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A systematic review on anti-Alzheimer's disease activity of prescription Kangen-karyu

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SUMMARY Traditional Chinese and Japanese medicines have become prime sources of drug discovery and there is a pressing need to investigate the effectiveness of these traditional medicines for modern drug discovery. Recently, among various traditional formulations, studies on Kangen-karyu (Guan-Yuan-Ke-Li), a mixture of six medicinal herbs (Salviae Miltiorrhizae Radix, Cnidii Rhizoma, Paeoniae Radix, Carthami Flos, Aucklandiae Radix, and Cyperi Rhizoma), have been growing to assess its neuroprotective role. This prompted us to undertake a thorough review of various targets of Kangen-karyu regarding its effectiveness against Alzheimer's disease, particularly focusing on cholinesterases, beta-site amyloid precursor protein cleaving enzyme 1, and glycogen synthase kinase 3β. This review provides new insights into Kangen-karyu medication as a prospective anti-Alzheimer's medication and indicates the need for in-depth *in vivo* investigation in the future.

Keywords Kangen-karyu, Alzheimer's disease, cholinesterase, BACE1, GSK3β

1. Introduction

With an increase in the elderly population in developing countries, the prevalence of Alzheimer's disease (AD) is rapidly increasing (*1*). Thirty-six million people suffer from AD worldwide and almost 50% of the cases of dementia are due to AD (*2*). Therefore, AD has become a major health problem and an economic burden for health systems. It is characterized by the occurrence of extracellular amyloid plaque deposits and neurofibrillary tangles of the microtubule-binding protein tau in the central nervous system (*3,4*), and clinical symptoms include compromised memory as well as cognition, orientation, and several emotional disturbances (*5*).

AD is a complex neuronal disease involving various factors. Although the exact etiology is still unclear, studies on the AD brain and AD animal models have proposed several hypotheses regarding AD: (a) Cholinergic hypothesis – loss of cholinergic neurotransmission in the cerebral cortex due to an increase in cholinesterase activity and degeneration of cholinergic neurons in the basal forebrain deteriorate cognition (*6,7*). (b) Amyloid hypothesis – proteolytic cleavage of the amyloid precursor protein (APP) by secretases forms amyloid-beta (Aβ) fibrils, and the accumulated Aβ amyloid fibrils develop into senile plaque, causing neurotoxicity, tauopathy, neuronal cell death, and neurodegeneration (*8,9*). (c) Tau hypothesis – tau is a microtubule-associated protein in axons that regulates the stability of tubulin assemblies; however, aggregation in a hyperphosphorylated state impairs axons of neurons (tauopathy) (*10,11*). (d) Glycogen synthase kinase 3β (GSK3β) hypothesis – hyperactive GSK3 leads to tau hyperphosphorylation, increased Aβ production, and memory impairment (*12,13*). Therefore, since there are several causes of AD, traditional Chinese medicine (TCM) or its combination therapy with various medicinal properties rather than a single agent mostly used in current AD therapy has the potential to be an effective treatment for AD.

TCM has been widely used in China for thousands of years. Recently, TCM has established its position in the Western world. Some TCM have been reported to be either ineffective or lethal due to hepatotoxicity at higher concentrations or herb-drug interactions (*14*). However, the majority of TCM, through controlled clinical trials, have been proven to be safe and effective (*15*). Herein, we systematically reviewed anti-AD activity of a herbal mixture Kangen-karyu (Guan-

Yuan-Ke-Li in Chinese has been developed in Japan *via* partial modification of the herbal constituents of the Chinese herbal prescription Guan-Xin No. 2), and its individual components (Salviae Miltiorrhizae Radix, Cnidii Rhizoma, Paeoniae Radix, Carthami Flos, Aucklandiae Radix, and Cyperi Rhizoma), particularly focusing on cholinergic, amyloid, tau, and GSK3β hypotheses of AD treatment. We also discuss *in vivo* studies on Kangen-karyu and its safety, highlighting its potential in neuronal drug discovery.

2. Cholinesterases as molecular targets of Kangen-karyu

Acetylcholine (ACh) is a neurotransmitter of all cholinergic neurons in the central and peripheral nervous systems that modulates neural functions in attention, learning, memory, stress responses, wakefulness and sleep, and responses to sensory information (*16*). Cholinergic neurotransmission relies on ACh synthesis, storage, transportation, and degradation. Acetylcholinesterase (AChE) is an enzyme responsible for ACh degradation *via* hydrolysis to acetate and choline. It is one of the most kinetically efficient enzymes because each molecule can hydrolyze 5,000 molecules of ACh/second (*17*). So, it is a highly effective therapeutic target for symptomatic relief in AD patients because cholinergic deficit is a consistent finding in early AD.

Impairment of brain AChE levels in diabetes is one of the reasons for diabetes-associated cognitive decline (*18*). In a previous study, dried powder of a boiled water extract of Kangen-karyu attenuated cognitive deficit in diabetic mice (*19*). Furthermore, the study was conducted *in vivo* using a cognitive deficit-diabetic mouse model by performing behavioral experiments, and evaluating the cholinergic marker protein choline acetyltransferase (ChAT) and muscarinic acetylcholine (M_1, M_3, and M_5) receptors in the hippocampus (*19*). In a behavioral study, 18-week-old *db/db* mice with a diabetic insult showed a marked decrease in spatial learning performance in terms of the escape latency compared with the same aged-matched non-diabetic mice. However, daily treatment of *db/db* mice with 200 mg/kg Kangen-karyu significantly and dose-dependently improved the spatial learning performance of old *db/db* animals in the training test. The administration of 100 mg/kg/day of Kangen-karyu also led to significant improvement in learning performance compared with a vehicle-treated group. In Western blot analysis, a vehicle-treated old *db/db* group showed significantly lower levels of ChAT, M_1 receptor, M_3 receptor, and M_5 receptor. However, the daily oral administration of Kangen-karyu extract (100 and 200 μg/mL) led to a significant increase in ChAT, M_1 receptor, M_3 receptor, and M_5 receptor levels. Decreases in levels of ACh, ChAT, and muscarinic and nicotinic ACh receptors are associated with cholinergic hypofunction with cognitive deficits (*20-22*), and the ability of Kangen-karyu to increase the reduced level of these markers in cognitive deficit mouse models demonstrates a neuroprotective role.

To our knowledge, there have been no reports on cholinesterase inhibition by the component Cnidii Rhizoma. From this component, ferulic acid, sinapic acid, 5-hydroxy ferulic acid, and chlorogenic acid have been reported (*23*). A previous study reported the effect of sinapic acid on the basal forebrain of an ibotenic acid-treated rat model (*24*). In that study, treatment of rats with ibotenic acid decreased ACh levels in the parietal and frontal cortices and ChAT activity in the parietal cortex. However, pretreatment of rats with sinapic acid (3 and 10 mg/kg) reversed the effect of ibotenic acid in a dose-dependent manner. A dose of 10 mg/kg of sinapic acid led to significant retention of the ACh levels and ChAT activity in the basal forebrain.

In a study conducted to identify novel AChE inhibitors derived from Salviae Miltiorrhizae Radix (*25*), two abietane diterpene dihydrotanshinone and cryptotanshinone showed promising inhibition of bovine erythrocyte AChE with IC_{50} values of 1.0 and 7.0 μM, respectively. However, tanshinone I and tanshinone IIA displayed weak inhibitory effects. Similarly, for human cloned cholinesterase (*26*), dihydrotanshinone and cryptotanshinone showed mixed non-competitive inhibition of AChE with IC_{50} values of 0.89 and 4.67 μM, respectively. For human butyrylcholinesterase (BChE), the inhibition mode was uncompetitive with IC_{50} values of 6.66 μM for cryptotanshinone and 5.51 μM for dihydrotanshinone. Also, Wong and colleagues (*26*) performed molecular docking studies to explore the binding mechanism of these diterpenes with human cloned cholinesterases. In the presence of human AChE, these compounds bound to the active-site gorge lining Trp86, Tyr124, and Tyr337 with different orientations. The penta ring of dihydrotanshinone faced the bottom of the gorge while cryptotanshinone faced the opposite direction – towards the gorge mouth. At the catalytic site, both compounds interacted with the prime catalytic triad residues Ser203 and His447. This suggested that additional interaction of dihydrotanshinone with Tyr337 and Gly120 *via* H-bonds plays a vital role due to its potency compared with cryptotanshinone. Interestingly, the docking of these diterpenes with hBChE led to a similar binding configuration involving aromatic residues, Trp430, Phe329, and Tyr332.

The crude extract of Paeoniae Radix and its major component, paeoniflorin, were previously reported to have beneficial effects on spatial cognitive deficits caused by the dysfunction of central cholinergic systems and aging in rodents (*27,28*). Also, in a previous report, an ethanol extract of Paeoniae Radix demonstrated good inhibition of AChE and BChE with IC_{50} values of 25.04 and 10.59 μg/mL, respectively (*29*).

3. Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1) as a molecular target of Kangen-karyu

BACE1 is a type 1 transmembrane aspartyl protease that is involved in the generation of Aβ peptides by neurons through proteolytic cleavage of APP (*30*). Cleaving of APP by β-secretase at the NH_2-terminus of Aβ to release a soluble ~100-kD NH_2-terminal fragment and a membrane-bound 12-kD COOH-terminal fragment initiates Aβ formation. The progressive formation of insoluble amyloid plaque and vascular deposits of Aβ peptide is a characteristic event in AD (*31*).

The effect of Kangen-karyu on BACE1 activity has yet to be reported. We performed a comparative study on BACE1 inhibition with a boiled water extract of Kangen-karyu and its components (Table 1) (data not published). The boiled water extract of Kangen-karyu demonstrated moderate inhibition. Among the fractions, moderate inhibition was also observed with a boiled water extract of Salviae Miltiorrhizae Radix and Cyperi Rhizoma followed by mild inhibition by Paeoniae Radix, Cnidii Rhizoma, and Carthami Flos. No noticeable inhibition was observed with a boiled water extract of Aucklandiae Radix.

Furthermore, depending upon the activity of individual components of Kangen-karyu, we further studied Salviae Miltiorrhizae Radix. We performed a comparative study on BACE1 inhibition by MeOH and water extracts of Salviae Miltiorrhizae Radix along with solvent-soluble fractions (*n*-hexane, CH_2Cl_2, EtOAc, *n*-BuOH, and water fractions) from each extract (data not published). As shown in Table 2, the MeOH extract of Salviae Miltiorrhizae Radix showed moderate inhibition of BACE1 with an IC_{50} value of 114.82 ± 3.00 μg/mL. Among the fractions of MeOH extract, *n*-hexane and CH_2Cl_2 fractions showed promising inhibition followed by EtOAc and *n*-BuOH fractions. However, the H_2O fraction had a mild inhibitory effect. Interestingly, the water extract of Salviae Miltiorrhizae Radix showed good inhibition (Table 3). Among the fractions, the EtOAc fraction demonstrated the most promising inhibition (IC_{50} = 0.20 ± 0.07 μg/mL), followed by CH_2Cl_2 (IC_{50} = 6.11 ± 0.53 μg/mL), *n*-hexane (IC_{50} = 11.83 ± 0.09 μg/mL), *n*-BuOH (IC_{50} = 49.32 ± 2.62 μg/mL), and H_2O (IC_{50} = 64.77 ± 7.59 μg/mL) fractions, respectively.

From a structural viewpoint, Salviae Miltiorrhizae Radix comprises water-soluble polyphenolic compounds – danshensu (3, 4-dihydroxyphenyllactic acid), salvianolic acid A, B, and C, protocatechuic aldehyde, and lipophilic compounds – tanshinones. HPLC analysis of a water extract of Salviae Miltiorrhizae Radix revealed tanshinone II and salvianolic acid B as dominant components (*32*). Because of the complex AD pathology, the neuroprotective components tanshinones and polyphenolics – salvianolic acids might be effective in treating AD (*33*). In our previous study (*34*), we evaluated the BACE1 inhibitory effect of tanshinones and salvianolic acid derivatives from Salviae Miltiorrhizae Radix along with enzyme kinetics and molecular docking simulations. Among the tested compounds, deoxyneocryptotanshinone and salvianolic acid C exhibited mixed modes of BACE1 inhibition with IC_{50} values of 11.53 ± 1.13 and 9.18 ± 0.03 μM, respectively. Similarly, salvianolic acid A inhibited the enzyme activity competitively with an IC_{50} value of 13.01 ± 0.32 μM. Most of the other tanshinones had moderate inhibitory effects with IC_{50} values ranging from 30 to 50 μM. Salvianolic acid A and C demonstrated higher binding affinity at the active catalytic site of BACE1 involving H-bond interaction with conserved aspartic acid residues Asp228 and

Table 1. BACE1 inhibitory potentials of water extract of Kangen-karyu and its constituents

Sample	IC_{50} values[a]
Kangen-karyu	77.40 ± 4.58
Aucklandiae Radix	> 400
Carthami Flos	233.34 ± 0.05
Cnidii Rhizoma	147.09 ± 0.93
Cyperi Rhizoma	91.16 ± 2.21
Paeoniae Radix	143.57 ± 1.79
Salviae Miltiorrhizae Radix	89.84 ± 1.87
Quercetin[b]	10.49 ± 0.28[*]

[a]The 50% inhibitory concentrations (IC_{50}, μg/mL) are expressed as the mean ± SD. [b]Used as positive control. [*]Values are expressed in μM.

Table 2. BACE1 inhibitory potentials of MeOH extract from Salviae Miltiorrhizae Radix and its various fractions

Sample	IC_{50} values[a]
MeOH extract	114.82 ± 3.00
n-Hexane fraction	11.79 ± 0.03
CH_2Cl_2 fraction	12.06 ± 0.58
EtOAc fraction	74.05 ± 0.64
n-BuOH fraction	115.79 ± 9.34
H_2O fraction	>150
Quercetin[b]	9.26 ± 0.36[*]

[a]The 50% inhibitory concentrations (IC_{50}, μg/mL) are expressed as the mean ± SD. [b]Used as positive control. [*]Values are expressed in μM.

Table 3. BACE1 inhibitory potentials of H_2O extract from Salviae Miltiorrhizae Radix and its various fractions

Sample	IC_{50} values[a]
H_2O extract	17.82 ± 0.35
n-Hexane fraction	11.83 ± 0.09
CH_2Cl_2 fraction	6.11 ± 0.53
EtOAc fraction	0.20 ± 0.07
n-BuOH fraction	49.32 ± 2.62
H_2O fraction	64.77 ± 7.59
Quercetin[b]	9.26 ± 0.36[*]

[a]The 50% inhibitory concentrations (IC_{50}, μg/mL) are expressed as the mean ± SD. [b]Used as positive control. [*]Values are expressed in μM.

Asp32. Prime interacting residue (Ser10) was observed for deoxyneocryptotanshinone and salvianolic acid C at the allosteric site. Oxygen groups of Ser229 and Glu310 interacted with the oxygen O11, O12, O15, and O16 of salvianolic acid B *via* hydrogen bonding interaction. The oxygen groups O6 and O15 of salvianolic acid C formed two H-bonds with Gly13 while O3, O6, and O7 interacted with Lys9, Gln304, and Asp318 *via* hydrogen-bonding. These findings reveal that the hydroxyl moieties in salvianolic acids play crucial roles in BACE1 interaction. Structure-activity relationships within caffeic acid derivatives reveal that the phenolic –OH group has a crucial effect on BACE1 inhibition. Activity was enhanced with an increase in phenolic –OH groups in rosmarinic acid (IC_{50}: 29.77 ± 0.70 μM) and magnesium lithospermate (IC_{50}; 30.35 ± 2.67 μM) compared with caffeic acid (IC_{50}; > 200 μM). The number of phenolic –OH groups in magnesium lithospermate is higher than in rosmarinic acid; however, the activity was similar. The arrangement of alkoxy groups and presence of magnesium might be responsible for this effect.

4. GSK3β as a molecular target of Kangen-karyu

GSK3 is one of the prime targets of AD and is responsible for the generation of paired helical filaments-tau, a major component of neurofibrillary tangles in the brain (*35*). It is a multifunctional proline-directed serine/threonine protein kinase for glycogen synthase phosphorylation involved in diverse biological processes (*36*). Cumulative evidence identifies glycogen synthase kinase as a potential target for neuroprotection because it contributes to the AD-associated hyperphosphorylation of tau and tau protein as a widely recognized substrate of GSK3 (*37*). The reduction of aberrant over-activity of this enzyme decreases various aspects of AD pathology (*38*). Two isomeric forms of GSK, GSK3α and GSK3β, share 98% homology in the catalytic domain, have similar biochemical properties, and are ubiquitously expressed in cells and tissues. Phosphorylation of serine residue (Ser21 in GSK3α and Ser9 in GSK3β) inhibits GSK3 (*36*).

In our previous study conducted to evaluate anti-AD activity *in vitro via* the GSK3 enzyme, boiled water extract of Kangen-karyu demonstrated potent inhibition of GSK3β with an IC_{50} value of 17.05 ± 1.14 μg/mL, as shown in Table 4 (*39*). Also, water extracts of all individual components showed good inhibition with IC_{50} values ranging from 7.77 to 93.61 μg/mL. Water extract of Salviae Miltiorrhizae Radix showed promising inhibition among the components followed by Cyperi Rhizoma. Other components showed mild inhibition with the following potency order: Paeoniae Radix > Cnidii Rhizome > Aucklandiae Radix > Carthami Flos (Table 4). Polar constituents (rosmarinic acid, magnesium lithospermate B, salvianolic acid A, salvianolic acid B, and salvianolic acid C) that were reported from water extract of Salviae Miltiorrhizae Radix were tested for GSK3β inhibition. The results demonstrated that these polar constituents inhibited GSK3β with IC_{50} values ranging from 6.97 to 135.5 μM. Among them, salvianolic acid B was the most potent ATP-competitive inhibitor of GSK3β with the lowest IC_{50} value (6.97 ± 0.96 μM). With IC_{50} values of approximately 30 μM, salvianolic acid A, salvianolic acid B, and magnesium lithospermate B showed good inhibition followed by the moderate activity of rosmarinic acid (IC_{50}: 135.35 ± 4.69 μM) and mild inhibition by caffeic acid (IC_{50}: 425.01 ± 7.61 μM). Although direct evidence of increased GSK3 activity in AD at present is still limited, studies have clearly demonstrated upregulated GSK3 expression in the hippocampus (*40*) and peripheral lymphocytes (*41*) of AD patients.

