in response to this adherence, the granules may fuse with the PMN outer membrane and release their contents, including HLE, directly onto the tissue [3].

Besides emphysema, HLE may also contribute to the pathogenesis of disease states such as adult respiratory distress syndrome, and its potential involvement in rheumatoid arthritis makes HLE inhibitors of considerable interest. It is known that some different antibiotics are acylating inhibitors of bacterial serine proteases which help synthesize the cell wall by performing a transpeptidation reaction on a peptidyl substrate bearing a D-Ala-D-Ala terminus.

To date, HLE has been investigated by other groups. A research team, from Merck [4], has synthesized azetidinones, as 1b structure [5], which seem to be able to inhibit HLE by forming a stable acyl-enzyme which does not undergo hydrolysis [6]. Another important monocyclic beta-lactam that underlies such diseases as emphysema [7], chronic bronchitis [8], cystic fibrosis [9] can be treated by inhibitor, which lacks the C-4 side chain in compound 1c [10,11] and 1d [12] (Figure 1). Taking these results into account, we decided to design a new type 1d structural model; it is an azetidinone in which some aromatic substituents have been replaced. We supposed that the characteristics of type 1d model should have made it capable of acting as an orally active anti-inflammatory drug and in particular as a serine proteinases inhibitor. We obtained it through cyclization of appropriate N-propionamides [13]; which gave the N-aryl-3, 3-dimetilazetidin-2-ones 1-6 showed in Figure 2. Preliminary *in vivo* tests were conducted to evaluate antinflammatory-activity (carrageenin-induced edema test) [14] and analgesic-activity (writhing test) [15,16] for the compounds 1-5.

Materials and Methods

a. Anti-edemigenic activity

Pretreating's effects rats with products 1-5. (30 min. previously), administered orally *via* gastric intubation (15 mg/kg), were studied on localized edematous reaction [14]; 0.1 ml of 1% suspension of carrageenin in distilled water was injected subcutaneously (s.c.) into the plantar sub-aponeurosis The paw volume was measured by a mercury plethysmograph (differential gaue, manufactured by Basile, Milano, Italy) at the time of carrageenin injection and then 1, 2, 3 and 4 hours later (always taking care to immerse the paw at the same level, that is the tibio-astragalic joint, assured and marked with a line before the test). Indomethacin (5 mg/Kg) was used as reference compound. Albino rats (180-200 g) of either sex were used, excepting pregnant females; each group comprised of six animals. The mean increase in paw volume, at the said time intervals of the control (receiving carragenin alone) and experimented groups was calculated. Results were expressed as mean percentage increases in paw volume *vs* basal value. ANOVA test was used in the statistical evaluation.

b. Analgesic activity

Analgesic activity was evaluated by acetic acid test (writhing test) as described by Winter et al.[15,16]. The activity of compounds **1-5** was evaluated in rats, administering the compounds (15 mg/Kg) orally (30 minutes before acetic acid, 0.25 ml intra peritoneum (i.p.), per rat, of a 0.5% aqueous solution). For each animal the number of writhing movements, in the 25 minutes immediately after administration of acetic acid, was observed. The mean number of writhes for each group of animals and the percentage variation compared with the control group were calculated. Mice (Mus musculus) of either sex were used, excepting pregnant females, weighing 20-25 g; each group comprised six animals. Results were expressed as percent of inhibition of the number of writhing movements in the control group treated with acetic acid. The number of writhing movements in the control group was 45± during the observing period (15 min). Wilcoxon test was used for the statistical evaluation.

Results and Discussion

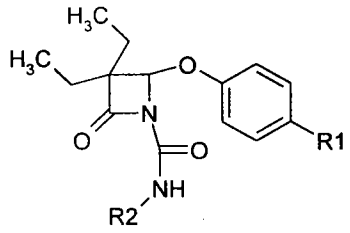
The results of our experiments indicate that all tested compounds showed a certain activity (Table I) and the results of compounds 1 and 3, indicate a marked anti-inflammatory activity in rats at a dose of 15 mg/Kg comparable to indomethacin. The anti-inflammatory activity of compounds 1 and 3 was significative at 1h and lasted for all the recording period (4h). Similar results were obtained with the second test where both compound 1 and 3 showed a good analgesic activity when compared to indomethacin (Table II).

Furthermore, the strong activity of the compounds 1-3 after oral administration, suggest a stability in the gastro-intestinal tract and in blood circulation, a problem commonly found in β-lactam structures and to many HLE inhibitors.

Table I. Evaluation of the anti-inflammatory activity of indomethacin and of compounds 1-5 by carrageenin paw edema test

Treatment	Dose (mg/kg/os)	Paw volume (ml)				
		0h Basal Value	1h	2h	3h	4h
Control		1.63±0.5	2.44± 0.3 (+149)	2.73±0.5 (+167)	2.95±0.4 (+181)	3.02±0.7 (+185)
Indomethacin	5	1.60±0.7	1.37± 0.6 (+56)[a]	1.34±0.5 (+83)[a]	1.47±0.7 (+91)[a]	1.57±0.5 (+98)[a]
1	15	1.60±0.7	1.35±0.6 (+84)[a]	1.23±0.5 (+76)[a]	1.22±0.4 (+76)[a]	1.35±0.3 (+84)[a]
2	15	1.69±0.6	2.47±0.3 (n.s.)	2.70±0.4 (n.s.)	2.97±0.5 (n.s.)	3.07±0.2 (n.s.)
3	15	1.58±0.2	1.09±0.4 (+68)[a]	1.13±0.5 (+71)[a]	1.19±0.7 (+75)[a]	1.22±0.5 (+77)[a]
4	15	1.72±0.3	2.33±0.2 (n.s.)	2.79±0.1 (n.s.)	2.99±0.6 (n.s.)	3.07±0.9 (n.s.)
5	15	1.75±0.6	2.39±0.9 (n.s.)	2.91±0.4 (n.s.)	2.97±0.3 (n.s.)	3.04±0.2 (n.s.)

Paw volume was measured after carrageenin administration, at the time (h) written at the top of the table. In brackets there are the mean percentage increases in paw volume vs basal value, n.s. is for not significative vs basal value. a) P<0.01 vs basal value (ANOVA)



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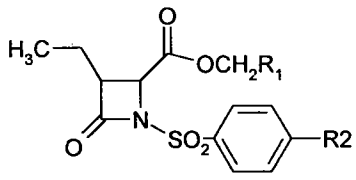

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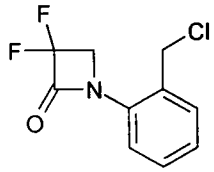

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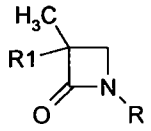

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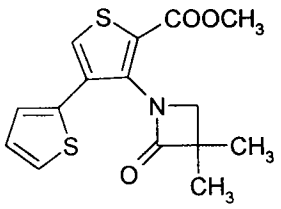
Fig. 1: *Chemical Structures for HLE inhibitors*



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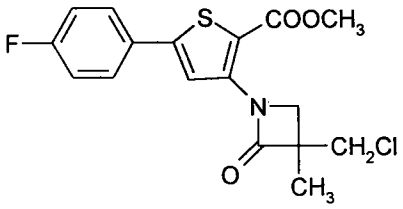

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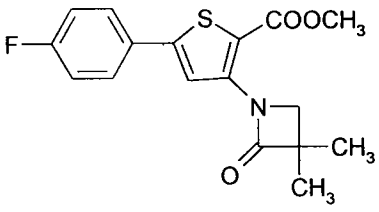

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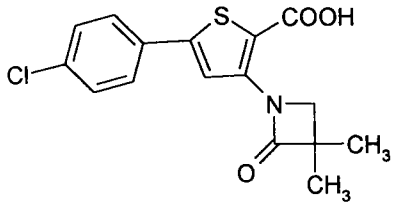

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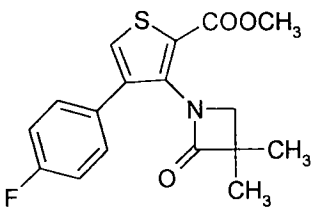

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Fig.2*: Chemical Structures for N-aryl-3,3-dimetilazetidin-2-ones: compounds 1-5*

Table II. Evaluation of analgesic activity of indomethacin and of compounds 1-5 by acetic acid test

Treatment	Dose (mg/kg)	Percent of Inhibition
Control	5	67.4[a]
Indomethacin		
1	15	48.2[a]
2	15	10.3
3	15	55.6[a]
4	15	9.7
5	15	8.3

a) P<0.01 vs control group (Wilcoxon test). Results were expressed as percent of inhibition of the number of writhing movements in the control group.

The present paper focuses on the antiinflammatory and analgesic activity of new azetidinones structurally very close to HLE inhibitors. Although we can not state that these new azetidinones are HLE inhibitors, we cannot exclude this possibility thus giving some useful suggestions to the strategy of design of HLE monocyclic β-lactam inhibitors.

HLE is a protease that is involved in the tissue destruction inflammation that characterize numerous diseases, including hereditary emphysema, chronic obstructive pulmonary disease, cystic fibrosis, adult respiratory distress syndrome, ischemic-reperfusion injury and rheumato-arthritis. Thus, elastase has been the object of extensive research to development potent inhibitors that target its destructive and pro-inflammatory action [7-9].

Although further studies are needed in order to establish their possible HLE inhibiting activity, it is of interest that these new azetidinones showed a good anti-inflammatory and analgesic activity.

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References

1. Finke PE, Shah SK, Fletcher DS, Ashe BM, Brause KA, Chandler GO, Dellea PS, Hand KM, Maycock AL, Osinga DG, Underwood DJ, Weston H, Davies P, Doherty J B. Orally Active beta-Lactam Inhibitors of Human Leukocyte Elastase. 3. Stereospecific Synthesis and Structure-Activity Relationships for 3,3-Dialkylazetidin-2-ones. J Med Chem 1996; 38: 2449-2462.

2. a)Snider GL. Protease-Antiprotease Imbalance in the Pathogenesis of Emphysema and Chronic Bronchial Injury. A Potential Target for Drug Development. Drug Deu. Res.,10,235-253, 1987. (b) Weinbaum, G., Giles R. E., Krell, R. D., Eds. Pulmonary Emphysema, the Rationale for Intervention. Ann N Y Acad Sci 1991a; 624: 1-370.

3. Bender JG, van Epps DE, Searles R, Williams RC Jr, Altered Function of Synovial Fluid Granulocytes in Patients with Acute Inflammatory Arthritis; Evidence for Action of Neutrophils and its Mediation by a Factor Present in Synovial Fluid. Inflammation. 1996; 10: 443.

4. Doherty JB, Ashe BM, Argenbright LW, Barker PL, Bonney RJ, Chandler GO, Dahlgren ME, Dorn CP Jr, Finke P E, Firestone RA. Cephalosporin antibiotics can be modified to inhibit human leukocyte elastase. Nature 1986; 322: 192-194.

5. Hagmann WK, Shah SK, Dorn CP, O'Grady LA, Hale J J, Finke PE, Thompson KR, Brause KA, Ashe BM, Weston H, Dalhgren ME, Maycock AL, Dellea PS, Hand KM, Osinga DG, Bonney RJ,,Davies P, Fletcher DS, Doherty J B. Prevention of human leukocyte elastase-mediated lung damage by 3-alkyl-4 Azetidinones. BioMed Chem Lett 1991; 1: 545-545.

6. Knight WB, Swiderek KM, Sakuma T, CalaycayJ, Shively JE, Lee TD, Covey TR, Shushan B, Green G, Chabin R, Shah S, Mumford R, Dickinson TE, Griffin PR. Electrospray ionization mass spectrometry as a mechanistic tool: Mass of human leukocyte elastase and a beta-lactam-derived E-I complex. Biochemistry 1993; 32: 2031-2035.

7. Snider GL, Ciccolella DE, Morris SM, Stone PJ, Lucey E C. Putative role of neutrophil elastase in the pathogenesis of emphysema. Ann NY Acad. Sci. 1991a; 624: 45-59.

8. Stockley RA, Hill SL, Burnett D. Proteinases in chronic lung infection. Ann NY Acad Sci 1991; 624: 257-266..

9. Jackson AH, Hill SL, Afford SC, Stockley RA. Sputum solphase proteins and elastase activity in patients with cystic fibrosis. J Resp Dis 1984; 65: 114-124.

10. Maillard JL, Fravreau C, Vergerly I, Reboud-Ravaux M, Joyeau R, Kobaiter R, Wakselman M. Protection of vascular basement membrane and microcirculation from elastase-induced damage with a fluorinated beta-lactam derivative. Clin Chim Acta 1992; 213: 75-86.

11. Vergely I, Laugaa P, Reboud-Ravaux M. Interaction of human leukocyte elastase with a N-aryl azetidinone suicide substrate: conformational analyses based on the mechanism of action of serine proteinases. J Mol Graphics 1996; 14: 158-167.

12. Lancelot JC, Letois B, Saturnino C, Perrine D, Robba M, Efficient synthesis of new beta-lactams. Synthetic. Comm 1993; 23: 1535-1542.

13. Lancelot JC, Saturnino C, El-Kashef H, Perrine D, Mahatsekake C, Prunier H, Robba M. A facile synthesis of new beta-lactams. J Heterocyclic Chem 1996; 3: 427-431.

14. Davies JE, Kellet DN, Pennington JC. The anti-inflammatory and analgesic effects of Norvedan, a novel, non-steroidal agent. Arch Int Pharmacodyn Ther 1976; 221: 274-282.

15. Winter CA, Nuss GW. Pyretogenic effects of bacterial lipopolysaccharide and the assay of antipyretic drugs in rats. Toxicol Appl Pharmacol 1963; 5: 247-256.

16. Winter CA, Risley EA, Nuss GW, Carrageenin-induced edema in hind paw of the rat as an assay for antiiflammatory drugs. Proc Exp Biol Med 1962; 111: 544-547.

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Prevalence of modifiable traditional coronary heart disease risk factors among young adult survivors of acute MI: A comparative study between Saudis and Non- Saudi patients

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Key words: Coronary heart disease, acute myocardial infarction, risk factors

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Abstract

A retrospective study of the prevalence of the traditional CHD risk factors among Saudi and non-Saudi patients aged 45years and younger with AMI was carried out. During the 6 years study period (May 1995 – April 2002), there were a total of 706 patients with diagnosis of AMI of whom 149 patients were young. 42.3% were Saudis while 64% of the non-Saudis were South Asian. There were only 8 young females with AMI, two of them were Saudis. Non-Saudis were significantly younger than Saudis (mean age 40 ± 5 vs 38 ± 5, P = 0.047, respectively).

The prevalence of AMI among the young Saudis was 12% compared to 52% among the young non-Saudis. Hypercholesterolemia (59%) was the commonest risk factor in both groups followed by smoking (55%), hypertriglyceridmia (37.9%), diabetes mellitus (26.8%), obesity (19%) and hypertension (18.8%). Multiple risk factors were significantly more prevalent among Saudis than non-Saudi patients (81% vs 65% P= 0.025). Saudis had significantly higher prevalence of hypertriglyceridemia, Diabetes mellitus and obesity than non- Saudis [(50.8% vs 27.8%, P = 0.005), (41.3% vs 16.3%, P = 0.001), (29.8% vs 10.3%, P = 0.006) respectively]. Smoking was the leading risk factor among non-Saudis (58%), though not significantly different from Saudis (50.8%).

Young Saudi patients with AMI had higher prevalence of multiple traditional CHD risk factors than non-Saudis. The commonest individual traditional risk factors among Saudis were hyperlipidemia, smoking, and diabetes mellitus respectively. Therefore, appropriate preventive measures were needed to control these conditions to prevent AMI in young active and productive population.

Introduction

Although acute myocardial infarction (AMI) is uncommon entity in young patients, it constitutes an important problem for the patient and the treating physician because of the devastating effect of this disease on the more active lifestyle of young patients. Evidence of increased risk of coronary heart disease CHD with the presence of specific risk factors has been documented in previous epidemiological studies, such as the Framingham Heart Study, the Multiple Risk Factor Intervention trial and others [1-3]. Although the incidence of CHD in western industrialized countries is declining, it has been increasing in developing countries[4]. Several studies from industrialized countries have identified the classic risk factors for CHD and their relation to case fatality. Attempts to modify these risk factors in the population may have contributed to the decline in mortality due to CHD in these countries [5,6].

Knowledge of the risk factors and methods of prevention of CHD are essential in order to reduce morbidity and mortality from them. Therefore, the aim of this study is to determine the prevalence of the traditional coronary heart disease risk factors among young adult Saudi survivors of AMI and to compare them with age matched non- Saudis.

Subjects and Methods

Most published studies have used an age cut-off point of 40 to 45years to define young patients with acute MI [7-16]. In this study I used the higher age of 45 years as the cut-off point. The medical records of all patients admitted to the coronary care unit

(CCU) of Aseer Central Hospital with the diagnosis of AMI over 6 years period (May 1995 – April 2002) were reviewed. Patients were classified into two groups: Saudis and non-Saudis. The diagnosis of acute MI was based on the presence of two of the followings criteria: ischemic chest pain for at least 30 min, ECG evidence of myocardial injury, an increase in serum creatine kinase level to more than two standard deviations above the upper limit of the normal range [17]

The presence of high level of serum cholesterol and triglyceride was noted according to the recommendations of the National Cholesterol Education Program Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adults Treatment Panel III) executive summary [18]. Patients were classified as obese if their body mass index (BMI) was greater than 30kg/ m2 [19].

Statistical analyses were performed by the use of a commercial software package.

Results

A total number of 706 patients with acute myocardial infarction (AMI) were admitted to ACH during the 6-years study period, of whom 76.5% were Saudis (Table 1). 21% of patients were 45 years and younger, and Saudis accounted for 42.3% of them. Among non-Saudis with AMI 52% were young adult. Majority (64%) of the non-Saudis were South Asian (SA). The mean age of the young adult Saudis was significantly higher than non-Saudis (40 ± 5 vs. 38 ± 5 P= 0.047). Bangladeshis, had the lowest mean age 35 ± 4.0. Only 2 out of 8 patients were young female Saudis. AMI with ST segment elevation occurred in 82.6%. In both groups, Hypercholesterolemia (59%) was the commonest risk factor followed by smoking (55%), Hypertriglyceridmia (37.9%), diabetes mellitus (26.8%), obesity (19%) and hypertension (18.8%).

The commonest risk factors among Saudis were hyperlipidemia (hypercholesterolemia and hypertriglyceridemia), smoking and diabetes mellitus in descending order, while smoking, was the leading risk factor among non-Saudis followed by hypercholesterolemia and hypertriglyceridemia (Table2). The incidence of hypertriglyceridemia, diabetes mellitus and obesity was significantly higher among Saudis than non-Saudis (P-value = 0.005, 0.001, & 0.006 respectively).

Majority of patients (94%) of both groups have at least one CHD risk factor, while the prevalence of two or more risk factors was significantly more among Saudis (81% vs. 65.1% P= 0.025).

Table 1: Shows the different nationalities and their mean ages

Nationality	All Patients with AMI No. = 706 (100%)	Age ≤ 45y	Age	
		No. =149 (100%)	Mean ± SD	Range
Saudis	---------540 (76.5)	63 (42.3)	40 ± 5.0	28-45
Non-Saudis	166 (23.5)	86 (57.7)	38 ± 5.0	28-45
Bangladeshis	30 (4.2)	21 (14.1)	35 ± 4.0	30-43
Indians	26 (3.7)	19 (12.8)	40 ± 5.0	30-45
Pakistanis	26 (3.7)	15 (10.1)	37 ± 5.6	28-45
Egyptians	27 (3.8)	15 (10.1)	42 ± 4.0	32-45
Others	57 (8.0)	16 (10.7)	38 ± 4.0	29-45

Table 2: Prevalence of risk factors among both groups

	No. of available data	All cases No. (%)	Nationality				P-Value
			Saudis		Non-Saudis		
			No.	%	No.	%	
Hypertension	149	28 (18.8)	14	22.2	14	16.3	0.240
Diabetes Mellitus	149	40 (26.8)	26	41.3	14	16.3	0.001
Hypercholesterolemia	140	83 (59.0)	39	63.9	44	55.7	0.210
Hypertriglyceridemia	140	53 (37.9)	31	50.8	22	27.8	0.005
Smoking	149	82 (55.0)	32	50.8	50	58.1	0.230
Obesity	125	24 (19.0)	17	29.8	07	10.3	0.006
One or more risk factors	149	140 (94.0)	60	95.2	80	93.0	0.423
Two or more risk factors	149	107 (71.8)	51	81.0	56	65.1	0.025

Discussion

In this study, the reported rate of non-Saudi patients with AMI is much less than Saudis, yet majority (57.7%) of non-Saudis are younger. The high rate of AMI among non-Saudis may not reflect exactly the true incidence in their homeland. This is mainly because non-Saudis constitute the main workforce in Saudi Arabia and tend to be younger. This difference in the population characteristics may explain some of the observed differences between the two groups admitted with AMI.
64% of the non-Saudis are South Asian (SA). Studies from the United Kingdom and Trinidad have shown a two- to four-fold greater incidence of AMI among SA compared with the native population[20-22] Indian immigrants to the United Kingdom have also been shown to have a higher rate of risk factors for coronary heart disease (CHD) compared to their siblings living in India [23]. In the United States, hospitalization and prevalence rates of CAD among SA is reported to be three to four times higher than that of other populations 24].

The rate of AMI among young Saudis is 12% which is not much different from the previous reports from the West [11,12,15]. This rate is probably high for Aseer region which is composed mainly of semi-urban and rural areas. However, the presence of higher rate of multiple risk factors (two or more risk factors) among the young Saudis (81% vs 65%, P=0,025) would explain this high rate. The effect of multiple risk factors on the extent of atherosclerosis is quite evident in the study by Berenson GS et al [25]. Furthermore, non-Saudis are younger than Saudis, yet the rate of AMI was very high (52%). Other non-traditional risk factors may play an important role mainly among the non-Saudis[20,23]. Increased blood level of homocysteine is one of the important non traditional risk factors known to affect predominantly young adult with CHD[26].

Most of the reported data showed that smoking is the most prevalent risk factor encountered in young patients with AMI [7-10]. In this study smoking and hypercholesterolemia are the leading risk factors among the non-Saudis and Saudis respectively. The reported incidence of hyperlipidemia, in young patients with AMI ranges from 12%-89% [11]. It is present in 64% of Saudis which is much higher than previously reported study from Saudi Arabia (33.8%) [24]. Al-Nuaim AR, reported higher prevalence of hypercholesterolemia among subjects living in rural than urban areas[27].

Diabetes mellitus which has bad influence in the outcome of patients with AMI is significantly more prevalent among Saudis than non-Saudis (41.3% vs 16.3%). This figure is much higher than reported from Germany, Singapore, and Saudi Arabia which showed a rate of 10%, 21% and 30.8% respectively [11,24]. Furthermore, young patients with AMI tend to have subtle problems with glucose metabolism. Glucose intolerance and hyperinsulinemia are common problems particularly among obese population [28].

Similarly, increased triglycerides level and obesity are significantly present in Saudis than non-Saudi population in this study. Both conditions are clearly independent risk factors for CHD particularly in young adult males [29]. In previous report high triglyceride level was, the most common lipid abnormality in young patients with MI [28]. It may be associated with glucose intolerance and a predominance of small atherogenic LDL particles, both of which predispose to atherosclerosis.

In Saudi Arabia, improved socioeconomic conditions in the past two decades have been followed by rapid changes in the lifestyle of the people brought about by urbanization and availability of housing, food and high purchasing power. Consequently, common risk factors such as diabetes mellitus, obesity, hyperlipidemia, hypertension and smoking have emerged [30-36].

Conclusion and Recommendations:

This study, showed that young Saudi patients with AMI have higher prevalence of multiple traditional CHD risk factors than non-Saudis. The commonest individual traditional risk factors among Saudis are hyperlipidemia, smoking, and diabetes mellitus respectively. Therefore, appropriate preventive measures are needed to control these conditions and subsequently AMI in young active population.

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References

1. Dawber TR. The Framingham Study: the epidemiology of atherosclerotic disease. Cambridge, Massachusetts, Harvard University Press, 1990.

2. Multiple risk factor intervention trial. Risk factor changes and mortality results. Multiple Risk Factor Intervention Trial Research Group. Journal of the American Medical Association, 1982, 248: 1465-77.

3. Semencw RM, Morrison HI, Mao Y, Johansen H, Davies JW, Wigle D T. Major risk factors for cardiovascular disease mortality in adults: results from the Nutrition Canada Survey cohort. International journal of epidemiology, 1988, 17: 317-324.

4. Cardiovascular disease mortality in the developing countries. Word Health Stat Q 1993; 46: 89-150.

5. Stern MP. The recent decline in ischemic heart disease mortality. Ann Intern Med 1979; 91: 630-640.

6. Frerichs RR, Chapman JM, Maes EF. Mortality due to all causes and to cardiovascular diseases among seven race-ethnic populations in Los Angeles County 1980. Int J Epidemiol 1984; 13: 291-298.

7. Imaazio M, Bobbio M, Bergerone S, Barlera S, Maggioni AP. Clinical and epidemiological characteristics of juvenile myocardial infarction in Italy: the GISSI experience. G Ital Cardiol 1998; 28 5: 505-512.

7. Kanitz MG, Giovannucci SJ, Jones JS, Mott M. Myocardial infarction in young adults: risk factors and clinical features. J Emerg Med 1996; 142: 139-145.

9. Siwach SB, Singh H, Sharma D, Katyal VK. Profile of young acute myocardial infarction in Harayana. J Assoc Physicians India 1998; 46: 424-426.

10. von Eyben FE, Bech J, Madsen JK, Efsen F. High prevalence of smoking in young patients with acute myocardial infarction. J R Soc Health 1996; 116 3: 153-156.

11. Choudhury L and Marsh JD, Myocardial infarction in young patients. Am J Med 1999; 107: 254-261.

12. Garoufalis S, Kouvaras G, Perdikouris K, Markatou P, Hatzisavas J, Kassinos N, Karidis K, Foussas S Comparison of angiographic findings, risk factors, and long term follow-up between young and old patients with a history of myocardial infarction. Int J Cardiol 1998; 67: 75-80.

13. Rumboldt Z, Rumboldt M, Pesenti S, Polic S and Miric D, Peculiarities of myocardial infarction at young age in Southern Croatia. Cardiologia 1995; 40: 407-411.

14. Doughty M, Mehta R, Bruckman D, Das S, Karavite D, Tsai T et al. Acute myocardial infarction in the young: the University of Michigan experience. Am Heart J 2002; 143: 4-6.

15. Brscic E, Bergerone S, Gagnor A, Colajanni E, Matullo G, Scaglione L, Cassader M, Gaschino G, Di Leo M, Brusca A, Pagano G F, Piazza A, Trevi G P. Acute myocardial infarction in young adults: prognostic role of angiotensin-converting enzyme, angiotensin II type I receptor, apolipoprotein E, endothelial constitutive nitric oxide synthase, and glycoprotein IIIa genetic polymorphisms at medium-term follow-up. Am Heart J 2000; 139: 979-984.

16. Conley M, Mcneer F, Lee M, Wagner A, Rosati. Cardiac arrest complicating acute myocardial infarction: predictability and prognosis. Am J Cardiol 1977; 39: 6-12.

17. Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III) Executed Summary. National Cholesterol Education Program. National Heart, Lung, and Blood Institute. National Institutes of Health, NIH Publication No. 01-3670, May 2001.

18. Donaldson LJ, Taylor JB. Patterns of Asian and non-Asian morbidity in hospitals. BMJ 1983; 286: 949-951.

19. Nieto-Garcia FJ. Bush TL. Keyl PM. Body mass definitions of obesity: sensitivity and specificity using self-reported weight and height. Epidemiology 1990; 2:146-152.

20. Hughes LO, Raval U, Raftery EB. First myocardial infarctions in Asian and white men. BMJ 1989; 298: 1345-1350.

21. Klatsky AL, Tekawa IS, Armstrong MA. Cardiovascular risk factors among Asian Americans. [Journal Article] Public Health Reports 1996; 111 Suppl 2: 62-64.

22. Bhatnagar D, Anand IS, Durrington PN, Patel DJ, Wander GS, Mackness MI, et al. Coronary risk factors in people from the Indian subcontinent living in west London and their siblings in India. Lancet 1995; 345: 405-409.

23. Beckles GL, Miller GJ, Kirkwood BR, Alexis SD, Carson DC, Byam NT. High total and cardiovascular disease mortality in adults of Indian descent in Trinidad, unexplained by major coronary risk factors. Lancet 1986; 1: 1298-1301.

24. Akram H. Al-Khadra, Clinical profile of young patients with acute myocardial infarction in Saudi Arabia. Int J Cardiol 2003; 91: 9-13.

25. Berenson GS, Srinivasan SR, Bao W, Newman WP, Tracy RE, Wattigney WA. Association between Multiple Cardiovascular Risk Factors and Atherosclerosis in Children and Young Adults. N Engl J Med 1998; 338: 1650-1656.

26. Welch GN and Loscalzo J. Homocysteine and atherothrombosis. N Engl J Med 1998; 338: 1042-1050.

27. Al-Nuaim AR. Serum total and fractionated cholesterol distribution and prevalence of hypercholesterolemia in urban and rural communities in Saudi Arabia. Int J Cardiol 1997; 58: 141-149.

28. Malmberg K, Bavenholm P, Hamsten A. Clinical and biochemical factors associated with prognosis after myocardial infarction at a young age. J Am Coll Cardiol 1994; 24: 592.

29. Rashid MN, Fuentes F, Touchon RC, Wehner PS. Obesity and the risk for cardiovascular disease. Preventive Cardiology 2003; 6: 42-47.

30. Sebai ZA. Health in Saudi Arabia, Vol. 2. Riyadh, Directorate of Scientific Research, King Abdul Aziz City for Science and Technology 1987: 81-126.

31. Abu-Zeid HAH, Al-Kassab ASK. Prevalence and health care features of hyperglycemia in semi-urban/rural communities in southern Saudi Arabia. Diabetes care, 1982; 15: 484-489.

32. Fatani HH, Mira SA, Al-Zubair AG. Prevalence of diabetes mellitus in rural Saudi Arabia. Diabetes care 1987; 10: 180-183.

33. El-Hazmi MAF, Warsy AS. A comparative study of hyperglycemia in different regions of Saudi Arabia. Annals of Saudi Medicine 1989; 9: 435-438.

34. Anokute CC. Epidemiology of diabetes mellitus in Saudi Arabia- retrospective study of 210 cases. Practical diabetes digest 1991; 3: 25-26.

35. Al-Nuaim AR. High prevalence of Metabolic risk factors for cardiovascular diseases among Saudi population, aged 30-64 years. Int J Cardiol 1997; 62: 227-235.

36. Abolfotouh MA, Abdel Aziz M, Alakija W, Al-Safy Abdullah, Khattab MS et al. Smoking habits of King Saud University students in Riyadh. Annals of Saudi medicine 1991; 11: 141-143.

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Major congenital anomalies in Southwestern Saudi Arabia

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Abstract

Currently, in the Arabian Peninsula, genetically determined disorders account for an increasing proportion of death, morbidity, chronic handicap, and disability. The pattern and classification of MCAs in Asir region, in a six-year period, in order to allow proper genetic counselling, early management and rehabilitation have been studied. The study included all neonates with congenital anomalies referred to Asir Central Hospital within the six-year period from 1997 to 2002. Cases with genetic syndromes were diagnosed by review of *Mandelian inheritance in man* and the *London dysmorphology database*. The major congenital anomalies were classified according to the ICD-10 system, and multiple MCAs were counted only once by the system of the most major anomaly. Of 1171 newborns admitted to neonatal intensive care unit (NICU) at Asir Central Hospital, 691 newborns were found to have congenital anomalies, constituting 59.1% of all admissions. According to ICD-10 classification of congenital anomalies, the systems involved in the MCAs investigated were as follows; digestive (28.6%), central nervous (26.1%), circulatory (16.5%), urogenital (7.1%), eye, ear, face and neck (4.1%), respiratory (6.2%), musculoskeletal (3.6%), chromosomal (3.3%), and other anomalies (4.5%). Congenital anomalies represent the main reason for referral to NICU in Asir region, and this implies that congenital malformations contribute significantly to perinatal and infant morbidity in the region. Premarital counselling is advised, especially in the presence of parental consanguinity and family history of a congenitally malformed child.

Introduction

Birth defects, (in 2%-4% of all neonates) present at birth [1]. These abnormalities may be caused by environmental factors that affect the mother, or are inherited via abnormal genes from the carrier or affected parent. Such disorders account for about 20% of deaths during the neonatal period and a higher percentage of morbidity in infancy and childhood [2]. With the dramatic decrease in infant mortality due to improvement in the control of infections and malnutrition-related disorders, chronic disabling conditions are an emerging challenge facing developing and industrialized nations. In Saudi Arabia, the incidence of major congenital anomalies is 22.7 per 1000 live births [3]. The Asir region is an area of about 80,000 Km^2, with a population of about 1.2 million people. Asir Central Hospital is the main referral hospital, and serves as the teaching hospital of the medical school in the region. All complicated and terminal cases from the 17 hospital in the region are routinely referred to this hospital. As such,neonates with major congenital anomalies (MCAs) referred to the hospital provide a fair reflection of such conditions in this area. It was decided, therefore, to study the pattern and classification of MCAs in Asir region, our a six-year period, in order to provide proper genetic counselling, early management and rehabilitation.

Material and Methods

The study included all neonates with congenital anomalies referred to Asir Central Hospital during the six-year period from 1997 to 2002. All neonates identified with congenital anomalies were admitted to NICU for observation, investigation, evaluation and management. Photographs, radiographs,necropsy reports and chromosomal studies were included when needed.

Cases with genetic syndromes were diagnosed by review of *Mandelian inheritance in man*(4) and the *London dysmorphology database* [5]. The major congenital anomalies were classified according to the ICD-10 system, and multiple MCAs were counted only once by the system of the most major anomaly [6].

Results

Out of 1171 newborns admitted to neonatal intensive care unit (NICU) at Asir Central Hospital, 691 newborns were found to have congenital anomalies, constituting 59.1% of all admissions. These congenital anomaly cases constituted significantly higher proportion of male admissions than female admissions ($p<0.001$) (Table 1).

Table 1: Number (%) of admissions in the neonatal unit at Asir Central Hspital within six year period (1997-2002) and the corresponding number of congenital anomaly cases

Sex	Admissions	New borns with anomalies	
		Number	%
Male	581	384	66.1
Female	589	317	53.8
Total	1170	691	59.1

According to ICD-10 classification of congenital anomalies, the systems involved were as follows; digestive (28.6%), central nervous (26.1%), circulatory (16.5%), urogenital (7.1%), ear, eye, face and neck (4.1), respiratory (6.2%), musculoskeletal (3.6%), chromosomal (3.3%), and other anomalies (4.5%) (Table 2).

Digestive system anomalies were commonest in the present study.

Table 2. Frequency of major congenital anomalies (MCAs)

MCAs by system (ICD-10)	No.	%	% of total
Q00-Q07 Central nervous system			
- spina bifida and encephaloceles	81	45.0	
- hydrocephalus without spinabifida	55	30.0	
- microcephaly	30	16.7	
- crynosyenstosis	6	3.3	
- porencephaly	5	2.8	
- anencephaly	3	1.6	
Subtotal	180	100	26.1
Q10-Q18 Eye,Ear,face and neck	28	100	4.1
Q20-Q28 Circulatory system			
- ventricular septal defect	60	52.6	
- hypoplastic single ventricle	14	12.3	
- tetralogy of fallot	13	11.4	
- endocardial cushion defects	12	10.5	
- transposition of great vessels	8	7.0	
- complex cardiac anomalies	7	6.2	
Subtotal	114	100	16.5
Q30-Q34 Respiratory system			
- tracheo-oesophageal fistula	43	100	6.2
Q35-Q37 Cleft lip and Cleft palate	25	100	3.6
Q38-Q45 Other digestive system			
- intestinal obstruction	56	32.4	
- imperforated anus	40	23.1	
- Hisrshsprung disease	39	22.5	
- Diaphragmatic hernia	38	22.0	
Subtotal	173	100	25.0
Q50-Q64 Urogenital system			
- posterior urethral valve	10	20.4	
- undescended testes	10	20,4	
- hypospadius	9	18.4	
- ambigeous genitalia	9	18.4	
- polycystic kidney	8	16.3	
- Poter syndrom	3	6.1	
Subtotal	49	100	7.1
Q65-Q79 Musculoskeletal system	25	100	3.6
Q80-Q89 Other anomalies			
- Cutaneous	15	48.4	
- hydrocele	11	35.5	
- tumor cyst	5	16.1	
Subtotal	31	100	4.5
Q90-Q99 Chromosomal (not classified elsewhere)			
- trisomy 21	15	62.2	
- trisomy 18	5	21.7	
- trisomy 13	3	13.1	
Subtotal	23	100	3.3
Total	**691**		**100**

Table 3: Rank order of MCAs in the present study in comparison with other studies in different countries [7]

System involved	Saudi Arabia	Libya	UAE	Nigeria	Current
Digestive	4	4	-	-	1
Central Nervous	3	-	-	2	2
Circulatory	1	2	1	-	3
Musculoskeletal	2	1	2	1	5
Chromosomal	4	3	-	-	6

Table represents the available data

Table 3 shows the rank order of different MCAs in the present study in comparison with other studies. Digestive system anomaly came first in the present study, while it ranked fourth in both Saudi Arabian and Libyan studies. On the other hand, circulatory system anomaly ranked first in both Saudi Arabia and UAE, but it ranked 3rd in the present study, after digestive and central nervous system anomalies.

Discussion

The social and economic conditions in the Arabian Peninsula have improved enormously in the last 3 decades. This led to the sharp decline in the incidence of infectious diseases and diseases related to malnutrition. Currently genetically determined disorders account for an increasing proportion of death, morbidity, chronic handicap, and disability[8]. During the period (1985-1989), 19% of pediatric inpatients - in King Khaled University Hospital in Riyad city of Saudi Arabia – had congenital or genetically-determined disorders(9). However, in the present study, neonates with MCAs constituted 59.1% of all neonates referred to NICU of Asir Central Hospital during a period of six years.

This high frequency of MCAs might have resulted from the consanguineous marriages practiced in this tribal area of Asir region, leading to the preservation of rare mutations kept in a genetically homogenous population. Several publications indicate that consanguineous marriages in the Kingdom of Saudi Arabia (KSA) are high (60%) and this has provided a background in which these genetic diseases abound [10,11]. This implies that congenital malformations constitute significantly to perinatal and infant morbidity in Asir region.

Cases with MCAs in the present study were observed significantly more among male admissions than that of females. This male proponderance is in agreement with the findings of other studies [12-14]. It may be speculated that either the females were afflicted with more lethal congenital malformations and could not survive till referred, or that their malformations were too mild to be referred.

Congenital anomalies in the present study- were classified by the systems according to the ICD-10 classification and multiple anomalies were counted only once based on the major system involved It is possible that some cases of congenital anomalies could have been undetected, particularly in asymptomatic neonates, in cases of internal anomalies and perinatal death. It is unlikely, however that any major, external or obvious malformations were unrecorded.

Digestive system anomalies were the commonest in the present study, athough in other studies, it was less common [7]. The most frequent lesions of this system were intestinal obstruction, tracheoesophageal fistula, imperforate anus and diaphragmatic hernia. This finding is in agreement with the results of Al-Qassim study in the central region of Saudi Arabia [15].

Central nervous system anomalies ranked second in frequency in the present study. However, spina bifida and encephalocele together ranked first among all reported congenital malformations. In the developed countries, following mandatory prescription of folic acid for pregnant women, the incidence of neural tube defect has markedly declined [14,16,17].

Throughout the world, chromosomal aberrations are among the least defined causes of congenital anomalies [18]. This was in concordance with the results of the present study, where these anomalies ranked six in frequency, However, Trisomy 21 constituted two-thirds of all these chromosomal anomalies. This syndrome is a major cause of mental retardation, congenital heart problems and gastrointestinal malformations [19].

In conclusion, congenital anomalies represent the main reason for referral to NICU in Asir region, and this implies that congenital malformations constitute significantly to perinatal and infant morbidity in the region. Our results probably underestimate the number of congenital anomalies in Asir region as a result of possible non-referral of minor anomalies, in addition to non-inclusion of stillbirths and neonatal deaths. However, the results of the present study aim at addressing the categories of MCAs that might bring the burden of the future handicapping conditions with their social and economical consequences.

Accurate and early diagnosis of congenital malformations is the key to proper management of cases. Premarital counselling is advised, especially in the presence of parental consanguinity and family history of a congenitally malformed child. Because of the high frequency of neural tube defects, proper prenatal diagnosis is recommended. However, cultural and religious factors are to be considered. If prevention is not possible, it becomes even more crucial to anticipate and plan accordingly for the management.

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References

1. El-Hazmi MAF. Early recognition and intervention for prevention of disability and its complications. East Mediterr Health J 1997; 3: 154-161.

2. Contribution of birth defects in infant mortality – United States, 1986, Morbidity and mortality weekly report, 1989, 38: 633-635.

3. Refat MYM. Major birth defects at King Fahd Hofuf Hospital: Prevalence, risk factors and outcome. Ann Saudi Med 1995; 15: 339-343.

4. McKusick VA. Mendelian inheritance in man: catalogs of autosomal dominant, autosomal recessive and X-linked phenotypes, 9th ed. Baltimore, Johns Hopkins University Press, 1994.

5. Winter R, Baraitser M. The *London dysmorphology database*. Oxford, Oxford University Press, 1995.

6. International statistical classification of diseases and related health problems (ICD-10), 10th rev. Geneva, World Health Organization, 1992.

7. Singh R, Al-Sudani O. Major congenital anomalies at birth in Benghazi, Libyan Arab Jamahiriya, 1995. East Mediterr Health J 2000; 6: 65-75.

8. Al-Odaib AN, Abu-Amero KK, Ozand PT, Al-Hellni AM. Anew era for preventive genetic programs in the Arabian Peninsula. Saudi Med J 2003; 24: 1168-1175.

9. Bahakim H. Pediatric inpatients at the King Khalid University Hospital, Riyadh, Saudi Arabia, 1985-1989. Ann Saudi Med 1993; 13: 8-13.

10. Al-Hussain M, Al-Bunyan M. Consanguineous marriages in a Saudi population and the effect of inbreeding on prenatal and postnatal mortality. Ann Trop Paediatr 1997; 17: 155-60.

11. Al-Abdulkareem AA, Ballal AG. Consanguineous marriages in an urban area of Saudi Arabia: rates and adverse health effects on the offspring. J Community Health 1998; 23: 75-83.

12. Mir NA, Galczek WC, Soni A. Easily identifiable congenital malformations in children : Survey of incidence and pattern in 32, 332 liveborn neonates. Ann Saudi Med 1992; 12: 366-371.

13. Khan SMD, Ahmad GS, Abu-Talib A. Are congenital anomalies more frequent in Saudi Arabia?. Ann Saudi Med 1990; 10: 488-489.

14. Daly LE, Kirke PN, Molly A, Weir DG, Scot J. Folate levels and neural tube defect: implication for prevention. JAMA 1995; 274: 1698-1702.

15. Hegazy IS, Al-Beyari TH, Al-Amri AH, Qureshi NA, Abdelgadir MH. Congenital malformations in the primary health care in Al-Qassim Region. Ann Saudi Med 1995; 15: 48-53.

16. Stone DH. The declining prevalence of anencephalus and spina bifida: its nature, causes and implications. Dev Med Neural 1978; 29: 541-549.

17. O'Dowa MJ, Connolly K, Ryan A. Neural tube defect in rural Ireland. Arch Dis Child 1978; 62: 297-298.

18. Hamamy H, Alwan A. Hereditary disorders in the Eastern Mediterranean Region. Bulletin of the World Health Organization 1994; 72: 145-154.

19. Korenberg JR, Bradley C, Disteche CM. Down syndrome: molecular mapping of the congenital heart disease and duodenal stenosis. Am J Human Genetics 1992; 50: 294-302.

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Intermittent sequential pneumatic lower limb compression and its relationship to prophylaxis of deep vein thrombosis

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Key words: venous blood flow velocity, deep vein thrombosis, intermittent sequential pneumatic compression

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Abstract

Femoral venous blood flow velocity was measured using Doppler ultrasonograph before, during and after application of intermittent sequential pneumatic lower limb compression. Both thigh and knee length pneumatic cuffs were used for comparison. The result showed a 400% and 240% increase in venous velocity for the thigh and knee length cuffs respectively. This suggests that higher compression may be required for different patients for optimal effect of this method of prophylaxis of deep vein thrombosis (DVT).

Introduction

Clotting disorders and stroke are responsible for mortality of about one third of blacks in a recent South African study [1]. About one-fifth of Americans die from stroke and clotting disorders [2]. Chronic crippling may arise from the post-phlebetic syndrome [3]. Prevention of clotting disorders offers the best approach to limiting morbidity and mortality from deep vein thrombosis and pulmonary embolism [4]. Graduated elastic stockings and intermittent sequential pneumatic compression applying graded external pressure on the legs have been found to be efficacious in the prevention of DVT [5-9], especially in surgical patients. External leg compression and most especially intermittent sequential pneumatic leg compression have been found to increase venous flow velocity [10-14].

Increase in venous flow velocity with or without flow pulsatility (which is a time variable of peak flow) enhances prophylaxis of deep vein throbosis probably by its stripping action of microthrombi in the valve pockets and on the vein wall. This stripping action is a consequence of the increase in shear rate (which probably also stirs stagnant blood in venous valve pockets) resulting from the velocity increase. Therefore, it is important to determine the increase in velocity arising from external compression as accurately as possible.

Increase in both peak velocity and pulsatility of the venous blood flow has been claimed to be affected by intermittent pneumatic compression thereby simulating the venous pump of the calf muscles. Intermittent sequential pneumatic compression, in addition, has been found to be more effective than uniform intermittent compression and optimal compression pressures have been recommended by Nicholaides et al [14], arising from the results of studies using venous blood velocity as determined by Doppler ultrasonic technique. The objective of the present measurement was to establish the response of femoral venous blood velocity to intermittent sequential pneumatic lower limb compression in an effort to elucidate the probable mechanism of action of this device in the prophylaxis of DVT. Furthermore, there was a need to confirm or establish any deviation on which the recommendation for optimal pressure requirement of sequential pneumatic compression was based in an effort to optimize therapy.

Subjects and Methods

Five subjects including one female and four males (average age 25 years) were used for this study. After inserting the leg into the pneumatic cuff, either the thigh or knee length respectively, the probe of the Doppler apparatus (Parks Model 806) was put into the probe-steady device designed and constructed by the author at the Bioengineering Unit. A coupling gel (Aquasonic) was placed on top of an area just medial to the pulsation of the femoral artery (located by palpation). The probe-steady device with the probe in-situ was attached to the femoral triangle by means of Velcro tape strapped around the thigh at femoral triangle. The Doppler apparatus was switched on and through the headphone, the femoral vein sound was located. By means of the probe holder, the probe was locked in the "best" femoral venous signal position after scanning.

The intermittent sequential pneumatic compression device (T.E.D.) Kendall) was then switched on and the compression pressure was consecutively adjusted and measurements made

for each compression pressure. Each consecutive compression pressure was allowed to act for an average of three cycles. The tracing of the venous signal was recorded on the chart recorder. The compression pressure of the device as registered on the integral dial of the equipment was noted visually and marked against the recording on the chart recorder. The venous signal as recorded in the chart recorder was analyzed and plotted against the compression pressure.

pression cycle, and decreased abruptly at the onset of the deflation cycle. Furthermore, there was almost a proportional increase in velocity with increase in compression pressure until the highest velocity was obtained at 8kPa compression pressure. Thereafter, there was no further increase of velocity with further increse in compression pressure.

$$V_{rel} = \frac{V_{max} - V_{mean}}{V_{mean}} \times 100$$

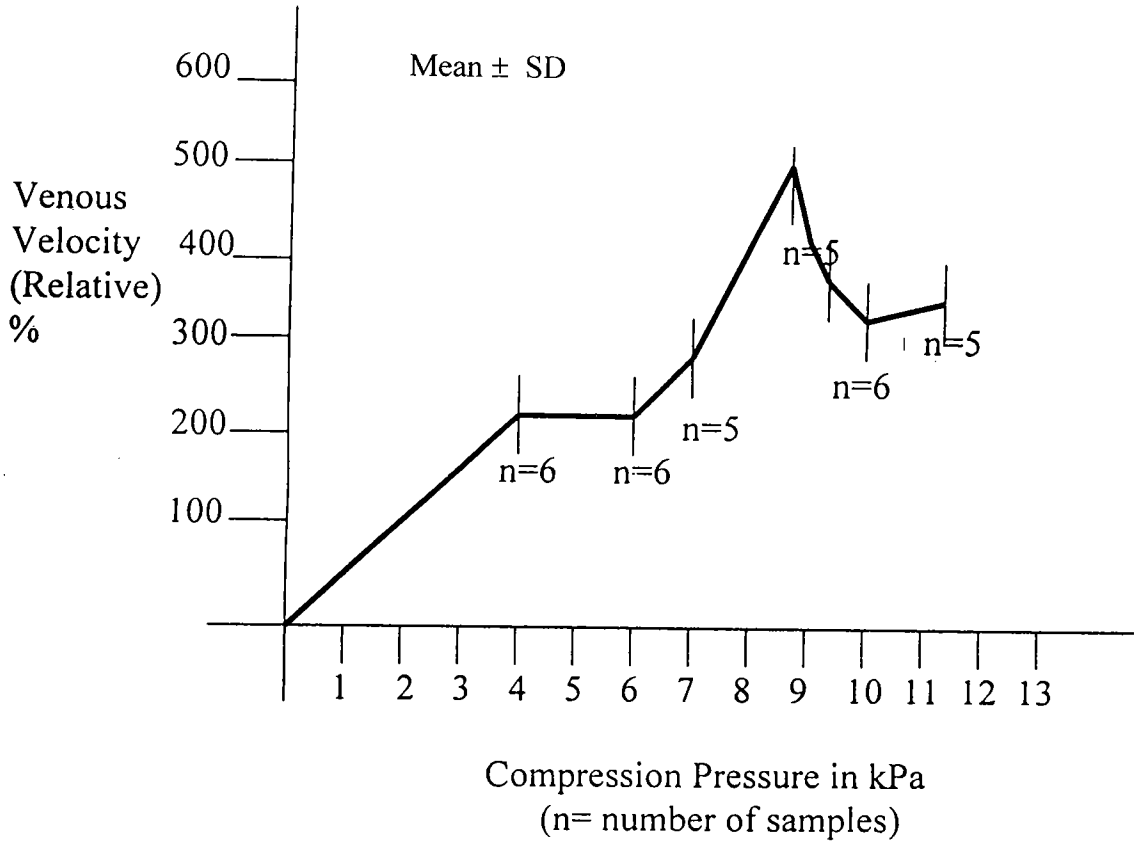

Fig. 1: *Venous Velocity change in response to external intermittent pneumatic sequential compression of the leg using thigh length cuf.*

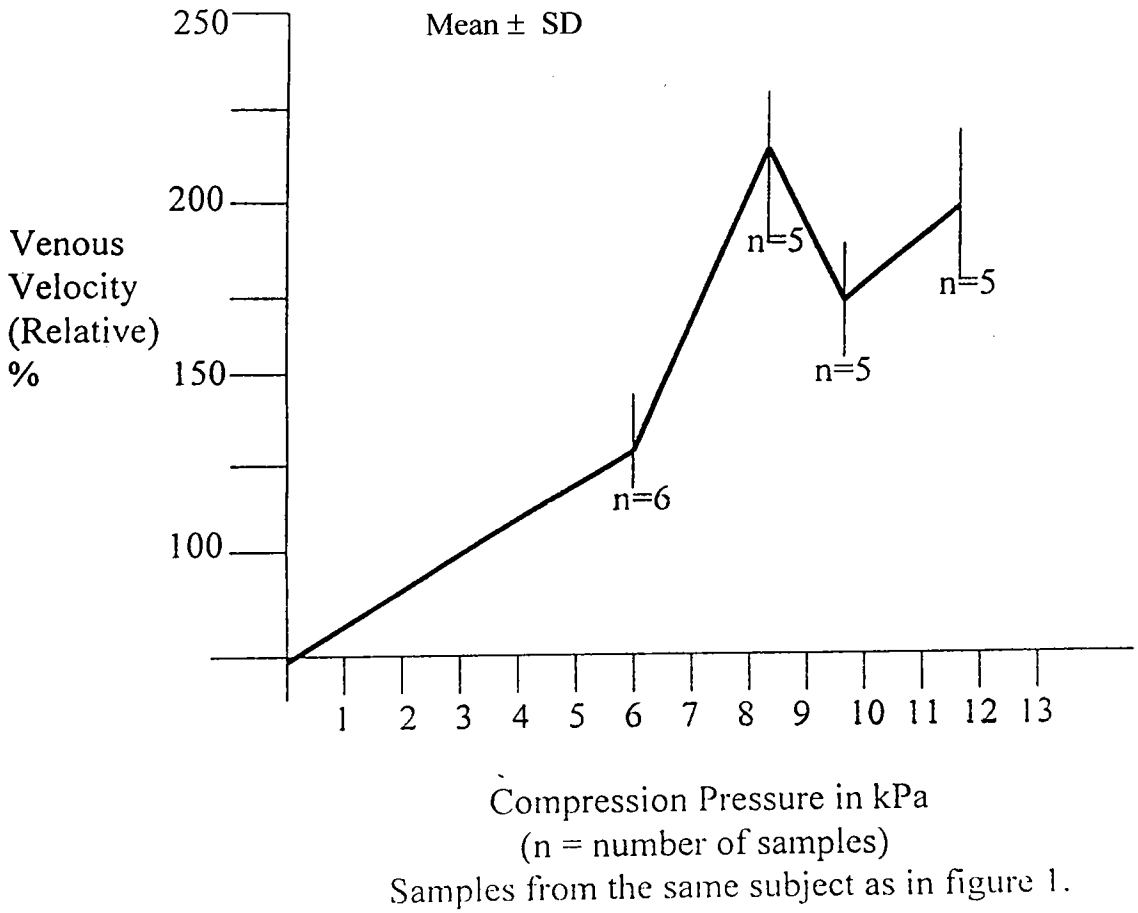

Fig. 2: Venous Velocity change in response to external intermittent pneumatic sequential compression of the leg using knee-length cuf.

