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Outpatient Clinical Building of The University of Tokyo Hospital, Tokyo, Japan

Cherry blossoms surrounding the Outpatient Clinical Building of The University of Tokyo Hospital, which boasts of the largest number of both inpatients and outpatients and the highest medical practice revenue among national university hospitals in Japan. SAKURA (桜, さくら) is the Japanese name for cherry trees and their blossoms. Annually, the Japanese go to parks, shrines and temples with family and friends and hold a "flower viewing party" known as "HANAMI" (花見). "HANAMI" signifies the beauty of the cherry blossom and gives the otherwise busy people a chance to let loose and relax. Also as symbolic figure, the tree is said to represent morality because of it extreme beauty and quick fall. As a symbol of good fortune, love, and affection, the cherry blossom is frequently used in Japanese life style.

(*by Huanli Xu*)

Review

Traditional Chinese medicine and related active compounds against hepatitis B virus infection

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Summary **Hepatitis B induced by hepatitis B virus (HBV) remains a major public health problem worldwide. Although several antiviral drugs have been approved for hepatitis B, they cause significant dose-dependent side-effects (interferon-α) and drug resistance (lamivudine, *etc.*). Safe and potent new anti-HBV drugs are urgently needed. Traditional Chinese medicine (TCM) is an established segment of the health care system in China and widely used for hepatitis B in China and many parts of the world. Many TCMs and related active compounds have been reported that have promising and potent anti-HBV activities, including *Phyllanthus*, *Salvia miltiorrhiza*, *Rheum palmatum* L., *Radix Astragali*, oxymatrine, artemisinin and artesunate, and wogonin. Thus, TCM is a potential candidate for anti-HBV drugs. More information is needed regarding TCMs, including preparation, standardization, identification of active ingredients, and toxicological evaluation. Therefore, TCM development needs to apply advanced and interdisciplinary methodology and technology and perform further rigorously designed experimental and clinical investigations.**

Keywords: Hepatitis B virus (HBV), traditional Chinese medicine, *Phyllanthus*, *Salvia miltiorrhiza*, oxymatrine

1. Introduction

Hepatitis B is a significant public health concern and it may develop into hepatic fibrosis, liver cirrhosis, and hepatocellular carcinoma, which result in one million deaths annually (*1*). According to the World Health Organization (WHO), there are two billion people worldwide infected by hepatitis B virus (HBV) at some time in their lives (*2*). Of these, more than 350 million people are estimated to chronically infect and become carriers of the virus (*3*). Although several anti-virus drugs have been approved for hepatitis B, they induce significant dose-dependent side-effects and drug resistance. Interferon-α (IFN-α) was the firstly approved therapy for chronic HBV infection around the world. However, its therapeutic effect is not satisfactory and is related with some side-effects such as influenza-like syndrome, and leukocyte and platelet decreases (*4*). Lamivudine (3TC) was the firstly approved nucleotide analog for HBV infection, but its efficacy resembles IFN-α and is associated with drug resistance following prolonged administration (*5*). Thus there exists an urgent need for safe and effective new anti-HBV drugs.

Traditional Chinese medicine (TCM) is an established segment of the health care system in China. There are many TCMs widely used for hepatitis B in China and many parts of the world. In China, Chinese medicine is used as an adjunct or alternative treatment and accounts for 30% to 50% of total medicine consumption, with low costs and low toxicity (*6*). The 2002 National Health Interview Survey (NHIS) of the

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United States suggested that 19% of adults used some form of herbal supplements within the past 12 months (*7*). McCulloch *et al.* (*8*) showed that Chinese medicine may have a potential therapeutic value for treatment of chronic hepatitis B using meta-analysis data. TCM is composed of complex mixtures of compounds. Although the active ingredients of many mixtures have not been completely identified, some ingredients have been isolated and identified as potential therapeutic agents. These natural active compounds offer major opportunities for finding novel active lead structures against a wide range of assay targets because they contain more characteristics of high chemical diversity and biochemical specificity than standard combinatorial chemistry. Moreover, biologically active small molecules derived from natural products have drug-like properties and they can be absorbed and metabolized by the body (*9*). Furthermore, TCM is easily available without the need for laborious pharmaceutical synthesis (*10*). Therefore, TCM may be a good candidate for special antiviral characteristics and it has drawn more attention from researchers making an effort to identify effective antiviral agents (*11*).

2. Virologic features of HBV

HBV is the prototype member of the Hepadnaviridae (hepatotropic DNA virus) family and HBV virions are double-shelled particles (*12*) with an outer lipoprotein envelope containing surface antigens (*13*). The viral nucleocapsid is within the envelope (*14*) and includes the viral genome (relaxed circular, partially double-stranded, 3.2 kb) and a polymerase for the synthesis of viral DNA in infected cells (*15*).

The HBV genome possesses only four long open reading frames: presurface-surface (preS-S) region, precore-core (preC-C) region, P coding region, and X open reading frame. Their translations ultimately yield the viral surface, e, core, and polymerase proteins, as well as the X polypeptides. All the HBV proteins play important roles in HBV transcriptional regulation, viral packaging, reverse-transcription, and viral DNA recycling. Therefore, serum HBV markers are the most important clinical data for epidemic screening and diagnosis of HBV infection (*16*). Among these markers, hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) are most commonly used in experimental and clinical studies.

3. TCM and related active compounds with potential anti-HBV activity

The progress of the major TCM and related active compounds for treatment of HBV infection, including *Phyllanthus*, *Salvia miltiorrhiza*, *Rheum palmatum* L., *Radix Astragali*, oxymatrine, artemisinin and artesunate, and wogonin, are summarized in this section. The main focus is on cell experiments, animal studies, clinical trials, and lack of cytotoxicity. Anti-HBV activities of these TCMs and related active compounds in cell experiments and in clinical trials are shown in Table 1 and Table 2, respectively. Therapeutic index (TI) is defined as the ratio between CC_{50} (drug concentration inducing 50% reduction in host cell viability) and IC_{50} (drug concentration inducing 50% inhibition in HBsAg or HBeAg or HBV DNA release) for the most sensitive parameters to detect reduction in HBV production in each case.

3.1. *Phyllanthus*

The plant genus *Phyllanthus* (Yexiazhu) is widely distributed in most tropical and subtropical countries and consists of approximately 550-750 species throughout the world. It has long been used in traditional medicine to treat chronic liver disease in China and India (*17*). In China, it is estimated that 33 species exist in more than 10 provinces, which is approximately the same number of species as are in India. The most widely studied species have been *P. amarus* (Kuweiyexiazhu), *P. nanus*

Table 1. Anti-HBV activities of TCM and related active compounds in cell experiments

Treatments	Duration (days)	CC_{50}[a]	IC_{50}[b]			TI[c]	*Ref.*
			HBsAg	HBeAg	HBV DNA		
Phyllanthus nanus ethanolic extract	7	100	> 200	-	> 50	< 2	*20*
PA from *Salvia miltiorrhiza*	9	> 96	3.94	2.46	4.17	> 39.02	*38*
Astragaloside IV from *Radix Astragali*	9	388	> 200	> 200	-	< 1.94	*41*
Rheum palmatum L. ethanol extract	8	1,628	292.42	1,435	212.36	7.67	*51*
Chrysophanol 8-*O*-β-D-glucoside from *Rheum palmatum* L.	8	> 10,000	237.4	183.41	36.98	> 270.42	*51*
Oxymatrine	3	> 2,000	-	-	< 1,000	> 2	*53*
Artesunate	21	7.69	0.88	-	0.19	40.47	*58*
Wogonin	9	> 200	4	4	> 20	> 50	*62*

[a] CC_{50}: drug concentration (μg/mL) inducing 50% reduction in host cell viability.
[b] IC_{50}: drug concentration (μg/mL) inducing 50% inhibition in HBsAg, HBeAg, and HBV DNA release.
[c] Therapeutic index (TI) was the ratio between CC_{50} and IC_{50} for the most sensitive parameters to detect reduction in HBV production (HBsAg or HBeAg or HBV DNA) in each case.
PA: protocatechuic aldehyde.

(Aiyexiazhu), and *P. urinaria* (Yexiazhu) (*18*). Many kinds of compounds, including alkaloids, flavonoids, lactones, steroids, triterpenes, lignans, and tannins, were isolated from *Phyllanthus*. These compounds were reported to be responsible for the pharmacologic actions of the plant (*19*).

Phyllanthus is currently used in preclinical and clinical evaluations. Its promising biological activities were shown by *in vitro* and *in vivo* assays. Lam *et al.* (*20*) showed that the ethanolic extract of *P. nanus* produced a suppressive effect on HBsAg secretion, HBsAg mRNA expression, and HBV replication *in vitro*. The TI of the ethanolic extract of *P. nanus* was less than 2 (Table 1). Moreover, Lee *et al.* (*21*) demonstrated that *P. amarus* inhibited HBV production in cell culture and HBV transgenic mice by affecting HBV polymerase and decreasing HBV mRNA accumulation. In the clinic, *P. amarus* was reported to significantly increase the negative conversion rate of serum HBeAg compared with control (*22*) (Table 2). Liu *et al.* (*23*) published a meta-analysis of the efficacy and safety of *Phyllanthus* for chronic HBV infection. Twenty-two randomized clinical trials (n = 1,947) were included. The combined results revealed that *Phyllanthus* had a positive effect on clearance of serum HBsAg compared with placebo or no intervention. There was no significant difference between *Phyllanthus* and interferon in clearance of HBsAg, HBeAg, and HBV DNA (*24*,*25*). *Phyllanthus* plus interferon was better than interferon alone (*26*,*27*) and *Phyllanthus* was better than nonspecific treatment or other herbal medicines for the negative conversion of HBsAg, HBeAg, and HBV DNA (*28*-*35*). No serious adverse reactions were reported. This meta-analysis showed that *Phyllanthus* might have an antiviral effect.

However, some papers reported that *Phyllanthus* had no demonstrable antiviral effect in chronic hepatitis B. A double-blind placebo-controlled study was conducted for treatment of chronic hepatitis B (*36*). After 6 months treatment, there was no difference between *P. urinaris* and placebo in HBV DNA reduction, HBeAg seroconversion, and alanine aminotransferase (ALT) normalization. The discrepancy in the clinical effect in these studies could be attributed to different species, different growing conditions and harvest seasons, and different processing methods. Therefore, standardization of the genus *Phyllanthus* and large-scale prospective, multicenter, randomized, controlled trials are needed.

3.2. *Salvia miltiorrhiza*

Salvia miltiorrhiza (SM, Danshen), a herb, is traditionally used to treat liver disease in China. SM is believed to be one of the most highly recommended and widely accepted medicines for the treatment of hepatitis B in China (*18*).

Like most herbal medicines, SM is not a single entity but comprises different ingredients. Both its lipophilic and hydrophilic fractions have biological activities (*37*). Zhou *et al.* (*38*) isolated and characterized a functionally unique anti-HBV water-soluble substance, protocatechuic aldehyde (PA), from SM. They found that in HepG2.2.15 cells PA (Figure 1A) significantly inhibited the production of HBV DNA with an IC_{50} of 4.17 μg/mL and suppressed the expression of HBsAg and HBeAg with an IC_{50} of 3.94 and 2.46 μg/mL, respectively. The TI of PA was more than 39.02 (Table 1). Moreover, their results showed that PA inhibited duck hepatitis B virus (DHBV) DNA replication in ducks.

In a clinical evaluation, 30 patients with chronic hepatitis B were treated with SM (*39*). After 3 months of treatment, the negative conversion rate of HBeAg was 16.7%. A follow up of 3 and 9 months after the end of treatment showed negative conversion rates of HBeAg were 22.7% and 25.0%, respectively. In another clinical trial (*40*), 123 cases were randomly divided into a treatment group (n = 63) and a control group (n = 60). The treatment group was treated with SM injections and *Radix Astragali* injections and the control group was treated with oral administration of Gankangning tablets and fufang yiganling tablets. The treatment group was significantly better than the control group in the negative conversion of HBeAg and HBV DNA (Table 2).

In summary, the active compound of SM, PA, has been isolated and characterized and clinical assays showed that SM possessed anti-HBV activity.

3.3. *Radix Astragali*

Radix Astragali (Huangqi) derives from the dried

Table 2. Anti-HBV response of TCM and related active compounds in clinical trials

Treatments	n	Control	Duration (days)	Negative conversion of serum HBV markers			*Ref.*
				HBsAg	HBeAg	HBV DNA	
Phyllanthus amarus	122	Vitamins, Hypoxanthosine	30	6/62 (9.7%)	21/48 (43.8%)**	-	*22*
Salvia miltiorrhiza	123	Gankangning, fufang yiganling	90	-	41/57 (71.9%)**	19/26 (73.1%)**	*40*
Astragali compound	208	other regular drugs	60	2/94 (2.1%)	13/47 (27.7%)**	14/50 (28.0%)*	*43*
Oxymatrine	100	Vitamins	182	-	21/50 (42.0%)**	22/50 (44.0%)**	*56*

*$p < 0.05$; **$p < 0.01$ compared with control group.

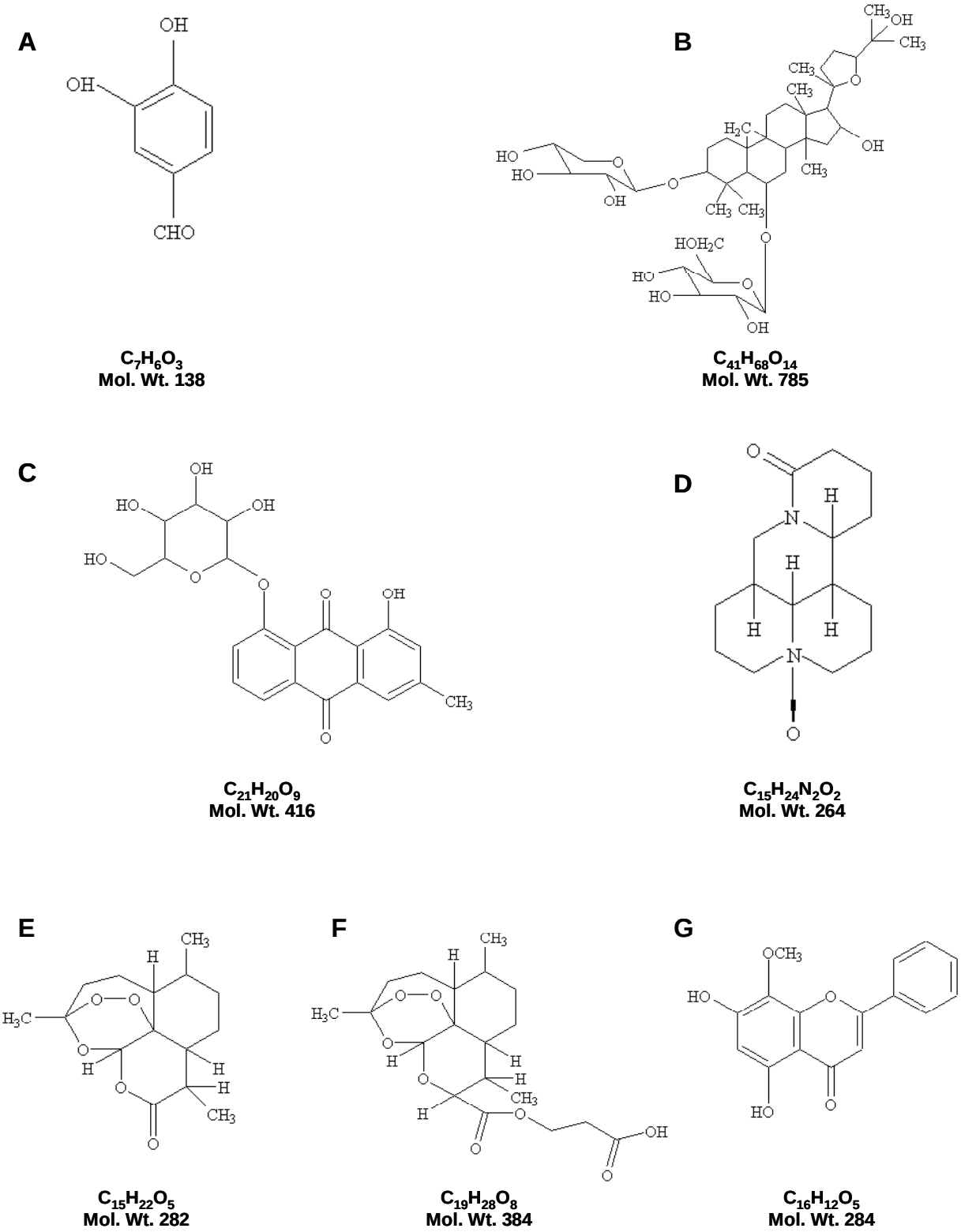

Figure 1. Chemical structures of various anti-HBV compounds in TCM. (**A**) protocatechuic aldehyde from *Salvia miltiorrhiza*, (**B**) astragaloside IV from *Radix Astragali*, (**C**) chrysophanol 8-*O*-β-D-glucoside from *Rheum palmatum* L., (**D**) oxymatrine, (**E**)artemisinin, (**F**) artesunate, and (**G**) wogonin. Mol. Wt. denotes molecular weight.

root of *Astragalus membranaceus* (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao (Mengguhuangqi) or *A. membranaceus* (Fisch.) Bge. (Mojiahuangqi). It has been widely used in Chinese medicine from ancient times and is one of the most widely prescribed Chinese herbs in many formulas. It has exhibited efficacy in treatment of immune disorders and liver diseases with an excellent safety record (*41*).

The major active constituents of *Radix Astragali* are believed to be the total saponins and the total flavonoids

(*42*). Wang *et al.* (*41*) showed that a major saponin of this herb, astragaloside IV (Figure 1B), suppressed both HBsAg and HBeAg secretion with inhibition rates of 23.6% and 22.9% at 100 μg/mL and possessed a more potent inhibitory activity than 3TC without significant cytotoxicity in HepG2.2.15 cells. Moreover, they found that astragaloside IV inhibited serum DHBV HBsAg by 64.0% at 120 mg/kg and reduced liver DHBV DNA levels in DHBV-infected ducklings. Their results suggested that astragaloside IV from *Radix Astragali* possessed anti-HBV activity and the TI of astragaloside IV was less than 1.94 (Table 1).

Furthermore, clinical evaluation of *Radix Astragali* was performed in 208 patients with chronic viral hepatitis B (*43*). The treatment group (n = 116) was treated with the Astragali compound (AC), containing *Radix Astragali* and adjuvant components, and the control group (n = 92) was treated with other drugs regularly used for viral hepatitis. Negative conversion rates of HBeAg and HBV DNA were significantly higher in the treatment group than in the control group (Table 2).

In summary, the major saponin of *Radix Astragali*, astragaloside IV, has been shown to possess anti-HBV activity and *Radix Astragali* appears to have a marked clinical efficacy in the treatment of patients with chronic viral hepatitis B.

3.4. *Rheum palmatum L.*

The herbal plant *Rheum palmatum* L. (Zhangyedahuang), a TCM, is widely distributed in mainland China and has a long history of treatment for gastroenteritic and liver diseases (*44*).

Some reports demonstrated that *R. palmatum* L. extracts could inhibit coxsackie virus and herpes simplex virus (*45,46*) and *R. palmatum* L. volatile oil could inhibit the expression of HBV antigens (HBsAg and HBeAg) (*47*). Moreover, some reports showed that the aqueous extracts of *R. palmatum* L. decreased the extracellular HBV DNA levels at concentrations ranging from 64 to 128 μg/mL, inhibited HBsAg secretion and HBV DNA polymerase activity *in vitro* (*48,49*), and showed a potential antiviral effect against duck hepatitis B virus (*50*).

Recently, Li *et al.* (*51*) evaluated the anti-HBV activities of *R. palmatum* L. ethanol extract (RPE) and its isolated anthraquinones in HepG2.2.15 cells. They found that RPE inhibited HBV-DNA production and HBsAg expression in a dose-dependent manner and its TI was 7.67 (Table 1). They also found that the only combined anthraquinone chrysophanol 8-*O*-β-D-glucoside (Figure 1C) exhibited significant activity against HBV DNA production with an IC_{50} of 36.98 μg/mL and antigen expression with an IC_{50} of 237.4 μg/mL for HBsAg and 183.41 μg/mL for HBeAg and its TI was more than 270.42 (Table 1). Furthermore, they observed that chrysophanol 8-*O*-β-D-glucoside was a potential inhibitior of HBV-DNA polymerase. Therefore, they concluded chrysophanol 8-*O*-β-D-glucoside is the major active compound in RPE and could be a promising candidate for the development of new anti-HBV drugs in the treatment of HBV infection.