Table 4. GSK3β inhibitory potentials of water extract of Kangen-karyu and its constituents

Sample	IC_{50} values[a]
Kangen-karyu	17.05 ± 1.14
Aucklandiae Radix	85.04 ± 6.32
Carthami Flos	93.61 ± 3.99
Cnidii Rhizoma	66.74 ± 2.05
Cyperi Rhizoma	20.68 ± 2.50
Paeoniae Radix	62.51 ± 1.89
Salviae Miltiorrhizae Radix	7.77 ± 1.38
Luteolin[b]	2.18 ± 0.13[*]

[a]The 50% inhibitory concentrations (IC_{50}, μg/mL) are expressed as the mean ± SEM. [b]Used as positive control. [*]Values are expressed in μM.

5. Future perspective and conclusion

Although there are reports on anti-AD activity of some compounds that are contained in either of the six components of Kangen-karyu, a detailed and systematic pharmacological study of Kangen-karyu and its individual components is lacking. Generally, traditional medicines are formulae (a mixture of herbs/herbal components), serving as combination therapy. As such, it is difficult to determine the precise pharmacology of the formulae because they can have multiple targets and might also have multiple actions along with synergistic, additive, and/or antagonistic effects. Owing to this and to search for therapeutics pursuing the 'one drug-fits-all' concept, the utility of combination therapy has been highlighted (*42*). This necessitates in-depth pharmacology of Kangen-karyu for the management of AD.

The present review highlights the possible role of Kangen-karyu in the management of AD *via* cholinesterase, BACE1, and GSK3β inhibition.

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Review

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Treatment of SARS-CoV-2: How far have we reached?

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SUMMARY The virus severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) is currently affecting more than 200 countries and territories worldwide. It has been declared as pandemic by World Health Organization (WHO) and the whole world is suffering from corona virus disease 2019 (COVID-19). Currently, no treatment for SARS-CoV-2 are approved because of lack of evidence, but a number of clinical trials are in process and we are expecting fruitful results very soon. This review focuses on various approaches of treatment and few of the most recent clinical trials carried out in this field.

Keywords SARS-CoV-2, pandemic, antiviral, corona virus, clinical trial

1. Introduction

The SARS-CoV-2 earlier called as 2019-nCoV (2019 novel corona virus) is known to cause corona virus disease 2019 (COVID-19) and has been declared pandemic by WHO. As of April 18, 2020 (20:50 GMT), total 2,322,033 cases have been reported so far worldwide causing 159,659 deaths. Currently, 1,512,066 patients are in mild conditions, whereas, 55,218 are in critical condition (*1*). There are several ways to combat the corona virus infection which include development of vaccine, but developing a vaccine can take at least 12-18 months in extraordinary circumstances and the first human clinical trial for corona virus vaccine has already started in US. The National Institute of Allergy and Infectious Diseases (NIAID) has already developed the vaccine in collaboration with Moderna Inc., a biotechnology-based company. There are other efforts to develop the vaccine too which include the major pharma giant GlaxoSmithKline in collaboration with Clover Biopharmaceuticals, China. Sanofi and Johnson & Johnson are working with the Biomedical Advanced Research and Development Authority for the same cause. But, in any case, the vaccine for human use would be available in at least 18 months and in the view of developing COVID-19 cases, we need to have a fast and effective treatment.

The other method to combat the virus is to develop a new drug that could target the virus or the host cell, but again this would take several years and we can not wait for that long. A new drug takes at least 14 years to get introduced to the market from the research and development phase and this remains an unlikely solution for this major problem. The third and most likely way to control the corona virus pandemic is to test the SARS-CoV-2 using existing drugs as most of the viruses share similar genome. In an attempt to treat the corona virus using this method, a number of different antiviral and other drugs are used and fortunately few drugs have shown a ray of hope as far as the reduction in duration of therapy and viral load is concerned. We summarized few important recent findings here.

2. Treatment approaches

2.1. Interferon-α (IFN-α)

The IFN-α, a broad spectrum antiviral drug approved for the treatment of viral hepatitis, is used to treat the COVID-19 at a dose of 5 million units through vapor inhalation two times a day alone or in combination with ribavirin (500 mg 2-3 times a day) and antiviral drugs lopinavir/ritonavir (400 mg/100 mg) for a period of 10 days (*2-4*). Previously, the combination of IFN-α 2a, ribavirin and lopinavir/ritonavir was used as a triple therapy for MERS-CoV in South Korea (*5*). It was seen that the SARS-CoV-2 is more susceptible to IFNs as compared to SARS-CoV as the inhalation of IFN-α 2b reduced the infection rate significantly (*6*) and it can be used for prophylaxis of SARS-CoV-2 infection (*7*).

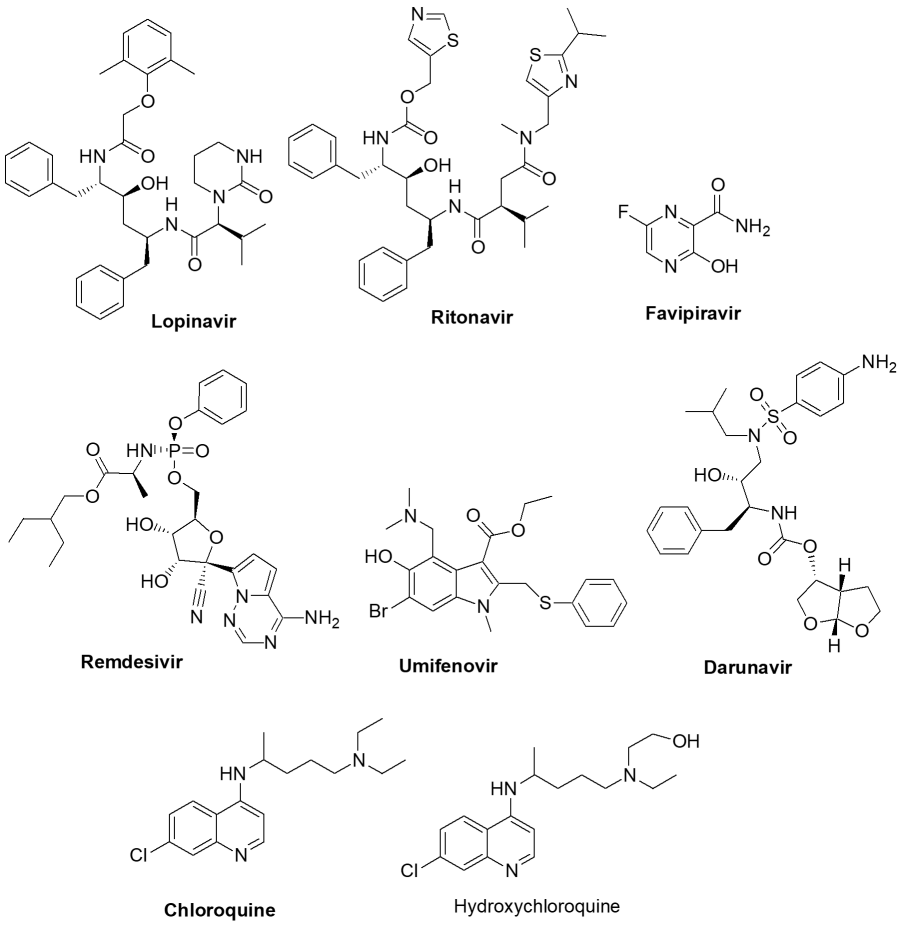

Figure 1. Structures of few drugs tested for anti-SARS-CoV-2 activity.

2.2. Interferon-β (IFN-β)

The other interferon, IFN-β was originally developed for chronic obstructive pulmonary disorder (COPD) and is known to improve the lung's condition and enhance its ability to fight the viral infections. Previously, it was reported that a decrease in the production of INF-β is directly linked to increased susceptibility of people to develop severe respiratory diseases resulting from viral infections (*4*). It was also observed that the SARS-CoV-2 infection suppresses the production of INF-β in body which results in protection from immune system (*2*). Recently, a UK biotechnology firm, Synairgen has been given approval to conduct a trial using IFN-β on patients with COVID-19 (*8*). The advantage with IFN-β is that it can be inhaled similar to IFN-α and can be administered by patients themselves.

2.3. Lopinavir/ritonavir

Lopinavir is an antiretroviral drug which inhibits the protease enzyme and can be formulated together with another protease inhibitor ritonavir which decreases the metabolism of the former by inhibiting the cytochrome (CYT) P4503A enzyme. Lopinavir/ritonavir drugs (Figure 1) combination (Kaletra) was approved for the treatment of human immunodeficiency virus (HIV) and was found to have *in vitro* anti-SARS-CoV efficacy (*9*). However, in a recent clinical trial conducted in China on the patients with severe COVID-19, the drug did not show any promising benefit as compared to the standard care. This randomized, controlled and open-labeled trial was conducted between January 18, 2020 and February 3, 2020 on 199 patients with confirmed SARS-CoV-2 infection. Out of the 199 patients, 99 patients were given the combination of lopinavir/ritonavir, whereas, 100 patients received standard care only. No significant clinical improvement and no reduction in mortality were observed for the lopinavir/ritonavir group along with standard care in comparison to the group receiving standard care only (*10*). Another trial on lopinavir was carried out on 44 participants with mild to moderate infection. Out of 44 patients, 21 patients received lopinavir for 14 days, 16 received umifenovir and 7 received standard care only (*11*). No significant differences in the clinical outcomes were observed for all three groups. The lopinavir group even showed deterioration of diseases conditions in 38.1% of patients compared to 12.5% and 14.3% for umifenovir and control groups respectively. However, Kaltera

can be useful in the early stages of the SARS-CoV-2 infection and might be beneficial for the milder disease conditions and a multi-country clinical trial is to be conducted for this combination.

2.4. Favipiravir

Favipiravir (Avigan) also known as T-705 was first approved for treatment of influenza virus in this February and is the inhibitor of RNA-dependent RNA polymerase in RNA viruses such as SARS-CoV-2 (*12*). Earlier, in February, a preliminary clinical trial on favipiravir was conducted in China on 80 patients and indicated better results for favipiravir in comparison to lopinavir/ritonavir with lesser adverse effects (*13*). Favipiravir was first developed in Japan by Fujifilm and was subjected to another clinical trial on 340 patients in China recently and resulted in very encouraging results as the patients receiving favipiravir with standard care showed cleared viral load in four days as compared to eleven days in patients receiving standard care only (*14*). Another multicentre, open labeled, randomized trial in China was conducted to compare the efficacy of favipiravir (1,600 mg × 2 on the first day followed by 600 mg × 2 for 9 days) and umifenovir (200 mg × 3 per day for 10 days) and the results revealed a higher recovery rate and better clinical outcomes in the patients treated with favipiravir at day 7 (*15*). A phase III trial is ongoing in Japan involving 100 patients and is expected to be completed in June. Another phase II trial is being conducted in the US at Massachusetts General Hospital, Brigham and Women's Hospital, and the University of Massachusetts. However, Avigan was not that much useful in severely ill patients and did not show much promising results. It has to be given before the viral load peaks in the body. Favipiravir would require government approval for usage against COVID-19 as it was earlier approved for the treatment of flu.

2.5. Remdesivir

Remdesivir is an antiviral drug originally developed for Ebola virus, but now is among the front runners for the therapy of novel corona virus, SARS-CoV-2. This drug has earlier shown very promising activity against SARS and MERS (Middle East respiratory syndrome), the two other forms of corona viruses which are more lethal but less contagious than SARS-CoV-2 (*16*). It is an inhibitor of viral replication and is much effective especially in the early stages of infection when the virus multiplies in the upper respiratory part of the body. It was invented by Gilead Sciences and had shown broad spectrum of activity against RNA viruses. Remdesivir resembles the RNA base adenosine and has several important features in its structure making it a strong inhibitor of viral RNA polymerase. It resembles the RNA building block and is taken up by the virus into its RNA strands causing chain termination. The results of compassionate-use of remdesivir were published recently by Gilead Sciences. This trial was conducted on 61 severely ill patients from the US, Canada, Europe and Japan who received intravenous remdesivir at a dose of 200 mg on first day followed by 100 mg for 9 days. Out of 61 patients receiving remdesivir, 8 patients were excluded from the study due to dosing error (1 patient) and lack of post-treatment data (7 patients). Out of the treated patients, 30 who were on mechanical ventilation showed significant improvement and 17 (57%) were extubated. A total of 25 patients (47%) were discharged and 7 (13%) died. Among the dead, 6 were on invasive ventilation, while 1 was not (*17*).

2.6. Umifenovir

Umifenovir (arbidol) was first invented by Pharmstandard and has shown efficacy in the treatment of influenza virus infection. It is claimed to be a viral entry inhibitor to the target cells. Interestingly, it does not have significant side effects and is patented for the treatment of SARS infection. It has shown very promising activity against SARS-CoV-2 *in vitro* showing inhibition of the virus at concentration as low as 10-30 μM (*18*). A randomized, open-labeled, multi-centered clinical trial was conducted in China during the period February 20, 2020 to March 12, 2020 to compare the efficacy and safety of favipiravir and arbidol on COVID-19 patients on 7 day's clinical recovery rate. 120 patients were assigned to each group receiving favipiravir and arbidol along with conventional therapy. The results were published on March 20, 2020 which revealed that the 7 day's recovery rate for arbidol group was 55.86% in comparison to 71.43% for favipiravir group (p = 0.0199). Patients with hypertension or diabetes also showed better improvement in case of favipiravir group in comparison to arbidol (*15*). Currently, three more phase IV clinical trials are planned for arbidol in the treatment of COVID-19. One clinical trial will compare the efficacy of arbidol on 380 patients at Jieming QU, Ruijin Hospital, China in comparison to the standard treatment (*19*), whereas, the other two would compare the efficacy of arbidol with oseltamivir (*20*) on 400 patients at Tongji Hospital, China, and carrimycin on 520 patients at Beijing Youan Hospital, China (*21*).

2.7. Darunavir

Darunavir (Prezista) is another antiviral drug used as HIV-1 protease inhibitor that was shown to have promising anti-SARS-CoV-2 activity *in vitro* earlier in February in a test carried out in China (*18*). It was shown to inhibit the viral replication at a concentration of 300 μM. However, Johnson and Johnson announced on March 18, 2020 that there is no any evidence to support the activity of darunavir against SARS-CoV-2.

Darunavir, marketed by its inventor company Janssen as Prezista was approved with a boosting agent such as ritonavir or cobicistat (*22*). A single center open labeled randomized and controlled phase III trial was conducted at Shanghai Public Health Clinical Center (SPHCC) for the evaluation of efficacy of darunavir/cobicistat combination on 30 COVID-19 patients and the results revealed that the combination was not effective in reducing the symptoms or the duration of treatment (*23*).

2.8. Sarilumab

Sarilumab (Kevzara) is a human monoclonal antibody against the interleukin-6 (IL-6) receptor. As IL-6 is the host target for SARS-CoV-2, its activation could result in severe respiratory symptoms due to lung inflammation. Sarilumab was first developed by Regeneron Pharmaceuticals Inc., US and has collaborated with Sanofi has announced on March 16 to conduct phase II/III clinical trials for the evaluation of Kevzara in around 400 patients hospitalized with COVID-19 infection. It is expected to reduce the overactive inflammatory response of the lungs by blocking the IL-6 receptor (*24,25*). The Feinstein Institute has also collaborated with Gilead Sciences and Regeneron Pharmaceuticals to conduct three clinical trials who are admitted to the Northwell Health Hospitals, US having moderate to severe infections. These trials would be conducted to test the efficacy and safety of sarilumab and remdesivir which is the investigational new drug (*26*).

2.9. Chloroquine (CQ) and hydroxychloroquine (HCQ)

Chloroquine is a widely used antimalarial drug that has shown good activity as antiviral drug in the year 2006 (*27*). In the *in vitro* studies carried out recently, CQ has shown good potential in inhibiting the SARS-CoV-2 at EC_{50} 1.13 μM and CC_{50} > 100 μM (*28*). CQ was reported to be superior in comparison to the control in inhibiting the exacerbation of pneumonia, improving the lung images as well as shortening of the duration of treatment (*29*) when tested on 100 patients. At least 16 clinical trials have been registered to check the efficacy and safety of CQ and HCQ in the treatment of COVID-19 patients.

A clinical trial is being planned in US to see the efficacy and safety of CQ on COVID-19 patients. A clinical study in France revealed that CQ derivative, HCQ when given alone and in combination with macrolide antibiotic azithromycin, have shown significant reduction in the duration of therapy (*30*). The study published on March 17, 2020 in the International Journal of Antimicrobial Agents confirmed that a clinical trial on COVID-19 patients during the period early March to March 16, 2020 showed significant reduction of the viral carriage and reduction of patients with PCR-positive samples for the HCQ treated patients at day 6 as compared to the control. Inclusion of azithromycin to the treatment further decreased the viral load significantly. Hydroxychloroquine was administered at a dose of 200 mg three times a day during ten days. However, this study had a smaller sample size as the HCQ was administered to 26 patients and 16 patients were kept as control. Out of those 26 patients, six patients were lost due to early cessation due to various reasons. Therefore, the data was reported for 20 HCQ administered patients and 16 control patients. However, use of both CQ and HCQ are questioned owing to the cardiovascular complications posed by both the drugs. Recently, worrying results came from Brazil where they needed to stop the high dose arm of CQ in six days as several patients died in the group. Two groups of COVID-19 patients were enrolled in the Manaus Public Hospital in Brazil, in which the high dose group of CQ were planned to receive 12 g total dose (600 mg × 2 × 10) over 10 days, whereas, the low dose received 2.7 g over 5 days (450 mg × 2 on the first day followed by 450 × 1 for 4 days). All the patients received antibiotics ceftriaxone and azithromycin also along with CQ. Unfortunately, 11 patients died in the study forcing the team to halt the high dose group as it caused more lethality. It revealed that high CQ dose for 10 days is not recommended for COVID-19 treatment due to potential toxic effects (*31*).

Similar results have emerged from France, where a University Hospital Centre of Nice has to stop the experiment involving HCQ as it posed a major risk leading to cardiac death. The trial which started on March 22 was experimenting four possible treatments of COVID-19, one of them was HCQ. The HCQ group of patients showed abnormally prolonged QTc resulting in abnormal heart rhythm as seen in the ECG of patients and one of the patients even died due to sudden cardiac arrest (*32*). Another study on HCQ and azithromycin revealed little difference in the clinical condition of COVID-19 patients receiving the combination with those receiving standard care only with a greater risk of cardiac rhythm related side effects (*33*).

