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Electromagnetic Fields Inhibit Endothelin-1 Production Stimulated by Thrombin in Endothelial Cells

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Electromagnetic field (EMF) radiation has been found to induce arteriolar dilatation, but the mechanism of action remains largely unknown. This study investigated the effect of EMF radiation on the production of endothelin-1 (ET-1), a potent vasoconstrictor, by cultured endothelial cells. EMF radiation reduced ET-1 basal levels in human umbilical vein and microvascular endothelial cells, but failed to reduce ET-1 basal levels in bovine and human aortic endothelial cells. EMF radiation significantly inhibited thrombin-stimulated ET-1 production in all four endothelial cell types in a dose-dependent manner. EMF radiation significantly inhibited thrombin-induced endothelin-1 mRNA expression in all four cell types. The inhibitory effect of EMF radiation on ET-1 production was abolished by the nitric oxide synthase inhibitor NG-monomethyl-L-arginine (10^{-3} mol/l). These results demonstrate that EMF radiation modulates ET-1 production in cultured vascular endothelial cells and the inhibitory effect of EMF radiation is, at least partly, mediated through a nitric oxide-related pathway.

KEY WORDS: ELECTROMAGNETIC FIELDS; ENDOTHELIN-1; NITRIC OXIDE; NORTHERN BLOT HYBRIDIZATION

Introduction

Irradiation by radio frequency (RF) burst-type electromagnetic fields (EMF) has been reported to induce arteriolar dilatation in the foot web of *Xenopus laevis* (frog foot web),[1] promote circulatory arterioles in the rabbit ear,[2] and increase the production of nitric oxide and cyclic guanosine monophosphate (GMP) in the rat cerebellum *in vitro*.[3] These effects have been utilized clinically for alleviating muscular stiffness[4] with a commercially available high-frequency therapeutic device (Matsushita Electric Works Ltd, Osaka, Japan), and the effectiveness of this device for easing lumbar pain has been reported.[5,6] There is also evidence that EMF promotes bone fracture healing,[7,8] affects the immune system,[9] reduces cell differentiation,[10] and stimulates migration of endothelial cells and capillary repair in culture models.[11] High magnetic flux densities of extremely low frequency EMF are also reported to exert acute effects

on leucocyte-endothelium interactions including cell adhesion *in vivo*.[12] Recently, EMF has been reported to augment angiogenesis primarily by stimulating endothelial release of fibroblast growth factor-β-2, inducing paracrine and autocrine changes in the surrounding tissue.[13] Although the clinical effectiveness of EMF therapy has been observed and the mechanism has been shown to involve radiation-induced nitric oxide synthesis,[3] the biological effects of EMF on the vasculature remain largely unknown.

Endothelin-1 (ET-1), a novel endothelium-derived peptide, has been recognized as a locally produced potent vasoconstrictor.[14] The production of ET-1 is up-regulated by thrombin, transforming growth factor-β1, interleukin-1, tumour necrosis factor-α, adrenaline, apolipoprotein A-I, shearing stress and hypoxia.[15 - 17] Its production is down-regulated by nitric oxide, cyclic GMP, prostaglandin E_2, prostacyclin and atrial natriuretic peptide.[16,18,19] ET-1 release from normal human neuronal culture cells is dramatically reduced after exposure (5 min) to a static magnetic field generated by a 0.2 T magnetic resonance tomograph.[20] As a result of these findings, there is considerable interest in whether EMF might improve the local circulation by affecting the production of ET-1.

The present study was performed to investigate the effect of RF burst-type EMF on basal and thrombin-stimulated ET-1 expression and production in cultured endothelial cells from a variety of sources.

Materials and methods

MATERIALS

Thrombin and bovine serum albumin were purchased from Sigma Chemical Co. (St Louis, MO, USA). Dulbecco's modified Eagles' medium (DMEM) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Endothelial cell basal medium-2 (EBM-2) was purchased from Sanko Pure Chemical Co. (Osaka, Japan). Fetal bovine serum was obtained from Flow Laboratories (North Ryde, Australia). All other chemicals used were commercial products of the highest grade available.

CELL CULTURE

Bovine aortic endothelial cells (BAEC) were isolated from bovine aortas according to the previously described method,[21] and grown in DMEM supplemented with 10% fetal bovine serum in 100-mm culture dishes at 37 °C in humidified 5% CO_2. Human umbilical vascular endothelial cells (HUVEC, Lot No. 16197, from a male neonate), human aortic endothelial cells (HAEC, Lot No. 13228, from a female donor aged 58 years old), and human microvascular endothelial cells (HMVEC, Lot No. 14828, from a male neonate) obtained from Sanko Pure Chemical Co. were cultured in EBM-2 medium supplemented with 10% fetal calf serum, 50 μg/ml gentamicin sulphate, 50 μg/ml amphotericin-B, 10 ng/ml epidermal growth factor and 1 mM hydrocortisone under standard conditions[22] at 37 °C in humidified 5% CO_2. Medium was refreshed every 2 days. Cells were passaged at confluence by treatment with 0.05% trypsin/0.02% EDTA in 10 mmol/l phosphate-buffered saline, followed by two washes with medium. BAEC at the fifth to ninth passage and HMVEC and HUVEC at the fourth to sixth passage were used.

ELECTROMAGNETIC FIELD RADIATION SYSTEM

As shown in Fig. 1A, the 96-well culture plate containing endothelial cells was placed on the generator coil. The generator coil of the coil

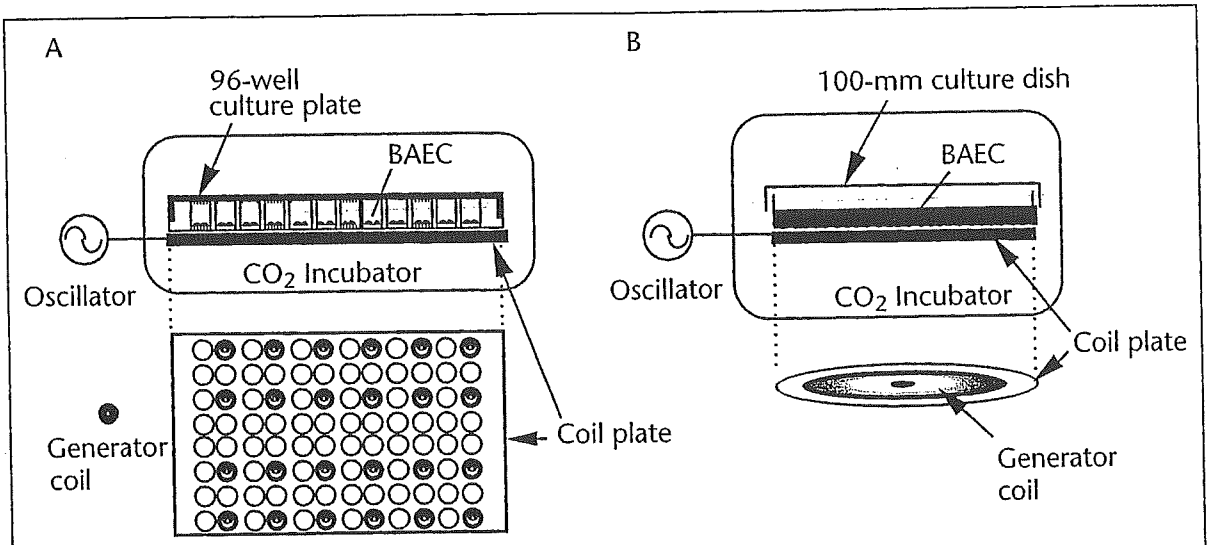

FIGURE 1: Experimental equipment used in the study. A, a 96-well culture plate containing endothelial cells was placed on the coil plate. B, a 100-mm culture dish containing endothelial cells was placed on the coil plate. BAEC, bovine aortic endothelial cells

plate consisted of a concentric nine-turn circular loop coil (i.d. 1.2 mm; o.d. 4.4 mm). The distance between the generator coil and the cells was 2.0 mm. When the amplitude of the oscillator (8116A, 50 MHz Pulse/Function Generator HPIB, Hewlett Packard, Telford, UK) was adjusted to 10 and 16 V peak to peak (Vpp), the intensity of the EMF became 1.25 V/m, 0.98 mW/kg and 1.92 V/m, 2.31 mW/kg, respectively. Fig. 1B shows the 100-mm culture dish containing endothelial cells placed on the coil plate. The generator coil of this coil plate consisted of a concentric ten-turn circular loop coil (i.d. 8 mm; o.d. 44 mm). The distance between the generator coil and the cells was 2.3 mm. When the amplitude of the oscillator was adjusted to 16 Vpp, the intensity of the EMF became 3.59 V/m and 8.06 mW/kg. The oscillator was able to emit various patterns of an oscillating burst-type EMF. As shown in Fig. 2, the frequency was fixed at 10 MHz with a pulse modulation of 10 kHz and 50% burst duty ratio. The EMF applied in the present study was established similarly in type and degree to that in the commercially available device (Matsushita Electric Works Ltd).

