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ORIGINAL ARTICLE

EAR INFECTION AND HEARING LOSS AMONGST HEADPHONE USERS

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The use of headphone has been thought to cause infection in the ear canal and contribute to hearing loss. In this study, we examined 136 Customer Service Representative from Celcom (Malaysia) Sdn. Bhd. who use headphone throughout their working hours. The purpose of this study was to determine the prevalence of ear canal infection and other related diseases of the ear, nose and throat. Their hearing thresholds were also determined using the Amplaid 309 Clinical Audiometer. We found no incidence of infection of the external ear canal amongst the subjects. There were 4 cases of chronic middle ear infection and 4 cases of impacted wax. Hearing impairment was found in 25 subjects (21.2%). However, there was no significant association between hearing loss and the exposure to sound from headphone usage because the high frequencies were not predominantly affected. There was also no association between hearing loss and duration of service.

Key words : *hearing loss, headphone users*

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Introduction

The use of headphone has been thought to create aural hygiene problems and infection in the ear canal. Not uncommonly the headphone user also express concern regarding the potential for this device to cause noise induced hearing loss. However, documented studies on the side effects of prolonged use of the headphone are rarely described in the literature. Among the prolonged user of the headphone are telephonists, radio deejays and mobile compact personal stereo.

Celcom (Malaysia) Sdn. Bhd. is a major telecommunication company in Malaysia. A total number of 141 customer service representatives are working in the company. Customer service representatives function as telephonists, which uses headphone to receive phone calls from Celcom customer. They work in shift of 8 hours with one-hour break. Therefore, all of them wear headphones and receiving calls continuously for 7 hours. Isolated incidence of ear problems has been reported among these customer service representatives. It is in the interest of both the employer and the employee that a proper study is carried out to determine ear problems including hearing loss among these customer service representatives.

Objective

The objectives of this study was to determine the prevalence of infection of the external ear canal as well as other ear, nose and throat diseases among customer service representatives and the hearing threshold level of these subject using Pure Tone Audiometry. The presence of hearing loss in relation to the duration of service was also analysed.

Methodology

Population of Study

118 randomly selected customer service representatives from Celcom working in Kuala

Table 1: Sex Distribution among the subjects

Sex	No. of subjects (n)	%
Male	40	33.9
Female	78	66.1
Total	**118**	**100.0**

Lumpur offices were included in this study from August 1999 to September 1999. The age of the subjects ranged from 18 years to 35 years.

Hearing Test

Clinical Audiometer machine, Amplaid 309 was used to determine the hearing threshold level of the subjects. Hearing thresholds were investigated in the 250 Hz to 80000 Hz range, which are the frequencies important for speech perception. Results were plotted on the audiogram, which showed the hearing threshold, in decibels hearing level (dB HL) against frequency in hertz (Hz). Audiometric testing was performed using TDH-39 headphones and a bone conductor. Background noise level of the sound proof room was 30dBA.

Ear examination

All subjects were examined only by the Ear, Nose and Throat Surgeon. Specific diseases of the ear canal were looked for and documented. Examination of nose and throat were also performed.

Table II: Duration of service among the subjects

Duration of service	No. of subjects (n)
1	4
2	55
3	31
4	11
5	9
6	4
7	1
8	3
Total	**118**

Table III: Disease of the Ear, Nose and Throat

Ear findings	**No. of subjects (n)**
Impacted wax	4
Active CSOM	2
Chronic Inactive OM	2
Nose findings	**No. of subjects (n)**
Chronic rhinosinusitis	1
Allergic rhinitis	1
Throat findings	**No.of subjects (n)**
Chronic tonsillitis	1
Total	**11**

Definition

Hearing threshold was defined as the lowest intensity level at which multiple representations are detected 50% of the time (1).

Normal hearing was defined as having hearing threshold between –10 dB HL to 20 dB HL for all frequencies tested (250 Hz to 8000 Hz).

Hearing impairment was defined as having hearing threshold of more than 20 dB HL in at least one frequency.

Data analysis

The hearing threshold levels for left and right ears were analyzed separately where the hearing impairment among the subjects were determined and the frequencies of sound divided into three categories: low frequencies-250 Hz and 500 Hz, mid frequencies-1 kHz and 2 kHz, high frequencies-4 kHz and 8 kHz

Results

Demographic Data

A total of 136 customer service representatives were analyzed from Celcom Call Centre. They worked on shift duty with an average duration of 8 hours per shift with one hour break.

Table IV : Number of subjects with normal and impaired hearing

Hearing Status	No. of subjects (n)	%
Normal hearing	93	78.8
Impaired hearing	25	21.2

The headphone was used on one ear only that is the preferred ear by the subjects, over 7 hours continuously.

Sex Distribution

Table 1 shows the sex distribution of the subjects. The majority of subjects were females (66.1%).

Race Distribution

Figure 1 shows the race distribution among the subjects. Majority (91.1%) of them were Malays.

Duration of Service

The duration of service among the subjects is shown in Table 2. Majority of the subjects (47%) have been working between 2-3 years with Celcom. The longest duration of service was 8 years in 3 subjects. However, in 18 subjects the duration of service could not be determined. Therefore, the 18 subjects were excluded from the research.

Diseases of the Ear, Nose and Throat

A total of 11 subjects were found to have diseases of ear, nose and throat, as shown in Table 3.

Four subjects were found have impacted wax. Another 4 subjects were found to have chronic otitis media. In 2 of them, there were active diseases and the other 2 were inactive. All of these subjects had perforated eardrum.

There was one case each of chronic rhinosinusitis, allergic rhinitis and chronic tonsilitis.

Hearing Impairment

The total number of subjects with normal and impaired hearing is shown in Table 4.

There were 93 (78.8%) subjects with normal hearing in both ears. Only 25 subjects (21.2%) were found to have hearing impairment in either one or both ears.

The 25 subjects with hearing impairment were further analyzed. The number of subjects according to ears with hearing impairment in low, mid and high frequencies are shown in Table 5.

The numbers of subjects with hearing impairment in the low, mid and high frequencies were almost equal in the left and right ear.

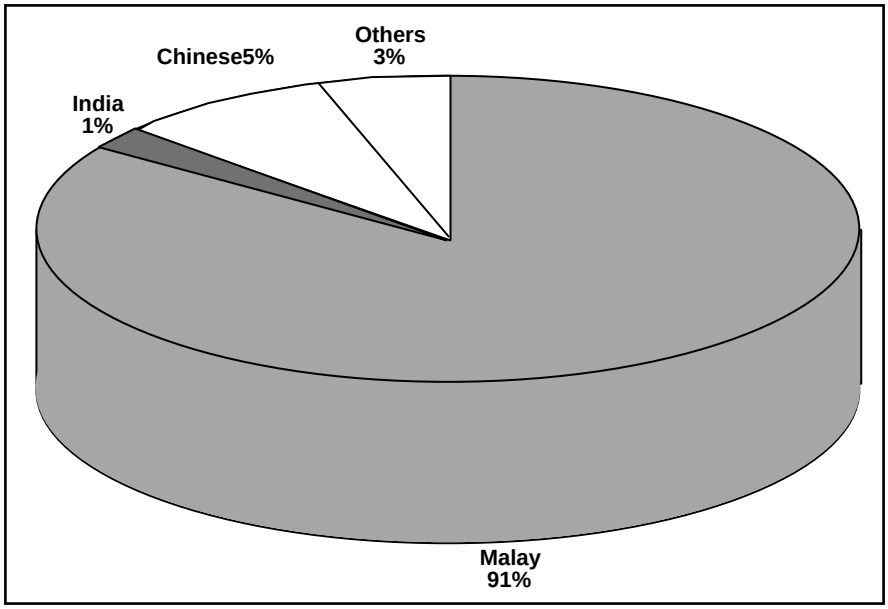
Duration of Service among the Hearing Impaired

Figure 2 shows the duration of service among the hearing impaired subjects. The majority of

Table V : Number of ear (left and right) with hearing impairment according tofrequency

Frequency	**Right Ear (n)**	**%**	**Left Ear(n)%**	
Low (.25 & .5 Hz)	24	20.3	26	22.0
Mid (1k & 2k Hz)	17	14.4	20	16.9
High (4k & 8k Hz)	19	16.1	16	13.6
Total	**60**	**50.8**	**62**	**52.5**

Figure 1: Race distribution among the subjects



subjects with hearing impairment were in the early years of service (2-3 years).