2.10. Convalescent plasma (CP)

The plasma of recovered patients can be used to treat the severely ill COVID-19 patients as it contains the antibodies developed by the body in response to the viral infection. It was tried earlier for SARS and doctors were successful in improving the condition of some patients (*34*) whose condition continued deteriorating despite treatment with methylprednisolone. Shorter hospital stay and lesser mortality rates were observed for the patients treated with CP as compared to the patients without receiving it (*35*). Similar resorts were employed for Ebola in the year 2014 (*36*) and MERS patients in the year 2015 (*37*). Therefore, it would be worthwhile to test the efficacy of CP taken from the recovered patients on the COVID-19 patients.

Since, CP has not yet been approved for use against COVID-19 by FDA, it is being regulated under the investigational product. Recently, a clinical trial was conducted between January 23, 2020 and February 19, 2020, where the plasma of 40 recovered COVID-19 patients were collected. For the study, 10 COVID-19 patients with severe infections were selected and were given the transfusion of 200 mL of CP with neutralizing antibody titre values above 1:640 in addition to standard care and other antiviral drugs (*38*). Safety of CP transfusion was kept the primary endpoint, whereas, the improvement in the clinical symptoms within 3 days of CP transfusion remained the second endpoint. Out of 10 patients, 5 showed rapid increase in the antibody titre values to 1:640 and the other four also showed high titre values. Importantly, the clinical symptoms improved significantly including better oxyhaemoglobin saturation, increased lymphocytes count and reduced level of C-reactive protein with no adverse effects.

However, there are various downsides to this approach which includes the difficulty in scaling up for widespread use as well as the risk of transmission of other diseases that would come along with the plasma of recovered patients. Also the antibodies present in the plasma generally are in lesser concentration that may not be sufficient for the treatment. Regeneron company from US is about to introduce two antibodies that could act against COVID-19 which can be synthetically produced and their clinical trial would be started later. This would be helpful as both prophylactic and as a treatment measure especially for high risk groups.

3. Conclusions

A number of trials have been and are being conducted to come up with a drug which shows significant efficacy and safety in the treatment of COVID-19. Few have shown encouraging results and few are in pipeline. Hopefully, we would be able to identify the most suitable approach to combat this deadly virus very soon and make this world a healthy place to live again.

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Mini-Review

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Rapid review for the anti-coronavirus effect of remdesivir

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SUMMARY The outbreak of SARS-CoV-2 rapidly spread across China and worldwide. Remdesivir had been proposed as a promising option for treating coronavirus disease 2019 (COVID-19). We provided a rapid review to critically assess the potential anti-coronavirus effect of remdesivir on COVID-19 and other coronaviruses based on the most up-to-date evidence. Even though remdesivir was proposed as a promising option for treating COVID-19 based on laboratory experiments and reports from compassionate use, its safety and effect in humans requires high-quality evidence from well-designed and adequately-powered clinical trials for further clarification.

Keywords SARS-CoV-2, COVID-19, remdesivir, safety, effect

1. Introduction

In early December 2019, a novel coronavirus named as severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) occurred in Wuhan city located in Hubei province, China (*1*). Similar to previously identified severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), SARS-CoV-2 is the third coronavirus that severely infects humans or even causes death (*2*). Initially, several SARS-CoV-2-infected pneumonia (coronavirus disease 2019, COVID-19) were identified in Wuhan. Shortly afterwards, the outbreak of COVID-19 has rapidly spread across China and worldwide, which now becomes a serious threat to global public health (*3,4*). While there is no specific therapy for COVID-19 available, supportive care and sometimes combined with broad-spectrum antivirals and corticosteroids remain the mainstay as the standard practice (*4*). Therefore, it is urgently needed to identify more effective therapeutic options in response to the rapid propagation of SARS-CoV-2. Several medications have been proposed to be tested for the prevention and treatment of COVID-19, among which remdesivir (GS-5734) has attracted substantial attention.

Remdesivir is a nucleotide analog prodrug that exhibits effective antiviral activity against a broad spectrum of human and zoonotic coronavirus in cell cultures and mouse models including SARS-CoV, MERS-CoV, and SARS-CoV-2. In addition, a recent case report of COVID-19 indicated the recovery of a 35-year-old patient probably due to the administration of remdesivir, while no adverse events related to remdesivir was found (*4*). Other evidence also implicated that remdesivir may be an effective option to treat COVID-19. Therefore, in this rapid review we aimed to summarize the evidence of antiviral effect of remdesivir on the coronaviruses, and to discuss the potential application to COVID-19, after systematically searching the databases of PubMed and MEDLINE with the keywords related to remdesivir.

The potential mechanism of remdesivir for coronavirus remains unclear. Several reasons have been proposed to interpret the effect of remdesivir. First, remdesivir can interfere with the nsp12 polymerase even when the exoribonuclease proofreading activity is intact (*5*). Furthermore, remdesivir can efficiently generate pharmacologically active nucleoside triphosphate (NTP) that acts as an alternative substrate and RNA-chain terminator. Subsequently, NTP can inhibit coronavirus by incorporating active triphosphates into viral RNA (*6*). Additionally, there is a high genetic barrier to achieve resistance of coronavirus to remdesivir, which suggests that remdesivir can maintain the effectiveness of coronavirus therapies (*7*).

As a broad-spectrum antiviral agent, remdesivir has been reported to be effective against a group of coronavirus including alphacoronavirus (NL63) and

Table 1. Antiviral effect of remdesivir on SARS-CoV, MERS-CoV and SARS-CoV-2

Virus type	Experimental studies	Human studies
SARS-CoV	• Remdesivir prophylactically inhibited SARS-CoV replication in human airway epithelial cell cultures. Prophylactic and early-stage therapeutic (within 1 day after infection) effects against SARS-CoV were demonstrated in animal experiments (*8*).	• No evidence in human was reported.
MERS-CoV	• Calu-3 cell cultures and animal experiments indicated the prophylactic and therapeutic effects of remdesivir on MERS-CoV, and the effects were found to be superior to the other anti-viral medications (*9*).	• No evidence in human was reported.
SARS-CoV-2	• In time-of-addition assay using Vero E6 cells, remdesivir therapeutically (administered 2 hours after infection) inhibited SARS-CoV-2 replication, while no prophylactic effect was found. Remdesivir could inhibit SARS-CoV-2 infection in human liver cancer Huh-7 cells (*10*).	• A case report in the United States of COVID-19 indicated the recovery of a patient probably due to the administration of remdesivir (*4*). • A study of compassionate-use remdesivir reported clinical improvement in 68% of the 53 recruited patients who had severe COVID-19 (*11*). • As of 28th April 2020, seven RCTs have been initiated, aiming to evaluate the benefit and harm effects of remdesivir for COVID-19 patients. No results have been available in the literature.

COVID-19: coronavirus disease 2019; MERS-CoV: Middle East respiratory syndrome coronavirus; SARS-CoV: severe acute respiratory syndrome coronavirus.

several SARS/MERS-CoV-like bat coronavirus (*8*). Below we summarized the most up-to-date evidence of remdesivir for SARS-CoV, MERS-CoV, and the SARS-CoV-2 of concern (Table 1).

2. MERS-CoV

A recent study compared antiviral effect on MERS-CoV of several medications including remdesivir, lopinavir (LPV), ritonavir (RTV) and interferon beta (IFN-β) and the combination of LPV/RTV-IFN-β (*9*). The prophylactic and therapeutic effect of remdesivir on MERS-CoV was found to be superior to the other medications. Specifically, results from the *in vitro* experiments showed that remdesivir had superior antiviral effect with a selectivity index (concentration causing a 50% reduction in replication/concentration causing a 50% cytotoxication) > 100 on Calu-3 cells, which was significantly higher than IFN-β (> 16), LPV (> 4.3) and RTV (> 2). Likewise, findings from the animal experiments demonstrated that prophylactic remdesivir could significantly inhibit MERS-CoV replication and diminish the pathological features of acute lung injury (ALI) in MERS-CoV-infected $Ces1c^{-/-}$ mice, while only minimal effect was found in other antiviral medications. When the agents were given on the first day after low-dose infection (5E + 04 pfu), improved pulmonary functions were observed in both remdesivir and LPV/RTV-IFN-β groups. Nevertheless, reductions in several indices (including virus lung titers, viral antigen labeling in lung tissue sections, body weight loss, lung hemorrhage and signs of ALI) were only detected in the group treated with remdesivir. Furthermore, when a lethal dose of MERS-CoV (5E + 05 pfu) was used, a reduced lung viral load in infected mice was only observed in remdesivir group, while no effect was found in other groups.

3. SARS-CoV-2

Wang *et al*. conducted an experiment to evaluate the anti-SARS-CoV-2 effect of remdesivir (*10*). In time-of-addition assay using Vero E6 cells, remdesivir was found to be effective when administered 2 hours after infection at a multiplicity of infection (MOI) of 0.05. However, no prophylactic effect was observed when remdesivir was administered prior to the SARS-CoV-2 infection. The concentration for 90% of maximal effect (EC_{90}) value of remdesivir against SARS-CoV-2 was found to be 1.76 μM. This study also revealed that remdesivir could inhibit SARS-CoV-2 infection in human liver cancer Huh-7 cells.

The recent case report recorded the administration of compassionate-use remdesivir on the 35-year-old man with COVID-19 in the United States (*4*). The patient had initial symptoms of mild cough and low-grade intermittent fevers; subsequently his nasopharyngeal and oropharyngeal swabs were tested positive for SARS-CoV-2 by real-time reverse-transcriptase-polymerase-chain-reaction assay. His vital signs and respiratory status remained largely stable before the 9th day of COVID-19 except for intermittent fevers and nonproductive cough. Since from day 9, the patient began to develop atypical pneumonia, with worsening chest radiograph, decreasing oxygen saturation values and substantial rales in both lungs. With remdesivir

administered on day 11, significant improvements in oxygen saturation values, rales and other symptoms were observed on day 12, indicating the rapid benefit of remdesivir. Subsequently, the patient returned to be afebrile, and all symptoms had resolved with the exception of mild cough. Besides, a recently published study revealed results of compassionate use of remdesivir for patients with severe COVID-19 (*11*). In 53 patients who received at least one dose of remdesivir, 36 (68%) had clinical improvements, including changes on oxygen-support and extubation of mechanical ventilation. The mortality of the patients was 13%, which was lower than the general mortality of severe patients with COVID-19 (over 50%), as reported by the WHO (*12*).

The current evidence on experimental studies and clinical observation indicated that remdesivir has the potential for treating COVID-19. Nevertheless, findings from the compassionate-use study were not adequately powered with a randomized controlled design to assess the safety and efficacy of remdisivir in patients with severe COVID-19. Therefore, more evidence from randomized clinical trials (RCTs) of high quality is eventually needed to confirm its safety and efficacy. Two phase III clinical trials had been launched in Hubei and Beijing in China in early February 2020, aiming to evaluate the safety and efficacy of remdesivir for adult patients with COVID-19 and with mild-moderate (NCT04252664, sample size: 308) and severe (NCT04257656, sample size: 452) symptoms. Subsequently, other five RCTs with similar objectives were further registered on *clinicaltrials.gov*. However, as of 28th April 2020, there has not been published results available in the literature. Therefore, it remains largely unknown currently regarding the benefit-harm profile of remdesivir for COVID-19.

In brief, remdesivir has been found to inhibit coronavirus and improve pulmonary functions prophylactically and therapeutically (in early stage of infection) based on evidence from both *in vitro* and *in vivo* experiments. However, evidence in patients with COVID-19 remained limited and sparse.

The ongoing clinical trials will provide more high-quality evidence on the benefit-harm effect of remdesivir. Nevertheless, there are several issues of concern regarding their protocols. First, the inclusion/exclusion criteria and the outcome measurements do not include chest radiography that is one of the key elements for disease diagnosis and criteria for recovery according to Guidelines for the Diagnosis and Treatment of Novel Coronavirus (SARS-CoV-2) Infection by the National Health Commission (Trial Version 5) published by National Health and Health Commission of the people's Republic of China (*13*). Thus, it may incur selection and reporting bias to weaken the results. Secondly, based on the previous experiments on SARS-CoV, remdesivir was effective only when it was administered at the early stage of infection (before the initiation of the immunopathological phase of pneumonia) (*8*). By contrast, another study showed that remdesivir was found to be functional for SARS-CoV-2 when administered 2 hours after infection (*10*). These results indicated the benefit of remdesivir may heavily depend on the time of administration. No predefined plans of trial designs or statistical analyses are given in their protocols related to the optimum time of administration. Thirdly, the current evidence was insufficient to support the safety of remdesivir in humans. Even though some cytotoxicity tests suggested that remdesivir could be effective at a relatively low micromolar concentration compared with its cytotoxic concentration (*8,9*), the safety test in humans is still ongoing currently. Moreover, a previous randomized controlled trial reported that in patients with Ebola virus disease, the overall mortality was even higher in remdesivir group (53%) than the control group (a triple monoclonal antibody agent; 50%), although without significance (*14*). Therefore, extreme cautions and monitoring should be taken in the ongoing trials for COVID-19 given the safety of remdesivir remains largely unconfirmed and unknown.

To summarize, even though remdesivir was proposed as a promising option for treating COVID-19 based on laboratory experiments and reports from compassionate use, its safety and effect in humans requires high-quality evidence from well-designed and adequately-powered clinical trials for further clarification. Similar to the inconclusive effect on SARS-CoV and MERS-CoV, the impact of remdesivir on the SARS-CoV-2 outbreak should not be overestimated in the current clinical practice. Further explorations remain urgently needed to treat the COVID-19 and bring the SARS-CoV-2 under control.

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Original Article

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Effects of the combined administration of risedronate and menatetrenone on bone loss induced by tacrolimus in rats

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SUMMARY Tacrolimus, a calcineurin inhibitor, affects bone metabolism and increases the risk of fracture due to marked bone loss. Bisphosphonates increase the bone mineral density (BMD) in osteoporosis patients. Menatetrenone has less positive effects on BMD but reduces the risk of fracture by improving bone quality. In this study, we investigated the effectiveness of the combined administration of risedronate and menatetrenone against bone loss induced by tacrolimus. Wistar rats were divided into four groups: [1] control, [2] tacrolimus at 1.5 mg/kg, [3] tacrolimus + risedronate at 1.0 mg/kg, and [4] tacrolimus + risedronate + menatetrenone at 20 mg/kg. After the drugs were administered for 4 weeks, bone histomorphometric analysis was performed and bone strength was evaluated using a three-point bending method. BMD was measured using quantitative computed tomography. Tacrolimus significantly reduced the BMD and strength properties of the lower limb bones. These tacrolimus-induced decreases were suppressed by risedronate treatment. The combined administration of risedronate and menatetrenone more significantly improved bone strength properties than risedronate alone. Bone histomorphometric analysis revealed a significant increase in bone resorption with tacrolimus. Risedronate alone significantly suppressed the tacrolimus-induced increase in bone resorption but simultaneously reduced bone formation. On the other hand, the combined administration of risedronate and menatetrenone suppressed the tacrolimus-induced increase in bone resorption, in addition to the significant risedronate-induced decrease in bone formation. This study suggests that the combined administration of risedronate and menatetrenone improves bone strength in tacrolimus-treated rats by preventing and ameliorating the risedronate-induced suppression of bone formation.

Keywords Bisphosphonate, menatetrenone, combined administration, bone loss, tacrolimus

1. Introduction

To further extend the healthy life expectancy in the super-aged societies of developed countries, fractures, which significantly reduce quality of life and vital prognosis in patients, should be prevented. Bone is a metabolically active organ that undergoes continuous remodeling, consisting of bone resorption by osteoclasts and bone formation by osteoblasts (*1*). An imbalance of bone remodeling induced skeletal disorders, such as osteopenia or osteoporosis (*2,3*). Aging and menopause are the main causes of bone fragility leading to fractures, whereas certain diseases and drugs significantly affect bone metabolism, resulting in an increased risk of fracture. We previously reported that tacrolimus, a calcineurin inhibitor, increased osteoclast activity, which regulates bone metabolism, and induced bone loss (*4*). To promote immunosuppressive therapy, which is essential for the prevention of rejection in recipients, prevention and treatment methods should be established using pharmacotherapy for tacrolimus-induced bone loss.

Bone strength is defined by bone mineral density (BMD) and bone quality, which refers to bone microstructure, bone turnover, micro-damage, and bone mineralization (*5*). Bisphosphonates, bone resorption inhibitors, are used worldwide for the treatment of osteoporosis. Bisphosphonates suppress bone resorption by inhibiting osteoclast activity (*6,7*). Of note, bisphosphonates have been demonstrated to increase

BMD in clinical studies (*8-11*) and osteoporosis animal models (*12-14*). However, long-term administration of bisphosphonates may degrade bone strength by markedly suppressing bone remodeling (*15-17*) and increase the risk of developing atypical femoral fractures (*18-21*).

Menatetrenone, vitamin K_2, acts as an essential coenzyme for the γ-carboxylation of glutamate residues in osteocalcin, a bone matrix protein (*22,23*), and significantly reduces the risk of fracture (*24,25*). As menatetrenone slightly increases BMD (*26*), its ability to reduce the risk of fracture is primarily explained by improved bone quality (*27*). Therefore, the combined administration of bisphosphonate and menatetrenone, which have different mechanisms of action on bone metabolism, may have beneficial effects on both BMD and bone quality, which determine bone strength, although its detailed effects remain unclear. In the present study, we investigated the effects of the combined administration of risedronate and menatetrenone on tacrolimus-induced bone loss in rats.

2. Materials and Methods

2.1. Animals

Four week-old male Wistar rats weighing 70-80 g were purchased from CLEA Japan Inc. (Tokyo, Japan). Animals were housed at 22 ± 2°C and 55 ± 5% humidity on a 12-h light-dark cycle with *ad libitum* access to standard chow (MF; Oriental Yeast Co., Tokyo, Japan) and water. All procedures were approved by the Animal Research Committee of Niigata University of Pharmacy and Applied Life Sciences in accordance with the Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals.