In summary, *R. palmatum* L. extracts possess anti-HBV activity in *in vitro* and *in vivo* assays and its major active compound may be chrysophanol 8-*O*-β-D-glucoside.

3.5. *Oxymatrine*

Oxymatrine (OM) is an alkaloid extracted from two kinds of Chinese plants, *Sophora alopecuraides* L. (Kudouzi) and the root of *Sophora flavescesn* Ait. (Kushen). The chemical structure of OM is shown in Figure 1D. OM was reported to possess antiviral, antifibrotic, hepatoprotective, and immunomodulating effects, especially against hepatitis B (*52*).

In vitro and *in vivo* assays suggested that OM possessed anti-HBV activity. Xu *et al.* (*53*) found that in HepG2.2.15 cells 1,000 μg/mL of OM inhibited HBV DNA production 79.6%. The TI of OM was more than 2 (Table 1). They also showed that OM inhibited the secretion of HBsAg and HBeAg from HepG2.2.15 cells according to dose- and time-dependence and the maximal inhibition rates were 93% and 63%, respectively. Furthermore, Chen *et al.* (*54*) demonstrated that in a complete genomic HBV transgenic mice model ICR (TgN, HBV 1.2 copy) OM decreased the intrahepatic HBsAg, HBeAg, and HBcAg concentrations and caused the intrahepatic HBsAg and HBeAg to become negative in six mice at a dosage of 200 mg/kg after a 30-day treatment. However, the intrahepatic HBsAg and HBeAg returned to positive with prolonged treatment, probably due to immune tolerance.

In a randomized double-blind and placebo-controlled multi-center trial (*55*), treatment with OM capsules resulted in seroconversion rates of 38.61% for HBV DNA and 31.91% for HBeAg and treatment with OM injections resulted in seroconversion rates of 43.33% for HBV DNA and 39.29% for HBeAg by the end of a 24 week treatment course. Both OM groups were significantly better than the placebo in seroconversion rates. There was no statistically significant difference among OM capsule, OM injection, and placebo in side-effects. In another trial (*56*), negative conversion rates of HBeAg (42.0%) and HBV DNA (44.0%) in the OM capsule treatment group were significantly higher than those (4.0%, 4.0%, respectively) in the control group (Table 2).

In summary, OM has many different activities and is much cheaper than INF-α for the treatment of chronic hepatitis B, which makes it an attractive therapeutic option and warrants further clinical trials.

3.6. *Artemisinin and artesunate*

Artemisinin (Figure 1E) is a sesquiterpene lactone derived from the TCM plant *Artemisia annua* (Qinghao) and has been used for centuries in TCM as a remedy for chills and fever (*57*). The semisynthetic derivative of artemisinin, artesunate (Figure 1F), had better anti-HBV effects than artemisinin. Romero *et al.* (*58*) showed that artesunate suppressed HBsAg secretion with an IC_{50} of 0.88 μg/mL and HBV DNA production with an IC_{50} of 0.19 μg/mL and its TI was 40.47 (Table 1). They also found that synergistic anti-HBV effects existed by combining artesunate and lamivudine. Moreover, there are no known serious side-effects with artemisinin and its derivatives because none have been seen in their use in large populations for their antimalaria properties (*59*). Therefore, artemisinin and artesunate deserve to be further investigated for their anti-HBV activities.

3.7. *Wogonin*

Wogonin (Figure 1G) is a monoflavonoid derived from the TCM herb *Scutellaria radix* (Huangqin), which has been widely used for treatment of inflammatory and liver diseases for thousands of years in Asia (*60*). In recent years, wogonin has been found to have antiviral activity. Huang *et al.* (*61*) demonstrated that wogonin suppressed HBsAg secretion in a HBV-transfected liver cell line without cytotoxicity. Guo *et al.* (*62*) showed that wogonin effectively suppressed the secretion of both HBsAg and HBeAg with an IC_{50} of 4 μg/mL and reduced HBV DNA levels in a dose-dependent manner in HepG2.2.15 cells. The TI of wogonin was more than 50 (Table 1). They also found that wogonin dramatically inhibited DHBV DNA polymerase with an IC_{50} of 0.57 μg/mL in DHBV-infected ducks and significantly improved duck livers in histopathological evaluations. Moreover, they observed that wogonin significantly reduced plasma HBsAg levels in human HBV-transgenic mice. Although only a limited number of observations have been performed on the anti-HBV activity of wogonin, preliminary results suggested that wogonin might be a candidate as a new antiviral drug.

4. Development strategy of TCM with potential anti-HBV activity

The use of TCM to treat HBV infections has a long tradition and is common in China and India. However, TCM has not yet become a widely acceptable treatment modality for hepatitis B around the world. This eventuality is held back by the lack of the following factors: standardization of TCM and identification of its active ingredient(s), randomized controlled clinical trials (RCTs), and toxicological evaluation (*63*). The above-mentioned drugs, including *Phyllanthus*, *Salvia miltiorrhiza*, *Rheum palmatum* L., *Radix Astragali*, oxymatrine, artemisinin and artesunate, and wogonin, to a greater or less degree, lack assessment in these areas.

Recently, enormous efforts have been directed towards the scientific basis and clinical evaluation of TCM (*64*,*65*) as a result of a growing interest in therapeutic agents derived from TCM. Some advanced and interdisciplinary technology and methodology can facilitate standardization of TCM and identification of its active ingredient(s) (*66*). Modern pharmacological disciplines, including phytochemistry, pharmacognosy, and phytotherapy, can promote more significant breakthroughs and scientific achievements through scientific technology and methodology (*67*). The information about all aspects (herbal formulations, constituent herbs, herbal ingredients, molecular structure and functional properties of active ingredients, therapeutic and toxic effects, clinical indications and applications) of TCM in several databases is available and makes the scientific evaluation of TCM easier (*68*). In addition, a herbogenomics approach, defined as the process during which functional genomics and proteomics can identify target molecules affected by TCM has been started. Thus researchers can study critical signaling pathway cascades resulting in effective recovery of patients with HBV infections, and the information described can be used to understand the mechanisms of action of TCM (*69*). Rigorously designed TCM treatment and long-term monitoring by a standardized and effective report system can promote the toxicological evaluation of TCM (*70*).

5. Conclusions

Continuous development of new agents to treat HBV infections is urgently needed because, to date, only a few drugs have been approved. Although the use of TCM provokes debate in its current and future role in health care and evidence for both efficacy and safety, many TCMs have been recognized for their promising and potent anti-HBV activities. More information is needed regarding TCM, including preparation, standardization, identification of active ingredients, and toxicological evaluation. Thus, TCM development needs to apply advanced and interdisciplinary technology and methodology. Further experimental and clinical investigations will allow a better understanding of mechanisms of action, therapeutic effects, and the safety profile of TCM.

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Review

Multi-drug resistant tuberculosis: An iatrogenic problem

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Summary **The occurrence of resistance to drugs used to treat tuberculosis (TB), and particularly multi-drug resistant TB (MDR-TB) defined as resistance to at least rifampicin and isoniazid, has become a significant public health dilemma in a number of countries and an obstacle to effective global TB control. HIV-associated MDR-TB understanding is vital in providing strategies for treatment of HIV and drug-resistant TB. Better understanding on the basis of drug action and resistance is a key to development of diagnostic strategies, novel drugs, and treatment programs, and to find an approach to study the pathogenicity of drug resistant strains. The effectiveness of strategies such as DOTS-Plus in the management of MDR-TB patients under program conditions should be tested in operational field clinical trials following strictly standardized definitions and nomenclature.**

Keywords: Tuberculosis (TB), multi-drug resistant TB (MDR-TB), anti-tuberculosis drug, DOTS

1. Introduction

Tuberculosis (TB) is the leading cause of death from a curable infectious disease (*1*). On the basis of results of surveys of the prevalence of infection and disease, assessment of the effectiveness of surveillance systems, and death registrations, there were an estimated 8.9 million new cases of tuberculosis in 2004, fewer than half of which were reported to public-health authorities and WHO. About 3.9 million cases were sputum-smear positive which is the most infectious form of the disease (*2-4*).

Drug resistance can be simply defined as the temporary or permanent capacity of organisms and their progeny to remain viable or to multiply in the presence of the concentration of the drug that would normally destroy or inhibit cell growth (*5*). Clinically, drug resistance can be divided into four types (Table 1).

Anti-tuberculosis drug resistance is classified according to the following three definitions (*6*): (*i*) Confirmed mono-resistance: Tuberculosis in patients whose infecting isolates of *M. tuberculosis* are confirmed to be resistant *in vitro* to one first-line anti-tuberculosis drug; (*ii*) Confirmed poly-resistance: Tuberculosis in patients who′s infecting isolates are resistant *in vitro* to more than one first-line anti-tuberculosis drug other than both isoniazid and rifampicin; and (*iii*) Confirmed multi-drug resistant TB (MDR-TB): Tuberculosis in patients whose infecting isolates are resistant *in vitro* to at least isoniazid and rifampicin.

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2. Site of drug-resistant tuberculosis disease (pulmonary and extrapulmonary) (*7*)

In general, recommended treatment regimens for drug-resistant forms of TB are similar, irrespective of site. Defining site is important primarily for recording and reporting purposes.

Pulmonary tuberculosis: Tuberculosis involving the lung parenchyma. Tuberculosis intrathoracic lymphadenopathy (mediastinal and/or hilar) or tuberculous pleural effusion, without radiographic abnormalities in the lungs, therefore constitutes a case of extrapulmonary TB. A patient with both pulmonary and extrapulmonary TB should be classified as a pulmonary case.

Extrapulmonary tuberculosis: Tuberculosis of organs other than the lungs, *e.g.* pleura, lymph nodes, abdomen, genitourinary tract, skin, joints and bones, meninges. The definition of an extrapulmonary

Table 1. Four types of drug resistance

Type of drug resistance	Definition
Natural drug resistance	When neither the patient with naturally resistant bacilli nor his source of infection has had chemotherapy in the past.
Primary drug resistance	The presence of drug resistance to one or more anti-TB drugs in a TB patient who has received either no or less than one month of prior TB chemotherapy.
Acquired drug resistance	Resistance to one or more anti-TB drug which arises during the course of treatment usably as a result of non adherence to the recommended regimen or faculty prescribing. This is found in a patient who has received at least one month of anti-TB treatment and was referred to as secondary resistance in the past.
Initial drug resistance	Drug resistance in a patient who denies history of previous chemotherapy. In reality it consists of true primary resistance and an undisclosed acquired resistance.

case with several sites affected depends on the site representing the most severe form of disease.

3. Epidemiology of drug resistance (*8-11*)

In India, it is reported that 1-3.4% of new patients have had multi-drug resistant TB (*8*). Studies on acquired resistance have shown rates of resistance to isoniazid ranging from 34.5-67%, for streptomycin around 25% and for rifampicin from 2.8-37.3%.

In western countries, the incidence of MDR-TB has gradually increased (*9*). However, drug resistant TB is not uniformly distributed in the US but more prevalent in large urban areas and coastal or border communities. At Bellevue Hospital Centre which treats approximately 10% of all the TB patients in New York City, combined resistance to isoniazid and rifampicin increased from 2.5% in 1971 to 16% in 1991. The hazards of even brief courses of monotherapy were illustrated in a study by the CDC which found resistance to isoniazid in 25% of isolates from patients who had previously received up to 2 weeks of isoniazid alone. Isoniazid resistance increased to more than 60% among patients receiving 6 months of monotherapy and to more than 80% after 2 years. The main contributing factors to the "epidemic" of MDR-TB in certain cities in the west has been the combination of HIV infection, homelessness, drug addiction and overcrowding leading to rapid spread of the disease.

4. Factors responsible for developing drug resistance

Factors responsible for the development of drug resistance can be clinical, biological or social (Table 2). It is to be noted that one of the most important factors is unreliable treatment regimens prescribed by doctors. These include giving fewer drugs or giving a single drug in a regimen which is failing. This leads to development of resistance to that drug and also other important factors. Out of ignorance and due to lack of proper health education and motivation some patients do not take drugs regularly, and irregular chemotherapy leads to the development of drug resistance.

Table 2. Type of factors associated with drug resistance

Type of factors	Factors associated with drug resistance
1. Clinical factors	Unreliable treatment regimens by doctors • Lesser number of drugs • Inadequate dosage duration Addition of a single drug to a failing regimen Easy availability of drug in private sector Poor drug supply Poor quality of drugs
2. Biological factors	Initial bacillary population Local factors in host favorable for multiplication of bacilli Presence of drug in insufficient concentrations
3. Sociological factors	Irregular intake/inadequate duration Neglect of disease Ignorance Lack of health education

5. Mechanisms of Resistance

Mycobacteria have unique characteristics which endow them with natural resistance to many commonly used antibacterial agents. The hydrophobic cell envelope of *Mycobacteria* poses a barrier to many drugs (*12*). The bacilli also have transporters which flush out the drugs (*13*). Moreover, they can hydrolyze or modify the drug by synthesizing necessary enzymes. This explains why only a few drugs are effective against *M. Tuberculosis* and why they can develop resistance to anti-TB drugs through chromosomal mutations. Resistance to isoniazid can occur due to mutations in the *katG, InhA*, and *kasA* genes, whereas resistance to rifampicin can be affected by mutations in the *rpoB* gene (*15-18*).

6. Multi-drug resistant TB (MDR-TB)

Multi-drug resistant TB is defined as the disease caused by *M. tuberculosis* that is resistant to at least isoniazid and rifampicin with or without resistance to other anti-TB drugs (*19*). Resistance to isoniazid and streptomycin only is probably the most common form of resistance world wide to more than one drug. This

is not strictly multi-drug resistance. Therefore, another separate term is needed to define this combination of resistances. Resistance to streptomycin, isoniazid and another drug or drugs other than rifampicin is probably very uncommon. MDR-TB is naturally a man-made problem, since poor prescriptions, poor case management, lack of coordinated education, and haphazard treatment result in drug resistance. Besides, poor patient compliance (*e.g.* patients' pick-and-choose attitude about medicines from prescribed regimens) is a major hurdle. If treatment failure is observed clinically and the sputum remains positive even after 3-4 months of treatment, then drug resistance should be suspected. Since a culture and susceptibility testing facility is available only in a few places, the exact epidemiological implication of MDR cannot be assessed.

7. Extensive drug resistant-tuberculosis (XDR-TB)

Extensive drug resistant-tuberculosis (XDR-TB) has been reported in all regions of the world (*20*). XDR-TB is defined as resistance to at least rifampicin, isoniazid, a second-line injectable drug (capreomycin, kanamycin or amikacin), and fluoroquinolone (*21*). Control of drug resistant tuberculosis requires a strong health infrastructure to ensure delivery of effective therapy coupled with surveillance and monitoring activities to enable timely intervention to limit transmission and spread of the disease.

8. Magnitude of the MDR-TB problem

The appearance of MDR-TB has followed the pervasive use of rifampicin since the 1970s. The number of incident cases (including new and re-treatment cases) occurring worldwide in 2003 alone was estimated to be 458,000 (95% confidence limits, 321,000-689,000) by The WHO Stop TB (*22*). Widespread cases of MDR-TB could be two or three times higher than the number of incident cases (*23*). Data on drug resistance using standard methodology to establish the global magnitude of resistance to four first-line antituberculosis drugs, such as, isoniazid, rifampicin, ethambutol, and streptomycin, were given by the WHO/IUATLD Global Project on Antituberculosis Drug Resistance Surveillance (*24*,*25*). The standard methodology includes representative sampling of patients with an adequate sample size, standardized data collection distinguishing between new and previously treated patients, and quality-assured laboratory drug sensitivity testing (DST) supported by a network of supranational TB reference laboratories. By 2003, three rounds of the global project had been completed covering 109 countries or regions within large countries (*26*). In spite of these surveillance data, the magnitude of drug resistance is not yet known in many areas of the world with high burdens of TB, such as, most of China, India, Indonesia, Nigeria, and countries of the former Soviet Union. Nevertheless, evidence from half the world's nations confirms that drug resistance is a serious problem worldwide. Many areas of the world face endemic and epidemic MDR-TB, and in some areas the incidence of resistance is alarmingly high as documented by a third global report on antituberculosis drug resistance surveillance. In patients never previously treated, the median prevalence of resistance to any of the first-line drugs, most commonly streptomycin and/or isoniazid, was 10.7% (range 0-57.1%); 20 survey sites exceeded 20%. The median prevalence of MDR-TB was 1.2% (range 0-14.2%); 11 sites exceeded the 6.5% threshold for extreme values, including in the former Soviet Union. In patients previously treated, the median prevalence of any resistance was 23.3% (range 0-82.1%) and of MDR-TB, 7.7% (range 0-58.3%).

Drug resistance was strongly associated with previous treatment. In previously treated patients, the probability of any resistance was over 4-fold higher, and that of MDR-TB over 10-fold higher, than that in untreated patients. The overall prevalence of drug resistance was often related to the number of previously treated cases in the country. Among countries with a high burden of TB, previously treated cases ranged from 4.4% to 26.9% of all patients registered in DOTS programs. In the two largest high-TB burden countries (China and India) re-treatment cases accounted for more than 20% of sputum smear-positive cases (*27*).

9. Extra pulmonary MDR-TB and MDR-TB treatment

Treatment of MDR-TB requires prolonged and expensive chemotherapy using second-line drugs of heightened toxicity. If resistance to the second-line drugs also arises then the disease becomes virtually untreatable. The treatment strategy is the same for patients with pulmonary and extrapulmonary MDR-TB. If the patient has symptoms suggestive of central nervous system involvement and is infected with MDR-TB, the regimen should use drugs that have adequate penetration into the central nervous system (*28*,*29*). Rifampicin, isoniazid, pyrazinamide, protionamide/ethionamide and cycloserine have good penetration; kanamycin, amikacin and capreomycin penetrate effectively only in the presence of meningeal inflammation; *p*-aminosalycilic acid and ethambutol have poor or no penetration.

10. MDR-TB in the era of HIV

An alarming increase in infection due to the human immunodeficiency virus (HIV) has accelerated this situation and it is believed that, as of now, about 3.5

million people in India are infected with HIV (*30*). There is a grave concern in India regarding the increase in HIV-associated TB and the emergence of MDR-TB in both magnitude and severity of the TB epidemic. HIV co-infection is an important challenge for the prevention, diagnosis and treatment of drug-resistant TB, especially in the case of MDR-TB. The local epidemiological prevalence of HIV, MDR-TB, and HIV-associated MDR-TB is important in guiding strategies for treatment of HIV and drug-resistant TB. All Drug Resistance-TB control programs are therefore strongly encouraged to determine the extent of the overlap between the MDR-TB and HIV epidemics (*31*).

11. Diagnosis of MDR-TB in HIV-infected patients

The appearance of MDR-TB in the HIV-infected patient does not differ from that of drug-susceptible TB in the HIV-infected patient (*32*). The diagnosis of TB in HIV-positive people is more difficult and may be confused with other pulmonary or systemic infections. The presentation is more likely to be extrapulmonary or sputum smear-negative than in HIV-uninfected TB patients. This can result in misdiagnosis or delays in diagnosis and, in turn, higher morbidity and mortality. The use of X-ray and/or cultures improves the ability to diagnose TB in HIV patients and is recommended where available. In areas where MDR-TB is known to be a problem in HIV-positive patients, and where resources permit, all HIV patients with TB should be screened for MDR-TB with DST. Rapid diagnostic techniques for MDR-TB should be employed when possible since HIV-infected patients with TB on inadequate antituberculosis treatment, or no treatment, for even short periods of time are at a high risk for death (*33*).

12. Diagnosis/rapid detection of MDR-TB

Multi-drug resistant TB should be suspected in patients if:

• Patient has taken treatment for tuberculosis in the past.

• Isoniazid resistance prevalence in community is more than 4%.

• There is likelihood of patient being exposed to MDR-TB.

• There is poor response to drug treatment as indicated by

a. Prolonged fever or cough,

b. Sputum conversion failure despite four months of standard short course chemotherapy.