DETERMINATION OF ET-1 PRODUCTION

Cells released from confluent stock cultures were seeded into 96-well culture plates at a density of 10^4 cells per well. At confluence, the cells were washed twice with serum-free

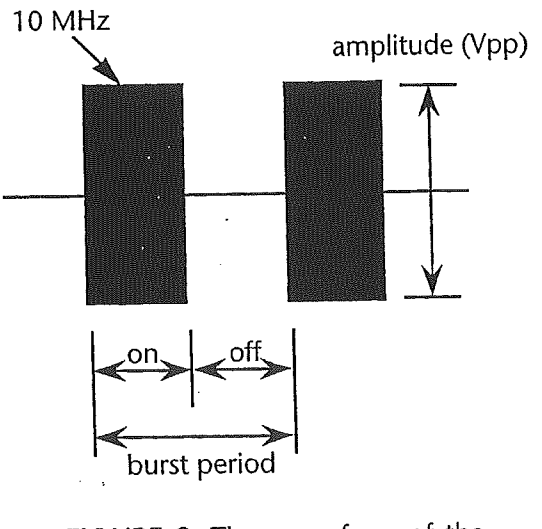

FIGURE 2: The wave-form of the generator coil. The frequency was fixed at 10 MHz with a pulse modulation of 10 kHz and 50% burst duty ratio. Burst duty radio (%) = on duration/burst period

medium and then cultured in medium containing 0.1% bovine serum albumin with or without thrombin. The responses of the cells to thrombin at doses of 0.625 – 10 U/ml were evaluated in our preliminary experiments. We found that thrombin treatment at 10 U/ml was ideal to investigate the effect of EMF. The cells were incubated with or without irradiation of burst-type EMF as described above for 8 h or 24 h. After incubation, the medium of each well was used to determine ET-1 levels using a sensitive sandwich-enzyme immunoassay as previously described.[23] The cells were then washed twice with 10 mmol/l phosphate-buffered saline, followed by the addition of 0.1 ml of 0.1 mol/l NaOH to dissolve the cells, in order to measure cell protein content by the method of Lowry *et al.*[24] using bovine serum albumin as a standard. ET-1 content was expressed in nmol/g of cell protein.

To examine whether nitric oxide may participate in the mediation of ET-1 production by EMF, NG-monomethyl-L-arginine (L-NMMA), a competitive inhibitor of nitric oxide synthase, was used to block the synthesis of nitric oxide in endothelial cell cultures. The EMF effects on ET-1 production in cultured endothelial cells were examined during exposure to L-NMMA (10^{-3} mol/l).

ANALYSIS OF ET-1 MRNA

For ET-1 mRNA analysis, BAEC released from confluent stock cultures were seeded on 100-mm culture dishes at a density of 5×10^4 cells/ml. At confluence, the medium was refreshed with DMEM containing 0.1% bovine serum albumin with or without 10 U/ml thrombin and with or without exposure to EMF radiation (16 Vpp) for 24 h. Total RNA extraction and Northern blot analysis were performed on vascular endothelial cells using 20 µg total RNA/lane, as previously described.[25] The probe used in the studies was a human prepro-ET-1 cDNA (1.17 kb) prepared from the EcoRI site of plasmid pUC18.[26,27] The membranes were rehybridized with a human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA probe (1.1 kb) (Clontech Laboratories Inc., Palo Alto, CA, USA). To correct for loading differences, the densitometric signal for each RNA sample hybridized to the ET-1 probe was divided by that hybridized to the GAPDH probe. The size of the ET-1 transcript was estimated from the positions of 28s and 18s ribosomal RNA.

STATISTICAL ANALYSIS

Results are expressed as mean ± SD. Statistical analysis was performed by unpaired Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

EFFECT OF EMF RADIATION ON ET-1 PRODUCTION IN ENDOTHELIAL CELLS

Basal and thrombin-stimulated ET-1 release from BAEC and human endothelial cells increased in a time-dependent manner (Fig. 3A). Thrombin (10 U/ml) enhanced ET-1 production above the basal levels in BAEC and human endothelial cells (Fig. 3B). EMF radiation had different effects on the basal secretion of ET-1 in the different endothelial cell types: EMF radiation (16 Vpp) reduced ET-1 basal levels in HUVEC and HMVEC, but failed to reduce endothelin-1 basal levels in BAEC; and EMF radiation enhanced the ET-1 basal level in HAEC (Fig. 4). However, EMF radiation significantly inhibited thrombin-stimulated ET-1 production in all bovine and human endothelial cell cultures in a dose-dependent manner (Fig. 4). The inhibitory effects of EMF radiation on ET-1 production in each endothelial cell culture were

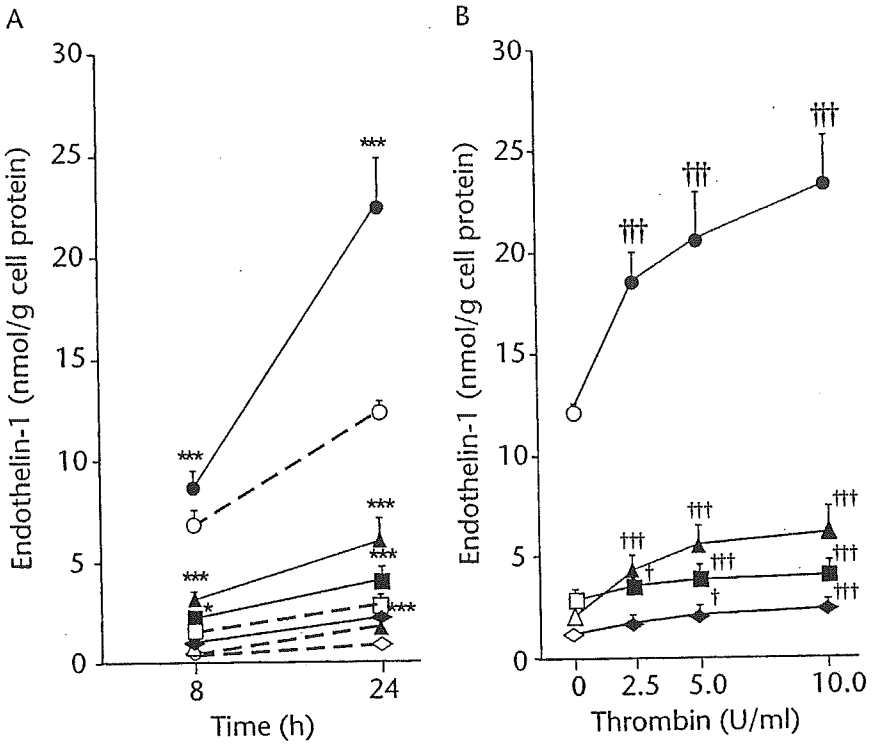

FIGURE 3: Time-dependent (A) and 24-h dose-dependent (B) secretion of endothelin-1 by cultured bovine aortic endothelial cells (BAEC: circles), human umbilical vein endothelial cells (HUVEC: squares), human aortic endothelial cells (HAEC: triangles), and human microvascular endothelial cells (HMVEC: diamonds) treated with (white symbols) or without (black symbols) thrombin (10 U/ml). Values are expressed as mean ± SD, $n = 8$. $^{*}P < 0.05$ and $^{***}P < 0.001$ versus respective basal levels at the same time point (A), and $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ versus cultures without thrombin (B)

abolished in the presence of L-NMMA, an inhibitor of nitric oxide synthase (Fig. 4).

EFFECT OF EMF RADIATION ON ET-1 MRNA LEVELS IN ENDOTHELIAL CELLS

Figure 5 shows the time course of endothelin-1 mRNA expression in response to thrombin (10 U/ml) in BAEC, HUVEC, HAEC and HMVEC. Northern blot hybridization showed that the maximal elevations of ET-1 mRNA levels after thrombin treatment were reached after 1 h in all the bovine and human endothelial cells (Fig. 5). EMF radiation (16 Vpp) did not affect basal level of ET-1 mRNA, but significantly inhibited thrombin-stimulated increase of ET-1 mRNA in all bovine and human endothelial cell cultures (Fig. 6).

Discussion

The present study demonstrated that EMF has an inhibitory effect on thrombin-stimulated ET-1 production in cultured BAEC and three types of human vascular endothelial cells – HAEC, HMVEC and HUVEC.