Result

The results of the measurements are given in figures 1 and 2. There was up to 400% and 240% increase in peak/ mean velocity with each compression cycle for thigh and knee length cuffs respectively. The highest peak occurred at the beginning of the compression of the first chamber (ankle). The peak velocity was maintained at a level slightly lower than the highest peak obtained at the onset of cuff inflation, throughout the duration of the com

Discussion

Increase in both peak velocity and pulsatility of the venous blood has been claimed to be affected by intermittent pneumatic compression thereby simulating the venous pump of the calf muscles. Intermittent sequential pneumatic compression in addition has been found to be more effective than uniform intermittent compression [10] and optimal compression pressure [11] have been recommended from results of studies using venous blood velocity as determined by Doppler ultrasonic technique as the objective criteria.

Nicolaides et al [11], found up to 240% increase in peak venous blood flow velocity. They therefore recommended optimal compression pressures of 4.61, 3.95, and 2.63 kPa respectively at the ankle, calf and thigh applied sequentially for 12 seconds. This was the lowest compression pressure gradient they found to give the highest increase in venous velocity. The result of the present study while confirming the increase in peak velocity as reported by Nicolaides et al [11], suggest that higher compression pressure could be applied for optimal increase in venous velocity with the application of the sequential intermittent compression. The discrepancy for the different results regarding optimal compression may be due to individual variability in response to the same input. Perhaps there might be the need to individualize the compression pressure gradient to suit the variations inherent in individual patients. Fortunately, the design of the sequential compression device used in this study incorporates such need for variation in compression pressure.

It is not certain as to whether or not increase in venous blood flow velocity totally prevent DVT. It seems plausible that there may be a common denominator of Virchow's triad of thrombogenesis through which the homeostatic balance between thrombogenesis

and fibrinolysis act to ensure the overall integrity of the entire vasculature [15]. Okoye et al [16], have found a significant reduction in the venous oxygen tension measured at the femoral vein on application of external pressure on the legs. Oxygen tension may, therefore, be a common denominator in the homeostatic mechanism responsible for the dynamic balance between thrombogenesis and fibrinolysis.

Intermittent compression of the arm has been shown to reduce the incidence of DVT of the leg [9] as well as increasing fibrinolysis [17]. Therefore external compression, in as much as it increases venous velocity, probably has a systemic effect on the dynamic balance between thrombogenesis and fibrinolysis. The delineation of the mechanism of action of these physical modalities of prophylaxis of DVT will no doubt lead to optimization of therapy. It requires further study in this regard to elucidate the probable mechanism of action of these physical devices for optimization of these therapeutic modalities as they are simple, portable and have not been shown to have any side effect.

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References

1. Jock F. New anticoagulant prompts bad blood between partners. Science 1996; 271: 1800-1801.

2. Pestana JA, Steyr K, Leiman A, Hartenberg GM. The direct and indirect costs of cardiovascular diseases in South Africa in 1991. South Africa Medical Journal 1996; 86: 879-884.

3. Cogo A, Lensing AN, Wells P, Prandoni P, Buller HR. Non-invasive objective test for the clinically suspected deep vein thrombosis. Haemostasis 1995; 25: 27-39.

4. Horn EH. Thrombosis and embolism: Balliere's Clinical Obstetrics and Gynaecology 1995; 9: 596-618.

5. Flame E, Bevry S, Coyle A, Dardik H, Raab L. Blood flow augmentation of intermittent pneumatic compression systems used for prevention of deep vein thrombosis prior to surgery. Am J Surg 1996; 17: 312-315.

6. Wilkins RW, Mixter G, Stanton JR, Litten J. Elastic stockings in the prevention for pulmonary embolism-a preliminary report. New England Journal of Medicine 1952; 246: 360-364.

7. Holford CP (BP) Bliss). The effect of graduated static compression on isotopically diagnosed deep vein thrombosis of the leg. Brit J Surg 1976; 63: 157.

8. Bolton J. The prevention of post-operative deep venous thrombosis by graduated compression stockings; in symposium on thrombosis. Scot Med J 1978; 23: 315-317.

9. Sigel B, Edelstein AL, Savitch L, ?Hasty JH, Felix WR. Type of compression for reducing venous stasis. Arch Surg 1975; 110: 171-175.

10. Olson DA, Kamm RD, Shapiro AH. Experimental studies of external pneumatic compression methods on a model leg. 32nd A.C.E.M.B. 1979, Denver, Colorado

11. Nicolaides AN, Fernandes E, Fernandes J, Pollock AV. Intermittent sequential pneumatic compression of the legs in the prevention of venous stasis and postoperative deep vein thrombosis. Surgery 1980; 87: 69-76.

12. Browse NL. The prevention of venous thromboembolism by mechanical methods. In: Venous problems eds. JJ Bergan, JST Yao. Yearbook Medical Publishers Inc.1978; pp 563-569.

13. Wright HP, Osborn SB. Effect of posture on venous velocity, measured with ^{24}NaCl. Brit Heart J 1951; 14: 325-330.

14. Clark C, Cotton LT. Blood flow in deep veins of the leg-recording technique and evaluation of methods to increase flow during operations. Brit J Surg 1968; 55: 211-214.

15. Doran FSA, Drury M, Sivyer AA. A simple way to combat the venous stasis which occurs during surgical operations in the lower limb. Brit J Surg 1964; 51: 486.

16. De Bruyne P, Dvorak T. The pressure exerted by an elastic stocking and its measurement. Medical and Biological Engineering 1976; 94-96.

17. Okoye GC. Potential for optimization of pressure therapy in the prophylaxis of deep vein thrombosis: A study of pressure therapy in the prophylaxis of deep vein thrombosis. A study on stroke patients, Ph.D. thesis. 1984, Bioenginering Unit, University of Strthclyde, Glasgow, UK.

18. Virchow RLK. Thrombose und embole. 1986, Frankfurt.

19. Sigel B, Edelstein AL, Savitch L, Hasty JH, Felix WR. Type of compression for reducing venous stasis. Arch Surg 1975; 10: 171-175.

20. Okoye GC. Incidence od dep vein thrombosis in stroke patients. Journal of Nigeria Medical Rehabilitation Therapists 1999; 4: 21-22.

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Activities of adenosine Triphosphatases (ATPases) in Sperm Cells: Implications for narrowing down treatment for Asthenospermia.

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Key words: ATPase, sperm cell motility, ssthenospermia, treatment.

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Abstract

The relationship between the activities of adenosine triphosphatases – ATPases (Ca^{2+} - ATPase, Mg^{2+} - ATPase and Na^{+}/K^{+} - ATPase) in human sperm cells and the spermatozoal motility was studied. A total of twenty-three (23) patients aged between 28 and 50 years attending the fertility clinic of the University of Nigeria Teaching Hospital, Enugu, Nigeria were involved in the study. The results showed that ther were positive correlations ($r=0.73$ for Ca^{2+} - ATPase; $r=0.42$ for Mg^{2+} - ATPase and $r=0.70$ for Na^{+}/K^{+}; $p<0.05$ for each) between the specific activities of the ATPases and the motility of the sperm cells. Also it was found that non-motile and sluggishly motile cells still had some residual enzyme activities, though the activities were significantly lower ($p<0.05$) than those of the actively motile ones. When grouped according to the percentage motility, there was also increasing enzyme activities as the percentage motility increased. Infact, with the exception of Mg^{2+} - ATPase, the activities of these enzymes in 61 – 90% motility group were significantly higher ($p<0.05$) than their activities in the other groups i.e 1 –30% and 31 – 60% motility groups, reaching twice the values of their activities in the later groups. More work to elucidate other biochemical causes of asthenospermia and possibility of using potent activators of these enzymes for designing drugs for the treatment of asthenospermia are advocated.

Introduction

Causes of male infertility are many and varied. They range from endocrine dysfunction, varicocele, testicular failure, obstruction of reproductive tract, cryptochordism, ejaculatory failure, semen viscosity, necrospermia, azoospermia to asthenospermia [1]. Good sperm motility in at least 60% of the cells and linear progression are required for the penetration of the cervical mucus and transport through the female genital tract, as well as for the penetration through the corona radiata and zona pellucida of the oocyte prior to fertilization.In a situation where all the other conditions necessary for normal fertilization are favourable, the highly motile spermatozoa will fertilize the ova more readily than others. Hence, more than four decades ago, motility has been generally recognized as the most important indicator of seminal quality [2]. Though the basic motility apparatus in the sperm cell – the axoneme, has been remarkably conserved during evolution, the mechanism that initiates and modulates the axonemal movement and thus sperm motility shows marked species variation [3]. In many patients with isolated asthenospermia, acquired or congenital lesions of flagella function may be likely. A review of the relationship between cAMP, Ca^{2+} ion and protein phosphorylation as major factors that modulate flagella motility in a variety of species revealed that activation of mammalian sperm motility requires cAMP – dependent phosphorylation [4]. On the other hand, Ca^{2+} ion is known to increase cAMP content of sperm cell as well as sperm cell adenyl cyclase (which catalyses the formation of cAMP from ATP). The actions of Na^{+}/K^{+}- ATPase which provides cellular ions and macromolecules (glucose and amino acids) and Ca^{2+}/Mg^{2+}- ATPase, which increases cAMP needed for phosphorylation for sperm motility points to possible relationship between the activities of these enzymes and sperm cell motility. Although the presence of these enzymes has been demonstrated in sperm cells [5], there is dearth of literature on their activities and sperm cell motility. For the first time, this study therefore investigated these relationships.

Materials and Methods

Subjects: A total of twenty – three male subjects on first visit to the Fertility Clinic of University of Nigeria Teaching Hospital

(UNTH), Enugu, Nigeria, were recruited for the study. They were apparently healthy, baring their primary complaint – suspected infertility. The subjects, whose consents were sought, were not on any spermatogenesis – enhancing drugs prior to the study. Approval for the study was given by the Ethics Committee of the University of Nigeria Teaching Hospital, Enugu.

Sample collection and estimation: Subjects were advised to abstain from sexual intercourse for at least three days before the study. A freshly voided semen sample was collected from each subject through coital interuptus, into a sterile universal container. Two drops of each semen sample were put on a clean microscope slide and covered with clean coverslip. This was focused on the microscope with x10 objective and then x40 objective. At x40 objective, the sperm cells in each field were counted while noting the number that were actively motile with linear progression. A percentage of the actively motile cells out of the total cells in each field was calculated. This was done for at least ten (10) fields and an average percentage motility taken for each sample. After estimating the percentage motility of the sperm cells, the samples were stored frozen for three days to reduce the viscosity of the semen.

Sample preparation: The samples were allowed to thaw at room temperature. After centrifuging at 5000 rpm for five minutes, the seminal plasma was discarded while the deposits (sperm cells) were washed four times using cold normal saline and then cold distilled water. The washed cells in each tube were then crushed using cold mortar and then homogenized with 1.0 ml of 50mM tris-Hcl buffer. Each homogenate was transferred into a clean test tube and stoppered with dry clean rubber cork and stored at –20°C until needed for biochemical analyses.

Analyses: Protein concentrations were estimated by method of Lowry et al [6] using bovine serum albumin as standard. Total ATPase activity was assayed by spectrophotometric method of Bonting [7] while the individual ATPases were assayed according to the method of Takeo et al [8] using potassium dihydrogen orthophosphate (KH_2PO_4) as standard. The incubation medium for the enzyme assay contained Nacl (1.0M), Kcl (100mM), $Mgcl_2$ (40mM), EDTA (10mM), Tris-Hcl (100mM), ATP (25mM) and 0.2ml of 1/3 dilution of the homogenate in a reaction volume of 1.1ml. The reaction was stopped by the addition of 0.4ml of 10% sodium dodecyl sulphate and the liberated inorganic phosphate (Pi) estimated by its reaction with ammonium molybdate as previously described [9]. Each result was multiplied by the dilution factor.

Correlation and regression analysis were used to analyse the data on the activities of the enzymes and the spermatozoal motility.

Results

The activities of the ATPases showed positive correlation ($p<0.05$) with the spermatozoal motility. The Ca^{2+} - ATPase activity showed the highest positive correlation with $r=0.73$ and $r^2=0.54$. This gives a regression equation of the enzyme activity (Y) on the sperm cell motility (X) as $Y=0.02X + 0.21$. The positive correlation between Na^+/K^+- ATPase activity and spermatozoal motility with $r=0.70$ and $r^2=0.48$ was less than that of Ca^{2+}- ATPase. The regression equation of the enzyme's activity (Y) on the sperm cell motility (X) is $Y=0.03X + 0.05$. The Mg^{2+}- ATPase has the lowest correlation with $r=0.42$ and $r^2=0.17$. The regression equation of Mg^{2+}- ATPase activity (Y) on the sperm cell motility (X) is $Y=0.01X + 0.40$.
Figure 1 is a bar–chart indicating the differences in the enzyme activities when grouped according to the percentage motilities. It also shows that the non-motile sperm cells also exhibited residual enzyme activities.

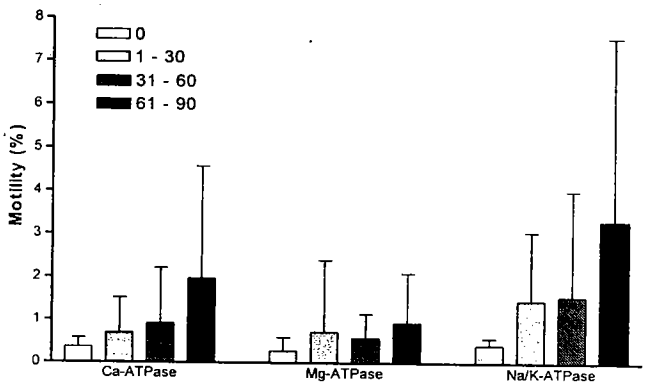

Fig. 1: *Bar-chart of ATPase activities at different percentage motilities of the sperm cells.*

Discussion

Our results showed positive correlation between the activities of ATPases in the spermatozoa and spermatozoal motility. Though there is dearth of literature on this relationship in human spermatozoa, our results are in line with previous results of studies on other species and organs [10,11] where it was found that dynein Ca^{2+}/Mg^{2+}- ATPase present in the microtubular doublets provides driving force for flagella and cilia movement. Moreover, it is known that Ca^{2+}/Mg^{2+} - ATPase system has tenacious affinity for Ca^{2+} ion [12] - the ion known to increase motility and cAMP content of sperm cell in a dose dependent manner [4]. In two separate inhibition studies, vanadate- a potent inhibitor of Ca^{2+}/Mg^{2+}- ATPase was found effective in inhibiting bovine sperm cell motility through its action on flagella dynein ATPase [13] while calcium channel blocker– verapamil, significantly decreased the motility and linear progression of human spermatozoa, in vtro, through its inhibitory effect on Ca^{2+}- ATPase and blocking of influx of extracellular Ca^{2+} ion [14]. These two findings signify that ATPase inhibitors that are safe for human consumption can be conveniently used as male contraceptives. In another study, L-carnitine, which has no inhibitory effect on the dynein ATPase activity but also inhibits flagella motility of fowl spermatozoa, does so only through its effect on axonemal phosphoproteins [15].

The positive correlation between the activities of Na^+/K^+- ATPase and spermatozoal motility is not unexpected. This is

because this enzyme is one of the major energy providers within cell membrane through its ability to hydrolyze ATP. Generally, mammalian sperm cells require high energy to enable them penetrate cervical mucus, corona radita and zona pellucida prior to fertilization. Na^+/K^+- ATPase readily provides this by facilitating transmembrane uptake of solutes like glucose and amino acids via the Na^+ symport systems coupled to ATP hydrolysis [16]. In their study, Saito et al [17] discovered that activation of ouabain – sensitive Na^+/K^+- ATPase has an important positive role in sperm motility. Also adenosine and 2'-deoxyadenosine have been demonstrated to have stimulation effect on both dynein ATPase and sperm cell motility [18]. One may postulate that the stimulation of the enzyme activity may have accounted for the increased sperm cell motility in that order. On the other hand, ouabain (potent inhibitor of Na^+/K^+- ATPase) was found to slightly decrease the respiration rate of motile spermatozoa [19], and hence their motility. This is most likely through its action on Na^+/K^+- ATPase, pointing to the potentials of inhibitors of these enzymes as male contraceptives. We believe that since the mentioned ATPase inhibitors have confirmed negative effects on the activities of these enzymes [19], their activators will invariably have positive effects on them. Thus, the discovery of potent activators of these enzymes, that are suitable for human consumption, will significantly narrow down the search for chemotherapeutic treatment for asthenospermia.

We tried to draw the percentage motilities into groups so as to study the required minimum percentage motility at which fertilization is most likely. In this study, striking things were revealed; (a) that even non-motile sperm cells still have residual enzyme activities, (b) that with exception of Ca^{2+}- ATPase, there were no significant differences ($p>0.05$) between the activities of the enzymes of sperm cells in 1-30% motility group and those in 31 – 60% motility group, and (c) that the differences between the activities of the enzymes of sperm cells with 6-90% motility and those of other groups are tremendously significant ($p<0.05$). This is in line with the previous study which suggested that a minimum percentage motility of 60% and good linear progression is required for the sperm cells to be able to penetrate the zona pellucida of oocytes. We therefore suggest that in asthenospermia, effort should be geared towards increasing the activities of these enzymes to 60% and above. Finally, our results also agree with the results obtained by Das et al [20] who established positive correlation between the activities of other phosphatases (acid and alkaline phosphatases) in human seminal fluid and spermatozoal motility. With these results obtained, we advocate that more work be done to elucidate other biochemical causes of asthenospermia and more activators of these enzymes. These will go a long way in solving some cases of infertility resulting from asthenospermia through the identification of a myriad of enzymes, receptors and metabolic pathways as targets for chemotherapy. This is because modern drug discovery relies more on purified enzymes or other molecular targets [21] such as these than it does with random sreening.

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References

1. Greenberg SH, ipshultz LI, Wein A. Experience with 425 subfertile male patients. J Urology 1978; 119: 507 -573.
2. Macleod J, Gold R. The male factor in fertility and infertility. Fertility and infertility 1953; 4: 10-19.
3. McConnel JO. Abnormalities in sperm motility: Techniques of evaluation and treatment. In, Infertility in the male. LI Lipshultz and S.S Howards eds. Mosby Year Book, St. Louis 1991; pp 254 – 277.
4. Tash J, Means A. Cyclic adenosine 3,5-monophosphate, calcium and protein phosphorylation in flagella motility. Biol. Reprod. 1983; 29: 75-104.
5. Sosa A, Calzada L, Alva S. Gonzalez-Angelo A. Distribution of ATPases in isolated human spermatozoa nuclei. Mex Int J Fert 1978; 24: 125-129.
6. Lowry HO, Rosebrough NJ, Farr AA, Randell RJ. Protein measurement with Folin-phenol reagent. J Biol Chem 1951; 193: 265-267.
7. Bonting SL. Sodium-potassium activated adenosine triphosphatase and cation transport. In, Membrane and ion transport. Bitter EE eds. Wiley Interscience, New York 1970; pp 257-363.
8. Takeo S, Fliegel L, Beamish RE, Dhalla NS. Effects of adrenochrome on rat heart sarcolemina ATPase activities. BioChem Pharmacol 1980; 29, 559-564.
9. Fiske CH, Subbarrow Y. The colorimetric determination of phosphorus. J Biol Chem 1925; 60: 275-400.
10. Gibbsons IR. Mechanism of flagella motility. In, The functional anatomy of the spermatozoa. Permagon press, New York 1975; pp 127-140.
11. Gibbsons IR. Dynein ATPase as microtubule motors. Minireview. J Biol Chem 1988; 263: 15837-15840.
12. Pietrebon D; Virgilo FD, Puzzan T. Structural and functional aspect of calcium homeostasis in eukaryotic cells. Euro J Biochem 1990; 193: 599-672.
13. Stegmayr B, Berggren PO, Ronquist G, Hellman B. Calcium. Magnesium and zinc contents in organelles of prostatic origin in human seminal plasma. Scand J Urol Nephrol 1982; 16: 199- 203.
14. Anand RJ, Kanwar U, Sanyel SN. Calcium channel antagonist- verapamil, modulates human spermatozoal functions. Res Exp Med Berl 1994; 194: 165-178.

15. Ashizawa K, Kamiya T, Tamura I,Tsuzuki Y. Inhibition of flagella motility of fowl spermatozoa by L– carnitine: its relationship with respiration and phosphorylation of axonemal proteins. Mol Reprod Dev 1994; 38: 318-125.

16. Parke HS; Kelly JM, Milligan LP. Energetics and cell membranes. In, Energy metabolism tissue determinants and cellular corollaries. JM Kinney and HN Tucker eds. Raven Press, New York 1992; pp 411-437.

17. Saito K; Kinoshita Y, Yumura Y; Iwasaki A, Hosaka H. Effect of extracellular ions on the reactivation of human spermatozoa preserved in electrolyte-free solution. Andrologia 1999; 31: 211-215.

18. Dreanno C, Cosson J, Suquet M, Seguin F, Dorange G, Billard R. Nucleotide content, oxidative phosphorylation, morphology and fertilizing capacity of turbot (Psetta maxima) spermatozoa during the motility period. Mol Reprod Dev 1999; 53: 230-243.

19. Romac P, Zanic-Grubisic T, Culic O, Ctitkovic P, Flogel M. Sperm motility and kinetics of dynein ATPase in astheno- and normozoospermic samples after stimulation with adenosine and its analogues. Human Reprod 1994; 9: 1474-1478.

20. Das RP, Roy S, Podder AK. Relation of phosphatases in human semen to count and motility. Ind J Med Research 1975; 63: 1323-1326.

21. Pink R. Molecular targets for anti-parasite drug screens. TDR News 2001; 65, pp 7.

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The involvement of nitric oxide in morphine-induced straub tail in mice

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Key words: Nitric oxide, straub tail response, cyclosporin A, L-NAME, mice

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Abstract

Many central neurotransmitter systems, such as dopaminergic, adrenergic and serotonergic are reported to be involved in the morphine-induced straub tail response (STR) in mice. Niric oxide (NO) is a potent mediator in the central and peripheral nervous system, which is involved in many aspects of opioid system. The object of this study was to clarify the role of nitrergic system in this phenomenon. Cyclosporin A (CsA), an inhibitor of catalytic activity of neuronal nitric oxide synthase(nNOS), and L-nitro-arginine-methyl ester (L-NAME), a non-selective nitric oxide inhibitor, both increase the morphine-induced straub tail when used alone, but co-administration of CsA and L-NAME decrease this phenomenon. In conclusion, these results may suggest the probable involvement of nitrergic system on the morphine-induced straub tail.

Introduction

Erection of mouse tail, a typical phenomenon induced by morphine, is a manifestation of morphine's central stimulation action, to end in spasm of the anal sphincter and sacrococcygeus muscle. Morphine-induced straub tail reaction (STR) is one of the main determinations of testing opioids activity [1]. Many central nervous neurotransmitter systems, such as dopaminergic [2,3] adrenergic and serotonergic [4] are reported to be involved in this phenomenon.

Nitric oxide (NO) is a potent mediator in the central nervous system and peripheral nervous system, which is involved in many aspects of opioid system. NO is suggested to play a role in pain perception [5-8] as well as in the modulation of opioid antinociception, tolerance and dependence [9-13]. Inhibitors of nitiric oxide synthase (NOS), have been shown to prevent or diminish the development of tolerance to opioid [11,14], and signs of withdrawal syndrome [13,14]. But, there has been no previous demonstration of nitric oxide involvement in morphine-induced straub tail reaction. In the present study we assessed the effect of a non-specific NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME) in morphine-induced STR in mice. Also our previous studies have shown that cyclosporin A (CsA), as a neuroimmunophilin ligand, can attenuate the development and expression of tolerance to and dependence on morphine in mice by decreasing NO production [17]. Cyclosporin A reduces the catalytic activity of neuronal nitric oxide synthase (nNOS), through inhibition the phosphatase activity of calcineurin [18-21]. These effects of CsA are in parallel with those of nitric oxide inhibitor, L-NAME. Thus, in this study we used CsA alone and along with L-NAME to clarify the role of immunophilin-calcineurin related pathways in morphine-induced STR, as an important phenomenon for assaying the acute action of morphine.

Materials and Experimental design

CsA (Sandimmune Sandos, Switzerland), L-NAME (Fluka, Switzerland) and morphine sulfate (Sigma, U.K.), were dissolved in 0.9% physiological saline, immediately before use. Male Swiss mice weighting 20 to 25g (5-6 weeks old) were used. Animals were housed six per cage in a room maintained at 22±1°C with an alternating 12h light-dark cycle. Animals had access to food and water. All procedures were carried out in accordance with institutional guidelines or laboratory animals care and use. Each treatment group consisted of 8 mice. Animals were divided into 4 main groups as following manner:

1) The animals were injected with CsA (1.25, 2.5, 5, 10 or 20 mg/kg) 30 min before the injection of saline and the straub tail reaction was scored 30 min later.
2) The mice were administered CsA (1.25, 2.5, 5, 10 or 20 mg/kg) 30 min before the injection of morphine (1.25 1.25, 2.5, 5 or 10 mg/kg) and the straub tail reaction was scored 30 min later.
3) The animals were injected with L-NAME (10 mg/kg) 30 min before the injection of saline or morphine (1.25, 2.5 or 5 mg/kg) and the straub tail reaction was scored 30 min later.

4) The mice were trated with CsA (10 mg/kg) and L-NAME (10 mg/kg) 30 min before the injection of morphine (1.25, 2.5 or 5 mg/kg) and the straub tail reaction was scored 30 min later.

Measurement of morphine-induced straub tail reaction

The straub tail reaction was scored 30 min after the i.p. administration of morphine. CsA and L-NAME were administered 30 min before the injection of morphine. The score was graded using a minor modification of the numerical scores of Kameyama [22] as follows: 0=0°, 0.5=1-44°, 1=45°, 1.5= 46-89°, 2=90o, 2.5=91-139°, 3>139°, the angle was measured above the horizontal table plane.

Statistical analysis

Comparisons between groups were done with two-way analysis of variance (ANOVA) followed by the Tukey posthoc. The limit for statistical significance was set at $P<0.05$.

Results

The effect of CsA on STR

CsA (1.25, 2.5, 5, 10, 20 mg/kg) alone did not cause STR, but pretreatment with CsA showed mean score increase in animals given morphine (1.25, 2.5, 5, 10 mg/kg). The effects of these findings were shown in figure 1.

The effect of L-NAME on STR

L-NAME (10 mg/kg) alone did not induce STR but administration of it before morphine increased the mean score of the morphine 1.25 mg/kg (0.5 ± 0.04)($P<0.05$) and 2.5 mg/kg (1.25 ± 0.16)($P<0.01$), significantly (Fig. 2).

The effect of co-administration of CsA and L-NAME on STR

Co-administration of CsA (10 mg/kg) and L-NAME (10 mg/kg) decreased STR induced by i.p. injected of morphine in doses 1.25 (0.43 ± 0.062), 2.5 (1.49 ± 0.012) and 5 mg/kg (1.65 ± 0.081)($P<0.001$), significantly.