Mycobacteria grow slowly and hence drug susceptibility tests take about 10-12 weeks. Rapid testing methods are necessary to detect drug resistance early and institute appropriate drug treatment. Culture sensitivity methods can detect drug resistance in about a week using a phage-based method with luciferase-incorporated phage (*34*,*35*); the BACTEC method is a radiometric technique based on detection of radio labeled CO_2 as a measure of growth index for microorganisms (*36*,*37*); and genotypic techniques involving rapid genotypic analysis of *Mycobacteria* to detect gene mutations causing drug resistance are also available (*38*,*39*).

13. Prevention of multi-drug resistant tuberculosis (*40*,*49*)

There are two main approaches to prevent multi-drug resistance:

(a) Identification and treatment of patients with multi-drug resistant tuberculosis. The aim is to identify their disease and to prevent further transmission.

(b) Identification of persons with tubercular infection and their prophylactic treatment. The aim is to prevent the 5-10% risk of subsequent development of disease.

The accepted guidelines for preventing the transmission of tuberculosis in health care settings with special focus on HIV related issues are:

(a) Patients with active tuberculosis must be identified quickly by using the most sensitive and rapid laboratory methods available.

(b) Confirmed or even suspected infectious tuberculosis patients should be placed immediately in isolation.

(c) Effective antitubercular therapy should he started immediately in diagnosed patients.

(d) Cough inducing procedures (*e.g*. bronchoscopy, sputum induction, administration of aerosol treatment) in confirmed or suspected tuberculosis patients should be carried out in isolated rooms.

(e) Patients and health care workers exposed to multi-drug resistant infectious tuberculosis patients should be evaluated regularly for the presence of infection/disease.

(f) Patterns of drug resistance should be evaluated regularly in the community.

14. Basic principle of chemotherapy in multi-drug resistant tuberculosis (*41-43*,*50*)

1. Treatment should be in a specialized center with standard laboratory facilities.

2. An appropriate regimen for individual patient needs design experience and skill.

3. The regimen should contain at least five drugs including three new drugs, depending upon potency of available drugs and the resistance pattern and previous history of treatment. Any first line oral agent to which the isolate is sensitive should be used. One injectable, one fluroquinolone and as many second-

line bacterostatic agents as needed should be used to make up the five drug regimen in the initial phase of treatment. When five adequate agents are given consider use of additional drugs. Previously used drugs to which bacilli may still be sensitive may be added to the regimen especially if they are powerful.

4. Never add a single drug to a failing regimen.

5. It is effective to combine two potentially ineffective drugs because of cross resistance. Cross resistance has been reported between thioamide and thioacetazone, kanamycin/amikacin with streptomycin (*41,42*). rifampicin with rifapentine, and rifabutin (> 70% strain) among various derivatives of fluoroquinolones. Cross resistance has also been reported between ethionamide and isoniazid, viomycin and kanamycin, viomycin and capreomycin/amikacin are still sensitive to capreomycin.

6. All the drug should be given in a single daily dose preferably, expert PAS is usually given in two divided doses in order to avoid the problem of intolerance. Among thioamides, prothioamide is better tolerated than ethionamide.

7. Intermittent therapy is usually not effective and should be avoided in multi-drug resistance tuberculosis.

8. No drug should be kept in reserve and the most powerful drugs (bactericidal) should be used initially and in maximum combinations to ensure that the first battle is won and won permanently.

9. Therapy should be under direct observation preferably for 3-4 months or until sputum conversion.

10. Surgical treatment should be considered as an adjunct to chemotherapy wherever applicable, as results of chemotherapy are very unpredictable.

11. All measures should be taken to persuade and encourage patients not to stop treatment despite all its discomfort as it is the last treatment that stands between the patient and death.

15. Drugs used in MDR-TB and their toxicities

The second-line drugs used for treatment of multi-drug resistance tuberculosis are given in Table 3 with their dosages in decreasing potency from top to bottom against mycobacterium tuberculosis. It is generally thought that, reserve drugs are frequently associated with very high rates of unacceptable adverse reactions, which need frequent interruption and change of regimen, but in clinical practice it is observed that they are not very toxic. The authors reported that 41%, experienced some side effects but only 21.1% of the patients required stoppage or change of drug in the study of 39% MDR-TB. Thus, it is practically possible to treat the MDR-TB patients with these drugs (*44*). Second-line reserve drugs, their toxicities and management are given in Table 4.

16. Regimen for multi-drug resistant tuberculosis

World Health Organization (WHO) has a recommended regimen (*41*,*42*) without availability of sensitivity results (Table 5) and with availability of sensitivity results (Table 6).

Table 3. Second-line drugs used for treatment of MDR tuberculosis

Drugs	Average daily dosage	Daily dosage (mg)		Type of antimycobacterial activity
		Minimum	Maximum	
Aminoglycoside	15 mg/kg	750	1,000	Bactericidal against activity multiplying organisms
Kanamycin				
Amikacin				
Capreomycin				
Thioamides	10-20 mg/kg	500	750	Bactericidal
Prothionamide				
Ethionamide				
Fluroquinolone				
Ciprofloxacin	15-20 mg/kg	1,000	1,500	Weakly bactericidal
Ofloxacin	7.5-15 mg/kg	600	800	
Sparfloxacin	6-8 mg/kg	400	600	
Levofloxcin		500	750	
Bacteriostatic second-line drugs				Bactericidal
Cycloserine	10-20 mg/kg	500	750	
p-Aminosalycilic acid	200-3,000 mg/kg	10 g	12,000	
Others drugs				
Clofazimine	4-5 mg/kg	100	200	Bacteriostatic
Coamaxyclav		750	2,000	Weakly bactericidal
Clarithromycin	10-15 mg/kg	1,000 mg/kg		Bacterial (pH dependent)
Azithromycin	10 mg/kg	500 mg/kg		
Rifabutin	May be used against some isolates of MDR-TB resistant rifampicin but sensitive to rifabutin.			
Thiacetazone	High rate of site effects in HIV patients.			
High dose Isoniazid	Animal model supports use but conflicting clinical data.			

17. Surgery for MDR-TB (*49*)

Surgery should be considered in patients with persistent culture positive MDR-TB despite effective medical treatment. If the patient has localized disease, reasonable lung function and only two or three (weak) drugs available, surgery should be seriously considered. Resection surgery is done as an adjunct to medical treatment (*45*). Published data has shown that overall cure rate was substantially higher (81-56%) when

Table 4. Toxicities and their management

Drug	Symptoms	Reaction
Kanamycin	Hearing loss	Change to capreomycin Lower the dose of drug Discontinue suspected drug if can be done without compromising regimen
Ethionamide Cycloserine	Psychotic symptoms	Initiate antipsychotic drugs Hold suspected agent for short period (1-4 weeks) lower the dose of drug Discontinue suspected drug if it can be done without compromising regimen
p-Aminosalycilic acid Ethionamide	Nausea and vomiting	Rehydration Initial anti-emetic therapy Lower the dose of drug Discontinue suspected drug if can be done without compromising regimen
Cycolserine	Seizures	Start anticonvulsant therapy Increase pyridoxine to 300 mg/day lower the dose of drug Discontinue suspected drug if can be done without compromising regimen

Table 5. Regimen before (or without) sensitivity results

Initial phase		Continuation phase	
Drugs	Minimum duration in months	Drugs	Minimum duration in months
Aminoglycoside[a]	6	Ethionamide	12-18
Ethionamide	6	Fluroquinolone[b]	12-18
Fluroquinolone[b]	6	Pyrazinamide	12-18
Pyrazinamide	6	Ethambutol +/–	12-18
Ethambutol +/–	6		

[a] Kanamycin, amikacin, or capreomycin. [b] Ciprofloxacin or ofloxancin.

Table 6. Regimen for multi-drug resistant tuberculosis when sensitivity results available

Resistance to	Initial phase		Continuation phase	
	Drugs	Minimum duration in months	Drugs	Duration in months
Isoniazid Rifampicin	Aminoglycoside[a] Ethionamide[b] Fluroquinolone[c] Pyrazinamide Ethambutol +/–	6 6 6 6 6	Ethionamide[b] Fluroquinolone[c] Pyrazinamide Ethambutol +/–	12-18 12-18 12-18 12-18
Isoniazid Rifampicin Streptomycinc[d] Ethambutol	Aminoglycoside[a] Ethionamide[b] Fluroquinolone[c] Cycloserine[e]	6 6 6 6	Ethionamide[b] Fluroquinolone[c] Cycloserine[e]	18 18 18
Resistance to all drugs	Aminoglycoside[a] Fluroquinolone[c] Two of these Ethionamide[b] *p*-Aminosalycilic acid Cycloserine[e]	6 6 6 6 6	Fluroquinolone[c] Two of these Ethionamide[b] *p*-Aminosalycilic acid Cycloserine[e]	18 18 18 18
Susceptibility test to reserve dugs available	Tailor regimen according to Susceptibility pattern[f]			

[a] Kanamycin or amikacin, or capreomycin. [b] If Ethionamide is not available or poorly tolerated (even at a dose of 500 mg/day) use ofloxacin. [c] Ciplofloxacin or ofloxacin. [d] Streptomycin, if still active, if resistant to streptomycin, use kanamycin or capreomycin. [e] *p*-Aminosalycilic acid if cycloserine is not available or too toxic. [f] Individualized regimen is feasible in designated centers of excellence.

Table 7. Relationship of the principles essential DOTS and the DOTS-Plus strategies

DOTS strategy	DOTS-Plus strategy
Political and administrative commitment	Sustained political and administrative commitment
Good-quality diagnosis by sputum microscopy	Accurate, timely diagnosis through quality-assured culture and drug susceptibility Testing
Directly observed treatment	Directly observed treatment
Systematic monitoring and accountability	Standardized recording and reporting system that enable performance monitoring and evaluation of treatment outcome
Uninterrupted supply of good-quality first-line drugs for standardized treatment through outpatient therapy	Uninterrupted supply of quality assured first and second-line drugs; appropriate treatment strategies utilizing second-line drugs under strict supervision

DOTS-Plus is an essential component of the presented National Tuberculosis Control Program to be implemented through program communications.

surgery was more frequently and aggressively applied (*46*). Feasibility and success of surgery appears to be substantially enhanced by nutrient support (*47*).

18. DOTS-Plus

The first WHO endorsed DOTS-Plus programs began in 2000 (*48*). The Green Light Committee (GLC) was established to promote access to high quality second-line drugs for appropriate use in TB control programs. The DOTS Plus strategy is part of the comprehensive DOTS strategy recommended by the WHO. The Revised National Tuberculosis Control Program (RNTCP) views the treatment of MDR-TB patients as a "standard of care" issue. Recognizing that the treatment of MDR-TB cases is very complex, the prescribed regimen follows the internationally recommended DOTS Plus guidelines and is available from designated RNTCP DOTS Plus sites. These sites will be located in a limited number of highly specialized centers, at least one in each large state, and will have ready access to a state level accredited culture and DST laboratory, and the Intermediate Reference laboratory (IRL) under RNTCP. These sites should have sufficient qualified staff available to manage MDR-TB patients, using standardized second-line drug regimens given under daily DOT, with consistent follow-up protocols.

19. Conclusion

The management of MDR-TB is a challenge that should be undertaken by experienced clinicians at centers equipped with reliable laboratory services for mycobacterial cultures and *in vitro* sensitivity testing because it requires prolonged use of costly second-line drugs with a significant potential for toxicity. The judicious use of drugs; supervised standardized treatment; focused clinical, radiological, and bacteriologic follow-up; and surgery at the appropriate juncture are key factors in the successful management of MDR-TB. Genotypic techniques involve rapid genotypic analysis of MDR-TB and newer effective anti-TB drugs are still a distant dream. Innovative approaches such as DOTS-Plus show promise for the management of MDR-TB patients and appear to be a hope for future.

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Brief Report

Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis

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Summary **Aminopeptidase N (APN)/CD13 is a widely expressed transmembrane protein and its altered expression has been detected in various cancer cells. Several APN inhibitors have been developed and some of them have been found to have effectiveness as anti-cancer agents. This article reports anti-cancer effects of a hydroxamic acid derivative 24F that was newly-synthesized as an APN inhibitor. 24F had the ability to inhibit the invasion of hepatocellular carcinoma (HCC) cell line HuH-7, although the growth of HuH-7 was not significantly inhibited at the analyzed concentrations of 24F and incubation times used. Furthermore, incubation of vascular endothelial cells with 24F was found to be effective for the suppression of the angiogenic phenomenon. These results suggest that the novel APN inhibitor 24F may work as an anti-cancer agent for HCC *via* inhibition of HCC cell invasion and angiogenesis.**

Keywords: Aminopeptidase N (APN), CD13, hepatocellular carcinoma (HCC), cancer growth, invasion, angiogenesis

1. Introduction

Aminopeptidase N (APN), which is also known as CD13, is a membranous glycoprotein expressed in a variety of cells and tissues (*1,2*). Several studies have suggested that APN plays important roles in several biological events during cancer progression such as cell proliferation and invasion. For example, overexpression of APN is detected in solid tumors and the expression level is likely to correlate with tumor malignancy (*3-6*). In addition, Yoneda *et al.* reported that APN functions to degrade extracellular matrix and thereby promotes cancer cell invasion and metastasis (*7*). Therefore, inhibition of APN function would have a significant role in the development of cancer chemotherapeutic agents.

Various natural or artificially-synthesized compounds with an ability to work as an inhibitor of APN have been developed (*8*). One well investigated APN inhibitor *N*-[(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutyryl]-L-leucine is named bestatin. Many researchers have analyzed the anti-cancer effects of bestatin and suggested that bestatin induced both the apoptotic effect in chronic myelogenous leukemia cells and the anti-angionic effect (*9,10*). In a recent study, Cui SX *et al.* developed a novel APN inhibitor named CIP13F that is a cyclic-imide peptidomimetic derivative and clarified the usefulness of CIP13F as an anti-proliferative agent of human ovarian carcinoma cells (*11,12*). In parallel with the progression of that study, the research group of Xu WF also synthesized a new compound named 24F that is an hydroxamic acid derivative with a free amino group ((*S*)-2-amino-*N*-((*S*)-1-(2-(hydroxyamino)-2-oxoethyl)-2,6-dioxopiperidin-3-yl)-3-phenylpropanamide, Figure 1). 24F was found to react strongly with the APN molecule *in vitro*, inhibit its enzyme activity in the preliminary study and therefore it is expected that 24F can contribute to suppression of cancer progression *via* the inhibition of APN function.

Hepatocellular carcinoma (HCC) is a common malignant disease, especially in eastern Asia. Various therapeutic strategies of HCC treatment including

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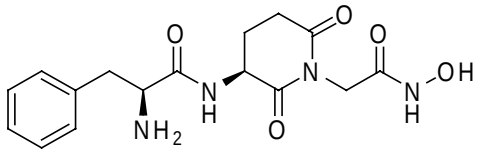

Figure 1. Chemical structure of 24F.

surgical techniques and some noninvasive treatment methods have been developed to improve the outcome of HCC patients. Recently, a novel molecular targeted agent named sorafenib, a multikinase inhibitor, has been developed as a chemotherapeutic agent for HCC (*13*), but discovery of a chemotherapeutic agent for the treatment of HCC has not progressed quickly. This study analyzed the effectiveness of 24F on suppression of HCC progression.

2. Materials and Methods

2.1. *Compound*

The hydroxamic acid derivative 24F was synthesized as one of a series of cyclic-imide peptidomimetics with a free amino group using a 3D-QSAR model (*11,12*). In the present study, this compound was provided by Prof. Xu in China-Japan Cooperation Center for Drug Discovery & Screen, Shandong University (Shandong, China), and dissolved in phosphate-buffered saline (PBS) for *in vitro* studies.

2.2. *Cell lines*

HCC cell line HuH-7 and human promyelocytic leukemia cell line HL-60 were obtained from Health Science Research Resources Bank (HSRRB; Osaka, Japan). HuH-7 and HL-60 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 supplemented with 10% fetal bovine serum (FBS), respectively. These media and reagents were purchased from Invitrogen, Carlsbad, CA, USA. Human umbilical vein endothelial cells (HUVEC) were maintained in EGM-2 medium. These cells and media were purchased from Sanko Junyaku Co., Ltd., Tokyo, Japan.

2.3. *Enzyme activity assay*

APN activity was measured using a spectrophotometric method with L-leucine-*p*-nitroanilide (Peptide Institute Inc., Osaka, Japan) as a substrate of APN (*14*). Continuously-cultivated HL-60 cells were collected in test tubes and washed with PBS. Cells (5×10^5) were resuspended in 200 µL of PBS with 0-2.7 mM of 24F and incubated at 37°C. APN enzyme activity was analyzed by measuring the absorbance at 405 nm using a micro-plate reader (Bio-Rad Laboratories) at 0, 15, 30, and 60 min after 1.6 mM substrate was added.

2.4. *Cell growth assay*

Continuously-cultivated HuH-7 cells were harvested in tubes and resuspended in DMEM containing 10% FBS after washing with PBS. Cells were seeded in triplicate in 96-well plates at a density of 2×10^3 cells in 100 µL with 0-200 µg/mL of 24F and incubated for 3 to 5 days at 37°C in a 5% CO_2 atmosphere. Cell viability was evaluated using a methylthiazole tetrazolium (MTT) cell proliferation assay kit in accordance with the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland).

2.5. *Invasion assay*

The cell invasion assay was performed using a BIOCOAT Matrigel invasion chamber (Becton-Dickinson, NJ, USA) according to the manufacturer's instructions. Continuously-cultivated HuH-7 cells were harvested and resuspended in serum-free medium, and then incubated for 24 h at 37°C in a 5% CO_2 atmosphere. The cells were harvested and resuspended at a density of 1×10^5 cells in 500 µL serum-free medium with 0-200 µg/mL of 24F. The cells were added to each chamber and allowed to invade the Matrigel for 48 h at 37°C in a 5% CO_2 atmosphere. After Matrigel, cells that had not penetrated the filter were removed with cotton swabs, and cells that had migrated to the lower surface of the filter were stained with a Diff-Quick stain kit (Sysmex International Reagents, Hyogo, Japan). After washing with water, the chambers were allowed to air-dry. The number of invading cells were counted under a light microscope.

2.6. *Tube formation assay*

Cultured HUVECs were harvested and resuspended in EGM-2 medium with 0-200 µg/mL of 24F. Cells were incubated for 72 h at 37°C in a 5% CO_2 atmosphere. After Matrigel-coated 24-well plates (Becton-Dickinson) were incubated for 30 min at 37°C, the harvested HUVECs were seeded at a density of 5×10^4 cells in 500 µL cultured medium with 0-200 µg/mL of 24F. After 15 h-incubation at 37°C in a 5% CO_2 atmosphere, the morphology of capillary-like structures was visualized using an inverted microscope (Olympus, Tokyo, Japan) and photographed.

3. Results and Discussion

The enzyme reaction assay was performed to confirm whether newly-synthesized compound 24F can inhibit the activity of aminopeptidase that is expressed on the surface of cell membranes. HL-60 cells are positive for APN expression (*15*), and therefore this

cell line is available to use as a positive control for detecting aminopeptidase activity. As a result, the aminopeptidase activity was inhibited in the presence of 24F in a dose-dependent manner (Figure 2A) and the inhibition rate of ΔA/min under the condition of 0.27 mM (100 μg/mL) 24F was around 25% compared to the condition without 24F. In this analysis, IC_{50} of 24F (the volume of 24F that displayed 50% inhibition of enzyme activity) was calculated to be 1.88 mM. Therefore, it was shown that the newly-synthesized compound 24F can be used as an aminopeptidase inhibitor.

Next, the effect of 24F on HCC cell growth was analyzed using HuH-7 cells that were confirmed to have positive expression of APN by flowcytometric analysis (data not shown). HuH-7 cell growth was inhibited by incubation with 24F, but there was no significant difference in the inhibition rate between 1-200 μg/mL of 24F. The inhibition rate of cell growth was 8.7% at a maximum which was detected in the sample incubated 120 h with 200 μg/mL of 24F. No acute cytotoxic effect was confirmed using microscopic observation in those analyzed concentrations of 24F. This result indicated that 24F might be workable as an anti-proliferative agent of HCC without acute cytotoxic effects, although a higher concentration of 24F than the inhibition of APN enzyme activity is required. Further analyses should be performed to clarify whether 24F can work as an anti-proliferative agent of other HCC cell lines using a shorter incubation period.