The main vascular effects of ET-1 are transient vasodilatation, and profound and sustained vasoconstriction, as well as proliferation of vascular smooth muscle cells.[17] Most of the ET-1 is released abluminally towards the vascular smooth muscle and less is released luminally, and it functions in an autocrine and/or paracrine manner.[17,28] The synthesis and release of ET-1 is up-regulated by thrombin,[15,17]

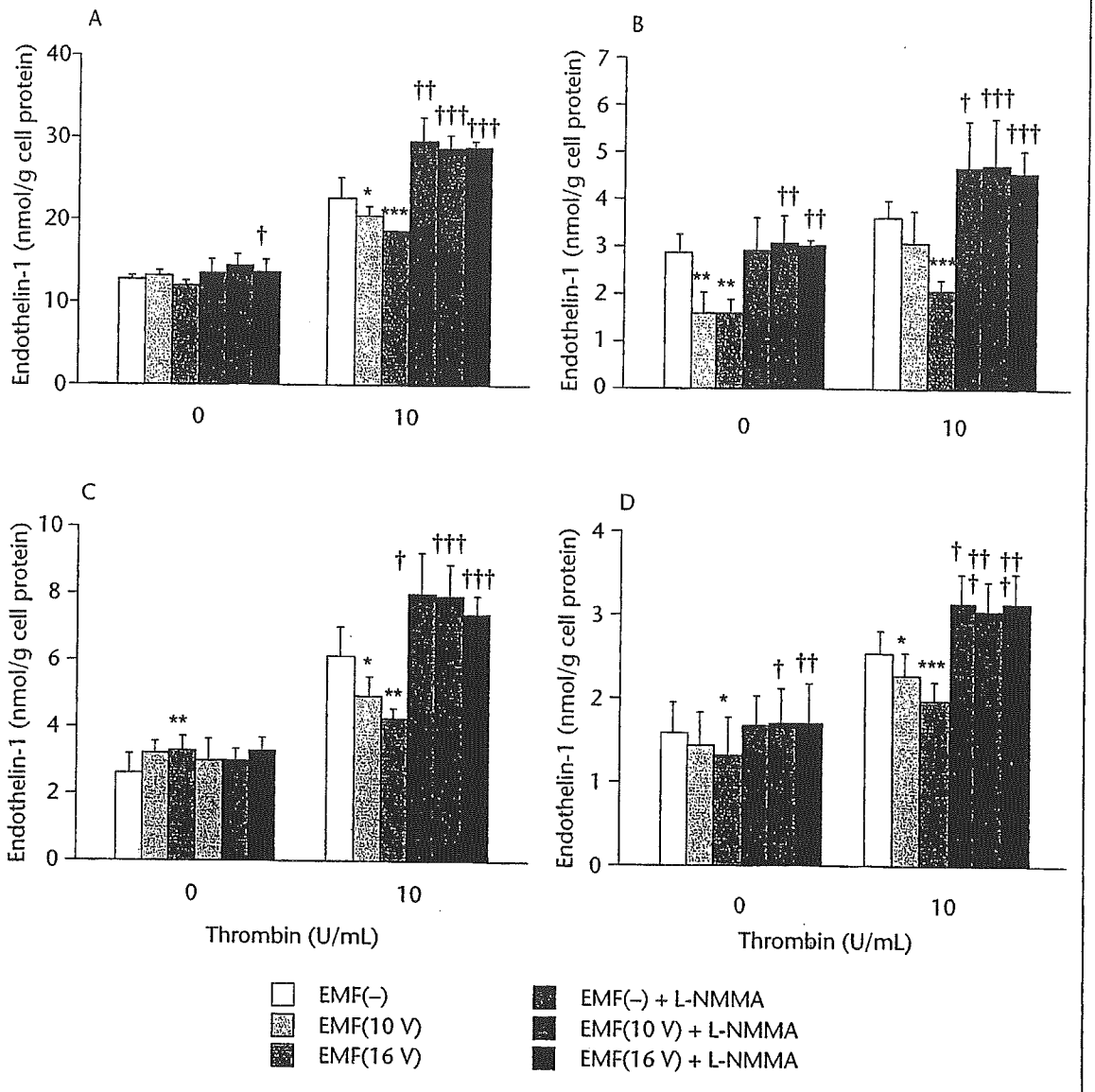

FIGURE 4: The effects of electromagnetic fields (EMF) on basal and thrombin-stimulated endothelin-1 production in cultured bovine aortic endothelial cells (BAEC, A), human umbilical vein endothelial cells (HUVEC, B), human aortic endothelial cells (HAEC, C), and human microvascular endothelial cells (HMVEC, D). Values are expressed as mean ± SD, $n = 8$, for each endothelial cell type. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus treated cultures not exposed to EMF radiation; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ versus treated cultures without addition of NG-monomethyl-L-arginine (L-NMMA)

hypoxia,[16] acidic pH[29] and certain cytokines.[15,17] The imbalance between ET-1 and other vasoactive substances may therefore be responsible for an alteration of the peripheral vascular resistance under physiological or pathological conditions. We propose that the inhibition of local production of ET-1 in the vasculature by EMF may improve local circulation. Miura *et al.*[3] have reported that EMF radiation can increase nitric oxide production in rat cerebellum, suggesting a mechanism for EMF-induced vasodilatation. Nitric oxide has been reported to inhibit ET-1 synthesis.[16,18]

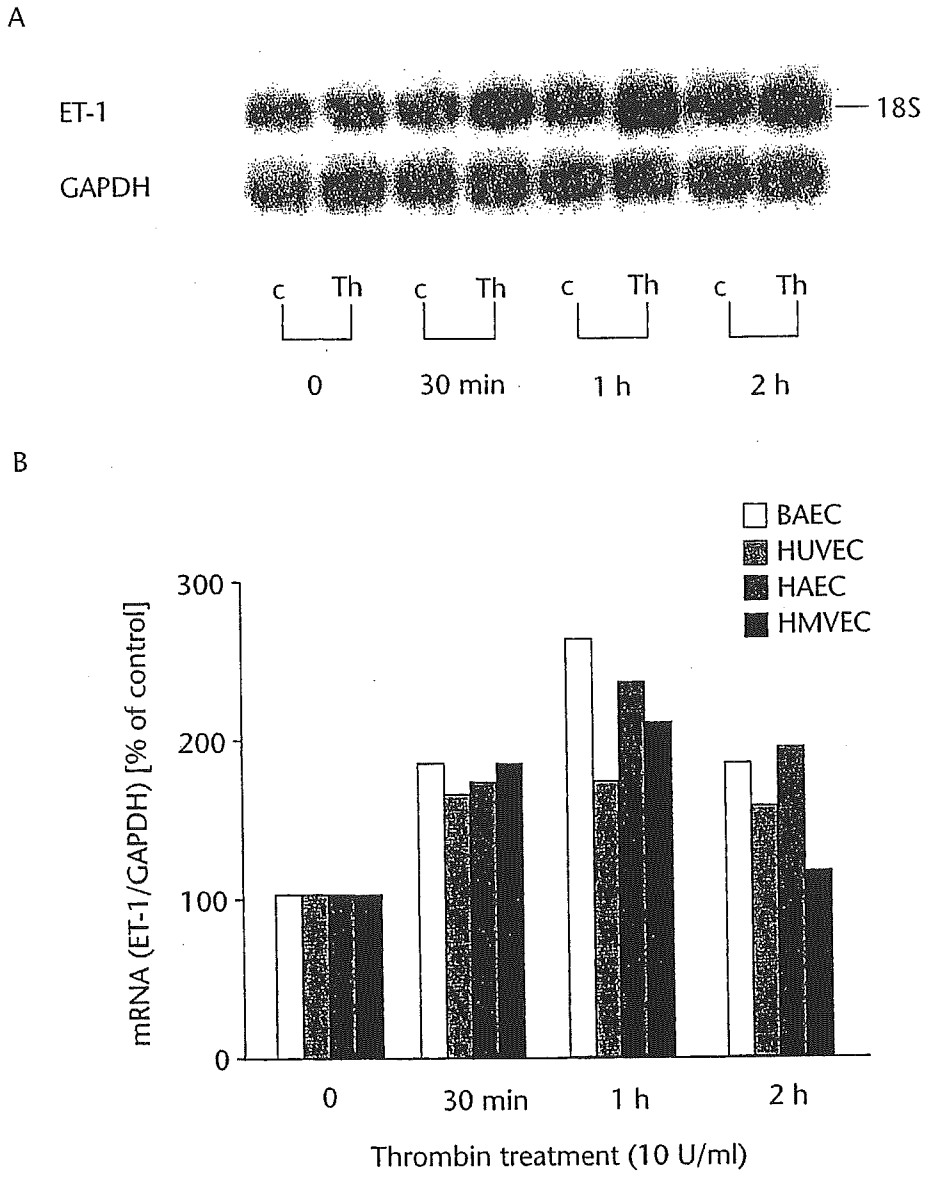

FIGURE 5: The time course of endothelin-1 (ET-1) mRNA expression in response to 10 U/ml thrombin (Th) in cultured bovine aortic endothelial cells (BAEC), human umbilical vein endothelial cells (HUVEC), human aortic endothelial cells (HAEC), and human microvascular endothelial cells (HMVEC). (A), representative blot of Th-treated HUVEC; c, control plates (vehicle only); (B), average multiples of induction of ET-1 mRNA levels in endothelial cells under control and Th-stimulated conditions over time. Data are expressed as the mean of three experiments. GADPH, glyceraldehyde-3-phosphate-dehydrogenase