Discussion

These data show that the acute administration of morphine produced the straub tail reaction dose dependently, which is in agreement with previous reports [1,3], and CsA caused significant increase in morphine-induced STR. NOS blocking by L-NAME, also, caused significant increase in this phenomenon. But, the concurrent use of CsA and L-NAME diminished morphine-induced STR.

Many central neurotransmitters are involved in STR. However, dopamine has a main role in this phenomenon and it is suggested that STR can be produced by activation of the central dopaminergic sysytem [2]. It is also suggested that concurrent D1/D2 dopamine receptor stimulation is necessary to produce STR in mice [23].

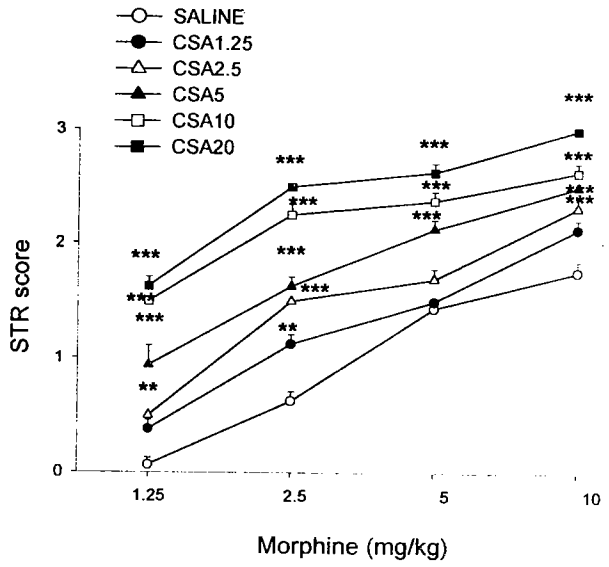

*Fig 1: The effect of CsA on the morphine induced straub tail reaction (STR). Different doses of CsA (1-20 mg/kg, i.p.) was administered separately 30 min before different doses of morphine (1.25 –10 mg/kg). STR was scored 30 min after morphine injection. Data are shown as mean ± S.E.M. *P<0.05, **P<0.01, ***P< 0.001 in comparison with morphine / saline group.*

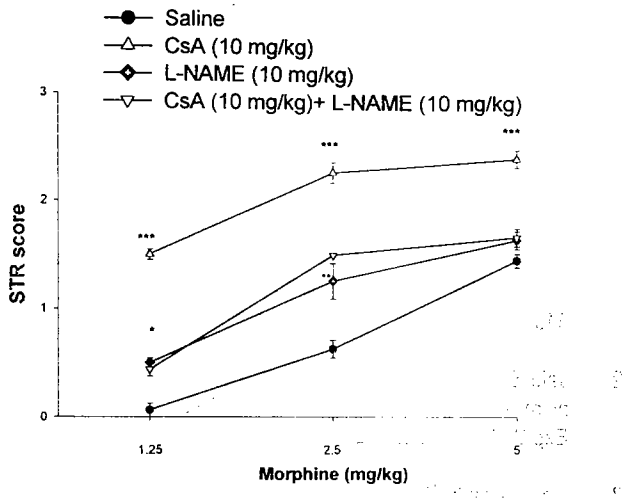

*Fig 2: The effect of L-NAME or concomitant administration of CsA and L-NAME on morphine - induced STR. L-NAME (10 mg/kg, i.p.) or CsA (10 mg/kg, i.p.) was administered 30 min before different doses of morphine (1.25-5 mg/kg, i.p.) and the STR was scored 30 min after morphine injection. In CsA group L-NAME (10 mg/kg, i.p.) was administered 30 min before CsA. Data are shown as means ± SEM. *P<0.05 and **P<0.01 in comparison with morphine/ saline group and ***P<0.001 in comparison with CsA/morphine Group.*

On the other hand, nitroprusside, a NO donor, inhibits the basal and potassium-stimulated release of dopamine [24]. Other results showed that NO plays an inhibitory role in N-methyl-D-aspartate (NMDA)-induced dopamine release in striatum and NOS inhibi-

tors potentiate NMDA-induced release of dopamine in striatum [25].

The immunosuppressive effects of CsA are due to its binding to its protein receptors, immunophilin [26]. Recently, it has been shown that the levels of immunophilin in the CNS are up to 50 times greater than the immune system [27]. Further research revealed that in the nervous system the complex of CsA with its protein receptor binds to and inhibits calcineurin which in turn leads to an increase in phosphorylated levels of nNOS. This in turn reduces the catalytic activity of nNOS and leads to important neuronal changes such as protection against stroke-induced damage and regulation of NO neurotoxicity by CsA and other immunophilin-binding ligands such as tacrolimus [18-20]. The inhibitory effect of nNOS causes increase in dopamine content, so morphine-induced STR is potentiated by CsA. We have reported that acute administration of CsA induced antinociception and anticonvulsant effects, suggesting the involvement of nitrergic system [28,29]. CsA may increase morphine-induced STR through inhibition of NO production. The induction of morphine-induced STR by L-NAME, also, comfirmed the inhibitory effect of NO in STR phenomenon.

It has been reported that NO may mediate both excitatory and inhibitory functions, according to the level of NO production in vivo [30,31]. There are evidences, suggesting that NO may have neurotoxic and neurprotective and also proconvulsant and anticonvulsant effects [30] Decreasing the morphine-induced STR with the concurrent use of CsA and L-NAME is in agreement with these reports. It seems that the different effects of CsA and L-NAME when used alone or together are dependent on the concentration of NO. In conclusion, our results suggest the probable involvement of nitrergic system on the morphine-induced STR.

References

1. Bilbey DL, Salem H, Grossman MH. The anatomical basis of the straub tail phenomemenon. Br J Pharmacol 1960; 15: 540.
2. Jaju BP, Strivastava YP. A study of dopaminergic mecha nism in morphine-induced straub tail response. Indian J Exp Biol 1984; 22: 242-430.
3. Hasegawa Y, Kurach M, Biggs SX. Dopamine D2 receptors and spinal cord examinaton in mice. Eur J Pharmacol 1990; 84: 207-212.
4. Kameyama T, Ukai M, Nabesshima T. Effects of various aminoacids and their related agents in association with neurotransmitter substances on morphine-induced straub tail reaction in mice. Chem Pharm Bull 1978; 26: 770-773.
5. Meller ST, Gebhart GF. Nitric oxide (NO) nociceptive processing in the spinal cord. Pain 1993; 52: 127-136.
6. Sousa AM, Prado WA. The dual effect of a nitric oxide donor in nociception. Brain Res 2001; 897: 9-19.
7. Machleska HD, Labuz D, Przewlosci R, Przewlcka B. Inhibition of nitric oxide synthase enhances antinociception mediated by Mu, Delta, Kapa opioid receptors in acute and prolonged pain in rat spinal cord. G Pharmacol Exp Ther 1997; 282: 977-984.
8. Duarte IDG, Ferreirl SH. The molecular mechanism of central antinociception induced by morphine or carbachol and the L-arginine-nitric oxide-cGMP pathway. Eur J Pharmacol 1992; 221: 171-174.
9. Kolesnikov YA, Pick CG, Pasternak GW. NG-nitro-L-arginine prevents morphine tolerance. Eur J Pharmacol 1992; 221, 399-400.
10. Kolesnikov YA, Pick CG, Ciszewska G, Pasternak GW. Blockade of tolerance to morphine but not to κ-opioids by a nitric oxide synthase inhibitor. Proc Natl Acad Sci USA 1993; 90, 5162-5166.
11. Adams ML, Kalicki JM, Meyer ER, Cicero TG. Inhibition of morphine withdrawal syndrome by a nitric oxide synthase inhibitor, NG –nitro-L-arginine methyl ester. Life Science 1993; 52: PL245-249.
12. Babey AM, Kolesnikov YA, Cheng J, Inturrisi CE, Trifilletti RR, Pasternak GW. Nitric oxide and opioid tolerance. Neuropharmacology 1994; 33: 1463-1470.
13. Dambisya YM, Lee TL. Role of nitric oxide in the induction and expression of morphine tolerance and dependence in mice. Br J Pharmacol 1996; 117: 914-918.
14. Kimes AS, Vaupel DB, London ED. Attenuation of signs of opioid withdrawal by inhibitors of nitric oxide synthase. Psychopharmacol 1993; 112: 521-524.
15. Majeed NH, Przelocka B, Machelska H, Przewlocki R. Inhibition of nitric oxide synthase attenuates the development of morphine tolerant and dependence in mice. Neuropharmacol 1994; 33: 189-192.
16. Highfiled DA, Grant S. NG–nitro-L-arginine, a NOS inhibitor, reduces tolerance to morphine in the rat locus coeruleus. Synapse 1998; 29: 233-239.
17. Homayoun H, Khavandegar S, Namiranian Kh, Dehpour AR. The effect of cyclosporin A on morphine tolerance and dependence: involvement of L-arginine/nitric oxide pathway. Eur J Pharmacol 2002; 452: 67-75.
18. Dawson TM, Steiner JP, Dawson VL, Dinerman JL, Uhl GR, Snyder SH. Immunosuppresant FK506 enhances phosphorylation of nitric oxide synthase and protect against glutamate neurotoxicity. Proc Natl Acad Sci 1993; 90: 9808-9812.
19. Sharkey J, Butcher SP. Immunophilins mediate the immunoprotective effects of FK506 in focal cerebral ischemia. Nature 1994; 371: 336-339.

20. Rao MR, Kanji VK, Salahudden MK, Wang C, Sekhar V. Inhibition of rat brain niric oxide synthase activiy by cyclosporin A. Indian J Exp Biol 1996; 34: 1265-1267.

21. Snyder SH, Sabatini DN, Lai MM, Steiner JP, Hamilton GS, Suzdik PD. Neural actions of immunophilin ligands. Trends Phamacol Sci 1998; 19: 21-26.

22. Kameyama T, Nabeshima T, Ukai T, Yamaguch K. Morphine induced straub tail reaction and spinal catecholamine metabolite content: Antagonism of naloxone to morphine-induced effects in mice. Chem Pharm Bull 1979; 27: 1063-1067.

23. Zarrindast MR, Bayat A, Shafaghi A. Involvement of dopaminergic subtypes in straub tail behavior in mice. Gen Pharmacol 1993; 24: 127-130.

24. Selicovich A, Lasaga M, Befumo M, Duvilanski BH, Diaz MBC, Rettori V, McCann SM. Nitric oxide inhibits the release of norepinephrine and dopamine from medial basal hypothalamus of the rat. Proc Natl Acad Sci 1995; 92: 1299-1302.

25. Segovia G, Mora F. Role of nitric oxide in modulating the release of dopamine, glutamate and GABA in striatum of the freely moving rat. Brain Res Bull 1998; 4: 275-279.

26. Schreiber SL, Crabtree GR. The mechanism of cyclosprine A and FK506. Immunol Today 1992; 13: 136-142.

27. Steiner JP, Dawson DM, Fotuhi M, Glatt CE, Snowman AM, Cohen N, Snyder SH. High brain densities of immunophilin FKBP colocalized with calcineurin. Nature 1992; 358: 584-587.

28. Homayoun H, Babaie A, Gharib B, Etminani A, Khavandgar S, Mani AR, Dehpour AR. The involvement of nitric oxide in the antinociception induced by cyclosporin A in mice. Pharmcol Biochem and Behav 2002a; 72: 267-272.

29. Homayoun H, Khavandegar S, Dehpour AR. Anticonvulsant effects of cyclosporin A on pentylentetrazole-induced seizure and kindling: modulation by nitric oxide system. Brain Res 2002b; 939: 1-10.

30. Segieth J, Getting SJ, Biggs CS, Whitton PS. Nitric oxide regulates excitatory amino acid release in a biphasic manner in freely moving rats. Neurosci Lett 1995; 20 : 101-104.

31. Getting SJ, Ahmad S, Biggs CS, Whitton PS. Biphasic modulation of GABA release by nitric oxide in the hippocampus of freely moving rats in vivo. Brain Res 1996; 22: 196-199.

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Rhegmatogenous retinal detachment: Protein in the subretinal fluid

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Abstract

The subretinal fluid of 26 patients subjected to *wet* retinal detachment repair surgery was analysed for total protein concentration and prominent (high molecular weight, medium molecular weight, low molecular weight) protein fractions by gel electrophoresis. The results of analysis of proteins in subretinal fluid were correlated with age, sex and phakic status of patients, duration and extent of retinal detachment, preoperative intraocular pressure, stage of proliferative vitreoretinopathy, and type and location of retinal break/s. The total protein concentration was higher in patients with total retinal detachment, longer duration of retinal detachment, lower preoperative intraocular pressure, and late stage of proliferative vitreoretinopathy and round breaks. Additionally the percentage of high molecular protein fraction was higher in total retinal detachment of longer duration and late stages of proliferative vitreoretinopathy. Thus, subretinal fluid can be considered as a window to retinal detachment pathophysiology since its chemical composition varies in relation to the causative factors and the progression of retinal detachment.

Introduction

Subretinal fluid accumulating between the pigment epithelial layer and the neurosensory retina is presumably derived from degenerated vitreous, pigment epithelium and choriocapillaris in rhegmatogenous type of retinal detachment [1,2,3].

Biochemical analysis of the subretinal fluid has shown the presence of proteins, lipids, carbohydrates, their metabolic products and trace elements [4,5,6].

Chemical composition of the subretinal fluid may, therefore, vary with chemical composition of the vitreous, type, size and duration of retinal detachment, size of retinal break/s, and permeability levels of choriocapillaris.

The present study is designed to correlate findings of gel electrophoresis of subretinal fluid with various clinical parameters of rhegmatogenous retinal detachment. The parameters include age, sex and phakic status of the patients, duration and extent of retinal detachment, preoperative intraocular pressure (IOP), grade of proliferative vitreoretinopathy (PVR), and type and location of retinal break/s.

Materials and Methods

This prospective study was carried on 26 eyes of 26 patients undergoing *wet* retinal detachment repair surgery from January 1998 –December 2001 at the AMU Institute of Ophthalmology, Aligarh. This included 20 male and 6 female patients. Age of the patients varied between 08 – 70 years (mean 40.6 $\pm$ 9.7 years). Duration of illness varied between 21 days to 34 months (mean 6.2 $\pm$ 4.7 months). Right eye was affected in 15 cases while left eye was involved in 11 individuals. Nine patients had retinal detachment of up to two months whereas seventeen cases had retinal detachment for more than two months. Preoperative intraocular pressure (IOP) was recorded below 10 mm of Hg in eight cases. Preoperative IOP was higher than 10 mm of Hg in 18 cases. Aphakia was noticed in 9 cases while 17 cases did not show any evidence of cataract surgery.

The retinal detachment was total (in extent) in seventeen cases and subtotal in nine patients. On indirect ophthalmoscopy, round holes in 18 eyes, horseshoe tears in 5 eyes and giant retinal tears in two eyes were observed. In one eye the retinal break/s could not be visualised. In accordance with proliferative vitreoretinopathy (PVR) classification (The Retina Society Terminology Committee, 1983) [7], seven patients had grade A, four patients had grade B, nine patients had grade C and six patients had grade D, proliferative vitreoretinopathy (PVR) changes. Due to smaller number of cases in the present study the patients of grade A and grade B proliferative vitreoretinopathy were grouped together as early PVR cases while patients of PVR grade C and D were grouped as late PVR cases.

During the course of retinal detachment repair surgery, subretinal fluid was collected through a sclerotomy using a 26 G needle

after careful heat coagulation of the sclera and the choroid to avoid blood contamination. The samples were kept in a deep freezer with the temperature maintained at –20°C and analysed within a fortnight.

Estimation of protein concentration in SRF was done by method described by Lowry et al [8]. Polyacrylamide gel electrophoresis was performed by the method described by Laemmelli et al [9] using a slab gel vertical electrophoresis system. On completion of electrophoresis, samples were stained with coomasie brilliant blue dye. The electrophoresed stained gels having an array of protein bands were scanned on a DU-640 spectrophotometer equipped with a densitometer at a fixed wavelength of 550 n.m. and the percentage of prominent high, medium and low molecular weight protein fractions were determined. The selected prominent protein fractions were; a high molecular weight (mol. wt.) protein fraction with molecular weight above 1,50,000; a medium mol. wt. protein fraction with molecular weight around 90,000; and a low mol. wt. protein fraction with molecular weight around 60,000. One of the samples was electrophoresed with protein molecular marker for identification of different protein bands (Fig.1). The correlation between various parameters of retinal detachment *vis- a - vis* total protein concentration and percentages of prominent protein fractions were analysed using Students- t - test.

Observation

The results of biochemical analysis of subretinal fluid performed in the present study viz. total protein concentration; and concentrations of prominent high molecular weight, medium molecular weight and low molecular weight protein fractions showed no statistically significant difference with the difference in age, sex and phakic status of the patients.

The results of analysis of proteins in SRF in patients with retinal detachment of up to two months and more than two months duration, is shown in Table 1. The mean total protein concentrations in RD of up to two months and more than two months duration were 28.4 mg/ml and 63.5 mg/ml respectively. This difference in total protein concentrations is statistically significant (p value less than 0.05). On gel electrophoresis, the high molecular weight protein fractions were 8% and 13% in RD of less than 2 months and more than 2 months duration respectively. This difference in high molecular weight protein fractions was statistically significant (p value less than 0.1) whereas the differences in medium and low molecular weight protein fractions of the two groups having different duration of RD were not.

Table 1: Proteins in SRF in patients with retinal detachment of up to two-month and more than two month duration

Duration of retinal detachment (No. of cases)	Results of analysis of proteins in SRF				
	Statistical values	Total protein concentration (mg/ml)	High molecular weight protein fraction (%)	Medium molecular weight protein fraction (%)	Low molecular weight protein fraction (%)
Less than or equal to 2 months (9 cases)	Mean	28.4± 9.1	08± 4.2	09± 1.2	69± 5.7
	Range	11.6 – 46.3	03 - 30	08 - 12	50 - 80
More than 2 months (17 cases)	Mean	63.5 ± 17.1	13± 4.4	10± 1.9	66± 6.2
	Range	7.0 – 112.4	02-30	08-12	50-80

Table 2: Proteins in SRF of eyes with subtotal and total retinal detachment

Extent of retinal detachment (No. of cases)	Results of analysis of proteins in SRF				
	Statistical values	Total protein concentration (mg/ml)	High molecular weight protein fraction (%)	Medium molecular weight protein fraction (%)	Low molecular weight protein fraction (%)
Subtotal (9 cases)	Mean	39± 9.2	06± 2.9	09± 2.2	70± 6.7
	Range	07 - 84	02 - 10	08 - 12	60 - 80
Total (17 cases)	Mean	57.7± 14.7	13± 4.6	10± 1.1	65± 8.2
	Range	11.5 - 112.4	03 - 30	08 - 12	50 - 80

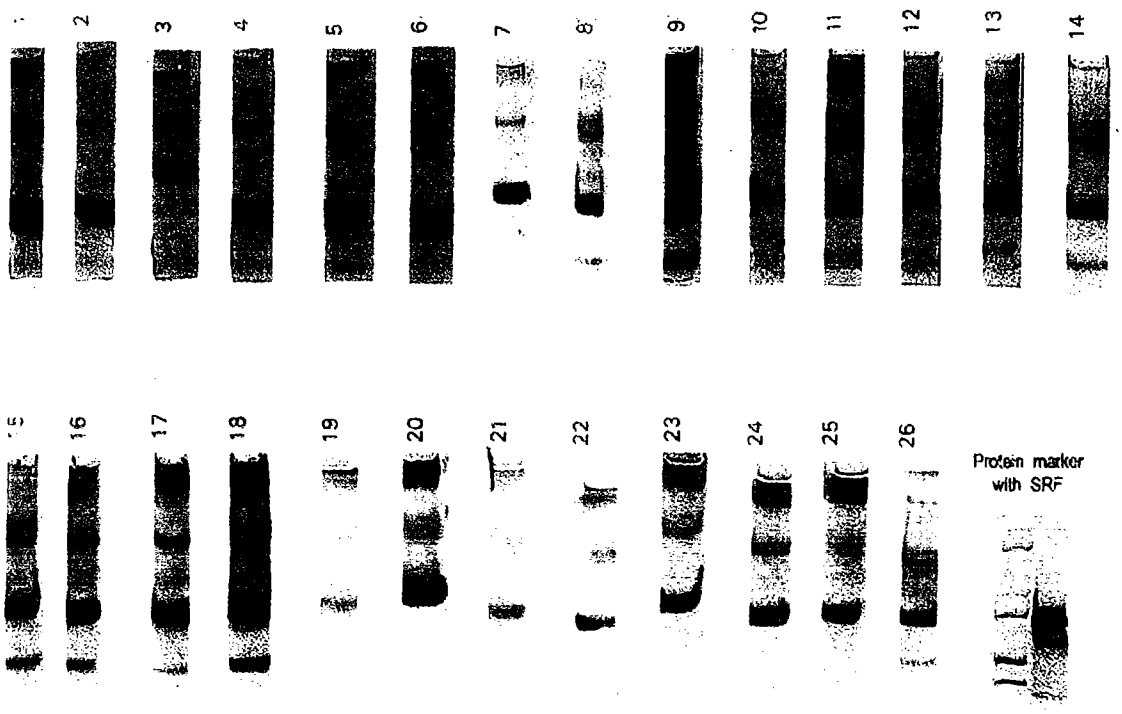

Fig. 1: Photograph showing electrophoresis pattern of different protein fractions in SRF of all the 26 studied cases.

The results of analysis of SRF proteins in eyes with subtotal and total retinal detachment is shown in table 2. The mean high molecular weight protein fractions were significantly higher (p value less than 0.05) in total retinal detachment whereas low molecular weight protein fractions were highly significantly higher in subtotal RD (p value less than 0.01). The differences in mean total protein concentration and mean medium molecular weight protein fractions of subtotal and total retinal detachment were statistically insignificant.

The results of analysis of proteins in SRF in patients with preoperative intraocular pressure up to 10 mm of Hg and more than 10 mm of Hg, is shown in Table 3. The mean total protein concentration in SRF of patients with IOP less than 10 mm of Hg was 76 mg/ ml (46.3 – 112.7) while mean total proteins in patients with IOP more than 10 mm of Hg was 44.6 (range 07 – 111). The higher concentration of proteins in the patients of retinal detachment with preoperative IOP less than 10 mm of Hg was statistically significant (p value less than 0.05) unlike the differences in various prominent (high molecular weight, medium molecular weight and low molecular weight) protein fractions. The results of analysis of proteins in SRF in the patients with early stages (grade A and B) of PVR changes and late stages (grade C and D) of PVR changes, is shown in Table 4. The mean total protein concentration in late stages of PVR was significantly higher (p value less than 0.05) than the mean total protein concentration in early stages of PVR. The mean low molecular weight protein fraction was significantly higher in early stages of PVR (p value less than 0.05). There were no statistically significant differences between mean concentration of medium and low molecular weight protein fractions in early and late stages of PVR.

The results of analysis of SRF proteins in eyes with different types of retinal break/s are shown in Table 5. The mean total protein concentration in SRF associated with round hole/s and horseshoe tear/s was 49.6± 13.6 mg/ml (range 07 –111.0) and 32.5± 3.3 mg/ml (range 18.0 – 42.9). This higher concentration of mean total proteins in SRF associated with round holes is statistically significant (p value less than 0.1). The mean total protein concentration was (highest) 73.4 mg/ml of SRF, in RD associated with giant tear/s. However statistical comparisons were not possible due to small (only two) number of cases. There was no statistically significant difference in the mean fractionated protein percentages with respect to type of retinal breaks.

The results of analysis of SRF proteins in cases with different locations of retinal breaks are shown in Table 6. There were no statistically significant differences in the mean total protein concentration and mean fractionated protein percentages with regards to the location of retinal breaks.

Table 3: Proteins in SRF in the patients with preoperative intraocular pressure up to 10mmof Hg and more than 10 mm of Hg

Preoperative intraocular pressure (No. of cases)	Results of analysis of proteins in SRF				
	Statistical values	Total protein concentration (mg/ml)	High molecular weight-protein fraction (%)	Medium molecular weight protein fraction (%)	Low molecular weight protein fraction (%)
Less than or equal to 10mm of Hg (8 cases)	Mean	76.1± 18.6	10± 2.9	10± 2.7	68± 3.8
	Range	46.3-112.7	07-15	08-12	60-75
More than 10 mm of Hg (18 cases)	Mean	44.6± 19.6	11± 2.8	9± 2.6	67± 4.2
	Range	07-111.0	02-30	08-12	50-80

Table 4: Proteins in SRF in different stages of proliferative vitreoretinopathy.

Stages of PVR (No. of cases)	Results of analysis of proteins in SRF				
	Statistical values	Total protein concentration (mg/ml)	High molecular weight protein fraction (%)	Medium molecular weight protein fraction (%)	Low molecular weight protein fraction (%)
Early (11 cases)	Mean	34.3 ± 9.9	5 ± 2.7	10± 2.7	72± 8.4
	Range	07-84	02-12	08-12	65-80
Late (15 cases)	Mean	62.6± 14.7	12± 4.2	10± 1.6	66± 9.9
	Range	11.6 -112.4	04 - 30	08 - 12	50 - 80

Table 5: Proteins in SRF of eyes with different types of retinal break/s.

Type of retinal break/s (No. of cases)	Results of analysis of proteins in SRF				
	Statistical values	**Total protein concentration** (mg/ml)	**High molecular weight protein fraction** (%)	**Medium molecular weight protein fraction** (%)	**Low molecular weight protein fraction** (%)
Round hole/s (18 cases)	Mean	49.6± 13.6	09± 4.2	09± 1.1	69± 7.2
	Range	11.6 - 111	02 - 25	08 – 12	50 –80
Horse shoe tear/s (5cases)	Mean	32.4± 3.3	13±2.6	10±1.2	64± 4.1
	Range	28 - 42.9	06 - 30	08 – 12	50 - 75
Giant tear/s (2 cases)	Mean	73.4± 4.0	10± 1.0	11± 1.0	67.5± 2.5
	Range	69.4 – 77.4	09 - 11	10 – 12	65 - 70

Table 6: SRF proteins in cases with different location of retinal break(s).

Location of retinal breaks (No. of cases)	Statistical values	Results of analysis of proteins in SRF: Total protein concentration (mg/ml)	High molecular weight protein fraction (%)	Medium molecular weight protein fraction (%)	Low molecular weight protein fraction (%)
Post - equatorial (9 cases)	Mean	50.6± 7.7	10± 3.9	10± 1.1	69± 4.4
	Range	11.5 - 111	04 - 30	08 - 12	55-80
Equatorial (11 cases)	Mean	42.4± 12.7	08± 4.7	09± 1.2	71± 6.3
	Range	07 - 84	03 - 25	08 -12	50 - 80
Pre – equatorial (5 cases)	Mean	64.1± 9.8	12± 4.3	10± 1.2	65± 3.7
	Range	36.9 - 88.3	04 - 20	08 - 12	55 - 75

Discussion

The sub retinal fluid has been the area of interest with many researchers as reported by Weber et al and Dorellu [3,10]. In the present investigation (Table 1) it was found that the total protein concentration was higher in retinal detachments of longer duration. In addition the high molecular weight protein fraction also increased with the duration of detachment. This can be explained by the fact that permeability of choroidal vessels increased with increase in duration of detachment. However, Smith et al, 1960 [11] did not find any significant difference in various fractions in relation to the duration of detachment.