Discussion

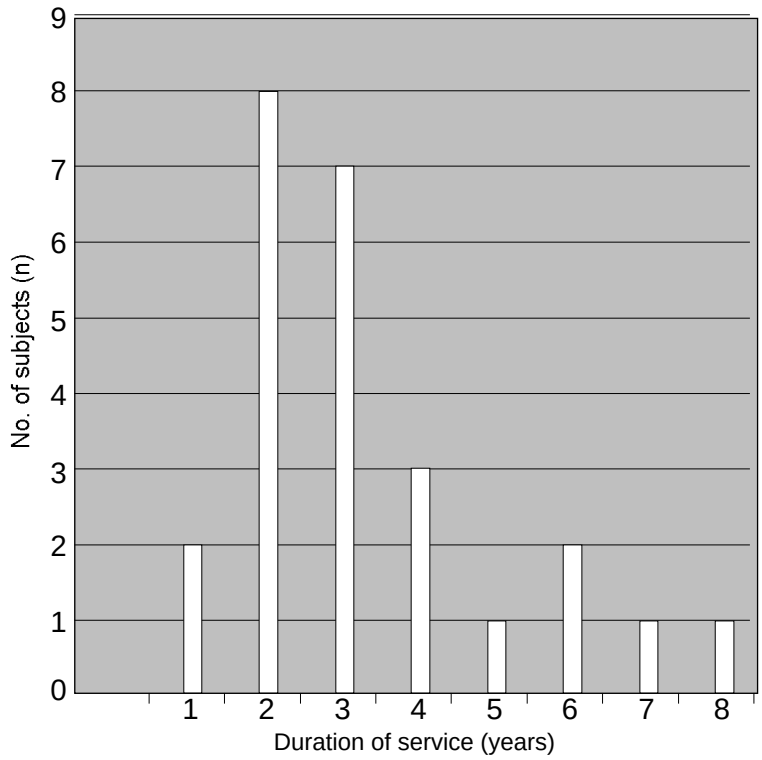
Documented instances in which headphones used by telephonists have been shown to create aural hygiene problems or are the cause for infections of the ear canal are rarely described in the literature. Nevertheless it is not uncommon for telephonists and other wearers of hearing protective devices to express concern regarding the potential for the headphones to cause ear infection.

Our study did not show any evidence of infection of the external ear canal amongst the 118 customer service representatives examined by the ENT Surgeons. Observable signs of infection that were looked for in the ear canal include swelling and reddening of the ear canal, discharge and foul odour of the ear canal. Symptoms included itching, pain and tenderness upon manipulation of the pinna and a feeling of fullness in the ear. None of the subjects had any of the above signs and symptoms. However amongst these subjects, 4 were noted to have impacted wax. Since this is a cross-sectional study with no control group, it is not possible to attribute the occurrence of impacted wax due to the use of headphones. This low percentage may represent the prevalence of impacted wax in normal population. Reports of external ear infection or impacted wax resulting directly from wearing headphones are exceedingly rare. Forshaw and Cruchley reported on a study of 60 long range patrol-aircraft crew members wearing earplugs, who were randomly divided into three groups; one wearing premould earplugs, the second using foam earplugs washed after each used, and (2) the third using earplugs washed only once per week (1). The study lasted 8 weeks and included examinations by medical officer as well as skin scrapping for bacterial culture and fungal examinations. The results indicated no fungal infection or clinically significant bacterial infections and no differences in positive bacterial cultures across the three groups of users. In another study, Cooper reported a study on 587 employees at five mid-western (United State of America) industrial facilities using otoscopy. The subjects were divided into premould earplug users, foam earplugs users and those who did not wear any device. The prevalence of external ear canal infections was less than 0.5% across all groups, with no statistically significant differences among the groups. Cooper also reported data on the presence of cerumen. The prevalence of partial cerumen blockage was 5.0% and total cerumen blockage was 5.1% (3).

Wearing headphones or earplugs has been suggested as a possible predisposing factor for external ear canal infection since their use can increase the temperature and humidity of the canal, create the potential for skin abrasion and provide a vehicle for the introduction of organisms into the canal skin (4). However, as discussed above, our study does not substantiate concern regarding the potential for headphones to increase the likelihood of developing an external ear infection.

We found 4 cases of chronic middle ear infection with perforation of the tympanic

Figure 2 : Duration of services among the hearing impaired subject



membranes. There were 2 active disease and 2 inactive diseases. These were incidental findings. It was most unlikely that the headphone is the cause of the middle ear infection without any external ear canal infection. However, in the 2 subjects with active chronic middle ear infection, they claimed that prolonged use of the headphone on the affected ear may cause itchiness and increased discharge from the ear. This was expected since it has been our experience that the use of ear mould for hearing aids in-patients with chronic middle ear infection would cause such complications. This finding has also been reported in other studies (5).

There was also concern amongst these users of headphones that prolonged use of the device may cause hearing loss. In this study, we examined the incidence of hearing impairment in the left and right ear separately by performing pure tone audiometry. A strict criteria for hearing impairment is used. Hearing impairment was defined as having hearing threshold of more than 20 dB HL in at least one tested frequency. Twenty-five subjects (21.2%) were found to have hearing impairment in either one or both ears. This prevalence was comparable to the prevalence of hearing loss in normal subjects used as controls in other studies (6).

The 25 subjects with hearing impairment in one or both eras were further analyzed to determine if the hearing impairment was due to noise exposure from prolonged use of the earphone. As one knows, noise induced hearing loss affects the 4 kHz frequency first before further affecting the other frequencies (7). This study did not show higher incidence of hearing impairment in the 4 kHz or other frequencies. In fact the number of subjects with hearing impairment in the low, mid and high frequencies were almost equal. Therefore, it was most unlikely that the use of headphone for a period of 8 hours per shift by these customer service representatives would have any effect on the hearing. This was further strengthened by our findings when comparing the association between hearing loss and duration of service. The majority of the subjects with hearing impairment were in fact in the early years (2-3 years). Thus, the longer the service and therefore more prolonged usage of the headphone does not predispose one to hearing impairment. This finding was expected since the sounds from these headphones are of low intensity. The average measurement of sound intensity from the headphone was found to be 58 dB HL. It was generally below 85 dB Hl, which was the threshold above which prolonged exposure of 8 hours or more which may caused a permanent hearing loss.

Conclusion

In this study, prolonged use of the headphones amongst customer service representatives did not predispose them to infection of the external ear canal. However in individuals who already had chronic middle ear infection, its use could cause increased itchiness and reactivation of the middle ear infection. There was also no evidence of noise induced hearing loss amongst those with prolonged exposure to the sound from headphones and the duration of service.

Recommendation

Examination of the ear and hearing test should be performed prior to employment, so that a more in-depth study can be done to establish or dismiss the association between the use of headphones and ear infection or hearing loss. Such examination should then be done yearly during employment. This will allow a reasoned approach and help to avoid an overreaction from the employee if they develop any ear infection or hearing loss. It is also useful to suggest regular hygienic wash or cleaning of the headphone in accordance with manufacturer's instruction, to prevent any reservoir of bacteria or fungus that can cause external canal infection. Sharing of headphones should be discouraged.

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ORIGINAL ARTICLE

PRELIMINARY SCREENING OF ENDOPHYTIC FUNGI FROM MEDICINAL PLANTS IN MALAYSIA FOR ANTIMICROBIAL AND ANTITUMOR ACTIVITY

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The screening of antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, yeast and fungi was carried out on isopropanol extracts prepared from 121 isolates of endophytic fungi isolated from medicinal plants in Malaysia. Sensitivity was found to vary among the microorganisms. *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Alternaria* sp. were susceptible to extracts from three, two and two isolates of endophytic fungi, respectively. None were found effective against *Salmonella typhimurium*. Sixteen endophytic fungal isolates tested were also found to exhibit antitumor activity in the yeast cell-based assay.