The present study demonstrated that L-NMMA, an inhibitor of nitric oxide synthase, completely abolished the inhibitory effect of EMF on ET-1 production, suggesting that EMF inhibits ET-1 production through a nitric oxide-related pathway. It is reported that thrombin receptor activation increases the release of nitric oxide,[30] raising the possibility that EMF may decrease thrombin-mediated ET-1 release through augmented release of nitric oxide. This possibility was confirmed by the observation that the EMF dose-dependent suppression of thrombin-stimulated ET-1 release was completely

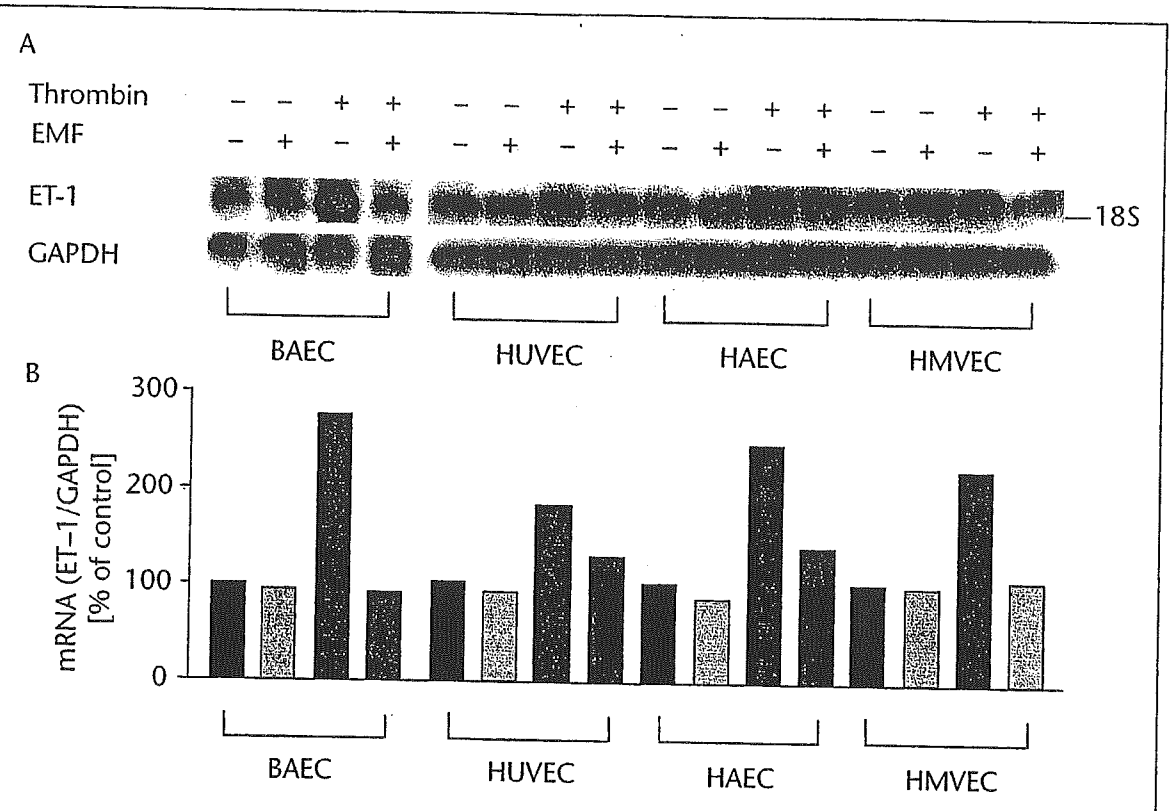

FIGURE 6: Inhibition of thrombin-stimulated endothelin-1 (ET-1) mRNA expression by electromagnetic fields (EMF) in cultured endothelial cells. Northern blot analysis was performed using 20 µg total RNA/lane: lane 1, control (■); lane 2, EMF radiation alone (▨); lane 3, thrombin alone (■); and lane 4, thrombin plus EMF radiation (■), are shown for bovine aortic endothelial cells (BAEC), human umbilical vein endothelial cells (HUVEC), human aortic endothelial cells (HAEC), and human microvascular endothelial cells (HMVEC). (A), representative blot of one of two identical experiments with a similar pattern in each endothelial cell. (B), average multiples of induction of ET-1 mRNA levels in endothelial cells. Data are expressed as the mean of three experiments

restored by the addition of L-NMMA in all the endothelial cell types studied. On the other hand, the basal production of ET-1 was not inhibited by EMF radiation in BAEC and HAEC cultures, but was inhibited in HMVEC and HUVEC. The reason for the different response to EMF radiation among the three human cell types is unclear. The HMVEC and HUVEC we used were from male neonates, while HAEC were from a female donor aged 58 years old. There is the possibility that the response to EMF radiation is age- or sex-related, or dependent upon the vascular source of the endothelial cells.

In a previous study,[15] the basal and thrombin-induced ET-1 production in endothelial cells varied according to the site of origin of the cells (microvascular endothelial cells > arterial endothelial cells > venous endothelial cells). In the present study, the basal and thrombin-stimulated ET-1 was highest in the HAEC, followed by HUVEC and HMVEC. The reason why the basal and thrombin-stimulated ET-1 production in our study was different from that in the previous study[15] remains unclear. Despite the different effects of EMF on the basal production of ET-1 among the four endothelial cell types, EMF similarly suppressed the thrombin-induced increase in

ET-1 production. These observations indicate that EMF might improve the local circulation under the vascular injury by affecting the production of ET-1.

The dose of thrombin used for this study (10 U/ml), which we also used in a previous study,[27] exerted almost maximal responses in ET-1 secretion in all of the bovine and human endothelial cell types. Moreover, the time-course of expression of ET-1 mRNA in the present study was comparable with that of our previous investigation.[27]

An interesting observation was the opposite effects of EMF on the basal secretion of ET-1 in different cultured endothelial cell types. EMF radiation substantially enhanced ET-1 secretion in HAEC, but considerably suppressed it in HUVEC. These effects suggest that EMF might reduce the shift of intravascular effusion into the extravascular space. However, EMF was also shown to inhibit the thrombin-induced increase in ET-1 production among every endothelial cell type studied.

In conclusion, the present study demonstrated that EMF radiation modulates endothelin-1 production in cultured vascular endothelial cells and that the inhibitory effect of EMF radiation is mediated through a nitric oxide-related pathway. The modulation of ET-1 production observed in the present study may represent an additional mechanism for the EMF regulation of vascular tone. Whether this mechanism could partly explain the effectiveness of EMF for clinically alleviating muscular stiffness[4] and lumbar pain,[5,6] however, requires further *in vivo* research.

Acknowledgment

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Conflicts of interest

No conflicts of interest were declared in relation to this article.

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ORIGINAL ARTICLE

Incidence of adverse drug reactions in geriatric units of university hospitals

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Background: Adverse drug reactions (ADR) in elderly people are often attributed to functional decline and polypharmacy.

Methods: In this study, a multi-institutional retrospective survey was undertaken to investigate the current status of ADR in geriatric units of university hospitals. The inpatient databases from 2000 to 2002 for five university hospitals were studied, and a total of 1289 patients were analyzed.

Results: The incidence of ADR, as determined by attending physicians, was 9.2% on average, but varied from 6.3 to 15.8% among the institutions. Factors significantly related to ADR were the number of diagnoses, the number of geriatric syndromes, the number of prescribed drugs, an increase of two or more drugs during hospitalization, longer hospital stay, emergency admission, depression and apathy.

Conclusion: These results are mostly consistent with previous reports and provide important information on drug treatment in elderly people.

Keywords: adverse drug reaction, elderly, medication error.

Introduction

Adverse drug reactions (ADR) in elderly people are common causes of admission to hospitals and are important causes of morbidity and mortality.[1,2] The risk of ADR has been shown to be related to the number of prescribed drugs and elderly people tend to receive more medications than younger people,[3] which are sometimes inappropriately prescribed.[4] Indeed, the risk of ADR is exponentially rather than linearly related to the number of medications taken.[5] Factors that predispose to pharmacological ADR include the dose, drug formulation, pharmacokinetic or pharmacodynamic abnormalities and drug interactions. Frail elderly patients may be more vulnerable because of impaired homeostatic reserve, multiple medication use, cognitive decline and impaired functional status. Drug therapy taking account of safety as well as effectiveness is still needed in the elderly, although there is accumulating evidence on drug therapy in the elderly with hypertension and hyperlipemia.[6,7]

Although the incidence of ADR for specific drugs can be obtained by large-scale examination and post-marketing surveillance studies by pharmaceutical companies, little data are available on ADR in the elderly as a whole. Previously, we reported the incidence of ADR in inpatients of the geriatric unit of the University of

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Tokyo Hospital, and showed that drug overdose and polypharmacy are important factors in ADR.[8,9] However, it is necessary to confirm whether similar results are obtained in geriatric units of other hospitals. Therefore, in this study, we analyzed the inpatient databases of five university hospitals with geriatric units, and examined the incidence of ADR and factors related to ADR.