2.2. Drugs

Commercially available tacrolimus (Astellas Pharma Inc., Tokyo, Japan) and risedronate (Eisai Co., Ltd., Tokyo, Japan) agents were obtained suspended in a 0.2% carboxymethylcellulose sodium solution (CMC-Na; Sigma-Aldrich, St. Louis, MO, USA). Menatetrenone (Eisai Co., Ltd., Tokyo, Japan) was suspended in olive oil.

2.3. Experimental procedure

Animals were randomly divided into four groups (10 animals/group): [1] control treated with the vehicle (0.2% CMC-Na), [2] tacrolimus at 1.5 mg/kg, [3] tacrolimus at 1.5 mg/kg + risedronate at 1.0 mg/kg, and [4] tacrolimus at 1.5 mg/kg + risedronate at 1.0 mg/kg + menatetrenone at 20 mg/kg. Drug doses were selected based on previous reports relevant to tacrolimus (*4*), risedronate (*12*), and menatetrenone (*27*). Drugs were administered *via* oral gavage in a volume of 0.1 mL/100 g of body weight once daily for 4 weeks. All animals were euthanized under CO_2 anesthesia 24 h after the final drug was administered. The femur and tibia were dissected, and soft tissue was removed.

2.4. Bone strength analysis

Bone strength of the femoral mid-diaphysis was evaluated *via* a three-point bending method using a mechanical testing machine (EZ-S; Shimadzu, Tokyo, Japan). The femur was positioned on two supports placed 10 mm apart. The bending load was vertically applied to the mid-diaphysis with a crosshead speed of 1.0 mm/min until fracture. The load deformation curves were calculated using operation software (Trapezium X; Shimadzu, Tokyo, Japan), and the maximum load, breaking energy, and stiffness were directly calculated from the load deformation curve.

2.5. BMD measurements

BMD of the whole femur and tibia was measured using quantitative computed tomography (LaTheta LCT-100; Aloka, Tokyo, Japan) with a pixel size of 250 × 250 μm and slice thickness of 1 mm. The values of BMD were calculated using LaTheta software (ver. 1.31; Aloka, Tokyo, Japan).

2.6. Bone histomorphometry

We prepared non-decalcified specimens from the proximal tibia metaphysis according to the following method: The tibia was fixed with 70% ethanol for 7 days, stained with Villanueva Bone Stain (basic fuchsin, fast green, orange G, and azure II; Merck, Darmstadt, Germany) in 70% methanol for 7 days, and embedded in a methyl methacrylate resin. The resin blocks were then sliced to 5-μm thickness on a microtome (Leica RM2255; Leica Inc., Nussloch, Germany). All bone histomorphometric parameters were measured at the secondary spongiosa region. To exclude the primary spongiosa, the measurement region was 0.42.1 mm distal to the lowest point of the growth plate and 0.2 mm from the lateral cortex.

Bone histomorphometric measurements were performed using a semiautomatic image analyzing system (Histometry RT CAMERA; System Supply, Nagano, Japan) with ×400 magnification. Bone structural parameters obtained included bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb. Sp). Bone formation parameters included the osteoblast surface per bone surface (Ob.S/BS). Bone resorption parameters included the eroded surface per bone surface (ES/BS), osteoclast surface per bone surface (Oc.S/BS),

and osteoclast number per bone surface (N.Oc/BS).

The dynamic parameter was measured using a double fluorescent labeling technique. For labeling, all rats were injected subcutaneously with 25 mg/kg of tetracycline (Sigma-Aldrich, St. Louis, MO, USA) and 10 mg/kg of calcein (Wako Pure Chemical Industries, Osaka, Japan) 5 and 2 days before they were euthanized, respectively. The labeled surface that reflected the calcification front at the time of tetracycline and calcein administration was visualized using a fluorescent microscope (Olympus BX50; Olympus America Inc., Center Valley, PA, USA). The parameters of single- and double-labeled surface (sLS and dLS) and inter-label thickness, and times (Ir.L.Th and Ir.L.t) were used in the calculation of the mineralizing surface per bone surface (MS [dLS+sLS/2]/BS]), mineral apposition rate (MAR; Ir.L.Th/Ir.L.t).

Figure 1 shows the scheme of the primary parameters (*28*). Standard bone histomorphometrical nomenclature, symbols, and units were based on those described in the report of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee (*29*).

2.7. Statistical analysis

Data are presented as the mean ± standard error (SE). Differences between groups were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons. $p < 0.05$ was considered significant.

3. Results

3.1. Bone strength properties

The maximum load and breaking energy in the tacrolimus group were significantly reduced (37% and 40%, respectively) compared with those in the control group (Figure 2). In addition, the maximum load and breaking energy in the risedronate and risedronate + menatetrenone groups were significantly higher than those in the tacrolimus group. Furthermore, the maximum load and breaking energy in the risedronate + menatetrenone group were significantly higher than those in the risedronate group.

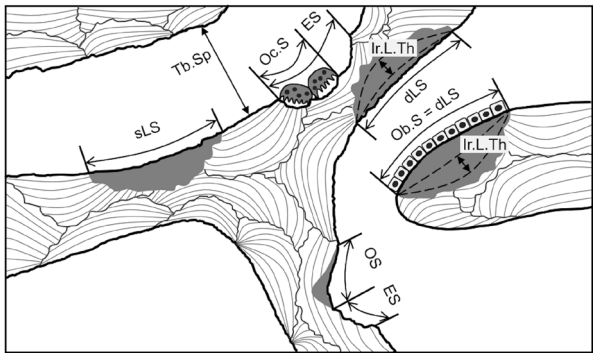

Figure 1. The scheme of the primary bone histomorphometric parameters. Tb.Sp; trabecular separation, dLS; double-labeled surface, sLS; single-labeled surface, Ir.L.Th; inter-labels thickness, Ob.S; osteoblast surface, OS; osteoid surface, ES; eroded surface, Oc.S; osteoclast surface.

3.2. BMD

Femoral and tibial BMD were significantly reduced (9% and 11%, respectively) in the tacrolimus group compared with those in the control group (Figure 3). The BMD of femur and tibia in the risedronate and risedronate + menatetrenone groups were significantly higher than those in the tacrolimus group. On the other hand, the BMD of femur and tibia in the risedronate + menatetrenone group were comparable with and not significantly different from those in the risedronate group.

3.3. Bone histomorphometric evaluation

Trabecular bone structural parameters according to the bone histomorphometry of the proximal tibia metaphysis are shown in Figure 4. Tacrolimus treatment significantly reduced the BV/TV, Tb.Th, and Tb.N (50%, 20%, and 66%, respectively), and increased the Tb.SP (57%) relative to those in the control group. These bone structural parameters in the risedronate and risedronate + menatetrenone groups were significantly higher than those in the tacrolimus group.

Bone formation parameters, Ob.S/BS, MS/BS, and MAR, in the tacrolimus group did not significantly differ from those in the control group, whereas bone resorption parameters, ES/BS, Oc.S/BS, and N.Oc/BS (69%, 70%, and 87%, respectively), were significantly increased (Figure 5). The tacrolimus-induced increases in ES/BS, Oc.S/BS, and N.Oc/BS were significantly suppressed by risedronate alone or risedronate + menatetrenone. However, Ob.S/BS, MS/BS, and MAR (83%, 70%, and 54%, respectively), were significantly reduced in the risedronate group compared with the other groups. In contrast, Ob.S/BS, MS/BS, and MAR were significantly increased in the risedronate + menatetrenone group compared with those in the risedronate group, and were comparable with those in the control group.

Typical fluorescence microphotographs of the slices assessed by bone histomorphometry are shown in Figure 6. These images confirmed the marked decrease in fluorescence labeled surface with tetracycline and calcein in the risedronate group compared with that in the control group. In contrast, bones in the risedronate + menatetrenone group had a marked increase in fluorescence labeled surface and inter-label thickness compared with those in the risedronate group.

4. Discussion

The present study examined the effects of the combined

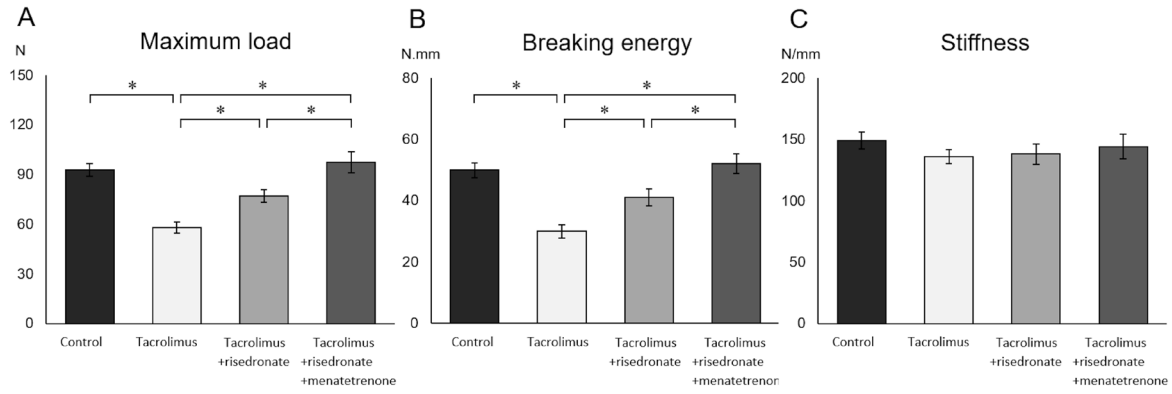

Figure 2. Bone strength properties (A: maximum load, B: breaking energy, C: stiffness) of the femoral mid-diaphysis in each group. Bone strength was evaluated by a three-point bending method. Data represents the mean ± SE (n = 10). $^{*}p < 0.05$.

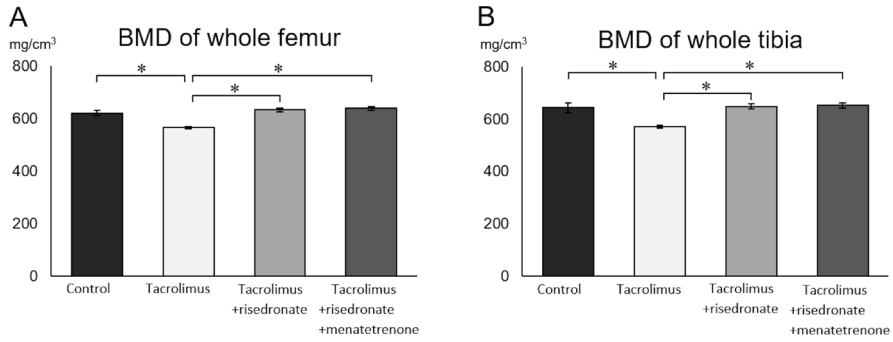

Figure 3. BMD of whole femur (A) and tibia (B) in each group. BMD was measured using quantitative computed tomography. Data represents the mean ± SE (n = 10). $^{*}p < 0.05$.

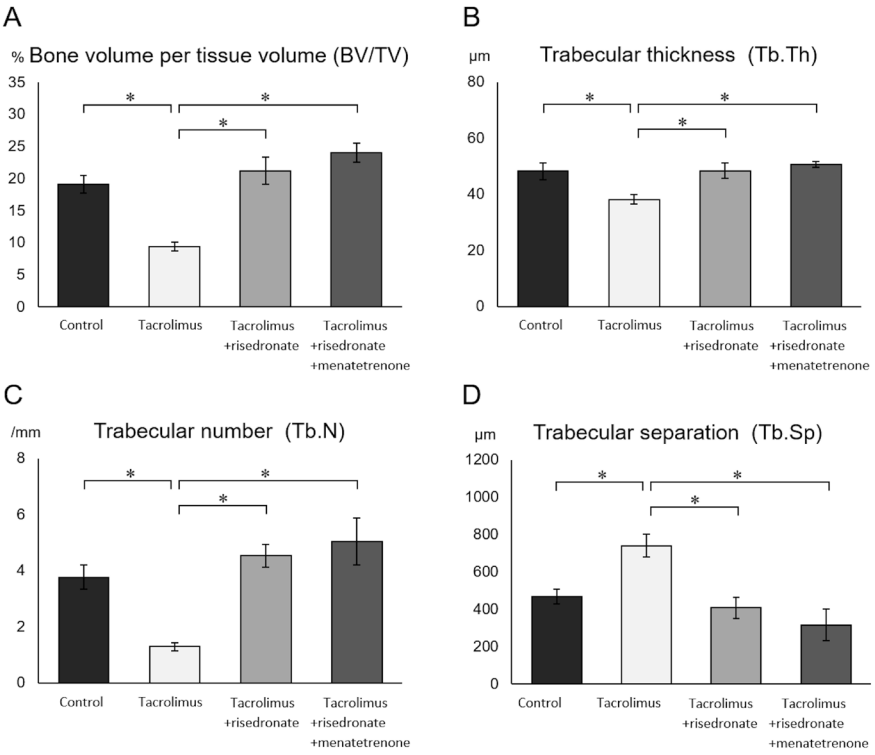

Figure 4. Trabecular bone structural parameters (A: bone volume per tissue volume (BV/TV), B: trabecular thickness (Tb.Th), C: trabecular number (Tb.N), D: trabecular separation (Tb.Sp)) according to the bone histomorphometry of the proximal tibia metaphysis. Data represents the mean ± SE (n = 10). $^{*}p < 0.05$.

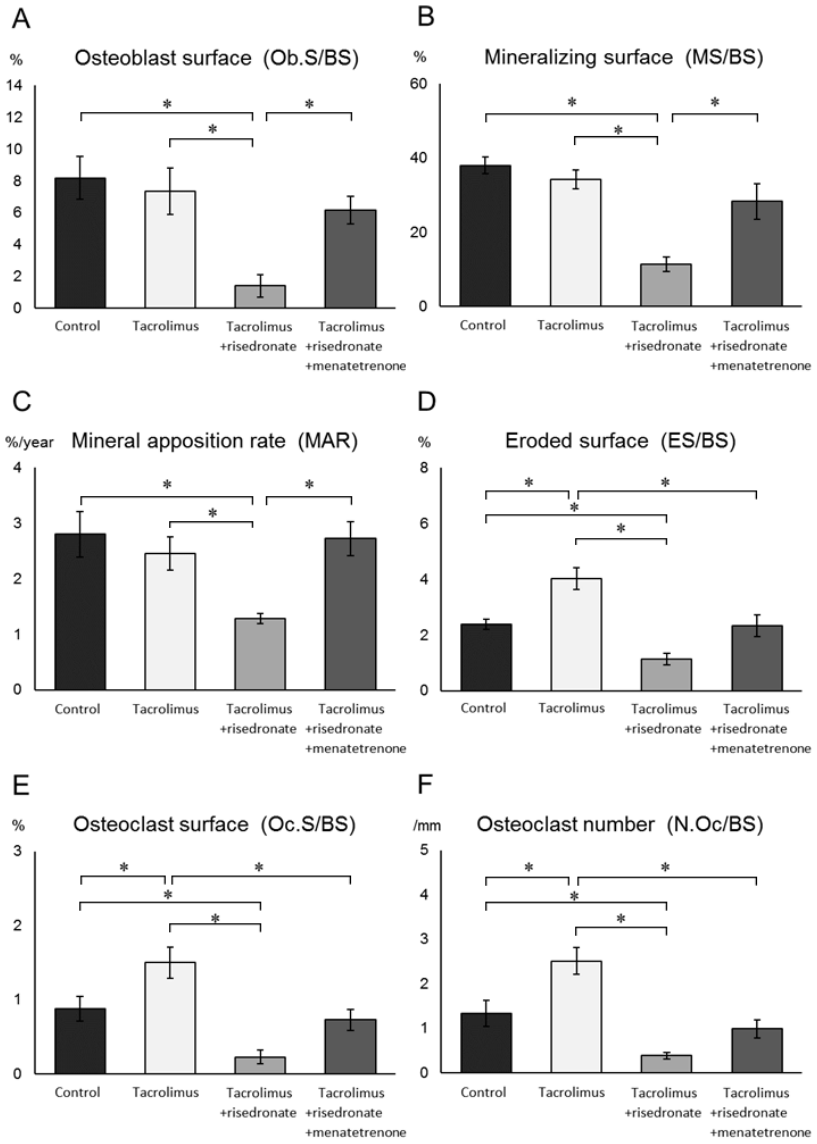

Figure 5. Bone formation parameters (A: osteoblast surface (Ob.S/BS), B: mineralizing surface (MS/BS), C: mineral apposition rate (MAR), D: eroded surface (ES/BS), E: osteoclast surface (Oc.S/BS), F: osteoclast number (N.Oc/BS)) according to bone histomorphometry of the proximal tibia metaphysis. Data represents the mean ± SE (n = 10). $^{*}p < 0.05$.

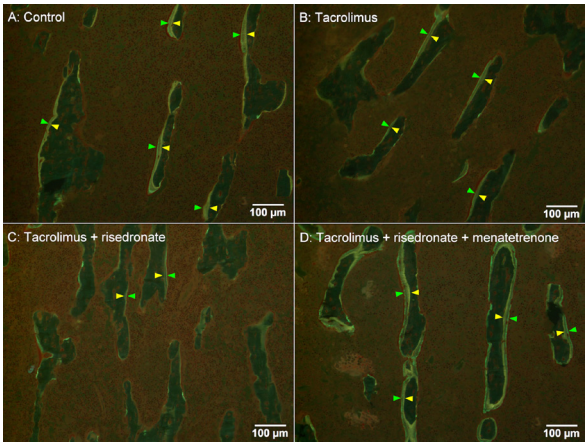

Figure 6. Typical micrographs of the slices assessed by bone histomorphometry under fluorescence. The labeling surface with tetracycline and calcein is indicated by the yellow arrows and the green arrows, respectively.

administration of risedronate and menatetrenone on tacrolimus-induced bone loss. The administration of tacrolimus at 1.5 mg/kg/day for 4 weeks significantly reduced the femoral and tibial BMD in rats. The tacrolimus-induced decreases in the BMD of the lower limb bones were significantly suppressed by risedronate alone, although no additive effects were achieved by the combined administration of risedronate and menatetrenone. On the other hand, the three-point bending test of bone strength properties demonstrated that risedronate was more effective in preventing and ameliorating the tacrolimus-induced decrease in bone strength properties when combined with menatetrenone than when administered alone.