Cell invasion is the essential event for cancer progression and metastasis (*16*). Therefore, for cancer therapy, inhibition of cancer cell invasion is an important strategy, along with inhibition of cancer cell growth. This study analyzed the effect of 24F on HuH-7 cell invasion by means of a Matrigel invasion chamber assay. Figure 2B displays typical photographs of stained cells that invaded Matrigel. The number of invading cells was significantly decreased in the presence of 100 μg/mL 24F (*right panel*) compared with that in non-treated cells (*left panel*). This result suggests that 24F has an ability to inhibit the invasion of HuH-7 cells, which displayed a 56% inhibition rate in the sample incubated with 100 μg/mL 24F. Previous studies showed that APN had an important role in the degradation of extracellular matrix and induced cancer cell invasion (*7,17*). Thus, 24F is suggested to suppress HCC cell invasion *via* inhibition of APN enzymatic reaction. Further studies should be performed to clarify the importance of APN in HCC cell invasion and the mechanism of the inhibitory effect of 24F.

Moreover, we also examined the inhibitory effect of 24F on angiogenesis by using the *in vitro* method. Several studies clarified that inhibition of APN seemed to contribute to suppression of angiogenesis

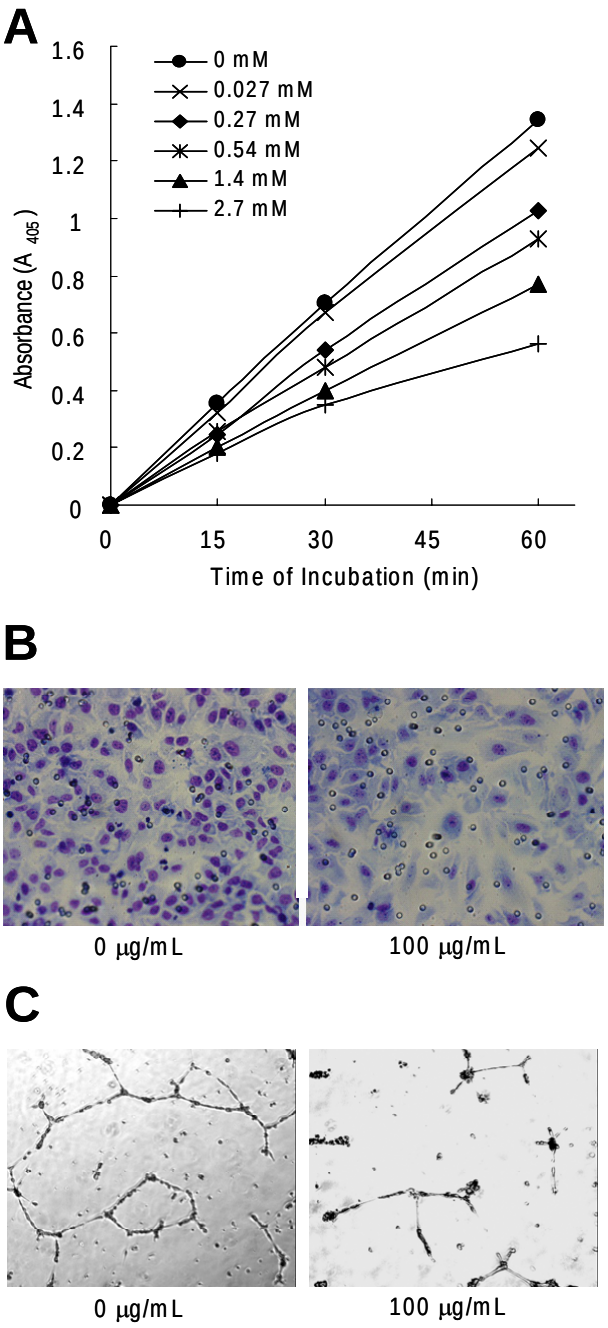

Figure 2. *In vitro* analyses of 24F. (**A**) Effect of 24F on the inhibition of APN enzyme activity. The absorbance, the level of enzyme reaction of APN, was decreased in samples with 24F in a dose-dependent manner. (**B**) Staining of HuH-7 cells that invaded Matrigel. The number of cells stained was decreased when incubating cells with 100 μg/mL of 24F (*right*) compared with incubating without 24F (*left*). Original magnification, ×200. (**C**) Typical example of tube-forming HUVECs on Matrigel. Tube formation was suppressed when incubating cells with 100 μg/mL of 24F (*right*) compared with incubating without 24F (*left*). Original magnification, ×40.

(*10,18*). Thus, our newly-synthesized compound 24F was expected to suppress migration and tube formation of vascular endothelial cells *via* inhibition of APN activity. The result of the tube formation assay showed that the number of tube-like structures of HUVECs on the surface of Matrigel was decreased

by incubating HUVECs with 100 μg/mL 24F (Figure 2C). Therefore, this result indicated that migration and tube formation of vascular endothelial cell can be inhibited by incubation with 24F. Additionally, in this analysis, HUVECs were incubated with 24F for 72 h before examination of the tube formation assay and there was no significant effect in the analysis without this incubation. Thus, it was suggested that persistent incubation with 24F has an ability to suppress angiogenesis by inhibiting the molecular mechanism of migration and tube formation of vascular endothelial cells. The mechanism of this inhibitory effect, however, seemed to be complex because the exact role of APN in angiogenesis is still unclear despite development of APN inhibitors which have an inhibitory effect on angiogenesis. Shim JS *et al.* clarified using DNA microarray analysis that their developed APN inhibitor affected the regulation of several angiogenesis-related genes in human fibrosarcoma cells (*19*). In subsequent study, alteration of expression of angiogenic factors in vascular endothelial cells should be examined in order to estimate the influence of 24F on the angiogenic pathway.

This study was performed with the aim of evaluating the effectiveness of the novel APN inhibitor 24F as an anti-cancer chemotherapeutic agent. The results clarified that 24F had an ability to inhibit HCC cell growth. Furthermore, several researchers showed that inhibition of APN activity can induce apoptosis in cancer cells (*9*). Thus, it is suggested that suppression of HCC cell growth by incubation with 24F is the result of the induction of apoptosis. Further study is required to clarify the mechanism of inhibition of HCC cell growth by 24F and its relation with apoptosis.

In conclusion, our newly-developed compound 24F can inhibit the activity of the targeted enzyme APN and suppress the invasive capacity of HCC cells. Furthermore, it was also suggested that 24F functions to suppress the angiogenic phenomenon of vascular endothelial cells, which are essential events for cancer progression. Novel APN inhibitor 24F is expected to work as a multi-functional anti-cancer chemotherapeutic agent.

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Original Article

Factors related to well-being among the elderly in urban China focusing on multiple roles

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Summary **Although studies have suggested that having multiple roles is beneficial to well-being in Western society, little is known about the effect of multiple roles in non-Western subjects. We explored predictive factors contributing to well-being, focusing on multiple roles, among elderly Chinese subjects. A cross-sectional survey was conducted among 356 adults aged 60 and older who retired from one university and lived in urban China; participants completed a self-administered questionnaire and returned it by mail. Well-being, the dependent variable, was measured by the Satisfaction with Life Scale. Independent variables included demographics, physical health, financial status, self-efficacy, and the number and frequency of multiple roles. Gender-segregated multiple linear regression analyses were performed. For males, factors related to better well-being were older age, absence of chronic diseases, better financial status, higher self-efficacy, absence of conflict with others, and having grandchildren. For females, factors relating to better well-being were absence of severe illness of a significant other, absence of conflict with others, more roles, more contact with neighbors, and engaging in more group and personal recreational activities. In conclusion, our results highlight predictive factors contributing to well-being among elderly Chinese subjects, and indicate the presence of gender differences. In terms of multiple roles, having more roles, having more contact with neighbors, and engaging in more group activities were significantly related to better well-being for women, but not for men; having grandchildren was significantly related to better well-being for men, but not for women. It is necessary to consider gender when providing livelihood support to elderly Chinese subjects.**

Keywords: Elderly, gender, multiple roles, well-being, urban China

1. Introduction

The number of aging people is rapidly increasing in China, and improving their quality of life (QOL) is an important task. According to the State Council Information Office, by the end of 2005, there were close to 144 million people older than 60 years in China, accounting for 11% of the entire population; urban areas are already ahead of this schedule (*1*). The Chinese government has established some policies to meet the social welfare needs of the elderly and improve their QOL in both urban and rural areas (*1*). For example, efforts have be made to improve and develop medical insurance and community health services as well as encourage the elderly to participate actively in society, including cultural education, work and learning programs, and exercise programs (*1*).

Kamitsuru suggests that QOL is defined as comprehensive well-being based on a personal standard, namely "subjective well-being" (*2*). In recent years, research into subjective well-being of the elderly has been attracting attention in China. Previous studies have identified several key factors that affect subjective well-being, such as age (*3*,*5*), gender (*3*,*5*), education (*3-5*), financial status (*3-4*,*6*), marital status (*4*,*5*), physical health (*5-7*), self-efficacy (*7*), personal activities (*8*), relationships with family members (*5-7*), and becoming a grandparent (*9*). In addition, subjective well-being changes during the various stages of life

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and is particularly affected by aging (*10*). The literature suggests that role loss, such as occurs with retirement, disease, injury, and death of a significant other, threatens the subjective well-being of both Western and Japanese elderly subjects (*11*,*12*). In contrast, acquiring new roles, such as participating actively in society and developing contacts outside the family, is thought to improve the subjective well-being of Western elderly subjects (*13*,*14*). However, little is known about how different roles might affect the subjective well-being of elderly Chinese subjects.

Two perspectives of multiple roles have been used frequently in previous studies: role strain (*15*) and role enhancement (*16*,*17*). These perspectives predict different outcomes for subjects who take on multiple roles. The role strain perspective suggests that multiple roles can make individuals feel overburdened, thereby having a detrimental effect on well-being. In contrast, the role enhancement perspective suggests that the accumulation of multiple roles can increase social integration, leading to an increase in "power, prestige, resources, and heightened sense of identity" (*16*). Based on this latter perspective, too few roles may be detrimental to mental well-being.

The role enhancement perspective has received great support from previous studies that indicate that multiple roles are beneficial for Western subjects' subjective well-being (*18*,*19*). However, few studies have been performed examining the effect of multiple roles on well-being in Chinese subjects.

The role strain perspective and the role enhancement perspective emphasize the total number of multiple roles, but ignore the frequency with which these roles are assumed. Several studies have indicated that the frequency of a specific role, such as engaging in paid work, volunteer, and care-giving activities, affects the well-being of middle- to late-aged Japanese or American adults (*20*,*21*). However, there are no reports regarding the frequency of multiple roles in China. On the other hand, previous studies suggested that multiple roles and well-being may be affected differently in different cultures (*6*,*20*). Therefore, investigating a relationship between multiple roles and well-being in Chinese subjects is important to gain information specific to Chinese subjects.

Chou's findings have suggested that predictors of life satisfaction vary by gender among older Chinese adults (*4*), and occupying multiple roles may be more beneficial to men's psychological well-being than to women's psychological well-being among Western and Japanese subjects (*18-20*). Because of these gender differences, separate analyses should be performed for men and women.

In addition to the factors listed above, other factors, such as physical health and financial status, should be considered when exploring predictive factors for well-being.

The purpose of the present study was to explore the predictive factors contributing to well-being among elderly Chinese subjects focusing on multiple roles. Furthermore, we examined gender differences in multiple roles and well-being. We tested the following hypotheses: (*i*) the number of multiple roles is associated with subjective well-being, and the more roles assumed, the higher well-being will be; and (*ii*) the higher the frequency of each role, the higher the well-being will be.

2. Methods

2.1. *Conceptual model*

Figure 1 depicts a conceptual model of our investigation, which examined the following categories: sociodemographic factors, personal resources, and environmental factors.

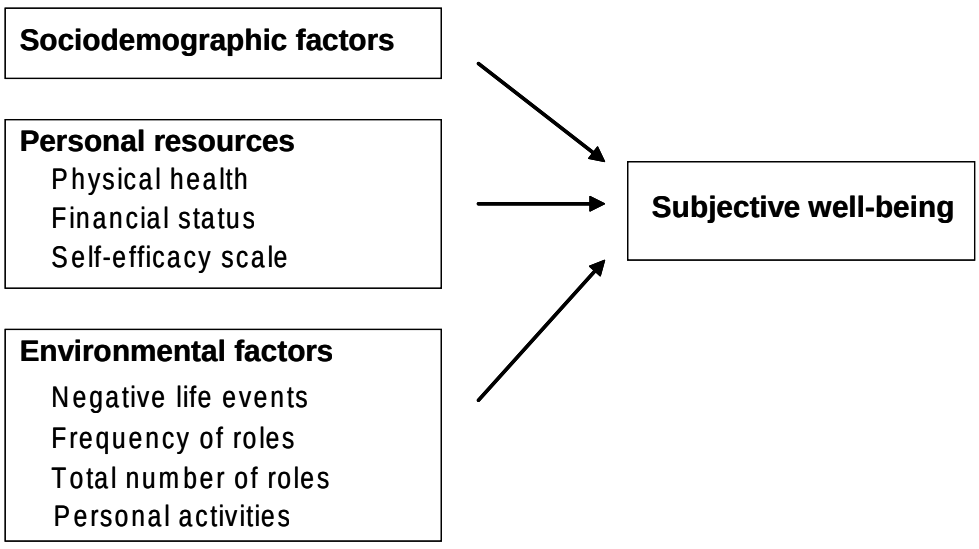

Figure 1. Conceptual model of the present study.

2.2. *Participants and procedures*

Participants were recruited from a register of members who retired from one comprehensive public university located in a city in Hebei Province, China. The main industry in this city was car manufacturing, chemical fiber manufacturing, and filmmaking, and the urban population was 950,000 in 2005 (*22*). According to the government office of the city (Committee on Age, personal communication, Aug 22, 2007), those aged 60 and older accounted for more than 13% of the population in 2006. Inclusion criteria were (*i*) aged 60 years and older, (*ii*) residing in an urban area, and (*iii*) having a clear registered address. Most participants resided in the same district.

An initial version of the questionnaire was tested with 10 community-dwelling elderly participants to identify potential problems; these participants retired from various offices and resided in the same district as present subjects, but were not included in the statistical analysis. A refined self-administered questionnaire and an invitation letter were distributed or mailed to each of the potential participants through the staff of the University between September and October 2007. Participants were instructed to return their voluntary and anonymous answers directly to the researcher in the prepaid envelope. Filling out the questionnaire constituted provision of informed consent. The present study was approved by the Ethics Committee of The University of Tokyo.

2.3. *Conceptual definitions*

The following conceptual definitions were used for this study. We defined "subjective well-being" as "satisfaction with life as a whole based on a personal standard". Drawing upon Nadel's role concept, we defined "role" as a "direct relation with others" and "role frequency" as "face-to-face contact frequency with others during the past year" (*23*). We defined 12 roles, drawing upon previous studies and Kurahashi's interpersonal role classifications: (*i*) child, (*ii*) spouse, (*iii*) parent, (*iv*) grandparent, (*v*) sibling, (*vi*) friend, (*vii*) relative, (*viii*) neighbor, (*ix*) worker, (*x*) group member, (*xi*) religious group member, and (*xii*) volunteer (*24*,*25*).

2.4. *Variables*

Subjective well-being was measured by the Chinese version of the Satisfaction with Life Scale (SWLS) (*26*). The SWLS is designed around the idea that one must make an overall judgment of his or her life to measure life satisfaction. The scale consists of five questions and each question is answered using a 7-point Likert scale, with possible scores ranging from 5 to 35, and higher scores indicating greater satisfaction. The Chinese version of the SWLS has high reliability and validity (*27*), and in the present sample, the scale's alpha reliability was 0.87.

Sociodemographic factors consisted of age, gender, marital status (currently married as 1, not currently married as 0), education (years of school completed), former occupation (teacher, office worker, skilled laborer), and family structure.

2.4.1. *Personal resources*

Personal resources included physical health, financial status, and scores on the self-efficacy scale. Physical health was assessed using two items: self-rated health and the presence of chronic diseases. Self-rated health was measured by a single item that asked "How would you rate your health at the present time?" with four response categories from "very bad" to "excellent". We coded "very bad" and "bad" as 1 and "good" and "excellent" as 0.

For chronic disease, participants were asked whether they had been told by a doctor that they had any of the following illnesses: hypertension, heart disease, stroke, diabetes, respiratory disease, hepatitis or liver cirrhosis, kidney or urinary tract disease, stomach or bowel disease, arthritis or rheumatism, or other disease. The presence of any one of these diseases was coded as 1, and the absence was coded as 0.

Financial status was measured by a single self-rated item that asked, "How would you rate your financial condition at the present time?" with five response categories from "very bad" to "excellent". We coded "good" and "excellent" as 1 and other three responses as 0.

Self-efficacy was assessed using the Chinese version of Generalized Self-efficacy Scale (*28*). This scale assesses the strength of respondents' belief in their effectiveness in dealing with prospective tasks or situations. The scale consists of 10 questions and each question is answered using a 4-point Likert scale, with possible scores ranging from 10 to 40, and a higher score indicating a high level of self-efficacy. The Chinese version of the scale has been validated in an earlier study (*29*), and in the present sample the scale's alpha reliability was 0.91.

2.4.2. *Environmental factors*

Environmental factors included negative life events, frequency of roles, total number of roles, and personal activities. Negative life events drawing upon previous studies included the following clusters and were also coded (yes as 1 and no as 0): death of significant other, severe illness or injury of significant other, severe illness or injury of self, separation from children, conflict with significant other, moving of significant other or self, economic change, legal difficulties,

newly retired, and other sudden unexpected events (*11*,*12*,*30*).

Multiple roles consisted of the total number of roles and the frequency of each role. Twelve roles were coded from the dataset. The first eight roles were determined based on the question, "Do you currently have parents, spouse, children, grandchildren, siblings, friends, relatives, or neighbors?" The second four roles were determined based on the question, "Are you working for pay, undertaking unpaid/volunteer work (*e.g*. public-interest activity, planting trees, supporting community activities), engaging in any group activities (*e.g*. hobbies, learning, recreational activities), or active in any religious or political party?" Responses regarding each role were considered dichotomous variables (yes as 1, no as 0), and the number of roles was summed, with scores ranging from 0 to 12.

The frequency of the 12 roles was based on the following questions: (*i*) "For the first eight roles, how often did you have face-to-face contact during the past year?" with response categories "more than once a week", "a few times a month", "a few times a year or less", and "never"; and (*ii*) "For the second four roles, how often did you engage in work, unpaid work, group activities, or religious or political activities?" with response categories "more than once a week", "a few times a month", "a few times a year", and "never".

2.4.3. *Personal activities*

Participants were asked two questions regarding their personal activities: (*i*) "Did you perform any unpaid housework (*e.g*. home maintenance, cooking)?" and (*ii*) "Did you take part in any individual recreational activities not performed with other people (*e.g*. caring for animals, growing plants, reading, calligraphy)?" with response categories "nearly every day", "a few times a week", "a few times a month", "a few times a year", and "never". We coded "nearly every day" as 1 and others as 0.

2.5 *Statistical analysis*

Descriptive statistics were used for demographic characteristics, total number and frequency of roles, and scores of the SWLS. The student *t*-test and Fisher's exact probability test were used to compare differences between genders. The SWLS was used as a dependent variable in two separate models by gender. Bivariate analysis was used to select independent variables, and multiple linear regression analysis was used to identify significant predictors of life satisfaction. Pearson's product-moment correlation coefficient was used to analyze the correlation with metric variables. Nonparametric statistics, including the Mann-Whitney *U* test, and the Kruskal-Wallis test were used to analyze differences between subgroups. Independent variables with p values less than 0.1 were considered candidate-associated factors of the SWLS, and were forced entered into the multiple linear regression model. Furthermore, multicollinearity among variables was checked. In this study, a p value less than 0.05 was considered statistically significant. All analyses were performed using the Statistical Package for the Social Sciences software, version 12.0 J (SPSS, Inc., Chicago, IL, USA) for Windows.

3. Results

A total of 562 questionnaires were distributed and 420 were returned, for a response rate of 75%. In China, the normal retirement age ranges from 50 to 60 years based on gender and occupation, and the term "elderly" refers to people aged 60 or older. Therefore, 57 participants aged 59 years and younger were excluded from the present study. Among 363 participants aged 60 years and older, seven participants were excluded because they did not fill out one third of the questionnaire, were in a care facility, or their questionnaires lacked information regarding age or gender.