Methods

Subjects

We performed a retrospective investigation of the hospital records of five university hospitals with geriatric units: Kyorin University Hospital, University of Tokyo Hospital, Kyoto University Hospital, Kanazawa Medical University Hospital and Tohoku University Hospital. We surveyed the records of inpatients from January 2000 to December 2002 in these hospitals, and a total of 1289 cases were used for analysis.

Investigation and analysis

We studied the incidence of ADR as judged by attending physicians during hospitalization, along with the number of medications taken on admission and on discharge. We also examined the number of final diagnoses on discharge, the length of hospital stay, age, sex and body weight of each patient, and whether or not the admission was emergent. We investigated the number of geriatric syndromes in the cases at Kyorin University Hospital and the University of Tokyo Hospital and performed comprehensive geriatric assessments (CGA). The 30 most significant of 51 geriatric syndromes are listed in Table 1. The CGA included Barthel Index on admission and discharge to evaluate activities of daily living (ADL), Hasegawa's Dementia Scale-Revised (HDS-R) to assess cognitive function, Geriatric Depression Scale 30-items (GDS-30) to assess depressive mood, and Vitality Index to assess energy.[10]

The data were expressed as means ± SD. The unpaired *t*-test was used to compare the data between two groups, and comparison among multiple groups was performed by ANOVA followed by Newman-Keuls' test. The incidences were compared using the χ^2 test. Correlation was analyzed according to Pearson's correlation coefficient. A value of $P < 0.05$ was considered statistically significant.

Results

Frequency of adverse drug reaction

In the analysis of a total of 1289 cases, the incidence of ADR was 9.2%. We analyzed the incidence at each hospital and found that the lowest incidence was 6.6%, while the highest was 15.8% among the five hospitals studied (Fig. 1).

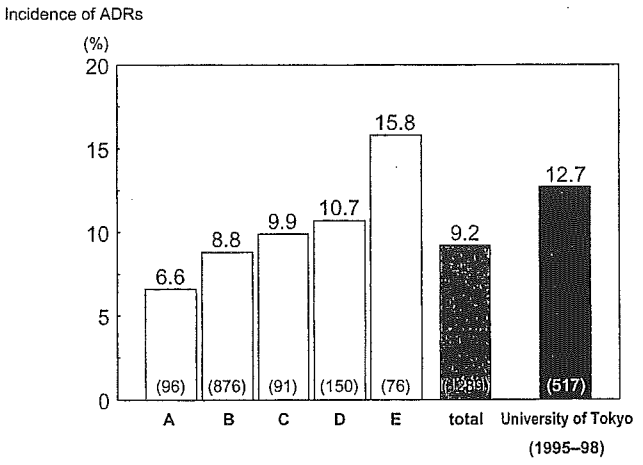

Figure 1 Incidence of ADR in inpatients of geriatric units of five university hospitals. The incidence of ADR in the geriatric unit of University of Tokyo Hospital in 1995–98 is shown as a reference.[9] The numbers of patients surveyed are shown in parentheses.

Table 1 List of major geriatric syndromes

Consciousness disturbance	Chest pain/chest oppression	Edema
Delirium	Palpitation/shortness of breath	Dehydration
Dementia	Arrhythmia	Hearing impairment
Insomnia	Abdominal pain	Motor disturbance
Depression	Constipation	Visual impairment
Dizziness/vertigo	Diarrhea	Back pain
Headache	Body weight loss	Fever
Anemia	Appetite loss	Arthralgia
Pressure ulcers	Nausea/vomiting	Osteoporosis
Falls	Malnutrition	Bleeding tendency
Hemoptysis	Dyspnea	Dysphasia
Urinary incontinence	Pollakisuria	Cough/sputum

Factors related to adverse drug reactions

Background factors related to ADR in cases with or without ADR are summarized in Table 2. There was no significant difference in sex, age or body weight between the two groups. However, patients with ADR had more diagnoses, were taking more drugs on discharge, and stayed longer in hospital than those without ADR ($P < 0.05$). They also showed a tendency to be taking more drugs on admission ($P = 0.08$). When we analyzed the relationship between ADR and the increase in medication during hospitalization, the incidence of ADR in patients with an increase of two or more drugs was 14.4%, which was significantly higher than in those with an increase of one drug (7.9%) and those without an increase (7.8%). Moreover, the incidence of ADR was higher in patients who received emergency admission than in those with scheduled admissions (12.5% vs 7.8%, $P < 0.05$).

The relationship between the factors related to ADR and the variation in ADR among the hospitals was analyzed. In hospital A, where the incidence of ADR was lowest, the number of diagnoses at discharge (2.8 ± 1.1 diseases), number of medications (4.3 ± 1.9 drugs), and the length of hospital stay (28.5 ± 6.8 days) were lowest among the five hospitals. Intriguingly, the mean age of the patients in hospital A was 82 years, while it was 67 years in hospital E, where the incidence of ADR was highest. The mean age of the patients was 71–72 years at other hospitals.

Age was positively correlated with the number of diagnoses ($r = 0.219$, $P < 0.001$) and the number of drugs at discharge ($r = 0.213$, $P < 0.001$), as previously reported.[8,9]

Geriatric syndrome and CGA were analyzed in relation to ADR in the cases at University of Tokyo Hospital and Kyorin University Hospital. The number of geriatric syndromes was significantly higher in patients with ADR than in those without ADR (Table 3). Patients with ADR showed depressed moods and apathy, as assessed by GDS and the Vitality Index, compared to those without ADR, while cognitive function and basic ADL, as assessed by HDS-R and Barthel index, did not differ between the two groups (Table 3).

Table 2 Characteristics of patients with or without adverse drug reactions (ADR)

	ADR (–)	ADR (+)
Number of patients	1170	119
Sex (female, %)	46%	50%
Age (years)	72 ± 14	73 ± 14
Body weight (kg)	56 ± 14	54 ± 14
Number of diagnoses	4.1 ± 2.0	4.9 ± 2.3*
Number of drugs on admission	5.0 ± 3.6	5.7 ± 4.1**
Number of drugs on discharge	5.3 ± 3.3	6.2 ± 3.7*
Length of hospital stay (days)	28 ± 27	38 ± 27*

*$P < 0.01$; **$P = 0.08$ by unpaired t-test.
Data are means ± SD.

Discussion

In this study, we surveyed ADR in the geriatric units of five university hospitals and found that the number of diagnoses, number of geriatric syndromes, number of prescribed drugs, an increase of two or more drugs during hospitalization, longer hospital stay, emergency admission, depression, and apathy were related to the incidence of ADR in elderly inpatients. Our study indicates that the number of diagnoses and drugs would be a better predictor for ADR in the elderly than age.

According to reports on ADR from the USA and Europe, the incidence of ADR in elderly inpatients is 6–15%.[11] The incidence was 1.5–2 fold higher in patients older than 70 years than in patients younger than 60 years. In nursing home residents, the incidence of ADR per year has been reported to be 15–20%.[11] In the outpatient setting, ADR were found in more than 10%

Table 3 Geriatric syndrome and comprehensive geriatric assessment in patients with or without adverse drug reactions (ADR)

	ADR (–)	ADR (+)
Number of geriatric syndromes	4.6 ± 3.8 (866)	6.4 ± 4.7** (85)
Barthel Index on admission	84 ± 28 (854)	80 ± 31 (82)
Barthel Index on discharge	86 ± 27 (840)	85 ± 28 (79)
HDS-R	23.0 ± 8.2 (358)	24.4 ± 6.3 (35)
GDS-30	10.2 ± 6.0 (325)	12.5 ± 6.8* (33)
Vitality index	9.0 ± 2.1 (535)	8.4 ± 2.6* (52)

*$P < 0.05$; **$P < 0.01$ by unpaired t-test. Data are mean ± SD. Numbers in parentheses indicate number of patients studied.
HDS-R, Hasegawa dementia scale-revised; GDS-30, Geriatric depression scale-30 items.

of elderly patients, although the study relied on self-reporting and review of medical records.[11] Only a few studies have been reported in Japan; the incidence was 12.7% in elderly inpatients of the geriatric unit of University of Tokyo Hospital.[9] In the present survey, the average incidence was 9.2%, ranging from 6.6 to 15.8% among facilities, but was similar to that reported previously.[9] Although the incidence varied among hospitals, it is important to note that the incidence of ADR was more than 5% in all hospitals.