Another interesting observation made in the present study was that eyes with low pre operative intraocular pressure (i.e. less than 10 mm of Hg.) had a significantly high total protein concentration (p value less than0.05) without any significant change in the proportion of the fractions. This may be related the reabsorption of the water from the subretinal fluid, giving rise to higher concentration of these proteins without altering the ratio of the protein fractions. Alternatively decreased aqueous secretion following the detachment may contribute to the observed increase. Similar observations were also made by Takeuchie et al [12] in experimental retinal detachment.

In the present study (Table 4) total protein concentration and high molecular weight protein fractions were found to increase with increase in stage of PVR. This was probably due to increased choroidal vascular permeability along with reduced pigment epithelial - blood retinal barrier in late stages of PVR. Some of the proteins in the fractions might be trigger proteins that induce the fibrosis. However, since the present study was time bound, isolation and characterisation of such proteins could not be carried out.

Another parameter of clinical interest was the extent of retinal detachment as reported by Weber et al [3]. In the present study (Table 2) there was higher concentration of high molecular weight protein fractions in total retinal detachments whereas low molecular weight proteins were highly significantly elevated in subtotal detachments. Greater breakdown of choriocapillaris subretinal barrier or more extensive degeneration of inter photoreceptor matrix associated with increase in the extent of retinal detachment may contribute to this difference.

The observed high total protein concentration in cases with round holes as compared to the horseshoe tears (Table 5) may be related to greater vitreous liquefaction and vitreous seepage into the sub retinal space in round holes as compared to the horseshoe tears. Significantly large proportion of this difference in protein concentration was probably contributed by the seeping vitreous. The difference in the degree of breakdown of blood retinal barrier in round holes and horseshoe tears was probably similar, as there was no significant difference in concentration of various fractions. This might also be the reason for highest total protein concentration in giant tears among the retinal breaks observed in the present study. Giant retinal tears due to their large size and associated greater vitreous degeneration probably allow much larger amounts of vitreous to seep into the subretinal space as compared to other breaks.

Location of breaks also had no bearing either on the total protein concentrations or prominent protein fractions of SRF (Table 6) suggesting that factors contributing to the variation of protein component of SRF work independent of location of break/s in the eye.

In conclusion, this study substantiates the observation that the sub retinal fluid could be considered as a window to retinal detachment pathophysiology with the constituents varying with the factors causing retinal detachment and its progression. Further, characterisation of individual proteins may enlighten regarding the role played by various factors in the causation and progression of the retinal detachment.

To isolate any prognostic protein markers, which may influence features like proliferative vitreoretinopathy and consequently the surgical failure, could be a future project of research.

References

1 Duke Elder WS, Dobree JA. Detachment of retina. In: WS Duke Elder, System of Ophthalmology Vol. X .1967: pp 771-785.

2 Manna F, Meyer J, Mielink I. The morphology of the subretinal fluid in spontaneous retinal detachment. Pd Med J. 1964; 3: 471-474.

3 Weber JC, Wilson FM. Biochemical studies of subretinal fluid. Invest Ophthalmol 1966; 5:323-328.

4 Heath H, Beck TC, Foulds WS. Chemical composition of subretinal fluid. Br. J. Ophthalmol. 1962; 46: 385-396.

5 Feeny L, Burns RP, Mixon MR. Human subretinal fluid, its cellular and subcellular components. Arch. Of Ophthalmol.1975; 93: 62-69.

6 Kaufman PL, Podas SM. The subretinal fluid in primary rhegatogenous retinal detachment. Surv phthalmol 1973; 2: 100-116.

7 The Retina Society Terminology Committee: The classification of retinal detachment with proliferative vitreoretinopathy. Ophthalmology 1983; 90: 121-125.

8 Lowry OH, Rosebrough NJ, Farr AL, Randall AJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-275.

9 Laemmelli UK. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature (London) 1970; 227: 680-685..

10 Dorello U. Electrophoretic research on the protein content of the subretinal fluid in idiopathic detachment of retina. Arch Ophthalmol. 1956; 59: 416-422.

11 Smith JL, Douty E. Electrophoresis of subretinal fluid. Arch. Ophthalmol. 1960; 64: 114-119.

12 Takeuchi A, Kicorian G. et al. The rate and source of albumin entry into saline filled experimental retinal detachment. Invest. Ophthalmol Visc Sci 1994; 35: 3792 - 3798.

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Genetic Molecular Marker Identification from DNA Polymorphism bands and Virulent Genes in *Streptococcus pyogenes* and characterization of isolates

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Key words: RAPD, Genetic marker, *Streptococcus pyogenes*, *emm1* and *speC* gene

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Abstract

The rapid and the correct identification of *S. pyogenes* that causes a variety of Streptococcal infection is necessary to initiate the appropriate antibiotic therapy. A method incorporating DNA amplification using arbitrary primers coupled with DNA marker band identification for the rapid identification of *Streptococcus pyogenes* is described. Random Amplified Polymorphic DNA (RAPD) and Polymerase Chain Reaction (PCR) techniques have been applied on ten isolates of *Streptococcus pyogenes* obtained from Hospital Tengku Ampuan Afzan (HTAA), Kuantan, Malaysia. All the isolates were isolated from the patient's specimens such as pus and blood. Five out of twenty primers screened yielded clear and reproducible bands corresponding to amplified products. Analyses were carried out using RAPDistance Software to generate a dendrogram. The dendrogram revealed that all the isolates from HTAA are close in the genetic relationships and is in accordance to the RAPD banding pattern. The banding patterns of most isolates for most of the primers are similar. Based on the banding patterns, a potential genetic marker band was detected. One band of the size 500 bp was consistently amplified in all isolates using primer OPAE-14 and thus could be selected as the genetic marker for identification of *S. pyogenes*. In the detection of virulent genes, it is found that five out of ten isolates screened carried *emm1* gene and only one isolate carried *speC* gene. The *emm1* gene is the M-protein gene and *speC* gene is the pyrogenic exotoxin C gene. In addition to the molecular characterization, antimicrobial susceptibility properties of most isolates showed high susceptibility to most antibiotics of choice but highly resistant to penicillin G.

Introduction

Streptococcus is a Gram-positive spherical bacterium that occurs in chains or pairs in gram staining. *S. pyogenes*, which is one of the species of Streptococcus, causes a wide range of infections like sinusitis, otitis, mastoiditis, pneumonia, joint or bone infections, myositis, meningitis or endocarditis. The pathogenicity of this organism is mainly associated with their ability to produce pyrogenic exotoxins types A, B and C that is encoded by the gene *spe*. The rapid and the correct identification of *S. pyogenes* is necessary for the appropriate antibiotic therapy. It is common to use phenotypic techniques (conventional methods and an tibiogram) for the characterization of bacteria but is time consuming and also have very limited discriminatory power [1]. The methods that are available currently for identification of *S. pyogenes* are not highly successful as each test has its own drawbacks and is also sometimes tedious. Therefore, to improve upon the rapid and correct identification of *S. pyogenes*, we sought to develop a method utilizing the amplification of *S. pyogenes* using arbitrary primers coupled with detection of the DNA marker. Nowadays, Arbitrarily primed PCR (AP-PCR) is a high-resolution genomic fingerprinting method that can be used to identify and compare microorganism at the genus, species level or even within a species. The successful application of this method to numerous bacterial species and strains [2,3] indicated that the method is very reliable, in addition no prior sequence information of the genomic DNA sequence is needed and also consumes less DNA than other genotyping methods. In addition to the RAPD coupled to DNA marker band, the detection of *emm* 1 gene was also carried out. The *emm* 1 gene encodes the M

surface protein of streptococcal group [4]. The M typing is one of the common typing method, but the method is serologically based [4,5]. The problem faced with this method is the unavailability of antiserum against many types of M protein. Therefore the main aim of this study is to develop a DNA marker for the rapid identification of *S. pyogenes* based on RAPD marker or virulent gene marker. The molecular characterization of 10 isolates of *S.pyogenes* obtained from Hospital Tengku Ampuan Afzan (HTAA), Kuantan, Malaysia by using the Random Amplified Polymorphic DNA was carried out. In addition, the antimicrobial susceptibility of isolates were characterized.

Materials and Methods

Sources of Bacterial isolates

Ten clinical isolates of *Streptococcus pyogenes* were obtained from Hospital Tengku Ampuan Afzan, Kuantan, Malaysia (Table 1). All isolates were reconfirmed as *S. pyogenes* by the colonies on blood agar, bacitracin sensitivity and by biochemical tests.

Table 1. List of isolates used in this study

S. No	Isolates no:	Source of isolate
1	P1	Pus
2	P2	Pus
3	P3	Throat swab
4	P4	Blood
5	P5	Blood
6	P6	Blood
7	P7	Blood
8	P8	Blood
9	P9	Blood
10	P10	Blood

DNA preparation

Few single isolated colonies were inoculated into Luria-Bertanii broth for DNA extraction. Genomic DNA from each isolate was extracted from an overnight culture using the Pure Gene (Bio-Syntech Technologies Inc.) bacterial genomic DNA extraction kit. The purity and quality of the DNA were determined by UV absorption with a UV spectrophotometer. The genomic DNA extracted was subjected to Random amplified Polymerase chain reaction.

RAPD primers

A total of 20 DNA primers were screened. The primers were obtained from Operon 10-mer Kit AE (Operon Technologies Inc.) containing 10-base oligonucleotides primers for use in genetic mapping and DNA fingerprinting.

RAPD finger printing

The amplification reactions were performed in a volume of 25µl containing 18.95µl ultrapure water, 2.5 µl of 10x BST, 0.45 µl $MgCl_2$, 0.5 µl dNTP mix , 15 picomoles of RAPD primer, 100-200ng of template, 1 unit of *Taq* polymerase. Amplification was performed in a DNA thermal cycler (Biometra-TRIO Thermoblock), the cycling parameters were as follows: 1 minute at 95°C (initial denaturation), 35 amplifications cycles of 1 minute at 95°C (second denaturation), 1 minute at 35°C (annealing step) and 2 minute at 72°C (extension) followed by one single extension cycle for 7 minutes at 72°C. PCR products were separated by agarose gel electrophoresis in 1.4% agarose in 1 x TBE, at 65V for 3-4 hours.

RAPD analysis

A data matrix of 1' s and 0' s (presence and absence of RAPD bands, respectively) was recorded and analyzed by RAPDistance software program. Dendrogram was generated from the distances matrices.

Amplification of emm1 and specC

The amplification reactions were performed in a volume of 25µl containing 18.3 µl distilled ultrapure water, 2.5 µl of 10x BST, 0.5 µl $MgCl_2$, 1 µl dNTP mix, 5 picomoles of each forward and reverse primer of either *emm1* or *speC* gene, 100-200ng of genomic DNA sample and 1 unit of *Taq* polymerase. Amplification was carried out in a DNA thermal cycler (Biometra-TRIO Thermoblock). The specific DNA fragments are amplified by PCR using the following cycling conditions: 3 minute at 95°C, 35 amplifications cycles of 15 seconds at 94°C, 15 seconds at 55°C and 20 seconds at 72°C followed by one single extension cycle for 5 minutes at 72°C. PCR products separated by electrophoresis in 1.4% agarose gels in 1 x TBE, at 65V.

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests for all isolates were performed by the disk diffusion method on Muller–Hinton agar. The antimicrobial agents tested in this study are Bacitracin (10 mg), Cefaclor (30), Cefepime (30), Cefixime (5), Cefotaxime (30 mg), Chloramphenicol (10mg), Erythromycin (15mg), Oxytetracycline (30mg), Penicillin G (10mg), Piperacillin (100mg), Tetracycline (30mg), Trimethoprim (25mg). The diameter of the zone of inhibition was measured and compared to the standard table.

Results

RAPD amplification and analysis

All the 10 isolates from HTAA were confirmed as *S. pyogenes* by the conventional methods. Agarose gel electrophoresis of amplified products revealed a range of 14 to 20 bands in each isolate between 250 to 7000 base pairs for each primer. The genetic relationships between the isolates are represented graphically

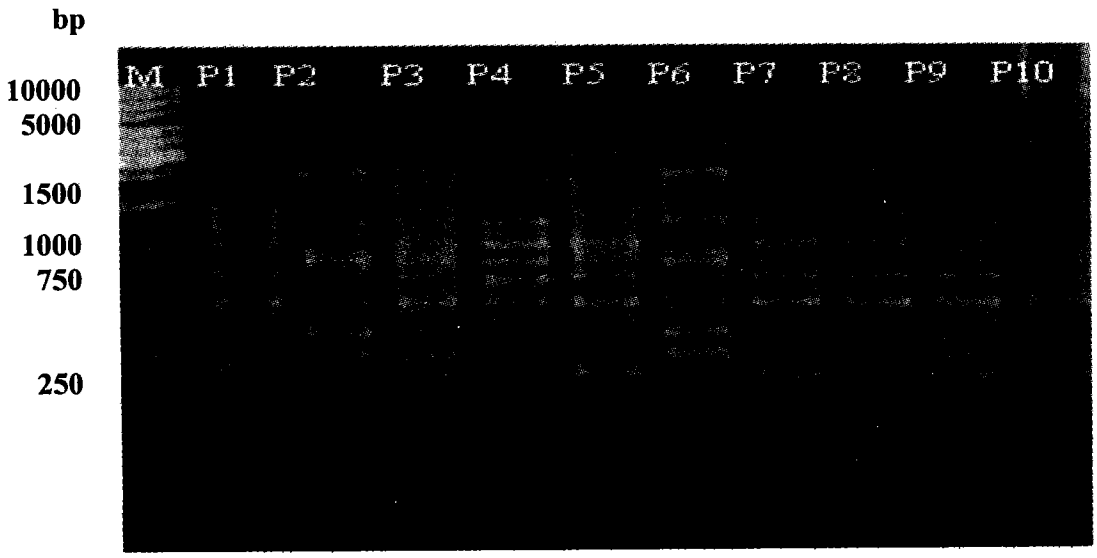

Fig. 1: RAPD genetic profiles of Streptococcus pyogenes isolates obtained with primer OPAE-06. Lane M is the 1kb molecular weight marker, while the rest of the lanes from P1 to P10 are the S. pyogenes isolates used in the genetic profiling.

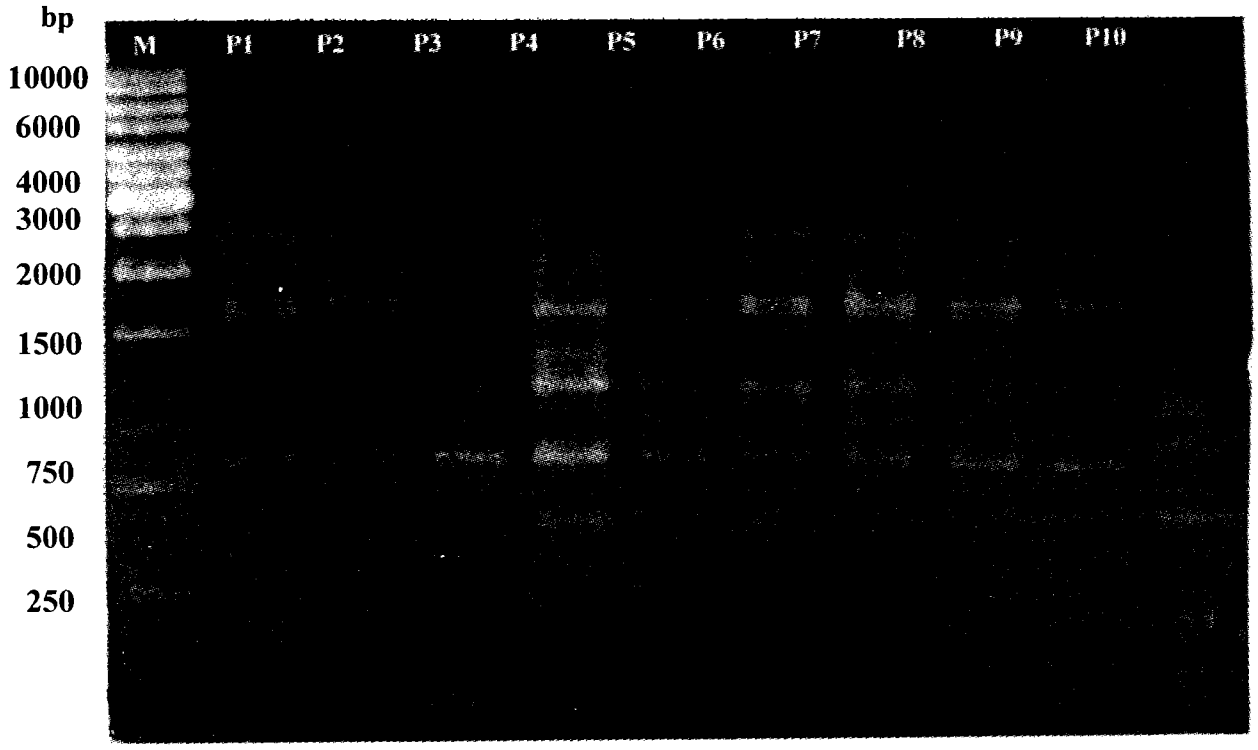

Fig. 2: RAPD genetic profiles of Streptococcus pyogenes isolates obtained with primer OPAE-10. Lane M is the 1kb molecular weight marker, while the rest of the lanes from P1 to P10 are the S. pyogenes isolates used in the genetic profiling.

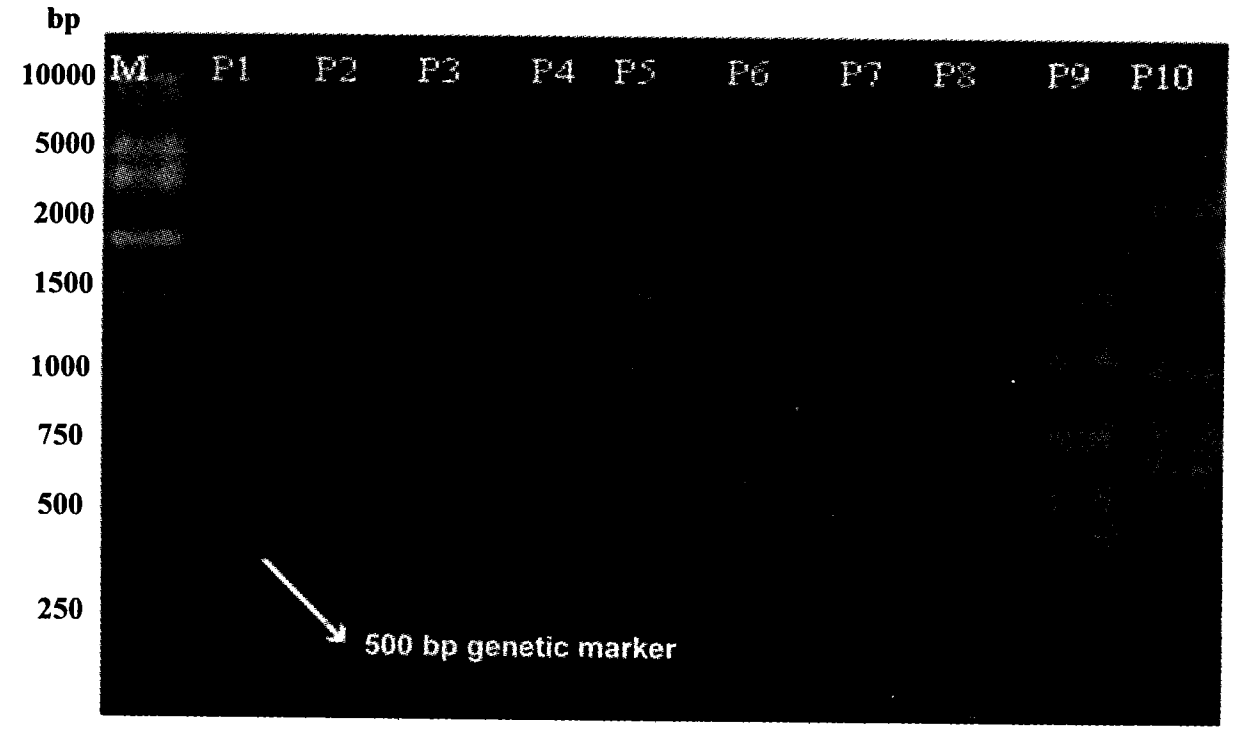

Fig. 3: RAPD genetic profiles of Streptococcus pyogenes isolates obtained with primer OPAE-14. Lane M is the1kb molecular weight marker, while the rest of the lanes from P1 to P10 are the S. pyogenes isolates used in the genetic profiling.

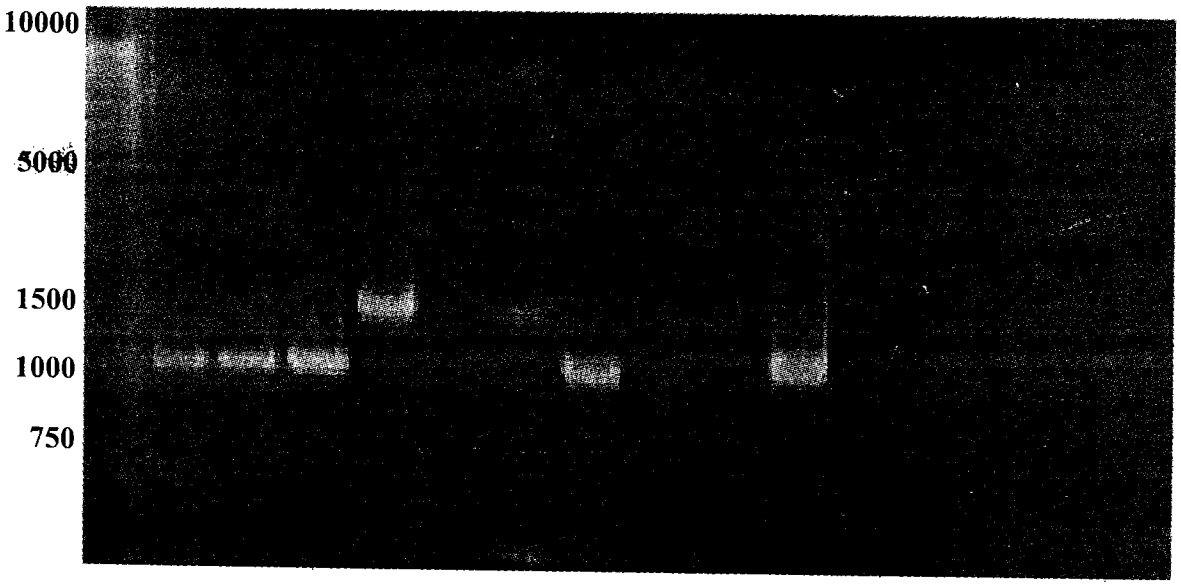

Fig 4: Lane M representing 1 Kb Marker. Lanes 1-10 are Streptococcus pyogenes isolates. Lanes 11-12: Escherichia coli, lanes 13-14: Staphylococcus aureus

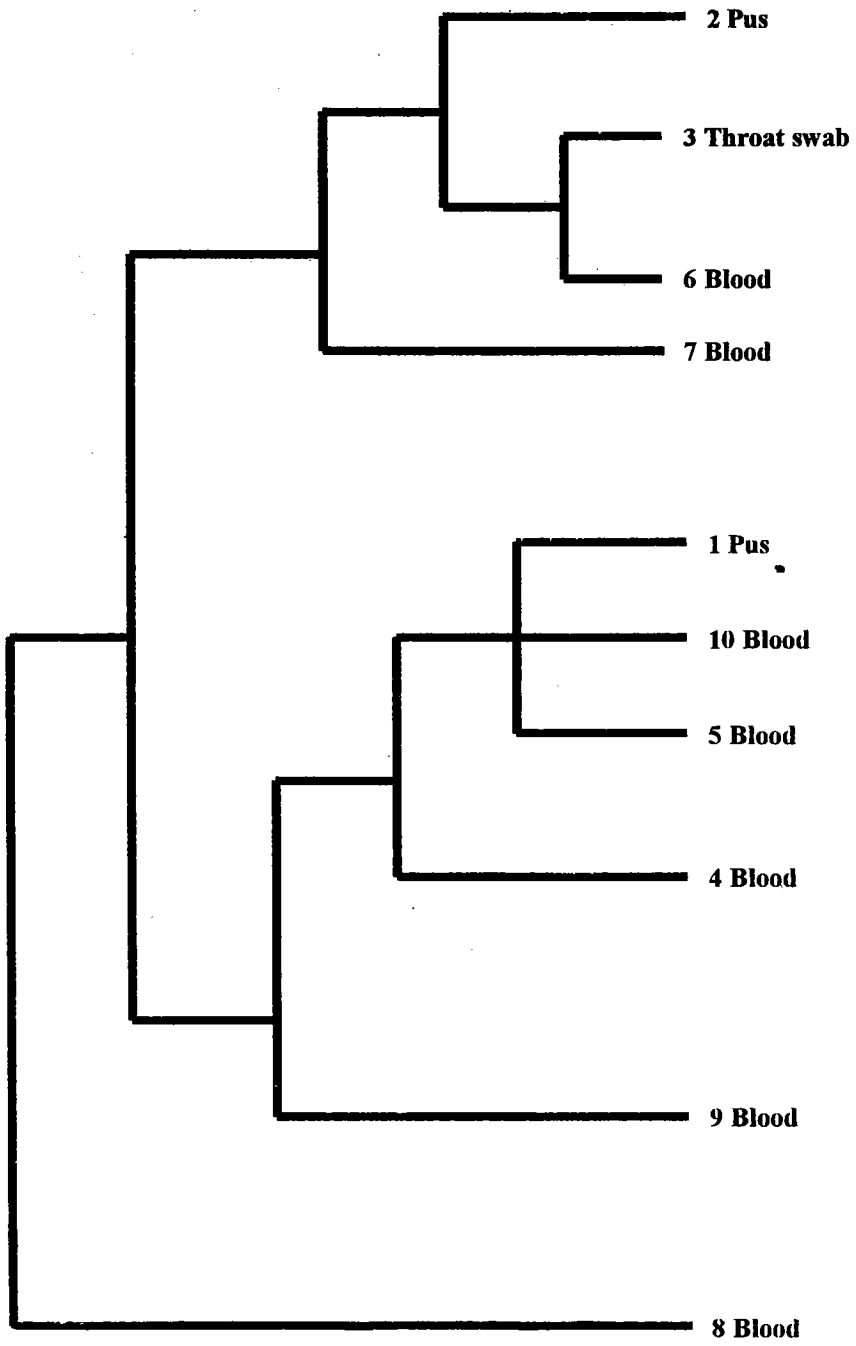

Fig 5: Representing the dendrogram obtained for 10 isolates of Streptococcus pyogenes with 5 RAPD primers

by the dendrogram. Figure 1-3 show the RAPD banding profiles obtained with OPAE-06 and OPAE-10, OPAE-14. Three blood isolates showed similar banding pattern. Primer OPAE-10 produced similar pattern of amplified products for 6 isolates (Figure 2). Three potential genetic marker bands were observed in 9 out of 10 isolates when amplified with primer OPAE-10. Another primer that produced potential genetic marker was primer OPAE-14, where a band with strong signal was observed at position 500 bp (Figure 3).