Key words : *endophytic fungi, antimicrobial, antitumor*

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Introduction

The nature and biological role of endophytic fungi with their plant host is variable. Endophytic fungi are known to have mutualistic relations to their hosts, often protecting plants against herbivory, insect attack or tissue invading pathogens (1-3); and in some instances the endophyte may survive as a latent pathogen, causing or quiescent infections for a long period and symptoms only when physiological or ecological conditions favors virulence (4-5). In Malaysia, extract from many types of local plants are used in traditional manner for treatments of various ailments (6-7). The question is whether they are produce by the plant itself or as a consequence of a mutualistic relationships with beneficial organisms in their tissue. Many reports showed that in a microbe-plant relationship, endophytes contribute substances that possess various types of bioactivity, such as antibacterial and antifungal. Thus in this study, we focus on the isolation of endophytic fungi and screening them for bioactivity.

Materials and methods

Isolation of endophytic fungi

A random sample from each plant consisting of asymptomatic leaves and branches was taken. Leaves and branches portion were thoroughly washed in running tap water, after which they were surface sterilized by submerging them in 75% ethanol for 2 min. The branch portions were further sterilized sequentially in 5.3% sodium hypochlorite solution for 5 min, and 75% ethanol for 0.5 min. After drying, each leaf was divided into three segments and placed on potato dextrose agar (PDA) supplemented with 50 mg/l chloramphenicol to suppress bacterial growth. Branch portions were cut to expose their inner tissue and placed on the same medium. All the plates were incubated at 27°C for up to 3 weeks. Emerging fungi were transferred to fresh PDA plates, incubated for 1 week and periodically checked for purity.

Table 1 : Endophytic fungi isolated from medicinal plants

No	Local name	Scientific name	First isolation	Second isolation
1	Asam jawa	*Tamarindus indica*	1B	b1L
2	Ati-ati	*Coleus blumei*	2L/B	-
3	Bangun-bangun/Sapooh	*Coleus camosus*	3L	-
4	Bawang putih/Garlic	*Alium sativum*	-	-
5	Bisa ular	*Barieria lupulina*	5L, 5B	b5L
6	Bunga melur	*Jasminum sambac*	6L, 6B	b6L1, b6L2, b6B
7	Bunga tahi ayam	*Lantana camara* L	7L1, 7L2, 7B	b7L, b7B
8	Cekam bumi	*Elephantopus scaber*	8L, 8ML	-
9	Cekur	*Kaemferia galanga*	9L	b9L
10	Cekur kuning	*Kaempferia angustifolia*	-	-
11	Celaka merah	*Plumbago indica*	11L1, 11L2	b11L

Continue from Table 1

12	Cemperai	*Champerela griffithii*	12L	b12L1, b12L2
13	Daun kepah	*Rhoeo discolor*	13L1, 13L2	b13L1, b13L2
14	Ervalanala	*Aerva lanata*	14L	b14L
15	Gandarusa	*Gendarussa vulgaris*	15L1, 15L2, 15B1, 15B2	b15L
16	Halia bara	*Zingiber minor*	16L, 16ML	-
17	Inai	*Lawsonia inermis*	-	b17B
18	Jarak pagar	*Ricinus communis*	18ML	-
19	Jarak untut gajah	*Jatropha podagrica*	19L	b19L
20	Jerangau	*Acorus calamus*	20L, 20ML	b20L
21	Kadok	*Piper longum*	21L, 21B1, 21B2	b21L
22	Kayu manis	*Cinnamomum zeylamicum*	22L	b22L
23	Kemangi	*Ocimum basilicum*	23B	-
24	Kesum	*Polygonum*	24L1, 24L2	b24L1,

Continue from Table 1

		minus		b24L2
25	Kucing galak	*Acalypha indica*	-	b25L
26	Kunyit putih	*Curcuma* sp.	26L, 26B	b26L1, b26L2
27	Lengkuas padi	*Languas conchigera*	17L1, 27L2	b27L1, b27L2
28	Lidah buaya	*Aloe vera*	-	-
29	Lidah mertua	*Sansevaieria trifasciata*	29L	b29L
30	Mengkudu	*Morinda citrifolia*	30L1, 30L2	b30L1, b30L2
31	Misai kucing	*Orthosiphon staminae*	-	b31L
32	Naga Buana	*Phyllanthus pulcher*	-	b32L
33	Nilam	*Coleus amboinicus*	33L	b33L
34	Pandan	*Pandanus odons*	-	b34L
35	Pasak bumi	*Andrographis paniculata*	35L	-
36	Pegaga segi	*Hydrocotyle*	-	-

Continue from Table 1

		patens		
49	Subong	*Blumae balsamifera*	-	b49L
50	Tembaga suasa besar	*Rinum aisiaticum*	50L, 50ML	b50L
51	Temu hitam	*Curcuma aeruginosa*	-	b51L1, b51L2
52	Temu merah	*Curcuma phaeocaulis*	-	b52L
53	Tongkat Ali	*Eurycoma longifolia*	-	b53L
54	Tulang-tulang	*Euphorbia tirucalli*	-	-
55	Ubi gadong	*Dioscorea hispida*	55L1, 55L2, 55ML	b55L1, b55L2
56	Ubi garut	*Maranta arundinacea*	56L	-
57	Bonglai	*Zingziber cassumunar*	-	B57L
58	Cekur manis	*Phylanthus frondosus*	-	b58L
59	Cotet mas	*Fleus jelsoidea*	-	b58L
60	Karipulei	*Murraya*	-	b60L, b60B

Continue from Table 1

		patens		
49	Subong	*Blumae balsamifera*	-	b49L
50	Tembaga suasa besar	*Rinum aisiaticum*	50L, 50ML	b50L
51	Temu hitam	*Curcuma aeruginosa*	-	b51L1, b51L2
52	Temu merah	*Curcuma phaeocaulis*	-	b52L
53	Tongkat Ali	*Eurycoma longifolia*	-	b53L
54	Tulang-tulang	*Euphorbia tirucalli*	-	-
55	Ubi gadong	*Dioscorea hispida*	55L1, 55L2, 55ML	b55L1, b55L2
56	Ubi garut	*Maranta arundinacea*	56L	-
57	Bonglai	*Zingziber cassumunar*	-	B57L
58	Cekur manis	*Phylanthus frondosus*	-	b58L
59	Cotet mas	*Fleus jelsoidea*	-	b58L
60	Karipulei	*Murraya*	-	b60L, b60B

Continue from Table 1

		koenigii		
61	Kemunting cina	*Catharantus roseus*	-	b61L
62	Lada hitam	*Piper nigrum*	-	-
63	Lemba	*Cucurtingo villosa*	-	b63L
64	Limau kasturi	*Citrus microcarpa*	-	b64L1, b64L2
65	Melati	*Telosma cordata*	-	b65L
66	Mengkudu hutan	*Morinda elliptica*	-	b66L
67	Pepulut	*Urena lobata*	-	b67L
68	Pinang makan	*Areca catechu*	-	-
69	Sambung nyawa	*Gynura procumbeus*	-	b69L1, b69L2
70	Serai kayu	*Eugenia polyantha*	-	b70L1, b70L2
71	Taji denak	*Zizyphus oenoplia*	-	b71L
72	Ulam raja	*Cosmos caudatus*	-	b72L, b72B

No endophytic fungi isolated.

Table 2 : Endophytic fungal isolates showing biological-activity against test organisms.

	Antimicrobial activity (mm)[a]				Antitumor activity (mm)[b]	
Endophytes	Bs	St	Sc	Al	UCS	UCK
12L	15	-	-	-	-	-
19L	19.5	-	-	20	-	-
22L	19.2	-	-	-	-	-
21L2	-	-	13.2	-	-	-
27L1	-	-	13.5	-	-	-
1B	-	-	-	21	-	-
5L	-	-	-	-	8	8.5
24L2	-	-	-	-	8	8
37L	-	-	-	-	9.2	9.1
41L1	-	-	-	-	8.3	8
50ML	-	-	-	-	10	10.3
b34L	-	-	-	-	7.2	7.1
b53L	-	-	-	-	8	8.1
b20L	-	-	-	-	7.5	7.5
b69L2	-	-	-	-	8.1	8
b9L	-	-	-	-	10	10.3
b30L	-	-	-	-	9	9
b7L	-	-	-	-	9.3	9.2
b70L2	-	-	-	-	8.6	8.3
b14L	-	-	-	-	8.2	8.2
b49L	-	-	-	-	8.7	8.5
b29L	-	-	-	-	8.3	8.1

[a] Test microorganisms: Bs, *Bacillus subtilis*; St, *Salmonella typhimurium*; Sc, *Saccharomyces cerevisiae*; and Al, *Alternaria sp*.