3.1. *Sociodemographic characteristic, roles, and gender differences*

Characteristics of the 356 participants are shown in Table 1. More than half the participants (61.8%) were male. The mean age of participants was 68.4 years, and men were about 2 years older than women ($p < 0.01$). Participants had a mean education level of 13 years, with men having 1 more year of education than women ($p < 0.01$). About half the participants (52%) had a former occupation as a teacher. In general, subjective well-being among this sample was high in both groups, with men reporting significantly higher scores than women (24.6 ± 5.0 *vs*. 23.5 ± 5.4, respectively; $p < 0.05$).

Men reported significantly better physical health than women ($p < 0.05$; Table 2). There were no significant differences in any environmental factor between men and women (Table 3). Table 3 also shows the frequency of fulfilling different roles. Except for paid work, which showed a significant difference between men and women ($p < 0.05$), there were no gender differences in the frequency of roles. The mean number of roles occupied was 7.7 ± 1.8. Men assumed significantly more roles than women (7.8 ± 1.7 *vs*. 7.4 ± 1.6, respectively; $p < 0.05$).

3.2. *Factors related to well-being*

Table 4 shows the multiple regression analysis results from men. All candidate-associated factors of the SWLS were included in the regression model.

Table 1. Characteristics and well-being of participants overall and by gender

	Total (*N* = 356)	Males (*N* = 220)	Females (*N* = 136)	*P*
	N (%) or Mean ± SD	*N* (%) or Mean ± SD	*N* (%) or Mean ± SD	
Age, y	68.4 ± 5.8	69.2 ± 6.0 * c)	67.0 ± 5.2	** a)
60-69	216 (60.7)	117 (53.2)	99 (72.8)	
70-79	125 (35.1)	91 (41.4)	34 (25.0)	
80-	15 (4.2)	12 (5.5)	3 (2.2)	
Marital status				n.s. b)
Married	318 (89.3)	199 (90.5)	119 (87.5)	
Single/divorced/widow/widower	38 (10.7)	21 (9.5)	17 (12.5)	
Education (Years of schooling completed)	13.2 ± 3.0	13.5 ± 3.0	12.6 ± 2.9	** a)
Junior high school or less	48 (13.5)	26 (11.8)	22 (16.2)	
High school	52 (14.6)	21 (9.5)	31 (22.8)	
Junior or technical college	65 (18.3)	37 (16.8)	28 (20.6)	
College degree or more	191 (53.7)	136 (61.8)	55 (40.4)	
Former occupation				n.s. b)
Teacher	185 (52.0)	118 (53.6)	67 (49.3)	
Office worker	124 (34.8)	77 (35.0)	47 (34.5)	
Skilled labor	47 (13.2)	25 (11.4)	22 (16.2)	
Family structure (multiple answers possible)				n.s. b)
Alone	14 (3.9)	8 (3.6)	6 (4.4)	
Other	342 (96.1)	212 (96.4)	130 (95.6)	
Parent	10 (2.8)	8 (3.6)	2 (1.5)	
Spouse/partner	318 (89.3)	198 (90.0)	120 (88.2)	
Child	142 (39.9)	87 (39.5)	55 (40.4)	
Grandchild	118 (33.1)	70 (31.8)	48 (35.3)	
Other	4 (1.1)	3 (1.4)	1 (0.7)	
Well-being				
Satisfaction with Life Scale	24.2 ± 5.2	24.6 ± 5.0	23.5 ± 5.4	* a)

* $P < 0.05$, ** $P < 0.01$.

a) Difference between males and females using *t* test; b) Difference between males and females using Fisher's exact probability test; c) Relationship between age and well-being using Pearson's product-moment correlation method.

Table 2. Personal resources of participants overall and by gender

	Total (*N* = 356)	Males (*N* = 220)	Females (*N* = 136)	*P*
	N (%) or Mean ± SD	*N* (%) or Mean ± SD	*N* (%) or Mean ± SD	
Physical health				
Self-rated health			* c)	* a)
Good	302 (84.8)	194 (88.2)	108 (79.4)	
Bad	52 (14.6)	24 (10.9)	28 (20.6)	
Chronic disease		* c)		* a)
Yes	279 (78.4)	164 (74.5)	115 (84.5)	
No	74 (20.7)	54 (24.5)	20 (14.7)	
Financial status				
Self-rated financial status		** c)	** c)	n.s. a)
Good	117 (32.9)	74 (33.6)	43 (31.6)	
Bad	236 (66.3)	144 (65.4)	92 (67.6)	
Self-efficacy scale	26.5 ± 6.5	26.9 ± 6.4 ** d)	25.9 ± 6.5 * d)	n.s. b)

* $P < 0.05$, ** $P < 0.01$.

a) Difference between males and females using Fisher's exact probability test; b) Difference between males and females using *t* test; c) Relationship of physical health, financial status, and well-being using the Mann-Whitney *U* test; d) Relationship between self-efficacy and well-being using Pearson's product-moment correlation method.

Table 3. Environmental factors of participants overall and by gender

	Total (*N* = 356)	Males (*N* = 220)	Females (*N* = 136)	*P*	
	N (%) or Mean ± SD	*N* (%) or Mean ± SD	*N* (%) or Mean ± SD		
Negative life-events (multiple answers)					
Death of significant other (yes)	120 (33.7)	79 (35.9)	41 (30.1)	n.s.	
Severe illness of significant other (yes)	95 (26.6)	60 (27.2)	35 (25.7) * c)	n.s.	
Severe illness of self (yes)	27 (7.5)	14 (6.3)	13 (9.5)	n.s.	
Separation from children (yes)	23 (6.4)	13 (5.9) * c)	10 (7.3)	n.s.	
Conflict with significant other (yes)	9 (2.5)	4 (1.8) * c)	5 (3.6) * c)	n.s.	
Moving of significant other or self (yes)	39 (10.9)	24 (10.9)	15 (10.9)	n.s.	a)
Economic change (yes)	11 (3.0)	8 (3.6)	3 (2.2) † c)	n.s.	
Legal difficulties (yes)	3 (0.8)	3 (1.3)	0 (0)	n.s.	
Newly retired (yes)	8 (2.2)	8 (3.6)	0 (0)	*	
Unexpected events (yes)	4 (1.1)	3 (1.3)	1 (0.7)	n.s.	
Frequency of roles					
Child				n.s.	a)
More than once a week	16 (4.4)	11 (5.0)	5 (3.6)		
A few times a month	10 (2.8)	6 (2.7)	4 (2.9)		
A few times a year or less	27 (7.5)	17 (7.7)	10 (7.3)		
Never	298 (83.7)	185 (84.0)	113 (89.6)		
Spouse				n.s.	a)
More than once a week	311 (87.3)	193 (87.7)	118 (86.7)		
A few times a month	4 (1.1)	3 (1.4)	1 (0.7)		
A few times a year or less	3 (0.8)	2 (0.9)	1 (0.7)		
Never	38 (10.6)	22 (10.0)	16 (11.7)		
Parent				n.s.	a)
More than once a week	205 (57.5)	121 (55.0)	84 (61.7)		
A few times a month	44 (12.3)	28 (12.7)	16 (11.7)		
A few times a year or less	95 (26.6)	64 (29.0)	31 (22.7)		
Never	3 (0.8)	2 (0.9)	1 (0.7)		
Grandparent		* d)		n.s.	a)
More than once a week	185 (51.9)	117 (53.1)	68 (50.0)		
A few times a month	66 (18.5)	41 (18.6)	25 (18.3)		
A few times a year or less	48 (13.4)	30 (13.6)	18 (13.2)		
Never	47 (13.2)	25 (11.3)	22 (16.1)		
Sibling				n.s.	a)
More than once a week	6 (1.6)	3 (1.3)	3 (2.2)		
A few times a month	41 (11.5)	24 (10.9)	17 (12.5)		
A few times a year or less	202 (56.7)	126 (57.2)	76 (55.8)		
Never	89 (25.0)	56 (25.4)	33 (24.2)		
Friend				n.s.	a)
More than once a week	78 (21.9)	42 (19.0)	36 (26.4)		
A few times a month	67 (18.8)	44 (20.0)	23 (16.9)		
A few times a year or less	166 (46.6)	105 (47.7)	61 (44.8)		
Never	22 (6.1)	15 (6.8)	7 (5.1)		
Relative				n.s.	a)
More than once a week	10 (2.8)	6 (2.7)	4 (2.9)		
A few times a month	47 (13.2)	32 (14.5)	15 (11.0)		
A few times a year or less	246 (69.1)	149 (67.7)	97 (71.3)		
Never	40 (11.2)	24 (10.9)	16 (11.7)		
Neighbor			* d)	n.s.	a)
More than once a week	210 (58.9)	126 (57.2)	84 (61.7)		
A few times a month	51 (14.3)	31 (14)	20 (14.7)		
A few times a year or less	65 (18.2)	41 (18.6)	24 (17.6)		
Never	14 (3.9)	11 (5.0)	3 (2.2)		

continued

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Worker				*	a)
More than once a week	36 (10.1)	29 (13.1)	7 (5.1)		
A few times a month	12 (3.3)	10 (4.5)	2 (1.4)		
A few times a year	19 (5.3)	13 (5.9)	6 (4.4)		
Never	288 (80.8)	168 (76.3)	120 (88.2)		
Group member			** d)	n.s.	a)
More than once a week	107 (30.0)	60 (27.2)	47 (34.5)		
A few times a month	49 (13.7)	35 (15.9)	14 (10.2)		
A few times a year	32 (8.9)	22 (10.0)	10 (7.3)		
Never	165 (46.3)	101 (45.9)	64 (47.0)		
Member of religious group			** d)	n.s.	a)
More than once a week	4 (1.1)	3 (1.3)	1 (0.7)		
A few times a month	16 (4.4)	12 (5.4)	4 (2.9)		
A few times a year	84 (23.5)	54 (24.5)	30 (22.0)		
Never	249 (69.9)	149 (67.7)	100 (73.5)		
Volunteer				n.s.	a)
More than once a week	11 (3.0)	8 (3.6)	3 (2.2)		
A few times a month	16 (4.4)	12 (5.4)	4 (2.9)		
A few times a year	61 (17.1)	44 (20.0)	17 (12.5)		
Never	265 (74.4)	155 (70.4)	110 (80.8)		
Total member of roles	7.7 ± 1.8	7.8 ± 1.7	7.4 ± 1.6 ** e)	*	b)
Personal activities					
Housework		† c)		*	a)
Nearly every day	277 (77.8)	159 (72.2)	118 (86.7)		
Others	76 (21.3)	60 (27.2)	16 (11.7)		
Recreational activities			** c)	n.s.	a)
Nearly every day	243 (68.2)	152 (69.0)	91 (66.9)		
Others	113 (31.7)	68 (30.9)	45 (33.0)		

† $P < 0.10$, * $P < 0.05$, ** $P < 0.01$.
a) Difference between males and females using Fisher's exact probability test;
b) Difference between males and females using *t* test;
c) Relationship of negative life-events, personal activities, and well-being using the Mann-Whitney *U* test;
d) Relationship between frequency of roles and well-being using the Kruskal-Wallis test;
e) Relationship between total number of roles and well-being using Pearson's product-moment correlation method.

However, the number of roles was not significantly related to well-being in the bivariable analysis of men. For men, significant predictors of better well-being were older age ($p < 0.05$), absence of chronic diseases ($p < 0.05$), better financial status ($p < 0.01$), higher self-efficacy ($p < 0.01$), absence of conflict with significant other ($p < 0.05$), and having contact with a grandchild more than once a week compared with not having a grandchild ($p < 0.05$). These factors explained 21% of the total variance in life satisfaction among men.

Table 5 shows the multiple regression analysis findings for women. Except for being a member of a religious group, which was excluded due to multicollinearity between that variable and the number of roles, all other candidate-associated factors of the SWLS were included in the regression model. For women, significant predictors of better well-being were absence of severe illness of significant other ($p < 0.05$), absence of conflict with significant other ($p < 0.05$), frequent contact with neighbors ($p < 0.01$), frequent involvement with group activities ($p < 0.05$), assuming more roles ($p < 0.01$), and taking part in more personal recreational activities ($p < 0.01$). These factors explained 34% of the total variance in life satisfaction.

4. Discussion

The present study identified factors related to life satisfaction among elderly Chinese subjects. Results of this study partly support the role enhancement perspective and the first hypothesis we considered. Among women, older adults occupying multiple roles experienced higher levels of subjective well-being than those with fewer roles. However, this relationship was not seen in men. These findings are consistent with Sugihara's findings in Japanese subjects, but not with Thoits and Adelman's findings in American subjects (*18-20*). Cheng found similar findings to this study, reporting that social relationships are a stronger determinant of life satisfaction in older Chinese women than in older Chinese men (*31*). It

Table 4. Regression of SWLS on sociodemographic variables and personal resources and environmental factors in men (*N* = 206)

	β	*P*
Sociodemographic variables		
Age	0.17	*
Personal resources		
Chronic diseases (yes = 1, no = 0)	–0.14	*
Financial status (good = 1, bad = 0)	0.24	**
Self-efficacy scale	0.22	**
Environmental factors		
Negative life events		
Conflict with significant other (yes = 1, no = 0)	–0.14	*
Separation from children (yes = 1, no = 0)	0.07	
Frequency of roles		
Grandparent		
More than once a week #		
A few times a month	–0.07	
A few times a year or less	–0.07	
Never	–0.16	*
Personal activities		
Housework (nearly every day = 1, other = 0)	–0.01	
R^2	0.25	
Adjusted R^2	0.21	**

* $P < 0.05$, ** $P < 0.01$. β: Standardized partial regression coefficient; #: Reference category.

Table 5. Regression of SWLS on sociodemographic variables and personal resources and environmental factor in women (*N* = 124)

	β	*P*
Personal resources		
Self-rated health (good = 1, bad = 0)	0.06	
Financial status (good = 1, bad = 0)	0.06	
Self-efficacy Scale	0.08	
Environmental factors		
Negative life events		
Severe illness of significant other (yes = 1, no = 0)	–0.19	*
Conflict with significant other (yes = 1, no = 0)	–0.18	*
Economic changes (yes = 1, no = 0)	0.14	†
Frequency of roles		
Neighbor		
More than once a week #		
A few times a month	0.00	
A few times a year or less	–0.25	**
Never	0.09	
Group member		
More than once a week #		
A few times a month	–0.21	*
A few times a year	–0.15	†
Never	–0.17	†
Number of roles	0.28	**
Personal activities		
Recreational activities (nearly every day = 1, other = 0)	0.25	**
R^2	0.41	
Adjusted R^2	0.34	**

† $P < 0.10$, * $P < 0.05$, ** $P < 0.01$. β: Standardized partial regression coefficient; #: Reference category.

is possible that gender differences in multiple roles and well-being might reflect characteristics of the different cultures studied. Another explanation for this finding is that there may be new specific role changes and role combinations in the present subjects, which may have a different effect on the subjects' well-being depending on gender. Sugihara and Menaghan's findings suggest that specific role combinations are meaningfully associated with the elderly well-being by gender (*20*,*32*). Future studies should examine these gender differences in specific role changes and role combinations among Chinese subjects.

Findings from this study also partly support the second hypothesis. Frequent contact with neighbors was a predictive factor related to better well-being among women. The result is in agreement with previous studies (*14*,*33*). Having frequent contact with neighbors allows women to share mutual concerns and understanding regarding their daily lives and helps confirm their own identity; it also provides a better sense of the neighborhood (*34*,*35*). In addition, the sample included in this study retired from the same university and many of them lived in the same apartment complex. It is likely that they knew each other for a long time and had multiple relationships, as both peers and acquaintances. These relationships may have led subjects to feel a greater emotional intimacy and gain a better sense of the neighborhood, therefore increasing their well-being.

Women who engaged in group activities more frequently had better well-being. This result is supported by the findings of Herzog, which showed that engaging in group activities could increase a person's well-being by improving their self-image (*36*). In addition, Boermel indicated that joining in group activities increased pleasure among the elderly and won them praise from their contemporaries (*37*).

In this study, being a grandparent was a predictive factor related to better well-being for men but not for women. One explanation for this finding is that a grandparent's role varies by gender. Although becoming a grandparent is a positive experience for Chinese subjects (*9*,*38*), the sense of responsibility and contribution to family and society that comes along with being a grandparent may be stronger in Chinese men than in Chinese women. Another explanation is that the beneficial gains of being a grandparent vary by gender. Filus' finding indicated that Chinese grandmothers engage in more activities concerning grandchildren than Chinese grandfathers (*9*). In this study, 73% of women were in their 60s and might have been looking after their grandchildren, taking on a greater caregiver burden than men. The beneficial gains of being a grandparent for women may therefore be partially offset by the burden they assume in this role. Future studies should examine these gender differences in terms of benefits and burdens related to being a grandparent among Chinese subjects.

Gender differences were observed between specific roles and well-being. One explanation for this finding may be that carrying out the same multiple roles has a different meaning to men and women, as shown by Simon (*39*).

4.1. *Implications for practice*

The current findings may provide useful information for public health programs and policies aimed at maintaining well-being in later life. The morbidity prevalence rate of chronic diseases was 78% in the present subjects, and chronic diseases significantly decreased the well-being of men. Therefore, it is critical that the Government improve health services and community care, such as providing regular checkups for free or at low cost; develop and perform effective health education programs for people of all ages to increase their ability to take care of themselves; and attempt to help prevent the development of chronic diseases. In addition, having healthcare professionals or family members assess an older adult's conflicts with significant others may help promote better communication and subsequently increase the well-being of the elderly. For elderly Chinese women, improving childcare services to decrease care burden, offering information and places to engage in group actives, increasing contact with neighbors, and expanding recreational activities may have a positive effect on well-being.

4.2. *Limitations and implications for future research*

The present study has several limitations. First, generalization of these findings to other groups is limited, as most of the participants had a high education level. For example, the mean education level was 13 years in the present participants, compared with 9 years in Tang's community participants in Beijing (*7*) and 6.6 years in Li's community participants in Shanghai (*8*). In addition, all of the participants came from one university, and thus do not accurately represent all older adults in China. Second, by using a mailed survey, we limited our respondents to subjects in their 60s or early 70s who were in fairly good health, as those who were not healthy may not have been able to complete and return the questionnaire. Performing face-to-face interviews in the future may help capture a larger portion of the population and increase the accuracy of our research. Third, this was a cross-sectional study and thus it is not possible to determine cause and effect; for example, whether women with better well-being scores engaged in more roles, or having more roles increased a woman's well-being score. Longitudinal

studies are necessary to clarify any causal relation between roles and well-being.

5. Conclusion

The present study highlights predictive factors contributing to well-being among elderly Chinese subjects, and indicates the presence of gender differences. Having more roles, having more contact with neighbors, and engaging in more group activities were significantly related to better well-being for women, but not for men. Having grandchildren was significantly related to better well-being for men, but not for women. Except for paid work, there were no gender differences in the frequency of the multiple roles, but gender differences between specific roles and well-being did exist. It is thus necessary to consider gender when providing livelihood support to elderly Chinese subjects.