Amplification of Emm1 and specC genes

Five isolates showed the presence of *emm1* gene with a single band of size 1125bp whereas *speC* gene was amplified in only one isolate producing a band of size 1450bp. Figure 4 shows the presence of *emm1* and *speC* gene in *S. pyogenes* isolates. Other genera like *Esherichia coli* and *Staphylococcus aureus* when tested for the presence of *emm1* and *speC* gene did not amplify any of these genes.

Antimicrobial susceptibility

The antimicrobial properties illustrated that most antibiotics tested are still effective to inhibit growth of *S. pyogenes*. The isolates are 100% resistant to penicillin G and 60% resistant to piperacillin.

Discussion

Amplification of DNA with arbitrary primers that involves the whole genome analysis is a powerful approach for the study of DNA polymorphisms. AP-PCR is widely used for the comparison of genomes from eucaryotes [6] or bacteria [7]. All 10 isolates of *Streptococcus pyogenes* amplified by RAPD technique had allowed the genotyping of the *S. pyogenes* isolates. A total of 5 primers out of 20 primers screened managed to amplify the DNA of *S. pyogenes*, thus producing finger print patterns that could be used to discriminate between isolates from different anatomical sites.

The highest number of bands in one display for each isolate ranged from 14-20, respectively with markers ranging in size from 250-7000 bp in all isolates generated with 5 primers. Primers generated reproducible and interpretable results based on the fact that every primer amplifies DNA through the genome during the RAPD-PCR reaction. The DNA banding pattern showed that 3 blood isolates are genetically very closely related since the pattern were similar when amplified by 3 primers. The blood isolates consistently showed different patterns from the pus or throat swab isolates. Primer OPAE-10 produced similar pattern of amplified products for 7 isolates (P1-P3, P5-P6, P8-P9). Three potential genetic marker bands of sizes, 800 bp, 1200 bp and 1600 bp could be detected, but all the 3 bands were observed in only 9 out of 10 isolates. Another primer that produced potential genetic marker was primer OPAE-14. A band with strong signal was observed at position 500 bp. This band could be better candidate as marker than the previous 3 bands since the signal was much stronger and consistently observed in most of the blood isolates (6 of 7), all pus and throat swab isolates. This genetic marker could be used for the rapid identification of *S. pyogenes*. The identification procedure, which are available so far for *S. pyogenes* are not 100% reliable and also time consuming. Therefore the genetic marker identified in this study will enable the development of DNA marker for the rapid identification of *S. pyogenes*. The dendrogram (Figure 5) generated reflects the banding pattern results in which isolates showing similar patterns are clustered together indicating close genetic relationship.

On the basis of utilizing virulent gene as the genetic marker, this study could not confirm the use of emm1 and *speC* gene since only 5 isolates showed the presence of *emm1* gene producing a single band of size 1125bp whereas *speC* gene was amplified in only one isolate producing a band of size 1450bp Figure 4). The *speC* gene is phage encoded and their presence is restricted to a limited number of strains [8]. Other genera like *Esherichia coli* and *Staphylococcus aureus* when tested for the presence of *emm*1 and *speC* gene did not amplify any of these genes, which indicates the absence of *emm1* and *specC* genes in *E. coli* and *S. aureus*. This data contribute to a better understanding of the local and global dynamics of *Streptococcus pyogenes* populations and the epidemiological aspects of Streptococcal infections occurring in tropical regions as reported by Teixeira and coworkers [5].

Besides the molecular characterization of the isolates, the antimicrobial susceptibility properties of *S. pyogenes* that shared similar banding pattern also showed similar antimicrobial properties. The results obtained in this study for the antibiotic sensitivity test indicated that most antibiotics tested are still effective against *S. pyogenes*. All the isolates were found to be 100% resistant to penicillin G and 60% resistant to piperacillin, which ensures that penicillin G can no longer be used to treat *S. pyogenes* infection.

Genotyping of *S. pyogenes* using RAPD revealed that most of the clinical isolates were genetically similar. This means that this technique can be used to study the clonal relationship between the isolates, in conclusion RAPD analysis provides a practical alternative for genomic typing of Streptococcal species as reported by many researchers [9]. Genetic marker identified from the DNA polymorphism can be applied for the rapid identification of *S. pyogenes*, by further characterization of this genetic marker, then this marker can be used in the clinical laboratory for the rapid and correct identification of *S. pyogenes*. The use of virulent gene as genetic markers cannot be confirmed in this study since among the 10 isolates of *S. pyogenes* from HTAA only one isolates carried *speC* gene, which is isolated from the blood sample, and 5 isolates from pus, throat swab and blood carried *emm1* gene.

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References

1. Martineau, F, Picard FJ, Roy PH *et al.* Species-specific and ubiquitous-DNA based assays for rapid identification of *Staphylococcus aureus*. J Clin Microbiol 1998; 36: 618-623.

2. Ralph D, Mclelland M, Welsh J *et al.* Leptospira species categorized by arbitrarily primed polymerase chain reaction (PCR) and by mapped restriction poymorphisms in PCR-amplified *rRNA* genes. J Bac 1993; 175: 973-981.

3. Neela V, Mariana NS, Somarny WMZ. Application of Randomly Amplified Polymorphic DNA Markers (RAPD) to distinguish isolates of *V. cholerae* and *V. alginolyticus*. Asia-Pacific J Mol Biol Biotech 2000; 8: 131-136.

4. Katsukawa C, Tamaru A, Morikawa Y *et al.* M protein gene (*emm*) typing of *Streptococcus pyogenes*. Kansenshogaku Zasshi (Article in Japanese) 2002; 76: 238-45.

5. Texeira LM, Barros RR, Angela CD *et al.* Genetic and phenotypic features of *Streptococcus pyogenes* strains isoated in Brazil that harbor new *emm* sequences. J Clin Microbiol 2001; 3290-3295

6. Welsh J, Petersen C, McClelland M. Polymorphisms generated by arbitrarily primed PCR in the mouse: applications to strain identification and genetic mapping. Nuc Acid Res 1991; 19: 303-306.

7. Tambic A, Power EG, Talsania M *et al.* Analysis of an outbreak of non-phage-typeable methicillin-resistant *Staphylococcus aureus* by using a randomly amplified polymorphic DNA assay. J Clin Microbiol 1997; 35: 3092-3097.

8. Ellen MM. Margriet J, Schouls LM *et al.*. Invasive and non-invasive Group A streptococcal Isolates with different *speA* alleles in Netherlands: Genetic relatedness and production of pyrogenic exotoxins A and B. J Clin Microbiol 1999; l.37: 3469-3473.

9. Seppala H, He Q, Osterblad M *et al.* Typing of group A *Streptococci* by random amplified polymorphic DNA analysis. J Clin Microbiol1994; 32: 1945-1948.

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Case Report:

Iron accumulation in hepatocellular carcinoma: A histochemical study of 135 cases

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Key words: iron hepatocellular carcinoma, malignant tumour, ferritin.

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Abstract

A liver biopsy study of 135 cases of well established hepatocellular carcinoma was carried out in the University of Nigeria Teaching Hospital (U.N.T.H.) Enugu over 5 years period (1997 – 2002). The level of accumulated stainable tissue iron in the tumour specimens was determined using the Perl's method. The prognostic significance of both age and sex in the patients was also determined. 50 (37.0%) of 135 cases were positive for stainable iron and 85 (63.0%) cases were negative. Five (3.7%) of 135 cases had both cirrhosis and hepatocellular carcinoma. Stainable iron was significantly less frequent ($P<0.05$) in the tumour (HCC). The highest incidence of HCC occurred in the 20-49 years age group with male: Female ratio of 3:2. Age and sex among others can be rated as non-significant variables.

Introduction

Hepatocellular carcinoma which is actually one type of malignant hepatoma is frequently found [1]. It is among the ten most common cancers in the world, and one of the three most prevalent in developing countries. Annual incidence of 1,000,000 with male to female ratio of about 4:1 cases have been estimated to occur each year [2].

The highest incidence of primary carcinoma of the liver has been recorded in tropical countries and in areas where poverty prevails [3]. This is congruent with the findings of Prates and Torres in, 1965 which pointed out that the highest incidence have been reported from Lourenco Marques where the occurrence in the 25 – 36 year-old age group is approximately 500 times that in North America. Notably, hepatoma is the most common malignant neoplasm among the young (15 to 34 years); comprising 24.48% of all cancers in males; and 11.22% in females; followed by leukemia with 20.14% in males and 21.03% in females as is obtainable in China. High incidence has also been reported in the South Africa Bantu, the Yourbas in Western Nigeria [4] and Uganda. Post mortem studies or relative ratio frequencies suggest that it is also very common in Senegal, Ghana [5] the Congo and Papua-New Guinea and Ethiopia, whereas the incidence is low in Egypt.

Hepatocellular carcinoma is considered a highly malignant tumour with a poor prognosis. It is usually not respectable [6,7] and there is no effective treatment [7] However, a slight deviation from this established that the pre-treatment immunological status of cancer patients has been shown to influence their prognosis[8].

Cancer causation and development involves, in part, the generation in the body of reactive compounds leading to the production of hydroxyl radicals, peroxides and similar reactive chemicals capable of damaging cells [9]. The role of iron in promoting the conversion of superoxide and hydrogen peroxide into the highly toxic free hydroxyl radicals is well documented. Increased lipid peroxidation is the most easily measurable effect and is usually regarded as the most significant event in pathogenesis of cellular damage [10].

A more recent work [11] presents evidence of iron being a hepatotoxin, more so, when aggravated or inhibited by a number of coexistent variable possibly leads to hepatocellular carcinoma [12]. A similar work [13] done independently shows that iron-induced DNA damage and inefficient repair in hepatocytes could be related to genotoxicity and most probably to hepatocarcinogenesis. Furthermore, a study on iron toxicity[10] shows that although both increased lysosomal fragility and impaired sar-

colemmal membrane function have been implicated in iron loaded liver cells; the most critical organelle in iron toxicity is the mitochondrion.

Although there is a plethora of literature on primary hepatic carcinoma both in the past [14,15] and present [16,17,18], we are not aware of any previous documented work done on iron accumulation in this type of tumour in this locality, hence the present investigation.

Materials and Methods

Liver biopsies from patients attending clinics and receiving treatment in the University of Nigeria Teaching Hospital (U.N.T.H), Enugu for well over 5 years period (1997 – 2002) were received in fixatives in the department of Morbid Anatomy, and histologically processed. Sections of 5μm thickness were cut from the paraffin-processed blocks and stained by both Haematoxylin and Eosin (H&E) [19] and Perls [20] staining techniques. Stainable iron in the tissue sections were evaluated and graded according to Bothwell and Bradlow [21]. As the specimens arrived they were processed and stained accordingly. Stained slides were evaluated using light microscopy and results recorded and kept. This procedure started in 1997 and continued as the specimens arrived through 2002. Intensity of staining varied, and influenced grading of iron stores. Grade O indicated no demonstrable iron; sparsely scattered stained cells, singly or in groups, were graded +; moderately stained cells were designated ++; +++ indicated sheets of reactive cells richly distributed. Negative control slides were obtained by using normal human liver from other sources. Age and sex of the patients was extracted from our laboratory records of the patients.

Results

A total of 142 biopsies of liver, were received during the study period. One hundred and thirty of 142 (91.5%) cases were his tologically diagnosed as frank HCC, whereas 5 others were diagnosed as hepatocellular carcinoma and cirrhosis (HCC + C). Seven of 142 cases were diagnosed as diseases other than HCC or HCC + C and were not included in the study.

Table 1 shows the age and sex distribution of patients with hepatoma. Eighty of the cases (59.3%) and 55 (40.7%) cases were males and females respectively. The highest incidence of the tumour (40.7%) occurred in the 40-49 years age group. Fifteen to thirty-four years age group had incidence of (51.9%) with male to female ratio of 4.3, whereas the patients in the 20-49 had the overall incidence of 125 (92.6%) with males to female ratio of 3:2. Malignancy did not occur in the 0-9, 10-19 and 50-59 years age groups, whereas only one case occurred in 60-69 and 70-79 years age groups respectively.

Table II shows grading of stainable iron. Fifty (37%) of 135 tumours showed stainable iron of varied intensity while 85 (63%) showed no demonstrable iron.

Table III displays the distribution pattern of stainable iron among the males and females. Fifty males and 35 females (63.0%) did not show any demonstrable iron in their biopsies, whereas 10 males and 10 females; 10 males and 5 females; 5 males and 10 females had +, ++ and +++ grades of stainable iron respectivley. All the 5 HCC + C cases were positive for iron staining

Table I: Age and sex distribution of 135 HCC patients

Age group	Male (n)	Female (n)	Total
0-9	0	0	0
10-19	0	0	0
20-29	15	20	35
30-39	25	10	35
40-49	35	20	55
50-59	0	0	0
60-69	5	0	5
70-79	0	5	5
Total	80	55	135

Table 2: Frequency and grading of stainable iron in HCC

Grades		Significance		
0	+	++	+++	
85	20	15	15	
63%	15%	11.0%	11.0%	P<0.05

Key: HCC = hepatocellular carcinoma; 0= none; Slight=+; Moderate= ++; Marked= +++

Table 3: Sex Distribution Pattern of Iron Grades in H.C.C.

Grades of Iron	0	+	++	+++
Total	50M.35F 75M.60F;	10M.10F 25M,25F	10M.5F (50 positives) and 50M. 35F (85 negatives)	5M.10F

Key: HCC= Hepatocellular Carcinoma; M = Males; F= Females

Discussion and Conclusion

Hepatocellular carcinoma is one of the health problems of the world. Nigeria is known to be among the area of high mortality rate concentration, but the forty-five HCC cases found in a review of approximately 7,500 surgical biopsies done on Nigerian Igbos during a period of 6 years calls for urgent attention to this intriguing tumour. In our study, 80 of the cases (59.3%) and 55 (40.7%) cases were males and females respectively. The highest incidence of the tumour (40.7%) occurred in the 40-49 years age group. It was also observed that the young patients (15-34 years age group) had incidence of 51.9% with male to female ratio of 4.3, whereas the patients in the 20-49 years age range had the overall incidence of 125 (92.6%) with male to female ratio of 3:2. These findings are compatible with those of other previous authors [22,23,24]. By contrast, most hepatocellular carcinoma patients are more than 60 years old in the United States and Western Europe. Our finding that the young patients in the age group of 15-34 years had a high incidence of the disease with male to female ratio of 4:3 is not far from that stated in the 1979 Editorial.[4] In our series, malignancy did not occur in the 0-9, 10-19 and 50-59 years age group, whereas only one case occurred in the 60-69 and 70-79 years age groups respectivley. Our finding in this respect is in conformity with the fact that males are more frequently affected than the females, though the differential tends to narrow over the age of 55 years [5].

The frequency and amount of stainable hepatic storage iron in the non-neoplastic liver and HCC has been reported.[12] In the non-neoplastic liver, 14 (35.0%) did not show presence of any stainable iron. In the same study, patients with HCC showed a striking difference with 36 (90.0%) showing absence of stainable iron, whereas only 4(10.0%) were positive for stainable iron. In the current study, only 50(37.0%) showed positivity for stainable iron, whereas 85(63.0%) showed no demonstrable iron. Both studies therefore may be interpreted as showing similar comparative findings. The present work has equally shown that 50 males and 35 females (63.0%) did not show any demonstrable iron in their biopsies while 10 males and 10 females, 10 males and 5 females and 5 males and 10 females had +, ++ and +++ grades of stainable iron respectively. As well, all the 5 HCC + c cases were positive for iron staining. Our findings in this case consonnates well with the hypothesis that sex could be possibly graded as non- significant variable in iron accumulation.

Iron overload has been shown to be a significant risk factor for the development of hepatocelluar carcinoma [18]. Iron metabolism may be required for the growth of cancer [26]. Furthermore, of sixteen [16] patients positive for ferritin in HCC, iron was found only in two [12]. This may indicate that immunohistologic ferritin in HCC is not due to increased stainable iron.

The apparent difference between stainable iron and patients with HCC, may reflect the complex nature of haemosiderin and ferritin synthesis and release. Studies [12] have shown that ferritin staining was significantly less frequent in HCC (40%) than in uninvolved liver (75%) ($P<0.01$). Furthermore, the ferritin synthesized by tumor cells may differ antigenically from that produced by hepatocytes (unpublished data). The iron contents of ferritin may vary greatly, with generally low quantity in ferritin isolated from tumor cells, and higher concentrations in ferritin extracted from hepatocytes.

The hardening of liver, due to any cause is generally accepted to possibly have a predisposition to HCC [27]. This condition has been found to be more frequent in geographic area of high incidence of HCC [14]. In a study [28], neither ascites nor cirrhosis affected the course of HCC. It is surprising because ascites or cirrhosis in patients with HCC are usually considered prognostic variables [7,27,29]. It might be postulated that the cancer and the cirrhosis are two independent result of a single agent, rather than that the cirrhosis gives rise to the cancer [5], although the evidence is equivocal. In our series, since all the five cirrhotic cases had iron deposition, it would then appear that iron is required in the formation of fibrosis.

A further study on the complex nature of ferritin synthesis and release is suggested. This will help to ascertain the product formed after the tumor cells have probably utilized the iron, if possibly a known product is formed a useful and more sensitive and specific staining technique can then be utilized, for its demonstration. The actual iron content in the tumor cell can now be estimated by summing up the quantities derived from Perl's method plus that from envisaged demonstration.

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References

1. Altman R, Serg MT. The cancer dictionary. Hamilton Printing Co. 1992, USA P.33.

.2. Kunio O. Early detection and treatment of liver cancer. Gann Monograph on cancer research (38). Japan scientific Society Press and Taylor and Francis Ltd London 1991

3. Alpert ME; Hatt MSR, Davidson CS. A study of geographic pathology. Lancet 1968; 1: 1265.

4. Editorial: Committee for the Atlas cancer mortality in the people's Republic of China, Shanghai, China Map Press 1979; 6-7.

5. Edington GM, Gilles HM. Pathology in the tropics 2nd ed. Spotciswoode, Ballantyne and Co Ltd Lond. 1969, P 588.

6. Okuda K.: Primary liver cancer in Japan Cancer 1980; 45: 2663 2669.

7. Okuda K, Obata H, Nakajima Y.: Prognosis of primary hepatocellular carcinoma. Hepatology 1984; 4: 35-65

8. Khandelwal C, Khanna S. Immunological status of patients of primary hepatic carcinoma. Indian J. Med. Res 1986; 83: 591-593.

9. Editorial: Can cancer risk be altered by changing Nutritional Traditions? Cancer 1998; 83: 1278-1281.

10. Ernest B. Scientific symposium 5: Hereditary and acquired Disorders of iron overload. XVth meeting of the international society of Haematology Education and symposium Programme Sept. 1999; 161-170

11. George DK; Powell LW, Losowsky MS. The haemochromatosis Gens: A co-factor for chronic liver diseases. J. Gastrenterol- Hepatol 1999, 14: 745-749

12. Xin-Da Zhou: Iron, Ferritin, Hepatitis B surface and core antigens in the livers of Chinese patients with hepatocellular carcinoma. Cancer 1987; 59: 1430-1437.

13. Abalea- V; Cilard-J; Dubos, MP; Sergeant-O; Cillard-P; and Morel, I. repair of iron-induced DNA Oxidation by the flavoniod myricetin in primary rat hepatocyte cultures. Free-Radic Bid Med 1999; 26: 1457-1466.

14. Elsner B, Jauregui EM. Autopsy study of primary liver carcinoma in Buenos Aires, Argentina Acta Hepato-Gastroenterol 1974; 21: 26.

15. Al-Sarraf M. Primary liver cancer. Caner 1974; 33: 574

16. Luo TY, Pan NQ. A study on the multiple factorial causation of Hepatoma among young patients 1997; 18: 37-39.

17. Deugnier YM, Gugader D, Grantock L, Lopez JM, Turlin B, Yaouana J, Jouanolle H, CampionJP, Launois B, Halliday JW. Primary liver cancer in genetic hemochromatosis: A clinical Pathological and pathogenic study of 54 cases. Gastroenterol 1993; 104: 228-234

18. Mandishona E, MacPhail AP, Gordenk VR, Kedda MA, Paterson AC, Rouault TA, et al. Dietary iron overload as a risk factor for hepatocellular carcinoma in Black Africans. Hepatol 1998; 27: 1563-1566.

19. Ehrlich P. Haematoxylin and Eosin staining method. Z Wiss Mikr 1886; 3: 150

20. Perls N. Perls' Prussian Blue Method for Haemosiderin. Virchows Arch Path Anat 1960; 39: 42.

21. Bothwell TH, Bradlow BA. Siderosisin the Bantu: a combination of histopathological and chemical study. Arch Pathol 1960; 70: 279-292

22. Onuigbo, W. I. B: Biopsy study of Hepatocarcinoma in Nigerian Igbos. Acta Hepato-Gastroenterol 1977; 24: 24-26

23. Antony, P.P: Primary Carcinoma of the liver: A study of 282 cases in Ugandan Africans. J Pathol 1972; 110: 37-48.

24. Kew MC, Geddes EW. Hepatocellular carcinoma in rural South African Blacks. Medicine 1982; 61: 98-108.

25. Sandlar DP, Sandlar RS, Horney LF: Primary liver cancer mortality in the United State J Chron Dis 1983; 36: 227-236

26. Weinberg CD. Iron in neoplastic disease. Nutrcancer 1983; 4: 223-233

27. Vogel CL, Linsell CA. Internation symposium on hepatocelluar carcinoma Kampala, Uganda. J Natl Cancer Inst 1972; 48: 567-571

28. Pierre A, Sylvie P; Gilles P, Laure P, Olivier Ink; Catherine B; et al. Prognostic factors in patients with hepatocellular carcinoma. Haematology 1987; 4: 35-65

29. Prinack, A; Vogel, C: A staging system for hepatocelllular carcinoma: prognostic factors in Ugandan patients. Cancer 1975; 35: 1257-1364

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Light microscopical details of perineuronal nets in the hippocampus of the mouse revealed by means of methylene blue labeling

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Key words: Cornu Ammonis, pyramidal cells, cationic dye, redox dye, extracellular substance, proteoglycans, glycoproteins, surface coats

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Abstract

Light microscopic results regarding the morphology of perineuronal nets in the hippocampus of the mouse achieved by means of methylene blue labeling are shown. Within the paraffin sections obtained from the dye-perfused tissue, an intense staining of extracellular fibrillary elements of the stratum pyramidale, primarily the CA1 field, could be identified; i.e. these structures formed perineuronal nets around the unstained cells and were also observed in contact to blood capillaries. The other layers of the cornu Ammonis and also the fascia dentata showed only a slight staining. The resulted images exhibited a certain similarity with those obtained by means of immunochemical labeling of phosphacan (6B4) as well as staining with colloidal iron enhanced by Bodian`s protein silver. In addition, they resembled those achieved by methylene blue staining of the stratum granulosum of the cerebellar cortex.The existence of perineuronal nets in the central nervous system supports some aspects of the old reticulum theory without contradicting the neuron theory, i.e. a reticular formation of extracellular matrix connects glial with nerve cells and is also associated via ankyrin and spectrin with the intracellular cytoskeleton of the cells. In conclusion, due to its selectivity for perineuronal nets, the technique used here supplement histochemistry in a helpful manner; moreover, regarding morphological details it appears to be superior compared with common histochemical procedures.

Introduction

Using his own silver impregnation technique, Golgi [1] described at first a delicate covering, mainly reticular in structure, which surrounds the cell bodies of nerve cells and expands along their dendrites. Later, this pericellular coat was also found by means of supravital methylene blue staining [2]. In the end of the 19th century, the central point of discussion was, whether these perineuronal nets represent delicate axonal networks or nonneuronal structures; in this context, it has to be referred to an excellent review given by Celio et al. [3]. Using the PAS-reaction for carbohydrate residues, it could be shown that these perineuronal nets are associated with anionic residues, i.e. carboxyl groups of acidic proteins or glycoproteins [4].

In the last decade of the 20th century, these molecules were investigated more in detail by means of lectin histochemistry and immunohistochemistry which led to a differentiation of two extracellulary located types of perineuronal nets: one of these is composed of glial cell, i.e. astrocyte, processes, whereas the other one consists of extracellular matrix molecules interposed between the tiny endfeet at the neuronal surface [3]. Manifold functions have been attributed to the perineuronal nets of extracellular matrix which include stabilization of synapses, concentration of growth factors around certain neurons, the generation of a polyanionic ion-buffering microenvironment, the formation of a link with the intracellular cytoskeleton via ankyrin and spectrin etc. [3].

In the hippocampus, solitary perineuronal nets were found around GABAergic interneurons (non-pyramidal cells) containing the calcium-binding protein parvalbumin by means of lectin histochemistry; furthermore, it was concluded that perineuronal nets of these fast-firing neurons serve as local buffers of excess cation changes in the extracellular spaces [5]. By means of an improved colloidal iron staining technique Hong et al. [6] observed a strong reaction of a diffuse perineuronal network mainly in the hippocampus of mice. Using immunohistochemistry for the chondroitine sulfate proteoglycan phosphacan, Okamoto et al. [7] obtained similar results in the rat.

Müller [8] introduced a new modification of the supravital methylene blue staining technique and demonstrated that the non-pyramidal neurons in the hippocampus exhibit a high affinity to the dye. Moreover, he published the first photographs of perineuronal nets of extracellular matrix around hippocampal non-pyramidal cells in mice [9]. In a further study, he could also visualize a diffuse perineuronal network in the stratum granulosum of the mouse cerebellar cortex [10]. Comparable

the mouse cerebellar cortex [10]. Comparable observations in the cerebellar cortex had been achieved by Mabuchi et al. [11] using different histochemical techniques.

The aim of the present study was to apply methylene blue labeling to the hippocampus of the mouse for the detection of perineuronal nets. The results were compared with published findings achieved by the use of other staining methods. In addition, more details on the morphology of the stained material should be elucidated.

Materials and Methods

General principles of animal care were applied throughout the experiments and all experiments and procedures complied with the German law on the protection of animals. Adult mice *(Mus Musculus)* were killed with tribromethanol. Further treatment of the tissues was carried out as described previously [10]:

Immediately after death, about 2 ml of an aqueous, 37°C warm dye solution (MB med. puriss., C.I. 52015; Chroma, Köngen, Germany) were injected into the left cardiac ventricle until the skin became blue. The dye was administered at a concentration of 20%.

After 1 h at room temperature (20°C), the hippocampi were removed, cut into approximately 1 mm-thick slices with a razor blade, and exposed to air in a moist chamber for 1 hour at room temperature (20°C). This led to a blueing of the specimens, i.e. oxidation of leuco-MB, since the dye had been primarily reduced to its colourless form in situ.

The first fixation was performed at 4°C (refrigerator) for 5 h (stock solution: 100 ml of 9% aqueous ammonium heptamolybdate solution with the addition of 9 drops of 25% hydrochloric acid and 0.9 ml 30% hydrogen peroxide). After a short rinse in distilled water, a second fixation took place for 2 h 30 min at 4°C [stock solution: 100ml of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) containing 1.8% phosphomolybdic acid and 0.1% hydrogen peroxide (final pH 5.0)]. Subsequently, the specimens were washed overnight in distilled water.