[b] Yeast test strain W303-1AY18 containing: plasmid pMR438-CyclinA Δ24-62 (UCK) or Yep51-SRX5 src (UCS).

- None detected.

Antimicrobial and antitumor activity tests

The endophytic fungi were grown at 27°C with shaking in 5 ml F-4 medium (Glycerol 40 g/l, Soy bean meal 25 g/l, Yeast extract 5 g/l, Corn steep liquor 1 g/l, NaCl 0.5 g/l) and PD-Y medium (Potato dextrose broth 24 g/l, Yeast extract 2 g/l) for 5 days. For extraction, an equal volume of isopropanol was added to the culture broth and vortexed vigorously for 1 min followed by a centrifugation at 3,000 rpm for 10 min. About 80 ml of supernatant was applied per sterile paper disc (5 mm diameter). After drying, the extract impregnated discs were used in a disc diffusion assays using *Alternaria* sp. in potato dextrose agar (PDA), *Bacillus substilis*, *Salmonella typhimurium* in PMg agar, and *Saccharomyces cerevisiae* in YPG agar as test microorganisms for antimicrobial activity. Five milliliter of spore suspension of *Alternaria* sp. grown in vegetable juice (tomato juice, 200 ml $CaCO_3$, 4.5 g; agar, 3.0 g in a total vol. of 300 ml) were used. *S. cerevisiae* was grown in YPG broth (yeast extract, 20 g/l; peptone 20 g/l; glucose, 20 g/l), and the *B. subtilis* and *S. typhimurium* were grown in PMg broth (peptone 10 g/l; $MgSO_4.7H_2O$, 2 g/l). Chloramphenicol (50 mg/ml) and nystatin (100 mg/ml) were used as positive controls.

To prepare assay plate in a 21.5 cm x 21.5 cm square plate, Yeast Nitrogen Base (YNB) broth (1.4 g) and Bacto agar (2.4 g) were dissolved in 150 ml of sterile distilled water. The pH was adjusted to 6.5 prior to autoclaving at 121°C for 15 min. When the agar is about 42°C, the following components were added: 20 ml of 50% galactose, 2 ml of 20% sucrose, 10 ml of 20x concentrated adenine (0.5 mg/ml), 2 ml of 100x concentrated histidine (2 mg/ml), 2 ml of 100x concentrated tryptophan (2 mg/ml), 2 ml of 100x concentrated uracil (2 mg/ml) or 2 ml of 100x concentrated leucine (2 mg/ml) when using the test strain UCK or UCS, respectively; 10x concentrated 4 dropout amino acid (containing each at 10x concentrated of arginine at 240 mg, methionine at 240 mg, tyrosine at 360 mg, isoleucine at 360 mg, lysine at 360 mg, phenylalanine at 600 mg, aspartic acid at 1000 mg, valine at 1500 mg and thymine at 2000 mg per 1000 ml sterile distilled water) and yeast glycerol (30-40%) stock of the yeast test strain W303-1AY18 containing either the plasmids pMR438-CyclinA D 24-62 (UCK) or Yep51-SRX5 src (UCS) (8), and poured into the square plate on a horizontal place. The 5 mm paper disc impregnated with the supernatant described above were placed on the agar plate and incubated at 30°C for 3-4 days. The growth circle around the disk which indicate positive results for anti-tumor activity was measured. Glucose (50%) was used as a positive control. All the screenings procedures were performed twice in duplicates. Inhibition zone (for antimicrobial test) or growth zone (for antitumor test) around the disk of 6 mm or more were defined as positive for biological activity.

Results and discussion

Plants have long provided mankind with a source of medicinal agents, with natural products once serving as source of all drugs (9). Though synthetic chemical also have long been used as active agents in reducing the incidence of plants, animals and humans diseases, they are costly, have potentially harmful effect on the environment and may induce pathogen resistance. Thus, biological controls or the use of microorganisms or their secretions to prevent diseases offer an attractive alternative or supplement to disease management without the negative impact of chemical control.

In natural product discovery programs, typical procedures included isolating microorganisms from samples, growing at various temperatures in a variety of selective or nonselective media and testing the extracts in a spectrum of targeted screens for activity for potential industrial or pharmaceutical applications. For a successful fungal screening, a varied and novel repertoire of either well-known or unexplored fungi is desirable. The most promising trend in isolating new fungi is the move towards investigating novel endophytes, with the idea that unusual endophytes may produce untapped natural products.

A total of 121 endophyte isolates were obtained from 62 of 72 (86.1%) different types of medicinal plants use by the local population in Malaysia (Table 1). The results of this study showed that endophyte fungi were more prevalent in the leaves (110/121 or 90.9%) than the branches. Further and more intensive samplings are necessary to clarify the fungal assemblages of the leaves and branches, as in traditional practice, the local population used mostly the extract from the leaves of the plants (6-7). Though there are still a lot of subjects to be explained in the mutualistic association of endophytic fungi and their plant host, more reports indicated the occurrence of endophytes in plants especially in relation to the possible origin of the plant metabolites detected (10-12). The culture residue of the isopropanol extract of the endophytes

cultures 12L and 22L in F-4 medium and 1B, 19L, 21L2 and 27L1 in PD-Y medium yielded impressive anti-fungal, anti-bacterial or anti-yeast activities. However, only extracts from endophytic fungal cultures of 5L, 24L2, 37L, 41L1, 50ML, b34L1, b53L, b20L, b69L2, b30L, b9l, b70L2, b14L, b49L and b29L in PD-Y broth showed positive activity for anti-tumor in the UCK/UCS yeast cell-based assay (Table 2). The basis of the anti-tumor screening using a yeast cell-based assay was that the hyperactivation of cylcin-dependent-kinase (CDK) resulted in growth arrest of the yeast harboring the genetically engineered recombinant plasmids, and a compound from the extract that can rescue the cyclinA1-induced growth arrest is viewed as a potential anti-tumor candidate.

In this study, we demonstrated that crude extracts from the culture broth of endophytic fungi grown aerobically in PD-Y or F4 medium displayed anti-bacterial, anti-fungal, anti-yeast or anti-tumor activity. These results suggest the presence of either good antimicrobial potency of the extract or of a high concentration of an active principle in the extracts of strains showing positive biological activities. Other endophytic fungal extracts which showed low anti-microbial or anti-tumor activity in the bioassay may have active compounds but probably in smaller amounts and/or the screened crude extracts could yield more potent compounds once they had undergone some purification (13). Also extracts which showed no anti-microbial or anti-tumor activity in the disc-diffusion bioassay may be active against other microbes which were not tested. Looking at the differing activity of test results obtained, additional modes of action should be explored for those isolates that do not have antimicrobial activity, as it is possible that some of these endophytes may produce substances that may ward off microbial infections by stimulating the host immune system rather than by antimicrobial activity. In addition, there is also the possibility that substances present in the extract can stimulate the growth of the microorganisms, as was evident by several isolates showing good bacterial growth forming wide zone of inhibition around the disk, thus counteracting the effect of inhibitory substances.

The observation that antibacterial and antifungal, although in crude extract, were detectable in several isolates may indicate, but not prove, that these isolates produce bioactive substances. In traditional natural products screening programs extracts that are 'hits' in a screen of interest require follow-up analysis, typically involving analytical chemists. This aspect will be further investigated as in any natural product screening, the "referm" problem (rare cultures that produce an activity of interest the first time they are grown often cannot be made to produce that activity again when they are refermented) need to be addressed to enhance production of the secondary metabolites of interest. Therefore, any information and/or research on endophyte-plant symbiosis, such as in this study is of value, especially taking into account the positive biological activity as anti-microbial and anti-tumor agents. Effective extracts could provide potential leads towards the development of novel and environmental friendly biologically active agents.