Only a few studies have examined the effects of anti-osteoporosis drugs on tacrolimus-induced bone loss. The clinical studies (*30,31*) and animal

studies (*32,33*) found significant protective effects of bisphosphonate on calcineurin inhibitor-induced bone loss. However, long-term administration of bisphosphonate increases microdamage accumulation in the bone (*15-17*) and is involved in the development of atypical femoral fractures (*18-21*). Specifically, the marked suppression of bone turnover by long-term bisphosphonate administration may degrade bone quality, resulting in bone fragility. To prevent the bone damage induced by immunosuppressive agents, care should be exercised not only to increase BMD, but also to improve bone quality.

Several studies using osteoporosis animal models also reported the positive effects of the combined administration of bisphosphonate and menatetrenone on bone metabolism (*34,35*). Menatetrenone is expected to reduce the risk of fracture primarily by improving bone quality (*27*) because of its negligible bone mass-increasing effects (*26*). Menatetrenone may improve the degradation of bone quality induced by bisphosphonate. Therefore, in the present study, in order to evaluate the bone quality, we performed a bone histomorphometry of the proximal tibia metaphysis. Bone histomorphometric analysis revealed the impaired microstructure of trabecular bone in rats administered tacrolimus. It also significantly increased the ES/BS, Oc.S/BS, and N.Oc/BS without significantly affecting bone formation. Thus, as previously reported (*4*), tacrolimus caused rarefaction of the trabecular bone by increasing bone resorption. Risedronate alone significantly suppressed the tacrolimus-induced increase in bone resorption and significantly reduced the bone formation parameters, Ob.S/BS, MS/BS, and MAR. MS/BS and MAR are considered an index of osteoblast differentiation and proliferation (*36*). Of note, Ob.S/BS, MS/BS, and MAR were significantly higher in the risedronate + menatetrenone group than in the control group. Thus, the suppression of bone formation by risedronate was significantly prevented and ameliorated by the combined administration of risedronate and menatetrenone. Menatetrenone promotes osteocalcin production and mineralization in osteoblasts (*37,38*), and suppresses osteoclast formation and bone resorption *in vitro* (*39*). The additive improving effects of the combined administration of risedronate and menatetrenone on the tacrolimus-induced decrease in bone strength may be partially explained by the menatetrenone-induced enhancement of bone formation.

In conclusion, the present study investigated the effectiveness of the combined administration of risedronate and menatetrenone against tacrolimus-induced bone loss. Risedronate alone and risedronate + menatetrenone significantly improved tacrolimus-induced decreases in bone strength properties and BMD. Moreover, the combined administration of risedronate and menatetrenone was highly effective in improving the tacrolimus-induced decrease in bone strength properties. The effectiveness of the combined administration of anti-osteoporosis drugs with different mechanisms of action may be caused by menatetrenone, which ameliorated the marked suppression of bone formation due to bisphosphonates.

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Brief Report

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Therapeutic effect of Chinese prescription Kangen-karyu in patients with diabetic nephropathy

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SUMMARY Diabetic nephropathy is the kidney disease that occurs as a result of diabetes. A number of new therapies have been developed based on the pathogenic factors of diabetic nephropathy such as intensive glycemic control, precise hypertension control, lifestyle modifications including exercise and an energy-restricted diet, and numerous novel agents. The utilization of traditional Chinese medicine for patients with diabetic nephropathy has also received increasing attention due to its wide availability, weak side-effects, and proven therapeutic mechanisms and benefits. In this paper, we report the case of patients with diabetic nephropathy, stage 2 or 3. Kangen-karyu extract (7.5 g/day) was administered three times per day for 6 months. The estimated glomerular filtration rate was increased at the 6-month follow-up. The serum creatinine level decreased following administration. At that time, somatic and subjective symptoms had partially disappeared. Here, we present evidence that Kangen-karyu exerts a renoprotective effect against the development of diabetic nephropathy.

Keywords diabetic nephropathy, traditional Chinese medicine, Kangen-karyu, case report

1. Introduction

Diabetes is the leading cause of end-stage renal disease (ESRD) in most developed countries, and has driven an increase in ESRD globally over recent decades (*1,2*). There is a strong economic and health imperative to improve outcomes for people with diabetes and kidney disease. A number of promising treatments have been found to be ineffective or harmful, many of which have now been abandoned in this population (*3-5*). One feature of these failures has been the emergence of unexpected adverse effects, highlighting the importance of safety monitoring in future trials and review of what is known about the safety of existing treatments in this patient population.

Traditional Chinese medicine has received much attention as a source of novel therapeutic agents due to their multiple beneficial effects and absence of toxic and/or side effects (*6*). Kangen-karyu (Guan-Yuan-Ke-Li in Chinese), one of our major interests among traditional Chinese medicine agents, has been developed in Japan by the modification of herbal constituents of Kan-shin No. 2 (Guan-xin No. 2 in Chinese) (*7*), and is composed of six herbal formulas (Salviae Miltiorrhizae Radix, Cnidii Rhizoma, Paeoniae Radix, Carthami Flos, Aucklandiae Radix, and Cyperi Rhizoma, as shown in Table 1). Kangen-karyu has been clinically used as a treatment for cardiovascular disease (CVD), known as a risk factor for the progression of chronic kidney disease (CKD) (*8,9*). Many studies demonstrated that Kangen-karyu exhibits favorable biological activity such as anti-aging effects, platelet aggregation inhibition, hypertension suppression, anti-dyslipidemia, aiding the recovery of learning and memory impairment induced by senescence, neuroprotection, and an anti-dementia effect in animal experiments (*10-17*). Although studies have proposed the pharmacological functions of Kangen-karyu to treat various diseases, we previously reported evidence supporting its preventive and/or therapeutic potential against diabetes-induced renal damage using *db/db* mice, a type 2 diabetic animal model (*18-20*). The results of our previous study provide important evidence that Kangen-karyu exerts a renoprotective effect against the development of diabetic nephropathy. We also provide evidence supporting the use of Kangen-karyu as a therapeutic agent in a patient with diabetic nephropathy in the early stage (*21*).

On the basis of these findings, we administered Kangen-karyu to diabetic nephropathy patients, stage 2 or 3, and report its therapeutic usefulness.

Table 1. Composition of Kangen-karyu

Common name	Botanical name	Family name
Salviae Miltiorrhizae Radix	*Salvia miltiorrhiza* Bunge	Labiatae
Cnidii Rhizoma	*Cnidium officinale* Makino	Umbelliferae
Paeoniae Radix	*Paeonia lactiflora* Pallas	Paeoniaceae
Carthami Flos	*Carthamus tinctorius* L.	Compositae
Aucklandiae Radix	*Aucklandia lappa* Dcne.	Compositae
Cyperi Rhizoma	*Cyperus rotundus* L.	Cyperaceae

2. Materials and Methods

2.1. Study population

This study was conducted according to the ethical guidelines for epidemiological research designated by the Japanese Ministry of Education, Culture, Sports, Science and Technology and Ministry of Health, Labour, and Welfare. Ethical approval was obtained from the Clinical Research Ethics Committees of Shinseikai Toyama Hospital. Written informed consent was obtained from all subjects at the time of enrollment for collection of clinical information and biosamples for archival and research purposes. The study cohort was previously diagnosed with diabetic nephropathy at Shinseikai Toyama Hospital. Both sexes (3 men and 2 women; 54-73 years, 64.0 ± 3.8 years) and stages of diabetic nephropathy (3, stage 2; and 2, stage 3) were represented. The patients continued to receive existing treatments: hypoglycemic agents (metformin: 750 mg/day, ipragliflozin: 50 mg/day), an antihypertensive agent (termisartan: 20 mg/day), antilipidemic agent (atrovastatin: 5 mg/day), and antacid-laxative (magnesium oxide: 990 mg/day). In addition, Kangen-karyu extract (7.5 g/day) was administered three times a day for 6 months. During the administration of Kangen-karyu extract, regular tests were performed to assess its effect on diabetic nephropathy. At that time, a medical interview including questions on the somatic and subjective symptoms was conducted during the study.

2.2. Measurements of study variables

All measurements were performed by the Department of Laboratory Medicine of Shinseikai Toyama Hospital using routine automated laboratory methods. Estimated GFR (eGFR) was based on the equation proposed by the Japanese Society of Nephrology (*22*). Body components were analyzed using an InBody 770 (InBody Japan Inc., Tokyo, Japan).

2.3. Assessment of somatic and subjective symptoms

The symptom checklist included the following symptoms: dizziness and palpitation, stiff shoulder and headache, coldness of the limbs and fatigability, mental stress, sleeping disorder, tension of the stomach and abdomen, pain, numbness of the waist and body, dark circles around eyes and lip symptoms, stains on face, aza skin, and tongue symptoms. The change in each symptom was assessed with a 3-point rating scale: "marked improvement" was 5 points, "improvement" was 4 points, and "slight improvement" was 2 points. The assessment of global improvement rating of subjective symptoms simply involved the addition of points.

Table 2. Laboratory data and physical characteristics on administration of Kangen-karyu for 6 months

Parameter	0 M	6 M
HbA1c (%)	7.52 ± 0.47	7.78 ± 0.39
Serum Cr (mg/dL)	0.71 ± 0.07	0.64 ± 0.09
eGFR (mL/min/1.73 m^2)	79.2 ± 8.8	88.2 ± 9.0
Urinary albumin (mg/g Cr)	56.1 ± 10.4	71.0 ± 45.4
Urinary protein (mg/g Cr)	425 ± 157	506 ± 193
BMI (kg/m^2)	28.2 ± 1.4	28.8 ± 1.3
SLM (kg)	45.8 ± 4.4	45.9 ± 4.2
BFM (kg)	25.0 ± 3.5	26.1 ± 3.3
VFA (cm^2)	119 ± 18	124 ± 19
PBF (%)	34.1 ± 3.6	35.1 ± 3.5
Score using the questionnaire	38.6 ± 2.7	27.2 ± 1.1*

M, months. Values are expressed as the mean ± SEM. of 5 patients. *$p < 0.01$ *vs.* 0 M values.

2.4. Statistical analysis

The data are expressed as the mean ± SEM. Significance was assessed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test (SPSS 11.5.1 for Windows, 2002, SPSS Inc., USA), with values of $p < 0.05$ considered to indicate significance.

3. Results and Discussion

3.1. Clinical characteristics

From samples obtained at the first timepoint, hemoglobin A1c (HbA1c) was 7.52%, showing poorly controlled blood glucose. The eGFR was 79.2 mL/min/1.73 m^2, and this corresponded to a serum creatinine (Cr) level of 0.71 mg/dL. The urinary albumin and protein levels were 56.1 and 425 mg/g Cr, respectively, indicating stage 2 to 3 diabetic nephropathy, as shown in Table 2. After the administration of Kangen-karyu extract for 6 months, eGFR was subsequently increased from 79.2 to 88.2 mL/

Table 3. Changes in eGFR and serum Cr before and after Kangen-karyu administration

Items	Kangen-karyu administration						
	-6 M	-3 M	-1 M	0 M	1 M	3 M	6 M
eGFR (mL/min/1.73 m^2)	84.6 ± 9.4	80.3 ± 7.9	79.2 ± 5.8	79.2 ± 8.8	80.7 ± 8.3	81.9 ± 7.9	88.2 ± 9.0
Serum Cr (mg/dL)	0.67 ± 0.14	0.70 ± 0.06	0.70 ± 0.05	0.71 ± 0.07	0.70 ± 0.07	0.68 ± 0.05	0.64 ± 0.09

M, months. Values are expressed as the mean ± SEM of 5 patients.

min/1.73 m^2 at the 6-month follow-up, and serum Cr was slightly decreased compared with the first timepoint. The urinary albumin level increased from 56.1 to 71.0 mg/g Cr. Urinary protein excretion also increased to 506 mg/g Cr. There was, however, no significant change in the HbA1c on the administration of Kangen-karyu extract, as shown in Table 2.

Moreover, to identify the therapeutic usefulness of Kangen-karyu extract to renal function of diabetic nephropathy patients, we investigated the eGFR and serum Cr levels in patients from the 6th month prior to Kangen-karyu extract administration. As shown in Table 3, the eGFR level of diabetic nephropathy patients was gradually decreased as time progressed until 6 months, indicating renal function decline. On the other hand, the administration of Kangen-karyu was increased from 79.2 to 80.7 mL/min/1.73 m^2 at 1 month, 81.9 mL/min/1.73 m^2 at 3 months, and 88.2 mL/min/1.73 m^2 at 6 months. When the rate of variability in eGFR was calculated using the formula shown in Table 4, its value was significantly recovered by Kangen-karyu administration. Additionally, a slight increase of serum Cr that progressed in diabetic nephropathy patients was progressively decreased by the administration of Kangen-karyu at the 6-month follow-up, as shown in Table 3. The rate of variability in serum Cr was significantly decreased by Kangen-karyu (Table 4).

Table 4. Rate of variability in eGFR and serum Cr before and after Kangen-karyu administration

Items	(At the start of administration – 6 months before administration)/6	(At the start of administration – 6 months after administration)/6
eGFR	– 0.894 ± 0.288	1.491 ± 0.406*
Serum Cr	0.007 ± 0.002	– 0.012 ± 0.003*

Significance: *$p < 0.01$ *vs.* (at the start of administration - 6 months before administration)/6 values.

3.2. Physical characteristics

There was no significant change in the physical parameters such as body mass index (BMI), soft lean mass (SLM), body fat mass (BFM), visceral fat area (VFA), or percent body fat (PBF) on the administration of Kangen-karyu extract, as shown in Table 2.

3.3. Somatic and subjective symptoms

At the 6-month follow-up of patients, the somatic and subjective symptoms such as stiff shoulder, headache, coldness of the limbs, and fatigability had disappeared. The score using the questionnaire had decreased from 38.6 to 27.2 at follow-up, being a significantly lower (30%) score, as shown in Table 2.

Diabetic nephropathy is the leading cause of ESRD, which is a threat to public health and a major financial burden for healthcare systems (*23,24*). The life expectancy of patients with ESRD has remained poor, and ESRD prevention is challenging. The prognosis of CVD patients with type 2 diabetes has markedly improved over the past 20 years, but the incidence of ESRD has decreased very little (*25*). Thus, early interventional treatment for diabetic nephropathy is important.

In the present study, we chose Kangen-karyu extract for the following reasons. Kangen-karyu was developed by the modification of herbal constituents of Kan-shin No. 2 in Japan (*7*). It has been clinically used as a treatment for CVD (*8*). Kangen-karyu has received much attention as a source of new therapeutic agents based on pre-clinical animal experiments related to various human diseases (*10-17*). To add to these findings, we report evidence supporting its preventive and/or therapeutic potential against diabetes-induced renal damage (*18-20*). The administration of Kangen-karyu reduced the increased serum glucose level in type 2 diabetic mice, and decreased the elevated oxidative and inflammatory biomarkers in the serum and kidney. The increased serum Cr and urea nitrogen levels, which reflect renal dysfunction, and renal structural changes, representing glomerular enlargement, were significantly improved by Kangen-karyu administration. The results of our previous study suggest that Kangen-karyu improves diabetes-induced renal damage through pleiotropic effects on the development of diabetic nephropathy. The utilization of traditional Chinese medicine to treat diabetic nephropathy has received increasing attention due to its wide availability, weak side-effects, and proven therapeutic mechanisms and benefits.

In the present patients, there was an improvement in diabetic nephropathy following the administration of Kangen-karyu for 6 months. Because of the short follow-up period, the effect of the long-term administration of Kangen-karyu on progressive nephropathy remains unknown. However, eGFR was subsequently increased

from 79.2 to 88.2 mL/min/1.73 m^2 at the 6-month follow-up. The serum Cr level decreased from 0.71 to 0.64 mg/dL. In addition, the score using the questionnaire was significantly decreased during the follow-up. We present the therapeutic option using Kangen-karyu to treat renal disease patients with diabetic nephropathy. Interesting findings were also obtained with regard to eGFR: the level of eGFR gradually decreased at 6, 3, 1, and 0 months prior to Kangen-karyu extract administration. The administration of Kangen-karyu for 6 months increased this level, and the rate of variability in eGFR was significantly recovered. There were, however, no improvement in the urinary albumin and protein levels on the administration of Kangen-karyu.

Albuminuria is characterized clinically as an early predictor for progression of diabetic nephropathy (*26*). Proteinuria is the universal finding in progressive renal disease, and viewed as a measure of the severity and determinant for diabetic renal disease progression (*27*), whereas eGFR is estimated using endogenous plasma or serum filtration markers, most commonly Cr (*28,29*). With regard to Cr, we have shown that Cr reacts with hydroxyl radical to quantitatively and non-enzymatically produce 5-hydroxycreatinine, which partially decomposes to methylguanidine, a stronger uremic toxin. These reactions have been reported to occur not only *in vitro* but also *in vivo* (*30*). Moreover, we suggested that the efficacy of Kangen-karyu on diabetic nephropathy in type 2 diabetic *db/db* mice was dependent on several oxidative stress-related parameters and exerted a renoprotective effect (*18-20*). Thus, Kangen-karyu may function as an ameliorator of oxidative stress and show beneficial effects to diabetic nephropathy patients.

Diabetic nephropathy is among the main causes of ESRD. Multiple factors such as metabolic and hemodynamic alterations, oxidative stress, activation of the renin-angiotensin system, and inflammation may interdepend on various levels, causing progressive nephropathy (*31,32*). In the present study, there was an improvement in diabetic nephropathy following the administration of Kangen-karyu extract for 6 months, although we cannot come to a conclusion on the pathway that was affected. In addition, the score using the questionnaire was decreased during the follow-up. Herein, we present a therapeutic option using Kangen-karyu in the early phase of diabetic nephropathy.

In conclusion, we report evidence supporting the use of Kangen-karyu as an adjunctive therapy in patients with diabetic nephropathy corresponding to stage 2 or 3. Kangen-karyu exhibits good efficacy in the treatment of patients with diabetic nephropathy.