Adverse drug reactions were judged by attending physicians in this study, whereas they were determined by objective review of the medical records in addition to judgment by attending physicians in the previous report from the geriatric unit of University of Tokyo Hospital. In the present study, the incidence of ADR in this facility was 8.8%, which was 30% lower than that in our last survey. This difference may be attributable to underestimation by the attending physicians rather than a decrease in ADR over this short period of 3 years. Therefore, if another authorized person judged the ADR strictly, the overall incidence rate might have been slightly higher.

Our results on the incidence of ADR in elderly patients may add important information. However, all the facilities in this survey were geriatric units of university hospitals, where most of the inpatients were older than 65 years and the doctors in those units are careful in prescribing medication to elderly patients. Therefore, our data might not be directly applicable to elderly patients in other hospitals or units. In fact, ADR were found in nearly half of elderly inpatients of the neuropsychiatry unit of University of Tsukuba Hospital (unpubl. obs, Mizukami *et al.*). In addition, our data in university hospitals, which are acute care hospitals, might not be applicable to chronic care facilities such as long-term care facilities. Since the introduction of the fixed payment system, Diagnosis Procedure Combination system, to university hospitals in Japan in 2003, drug treatment in university hospitals might be changing in the future. Therefore, the incidence of ADR in various types of hospitals in Japan needs to be studied.

In this study, depression and apathy were found to be associated with ADR in addition to the accumulation of diseases and geriatric syndromes, polypharmacy, an increase of prescribed drugs during hospitalization, longer hospital stay and emergency admission. This result is consistent with other reports.[9] However, the causal relationship remains unknown. A higher number of diseases or geriatric syndromes can lead to an increase in ADR through polypharmacy[8,9] while ADR themselves may increase diseases or geriatric syndromes. Similarly, longer hospital stays can increase the risk of ADR, while ADR prolong the duration of hospitalization. The latter point is critical to medical economics as well. Age was not associated with ADR in this study, inconsistent with other studies. This might be due to effects of education on pharmacotherapy in elderly patients for several years at university hospitals. Although we did not analyze the types or classes of ADR in this survey, it has been reported that severe ADR such as neuropsychiatric disorders or cardiovascular injury occur in elderly patients.[9]

Recently, evidence has been accumulating on drug therapy in the elderly. However, there are very few data available in people aged 75 years and older or in frail elderly people. Therefore, it is necessary to establish the safety and effectiveness of drug therapy in these patients in the future. Evidence-based medicine in the elderly aims to discontinue unnecessary drugs and to avoid polypharmacy. On the other hand, a fixed payment system such as the long-term care insurance system in Japan forces doctors to reduce prescribed drugs from a business viewpoint. Indeed, it has been reported that 0.6 drugs were on average discontinued within a month after admission to long-term care facilities, although adverse drug withdrawal events were very few.[12] Because minimally prescribed drugs have not increased ADR in patients with dementia and a low capacity for medication management,[13] it is necessary to cut down unnecessary drugs in frail elderly patients based on evidence-based medicine. In the USA, Beers' criteria are available to identify potentially inappropriate medication use, in order to reduce drug-related problems.[14] In Japan, however, we do not have such guidelines for drug treatment in the elderly. Because the drugs and medical situation in Japan are different from those in the USA, we need to establish our own guidelines, which will be published this year. In addition, we need to accumulate clinical evidence to support the guidelines. We also need to utilize pharmacists more efficiently, because they are an underused resource in avoiding medication errors and can provide important safeguards for elderly patients in hospitals and nursing homes.

Elderly patients are exposed to more medications and have an increased risk of ADR, many of which are avoidable. Knowledge of pharmacological principles and age-related effects on pharmacokinetics/pharmacodynamics is essential to promote safe prescribing. Other factors related to ADR such as polypharmacy, long admission and depression should also be evaluated during hospitalization.

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Original Article

Beneficial Effect of Brewers' Yeast Extract on Daily Activity in a Murine Model of Chronic Fatigue Syndrome

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The aim of this study was to assess the effect of Brewers' yeast extract (BYE) on daily activity in a mouse model of chronic fatigue syndrome (CFS). CFS was induced by repeated injection of *Brucella abortus* (BA) antigen every 2 weeks. BYE was orally administered to mice in a dose of 2 g per kg per day for 2 weeks before injecting BA and for 4 weeks thereafter. We evaluated daily running activity in mice receiving BYE as compared with that in untreated mice. Weekly variation of body weight (BW) and survival in both groups was monitored during the observation period. Spleen weight (SW), SW/BW ratio, percent splenic follicular area and expression levels of interferon-γ (IFN-γ) and interleukin-10 (IL-10) mRNA in spleen were determined in both groups at the time of sacrifice. The daily activity during 2 weeks after the second BA injection was significantly higher in the treated group than in the control. There was no difference in BW between both groups through the experimental course. Two mice in the control died 2 and 7 days after the second injection, whereas no mice in the treated group died. Significantly decreased SW and SW/BW ratio were observed in the treated mice together with elevation of splenic follicular area. There were suppressed IFN-γ and IL-10 mRNA levels in spleens from the treated mice. Our results suggest that BYE might have a protective effect on the marked reduction in activity following repeated BA injection via normalization of host immune responses.

Keywords: Brewers' yeast extract – chronic fatigue syndrome – daily activity – spleen

Introduction

Fatigue is a common clinical feature in subjects with various immunologic disorders or infectious diseases (1). However, the pathophysiology of fatigue status is still unclear. Several cytokines produced during the immune response to infection are described to be mediators of some symptoms including fever, somnolence, lymphadenopathy and appetite loss (2). It is suggested that these cytokines also have a role in eliciting such fatigue condition (3).

Chronic fatigue syndrome (CFS) is an incapacitating illness defined by disabling chronic fatigue and characteristic accompanying signs (4). A reduction in daily activity >50% for at least 6 months is a major criterion for diagnosis of CFS (5). Hypotheses about the etiology of CFS propose the involvement of a specific bacterial or viral infection and immune dysfunction associated with the infection. Previous research described an immunologic model of CFS induced by intraperitoneal administration of bacterial antigen; however, the duration of fatigue condition evaluated by wheel running was shown to be short (6). Ottenweller *et al.* (7) have reported the establishment of a mouse model of CFS which could be induced by *Brucella abortus* (BA) administration. In this model, the mice were found to diminish their voluntary

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activity in the running wheel after intravenously injecting the fixed killed whole BA ring test antigen (7). The advantage of using this model is that the experiment uses voluntary exertion, and that the ability to run for longer periods characterizes recovery. Thus, the BA-induced mouse model is a good one for studying the biological underpinnings of chronic fatigue. We have recently demonstrated the effectiveness of Hochu-ekki-to (TJ-41), a Japanese herbal medicine for the daily running activity in the same model of CFS (8). BA administration was also known to induce changes in cytokine expression characterized by elevated interleukin-10 (IL-10) and interferon-γ (IFN-γ) in $CD4^+$ T cells in mice (9).

Beer is a complex alcoholic beverage made from malt, hops, water and Brewers' yeast. Scientific evidence has accumulated over the past 10 years pointing to the cancer preventive potential of selected hops-derived beer constituents, i.e. prenylflavonoids including xanthohumol and isoxanthohumol, and hop bitter acids (10). Generally, a strain of Brewers' yeast such as *Saccharomyces pastorianus* or *Saccharomyces cerevisiae* is applied as yeast for beer fermentation, and after brewing, a part of the yeast is recovered from the brewing instrument and used for additional yeast products including the Brewers' yeast extract (BYE) as well as the dried yeast. This extract has been widely used as a food ingredient and a seasoning. Although BYE includes many soluble components of vitamin B group, amino acids, peptides and mineral materials, nutritional or pharmacological aspects of BYE have yet to be fully understood. Since the vitamins and microelements are frequently utilized for the prevention and therapy for CFS, BYE is a candidate of the nutritional supplement for CFS.

Puri *et al.* (11) have reported eicosapentaenoic acid-rich essential fatty acid supplementation in CFS associated with symptom remission and structural brain changes. We describe the effect of BYE on daily running activity in a murine model of CFS. In addition, the differences in pathological modification and cytokine gene expression in spleen between BYE-treated mice and control are determined.

Materials and Methods

Living Conditions

Female BALB/c mice, 8 weeks of age, were obtained from Charles River (Kanagawa, Japan), and housed singly in cages (230 × 100 × 100 mm) including running wheels (230 mm in diameter), counters showing running wheel activity and water taps, which were obtained from Natsume Seisakusho Co., Ltd (Tokyo, Japan). These cages were maintained under a light–dark photoperiod (10 h versus 14 h) provided by fluorescent bulbs fitted in the cage floor. We fed all the mice ($n = 20$) every day during the course of the experiment. Environmental air temperature was maintained at 24–25°C. The daily running activity of mice was defined as the number of wheel complete turns per 24 h. The running activity was measured at 9 o'clock when the environmental lighting was turned on. Approval for this experiment was obtained from the animal experiment committee in Kanazawa Medical University.