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ORIGINAL ARTICLE

ROLE OF VITAMIN E ON OXIDATIVE STRESS IN SMOKERS

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Cigarette smoke contains numerous oxygen free radicals that when inhaled, overwhelm antioxidant defenses and produce a condition of oxidative stress. This study investigated whether or not supplementation with vitamin E can affect the state of oxidative stress in healthy smokers. In this randomised double blind trial, 32 smokers received 200 mg of vitamin E or placebo daily for 8 weeks. All smokers in the vitamin E group completed the trial whilst only nine in the placebo group completed the trial. Plasma vitamin E concentrations increased significantly [P<0.02] in the vitamin E group. The release of malondialdehyde [MDA] from erythrocytes was not significantly different between the two groups at baseline and was clearly reduced [P<0.01] after 8 weeks of vitamin E supplementation. Vitamin E increased erythrocyte superoxide dismutase activity [P<0.02] and decreased gluthathione peroxidase activity [P<0.02]. No changes were detected in plasma MDA. We conclude that daily supplementation with 200 mg of vitamin E for 8 weeks improved the oxidative stress state in smokers.

Key words : *Vitamin E, smokers, oxidative stress, antioxidants*

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Introduction

Cigarette smoke has an abundance of oxidants that lead to a tremendous production of free radicals. There is overwhelming evidence from epidemiological and experimental studies showing the detrimental effects of free radicals on health. The body has the ability to produce endogenous antioxidants such as superoxide dismutase, catalase and glutathione peroxidase.

Under normal circumstances, there is a balance between these endogenous antioxidants and the production of free radicals in the body. In the instance where there are excessive free radicals, the available tissue antioxidants may become depleted, leading to oxidative damage as in the case of cigarette smoke. The most logical course to defeat smoking is the implementation of smoking cessation programmes, but given the poor success rates of these programmes, there is compelling evidence for a beneficial role of exogenous antioxidants to minimise smoking-related oxidative damage.

Compounds, which possess antioxidant properties, have the potential to decrease oxidative stress and thus may protect against smoking-induced pathology. Compounds that have been investigated are lipoic acid, taurine, ubiquinone, selenium, garlic, ginkgo biloba and polyphenols (1 – 6). Other compounds, which have been widely tried for their antioxidant properties, are vitamins such as alpha-tocopherol (7,8), and ascorbic acid (9, 10). Vitamin C doses of between 500 mg and 2000 mg have produced controversial results (9, 10). Despite these antioxidative and anti atherogenic effects, the clinical results of vitamin E supplementation in human subjects varied (7, 8). The Cambridge Heart Antioxidant Study using alpha-tocopherol showed that a higher dose of 800 iu daily did not have additional advantage over a dose of 400 iu daily (7).

Of the tocopherols, alpha-tocopherol exhibits the greatest antioxidant potential *in vivo*. Although there is no clear evidence that the antioxidant capacity of tocotrienols is superior to tocopherols, tocotrienols have been found to be more potent

antioxidants (11). Therefore, the objective of this study was to assess the effect of a tocotrienol-rich mixture on the status of oxidative stress in otherwise healthy smokers.

Methods

1) Study subjects

a) Recruitment of study subjects

Subjects were volunteers who responded to an advertisement to participate in this study. Thirty two volunteers who satisfied all the criteria for inclusion were included in the study.

b) Inclusion criteria

The criteria for inclusion were males, aged between 25 - 45 years, who are current smokers and had been smoking 20 cigarettes/day for at least 5 years.

c) Baseline investigations

All subjects underwent a clinical examination. Blood samples (10 ml) were drawn after 12 hour of fasting for lipid profile, blood sugar, liver and renal function tests. Lung function test was performed and FEV_1, FVC and FEV_1/FVC were determined. Chest X-ray and an electrocardiogram [ECG) were done.

d) Health status

All volunteers did not have a history of asthma or other respiratory illnesses. All volunteers had normal biochemical parameters, lung function test, chest X-ray and ECG.

e) Drug and additional vitamin E restriction

During the study, drugs such as aspirin and allopurinol as well as additional intake of vitamin C or vitamin E were not permitted.

2) Study design

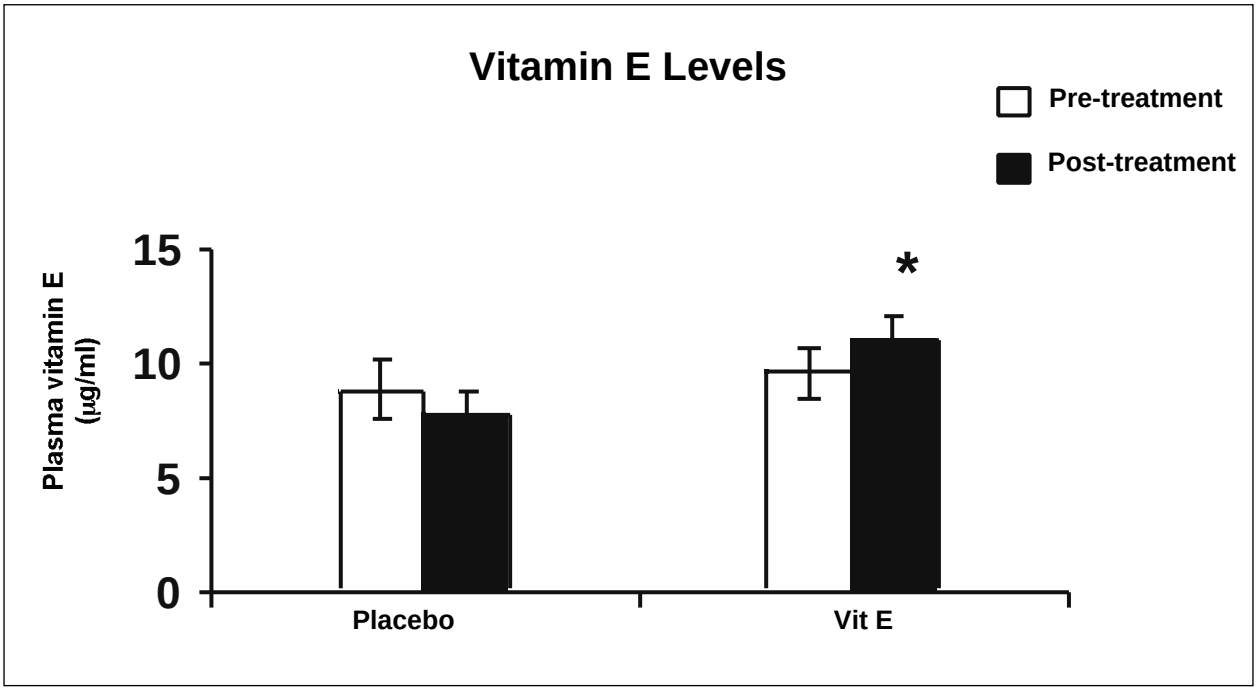
a) Ethical clearance

This study was approved by the Ethics committee of the Faculty of Medicine, Universiti Kebangsaan Malaysia.

b) Randomisation

The volunteers were randomised into two experimental groups of 16 subjects.

*Figure 1 : Effect of 8 weeks of supplementation with 200 mg daily of vitamin E or placebo on plasma vitamin E concentrations *(p<0.02). There is no difference in plasma vitamin E levels before and after treatment in the placebo group.*



c) Vitamin E dose

The volunteers were given either 200 mg soft gel capsules of vitamin E containing approximately 30 % tocopherol [TF] and 70 % tocotrienol [TT] or placebo.

d) Placebo

The placebo was visually identical to the vitamin E soft gels and contained palm olein of negligible vitamin E content.

e) Treatment and duration

The period of treatment for both groups were 8 weeks. The groups consisted of all males, with a mean age of 31.6 ± 8.9 years. All subjects signed an informed consent form, which was approved by the Universiti Kebangsaan Malaysia.