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Brief Report

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Change of serum cytokine profiles by propranolol treatment in patients with infantile hemangioma

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SUMMARY Infantile hemangioma is a benign cutaneous tumor, which sometimes rapidly enlarges, causes cosmetic problem, destroys normal tissue, and possibly threatens life. Dye lasers, steroid administration, and watchful waiting had been the treatment options for infantile hemangioma, but in recent years propranolol therapy has become available. The mechanism underlying the action of propranolol, however, is still unknown. We hypothesized that cytokines whose expressions change before and during the treatment are responsible for the efficacy of the drug. This study aims to prove the hypothesis using patients' sera and membrane array. In this study, the serum cytokine concentrations of five patients with infantile hemangioma were measured using membrane array of 20 angiogenic cytokines. We compared them before and during propranolol treatment to identify the cytokines responsible for the effect of propranolol. Signals for angiogenin, epidermal growth factor (EGF), platelet-derived growth factor-BB (PDGF-BB), regulated on activation, normal T-cell expressed and secreted chemokine (RANTES), tissue inhibitor of metalloproteinases 1 (TIMP-1), and tissue inhibitor of metalloproteinases 2 (TIMP-2) were evident in all five cases before treatment. Furthermore, PDGF-BB was the only cytokine of which concentration was decreased during treatment with statistically significant difference. This report is a pilot study with a small number of samples, and further detailed research with increased number of samples is necessary. Nonetheless, our results suggest that PDGF-BB may be involved in the action of propranolol. In addition, its serum concentration can be utilized as a potential marker of the therapeutic effect.

Keywords infantile hemangioma, propranolol, PDGF-BB, cytokine, angiogenesis

1. Introduction

Infantile hemangioma is a benign cutaneous tumor caused by proliferation of vascular endothelial cells. The lesions typically appear on the head or face around 1-2 weeks after birth. They tend to grow until one year (proliferating phase), after which they start to involute (involution phase) (*1*). Larger lesions, however, may cause organ failures (*e.g.* visual impairment, eating disorder, airway obstruction, or heart failure), and sometimes even threaten life. They also become cosmetically problematic, leaving capillary dilation, skin relaxation, skin atrophy, and scarring.

Dye lasers, steroid administration, or watchful waiting had been the most common treatment options for infantile hemangioma (*2*). Since propranolol recently became available, its dramatic effects have been reported. However, the mechanism underlying its action is still unknown. We hypothesized that cytokines whose expressions change before and during the treatment are responsible for the efficacy of the drug. This study aims to prove the hypothesis using patients' sera and membrane array.

2. Materials and Methods

2.1. Clinical assessment and patient material

Serum samples were obtained from five patients with infantile hemangioma (Table 1) before and during propranolol treatment. The age of the patients at the first visit was between three and six months. Three cases had hemangiomas on the head or face, and one case had lesion on the trunk. Case 2 had lesions on both the face and trunk. The clinical subtype according to the depth of soft tissue involvement is as follows: Two cases of

Table 1. Clinical features of infantile hemangioma cases in this study

Case	age (months)	sex	site	type	period of treatment (months)	Dose of propranolol
1	3	M	cheek	mixed	3	3 mg/kg/day
2	3	M	forehead	mixed	5	3 mg/kg/day
			abdomen	mixed		
3	6	F	head	superficial	4	3 mg/kg/day
4	4	F	buttock	superficial	3	3 mg/kg/day
5	4	M	forehead	deep	4	3 mg/kg/day

superficial type, one case of deep type, and two cases of mixed type.

Propranolol administration was initiated at a dosage of 1 mg/kg/day orally divided twice daily, and then increased to 3 mg/kg/day in all cases. Cardiac screenings, such as chest X-ray, electrocardiogram, and 2-dimension echocardiogram, were performed before and during propranolol treatment. The serum samples were obtained within three hours after morning intake of propranolol. Propranolol successfully reduced the coloration and/or size of tumors in all cases at the time of serum sampling (3-5 month after the initiation, Table 1).

This study was approved by the Research Ethics Committee of Wakayama Medical University (No. 2730). Informed consent was obtained according to the Declaration of Helsinki.

2.2. Measurement of serum cytokine levels

Serum levels of 20 cytokines (angiogenin, epidermal growth factor (EGF), epithelial neutrophil activating peptide-78 (ENA-78), basic fibroblast growth factor (bFGF), growth-related oncogene (GRO), interferon-γ (IFN-γ), insulin-like growth factors-1 (IGF-I), interleukin-6 (IL-6), interleukin-8 (IL-8), leptin, monocyte chemotactic protein 1 (MCP-1), platelet-derived growth factor-BB (PDGF-BB), placental growth factor (PIGF), regulated on activation, normal T-cell expressed and secreted chemokine (RANTES), transforming growth factor-β1 (TGF-β1), tissue inhibitor of metalloproteinases 1 (TIMP-1), tissue inhibitor of metalloproteinases 2 (TIMP-2), thrombopoietin, vascular endothelial growth factor (VEGF), and VEGF-D) were measured using human angiogenesis antibody Array-Membrane (Abcam, Cambridge, UK) (*3*). Biotin-conjugated monoclonal antibody of each cytokine was precoated on the membranes.

As reported previously (*3*), an aliquot of serum was reacted with the antibodies on each membrane. The membrane was then incubated with labeled streptavidin, and detection buffer was added. Images were obtained using WSE-6,100 Lumino Graph (ATTO, Tokyo, Japan).

2.3. Statistical analysis

Statistical analysis was carried out with Mann-Whitney's U test for the comparison of medians. p values < 0.05 were considered significant.

3. Results and Discussion

3.1. Clinical features of infantile hemangioma patients in this study

In this study, propranolol was administered to various types of patients with infantile hemangioma (Table 1). The clinical images of all patients are shown in Figure 1. Hemangiomas were present on a three-month-old boy's cheek (mixed type), a three-month-old boy's forehead and abdomen (mixed type), a six-month-old girl's head (superficial type), a four-month-old girl's buttock (superficial type), and a four-month-old boy's forehead (deep type). Propranolol successfully reduced the coloration and/or size of tumors in all cases. No cardiac abnormalities were detected in all five cases before and during propranolol treatment.

3.2. Serum cytokine expression profiles of infantile hemangioma

Cytokine expression profiles in sera of patients with infantile hemangioma were analyzed using commercially available membrane array kits. As shown in Figure 2A, biotin-conjugated monoclonal antibodies of 20 cytokine (angiogenin, EGF, ENA-78, bFGF, GRO, IFN-γ, IGF-I, IL-6, IL-8, Leptin, MCP-1, PDGF-BB, PIGF, RANTES, TGF-β1, TIMP-1, TIMP-2, thrombopoietin, VEGF, and VEGF-D) were precoated on the membrane. Aliquots of serum were added to the membrane, and then allowed to react with the antibodies at room temperature, after which signals were developed by labeled streptavidin. The signal densities of each antigen-specific antibody spot were obtained using 2-D densitometry and then quantified.

Signals for angiogenin, EGF, PDGF-BB, RANTES, TIMP-1, and TIMP-2 were evident in all five cases before treatment, whereas ENA-78, bFGF, GRO, IFN-γ, IGF-I, IL-6, IL-8, PIGF, TGF-β1, thrombopoietin, VEGF, or VEGF-D were absent in all cases (Figure 2B). Fluctuations in expression were observed in the levels of Leptin and MCP-1.

Figure 3 shows percentage changes of cytokine expression during treatment compared with the values before treatment: Changes in the expression of eight cytokines were shown, excluding those that were not expressed both before and during treatment. The levels of EGF, RANTES, TIMP-1, and TIMP-2 tended to

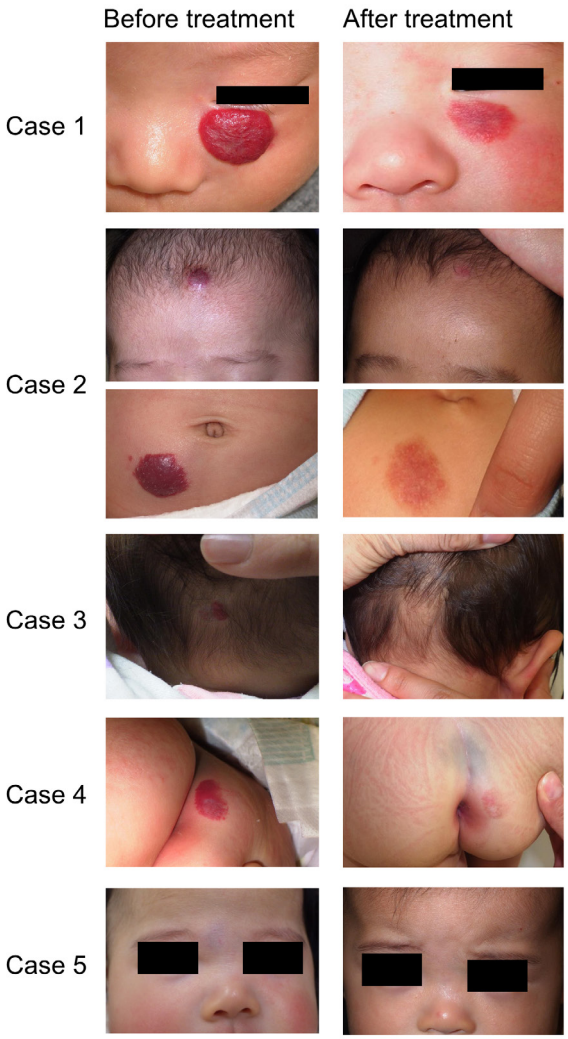

Figure 1. Clinical images of patient with infantile hemangioma included in this study. (left) Cases 1-5 before the treatment with propranolol, **(right)** Cases 1-5 during the propranolol treatment.

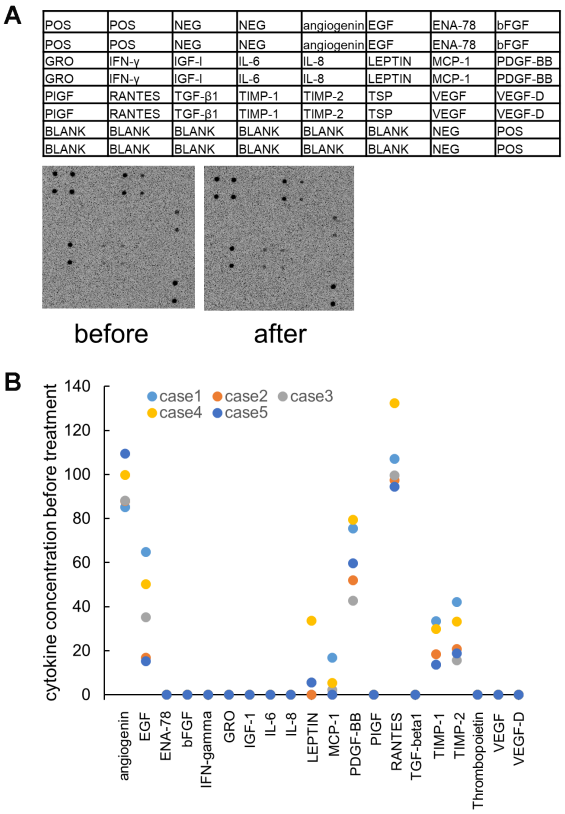

Figure 2. (A) Results of human angiogenesis antibody array. (upper panel) Array maps used in this study, **(lower panel)** Set of representative membranes before and during the propranolol treatment. **(B) Cytokine concentration before the treatment.** The relative expression levels of twenty cytokines measured by the array using sera of five patients with hemangioma before the treatment are shown on the ordinate. The density of positive control in each membrane was set at 100.

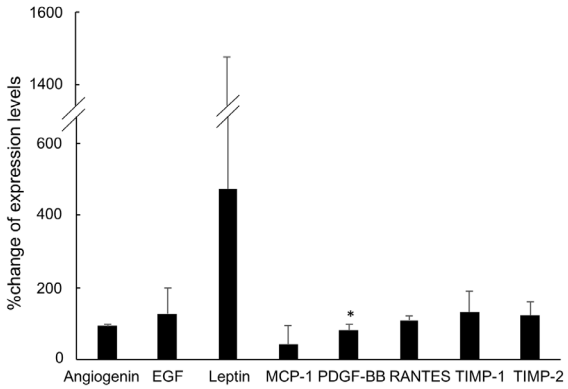

Figure 3. Change of cytokine expression levels measured by the array. The y-axis indicates the change of each cytokine level before and during the propranolol treatment (%). Levels of cytokines unexpressed both before and during the treatment was not show. The bar graphs represent the mean values, and the error bars indicate standard deviations. $^{*}p < 0.05$. When signals were not detected, cytokine level was set at 1 for the statistical analysis.

be increased during the treatment, albeit statistically insignificant. We found significant difference in the decrease of PDGF-BB levels during the treatment (Figure 3, $p < 0.05$).

Various cytokines may participate in the tumorigenesis of infantile hemangioma. The involvement of VEGF, angiopoietin (*4*), TGF-β (*5*), TNF-α, and IL-1 (*6*) have already been described. Taran *et al.* also found that significantly higher TIMP-2 levels were observed in infantile hemangioma tissues at the mRNA level (*7*). Furthermore, serum cytokine levels have also been evaluated for their potential as tumor markers in this disease. For example, serum levels of VEGF were increased in infantile hemangioma at the proliferative or involution phase. In addition, serum MCP-1 and macrophage inflammatory protein-1β (MIP-1β) may be utilized as the marker of regression (*8*). Similarly, propranolol treatment for proliferating infantile hemangioma can reduce the peripheral serum and urinary concentrations of epidermal growth factor-like protein 7 (EGFL7) levels (*9*).

In this study, we aimed to determine multiple serum cytokines at the same time using array experiments before and during administration of propranolol. Before the treatment, signals for angiogenin, EGF, PDGF-

BB, RANTES, TIMP-1, and TIMP-2 were evident in all five cases. The levels of Leptin and MCP-1 were not consistent among five patients. The other cytokines were not expressed in all cases. Consistently, Jiang *et al.* reported that angiogenin levels are increased in the sera of proliferative hemangioma (*10*), and suggested them as the biomarker. Yamashita *et al.* also described that serum RANTES levels were significantly down-regulated in patients with progressive infantile hemangioma than those in non-progressive hemangioma group (*3*).

The second novel finding is that PDGF-BB was the only cytokine whose concentration was changed during treatment with statistically significant difference. PDGF is an angiogenic factor produced by various cell types including monocytes and macrophages, and is known to promote endothelial proliferation. Roach *et al.* reported that PDGF-BB inhibits differentiation into adipocytes in infantile hemangiomas and suggested the cytokine as a negative regulator of hemangioma regression (*11*). On the other hand, no relationship between propranolol and PDGF-BB has yet been reported.

Taken together, our results suggested two possibilities: (1) Propranolol suppresses PDGF-BB production from monocytes and macrophages, which promotes regression of hemangioma. (2) The total amount of PDGF produced from decreased number of tumor cells was reduced due to administration of propranolol. Considering the first possibility, PDGF-BB may be involved in the action of propranolol. Furthermore, its serum concentration can be utilized as a marker of therapeutic effect. This report is a pilot study with a small number of samples, and there is limitation: In babies, growth may dramatically affect serum cytokine levels. However, control group without hemangioma for comparing the baseline levels and the subsequent cytokine changes as babies grow up were not included in this study. Further detailed research with larger number of samples and control patients (*e.g.* patients without hemangioma or propranolol administration) is necessary.

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Case Report

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Brain abscess in patients with chronic kidney disease: A case-based approach to management in resource-limited settings

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SUMMARY The management of patients with brain abscess poses a significant challenge to clinicians in patients with chronic kidney disease. Obtaining a biopsy sample from the affected area is the mainstay in the diagnosis, but it is often unavailable. In most cases, therapy is guided by clinical findings and imaging alone. We discuss three cases of brain abscess- each with a different scenario and discuss the issues faced in management. The first case was a 32-year-old post-renal transplant male patient with a brain abscess due to dematiaceous fungi and was treated with amphotericin. The second case was a 42-year-old female patient with stage 5 chronic kidney disease on maintenance hemodialysis who presented with a brain abscess due to suspected fungal infection based on imaging findings and was managed with antibiotics and voriconazole. The third case was a 42-year-old post-renal transplant male patient who presented with a brain abscess due to nocardiosis and was managed with cotrimoxazole, meropenem and linezolid. We also summarize the approach to the management of brain abscess in resource-limited settings.

Keywords Fungal, dematiaceous, *Cladophialophora*, nocardiosis

1. Introduction

Brain abscess is a focal, intracerebral infection that begins as a localized area of inflammation and develops into a collection of pus surrounded by a rim (*1*). A total of 8% of intracranial mass turn out to be brain abscess in developing countries compared to 1-2% in developed countries (*2*). It can be a result of direct contiguous or hematogenous spread or a complication of trauma or neurosurgical procedures. Identifying the cause and source of infection may help select antibiotic therapy for the patients. The definitive diagnosis of brain abscess requires invasive techniques and therefore, more often than not, diagnosis is established based on clinical findings and imaging. For example, multiple abscesses on imaging in middle cerebral artery distribution points towards bacteremia from another source, abscess in frontal lobe indicate a contiguous dental or sinus source and abscess in temporal lobe/cerebellum indicates an otogenic source. Similarly, in patients with immunosuppression, tuberculosis, toxoplasmosis, nocardiosis and fungal etiologies should be kept in the differential. There are no definite guidelines on the management of brain abscess in resource-limited settings. We report three cases of brain abscess and discuss the challenges and approach to the management of such cases in resource-limited settings.

2. Case report

2.1. Case 1

A 32-year-old male patient from Bihar (a state in northern part of India) presented one year after renal transplant with two episodes of sudden onset tonic-clonic posturing followed by clonic movements of all four limbs. He was admitted to a local hospital where he was stabilized and given intravenous antimicrobials (details unknown). The patient was referred to our centre for further management. At presentation, he was conscious and co-operative without any focal deficits on detailed neurological examination. A magnetic resonance imaging (MRI) was done, which showed a lobulated lesion of 2.7 × 2.5 × 2.5 cm showing restricted

diffusion in the right anterior and basi-frontal lobe. He underwent right frontal craniotomy and excision of the right basi-frontal abscess. Histopathological examination showed pigmented septate hyphae with acute angle branching (Figure 1). He was started on liposomal amphotericin B (3 mg/kg) for the fungal brain abscess. He was incidentally detected to be positive for anti-hepatitis C antibodies. Hepatitis C virus (HCV) genotype was 1a with ribonucleic acid (RNA) levels of 38,237 IU/mL. He was started on sofosbuvir and ledipasvir for this. He was discharged in a stable condition and was advised to complete four more weeks of amphotericin B at a hospital near his home.