Induction of CFS by BA

Fixed killed whole BA ring test antigen (BA strain 1119-3, lot no. 302) was obtained from the National Veterinary Services Laboratories in the United States Department of Agriculture. CFS was induced by two repeated injections of original BA antigen solution (0.2 ml per mouse) via the tail vein every 2 weeks (8). In the pilot experiment, we had already confirmed that the mice with a single administration of BA showed less running activity for 2–3 weeks after injection and recovery from the reduced activity thereafter (8). The criteria for establishing induction of CFS were statistically significant decreases of the running activity after first or second BA injection as compared with the baseline levels.

Treatment of Mice with Brewers' Yeast Extract

We obtained BYE (lot no. 909031) from Asahi Breweries, Ltd (Tokyo, Japan). The components in BYE are shown in Table 1. This agent was dissolved in distilled water and diluted with water to the appropriate concentration. The BYE solution was administered orally in a dose of 2 g per kg once daily through a feeding cannula inserted down the throat of the

Table 1. Components of brewing yeast extract used in the study

Components	Amount (per 100 g extract)
Vitamin B_1	13.11 mg
Vitamin B_2	5.10 mg
Vitamin B_6	4.73 mg
Pantothenate	4.48 mg
Zinc	6.76 mg
Glutathione	180 mg
Arginine	4.66 g
Lysine	5.36 g
Histidine	1.65 g
Phenylalanine	2.60 g
Tyrosine	0.83 g
Leucine	3.95 g
Isoleucine	3.06 g
Methionine	1.03 g
Valine	3.89 g
Alanine	4.55 g
Glycine	3.25 g
Proline	2.42 g
Glutamic acid	7.44 g
Serine	3.12 g
Threonine	3.07 g
Asparaginic acid	6.90 g
Tryptophan	0.87 g
Cystine	0.58 g

mice ($n = 10$) for 2 week before the induction of CFS and for 4 weeks thereafter. The dose of the BYE was determined on the basis of findings of another research applying the same BYE for the fatigue mice (12). Untreated mice ($n = 10$) were given saline during the same period. The mice in this experiment were randomly assigned to the BYE-treated or the control group.

Daily Running Activity, Body Weight and Survival in Mice

We started to examine the running activity during 2 weeks at baseline levels after 2 weeks of housing, since the activity was stabilized after 2–3 weeks of housing (7,8). Daily activity during 2 weeks after each injection of BA was evaluated in the mice receiving BYE as compared with that in the untreated mice. We measured body weight (BW) in both groups weekly from the start of treatment with the extract to the end of experiment. Survival in both groups was also monitored during the observation period.

Organ Weights and Pathological Examination in Spleen and Thymus

The mice in both groups were sacrificed by cervical dislocation 4 weeks after the first BA injection. Ratios of spleen weight (SW) (mg) to BW (g) (SW/BW), thymus weight (TW) (mg) to BW (TW/BW), heart weight (HW) (mg) to BW (HW/BW) and lung weight (LW) (mg) to BW (LW/BW) as well as the weights of the organs and the body were assessed between both groups at the time of sacrifice. One half of spleen and thymus was fixed in 10% buffered formalin and stained with hematoxylin–eosin; the other half was frozen and stored at −80°C until analysis of cytokine gene expression. We performed measurements of the splenic lymphoid follicular area and thymic medullary area to be expressed as a percentage of total splenic or thymic area in the long-axis sections (13). The splenic follicular area and thymic medullary area were examined in a normal female BALB/c mouse. Each tissue was evaluated blindly by an experienced pathologist who had no knowledge of the study design.

Expression Levels of INF-γ and IL-10 mRNA in Spleen

RNA extraction for each frozen splenic tissue was performed as described by the manufacturer (RNeasy Mini Kit, QIAGEN Inc., Tokyo, Japan). Procedure of DNase was performed during the RNA extraction to avoid DNA contaminations. The total RNA concentrations were determined by measuring the optical density at 260 and 280 nm. Aliquots of 20 μl RNA from each tissue were applied for production of cDNA. Comparative expression levels of IFN-γ (proinflammatory cytokine) and IL-10 (anti-inflammatory cytokine) mRNA in spleens from both groups were determined by using real-time quantitative RT–PCR as described previously (7,8). We applied TaqMan MGB Probe (Applied Biosystems Inc., CA, USA) for the RT–PCR. Commercially available kits for IFN-γ and IL-10 RT–PCR (Mm00801778_m1 and Mm00439616_m1, Applied Biosystems Inc.) were used. Each threshold cycle number up to 50 cycles (C_t value) within the RT–PCR was examined for the IFN-γ and IL-10 mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous internal standard, and was amplified with specific primers for the number of cycles. A negative control without template cDNA was always included. ΔC_t values referred to differences between the C_t values for each target gene and the GAPDH gene. After confirming that efficiencies of amplification of each molecule and GAPDH transcripts were approximately equal, amount of the IFN-γ or IL-10 transcript relative to the GAPDH transcript was determined using the comparative C_t method described in Perkin Elmer Applied Biosystems User Bulletin #2 (1997). Data are expressed as fold-increases relative to the baseline (value = 1) in spleen from a normal female BALB/c mouse.

Statistical Analyses

Data are expressed as mean values ± SD. Significant changes of the activity after first or second BA injection as compared with the baseline levels were evaluated by the paired Student's t-test. Data differences between the mice treated with the yeast extract and the control were analyzed by the unpaired Student's t-test. A P-value of <0.05 was considered to be statistically significant.

Results

Daily Running Activity, Body Weight and Survival in Mice

Daily running activity in both mouse groups before and after repeated BA injection is indicated in Fig. 1. Significant decreases of the activity after first or second BA injection as compared with the baseline levels were observed in the treatment and control groups, showing the induction of CFS. The baseline activity and the activity during 2 weeks after the first BA injection were not significantly different between both groups (Fig. 1). However, the activity during 2 weeks after the second BA injection was significantly higher in the group treated with BYE than in the control (8966 + 1647 versus 4821 + 1355, respectively, $P < 0.05$). There was no significant difference in BW between both the groups through the observation course, although a transient decline in BW was found in the control (Fig. 2). Two mice in the untreated group died 2 and 7 days after the second BA injection, whereas no mice in the group treated with BYE died.

Organ Weights and Pathological Examination in Spleen and Thymus

Ratios of each organ to BW as well as BW, SW, TW, HW and LW in both groups are shown in Table 2. Significant reduction in SW was found in the mice treated with BYE as compared with that in the control (329 ± 73 mg versus 517 ± 98 mg,

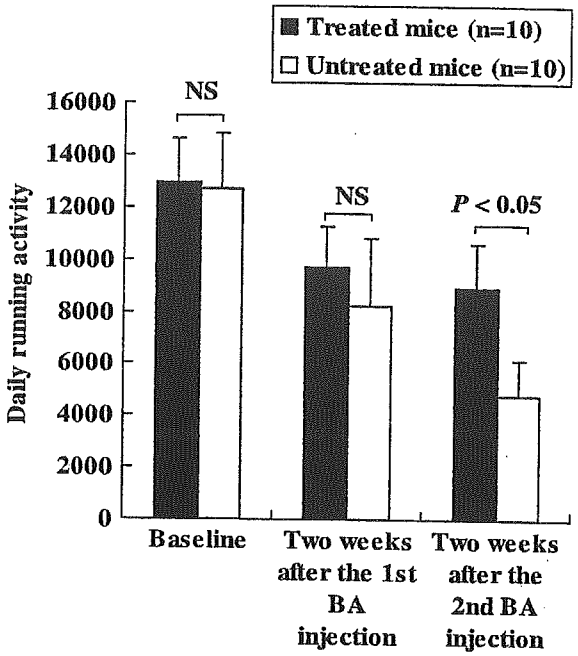

Figure 1. Effect of brewing yeast extract on daily running activity in a mouse model of chronic fatigue syndrome. Data are expressed as means ± SD. NS, not significant; BA, *Brucella abortus*. Daily running activity was defined as the number of wheel turns per 24 h. Asterisk represents two mice in the untreated group died 2 and 7 days after the second injection of BA, while no mice in the treated group died.