3) Chemical analyses

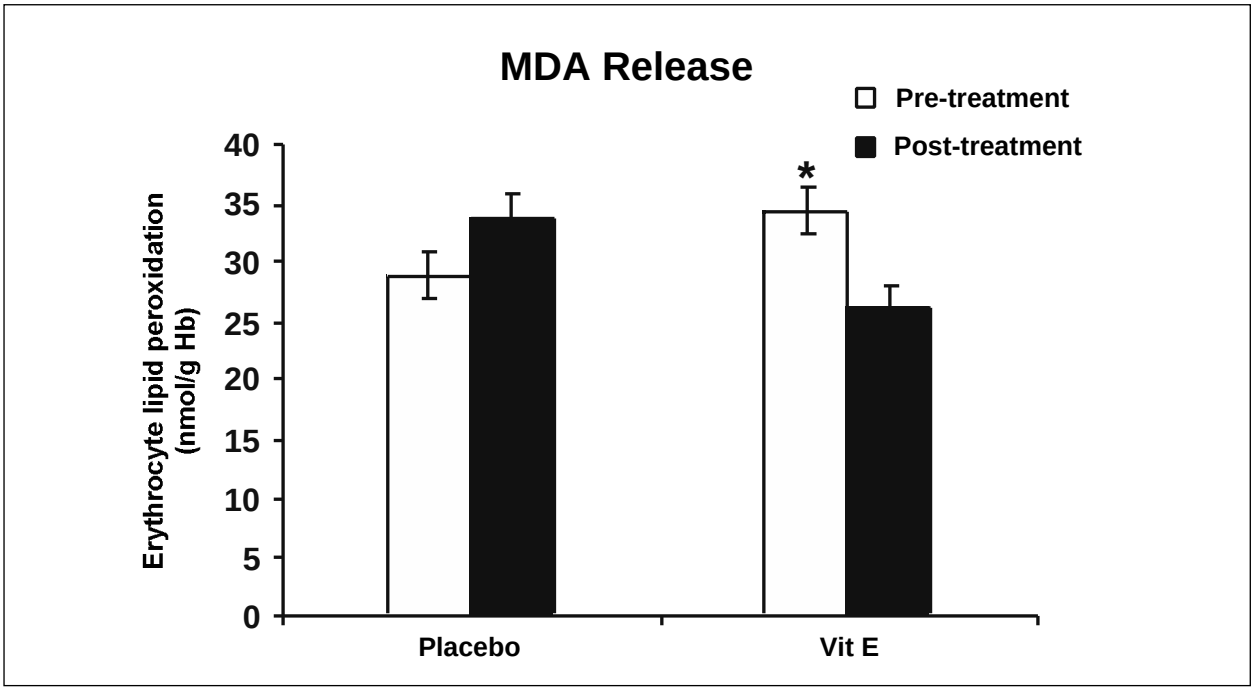
The release of MDA from erythrocytes *in vitro* was used as a functional measure of vitamin E status in the subjects. The plasma vitamin E levels were determined using HPLC. The indicators measured to reflect the status of oxidative stress were plasma MDA, antioxidant enzymes such as superoxide dismutase and glutathione peroxidase. All parameters were measured at the beginning and at the end of the treatment period.

a) Measurement of MDA release from erythrocyte

The determination of MDA release from erythrocytes *in vitro* was done according to a method described previously (12). Isolated erythrocytes were washed twice with phosphate buffered saline at a pH of 7.4. Two aliquots of 0.2 ml erythrocytes were placed in two separate test tubes. One aliquot was placed in a test tube (test tube 1) containing 3.8 ml phosphate buffered saline and the second aliquot of packed RBCs was suspended in another test tube (test tube 2) containing 3.8 ml phosphate buffered saline to which sodium azide had been added. Both suspensions were vortexed for 15 seconds.

One ml of the erythrocyte suspension was taken from test tubes 1 and 2 and mixed with 1.0 ml of 3 % hydrogen peroxide and 0.75 % hydrogen peroxide, respectively. These samples were prepared for duplicate incubation. All test tubes were vortexed for 10 seconds prior to incubation at 37°C in a shaking water bath. After an hour, 1 ml of trichloroacetic acid (TCA) in sodium arsenite was added to all tubes. Thiobarbituric acid (1 ml) was added to 2 ml of supernatant that was removed from each tube following centrifugation (3000 x g for 10

*Figure 2 : Effect of 8 weeks of supplementation with 200 mg daily of vitamin E or placebo on erythrocyte lipid peroxidation.The group supplemented with 200mg vitamin E for 8 weeks had significantly lower percentage of MDA release from erythrocyte compared to baseline values *(p< 0.01).*



minutes). These specimens were then boiled for 10 minutes in a water bath, cooled to room temperature and absorbance at 535 determined using a spectrophotometer. The concentration of MDA in the samples was obtained from a standard absorption curve for MDA.

b) Determination of plasma vitamin E levels

The sample preparation and the analysis of vitamin E by HPLC according were performed to a method described by Lang et al. (13). The Gilson HPLC system was used with a fluorescent detector. Vitamin E was detected at an excitation wavelength of 294 nm and an emmision wavelength of 330 nm. The concentration of vitamin E was determined using a standard curve.

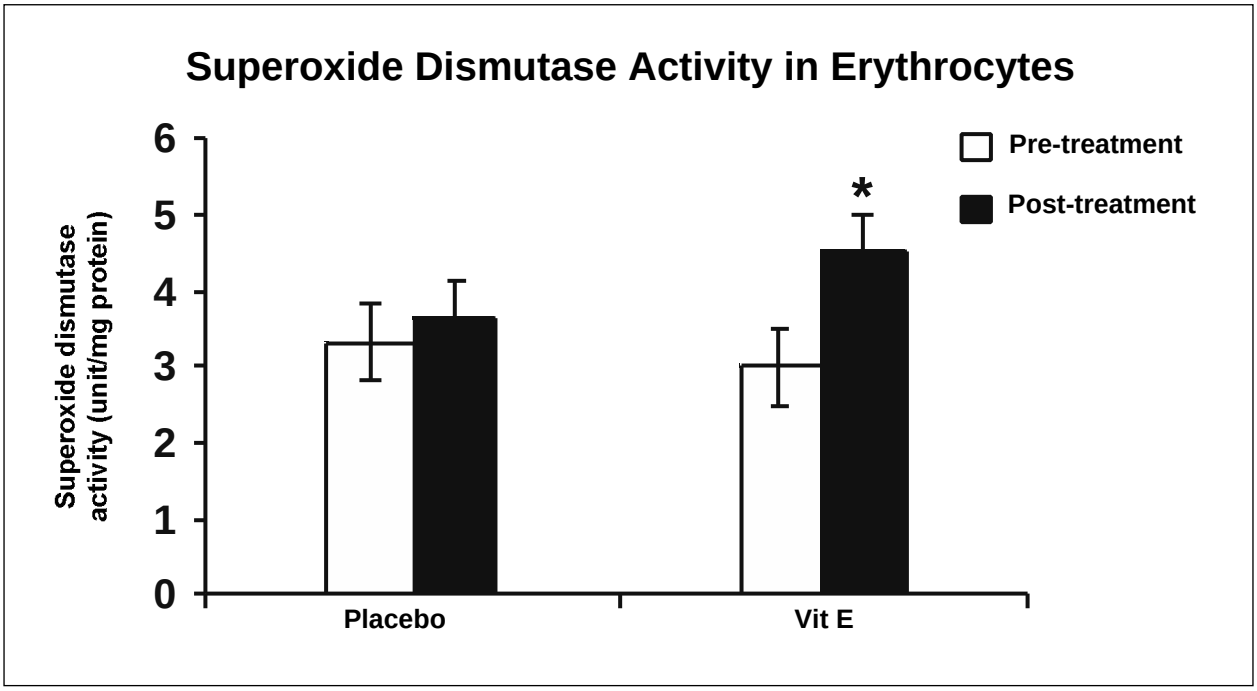
c) Determination of plasma MDA

The plasma content of MDA was determined using a method described by Ledwozyw *et al*. (14) with some modifications. A plasma sample of 0.5 ml was acidified with 2.5 ml of 1.22 M trichloroacetic acid /0.6 M HCl and left to stand at room temperature for 15 min after which 1.5 ml of 0.67% thiobarbituric acid/0.05 M NaOH was added. The samples were incubated in a 100°C water bath for 30 min. Subsequently it was left to cool at room temperature before the addition of 4 ml of n-butanol. After thorough mixing, the mixture was centrifuged for 10 min. at 1500 x g. The absorbency of the upper phase was read at 535 nm. Various amounts of MDA standard, freshly prepared by acidification of 1,1,3,3-tetraethoxypropane were subjected to the identical test procedure as the basis for constructing a standard curve for thiobarbituric acid reactivity as MDA equivalent.