2.2. Case 2

A 42-year-old female patient from Bihar, a known case of stage 5 chronic kidney disease on maintenance hemodialysis for the last one year presented with complaints of intermittent high-grade fever with chills and rigours for one month. It was associated with a diffuse, throbbing headache of severe intensity and frequent episodes of non-bilious, non-projectile and non-blood stained vomiting. The patient developed insidious onset progressive weakness in the right half of the body for the past seven days, which started in the lower limbs and progressed to involve the entire right half of the body. She had a history of provoked thrombosis in internal jugular vein due to multiple double-lumen jugular catheter insertions. She also had a history of contact with a tuberculosis patient (husband, diagnosed one year back, completed treatment). On examination, she was conscious and oriented. She had increased tone and power of 0/5 grading in both the right upper and lower limbs. Her right biceps, triceps, supinator and ankle jerks were exaggerated. Plantar on the right side was extensor. Examination in the left half of the body was essentially normal. Sensory exam was normal. Rest of the systemic examination was essentially normal. Non-contrast MRI revealed a well-defined lesion with crenated margins and intra-cavitary projections measuring 2.4 × 1.6 × 2 cm having T2 hypointense rim with a hyperintense core in the periventricular location on the left side with extensive perilesional oedema (Figure 2). The features were consistent with a fungal abscess. She was empirically started on ceftriaxone, metronidazole, linezolid, voriconazole and steroids. Cerebrospinal fluid examination (CSF) showed neutrophilic predominant pleocytosis. CSF cryptococcal antigen was negative, but galactomannan was raised with an optical density value of 2.08. Transthoracic echocardiography was normal. Positron emission tomography (PET) scan was done, which showed isolated uptake in the abscess region only. Anti-tubercular drugs were not started because of isolated brain abscess, no meningeal enhancement and no uptake in any other part of the body. The patient started improving gradually and power improved to 3/5 in right upper limb and 2/5 in the right lower limb after two weeks of therapy. Non-contrast computed tomography (NCCT) head was done on follow up, which showed a significant reduction in the size of abscess and oedema. Brain biopsy was deferred from the neurosurgery side because of improving power. The patient developed in-hospital thrombocytopenia after which linezolid was continued. The patient was discharged on ceftriaxone, metronidazole and voriconazole after three weeks of intravenous therapy in a stable condition and was advised to continue these drugs from a local hospital. She was advised to follow up after four weeks of therapy but was lost to follow up.

2.3. Case 3

A 42-years-old male presented with a history of fall while after which he complained of weakness in the

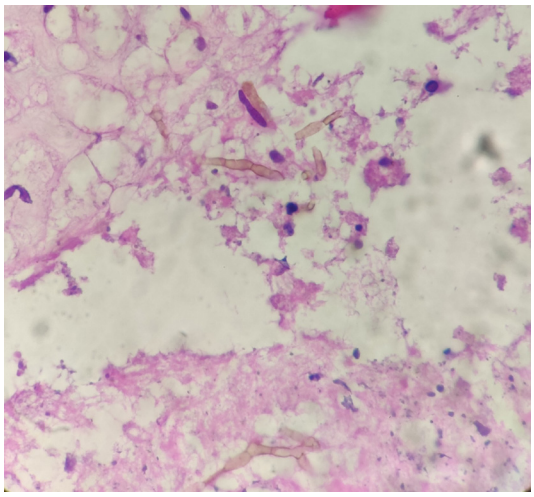

Figure 1. Histopathological examination showing pigmented narrow septate hyphae with acute-angled branching.

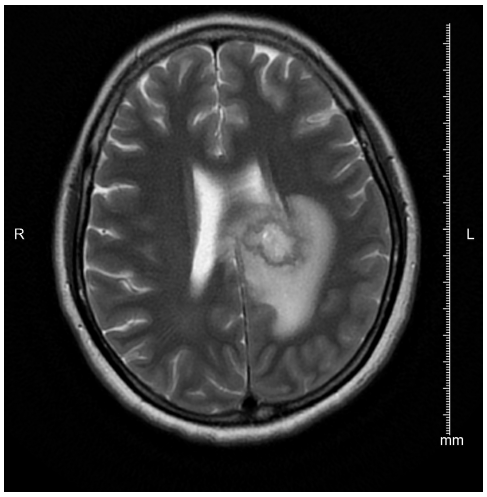

Figure 2. T2 weighted axial image showing a well-defined crenated lesion with a T2 hypointense rim in a left periventricular location with extensive perilesional oedema suggestive of a fungal abscess.

right half of the body and deviation of angle of the mouth towards the left side. He also had a history of low-grade fever and generalized weakness along with loss of appetite and loss of weight for one month. He was a diagnosed case of type 2 diabetes mellitus and hypertension with stage 5 chronic kidney disease and underwent a heterotopic renal transplantation (unrelated matched donor) one year back. Examination revealed features of right hemiplegia with right-sided upper motor neuron facial weakness. A NCCT of the head was suggestive of a left-sided brain abscess. A brain biopsy could not be arranged for him. On re-examination, soft, fluctuant swelling of size 3 × 3 cm with poorly defined margins was noted over the right 9th and 10th intercostal spaces in the mid-axillary line. Contrast-enhanced CT of the abdomen revealed a hypodense collection in the right sub-diaphragmatic and the right sub-hepatic space communicating with the superficial collection. Gram stain of the aspirated pus from the collection revealed gram-positive thin filamentous branching structures that were acid-fast on modified Ziehl Neelsen stain (Figure 3). With a diagnosis of nocardiosis, he was started on cotrimoxazole, meropenem and linezolid and his immunosuppression were reduced. He improved significantly on treatment.

3. Discussion

All three cases of brain abscess highlighted different challenges in diagnosis and management. The first case was relatively stable as he had already undergone a renal transplant, received some empirical antifungals and had no neurological deficits at presentation. The availability of brain abscess in resource-limited settings is a matter of concern. Ours was a referral care centre where the facility of brain biopsy is available, but the procedure is often delayed because of the heavy burden of such cases. Antimicrobial therapy in this patient could be deferred until a brain biopsy was performed because of his stable condition. A definitive diagnosis of fungal brain abscess due to fungal aetiology was established.

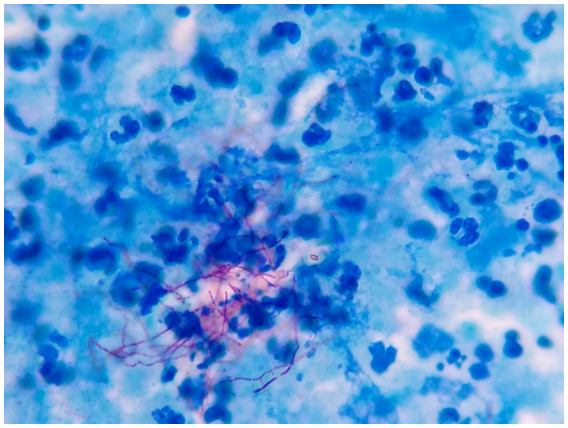

Figure 3. Modified Ziehl Neelsen stain showing long branched filamentous acid-fast bacilli suggestive of nocardiosis.

The likely organism was a dematiaceous fungus because of the pigmented nature of hyphae, and he was started on amphotericin. Voriconazole was avoided in this patient because of possible drug-drug interactions with the immunosuppressants.

In the second patient, the patient was on alternative day maintenance hemodialysis, and since brain biopsy could not be urgently arranged for her, she was started on empiric antimicrobials. Although MRI findings and CSF galactomannan pointed towards a possible fungal aetiology, the patient was initiated on both anti-bacterial and antifungals. Voriconazole was chosen as the anti-fungal of choice as amphotericin was avoided because of its nephrotoxicity.

Fungal abscesses are less common compared to other causes but are very difficult to manage. The optimal therapy for fungal brain abscesses usually requires a combined medical and surgical approach; surgery involves either excision or drainage of the abscess. The therapy in fungal brain abscess is guided by the type of fungus involved. In cryptococcal brain abscess, basal ganglia are commonly involved. CSF shows mild cellularity (predominantly lymphocytic) and raised protein. CSF may be positive for India ink, cryptococcal antigen or culture. Liposomal amphotericin B, along with flucytosine, is used for induction therapy followed by fluconazole for consolidation and maintenance therapy. If the initial KOH or biopsy shows septate hyphae (*Aspergillus* spp., *Scedosporium* spp., *Fusarium* spp., dematiaceous fungi including *Cladophialophora bantiana*), the patients should be started on voriconazole. On the other hand, if initial microscopy shows hyaline aseptate hyphae (mucormycosis), liposomal amphotericin B should be used at high doses (at least 5 mg/kg once daily) with close monitoring of renal function and electrolytes. After stabilization of the initial condition, step down therapy with oral posaconazole can be tried.

In the third patient, the patient was post-transplant, and the microbiological diagnosis could be established by sampling from an accessible site (superficial skin collection). Brain biopsy was, therefore, not needed in this case, and with a diagnosis of disseminated nocardiosis, the patient was adequately treated. Diagnostic and therapeutic aspiration is essential in patients with nocardial brain abscess, but aspiration or biopsy of other accessible sites may be tried if available. Modified acid-fast stain shows branched filamentous bacilli. Isolated nocardial brain abscess is treated with initial inpatient parenteral therapy consisting of cotrimoxazole (15 mg/kg *i.v.* of the trimethoprim component per day in three or four divided doses) along with a carbapenem (imipenem or meropenem). After the initial six weeks of parenteral therapy and improving clinical and imaging profile, patients can be shifted on oral therapy (cotrimoxazole +/– amoxicillin/clavulanic acid for one year at least). In patients with brain abscess

Table 1. Radiological features of Brain abscess

MRI features	Pyogenic	Tubercular	Fungal	Toxoplasmosis
Morphology	T1 hypointense, T2 hyperintense, smooth/lobulated thin-walled margins	T1hypointense, T2 hyperintense, smooth/lobulated thin-walled margins	T1 isointense, T2 hypointense rim, crenated margins, Intra-cavitary projections not showing contrast enhancement	Eccentric target sign on post-contrast images, concentric target appearance on T2-weighted images, multiple abscesses in varying stages
Diffusion-weighted imaging	Central diffusion restriction	Central diffusion restriction	Diffusion restriction in-wall and intra-cavitary projections only. No central diffusion restriction	No central diffusion restriction
MR Spectroscopy	Amino acid peak (0.9 ppm)	Lipid lactate peak (1.3ppm)	Trehalose peak (3.6-3.8 ppm)	Non-specific
Associated features		Infarcts in tubercular vasculitis	Infarcts in angioinvasive fungal infection	Perilesional haemorrhage Basal ganglia location

*MRI, magnetic resonance imaging; MR, magnetic resonance; ppm, parts per million.

and multi-organ involvement (*i.e.*, at least one other site), initial inpatient parenteral therapy can also be supplemented with a third drug, amikacin.

Patients with brain abscess usually present with fever, headache, and focal neurologic deficits. However, this classical presentation is observed in less than half of the patients. In immunocompromised patients, the clinical symptoms are even more non-specific due to diminished inflammatory response in these patients (*3*). In the presence of focal symptoms & signs or features suggestive of increased intracranial pressure, lumbar puncture should be performed only after imaging. MRI with Magnetic Resonant Spectroscopy (MRS) is the preferred diagnostic modality. The imaging finding of brain abscess due to different etiologies has been summarized in Table 1. In those patients where MRI cannot be done, computed tomography can be used for diagnosis.

Stereotactic brain biopsy/aspiration should ideally be done in all lesions greater than 2.5 cm (*4*). In resource-limited overburdened settings, it is challenging to obtain brain biopsy in most cases. The treatment of brain abscess without diagnosis has to be empiric in such cases taking clues from radiological findings and other supportive investigations. The empiric regimen is further complicated in patients with renal impairment because of nephrotoxicity of certain antibiotics, need for dosing modifications and possible drug-drug interactions.

The approach to empirical management of brain abscess in resource-limited settings is summarized below based on the available literature and our experience. The empiric management of patients with brain abscess from contiguous sources should consist of ceftriaxone and metronidazole (*5,6*). This would cover for the most common organisms responsible for brain abscess in Indian studies, *i.e. Streptococcus* spp. and anaerobic organisms (*7,8*). Vancomycin may be added if there is a history of trauma or neurosurgery as these brain abscesses are often caused by methicillin-resistant

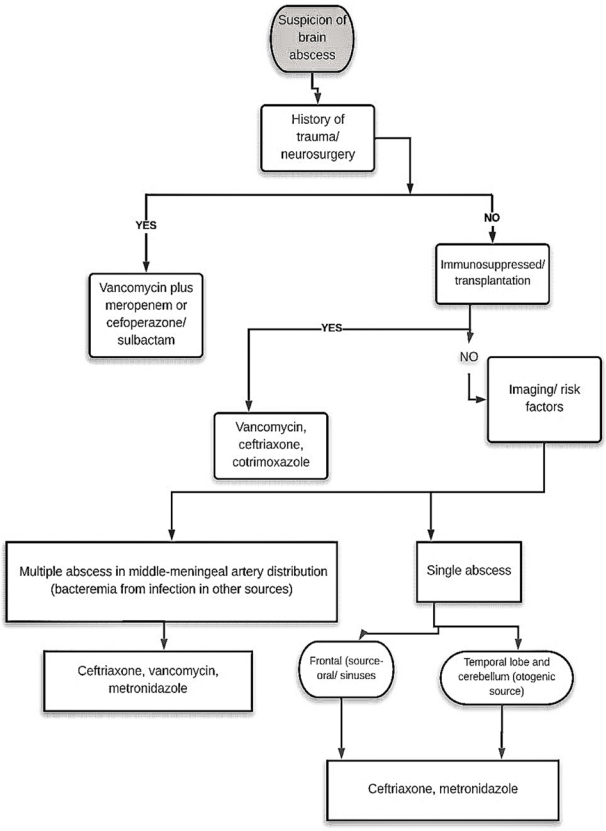

Figure 4. Flow chart showing approach to empirical management of brain abscess.

Staphylococcus aureus (MRSA). In these patients, meropenem or sulbactam containing combinations should replace ceftriaxone and metronidazole to cover for the multi-drug resistant gram-negative organisms, including *Acinetobacter* spp.

In patients with immunosuppression (especially HIV/AIDS and solid organ transplant), cotrimoxazole should be added to cover for toxoplasmosis. The empirical management has been further summarized in a flow chart (Figure 4). The minimum duration of

treatment is six to eight weeks. Duration of therapy beyond that period is primarily individualized depending upon response. Contrast-enhanced CT Scans or MRI can be repeated every two weeks to see the response to treatment. The therapy has to be eventually narrowed down based on the microbiological diagnosis. In patients living with HIV/AIDS (PLHA) having low CD4 count ($\leq 100/mm^3$), toxoplasmosis is a strong differential (*9*). Positive toxoplasmosis serology (IgG) positive and response to cotrimoxazole point towards the diagnosis of toxoplasmosis. Tuberculosis may be one of the most common causes of brain abscess in India, according to some earlier reports (*2*). Characteristic CSF picture (high protein, increased cellular response with lymphocytic predominance and higher values of adenosine deaminase) with microbiological evidence by cartridge-based nucleic acid amplification test or liquid culture from CSF is used for making the final diagnosis. The patients should be treated with at least nine months of anti-tubercular therapy with at-least four weeks of steroids (*10-12*).

Brain abscess is an important cause of morbidity and mortality in developing countries like India. Besides surgical management, prompt initiation of empirical or microbial directed therapy should be initiated. There is an urgent need for the development of evidence-informed local guidelines based on the epidemiology.

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Case Report

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Visual improvement in a patient with paracentral acute middle maculopathy treated with prostaglandin E1

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SUMMARY The authors present the use of prostaglandin E1 (PGE1) for the treatment of an acute paracentral acute middle maculopathy (PAMM). A 78-year-old white female was seen with a sudden loss of vision in her left eye (OS) to 20/200 noted upon awakening. The right eye (OD) saw 20/20. A complete eye exam was done and an ocular coherent tomography revealed retinal thickening and a whitening of the inner nuclear layer in the area of the macula OS. A diagnosis of PAMM in the OS was made. Treatment was immediately started with 70 μg of PGE1 administered over 1.5 hours in the form of a skin cream. A volume of 3.5 cc of skin cream was applied in divided doses to the inner surface of the forearm, rubbed into the skin and allowed to dry. The same 70 μg of PGE1 in 3.5 cc of skin cream was repeated once the next morning. The patient began to see better the second day of treatment with a final visual acuity of 20/20. The OD was unchanged. After 14 months she was stable with no further treatment. PAMM is an ischemic process of the inner retina. PGE1, a potent vasodilator of the microcirculation, when given immediately seemed to be useful in restoring vision in this form of retinal ischemia. Treatment was immediately started with PGE1 in the form of a skin cream with visual improvement. The authors normally use PGE1 intravenously for acute ocular ischemia and would have preferred that here. Intravenous PGE1 was not available and was substituted with the skin cream of PGE1 that worked well for the patient.

Keywords paracentral acute middle maculopathy, acute retinal ischemia, prostaglandin E1

1. Introduction

Paracentral acute middle maculopathy (PAMM) is the spectral domain-optical coherent tomography (SD-OCT) finding of a hyperreflective band involving the inner nuclear layer (INL) resulting from ischemia at the level of the deep vascular complex which is made up of the intermediate and deep retinal capillary plexus of the retina (*1*). Perivenular retinal whitening can be seen in the macula. The final visual acuity can vary and there is no known therapy for the ischemic lesion in the retina.

Prostaglandin E1 (PGE1), a powerful vasodilator of the microcirculation, improves ocular blood flow in the presence of peripheral vascular disease and diabetes (*2*). In cases of acuity ocular ischemia, intravenous (*i.v.*) PGE1 given 1 μg/kg leads to visual improvement. In nonarteritic anterior ischemic optic neuropathy (*3*) and nonarteritic posterior ischemic optic neuropathy (*4,5*), *i.v.* PGE1 with steroids was shown to improve the visual acuity as well as the ocular and retrobulbar blood flow. An acute branch retinal arterial occlusion has also been successfully treated (*6*). In this case report PGE1 was not immediately available for *i.v.* administration but only in the form of a skin cream. Since treatment could not be delayed, PGE1 was given in the form of a skin cream in the same dosage that would be used *i.v.* This appeared to be successful which will be explained in the paper.