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Original Article

A novel model for prognosis of Meniere's disease using oxidative stress susceptibility of lymphoblastoid cell lines

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Summary **The aim of this study was to examine differences of susceptibility to oxidative stress of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) established from Meniere's disease (MD) patients and to examine the effect of ATP treatment in the prognosis and treatment MD. LCLs were established from 10 patients with MD and 10 healthy donors by EBV. Cell viabilities were calculated after treatment of H_2O_2 with or without ATP. The relationship between the sensitivity of H_2O_2-treated LCLs to ATP and the staging scale of MD was examined. The nuclear morphological changes of Hoechst 33258-stained LCLs after H_2O_2-treatment were observed under a fluorescence microscope. LCLs from MD were significantly more sensitive ($p < 0.001$) to H_2O_2 than LCLs from healthy donors after 3 h of H_2O_2 treatment. All of the ATP-sensitive LCLs were categorized as Stage 1 or 2, while others categorized as Stage 3 or 4 were not sensitive to ATP. There were significant differences ($p < 0.01$) of cell viabilities after addition of ATP between H_2O_2-treated LCLs classified as Stage 1 or 2 and as Stage 3 or 4 in MD. Both chromatin condensation and swelling of the cell body were observed in H_2O_2-treated LCLs. Our findings indicate that LCLs established from MD patients might be used as a unique model to detect susceptibility to oxidative stress and ATP treatment in MD patients. Also, the difference of the sensitivity of H_2O_2-treated LCLs to ATP might relate to prognosis and treatment of MD. This system may form the basis of tailor-made therapy for MD.**

Keywords: Meniere's disease (MD), lymphoblastoid cell line (LCL), oxidative stress, adenosine 5'-triphosphate (ATP)

1. Introduction

Meniere's disease (MD), described by Prospero Meniere in 1861, is typically characterized by fluctuating hearing loss, episodic vertigo, tinnitus and a sensation of pressure. The histopathological hallmarks of the disease, at the bone level, are endolymphatic hydrops, atrophy and erosion of the endolymphatic sac. Despite a rigorous pathological definition, the etiology of MD, which is usually defined as idiopathic, is ascribed to a variety of causes, such as alterations of ionic homeostasis, vascular disorder, trauma, viral infections and immunological disorder. However, until recently it has been difficult to estimate the prognosis of MD clinically. Current studies have reported that oxidative stress may play a crucial role in the pathogenesis of a variety of inner ear diseases, such as noise-induced hearing loss (*1*), ischemic impairment (*2*) and age-related hearing loss (*3*). Concerning MD, Horner and Guilhaume suggested that oxidative insult was likely to contribute to the pathology associated with endolymphatic hydrops and therefore free radical scavengers might be useful in the treatment of MD patients (*4*). Takumida *et al*. demonstrated that edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), an

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inhibitor of reactive oxygen species (ROS), attenuated the formation of endolymphatic hydrops in the guinea pig cochlea (*5*). In a clinical trial, treatment using such radical scavengers was reported to have the potential to become an effective new therapy for patients with MD (*6*). However, the direct effect of oxidative stress for MD inner ear tissue cells is still unknown, because the normal inner ear tissue can not be obtained. Therefore, we focused on Epstein-Barr virus (EBV)-transformed B-lymphocytes (lymphoblastoid cell lines; LCLs) as a cellular model for MD like hypertension (*7*), diabetes mellitus (*8*), Alzheimer's disease (*9*), Huntington's disease (*10*), and bipolar disease (*11*), because we hypothesized that LCLs could be used in place of inner ear cells of patients with MD. In addition, LCLs can be easily established from B-lymphocytes obtained from patients using EBV infection and can be maintained for a long time.

Hydrogen peroxide (H_2O_2) is an intermediate product of the degradation of ROS and a highly reactive molecule. The treatment of cells with H_2O_2 induces oxidative stress *via* an increased production of ROS and may subsequently lead to cell damage or cell death. Extracellular H_2O_2 is able to cross cell membranes and directly alters their intracellular concentration (*12*). The loss of adenosine 5'-triphosphate (ATP) has been reported to be an early step after initiation of H_2O_2-induced oxidative stress in non-neuronal (*13*) and neural systems (*14*). Teepker *et al*. reported that ATP-decline under H_2O_2-induced oxidative stress might point to a relevant ATP consumption related to apoptosis (*15*). In this study, we evaluated the difference of the susceptibility of the LCLs to H_2O_2-induced oxidative stress from patients with MD and healthy donors. We also investigated the ability of ATP treatment to modulate the cell viability of LCLs loaded with H_2O_2 and considered the relationship between the sensitivity of the H_2O_2-treated LCLs to ATP and the staging scale of MD. The aim of this study was to examine whether the difference of the susceptibility of LCLs to H_2O_2-induced oxidative stress and the effect of ATP treatment reflects the prognosis of MD. To our knowledge, this is the first report in which LCLs established from the patients were used as a cellular model for MD.

2. Materials and Methods

2.1. *Materials*

Ten patients with MD as defined by the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) (4 males and 6 females, 54.7 ± 13.7 years old) and 10 healthy volunteers (5 males and 5 females, 53.5 ± 11.4 years old) were studied. Informed consent was obtained from all cases. Sensory hearing threshold was classified on the four-way classification of the American Academy of Otolaryngology (Committee on Hearing and Equilibrium, 1995): Stage 1 (mean threshold < 26 dB), Stage 2 (mean threshold 26-40 dB), Stage 3 (mean threshold 41-70 dB), and Stage 4 (mean threshold > 71 dB). Mean threshold was in each case calculated as the arithmetic mean of the threshold at 4 frequencies (500, 1,000, 2,000, and 3,000 Hz) measured on the same day as the dynamic posturography session.

2.2. *Cell Culture*

Peripheral blood lymphocytes (PBMC) were obtained from patients with MD and normal controls and transformed by Epstein-Barr virus (B95-8 strain) for establishing LCLs as described elsewhere (*16*,*17*). The LCLs were grown in complete medium consisting of RPMI 1640 (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10 % fetal calf serum (FCS).

2.3. *Viability assay*

Cells were suspended at a density of 4.0×10^6 cells/mL in complete medium and seeded at 1.0×10^5 cells per well of a 96-well UV plate (Nunc, Roskilde, Denmark). An equal volume of 0.04 mM H_2O_2 was added to each cell suspension and the mixtures were incubated at 37°C. The adequate concentration of H_2O_2 in this study was 0.02 mM at the final concentration by determining the results of the preliminary experiments and on the basis of the effects of H_2O_2 in HeLa cells (*18*), fibroblasts (*19*), cardiac myocytes (*20*), or human T-lymphoma (*21*). ATP (GE Healthcare, Salt Lake City, UT, USA) was also added as 20× stock solution to 5 mM at the final concentration. Cell viability was determined by trypan blue exclusion. Cells that were treated with H_2O_2 with or without ATP and incubated at 37°C for the times indicated were suspended in an equal volume of 0.4% trypan blue (Invitrogen, Carlsbad, CA, USA). Dead (blue) and live (clear) cells were counted using a hemocytometer. The percentage of viability was defined as the number of live cells divided by the number of live and dead cells. All experiments were performed in triplicate.

2.4. *Morphological examinations of cells*

LCLs exposed to the medium containing 0.02 mM H_2O_2 for 5 h were observed under a phase-contrast microscope. For determination of nuclear morphological change, the cells were additionally incubated with 1 μL of Hoechst 33258 (Dojido, Kumamoto, Japan) (1 mg/mL) for 10 min. After staining, the cells were washed with PBS and analyzed under a fluorescence microscope.

2.5. *Statistical Analysis*

All statistical analysis was performed using Ystat 2004.

xls program for Windows/Macintosh. Descriptive statistics were used to describe the response, and paired *t*-test or unpaired *t*-test was used where appropriate. Continuous data were displayed as the mean ± SD. Statistical significance was accepted when the *p* value was less than 0.05.

3. Results

3.1. *Clinical trial*

The results are summarized in Table 1. AAO-HNS Staging scale identified two patients (Me1 and Me10) with Stage 1, three patients (Me3, Me4, and Me7) with Stage 2, one patient (Me8) with Stage 3, and four patients (Me2, Me5, Me6, and Me9) with Stage 4.

3.2. *Susceptibility of LCLs to killing by H_2O_2*

After treatment with 0.02 mM H_2O_2, the viabilities of LCLs from MD patients and from normal controls were compared (Figure 1). These data showed that LCLs from MDs were significantly more sensitive ($p < 0.001$) to H_2O_2 than LCLs from healthy donors after 3 h of H_2O_2 treatment. These results indicate that control cells are resistant to H_2O_2 treatment, while MD cells are sensitive.

Table 1. Demographic and clinical characteristics

	Age	Gender	AAO-HNS Staging
Me1	40	F	1
Me2	70	F	4
Me3	68	M	2
Me4	38	M	2
Me5	66	M	4
Me6	57	F	4
Me7	66	F	2
Me8	44	F	3
Me9	36	F	4
Me10	62	M	1

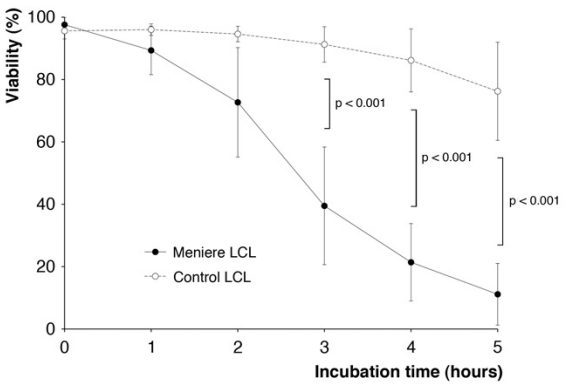

Figure 1. Comparison of the viabilities of LCLs from MDs and healthy donors after H_2O_2 treatment. LCLs from MDs were significantly more sensitive ($p < 0.001$) to H_2O_2 than LCLs from healthy donors after 3 h of H_2O_2-treatment.

3.3. *Effects of ATP addition on H_2O_2 cell damage in LCLs*

Figure 2 showed that the viability after 5 h of 0.02 mM H_2O_2 treatment were 24.6% in Me1 cells, 3.3% in Me2 cells, 27.8% in Me3 cells, 8.2% in Me4 cells, 5.9% in Me5 cells, 0% in Me6 cells, 5.6% in Me7 cells, 9.4% in Me8 cells, 4.3% in Me9 cells, and 22.2% in Me10 cells. Addition of ATP could obviously recover the viabilities of the H_2O_2-treateted LCLs from Me1, Me3, Me4, Me7, and Me10 patients (Figures 2A, 2C, 2D, 2G, and 2J). In contrast, the viabilities of H_2O_2-treated LCLs from Me2, Me5, Me6, Me8, and Me9 patients decreased in a similar manner as when ATP was not added (Figures 2B, 2E, 2F, 2H, and 2I). The viabilities after 5 h of treatment of 0.02 mM H_2O_2 and the addition of 0.05 mM ATP were 89.9% in Me1 cells, 7.2% in Me2 cells, 84.8% in Me3 cells, 61.1% in Me4 cells, 10.0% in Me5 cells, 0% in Me6 cells, 80.1% in Me7 cells, 7.8% in Me8 cells, 4.4% in Me9 cells, and 79.3% in Me10 cells. These data indicate that Me1, Me3, Me4, Me7, and Me10 cells might be sensitive to ATP. On the other hand, Me2, Me5, Me6, Me8, and Me9 cells are not as strongly affected by ATP.

3.4. *The relationship between the sensitivity of H_2O_2-treated LCLs to ATP and the staging scale of MD*

As shown in Figure 3, the ATP-sensitive LCLs (Me1, Me3, Me4, Me7, and Me10) were classified as Stage 1 or 2, while the ATP-insensitive LCLs (Me2, Me5, Me6, Me8, and Me9) were classified as Stage 3 or 4. There were significant differences ($p < 0.01$) in the viabilities of H_2O_2-treated LCLs classified as Stage 1 or 2 and as Stage 3 or 4 in MD after the addition of ATP.

3.5. *Effects of H_2O_2 on membrane integrity and chromatin structure*

Both chromatin condensation and swelling of the cell body were observed after treatment with 0.02 mM H_2O_2 (Figure 4).

4. Discussion

Treatment of cells with H_2O_2 induces oxidative stress, accompanied by lipid peroxidation, DNA and protein damage (*22*), and finally cell death (*23*). In addition, oxidative stress is able to disturb cellular energy metabolism as a result of the decrease of ATP in a variety of cells (*24*). These studies were based on the hypothesis that the susceptibility of individual cells to oxidative stress was different from one person to another. Our results strongly demonstrated that LCLs from patients with MD were significantly more sensitive ($p < 0.001$) to oxidative stress than LCLs

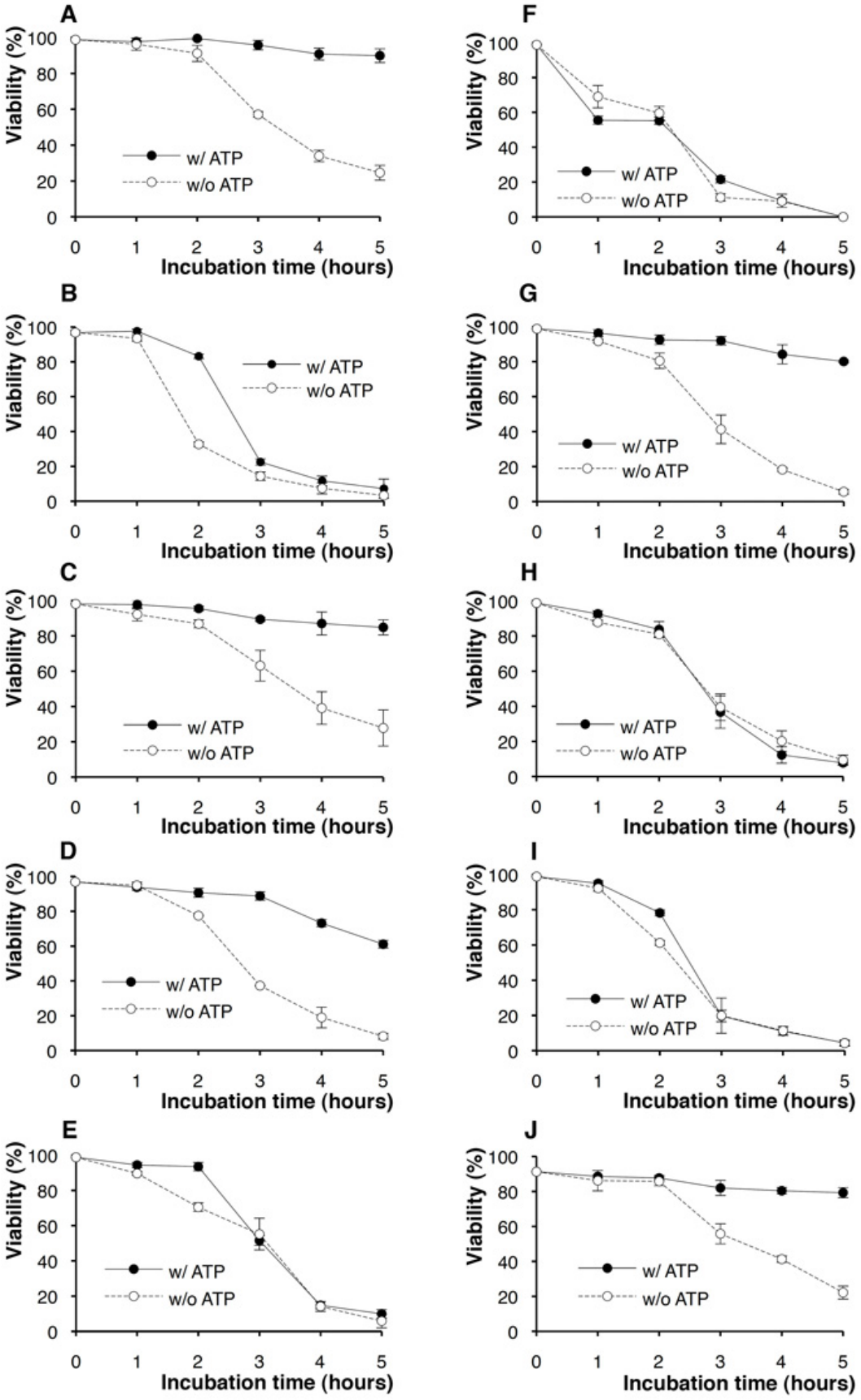

Figure 2. Effect of ATP addition to the viabilities of LCLs after H_2O_2 treatment. Addition of ATP could obviously recover the viabilities of H_2O_2-treated LCLs from Me1, Me3, Me4, Me7, and Me10 patients (**A**, **C**, **D**, **G**, and **J**, respectively). In contrast, the viabilities of H_2O_2-treated LCLs from Me2, Me5, Me6, Me8, and Me9 patients decreased in a similar manner as when ATP was not added (**B**, **E**, **F**, **H**, and **I**, respectively).

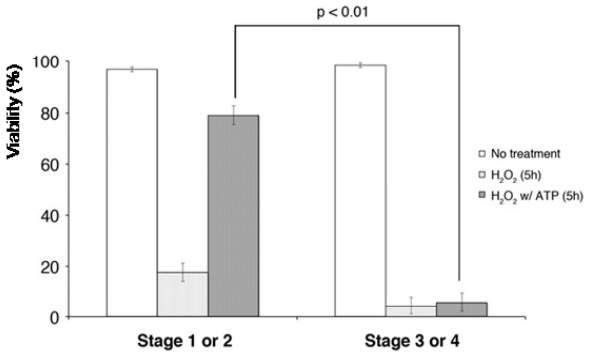

Figure 3. Comparison of the viabilities of H_2O_2-treated LCLs from MD classified on the four-way classification. There were significant differences ($p < 0.01$) of cell viabilities of H_2O_2-treated LCLs classified as Stage 1 or 2 and as Stage 3 or 4 after the addition of ATP.

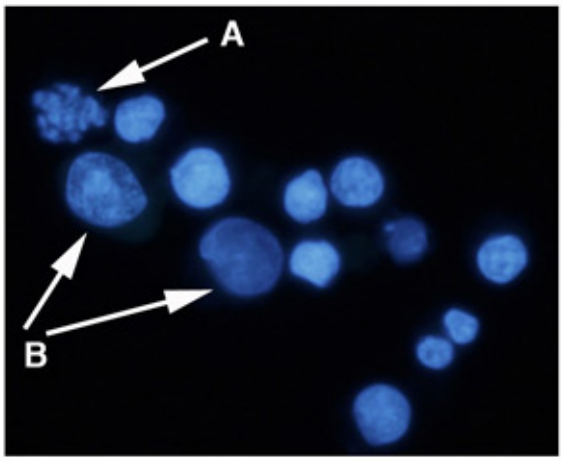

Figure 4. Effects of H_2O_2 on membrane integrity and chromatin structure in LCLs. LCLs treated with H_2O_2 for 5 h showed either chromatin condensation (**A**) or swelling of the cell body (**B**).

from healthy donors (Figure 1). In other words, LCLs from healthy donors were resistant to H_2O_2, while LCLs from MD patients were not. This finding also suggests that patients with MD can be diagnosed by the difference of susceptibility of established LCLs to oxidative stress.

Next, we investigated the effect of ATP treatment on H_2O_2-treated LCLs. At an early stage of cell damage, ATP-depletion and intracellular Ca^{2+} alteration may occur under H_2O_2-induced oxidative stress (*25*). The concentration of extracellular ATP regulates various signaling systems including propagation of intercellular Ca^{2+} signals. To reveal the cause of ATP-depletion after the exposure of cells to H_2O_2, the oxidative inactivation of mitochondrial ATP synthetase was examined (*26*). Lee *et al*. reported that epithelial cells of the inner ear coordinated their ion transport activity through the autocrine and paracrine signal pathway among neighboring cells in the ear *via* ATP (*27*). In addition, ATP is one of the commonly used medications for the treatment of MD in Japan (*28*). Our results demonstrated that the addition of ATP to H_2O_2-treated LCLs clearly recovered the viabilities in Me1, M3, M4, Me7, and Me10 cells, although the cells from Me2, Me5, Me6, Me8, and Me9 did not recover their viability after ATP treatment. Therefore, we thought that Me1, M3, M4, Me7, and Me10 cells were sensitive to ATP treatment, whereas Me2, Me5, Me6, Me8, and Me9 cells, by contrast, were not. Interestingly, as shown in Table 1, all ATP-sensitive cases were classified as AAO-HNS Stage 1 or 2 and all ATP-insensitive cases were classified as AAO-HNS Stage 3 or 4. After the ATP treatment, there was a significant difference ($p < 0.01$) of the viabilities of the H_2O_2-treated LCLs classified as Stage 1 or 2 and Stage 3 or 4 in MD (Figure 3). These results demonstrated that the sensitivity of H_2O_2-treated LCLs to ATP might represent a method for prognosis and treatment of MD. Clinically, some of patients staged 3 or 4 experiences poor control of vertigo, the progressive sensorineural hearing loss and the worsening of tinnitus even after several years treatment. The treatment of MD mainly aims to reduce these symptoms, because all three symptoms, either separately or in combination, cause great distress and have a considerable impact on the patients quality of life (*29*). Therefore, the prognostic expectation of MD is very profound for the quality of life of patients with MD. Additionally, these LCLs established from patients may be used for the drug susceptibility test in MD.

We also investigated morphological changes of the LCLs treated with H_2O_2. Figure 4 showed that after 5 h treatment, H_2O_2-treated LCLs showed either chromatin condensation or swelling of the cell body. Chromatin condensation indicates apoptosis of LCLs and swelling of cells body indicates necrosis. Since apoptosis is a highly regulated and energy-dependent process (*30*), the ATP decline may point to a relevant ATP consumption related to apoptosis. Saito *et al*. reported that prevention of intracellular ATP loss significantly activated caspases and changed the mode of cell death from necrosis to apoptosis, and therefore ATP-dependent apoptosome formation determined whether H_2O_2-induced cell death was due to apoptosis or necrosis (*21*). We previously reported oxidative stress in the form of H_2O_2-induced morphological changes in vestibular hair cells (*31*). This finding suggested oxidative stress caused by H_2O_2 might affect the morphology and survival of inner ear cells.