d) Analysis of superoxide dismutase

The measurement of this enzyme was done in erythrocytes according to previously described methods (15). This method was based on the ability of superoxide dismutase (SOD) to inhibit the reduction of nitro blue tetrazolium whereby 1 unit of SOD was taken as a 50% inhibition of reduction of nitro blue tetrazolium. A mixture that consisted of a substrate solution (L-methionine, Triton X-100 and nitro blue tetrazolium) and 20 ml sample of lysed erythrocytes or PBS (control) were vortexed. A riboflavine mixture (riboflavine, 0.1 ml of EDTA in PBS, pH 7.8) was then added. The tubes were then placed in a box lined with aluminium paper and lighted with a flourescent lamp (20 W). After 7 minutes, the absorbance of the mixture at 560 nm

*Figure 3 : Effect of 8 weeks of supplementation with 200 mg daily of vitamin E or placebo groups on erythrocyte superoxide dismutase activity. The vitamin E group had a significant increase in the erythrocyte SOD activity *(p< 0.02) at the end of the supplementation period. There was no difference before and after treatment in the placebo group.*



was determined using a spectrophotometer (Shimadzu UV-160 A) against a blank without the sample.

e) Analysis of glutathione peroxidase

The method used was that of Lawrence & Burk (16). A 0.1 ml sample of lysed erythrocytes and a buffered solution 1 mM NaN_3, 1mM EDTA, 0.2 mM NADPH, 50 units of glutathione reductase (GSSG) and 1mM GSH, 50mM PBS, (pH 7) was incubated at room temperature for 5 minutes, then 0.1 ml 0.25 mM hydrogen peroxide was added. The absorbance of the mixture at 340 nm was determined using a spectrophotometer (Shimadzu UV-160 A) against a blank without the sample.

4) Determination of lung function

Spirometric measurements were made using a Cosmed Pony Graphic 3.5 spirometer with subjects in a sitting position. After practice blows, recordings were repeated until three satisfactory tracings were obtained. Analysis was based on the average of these three readings. The measurement that was considered in this study was the percent ratio of FEV_1 and FVC.

5) Statistical Analysis

All values are expressed as mean ± S.E.M. Wilcoxon's nonparametric signed rank test for paired observations was used for comparison of all parameters before and after supplementation with vitamin E. A p value <0.05 was considered significant.

Results

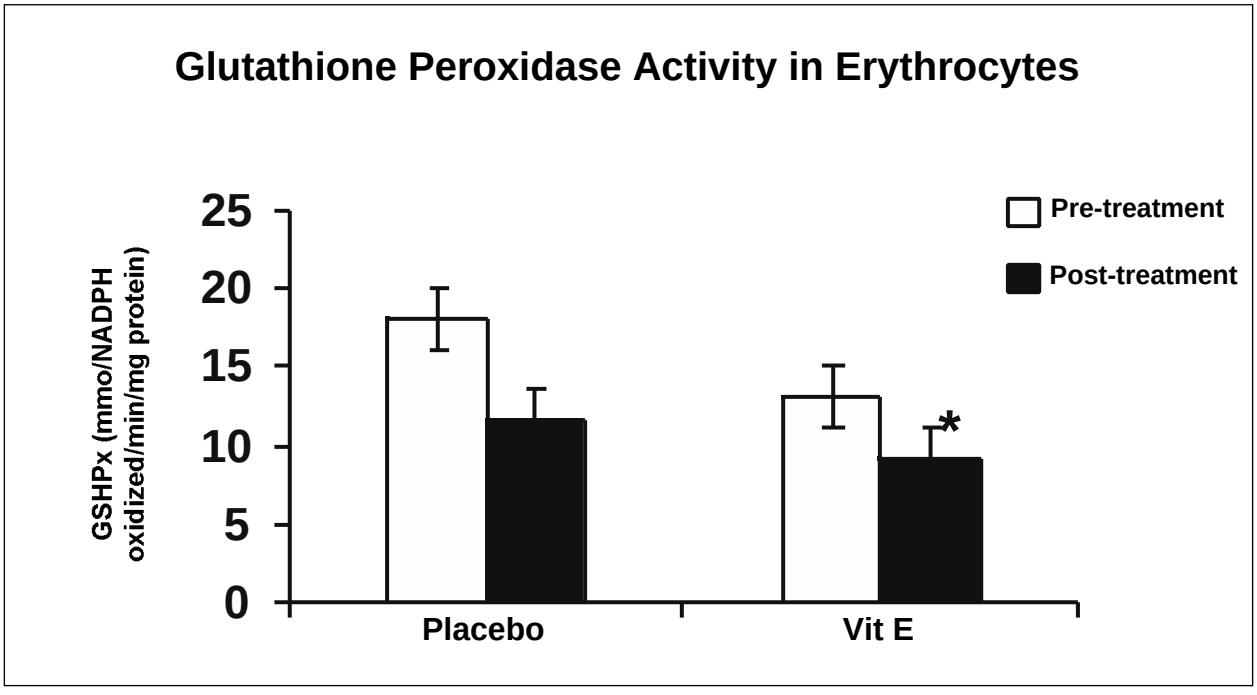
Plasma Vitamin E levels

There was no significant difference in total plasma vitamin E levels between the two groups at baseline (fig. 1). The plasma levels increased significantly ($p<0.02$) fig. 1 or fig 2 between the two groups after 8 weeks of supplementation with vitamin E. There was no difference in plasma vitamin E levels in the placebo group.

Effects of Vitamin E Supplementation on Erythrocyte Antioxidant Defence

There was no significant difference in the extent of erythrocyte susceptibility to peroxidation between the two groups at baseline (fig. 2). Vitamin

Figure 4 : Effect of 8 weeks of supplementation with 200 mg daily of vitamin E or placebo groups on erythrocyte GSHPx oxidised activity. The vitamin E group had significant decrease in the erythrocyte GSHPx activity(p< 0.02) at the end of the supplementation period. There was no difference before and after treatment in the placebo group.*



E supplementation reduced erythrocyte release of MDA compared to the respective baseline levels ($p<0.01$]. Erythrocytes from the placebo group showed a greater susceptibility to hydrogen-induced lipid peroxidation than those from the vitamin E supplemented group ($p<0.030$). Before supplementation, erythrocyte GSHPx and SOD activities were comparable. After supplementation with vitamin E, the erythrocyte SOD activity was increased ($p<0.02$) and the erythrocyte GSHPx activity decreased ($p<0.02$) compared to the respective baseline levels.

Plasma MDA Levels

There was no difference in baseline levels of plasma MDA levels between the two groups. Vitamin E supplementation did not affect the plasma MDA levels.

Lung Function Test

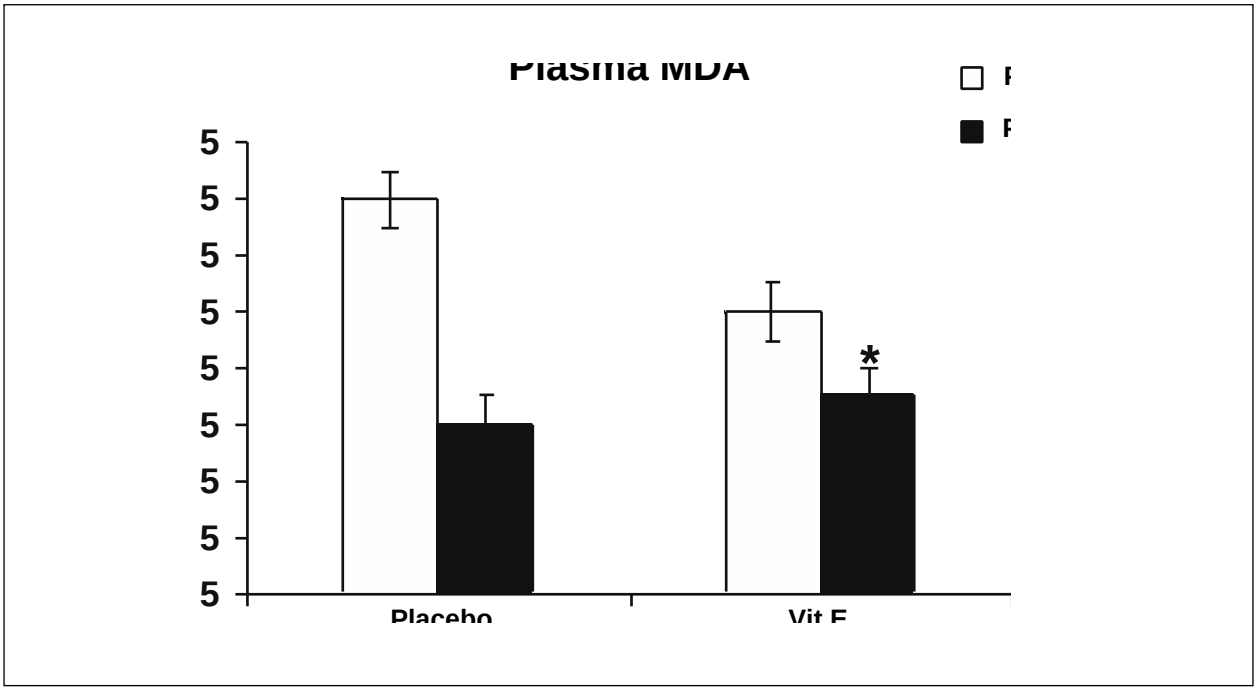
There was no difference in FVC (fig. 6), FEV_1 (fig.7) and the percent ratio of FEV_1 and FVC (fig. 8) before or after supplementation with vitamin E between the two groups. There was also no difference in the FVC, FEV_1 and the percent ratio of FEV_1 and FVC after vitamin E supplementation compared to the baseline values.