2. Case Report

A 78-year-old white female was seen in the morning of December 12, 2018 with a sudden loss of vision in her left eye (OS) noted upon awakening. She was under treatment for ocular hypertension and anemia. She was 20/20 in her right eye (OD) and 20/200 in the OS measured using the Early Treatment Diabetic Retinopathy Study letter scoring. There was a white inferior parafoveal semicircular lesion 1 disc diameter in width OS. SD-OCT revealed retinal thickening and a hyperreflective band in the INL extending into the inner retina (Figure 1 panel A). A diagnosis of PAMM in the OS was made. After explaining the urgency of the situation, written informed consent was obtained for

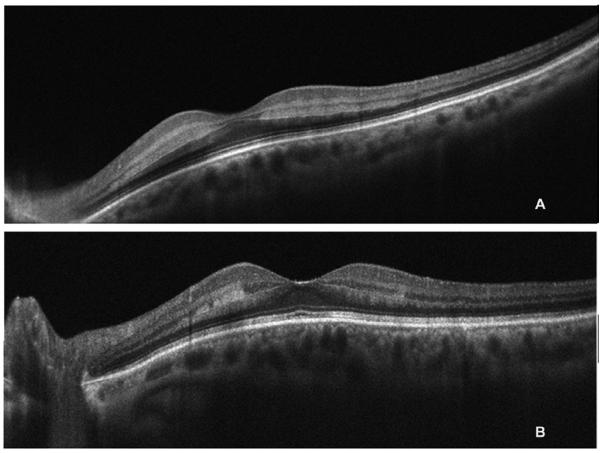

Figure 1. Spectral domain-ocular coherent tomography (SD-OCT) of the left eye of the patient. (panel A) before treatment revealed retinal thickening and a whitening of the inner nuclear layer (INL); **(panel B)** 9 days after treatment showed decreased retinal thickening with a reduction of the white lesion of the INL.

treatment and for the collection of clinical information for archival and research purposes. The patient weighed 70 kg. The normal dosage of *i.v*. PGE1 to treat acute ocular ischemia is 1 µg/kg or 70 µg in this case. IV PGE1 was not available so treatment was immediately started with 70 µg of PGE1 administered over 1.5 hours in the form of a skin cream. A quantity of 3.5 cc of skin cream was applied in divided doses to the inner surface of the forearm, rubbed into the skin and allowed to dry. The same 70 µg of PGE1 in 3.5 cc of skin cream was repeated once the next morning. The patient noted visual improvement during the second day of treatment but was seen 9 days later on December 21, 2018. The acuity of the OD was stable and in the OS improved to 20/20. At that time the SD-OCT showed decreased retinal thickening with a reduction of the white lesion of the INL. (Figure 1 panel B). No further treatment with PGE1 was done. She was last seen on February 12, 2020 with 20/20 OS.

3. Discussion

The patient was seen with an acute ischemic episode of PAMM in the OS. Treatment was immediately started with PGE1 in the form of a skin cream with visual improvement. The authors normally use *i.v*. PGE1 for acute ocular ischemia (*3-6*) and would have preferred that here. With *i.v*. treatment in acute ocular ischemia, the authors are more certain to achieve clinically significant therapeutic levels. IV PGE1 was not available and was substituted with a topical skin cream of PGE1 which worked well for the patient. The authors however still recommend *i.v*. treatment in this form of acute ocular ischemia.

It is also important to note that treatment was started immediately and that this case of PAMM was treated only once over 2 days. Good visual acuity was maintained for 14 months without further treatments.

Conflict of Interest:

The author, RDS, has a financial interest in the prostaglandin E1 skin cream mentioned in the case report. MN has no financial interest.

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Commentary

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Treat 2019 novel coronavirus (COVID-19) with IL-6 inhibitor: Are we already that far?

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SUMMARY The world is in the midst of the coronavirus disease 2019 (COVID-19) pandemic. Interleukin 6 (IL-6) inhibitor (tocilizumab) had been suggested for the treatment of acute respiratory distress syndrome (ARDS) patients based on the concept of "cytokine storm" in COVID-19. However, we still lack reliable studies to verify "cytokine storm" in COVID-19 pneumonia. Furthermore, IL-6 inhibitor has potential hazards of inducing infectious diseases. The efficacy of IL-6 monoclonal antibody-directed therapy remains to be fully evaluated.

Keywords coronavirus, cytokine storm, pneumonia, tocilizumab

The pandemic of coronavirus disease 2019 (COVID-19) is a highly contagious respiratory disease resulting from a life-threatening novel coronavirus, SARS-CoV-2. Since its outbreak in Wuhan, China in December 2019, it has now spread to 213 countries, areas or territories, causing a severe public health burden (*1*). There is an urgent need for effective treatment. Current focus has been on the development of novel therapeutics, including antiviral drugs and vaccines. Remdesivir, which has been recognized as a promising antiviral drug against a wide array of RNA virus infections, has been revealed to be highly effective in the control of COVID-19 infection in vitro (*2*). A compassionate-use cohort suggests that remdesivir may have clinical benefit in patients with severe COVID-19 (*3*). However, there are still no randomized, placebo-controlled trials of antiviral therapy for COVID-19.

The most common clinical presentation of COVID-19 infection is fever, fatigue, and dry cough. Notably, 11-53% of pneumonia patients developed acute respiratory distress syndrome (ARDS) in different cohorts, and respiratory failure from ARDS is the leading cause of mortality (*4-7*). The pathophysiology of COVID-19 has not been completely understood. In other respiratory infections like influenza and Middle East respiratory syndrome coronavirus (MERS-CoV), severe "cytokine storm", with markedly higher levels of proinflammatory cytokines including interferons (IFNs), tumor necrosis factors (TNFs), interleukins (ILs), and chemokines, has been reported in severe hospitalized patients (*8,9*). Following previous experiences in severe acute respiratory syndrome (SARS) infected subjects, the Chinese National Health Commission (NHC) has recommended steroids as standard therapy for severe COVID-19 pneumonia although their role remains controversial. A new approach of targeting "cytokine storm" in severe COVID-19 patients emerged from clinical experiences in China. Recently, interleukin 6 receptor (IL-6R) monoclonal antibody (tocilizumab)-directed COVID-19 therapy has been used in a clinical trial in China (No.ChiCTR2000029765), and it has been incorporated into COVID-19 management guidelines generated in China based on the concept of "cytokine storm" in COVID-19 pneumonia (*10*). However, the role of interleukin 6 (IL-6) in COVID-19 is still unknown, and the efficacy of IL-6 monoclonal antibody-directed therapy remains to be fully evaluated.

One study that was conducted in the early epidemic period of COVID-19, reported that patients requiring ICU admission had higher concentrations of proinflammatory cytokines (GCSF, IP10, MCP1, MIP1A, and TNFα) than did those not requiring ICU admission, suggesting that the cytokine storm was associated with disease severity. However, they also found increased secretion of T-helper-2 (Th2) cytokines (*e.g.* IL-4 and IL-10) that suppress inflammation, which couldn't be elucidated well (*11*). Some recent studies also show that IL-6 level increased in severe patients (*7,12,13*). Serum viral load detected by RT-PCR is closely correlated with IL-6 level in critically ill COVID-19 patients (*14*). These reports provide valuable information on the immunopathology of COVID-19. However, it is important to bear in mind that it is hard to make a conclusion of "cytokine storm" based solely

on elevated IL-6 level. Importantly, PCR positivity does not necessarily indicate viable virus, and additional data are needed to better understand the infectious period of COVID-19 and implications for treatment and infection control. A preprint article reported high levels of three cytokines (CXCL10, CCL7 and IL-1 receptor antagonist), rather than IL-6, were associated with increased viral load, loss of lung function, lung injury and a fatal outcome in clinically moderate and severe COVID-19 patients (*15*). Furthermore, high levels of a particular cytokine that strongly correlates with disease activity do not necessarily constitute causality. It is presently unclear if elevated IL-6 levels are detrimental or beneficial in COVID-19 pneumonia. In experimental model systems, IL-6 can either suppress or facilitate viral replication (*16*), so studies on COVID-19 are urgently needed. Timing of anti-IL-6R, if too early might adversely affect viral clearance, which needs to be assessed in trials (*17*). Some scientists may refer to the postmortem pathology findings of COVID-19 as one of evidence for cytokine storm (*18*). In this study, they reported there was an increased concentration of highly proinflammatory CCR4+CCR6+ Th-17 in CD4 T cells which implied that overactivation of T cells accounts for the severe immune injury. However, they did not mention the time point when the peripheral blood was prepared for flow cytometric analysis. If the blood was taken right before the patient's death rather than before the onset of ARDS, using the result to account for immune injury would be unpersuasive. In conclusion, the available studies may be helpful for encouraging further, more in-depth research, but they should not be regarded as definitive evidence of tocilizumab therapy.

Indeed, "cytokine storm" had been reported in many diseases including infections and rheumatic diseases. Macrophage activation syndrome (MAS), for example, refers to acute overwhelming inflammation caused by a "cytokine storm" (*19*). The fact that biologic agents neutralizing IL-6 and IL-1 are highly effective treatments for sJIA – a rheumatic disease strongly associated with MAS – raised hopes that the same strategies would be successful to prevent cytokine storm in inflammation. However, we should be very cautious about these biologics since they have potential hazards of inducing infectious diseases.

In conclusion, we now have a better understanding of the IL-6 change and cytokine storm in COVID-19 pneumonia, but more data are needed on treatment options that improve survival.

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Commentary

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Ultra-low price of generic agents in China may weaken patients' drug recognition and compliance

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SUMMARY Drug quantity purchase in China has reshaped the pattern of drug market with lower price of generic drugs and relatively higher price of original brand-name drugs. However, most Chinese people do not trust the safety and efficacy of the generic drugs that sold at a very low price. Ultra-low price of generic agents may weaken patients' drug recognition and compliance, and affect the implementation of drug quantity purchase policy.

Keywords Drug quantity purchase, drug price, brand recognition, medication compliance

Under the spotlight, China's drug price negotiation was finally settled at the end of 2019 (*1,2*). It is encouraging to see the results of drug pricing negotiations help patients reduce the economic burden. Drug quantity purchase policy in China is executing, and is to expand from its major 4+7 cities to all over the country. The intention of the quantity purchase policy is to improve the medication accessibility for ordinary peoples, meanwhile to lower national healthcare costs and to contain the too fast rising medical expense. The generic drug advocation now is in full swing in this country since China Food and Drug Administration (CFDA) published *Opinions on Evaluation of Consistency of Quality and Efficacy of Generic Drugs* in 2016. CFDA originally scheduled to complete quality and efficacy consistency evaluation of 289 generic drugs with original brand name drugs in late 2018, however, since the workload is large and cost is high, it is an impossible mission.

Up to now, a few generic agents have passed the consistency evaluation of the quality and efficacy with original branded drugs, and are qualified for quantity purchase in the whole country. The centralized drugs quantity purchase project has significantly reduced the prices of drugs, which reshaped the pattern of drug market with lower price of generic drugs and relatively higher price of original branded drugs. For example, the price of original brand-name glimepiride (Amaryl, Sanofi Aventis) is 64 CNY for 2 mg × 15 tablets in China market, while the price is as low as only 0.0198 CNY (about 0.00283 USD) for a piece of generic counterpart tablet.

In order to push the implementation of policy, the National Healthcare Security Administration (NHSA) requires that prescription priority should be given to generic drugs in hospitals of all levels. Will ultra-low price of generic agents obtain satisfaction from all levels of people? Based on our pilot interview with outpatients, we believe the situation perhaps is not optimistic.

During the interview, most patients claim that they sincerely believe in "the higher the price, the better the quality of the merchandise", "you get what you pay for". Therefore they do not trust the safety and efficacy of generic drugs that are sold at a very low price. Many patients express that they are rich and affordable to everything, not to mention "life-saving medicine". In their opinions, price differences are usually used as a proxy for differences in quality. Generic drugs with too low price seem to be impossible to be with good quality. "Can you image that a drug as cheap as cabbage could cure your disease?" Some patients made such comments. The deep-rooted reason is related to consumer psychology. A few Chinese people worship and have blind faith in things foreign (*3,4*). Furthermore, the most leisured class prefer to use the high price original brand-name drug just out of show-off psychology (*5*). Facing disease treatment and medicine choice, even low-income Chinese parents would choose original branded drugs for their sick baby and child in spite of emptying their purse (*6*). In fact, a strong brand loyalty has been built among ordinary Chinese people. It is difficulty to change the current situation.

There is still a long way to go to persuade patients to use extremely cheap generic drugs. In the generic

drug advocate in China, we suggest that NHSA should guarantee appropriate profit for those generics instead of just blindly lowering the price of drugs. It is a truth universally acknowledged that appropriate profit is better than small profit and/or no profit to guarantee the quality of drugs. As the Chinese saying goes, "Cheap goods are not good, while good goods are not cheap". Generic drug with appropriate price might give patients a little more confidence, enhance patients' drug recognition and compliance than those dirt cheap counterparts.

Acknowledgements

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Letter

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Facts and reflections on COVID-19 and anti-hypertensives drugs

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SUMMARY Based on some publications that associate SARS-CoV-2 infection with the use of anti-hypertensive drug groups such as angiotensin-converting-enzyme inhibitors (*e.g*. enalapril) or angiotensin II receptor blockers (*e.g*. losartan), many patients from South America, Central America or Spain, have stopped or intend to interrupt their treatments with these drugs. Hence, it may exist ominous consequences due to this drop out. For this reason, it is necessary to quickly warn about this situation and the risks associated with it.

Keywords SARS-CoV-2, COVID-19, Anti-hypertensive drugs, ACE, ATII-RB

We would like to contribute with some reflections regarding the association of SARS-CoV-2 infection, hypertension and antihypertensive drugs.

It is a fact that SARS-CoV-2 enters to the cells by the same route as the SARS-CoV that is, through a binding with the angiotensin II converting enzyme (ACE-II) (*1-4*). Cells with large amounts of ACE-II are present in the salivary glands of the mouth, along all the respiratory tract, epithelial cells of the lung, intestine, kidney, and blood vessels (*5*). ACE-II expression increases when patients are treated with drugs that inhibits this enzyme or with angiotensin II receptor blockers (ATII-RB) (*5*). This is due to the properties of both groups to significantly increase mRNA expression for ACE, increasing in this way the amount of enzyme available in the cells (*6*). Consequently, some authors hypothesize that ACE-II or ATII-RB groups, increasing the expression of ACE II in some cells (particularly alveoli) may rise the risk of infection with SARS-CoV-2 (*4-6*). Likewise, 2 studies in patients with confirmed coronavirus disease 2019 (COVID-19), showed that up to 30% of people affected had chronic arterial hypertension disease hypothesizing that hypertension may favor infection or aggravation of COVID-19 symptoms (*7-9*).

From these data we can conclude that there is a "potential/theoretical" risk that drugs from the ACE inhibitors group (such as enalapril, ramipril, captopril, and lisinopril) and ATII-RB (such as losartan, candesartan, valsartan, ibersartan, and telmisartan) may favor the internalization of COVID-19 inside the cell. However, we must recognize that the scientific foundation of this theory is very weak to date (*10*). About the reports associating hypertension with COVID-infection, it could be said that although 23 to 30% of the patients hospitalized because of the virus, had hypertension, it should be recognized that the prevalence of hypertension in adults is around that same percentage; so, this epidemiological association is clearly objectionable.

The abrupt drop-out of anti-hypertensive treatment could be associated with serious risks such as acute myocardial infarction (AMI), stroke and death from cardiovascular causes. So, withdraw of hypertension therapy, might cause even more morbidity and mortality than COVID-19 itself. If patients or their doctors wish to change ACEII-ATII-RB therapeutics, it would be time to remember that other anti-hypertensive groups like thiazide diuretics are a rational, effective, cheap and safe option.

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Letter

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Is GSK3β a molecular target of chloroquine treatment against COVID-19?

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SUMMARY The recent clinical trial reports pertaining to the efficacy of chloroquine and hydroxychloroquine against COVID-19 albeit yet to be validated with larger clinical trials, have sparked much interest globally to evaluate whether this anti-malarial drug can be repurposed for the treatment of COVID-19. In addition to its anti-viral activity, the anti-inflammatory activity of chloroquine may also contribute to its efficacy. Based on our data obtained from an animal infection model of melioidosis (a disease caused by the bacteria *Burkholderia pseudomallei*), treatment with chloroquine can result in the phosphorylation and consequent inhibition of glycogen synthase kinase-3β (GSK3β). This serine/threonine protein kinase is now recognised as a point of convergence for host inflammatory response. In view of this, it is plausible that the mechanism for the anti-inflammatory effect of chloroquine against COVID-19 involves inhibition of host GSK3β.

Keywords Chloroquine, COVID-19, GSK3β, anti-inflammatory

The molecular basis by which chloroquine dampens the host overwhelming inflammatory response (cytokine storm) during infection is still not fully understood. As in malaria, pathogenesis in viral infection may also be related to dysfunction in the regulation of pertinent signalling pathways; for example aberrant GSK3β signalling. Our notion is based on the understanding that GSK3β is a molecular hub linking numerous signalling pathways in the cell, including host-directed inflammatory response. Lithium chloride (LiCl) , a well-known GSK3 inhibitor, has been reported to suppress avian coronavirus infectious bronchitis where the anti-viral activity of lithium was attributed to its cellular effect (*1*). Most recently, Nowak & Walkowiak (2020) (*2*) proposed LiCl to be further explored as a potential therapeutic for the treatment of COVID-19. The reported efficacy of chloroquine in recent clinical trials to treat COVID-19 (*3,4*) may also be attributed to a mechanism of action that involve inhibition of GSK3β. In our laboratory, we have shown that chloroquine treatment in experimental animal melioidosis modulated cytokine levels and increased animal survivability *via* inhibition of GSK3β (*5*). Our analysis revealed that chloroquine resulted in phosphorylation and consequent inhibition of GSK3β in the liver. In a subsequent study (*6*), we concluded that chloroquine is a plausible candidate for repurposing in the treatment of melioidosis. It is possible that the mechanism for the anti-viral activity of chloroquine, specifically its anti-inflammatory effect involves inhibition of GSK3β in lung epithelial and immune cells. The lead author's 1980 publication (*7*) first identified GSK3 forty years ago. This kinase, initially described as a key enzyme involved in glycogen metabolism, is now known to regulate a wide array of cellular processes. Dysregulation of this kinase is implicated in several diseases including bipolar disorder, diabetes mellitus, Alzheimer's disease, inflammation, and cancer (*8,9*). Further research to better understand the molecular basis of the anti-viral effects of chloroquine can have far-reaching clinical implications.

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