The tissues were dehydrated in 100% tertiary butanol (melting point: 25°C) for 48 h. The first alcohol change was performed after 15 min, the second after 1 h and the third after 7 h. For these three preliminary dehydration steps, phosphomolybdic acid was added to the alcohol in a concentration of 0.05%. The tissues were then transferred into pure tertiary butanol. After dehydration, they were stored for 1 h in a mixture of 8 parts decahydronaphthalene (Dekalin®; Chroma, Köngen, Germany) and 2 parts methyl benzoate. Before being embedded in paraffin, they were immersed for another hour in 100% decahydronaphthalene.

20 µm-thick microtome sections were mounted on glass slides. After drying, they were deparaffinized in xylene and coverslipped with DePeX® (Serva, Heidelberg, Germany). Subsquently, the paraffin sections were focused under the light microscope and digitally photographed.

Results

In the deeper regions of the slices (more than 200 µm distant from the cut surface), a selective staining of components of the extracellular matrix could be achieved (Figs. 1 and 2). In the stratum pyramidale of the cornu Ammonis, the extracellular matrix was found to be intensely labeled; i.e. a dense network of extracellular fibrillary structures could be identified. The intermingled perikarya remained more or less unstained. These perineuronal nets were mainly stained in the CA1 field; this inhomogeneous staining pattern was obviously caused by differences regarding the dye perfusion. The other layers of the cornu Ammonis, i.e. stratum oriens, radiatum and moleculare, showed only a slight extracellular labeling. The fascia dentata was also only slightly stained. Moreover, the extracellular fibrils were also seen contacting blood capillaries (Fig. 2).

Discussion

An important observation of the present study was the finding that perineuronal nets were not only found covering non-pyramidal cells; i.e. they formed a diffuse network intermingled between the cells of the stratum pyramidale. Therefore, the resulted were different to those obtained by means of lectin histochemistry, for example Wisteria floribunda agglutinin [12]. Moreover, they resembled those revealed by colloidial iron in combination with enhancement by Bodian's silver in the murine hippocampus [6]. Similar results were also obtained by immunohistochemical visualization of phosphacan (6B4) in the rat [7]. This leads to the conclusion that different kinds of perineuronal nets exist regarding their chemical compositions; this phenomen would also suggest varying functional characteristics of the nets.

In the present studys, MB-labeled nets were primarily seen in the CA1 field of the cornu Ammonis. This phenomenon was probably caused by an inhomogeneous dye perfusion.

Comparable results had been obtained by colloidal iron and subsequent Prussian blue reaction in combination with enhancement by Bodian's silver [6]. Nevertheless, it has to be pointed out that only in the CA3 field in humans the reaction was positive. In contrast to this finding, MB-labeled nets were primarily seen in the CA1 field of the mouse. Although interspecies-differences cannot be completely excluded, it appears to be more likely that such diffuse networks of extracellular networks are distributed throughout the hippocampus. This conclusion is supported by the results of Okamoto et al. [7] demonstrating a similar staining in the CA2 stratum pyramidale using immunohistochemistry for the chondroitine sulfate proteoglycan phosphacan.

Regarding the staining mechanism, the opinions differ: There is some evidence that methylene blue is reduced to its uncharged colourless lipophilic leuco-form immediately before passing the blood brain-barrier followed by a subsequent reoxidation to the blue positively charged, i.e. cationic, form. This phenomenon

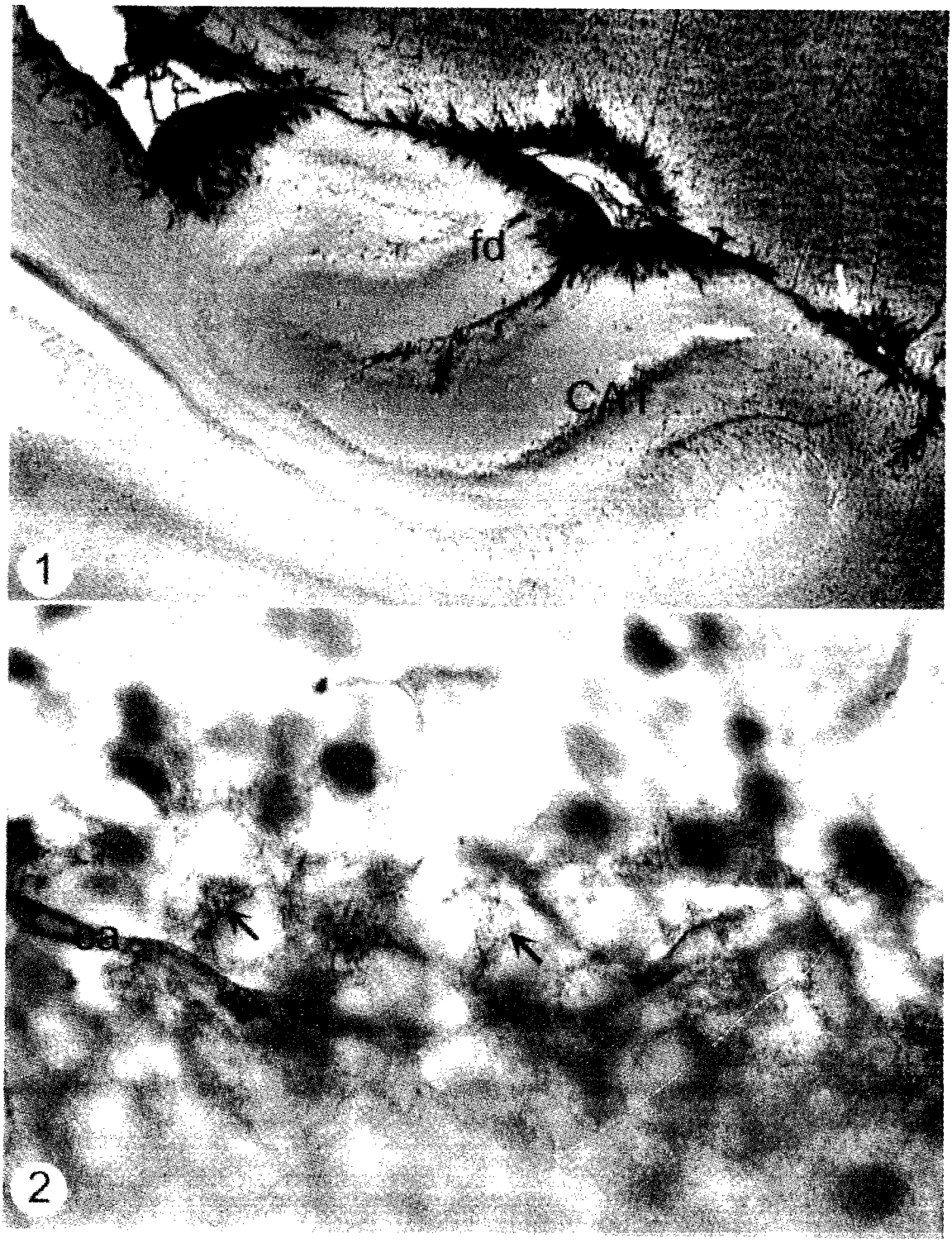

Fig. 1: *The overview shows the dye distribution in the hippocampus including the fascia dentata (fd); note that the stratum pyramidale of the CA1 field (CA1) in the cornu Ammonis appears to be more intensely stained, whereas other regions show only a slight dye labeling. Due to excessive dye-supply, crystals of methylene blue precipitate are also visible (white arrows). x 40*

Fig. 2: *In the higher magnification, perineuronal nets of fibrillary elements (arrows) around the more or less unstained perikarya can be identified. These structures are also seen contacting blood capillaries (ca). x 600*

leads to a possible binding mechanisms of the dye: Since perineuronal nets of extracellular matrix are known to be rich in strong anionic residues; these negatively-charged groups might attract the dye in its cationic form after reoxidation [9]. Here, it has to be emphasized that according to the conclusions of Murakami et al. [13] ligand-proteoglycans which connect the perineuronal proteoglycan surface coat with cell surface glycoproteins are considered to be the targets of the dye.

Moreover, it has to be pointed out that the existence of perineuronal nets in the central nervous system supports in a certain kind of manner the old reticulum theory without contradicting the neuron theory, i.e. a reticular formation of extracellular matrix connects glial with nerve cells and is also associated via ankyrin and spectrin with the intracellular cytoskeleton of the cells. However, this does not mean that axons and dendrites anastomose in a continuous network. Therefore, from the present point of view the opinion of the moderate reticularist Bethe [14] has to be confirmed; in contrast to Meyer [15], Bethe did not interpret perineuronal nets as delicate axon terminals, he attributed other properties and functions to these structures.

In conclusion, the MB-method used in the present study is highly selective, easy to handle, and visualises morphological details. In contrast to common histochemical techniques, the staining method can also successfully be applied to the mouse hippocampus. When histochemical reactivity for a specific marker or several markers is absent, the methylene blue-method may give the decisive answer whether perineuronal nets are really present or not. Therefore, this technique represents a helpful supplement to histochemistry in neuroanatomical research.

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References

1. Golgi C. Sulla struttura della sostanza grigia del cervello. Gazzetta Med Ital-Lomb 1873; 6: 244-246.

2. Ramon y Cajal S. La red superficial de las células nerviosas centrales. Rev Trim Micrográf 1897; 3: 199-204.

3. Celio MR, Spreafico R, De Biasi S, Vitellaro-Zuccarello L. Perineuronal nets: past and present. Trends Neurosci 1998; 21: 510-515.

4. Brückner G, Gogala M, Zei M, Biesold D. Histochemical detection of anionic components in the cephalopod brain. Acta Histochem 1984; 74: 91-102.

5. Härtig W, Derouiche A, Welt K, Brauer K, Grosche J, Mäder M, Reichenbach A, Brückner G. Cortical neurons immunoreactive for the potassium channel Kv3.1b subunit are predominantly surrounded by perineuronal nets presumed as a buffering system for cations. Brain Res 1999; 842: 15-29.

6. Hong JL, Mubarak WAE, Sunami Y, Murakami S, Fuyama Y, Ohtsuka A, Murakami T. Enhanced visualization of weak colloidal iron signals with Bodian's protein silver for demonstration of perineuronal nets of proteoglycans in the central nervous system. Arch Histol Cytol 2000, 63: 459-465.

7. Okamoto M, Sakiyama J, Kurazono S, Mori S, Nakata Y, Nakaya N, Oohira A. Developmentally regulated expression of brain-specific chondroitin sulfate proteoglycans, neurocan and phosphacan, in the postnatal rat hippocampus. Cell Tissue Res 2001; 306: 217-229.

8. Müller T. Light and electron microscopic demonstration of methylene blue accumulation sites in the murine cornu Ammonis after supravital staining. Ital J Anat Embryol 1995; 100: 179-187.

9. Müller T. Methylene blue supravital staining: an evaluation of its applicability to the mammalian brain and pineal gland. Histol Histopathol 1998; 13: 1019-1026.

10. Müller T. Light microscopical details of perineuronal nets in the cerebellar cortex of the mouse revealed by means of methylene blue labeling. Biomed Res 2003; 14: 151-156.

11. Mabuchi M, Murakami S, Taguchi T, Ohtsuka A, Murakami T. Purkinje cells in the adult cat cerebellar cortex possess a perineuronal net of proteoglycans. Arch Histol Cytol 2001; 64: 203-209.

12. Brückner G, Grosche J. Perineuronal cells show intrinsic patterns of extracellular matrix differentiation in organotypic slice cultures. Brain Res 2001; 137: 83-93.

13. Murakami T, Murakami T, Sato H, Mubarak WA, Ohtsuka A, Abe K. Perineuronal nets of proteoglycans in the adult mouse brain, with special refererence to their reactions to Gömöri's ammoniacal silver and Ehrlich's methylene blue. Arch Histol Cytol 1999; 62: 71-81.

14. Bethe A. Über die Neurofibrillen in den Ganglienzellen von Wirbelthieren und ihre Beziehungen zu den Golginetzen. Arch Mikrosk Anat 1900; 55: 513-558.

15. Meyer S. Ueber centrale Neuritenendigungen. Arch Mikrosk Anat 1899; 54: 296-311.

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Antinociceptive Effects of *Coscinium fenestratum* (Gaertn) on mouse formalin test

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Key words : *Coscinium fenestratum*, antinociception, formalin test, mice, plant.

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Abstract

In this study, the effect of *Coscinium fenestratum* (Gaertn) a woody climbing shurb, belonging to the family *menispermaceae* and found in Srilanka was studied on inflammatory pain induced by formalin in mice. The total extract and its polar and non polar fractions were administered intraperitoneally 30 minutes before formalin injection. Total extract in doses (mg/kg of body weight) used, induced significant reduction in response as compared to control as follows: 40 (30.52%, P<0.05), 80 (42.50%, P<0.05) and 160 (68.28%) P<0.01) in the early phase and 40 (62.48%, P<0.01), 80(45.42%, P<0.05) 160(48.38%, P<0.05) in late phase. Fraction I (water phase) in doses (mg/kg) of 40(60.22%, p<0.01), 80(65 28% p<0.01) and 160 (72.23% P<0.01) in early phase and 40(75.83% P<0.01), 80(62.83% P<0.01), 160(53.83%, p<0.01) in the late phase induced antinociception when compared to controls. Fraction II (chloroform phase), Fraction III (hexane phase) did not induce significant antinociception in comparison to that of control group. It is concluded that the polar ingredients of the extracts are responsible for the analgesic and anti-inflammatory properties of *Coscinium fenestratum* (Gaertn). To elucidate the mechanisms relating in this historical plant, antinociceptive effect and regarding the mediators involving in the early and chronic phases of formalin test, further studies may focus to explore the interaction of the plant with histamine, kinin, serotonin and prostaglandins.

Introduction

Since the available analgesic drugs exert a wide range of side effects and are either two potent or too weak, the search for new analgesic compounds has been a priority of pharmacologists and pharmaceutical Industries[1]. Medicinal plants are believed to be an important source of new chemical substances with potential therapeutic effects [2,3]. Thus study of plant species that traditionally have been used as pain killers should still be seen as logical research strategy, in search for new analgesic drugs [4].

The *Coscinium fenestratum* (Gaertn) is a woody climbing shrub found in western ghats of Tamilnadu, Kerala and also in Srilanka. The stem portion of the plant is suggested to have thermogenic, anti-inflammatory, antiseptic and tonic effects and is used against ophthalmopathy, inflammations, ulcers, jaundice, and general debility [5-8]. Singh et al [9] revealed the hypotensive activity of stem extracts of this plant in experimental dogs, rats, and guinea pigs.

Among the several models of persistent nociception, formalin has been well established as a valid model to study the central sensitisation events at the spinal level after pheripheral inflammatory states. In this test, two types of pain were postulated; a short lasting pain caused by a direct effect on nociceptors followed by a long lasting pain due to inflammation. Since the formalin test measures the response to a long lasting nociceptive stimulus, thus it has a closer resemblance to a clinical pain too [10].

To examine the effects in more detail, the present experiment was designed to investigate the effects of alcohol extracts of *C. fenestratum* and its aqueous, hexane and chloroform extracts on the formalin test in mice.

Materials and Methods

Plant material

The woody climbing stem of *Coscinium fenestratum* was collected from Kathnapura District of Srilanka during April - May 2002 were used in this investigation. It was chopped, air dried at 35-40°C for 2 months and pulverized in an electric grinder.

Extract Preparation

The dry stemmy woody part of *Coscinium fenestratum* (300 g) were grinded and then were extracted with 80% ethanol for 24 hr in a continous extraction (Soxhlet) apparatus. The extract was filtered, and ethanol was evaporated on a rotatory evaporator under vaccum at a temperature of 45°C to a small volume. Then the rest of the solvent was evaporated in the air to make the extract. Ten grams of extract was added to 100ml of water. Then thirty-three ml of hexane was added and slowly shacked until the non polar materials separated from water and enter to the hexane phase. Then hexane phase was repeatedly decanted to be separated from water phase. Then thirty three ml of chloroform was added and slowly shacked until the moderately polar materials separated from chloroform and enter to the chloroform phase. Fraction I was separated from water in the same way of total extract. Fraction II was separated from hexane Fraction III was separated from chloroform.

Animals

Male albino mice weighing 25-30g from Central Animal Facility, SRMC&RI was used in the experiements. The animals were housed in standared stainless steel cages in a temperature controlled room (22±2°C) with a 12-12 h light-dark cycle. The mice were randomly distributed into groups of six as control and test subjects of 6 each. All animals had enough access to food and water throughout the experiements. All nociceptive testing was done in the morning, within 4 hr after the onset of the light cycle. For antinociception recording, mice were allowed to acclimatize for 30 min before any injection.

Preparation of Dose

The doses of 20, 40, 80 and 160 mg/kg of body weight of the extract and 40, 80 and 160 mg/kg of body weight of fractions I, II and III were used. Doses were selected to be at the range of 0.1 of extract's LD_{50} [11] Sodium chloride 0.9% was used as solvent. All doses were administered intraperitoneally 30min before formalin injection to animals.

Formalin test

As described previously [12,13] each mouse received a single subcutaneous injection of 25□ □l of formalin (0.5%) into the dorsal surface of the right hind paw using a micro syringe with a 26 gauge needle. Immediately after formalin injection, animals were placed individually in a glass clinder (20cm wide, 25cm long) on a flat glass floor and a mirror was arranged at an angle of 45°C under the cylinder to allow clear observation of the paws of the animals. In both phases, only licking or biting of the injected paw was defined as a nociceptive response, and the total time of the response was measured during periods of 0-5min (early phase) and 15-40 min (late phase)

Statistical analysis:

Comparison between groups was made by one-way analysis of variance (ANOVA) followed by Newman Keul's test. Differnces with P<0.05 between experimental groups were considered statistically significant.

Results

Antinociception induced by different doses of extract on the formalin test in mice is shown in fig 1. Total extract in doses (mg/kg), used induced in doses (mg/kg). used induced significant reduction in pain response as compared to control as follow: 40 (30.52%, p<0.05), 80(42.50%, P<0.05) and 160 (68.28% p<0.01) in the early phase and 40 (62.48% p<0.01) , 80 (45.42% p<0.05) 160 (48.32% p<0.05) in late phase. In comparison to saline no significant difference was observed in animals treated by dose of 20 mg/kg of extract in both phases of formalin test (p>0.05)

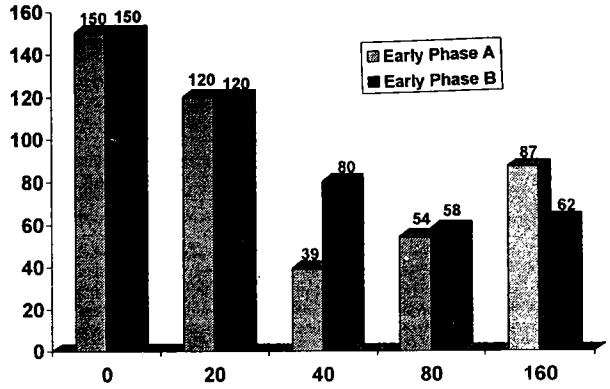

Fig. 1: Effect of Coscinium fenestratum total extract on formalin test in mice. *Saline (O) or different doses of total extract (20,40,80,160 mg/kg) were administered intraperitoneally 30 minutes before formalin injection to mice. Antinociception was recorded 0-5 (early phase, A) and 15-40 (late phase B) minutes after formalin injection. Each point is the Mean ± SE of 6 animals. * and ** means difference betewen control and treated groups is significant respectively at p<0.05 and p<0.01*

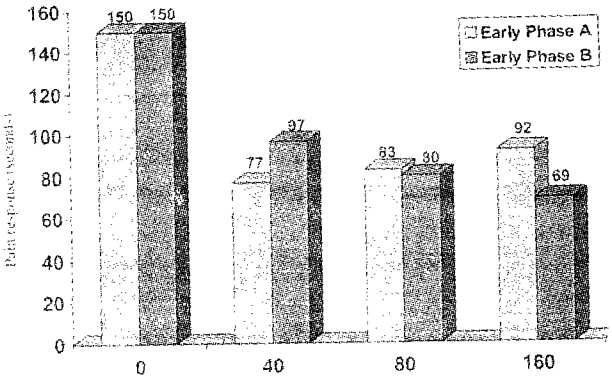

Fig. 2: Effects of fraction I (water) on formalin test in mice. *Saline or different doses of fraction I (40, 80 and 160mg/kg) were administered intraperitoneally 30 minutes before formalin to mice. Antinociception was recorded 0-5 (early phase, A) and 15-40 (late phase, B) minutes after formalin injection. Each point is the mean ±SE of 6 animals. ** Difference between control and treated group is significant at p<0.01*

Antinociception induced by different doses of fraction I on the formalin test in mice is shown in figure 2. Fraction I in doses (mg/kg) used induced significant reduction in pain response as compared to control as follow: 40 (60. 22%, p<0.01), 80 (65.28%, P<0.01) and 160 (72.73%, p<0.01) in early phase and 40(75.83%, P<0.01), 80 (62.83%) p<0.01), 160 (53.83%, p<0.01) in the last phate (p<0.01). Differences between animals treated with 40, 80 and 160 mg/kg of fraction II, fraction III in the early phase and late phase were not significant in comparison to that of control (p>0.05).

Discussion

The results of the present experiment demonstrate the significant antinociceptive effects of total extract in both phases of the formalin test at doses of 40, 80 and 160 mg/kg. The data obtained also indicate that only the water fraction induces antinociception in both phases of formalin list. This finding is interesting and shows that polar compounds of the extract are responsible for antinociceptive effects of total extract. As shown in figure 1 and figure 2, it is indicated that water fraction of the extract shows better antinociception than total extract. Also results indicate that when the dose of extract increases more than 40 mg/kg the antinociception effect is decreased. These findings suggest that extract has different components which when dose is increased, they can interact together negatively in induction of antinociception. This plant is known to contain alkaloids, 12, 13-dihydro-8-oxo berberine, berberine, oxy berberine, tetrahydro berberine, sitosterol (5%) and stigmasterol.

In the formalin test the initial pain (early phase) is explained as a direct stimulation of nociceptors and the late phase is thought to be secondary to the inflammatory reactions. Several mediators such as histamine, kinin, serotonin and prostaglandins are released from damaged cells. These mediators take part in the inflammatory response and are able to stimulate nociceptors and thus induce pain [4]. Based on bibliography no study has been conducted on interactive effects of Coscinium with these mediators. However this study confirm the folk medicine use of Coscinium as analgesic and anti inflammatory but its mechanism of action remain to be elucidated by further studies on the properties of Coscinium to interact with mediators like histamine, kinin, serotonin and prostaglandins. More over the active compounds responsible for these pharmacological actions also remain to be identified.

References

1. Mattison N, Trimple AG, Lasagna I. New drug development in the United states, 1963 through 1984. Clin Pharmacol Ther 1998; 43(3): 290-301.
2. Farnsworth NR. Screening plants for new medicines. In: Wilson EO, editor, Biodiversity, Part II. National Academic Press, Washington 1989: PP 83-97.
3. Blumenthal M. 2000. Herbal medicines. Integrative Medicine Communications Austin 2000: pp 419-423.
4. Rang HP, Dale MM, Ritter JM. Pharmacology. Churchill Livingston, New York 1998: PP 614-616.
5. Kiritikar KR, Basu BD Indian Medicinal Plants 2nd Edition, Vol I, 1933 P 84.
6. Chopra RN, Bhadwa RL and Ghose S. Poisonous Plants of India. Ist edition, Vol I, ICAR, Calcuttta, 1949, P148.
7. Nadkarani AK. Indian Materia Medica. 3rd Edition, Vol II, Poppular Press, Bombay 1954, P 318.
8. Cains JE, The Medicinal and Poisonous Plants of India, 1st edition, Scientific Publishers, Jhodhpur, India. 1986, P. 171.
9. Singh GB, Singh S, Bani S, Malhotra S. Hypotensive action of a *Coscinium fenestratum* stem extract. J. Ethnopharmacol 1990; 30: PP 151-155.
10. Hunskaar S, Fasmer OB, Hole K. Formalin test in mice, a useful technique for evaluating mild analgesics. J Neurosci methods 1985; 14: 69-76.
11. Venukmumar MR and Latha MS. Anti oxidant effect of *Coscinium fenestratum* in carbontetrachloride treated rats Indian J Physiol Pharmacol 2002; 46: 223-228.
12. Dubuisson D, Dennis SG. The formalin test: A quantitative study of the analgesic effect of morphine, meperidine and brain stem stimulation in rats and cats. Pain 1977; 4: 74-161.
13. Shibata M, Ohcubo T, Taksash; H, Inoki R. Modified formalin test, characteristic biphasic pain response, Pain 1989; 38: 347-352.

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Antibody response to *M.tb* $H_{37}Ra$ excretory secretory ES-43 and ES-31 antigens at different stages of pulmonary tuberculosis

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Key words: Excretory secretory proteins ES-31, ES-43, tuberculosis.

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Abstract

The secreted protein antigens provide first stimulus *in vivo* for humoral and cellular immune response to mycobacteria and may play useful role in serodiagnosis. A secretory protein ES-31 isolated from *Mycobacterium tuberculosis* $H_{37}Ra$ culture filtrate has been earlier shown diagnostically useful in pulmonary tuberculosis. Similarly ESAS-6 antigen found to be reactive in our earlier studies, was further fractionated by Fast Protein Liquid Chromatography (FPLC) and obtained a 43 kDa protein labelled as ES-43 antigen. The immunoreactivities of purified ES-31 kDa and ES-43 kDa antigens were assessed in sera at different stages of pulmonary tuberculosis viz. Fresh, chronic and relapse cases by indirect stick penicillinase ELISA. The ES-31 antigen showed higher reactivity in chronic cases, while ES-43 antigen was primarily recognized by serum antibodies in relapse cases, whereas both antigens showed comparatively decreased antibody response in fresh cases. The study does show that the immune response varies with different antigens at different stages of tuberculosis. It will be of interest to monitor tuberculosis patients by using antibody response to ES-31 and ES-43 antigens, as a predictive tool for relapse cases.

Introduction

Tuberculosis (TB) currently possesses a problem worldwide. The world health organization estimates that at least one-third of the world population (or 1.9 billion people) are infected with TB. India accounts for nearly a third of all TB cases in the world [1].
Overwhelming importance in immunological tests have been seen in recent years, since a serodiagnostic test can potentially satisfies all the requirements for an optimal diagnostic test for TB. Although numerous *Mycobacterium tub*erculosis (*M.tb*) antigens capable of generating specific antibody titers in TB patients have been identified, no single antigen appears to be ideal for serodiagnostic use. Extensive studies have been done by researchers on various purified antigens like antigen A60, 38 kDa, antigen 85 A and B, and found that sensitivities varied with the antigens for different study cohort [2,3].

In this study, we have analyzed the antibody response to already reported antigen ES-31 kDa and another purified antigen ES-43 kDa in different stages of pulmonary tuberculosis viz. Fresh, chronic and relapse cases.

Material and Methods

Subject: Blood samples were collected from confirmed pulmonary tuberculosis patients from district tuberculosis centre, Wardha and patients attending Kasturba hospital, Sevagram, Dist. Wardha, Maharashtra State, India. According to RNTCP criteria the cases were further classified into fresh, chronic and relapse.

Fresh cases (n=25)

A patient who has never had treatment for TB or who is freshly diagnosed as TB case and taken antitubercular drug for less than four weeks.

Patients in this category were considered to have signs and symptoms of TB for less than four weeks.

Relapse case (n=30)

A patient who has been declared cured of any form of TB in past by a physician after one full course of chemotherapy and has again become sputum smear positive.