Discussion

Presently, it is widely known and accepted that free radical mediated lipid peroxidation has a crucial role in the pathogenesis of many disease processes such as atherosclerosis, diabetes mellitus, carcinogenesis, inflammation and many other conditions. Hence, the use of various antioxidant supplements to prevent or reduce damage to biological tissues are currently extensively investigated in various disease conditions. The amount of exogenous antioxidants required by individuals will be influenced by the oxidative stress status of the individual as this will affect the endogenous cellular antioxidant defence system. Antioxidant defences in the lung are provided by endogenous enzyme systems and by dietary antioxidants, particularly vitamin C and E (16 – 18). Smokers have a higher index of oxidative stress resulting from inhalation of cigarette smoke that is known to contain oxidizing substances. Consequently, smokers have been shown to have a lower antioxidant status (19 – 24) that predisposes them to developing free radical mediated lung injury.

In this study, we assessed the antioxidant defences [in individuals with high oxidant stress] in response to supplementation with vitamin E as an antioxidant and compared this response to a placebo intake. It has previously been shown that

Figure 5 : Effect of 8 weeks of supplementation with 200 mg daily of vitamin Eor placebo on plasma MDA concentration. There was no difference in theplasma MDA concentration in both groups.Fig. 6 There is no difference in FEV1 /FVC in the vitamin E supplementedgroup and the placebo group after 8 weeks of treatment.



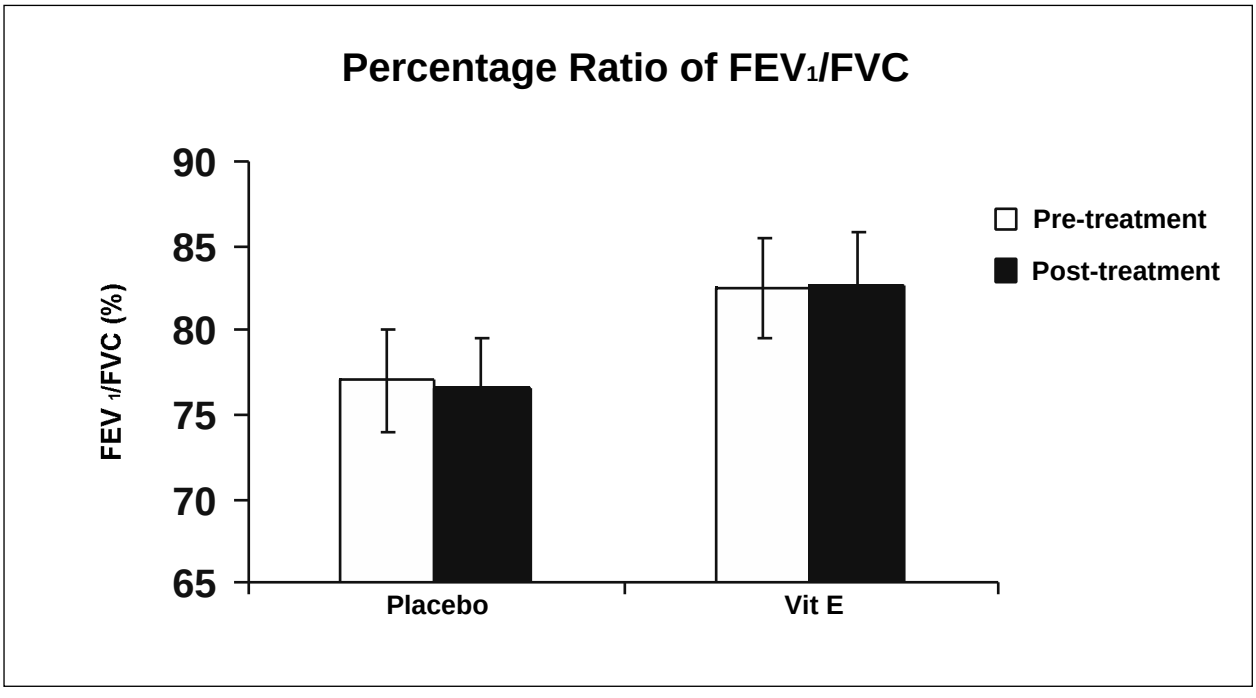
erythrocytes from smokers are more susceptible to lipid peroxidation (25). This study demonstrated that vitamin E reduces the susceptibility of erythrocytes to lipid peroxidation. The end product of lipid peroxidation, MDA was measured by fluorometric determination. Although this assay is not specific only for MDA and measures other substance that can react with thiobarbituric acid and give rise to the colored end points detected, the modifying effect of vitamin E on the generation of TBA-reacting species implies that it is a valid indicator of peroxidation *in vitro.*

It has also been shown that erythrocyte activity is increased in smokers and this may reflect an increased spontaneous regeneration of superoxide in the cells (26). We found that vitamin E supplementation can further increase the SOD. The function of SOD is to metabolise the oxygen radical (O_2^-). Vitamin E is also a scavenger for superoxide. Therefore, in the presence of vitamin E, SOD is utilized less thus the pool of SOD is increased. On the other hand, the constant intake of vitamin E ensures its continued presence and increases the process of scavenging O_2^-, thereby generating hydrogen peroxide, the substrate for GSHPx. With increased production of its substrate, the utilization of GSHPx is more. There is a consequent fall in the reserves of GSHPx as the cellular utilization of GSHPx exceeds the production of this enzyme. Other studies have shown that there is a decline in glutathione (GSH) concentration with increasing erythrocyte vitamin E concentration and this may indicate an increased utilization of GSH in the reductive regeneration of the vitamin E its peroxyl radical (25). Glutathione depletion studies in mice demonstrate that the rate of decline of total GSH in tissues and blood reflects its rate of utilization (27).

There have been various reports on the intake of antioxidants and lung function (28 –30). Although this study demonstrates that vitamin E can improve the antioxidant status, there was no improvement of lung function. Britton *et al.* showed that a combined intake of vitamin C and E correlated positively with FEV_1 and FVC. It is possible that a higher dose of vitamin E is necessary to achieve an improvement in lung function, hence the improvement seen when vitamin E intake is combined with vitamin C (30). The fat-soluble vitamin E and water-soluble vitamin C are thought to act cooperatively in a system whereby vitamin E, which is mainly sequestered in cell membranes and other lipid structures, is maintained in a reduced state by interacting with water-soluble vitamin C.

The endogenous and exogenous factors influencing the cellular protection against free radicals are many and varied. Our study suggests that one such factor is the constant availability of vitamin E in the body. Although vitamin E improved the integrity of erythrocytes membrane shown by improved protection against lipid peroxidation *in*

Figure 6 : There is no difference in FEV1 /FVC in the vitamin E supplemented group and the placebo group after 8 weeks of treatment.



vitro, it also affected the regulation of the endogenous antioxidant enzymes. Until the biological relevance of this is made clear, the recommendation of vitamin E supplements against smoking induced lipid peroxidation should be done cautiously.

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