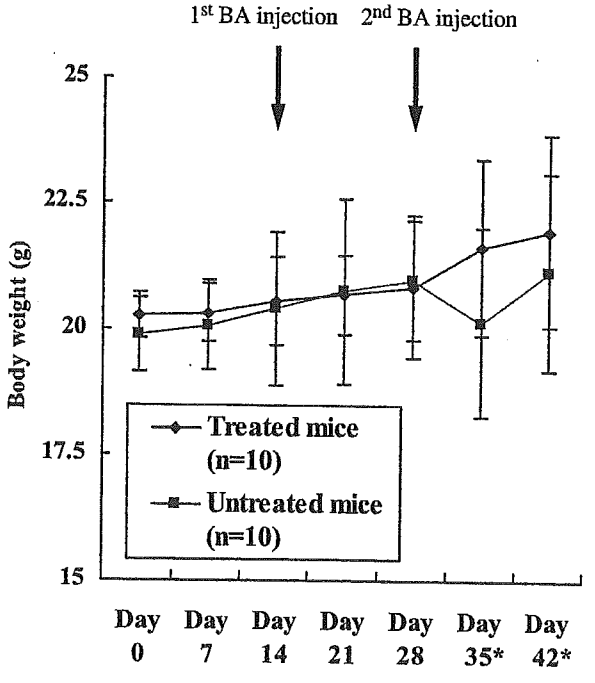

Figure 2. Weekly variation of body weight between different mice from the start of treatment with brewing yeast extract to the end of experiment. Data are expressed as means ± SD. NS, not significant; BA, *Brucella abortus*. Asterisk represents two mice in the untreated group died 2 and 7 days after the second injection of BA, while no mice in the treated group died.

Table 2. Effect of brewing yeast extract on body weight and weight of organs including spleen, thymus, heart and lungs at the time of sacrifice

	BW (g)	SW (mg)	TW (mg)	HW (mg)	LW (mg)
Treated mice	22.0 ± 1.9	329 ± 73*	44 ± 7	114 ± 15	158 ± 22
(n = 10)		(15.0 ± 3.4*)	(2.1 ± 0.2)	(5.2 ± 0.7)	(7.2 ± 1.0)
Untreated mice	21.2 ± 2.0	517 ± 98	42 ± 4	111 ± 10	152 ± 20
(n = 8)**		(24.5 ± 4.7)	(2.0 ± 0.2)	(5.1 ± 0.5)	(7.1 ± 0.9)

Data are expressed as means ± SD. BW, body weight; SW, spleen weight; TW, thymus weight; HW, heart weight; LW, lungs weight. Parentheses show ratio of each organ weight to body weight.
*$P < 0.05$ compared with spleen weight in the untreated mice.
**Two mice in the untreated group died 2 and 7 days after the second injection of BA.

respectively, $P < 0.05$), whereas there were no differences in the weights of other organs between both the groups (Table 2). SW/BW ratio was significantly lower in the group treated with BYE than in the untreated group at the time of sacrifice (15.0 ± 3.4 versus 24.5 ± 4.7, $P < 0.05$), while TW/BW, HW/BW and LW/BW ratios were not significantly different between both the groups (Table 2). The percent follicular area and medullary area were 31 and 28% in spleen and thymus of the normal female BALB/c mouse. Significantly, increase of splenic follicular area was observed in the treated mice as compared with that in the untreated group (24 ± 8% versus 14 ± 6%, respectively, $P < 0.05$). As shown in Fig. 3B, the lymphoid follicles were found to be impaired in spleens of the untreated mice. However, there was no difference in thymic medullary area between both the groups (Fig. 3A).

Expression Levels of IFN-γ and IL-10 mRNA in Spleen

Comparative expression levels of IFN-γ and IL-10 mRNA in spleen are shown in Fig. 4A and B. The IFN-γ and IL-10 mRNA levels in spleens of the mice treated with BYE were significantly lower than those in the control (7.67 ± 5.97 versus 13.87 ± 4.77 and 5.18 ± 1.36 versus 8.45 ± 1.94 in IFN-γ and IL-10 mRNA levels, respectively, $P < 0.05$).

Discussion

Vitamins and microelements such as high amounts of vitamins B, K and essential amino acids are very important for the prevention and treatment for individuals with fatigue. In a clinical study, a nutritional supplement 'Nagipol' made on the basis of Brewers' yeast in Russia was applied for the prevention and treatment of CFS patients (14). The results showed that this food was useful in CFS because of the clinical status improvement, positively influencing cognitive CNS functions and symptoms of emotional instability and normalizing blood biochemical parameters, suggesting recommendation of the food as preventive medical dietetic means for this pathology. We have also found the beneficial effect of BYE on daily running activity in a murine model of CFS. It might be worth

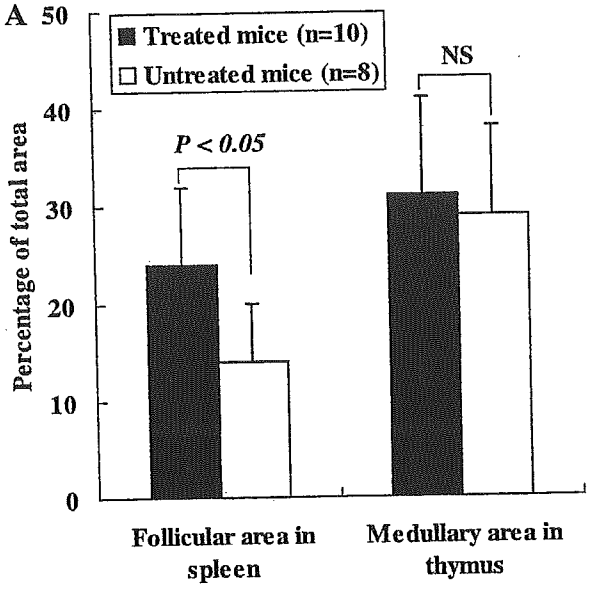

Figure 3. Percentages of splenic lymphoid follicular area and thymic medullary area between different mice (**A**) and pathological findings of the follicular area in spleen (**B**, upper image in the treated mice and lower image in the control). Data are expressed as means ± SD. NS, not significant; BA, *Brucella abortus*. Arrows show the follicular area in the spleen.

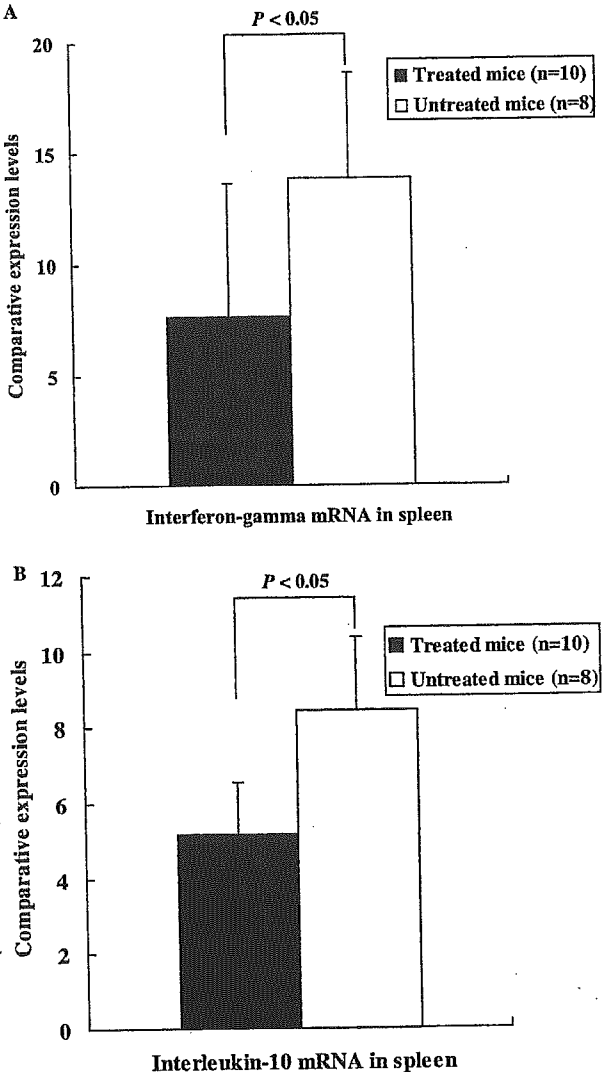

Figure 4. Comparative expression levels of interferon-γ (INF-γ) (**A**) and interleukin-10 (**B**) mRNA in spleen between different mice. Data are expressed as means ± SD. The INF-γ and interleukin-10 mRNA levels were calculated as comparative values, which were normalized to the cytokines' mRNA in the spleen from normal female BALB/c mouse (value = 1).

investigating the effectiveness of BYE on symptoms in the CFS or fatigue subjects with other various disorders in future clinical study.

Previous study reported clinical aspects of 21 individuals with chronic brucellosis (15). Seventeen patients suffered from chronic disease and had no history of any acute episode of brucellosis. The most common symptoms in the patient population were tiredness, fatigue, depression, arthralgia and muscular pains. Most of these subjects had already been receiving psychiatric treatment. Clinical examination was largely negative, but lymphadenopathy was found in nine cases. Based on these clinical manifestations, the fatigue induced by BA administration which was used in the present research might be applicable to a mouse model of CFS.