In this study, 10 EBV-transformed LCLs derived from patients with MD were examined. It is not clear how these cells accurately represent the endogenous condition of inner ear cells, because various genes are expressed in each cell. In spite of these restrictive conditions, our results suggested that patients with MD might be unable to cope with oxidative stress.

In conclusion, LCLs established from MD patients could be used as a unique model to detect the susceptibility to oxidative stress and the effect of ATP treatment in MD patients. The difference of the sensitivity of H_2O_2-treated LCLs to ATP might relate to the prognosis of MD. This system may form the basis of tailor-made therapy for MD.

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Original Article

Study of the hepatotoxicity induced by *Dioscorea bulbifera* L. rhizome in mice

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Summary ***Dioscorea bulbifera* L. is a medicinal plant. The present study was undertaken to investigate the hepatotoxicity induced by *D. bulbifera* in mice. Through the acute toxicity of various extracts including the EtOAc fraction (EF) and the non-EtOAc fraction (Non-EF) from ethanol, and the ethanol itself, we found that the EF contains the toxic ingredients of *D. bulbifera* rhizome. On this basis, to study the hepatotoxicity induced by the toxic ingredients, mice were treated with 0.5% sodium carboxymethyl cellulose (CMC-Na) alone or the EF of *D. bulbifera* rhizome at doses of 80, 160, 320, and 480 mg/kg once daily *i.g.* for fourteen consecutive administrations. Serum samples were collected for determination of the biomarkers for liver injury, such as, alanine transaminase (ALT) and aspartate transanimase (AST). Hepatic tissues were used to assay for the level of lipid peroxide (LPO), amounts of antioxidants such as glutathione, and activities of antioxidant-related enzymes for liver oxidative-antioxidative status in mice. The results showed that ALT and AST were significantly elevated after fourteen consecutive administrations of the EF of *D. bulbifera* rhizome. In addition, the level of LPO increased remarkably, while the glutathione amounts, and the activities of the antioxidant-related and glutathione-related enzymes including superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR) and glutamate-cysteine ligase (GCL) of hepatic tissues all decreased conspicuously, in livers of mice treated with the EF of *D. bulbifera* rhizome. Taken together, our results indicate that the EF contains the main toxic ingredients of *D. bulbifera* rhizome, and the mechanism of hepatotoxicity induced by it may be due to liver oxidative stress injury in mice.**

Keywords: *D. bulbifera*, EtOAc fraction, hepatotoxicity, oxidative stress

1. Introduction

The rhizome of *Dioscorea bulbifera* (Dioscoreaceae) is widely distributed in Asia and traditionally used to treat various diseases including thyroid disease, tumors, *etc.* (*1-2*). Research results have demonstrated that *D. bulbifera* rhizome could induce hepatotoxicity in mice and rats (*3-4*). However, both the chemical compounds and mechanism of induced liver toxicity are still not very clear.

It is reported that oxidative stress plays a critical role in liver toxicity induced by alcohol, carbon tetrachloride, acetaminophen, chemotherapeutic agents and so on (*5-9*). Reactive oxygen species (ROS), produced in the process of oxidative stress, are extremely reactive, which may modify and inactivate lipids, proteins, DNA, and RNA, and thus induce cell injury. To prevent ROS-induced cell injury, the body has developed antioxidant systems including antioxidants and antioxidant enzymes. Among them,

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glutathione is one of the common antioxidants (*10*). Superoxide dismutase (SOD) and catalase (CAT) are two important antioxidant-related enzymes, while glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR), and glutamate-cysteine ligase (GCL) are all glutathione-related antioxidant enzymes. Such antioxidant systems protect the organism from injury induced by ROS. However, with regard to the liver toxicity induced by *D. bulbifera* rhizome, there is no related report about the involvement of ROS in induced liver injury.

The present study was designed to evaluate the toxic ingredients of *D. bulbifera* rhizome through acute toxicity experiments, and then to explore the mechanism that oxidative stress injury has in such hepatotoxicity by measuring the lipid peroxide (LPO) level, glutathione amounts, and activities of antioxidant enzymes including SOD, CAT, GPx, GST, GR, and GCL in mice livers.

2. Materials and Methods

2.1. *Experimental animals*

ICR male and female mice (20 ± 2 g) were purchased from Shanghai Slac Laboratory Animal Co. Ltd. (Certificate No. SCXK 2007-0005, Shanghai, China). The animals were reared in the animal house of Regional Center Animals. They were given rodent laboratory chow and water *ad libitum*, and maintained under controlled conditions with a temperature of 22 ± 1°C, relative humidity 65 ± 10% and a 12/12 h light/dark cycle (lights on at 7:00 am). All the procedures were in strict accordance with the P. R. China legislation on the use and care of laboratory animals using the guidelines established by Institute for Experimental Animals of Shanghai University of Traditional Chinese Medicine and were approved by the university committee for animal experiments.

2.2. *Materials*

NADPH, reduced glutathione (GSH) and oxidized glutathione (GSSG) were purchased from Roche (Basel, Switzerland). 5,5'-Dithio-bis(2-nitrobenzoic acid) (DTNB), glutathione reductase, 2-thiobarbituric acid (TBA), and other reagents unless indicated were from Sigma Chemical Co. (St. Louis, MO, USA).

2.3. *Samples and preparation of various extracts*

The rhizomes of *D. bulbifera* were collected in Qingyang, Anhui Province and authenticated by Prof. Shou-Jin Liu (Anhui College of Traditional Chinese Medicine, Anhui, China). A voucher specimen was deposited in the herbarium of Institute of Traditional Chinese Medicine, Shanghai University of Traditional Chinese Medicine. Preparation of various extracts is described as follows.

Water or ethanol extract: The powder was soaked in water or 80% ethanol (w/v = 1:10), and incubated at room temperature for 120 min, respectively. The mixtures were extracted three consecutive times, with constant stirring at 100°C once for 60 min for the water extract, and with a rotary evaporator at 85 ± 5°C and once for 180 min for the ethanol extract, respectively. The combined extracts were centrifuged at 4,000 × g for 10 min and the supernatant was decanted to a glass container and concentrated under vacuum using a rotary evaporator under reduced pressure at 45 ± 5°C. Thus, the water (about 53 g) and ethanol (about 78 g) extracts were obtained.

EF and Non-EF from ethanol extract: Half of the above ethanol extract was extracted eight times using EtOAc (v/v = 1:1) at room temperature. After evaporation of solvents, EtOAc (about 16 g) and Non-EtOAc (about 62 g) layers were achieved. The yield of EF from the ethanol extract was about 1.6% of raw medicinal materials.

The above extracts were diluted with 0.5% CMC-Na into different doses for the experiment. All extracts were stored at 4°C before use.

2.4. *Acute toxicity*

Healthy ICR mice, weighing 20 ± 2 g, were randomly divided into groups of 10 animals (5 males and 5 females). They were fasted from food, but not water 12 h prior to the administration of the test suspension. The control group received water containing 0.5% CMC-Na (vehicle) administered orally *i.g.*. The water extract of *D. bulbifera* rhizome suspended in 0.5% CMC-Na was administered orally at a dose of 14,000 mg/kg, while the ethanol suspension in 0.5% CMC-Na was administered orally at doses of 2,700, 3,200, 3,800, 4,500, and 5,400 mg/kg. The Non-EF ethanol extract of *D. bulbifera* rhizome suspended in 0.5% CMC-Na was administered orally at a dose 13,800 mg/kg, while the EF suspension in 0.5% CMC-Na was administered orally at doses of 320, 480, 640, 761, 905, 1,077, and 1,280 mg/kg. Toxic symptoms such as piloerection, inactiveness, dizziness, hypothermia, loss of righting reflex, *etc.*, and mortality was observed twice a day for fourteen consecutive days after the treatment. The toxicological effect was assessed on the basis of mortality, which was expressed as the median lethal dose (LD_{50}) (*11*).

2.5. *Hepatotoxicity*

To determine the hepatotoxicity induced by *D. bulbifera* rhizome, groups of 10 male mice each were administered, once daily 80, 160, 320, or 480 mg/kg, *i.g.*, of the EF (suspended in 0.5% CMC-Na) of the ethanol extract from *D. bulbifera* rhizome for 14 days. Mice in

the control group (Normal) received the vehicle in an identical manner. After the treatment period, mice were sacrificed, blood was collected and livers were removed. Serum samples from blood were used for the assay of biomarkers for liver injury and the liver tissues of partial groups for assay of the mechanism of liver toxicity.

2.6. *Serum biomarkers for liver injury*

Blood samples were obtained from mice of all groups (10 mice per group) for determination of serum biomarkers for liver injury. Serum alanine transaminase (ALT) and aspartate transanimase (AST) were assayed according to the reported method (*12*).

2.7. *Assay for the mechanism of hepatotoxicity induced by the EF of the D. bulbifera rhizome*

LPO level, glutathione amounts, and activities of antioxidant-related enzymes including SOD, CAT, and glutathione-related enzymes such as GST, GPx, GR, and GCL in mice liver tissues of the partial groups were assayed according to the following descriptions.

2.7.1. *Assay for liver tissue LPO*

Liver tissue was homogenized in cold phosphate-buffered saline (PBS). LPO was determined using the previously reported method (*13*). Malondialdehyde (MDA) formed as an end product of the LPO and served as an index of the intensity of LPO. MDA reacts with TBA to generate a pink colored product, which has an absorbance at 532 nm. The standard curve of MDA was constructed over the concentration range of 0-40 μM. The LPO level was expressed as micromoles of MDA per milligram of protein based on tissue protein concentration.

2.7.2. *Assay for liver tissue glutathione*

The quantity of glutathione was measured immediately as described in a previous study (*14*). The reaction mixture contained: samples, 150 μL of a working solution (100 mM sodium phosphate buffer, pH 7.0, containing 0.53 U/mL of glutathione reductase, 40.7 μg/mL of DTNB, and 1 mM EDTA), and 50 μL of 0.16 mg/mL of NADPH solution. The change in absorbance was determined at 412 nm against the reagent blank after standing at room temperature for 30 min. Glutathione amounts were determined from a standard curve. The glutathione amounts from mouse liver tissue were calculated based on tissue protein concentration.

2.7.3. *Assay for liver tissue SOD and CAT activity*

Tissue was homogenized in cold PBS, centrifuged at 5,000 × g for 5 min and the supernatant was transferred to new tubes for assay. Liver tissue activity of SOD and CAT was determined using methods from previous studies (*15-16*) and calculated based on tissue protein concentration.

2.7.4. *Assay for liver tissue GPx activity*

GPx activity was measured by the utilization of glutathione as reaction substrate according to the previously reported method (*17*). Mouse liver tissue was homogenized in cold PBS, centrifuged at 5,000 × g for 10 min and the supernatant was transferred to new tubes for GPx assay. Tissue activity of GPx was calculated based on tissue protein concentration.

2.7.5. *Assay for liver tissue GST activity*

GST activity was measured according to the previously reported method (*18*) using 1-chloro-2,4-dinitrophenol (CDNB) as substrate. Mouse liver tissue was homogenized in cold PBS, centrifuged at 5,000 × g for 10 min and the supernatant was transferred to new tubes for GST assay. Tissue activity of GST was calculated based on tissue protein concentration.

2.7.6. *Assay for liver tissue GR activity*

GR activity was assayed spectrophotometrically by following the oxidation of NADPH at 340 nm (*19*). Briefly, mouse liver tissue was homogenized in cold PBS, centrifuged at 5,000 × g for 10 min and the supernatant was transferred to new tubes for GR assay. The reaction mixture (0.2 mL) contained 0.1 M phosphate buffer (pH 7.0), 1 mM GSSG, and 0.1 mM NADPH and was initiated by addition of supernatant. The activity of GR was calculated based on tissue protein concentration and expressed as mU/mg protein, where 1 unit of GR activity is defined as 1 mmol GSSG catalyzed per minute.

2.7.7. *Assay for liver tissue GCL activity*

GCL activity was assayed according to the previously reported method (*20*) with minor modifications. Briefly, mouse liver tissue was homogenized in cold PBS, centrifuged at 5,000 × g for 10 min and the supernatant was transferred to new tubes for GCL assay. Enzyme activity was determined at 37°C in reaction mixtures (final volume, 0.2 mL) containing 0.1 M Tris-HCl buffer, 150 mM KCl, 5 mM ATP, 2 mM phosphoenolpyruvate, 10 mM L-glutamate, 10 mM L-α-amino-butyrate, 20 mM $MgCl_2$, 2 mM EDTA, 0.2 mM NADPH, 17 μg of pyruvate kinase, and 17 μg of lactate dehydrogenase. The activity of GCL was calculated based on tissue protein concentration and expressed as mU/mg protein, where 1 unit of GCL activity was equal to the oxidation of 1 mM NADPH per min.

2.8. *Statistical analysis*

All experimental data were expressed as mean ± standard error of mean (SEM). Significant differences between experimental groups were compared by One-Way ANOVA (analysis of variance) followed by Least Significant Difference (LSD) ($p < 0.05$) using the Statistics Package for Social Science (SPSS) program Version 11.5.

3. Results

3.1. *Acute toxicity*

Mice were observed for fourteen consecutive days twice a day for toxic symptoms and mortality after oral administration of a single dose of various extracts of *D. bulbifera* rhizome, respectively. The results indicated that none of the mice in the group of the water extract (at a dose of 14,000 mg/kg) exhibited any toxic symptoms, while some in the ethanol extract (at a dose of 2,700 mg/kg) showed toxic symptoms. The results suggest that the ethanol extract is the toxic fraction of *D. bulbifera* rhizome. Meanwhile, further results indicated that none of the mice in the group of the Non-EF of the ethanol extract (at a dose of 13,800 mg/kg) exhibited any toxic symptoms, while mice in the EF of the ethanol extract (at a dose of 640 mg/kg) showed some toxic symptoms. All these results suggest that the EF is the toxic fraction of *D. bulbifera* rhizome.

The toxic symptoms appeared almost 2 h after the EF of the ethanol extract or the ethanol administration itself and slowly progressed to some extent. Most of the animals died on the second or third day after a single administration. The surviving mice could almost come back to the normal state in a week. The LD_{50} values for a single oral dose of the EF of the ethanol extract and the ethanol itself for ICR mice were 922 and 3,800 mg/kg, respectively (Table 1).

3.2. *Hepatotoxicity*

3.2.1. *Serum biomarkers for liver injury*

As for liver injury, serum ALT and AST activities are the generally accepted biomarkers. The obvious elevation of them often reflects liver injury. This study revealed that ALT increased significantly ($p < 0.05$) in groups of mice at and above the dose of 160 mg/kg after fourteen consecutive administrations of EF of *D. bulbifera* rhizome. Meanwhile, AST was found to be elevated in groups of mice after treatment at and above 320 mg/kg for fourteen consecutive days. This suggests that EF of *D. bulbifera* rhizome has induced liver injury in mice at and above the dose of 160 mg/kg for fourteen days (Figure 1).

Table 1. Acute toxicity of various extracts of the *D. bulbifera* rhizome in mice

Groups	LD_{50} values (mg/kg)
Water extract	> 14,000
Ethanol extract	3,800
EF of the ethanol extract	922
Non-EF of the ethanol extract	> 13,800

3.2.2. *Assay for liver tissue LPO level*

As one of the main end products of LPO, MDA amounts reflect the LPO level (*21*). Figure 2A showed that MDA amounts increased significantly ($p < 0.05$) in liver tissue of mice. This result demonstrated that EF of *D. bulbifera* rhizome could induce liver LPO injury in mice.

3.2.3. *Assay for liver tissue glutathione*

Glutathione is an antioxidant which helps protect cells against ROS such as free radicals and peroxides (*22*), and the excessive exhaustion can induce oxidative stress injury. In the present study, glutathione amounts decreased significantly ($p < 0.05$) in liver tissue of mice after given the EF of *D. bulbifera* rhizome at a dose of 480 mg/kg for fourteen consecutive days of administration (Figure 2B). The result suggests that EF of *D. bulbifera* rhizome can destroy the balance between cellular oxidants and antioxidants through exhausting cellular glutathione and thus can likely induce liver oxidative stress injury.

3.2.4. *Assay for liver tissue SOD and CAT activities*

SOD and CAT are both intracellular antioxidant-related enzymes, which participate in the process of oxidative stress. The results in this study showed that the SOD activity decreased significantly (Figure 3A) in livers of mice after treatment with *D. bulbifera* rhizome, but the CAT activity did not (Figure 3B). Meanwhile, the

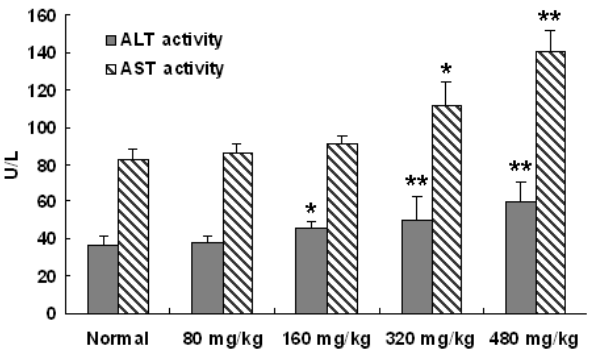

Figure 1. Effects of the EF of ethanol extract of *D. bulbifera* rhizome on serum ALT and AST activities in mice. Data are presented as mean ± SEM ($n = 10$). Significant differences with the normal group were designated as * $p < 0.05$ and ** $p < 0.01$.

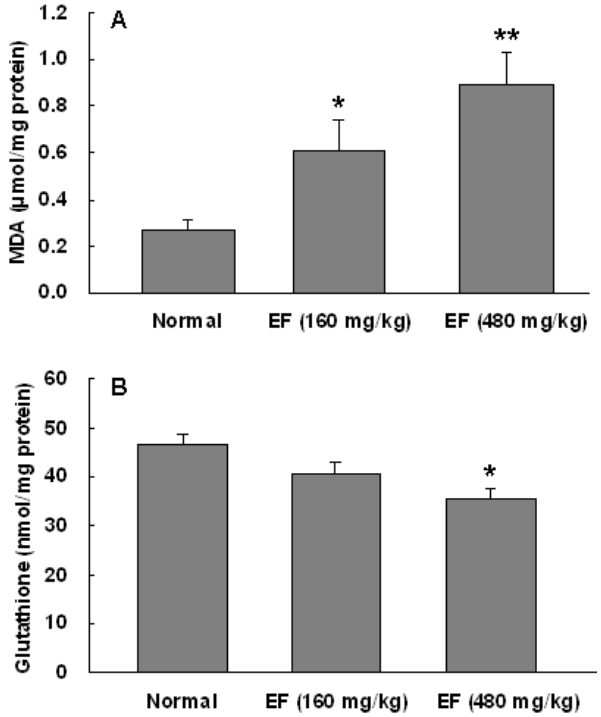

Figure 2. Effects of the EF of ethanol extract of *D. bulbifera* rhizome on the MDA and glutathione amounts in mice liver tissue. Data are presented as mean ± SEM (n = 10). Significant differences with the Normal group were designated as * $p < 0.05$ and ** $p < 0.01$.

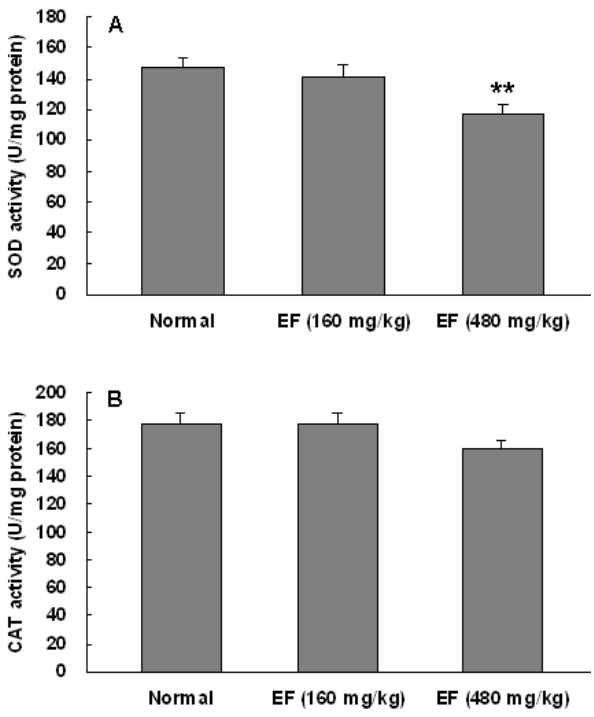

Figure 3. Effects of the EF of ethanol extract of *D. bulbifera* rhizome on the activities of SOD and CAT in mice liver tissue. Data are presented as mean ± SEM (n = 10). Significant differences with the Normal group were designated as * $p < 0.05$ and ** $p < 0.01$.

results further confirmed the oxidative stress injury induced by *D. bulbifera* rhizome in mice.