Chronic cases (n=25)

A patient who remained or became again smear positive after completing a fully supervised retreatment regimen.

Blood samples were also collected from 24 cases of nontubercular pulmonary disease as disease control and 25 from healthy individuals of this locality served as healthy control. Sera were separated and stored at -20^{0}c with 0.1% sodium azide.

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Isolation of M.tb $H_{37}Ra$ ES-31antigen

M.tb ES-31 antigen is isolated from *M.tb* crude culture filtrate Excretory Secretory (ES) antigen by affinity chromatography using affinity purified anti ES-31 antibody coupled sepharose 4B column as described earlier [4].

Isolation of M.tb $H_{37}Ra$ ES-43 antigen

Mycobacterium tuberculosis $H_{37}Ra$ Excretory Secretory Ammonium Sulphate solubilized (ESAS) antigen was prepared by 50% ammonium sulphate, as described earlier [5]. The ESAS antigen was further fractionated by Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS-PAGE) using 10% nongradient gel. The gel was sliced horizontally (in to 12 fractions) at 1 cm intervals and proteins from 6th gel slice (ESAS-6) containing antigen was recovered by electroelution. The 6th fraction was further fractionated by fast Protein Liquid Chromatography (FPLC) using anion exchange resource 'Q' 1 ml column following the manufacturer's instructions. In brief separation of antigen was accomplished by a salt gradient obtained by mixing buffers A (20 mM Tris HCl pH 8.0) and B (20 mM Tris HCl + 0.7 M NaCl). The flow rate was 1 ml / min and fractions of 0.5 ml were obtained. The column was equilibrated with 2 ml of buffer B followed by 5 ml of buffer A. All the fractions obtained were checked for antigenic activity by indirect ELISA using pooled tuberculosis positive and healthy normal negative control sera.

Stick Indirect Penicillinase ELISA

Stick indirect penicillinase ELISA, was carried out as described earlier [5], to evaluate reactivity of FPLC fractions and purified antigens for detection of tuberculous IgG antibodies in sera samples. Five µl volume of optimally diluted antigenic fractions ES-31 or ES-43 (0.2µg / ml), and serial ten fold dilutions of FPLC fractions (ESAS 6 A,B,C and D) with starting concentration of 200µg / ml) was applied on to cellulose acetate membrane fixed on to plastic strips. Sticks were further incubated with 0.5 ml optimally diluted serum (1:600) and serial two fold diluted sera in PBS / T at 37^0 for 1hr. Anti human IgG penicillinase conjugate (1:1000) were used in this assay. The sera showing complete decolorization of blue colour of starch iodine penicillin 'v' substrate at least 5 min earlier than negative control denoted positive reaction. Geometric mean titre (GMT) of tuberculous IgG antibody was calculated and data were analyzed by student 't'- test.

Result

Fractionation of ESAS-6 by FPLC using resource 'Q' 1 ml anion exchange column yielded four protein fractions as shown in figure 1. Analysis of these fractions by indirect ELISA, showed ESAS-6A compared to other three fractions, highly reactive to tuberculous antibodies in pooled sera, at a dilution of 1:1000 i.e. protein conc. of 1 ng / stick (Table 1). ESAS-6A has been found to be single 43 kDa protein on 10% nongradient SDS- PAGE followed by silver staining (Fig. 2) hence labelled as ES-43 kDa antigen.

The ES-43 and ES-31 antigen fractions were employed in stick indirect ELISA for detection of tuberculous IgG antibody (Table 2). Using ES-31 antigen 23 of 25 (92%) chronic cases turned out

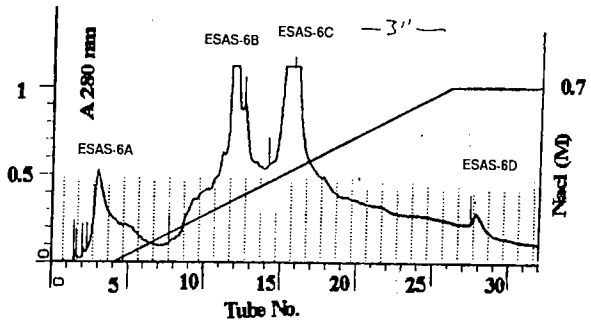

Fig.1: *Elution profile of FPLC separation of M.tb. $H_{37}Ra$ excretory secretory antigen fraction ESAS-6 on Resource 'Q' anion exchange 1ml column. For elution 20 mM Tris HCL pH 8.0 and gradient of 0.0 to 0.7 NaCl were used at a flow rate of 1 ml/ min*

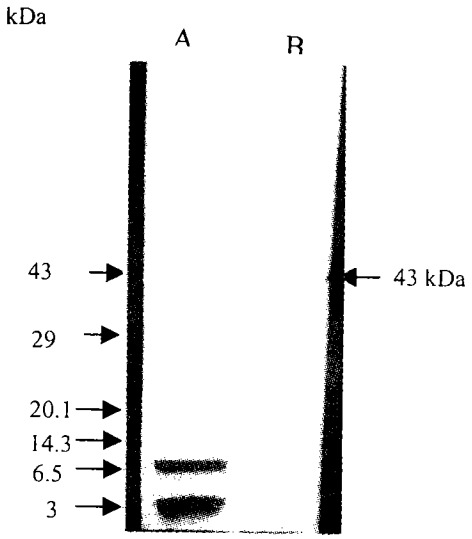

Fig. 2: *Silver stained SDS - PAGE profile of M.tb ESAS- 6A antigen using 10% non gradient slab gel.* ***A:*** *Molecular weight markers: Ovalbumin (MW-43 kDa), Carbonic Anhydrase (MW - 29 kDa), Soyabeen Trypsin Inhibitor (MW-20.1 kDa), Lysozyme (MW-14.3 kDa), Aprotinin (MW-6.5 kDa), Insulin (MW-3 kDa)* ***B:*** *Purified ESAS-6A antigen*

Table 1: Differential reactivity of anion exchange FPLC fractions of ESAS-6 antigen fraction by Indirect ELISA.

Anion exchange FPLC fractions of ESAS-6	Maximum antigen dilution showing antigenic activity*
ESAS-6 A	1000
ESAS-6 B	10
ESAS-6 C	10
ESAS-6 D	10

**antigenic activity indicated by positive reaction with tuberculous serum by indirect ELISA with initial protein concentration of 200 µg/ml (1µg / stick) and serially diluted (10, 100, 1000 times).*

positive, while using ES-43 antigen 19 of 25 (76%) chronic cases showed positive reaction. On the other hand, ES-43 antigen showed 90% (27/30) positivity in relapse cases compared to ES-31 antigen, which showed positivity of 73% (22/30). Both the antigens showed comparatively less positivity of 76% (19 of 25) in fresh cases compared to chronic and relapse cases.

Table 2: Analysis of seroreativity of ES-43 and ES-31 antigens in different stages of pulmonary tuberculosis using stick indirect penicillinase ELISA.

Group	No. Screened	No. (%) positive* with	
		ES-34	ES-31
Fresh cases	25	19(76)	19(76)
Chronic cases	25	19(76)	23(92)
Relapse cases	30	27(90)	22(73)
Healthy control	25	3(12)	2(8)
Disease control	24	3(12)	3(12)
Leprosy	04	-	-
COAD**	06	1	1
PUO***	02	-	-
Bronchial asthma	04	1	-
Pneumonia	04	-	1
Pleural effusion	04	1	1

** Sera showing positive reaction at 1: 600 sera dilution.*
***COAD - Chronic Obstructive Airway Disease*
****PUO - Pyrexia of Unknown Origin*

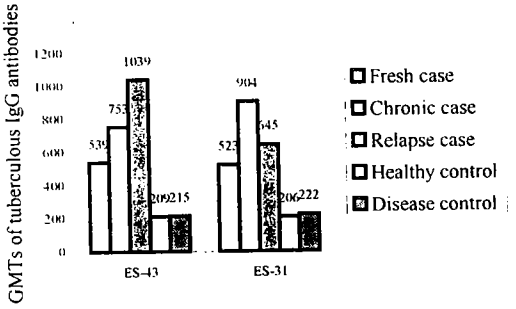

Fig. 3: *Geometric mean titres of tuberculous IgG antibody in different stages of sera using ES-43 kDa and ES-31 kDa antigen.*

Geometric mean titre (GMT) of tuberculous IgG antibody was analyzed to antigen ES-43 and ES-31 in all the groups. In chronic cases the GMT of tuberculous IgG antibody to ES-43 and ES-31 antigen was 904 and 753 respectively (Fig. 3). The difference in GMT to antigen ES-43 Vs ES-31 was statistically significant ($P < 0.02$) in this group by student 't' test. While in relapse cases the ES-43 antigen showed significantly ($P<0.001$) higher antibody titre (GMT-1039) compared to ES-31 antigen (GMT-645). GMT of tuberculous IgG antibody was not significantly different in fresh cases to antigen ES-43 (539) Vs ES-31 (523). The ES-43 antigen detected IgG antibody in 3 of 24 disease control (GMT-215) and 3 of 25 healthy control (GMT-209), while ES-31 antigen showed positivity in 3 of 24 disease control (GMT-222) and 2 of 25 healthy controls (GMT-206).

Discussion

Proteins which are actively secreted in culture medium by *M.tb.* are of immunodiagnostic importance and are well defined in terms of immunodominance and function compared to other antigens in tubercle bacilli. Till date extensive efforts have been made by researchers to isolate and evaluate the antigens of *M.tb.* for detection of tuberculous antibodies in TB, but their diagnostic potential found varying from one study to another [6,7,8,9]. In our earlier studies we have shown the diagnostic potential of ES-31 antigen in pulmonary tuberculosis [5] and in certain groups of extrapulomanry tuberculosis *viz.* tuberculous lympadenopathy and tubercular meningitis [10]. The ESAS-6 fraction has shown reactivity in our earlier studies [11]. In the present study, we have isolated and further purified another excretory – secretory antigen ES-43 kDa by FPLC, from ESAS-6 antigen fraction. Further the seroreactivity of two purified antigens viz. ES-31 and ES-43 kDa were compared in different stages of tuberculosis to analyze the antibody response during the disease progression.

Very few studies are available on comparative analysis of reactivity of antigen in different stages of disease. Studies using 38 kDa antigen have shown the presence of anti-38 antibodies primarily in patients with recurrent, advanced and chronic disease [6,12,13]. However the 38 kDa antigen was poorly recognized by serum antibodies from HIV infected TB patients [14]. Samaniach *et al.* has also demonstrated that, profile of antigen recognized, changes with disease progression [2] in HIV- negative TB patients and HIV- positive TB patients.

In our study, using both the antigens for seroactivity, ES-31 antigen showed comparatively higher positivity of 92% in chronic cases to that of ES-43 antigen (76%), whereas sera from patients with relapse TB showed significantly higher percentage of antibodies to ES-43 antigen (90%), compared to ES-31 (73%). Both the antigens were comparatively less reactive in fresh cases (76%) compared to chronic and relapse cases. The variation in antibody response which is observed, may be due to the differential antigen expression in different disease condition. Our earlier studies have also reported, another purified antigen ES-41 to be more reactive in abdominal and bone and joint TB sera [10] & its elevated level could be demonstrated *in vivo* [15].

To the best of our knowledge, the analysis of antibody response to ES antigens in patients with fresh, chronic and relapse TB has not been studied previously. A study by Kaplan *et al.* [16] showed difference in production of antibody in patients of new and relapse TB, using crude culture filtrate antigen by immunodiffusion test. While in other studies [17,18] using 38 kDa antigen, sera from only new cases and chronic cases were analyzed and the variation in reactivity among these cases were observed. Chronic cases showed more sensitivity of 70% compared to 40% in new cases. It is reported that the immune response in mycobacterial disease appears to be associated with HLA class II allotypes and different patients appear to recognize different antigens [19]. Our study includes all the groups namely fresh, chronic

and relapse cases which are important in early diagnosis, prevention of spread of disease and its recurrence aspects. Thus our study suggests that the generally observed variation in sensitivity and specificity of the test may depend on the disease status and antigen used and ES-43 antigen may be useful in predicting relapse cases.

The study does show that the immune response varies with different antigens at different stage of tuberculosis and it will be of interest to monitor antibody response to ES-31 and ES-43 antigens in tuberculosis patients to predict possible recurrence of disease.

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References

1. TB India 2002. RNTCP Status Report .

2. Samanich KM, Keen MA, Vissa VD, Hasder JD, Spencer JS, Belisle JT, Zalla-Pazner S and Laal S. Serodiagnostic potential of culture filtrate antigen of *Myobacterium tuberculosis*. Clin Bio lab Immunol 2000; 7: 662-668.

3. Daniel TM. Immunodiagnosis of tuberculosis. In: Tuberculosis. WR Rom and S Garay (ed). Little brown and company, Inc., Boston, Mass 1996. PP-223-231.

4. Nair ER, Banerjee S, Reddy MVR, Kumar S & Harinath BC. Isolation of *Mycobacterium tuberculosis* 31 kDa antigen protein of diagnostic interest from culture filtrate using anti ES-31 antibody by affinity chromatography. Ind J Clin Biochem 2001; 16: 132-135.

5. Nair ER, Banerjee S, Kumar S, Reddy MVR & Harinath BC. Purification and characterization of a 31 kDa mycobacterial excretory-secretory antigenic protein with a diagnostic potential in pulmonary tuberculosis. Indian J Chest Dis Allied Sci. 2001; 43: 81-90.

6. Daniel TM and Debanne SM. The serodiagnosis of tuberculosis and other mycobacterial disease by enzyme linked immunosorbent assay. Am Rev Resp Dis 1987; 135: 1137-1151.

7. Mathai A, Radhakrishnan VV and Sehgal S (1990). IgG antibody to *M. tuberculosis* antigens in cerebrospinal fluid and its diagnostic application. Ind J Exp Biol 1990; 28: 816-820.

8. Andersen P. Askgaard D, Ljungyuist L, Bennedsen J, Heron I. Protein released from *Mycobacterium tuberculosis* during growth. Infect Immun 1991; 59: 1905-1910.

9. Benjamin RG, Debanne SM, Ma Y, Daniel TM. Evaluation of mycobacterial antigens in an enzyme-linked immunosorbent assay (ELISA) for serodiagnosis of tuberculosis. J Med Micro Biol 1984; 18: 309-318.

10. Banerjee S, Gupta S, Kumar S, Reddy MVR, Harinath BC. Seroreactivity of 31 kDa and 41 kDa mycobacterial secretory proteins isolated from culture filtrate in extra pulmonary tuberculosis. Indian J Patho Microbiol 2003; 46: 261-264

11. Nair ER, Kumar S, Reddy MVR and Harinath BC. *Mycobacterium tuberculosis* H_{37} Ra ESAS-7 an excretory-secretary antigen fraction of immunodiagnostic potential in pulmonary tuberculosis. Ind J Clin Biochem 1998; 13: 98-105.

12. Bothamley GH, Rudd R, Festenstein F and Ivanyi J. Clinical value of the measurement of *Mycobacterium tuberculosis* specific antibody in pulmonary tuberculosis. Thorax 1992; 47: 270-275.

13. Espitia I, Cervera I, Gonzalez R and Mancilla R. A 38 kDa of *Myobacterium tuberculosis* antigen associated with infection it's isolation and serological evaluation. Clin Exp Immunol 1989; 77: 373-377.

14. Thybo S, Richter C, Wachmann H, Maselle SY, Mwakyusa DH, Mtoni I, and Anderson AB. Humoral response to *Myobacterium tuberculosis* specific antigens in African tuberculosis patients with high prevalence of human immunodefiency virus infection. Tuberc Lung Dis 1995; 76: 149-155

15. Banerjee S, Kumar S and Harinath BC. Isolation and characterization of *in vivo* released 41kDa mycobacterial antigen in pulmonary and bone and joint tuberculosis and its identification with H_{37}Ra *in vitro* released antigen. Int J Tuber Lung Dis 2003; 7: 278-283.

16. Kaplan MH, Chase MW. Antibodies to Mycobacteria in human tuberculosis. I. Development of antibodies before and after antimicrobial therapy. J Infect Dis 1980; 142: 825-834.

17. Demkow U, Zielonka TM, Strzalkowski J, Michalowska- Mitczuk D, Augastynowicz – kopec E, Bialas- chromiec B, Kus J, Skopinska- Rozewska E, Zwolska Z. Diagnostic value of IgG antibody levels against 38 kDa mycobacterial antigen. Pneumonol Alergol pol 1998; 66: 509-516.

18. Demkow U, Zielonka TM, Filewska M, bialas- chromiec B, Michalowska-Mitczuk D, Zaleska J, Radziukiewicz– Byszewska D, strazalkowski J, Augustynowicz- Kopec E, kus J, Zwolska Z, Rowinska- zakrzewska E, Skopinska- Rozewska E. Value of the immunochromatographic assay for detecting IgG antibodies against 38kDa mycobacterial antigen in diagnosis of tuberculosis. Pneumonol Alergol pol 2000; 68: 355- 362.

19. ICMR Bulletin, Aug 2002. 32(8): 72.

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CONTENT

103-109 **Ca^{2+}-handling in airway smooth muscle: A historical perspective and novel develoments**..... *LJ Janssen (McMaster Univ. Hamilton)*

111-117 **Relationship between muscarinic receptor reserve and mode of excitation-contraction coupling in bovine tracheal smooth muscle**.....*S Shen/ JP Bourreau (Univ. Hong Kon,, Hong Kong)*

119-129 **The other endothelium-derived relaxing factor – a review of recent findings concerning the nature and cellular actions of endothelium-derived hyperpolarizing factor (EDHF)**.....*H Ding/ J McGuire/ C Triggle (Univ. Calgary, Calgary)*

131-136 **Actions of cardiotoxin on cytosolic free Ca^{2+} in the presence of Ca^{2+} -free environment, Ca^{2+} channel blockers and the polyamine spermine in vascular smooth muscle and endothelial cells**.....*S Cheung/ SJ Huang/ CY Kwan (McMaster Univ. Hamilton)*

137-147 **Arterial smooth muscle tone and its modification by endothelium in spontaneously hypertensive rats**.....*S Sunano/ F Sekiguchi/ K Shimamura (Kinki Univ. Osaka)*

149-162 **Diabetes-associated alterations in signal transduction pathways in smooth muscle** .....*Y Sakai/ Y Maruyama (Showa Univ. Yukohama)*

163-171 **Functional AT_1- and α_1-adrenocpetors in cultured smooth muscle cells from WKY and SHR rats**.....*ES Werstuik/ JJH Kim/ RMKW Lee (McMaster Univ. Hamilton)*

173-177 **The influence of chronic hypoxia on the functional response to endothelin of the pulmonary artery from adult rats**...*R Das/ ML Fung/ HJ Ballard (Univ. Hong Kong, Hong Kong)*

179-188 **Cuff-induced intimal thickening in the carotid artery of the marmoset**.....*B Badorrek/ B Rolfe/JH Campbell/ GR Campbell (Univ. Queensland,, Australia)*

189-196 **Differences in response of rat and rabbit arteries to injury** .....*AC Thomas/ JH Campbell(Univ. Queensland,, Australia)*

197-199 **Not superoxide but an impurity in xanthine oxidase increases endothelin binding to pig coronary artery smooth muscle**.....*AB Elmoselhi/ AK Grover (Instit. Cardiovas Sci, Winnipeg)*

201-207 **Identification of 5-HT receptor subtype mediating 5-HT-induced relaxation of porcine pial veins**.....*TJF Lee (Southern Illinois Univ. Springfield)*

209-212 **Leonurine, an alkaloid isolated from *Leonurus artemesia* induces contraction in mouse uterine smooth muscle but relaxation in vascular smooth muscle of rat portal vein**...*ZS.Chen/ CX Chen/ CY Kwan (McMaster Univ., Hamilton)*

213-218 **Barium-induced oscillatiion of tension in rat vas deferens smooth muscle**.....*Y Huang/ XQ Yao (Chinese Univ. Hong Kong, Hong Kong)*

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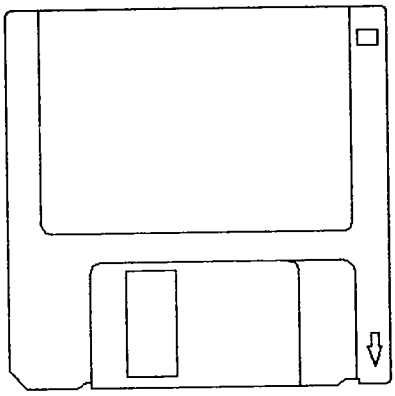

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5-10 **Effects of quaternary ammonium ions on vasorelaxation induced by endothelial and exogenous nitric oxide** .....*Y. Huang, C.W. Lau, F.L. Chan, X.Q. Yao (Hong Kong, China)*

11-16 **Scintigraphic imaging of neuroadrenergic cardiac function: An in vitro and in vivo study** .....*S.L. Nori, M.L. Calcagni, M. Martire et al (Rome, Italy)*

17-19 **Non-adrenergic non-cholinergic neurotransmission at the hamster lower urinary tract**..... *Christian Pinna (Milan, Italy)*

20-22 **P2 receptor-mediated responses in pregnant human uterus**.....*Airat U. Ziganshin, Alexander P. Zaitzev, Julia T. Zefirova, Lilia E. Ziganshina((Kazan, Russia)*

23-29 **Changes of P2X-purinoceptors in rat vas deferens and spleen caused by long term guane thidine treatment** .....*M. Zhao, P. Milner (Aberdeen/ London, U.K.)*

30-36 **The cervical sympathetic trunk- submandibular gland neuroendocrine axis: Its role in immune regulation**.....*J.S. Davisoin, A.D. Befus, R.D. Mathison (Alberta, Canada)*

37-45 **Proliferation of retinal glial (Müller) cells: Role of P2 receptors and and potassium channels**.....*A. Bringmann, F. Francke, et al* (Leipzig, Germany)

46-60 **Signal transduction pathways for $P2Y_2$ and $P2X_7$ nucleotide receptors that mediate neuroinflammatory responses in astrocytes and microglial cells** .....*F. Gendron, N.L. Newbold, P.E. Vivas-Mejia et al (Columbia/ Miami/ Puerto Rico, USA).*

61-68 **Gastric relaxation in hypertensive rat** .....*K. Shimamura, S. Kimura, F. Sekiguchi et al (Hokkaido/Higashi-Osaka, Japan)*

69-73 **Expression of carbonic anhydrase-related protein VIII, X and XI in the enteric autonomic nervous system**.....*I. Nishimori, T. Takeuchi, K. Morimoto et al (Kochi, Japan)*

74-78 **The in vivo effect of intravenous ATP on the activity of smooth muscle in the canine, rat and human stomach and intestine** .....*Lubo Kasakov, Milo Vlaskov (Sofia, Bulgaria)*

80-86 **Ontogenesis of $P2X_3$ receptor-expressing nerve fibres in the rat lung, with special reference to neuroepithelial bodies**.....*Inge Brouns, Jeroen Van Genechten, et al (Antwerp/ London, Belgium, UK)*

87-91 **Are animals a suitable model for motility disorders? Current views upon ENS development and own experiences with a FGF-knock-out model**.....*C.I. Hagl, E. Wink, K. Kränzle et al (Giessen/ Zweibrücken, Germany)*

92-99 **Three dimensional cultures of dissociated myenteric plexus as model for the development of the enteric system** .....*S. Holland-Cunz, C. Hagl, H. Krammer et al (Mannheim/ Zweibrucken, Germany)*

100-108 **The fine structure of the Giant Serotonin-containing Cells of *Helix aspersa***.....*A.H. Sathananthan, M.A. Qayyum (Melbourne, Australia)*

109-115 **Ultrastructural observations on the effects of hydroxylated tryptamines on the giant serotonin- Containing Cell (GSC) of *Helix aspersa*** ...*M.A. Qayyum, A.H. Sathananthan (Melbourne, Australia)*

CONTENTS

Biomedical Research

Volume 14 Number 2 October 2003

117-120 **Effects of alendronate on the expression of osteoclasts in the transplanted bone**.....*T. Kawata, K. Tenjo, C. Tokimasa, T. Fujita, M. Kaku, A. Matsuki, S. Kohno, K. Tsutsui, J. Ohtani, M. Motokawa, M Shigekawa, Y. Tohma, K. Tanne (Hiroshima, Japan)*

121-125 **Attraction of phagocyte to dying cells in the uterine epithelium of mouse during postpartum involution: A ultrastructural study**.....*K. Shimizu, J. Yamada (Tokyo, Japan)*

126-132 **Okadaic acid stimulates the expression of receptor activator of nuclear factor-kappa B ligand (RANKL) in mouse osteoblastic cells**.....*K. Yoshida, H. Okamura, H. Morimoto, T. Nagata, T. Haneji (Tokushima, Japan)*

133-137 **Assessment of body fat in young Indian males and females by two and four skinfold methods**.....*B. Kumar, A. K. De (Varanasi, India)*

138-146 **Expression and distribution of aromatase mRNA in developing mouse brain**.....*L.Xiao, W. Cai (Chongqing, China)*

147-150 **Fluorescence study of motor neurons of mylohyoid muscle**.....*Amin A. Rufai (Riyadh, Saudi Arabia)*

151-156 **Light microscopical details of perineuronal nets in the cerebellar cortex of the mouse revealed by means of methylene blue labelling**.....*Thomas Müller (Mainz, Germany)*

157-160 **Effect of lead acetate on wound contraction in Wistar rats**.....*S.alah Eldin O. Elsayed (Riyadh, Saudi Arabia*

161-165 **Quality of nutrition in critically ill patients**.....*M. Mojtahedzadeh, H. Khalili, M.R. Oveisi, F. Tavakolli, M. Abdollahi (Tehran, Iran)*

166-170 **The toxicity, mutagenicity and carcinogenicity of formaldehyde used in histology and histochemistry: A review**.....*AA Ngokere, PM Ofordile (Enugu, Nigeria)*

171-178 **Morphometric analysis of the lumbar intervertebral foramina in Saudi population**.....*Amin A. Rufai (Riyadh, Saudi Arabia)*

179-184 **Ultrastructural aspects of chronic iron-sorbitol overload in rat liver**.....*M. Özgüner, Ö. Dabak, N. Sayın(Ankara, Turkey)*

185-188 **Antisickling properties of *Parquetina nigrescens***.....*I. J. Kade, O. O. Kotila, A. O. Ayeleso, A. A. Olaleye, T. L. Olawoye (Akure, Nigeria)*

189-192 **Effect of hemodialysis on ventricular repolarization in patients with end stage renal disease: A hospital based study**.....*A.S. Khan, S.F. Haque, Anshul Gupta, M. Singh(Aligarh, India)*

193-198 **Referral pattern in primary care using the ICPC system and referrability rate**.....*Yousef Al-Jameel, Khalid S. AL Gelban (Riyadh/ Abha, Saudi Arabia)*

199-202 **The role of central noradrenergic system on FSH Secretion induced by excitatory amino acid** ....*S. Shahabi and A. A. Moghadamnia.* (Babool, Iran)

203-210 **Vascular endothelial Growth Factor (VEGF) and Parathyroid Hormone (PTH)/PTH-Related Peptide (PTHrP) receptor in the femoral head of the growing rat**.....*M. Hyakutake, T. Saga, K. Yamaki, M. Yoshizuka (Kurume, Japan)*

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