3.2.5. *Assay for liver tissue GST, GPx, GR and GCL activity*

GST, GPx, GR, and GCL are all intracellular glutathione-related enzymes, cooperating with glutathione in participating in the course of oxidative stress. Our results showed that GST, GPx, GR, and GCL activity were all significantly ($p < 0.05$) decreased in liver tissue of mice (Figure 4). Our results further confirmed that *D. bulbifera* rhizome destroyed the balance between cellular oxidants and antioxidants.

4. Discussion

The *D. bulbifera* rhizome has many bioactivities including anti-goiter, antitumor and so on (*1*). However, toxicity, especially hepatotoxity induced by it greatly affects its use in the clinic (*4*). The results of the present study demonstrated that the EF of the ethanol extract was the main toxic ingredient of *D. bulbifera* rhizome.

After the liver is injured by hepatotoxins, ALT and AST can leak from the damaged liver into the serum. Therefore, the obvious elevation of serum ALT and AST generally reflects liver injury (*12*). In the present study, ALT and AST activities both increased significantly in the serum of mice after treatment with the EF of the *D. bulbifera* rhizome for fourteen consecutive days. These results suggest that *D. bulbifera* rhizome can induce mice liver injury.

Many studies have demonstrated that ROS plays an important role in various hepatotoxins-induced liver injury (*5,6,23*). Hepatic cellular oxidative stress often takes place during the occurrence of the imbalance between oxidants and antioxidants. Moreover, many non-enzymatic antioxidant and glutathione-related enzymes may be changed during this process (*24-27*). Of them, LPO is a free radical-related process (*28*). In this study, MDA amounts increased significantly, which indicated potential oxidative stress injury induced by LPO.

There are superoxide anions produced in the process of oxidative stress. As a metalloenzyme, SOD can convert such superoxide anions to hydrogen peroxide (*29*). The results in Figure 3A showed that SOD activity significantly decreased in livers of mice after given *D. bulbifera* rhizome for fourteen consecutive days, which confirmed that it induced oxidative stress injury in livers. CAT can further detoxify hydrogen peroxide (*29*). However, the results in Figure 3B indicated that there was no significant difference found in CAT activity in livers of mice treated with *D. bulbifera* rhizome.

Cellular glutathione plays a critical role in protecting hepatocytes against exogenous toxins. Its amounts are related to oxidative damage (*30,31*). The results showed that *D. bulbifera* rhizome caused excessive exhaustion of liver glutathione amounts and thus likely induced

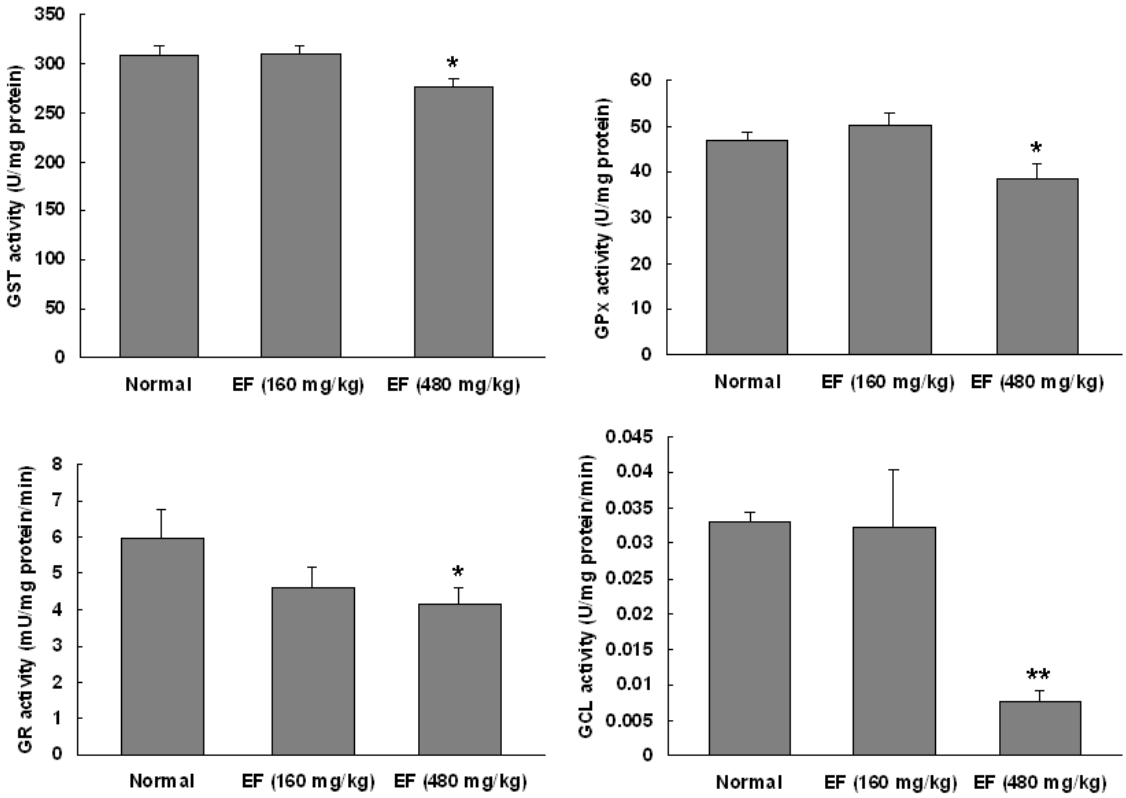

Figure 4. Effects of the EF of ethanol extract of *D. bulbifera* rhizome on the activities of GST, GPx, GR, and GCL in mice liver tissue. Data are presented as mean ± SEM (n = 10). Significant differences with the Normal group were designated as * p < 0.05 and ** p < 0.01.

damage of liver normal antioxidant oxidant balance and caused oxidative stress injury in mice.

GST, GPx, GCL, and GR are all glutathione-related enzymes. Among them, the cytosolic GSTs exist in almost all aerobic species. It can catalyze the conjugation of electrophilic compounds produced during oxidative stress with glutathione. In the present study, *D. bulbifera* rhizome significantly decreased GST activity in livers of mice and thus contributed to liver oxidative stress injury. GPx catalyzes hydrogen peroxide decomposition to the stable form of hydroxides, specifically using reduced glutathione as the electron provider (*32*). GCL regulates glutathione as the first and rate-limiting enzyme in GSH *de novo* synthesis, which protects against free radical damage (*20*). GR catalyzes the reduction of GSSG to GSH using NADPH resulting from the pentose phosphate pathway (*22*). The results in the present study indicated that *D. bulbifera* rhizome induced a significant decrease in the activity of GPx, GR, and GCL in mice livers. All of these results confirmed damage on the balance between cellular oxidants and antioxidants induced by *D. bulbifera* rhizome.

In conclusion, the present study shows that the EF of the ethanol extract is the main toxic ingredient of *D. bulbifera* rhizome. Moreover, it also demonstrates that the EF of the *D. bulbifera* rhizome can induce liver toxicity and the mechanism of such hepatotoxicity may be related to liver oxidative stress injury in mice. All of these results remind us to pay attention to liver toxicity induced by *D. bulbifera* rhizome in the clinic. Further studies are in progress in our laboratory to find specific hepatotoxic chemical compounds in *D. bulbifera* rhizome and to explore the molecular mechanisms of its induced hepatotoxicity.

Acknowledgements

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Original Article

The expression of human high molecular weight melanoma-associated antigen in acral lentiginous melanoma

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Summary **The high molecular weight melanoma-associated antigen (HMW-MAA) is a membrane-bound chondroitin sulphate proteoglycan that is highly expressed on the surface of melanoma cells. It represents an attractive target for immunotherapy of malignant melanoma. Previously, it was reported that HMW-MAA was detected in about 20-30% of primary acral lentiginous melanoma (ALM) lesions by immunohistochemical staining (IHC) of frozen sections with monoclonal antibodies (mAbs). In the present study, we examined the expression of HMW-MAA in 95 paraffin-embedded, primary ALM lesions and 13 primary superficial spreading melanoma (SSM) lesions. A total of 51 primary ALM lesions (53.6%) were positive for HMW-MAA. Almost all of these positive cases showed a weak staining intensity. On the other hand, all 13 primary SSM lesions were strongly positive for HMW-MAA expression. Our data showed that the staining intensity of HMW-MAA ALM lesions was weaker than that of SSM. Furthermore, the percentage of HMW-MAA positive staining in ALM lesions was higher than previously reported.**

Keywords: High molecular weight melanoma-associated antigen (HMW-MAA), acral lentiginous melanoma (ALM)

1. Introduction

The high molecular weight melanoma-associated antigen (HMW-MAA) is a membrane-bound chondroitin sulphate proteoglycan that is highly expressed on the surface of melanoma cells. Recent findings have shown that HMW-MAA is involved in the activation of several signaling pathways modulating melanoma cell adhesion, spreading, migration and invasion (*1*). The HMW-MAA mediates the interaction of melanoma cells with the extracellular matrix, appears to play a role in the metastatic potential of melanoma cells, and has been shown to promote melanoma invasion through cytoskeletal rearrangements (*1*).

The HMW-MAA has been used as a target for immunotherapy of melanoma. Induction of humoral anti-HMW-MAA immunity following immunization with anti-idiotypic mAb MK2-23, which mimics the HMW-MAA, is associated with statistically significant survival prolongation in patients with stage IV melanoma (*2*). It was also reported that elicited HMW-MAA-specific Abs were able to mediate cell-dependent cytotoxicity and inhibited several HMW-MAA-dependent cellular functions including: spreading, migration, and invasion upon binding to HMW-MAA$^+$ melanoma cells (*3*).

The HMW-MAA is expressed in over 80% of human melanoma lesions and in the majority of human melanoma cell lines (*1*). The level of HMW-MAA expression is similar among lentigo maligna, nodular and superficial spreading melanoma lesions, but is lower in ALM lesions (20-30%) as detected by immunohistochemical staining (IHC) of frozen sections (*1*,*4*).

In Japan, ALM is the most common type of malignant melanoma, accounting for nearly 50% of melanoma patients (*5*). In this study, we investigated HMW-MAA expression in paraffin embedded, primary ALM and SSM lesions.

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2. Materials and Methods

2.1. *Melanoma specimens*

Formalin-fixed, paraffin-embedded archival tissue (PEAT) specimens were obtained from a total of 95 primary ALM (42 males and 53 females, mean age 70.9 years, age range 37-97 years) and 13 primary SSM (6 males and 7 females, mean age 62.1 years, range 30-83 years). With respect to ALM cases, 30 patients had stage I melanoma, 45 had stage II, 15 had stage III, and 5 had stage IV. With respect to SSM cases, 4 patients had stage I melanoma, 5 had stage II, and 4 had stage III.

A total of 60 and 13 patients with ALM and SSM, respectively, were from the Department of Dermatology & Plastic Surgery, Kumamoto University Hospital, while 35 patients with ALM were from the Department of Dermatology, Shinshu University Hospital; these patients underwent surgery between 1989 and 2006.

2.2. *Monoclonal antibody*

The mAb D2.8.5-C4B8 against distinct determinants of HMW-MAA was developed and characterized as described previously (*1*,*6*). The mAb is of mouse origin.

2.3. *Immunohistochemistry*

IHC was performed on 4-μm sections that had been incubated overnight at 50°C and deparaffinized in xylene. We used the CSA II System (Biotin-Free Catalyzed Amplification System, Dako, Carpinteria, CA, USA) with modifications as previously described (*7*). Tissue sections were incubated overnight at 4°C with HMW-MAA-specific mAb at 5 μg/mL. After development with the substrate (VIP Substrate Kit, Vector Labs, Burlingame, CA, USA), tissue sections were counterstained with Mayer's hematoxylin 1× (Muto Pure Chemicals, Tokyo, Japan) for 1 min at room temperature, dehydrated and mounted.

Negative controls were performed by replacing the primary antibody with TBS containing Tween 20 buffer (Tris-HCl/NaCl/0.1% Tween 20).

Stained sections were scored according to the percentage of stained melanoma cells: 100-75%, 74-50%, 49-25%, 24-1%, or negative. The staining intensity was scored as strong, intermediate, weak or negative. The staining intensity and percentage of stained melanoma cells in the each section were estimated independently by three investigators (H.N., Y.I., and T.K.).

2.4. *Statistical analysis*

Chi-square tests were used to evaluate the percentage of positive HMW-MAA in ALM and SSM lesions. $P < 0.05$ was considered statistically significant.

3. Results

Representative staining patterns are shown in Figure 1. Positive immunoreactivity for HMW-MAA was indicated by purple staining. The staining was observed on the membrane of melanoma cells; there was no

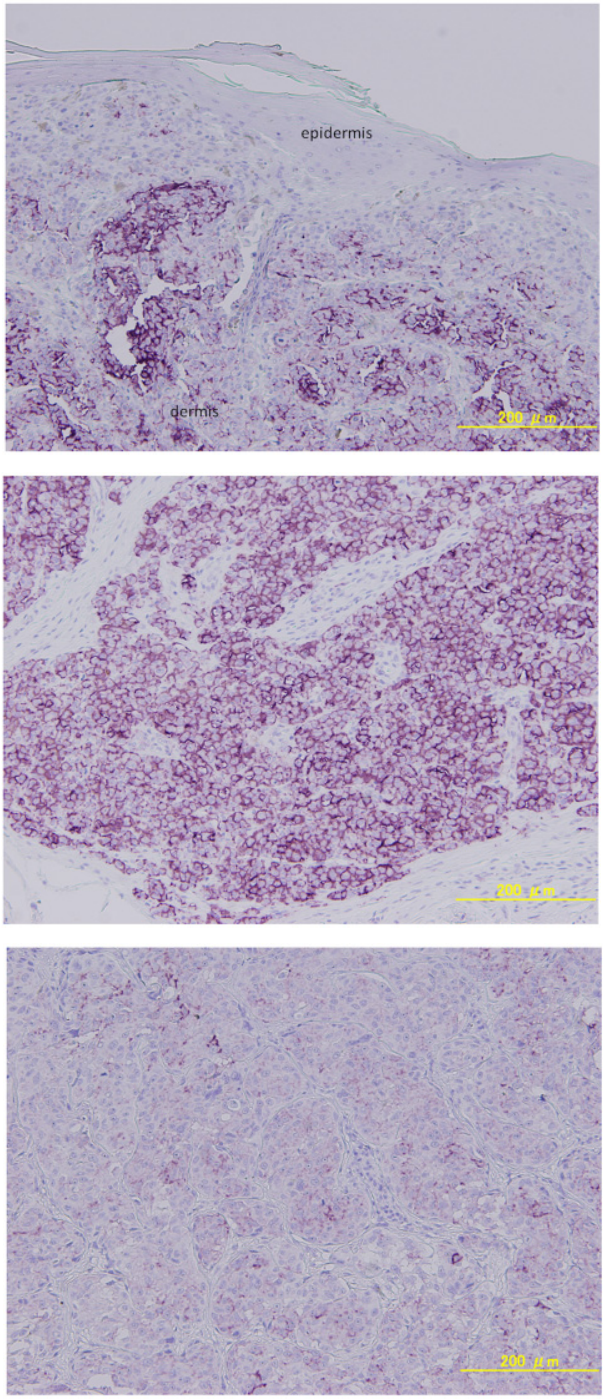

Figure 1. Representative staining patterns for HMW-MAA in primary ALM lesions. (**A** and **B**) Immunohistochemical staining for HMW-MAA of primary ALM lesions showing strong staining intensity. (**C**) The weak staining intensity of HMW-MAA-positive cells in primary ALM lesions.

Table 1. Positive reactivity with anti-HMW-MAA mAb and the percentage of stained melanoma cells in ALM lesions

	Negative	< 25%	25-49%	50-74%	≥ 75%	Total of positive cases (%)
Stage I (*n* = 30)	11 (36.7%)	2 (6.7%)	8 (26.7%)	3 (10%)	6 (20%)	19/30 (63.3%)
Stage II (*n* = 45)	25 (55.6%)	7 (15.6%)	5 (11.1%)	5 (11.1%)	3 (6.67%)	20/45 (44.4%)
Stage III (*n* = 15)	5 (33.3%)	6 (40%)	0	2 (16%)	2 (16%)	10/15 (66.7%)
Stage IV (*n* = 5)	3 (60%)	0	0	1 (20%)	1 (20%)	2/5 (40%)
Total						51/95 (53.6%)

staining of the surrounding lymphocytes. The staining was heterogeneous in almost all lesions.

The results of IHC of the 95 primary ALM lesions using the anti-HMW-MAA mAb are summarized in Table 1. A total of 51 primary ALM lesions (53.6%) were positive for HMW-MAA; there were 19 (63.3%) positive stage I ALM, 20 (44.4%) positive stage II ALM, 10 (66.7%) positive stage III ALM, and 2 (40%) positive stage IV ALM. The percentage of stained melanoma cells in ALM lesions is also shown in Table 1. A total of 23 (45%) out of the 51 showed positive staining in more than half of the melanoma cells. There was no significant difference among the stages. The intensity of staining of ALM lesions is shown in Table 2. Almost all of the positive cases had a weak staining intensity.

On the other hand, all of the 13 primary SSM lesions reacted with the anti-HMW-MAA mAb. The intensity of staining of SSM lesions is shown in Table 2. The staining intensity of SSM lesions was higher than that in ALM lesions. The percentage of stained melanoma cells in SSM is shown in Table 3. The percentage of stained melanoma cells was < 25% in 2 out of 4 lesions of stage I SSM, and the prevalence of positive HMW-MAA in SSM lesions was higher than that in ALM lesions ($P < 0.01$).

4. Discussion

We showed that 53.6% of PEAT from ALM expressed HMW-MAA, and that almost half of the ALM lesions showed a weak staining intensity. It has been reported that HMW-MAA was detected in approximately 30% of frozen tissue of primary ALM lesions by IHC using mAb (*8*). HMW-MAA was detected in a greater percentage of ALM lesions than reported previously; this may be due to the difference in the immunohistochemical staining method (*7*). It was reported that the staining intensity achieved using HMW-MAA-specific mAbs was stronger than that from MART-1 mAbs for both macro- and micrometastases (*7*).

In this study, the percentage of positive HMW-MAA in SSM lesions was higher than that in ALM lesions. Furthermore, the immunostaining intensity of HMW-MAA in SSM lesions was higher than that in ALM lesions. This is similar to a previous report that describes a greater percentage of HMW-MAA-positive SSM lesions compared to ALM (*1*), and that its expression was demonstrated in 80% of primary melanomas except ALM (*1*).

Our data showed that the staining intensity of HMW-MAA in ALM was weaker than that of SSM, and the percentage of HMW-MAA positive ALM was higher than that previously reported.

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Table 2. Staining intensity of ALM and SSM lesions

Staining intensity	Cases of ALM (%)	Cases of SSM (%)
Strong	4 (4.2%)	4 (30.8%)
Intermediate	9 (9.5%)	6 (46.2%)
Weak	38 (40%)	3 (23.1%)
Negative	44 (46.3%)	0 (0%)

Table 3. The percentage of stained melanoma cells in SSM lesions

	Negative	< 25%	25-49%	50-74%	≥ 75%	Total of positive cases (%)
Stage I (*n* = 4)	0	2	0	0	2	4/4 (100%)
Stage II (*n* = 6)	0	0	1	0	5	6/6 (100%)
Stage III (*n* = 3)	0	0	1	1	1	3/3 (100%)
Total	0	2/13 (15.4%)	2/13 (15.4%)	1/13 (7.7%)	8/13 (61.5%)	13/13 (100%)

antigen (HMW-MAA) mimicry by mouse anti-idiotypic monoclonal antibody MK2-23: induction of humoral anti-HMW-MAA immunity and prolongation of survival in patients with Stage IV melanoma. Proc Natl Acad Sci U S A. 1992; 89:466-470.
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