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Editorial

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Insights into Research on Natural Products

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Small, naturally derived molecules are important targets of drug research and development and are models of chemical synthesis. Such molecules accounted for over 60% of the approved drugs and pre-New Drug Application candidates from 1989 to 1995 (*1*). Of the top 35 ethical drug sales worldwide, drugs derived from natural products accounted for 24% in 2001 and 26% in 2002 (*2*). Drug research and development is gaining insights from research on natural products, a new trend that combines traditional methods with new techniques in life science. In addition to research seeking to discover natural compounds through use of isolation techniques and structural determination methods, research to identify new sources of natural compounds and classify their functions and research to modify the structures of those compounds by biosynthetic or chemical manipulation will definitely expand the field of natural product chemistry.

New Sources of Medicines — Natural products were originally small molecules derived from higher plants and microorganisms on land. Plant endophytes and marine organisms represent the dominant source of compounds of pharmacological interest. Ecteinascidin 743 from *Ecteinascidia turbinata* has been developed into an antitumor agent while ω-conotoxin MVIIA from cone snails has been developed into an analgesic, and numerous metabolites have been evaluated in clinical trials (*3*).

Manipulation of Secondary Metabolite Biosynthesis — Organisms biosynthesize specific secondary metabolites catalyzed by gene-regulated enzymes. Elucidation of the pathways of biosynthesis has allowed scientists to manipulate the production of metabolites or increase molecules of interest by either supplying alternative substrates or by artificially altering those organisms. Modifying organisms to improve secondary metabolite production has been an effective means of increasing various natural products and producing vast amounts of specific constituents. The *ttmM* gene, for instance, encodes a C3' hydroxylase in the biosynthesis of tautomycin. Artificial inactivation of this gene has yielded a mutant SB6005 bacterial strain, and fermentation of this strain has yielded three new 3'-deshydroxy tautomycin derivatives (*4*).

Chemical Synthesis — Chemical synthesis of a complex natural product from simple chemicals is a challenge because of the instability, multiple functional groups, multiple stereocenters, and complex framework of target structures (*5*). This challenge has encouraged chemists to identify efficient methods for and strategies of synthesis and has thus encouraged the development of organic chemistry. A large number of molecules and their derivatives have been synthesized, increasing their potential for development into drugs. The synthesis of camptothecin and its derivatives is a topic of great interest in order to establish high-yield strategies and identify potent anti-tumor agents with low toxicity. The camptothecin derivatives topotecan and irinotecan have been approved by FDA and belotecan has been marketed in Korea; some derivatives are in different phases of clinical studies (*6,7*). Chemical synthesis

has now become an active branch of research on natural products. Taxol, epothilone derivatives, and tubulin-targeting drugs have also been synthesized, but they still have a ways to go until they are ready for commercial use.

Biomimetic Synthesis — Nature is an outstanding 'chemist' that promotes highly efficient reactions under different conditions and in a regio- and stereo-selective manner in order to produce complex secondary metabolites. Like of nature's students, biomimetic synthesis prepares natural metabolites by mimicking nature's steps of biosynthesis. Important reactions in biosynthesis such as the Diels-Alder reaction, cyclization of isoprenoids, and oxidative coupling of phenols have been widely used to synthesize natural products (*8,9*).

Learning How Organisms Function — The official goal of research on natural products was to identify bioactive compounds for use in treating human diseases, and this research did not bother with the physiological functions of these compounds in target organisms themselves. However, this research now seeks to determine why such compounds exist and what significance they have. A typical example is resveratrol, a stilbene known for its cardioprotective and chemopreventive effects in humans (*10*). Resveratrol is essentially a toxin produced by several plants in response to infection or other stresses (*11*).

Nature knows best. Natural molecules produced by natural organisms will have greater biological significance as they are used in drug development. Future research on natural products will be multi-disciplinary, including fields such as phytochemistry, organic chemistry, microbiology, pharmacology, chemical ecology, and molecular biology.

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Policy Forum

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Prospects for using pre-exposure prophylaxis to control the prevalence of HIV/AIDS in China

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ABSTRACT: Although important progress has been achieved in combating HIV/AIDs over the past 30 years, HIV/AIDs is still a serious threat to today's world. In China, figures on the incidence of this disease have painted a less than optimistic outlook. As the prevailing methods of preventing HIV/AIDS are all partially effective, novel and effective preventive interventions are needed in order to control the spread of the disease. Pre-exposure prophylaxis (PrEP) is one of the most promising prevention strategies and has garnered great attention worldwide. Current clinical trials on the efficacy and safety of this strategy have had some favorable results though major challenges around the world remain. Thus, China has taken an active part in the PrEP study to limit the prevalence of HIV/AIDS. This article describes the status of the PrEP study and discusses the opportunities and challenges encountered when implementing this strategy in China.

Keywords: Pre-exposure Prophylaxis (PrEP), HIV/AIDS, ethical dilemmas, financial capacity

Thirty years after its discovery, HIV/AIDS remains one of the world's most significant public health challenges, particularly in low- and middle-income countries. Recent data suggest that the levels of new infections worldwide remain high (about 2.6 million in 2009), although the overall growth of the global HIV/AIDS epidemic appears to have stabilized (*1*). The "prevention first" principle is broadly followed when controlling the prevalence of HIV/AIDS. However, the high incidence of HIV infection and partial effectiveness of current prevention strategies such as condom use, male circumcision, and distribution of sterile needles and injection equipment highlight the need for new and effective interventions to complement existing strategies. One preventive option currently being studied is pre-exposure prophylaxis (PrEP). PrEP refers to daily or intermittent administration of antiretroviral drugs (ARVs) such as tenofovir disoproxil fumarate (TDF) and emtricitabine (FTC) to protect high-risk HIV-negative individuals from infection. Satisfactory preclinical findings with regard to prevention of HIV infection by PrEP and confirmation of the long-term safety of ARVs used to treat AIDS (*2*,*3*) have fueled interest in using PrEP to prevent HIV infection in humans.

In China, the first AIDS case was reported in 1985, and the disease spread quickly in the 1990s. However, the scale of China's HIV/AIDS epidemic did not attract attention until 2003. Since then, annual morbidity and mortality rates have been showing obvious upward tendencies (Figure 1) (*4*). By October 31, 2009, a total of 319,877 individuals was reported to be HIV-positive; this figure included 102,323 cases of AIDS and 49,845 recorded deaths (*5*). By the end of 2009, approximately 740,000 individuals (range: 560,000-920,000) were estimated to be HIV-positive, with 48,000 new HIV infections (range: 41,000-55,000) in 2009 (*5*). Although the prevalence of HIV/AIDS in China remains low overall, pockets of greater prevalence among specific sub-populations and in some localities have appeared and the HIV/AIDS epidemic is spreading from high-risk groups to the general population. Since HIV/AIDS prevention and control remains crucial, universal, effective, and more accessible intervention strategies such as PrEP are

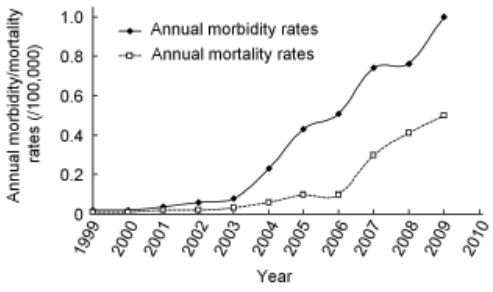

Figure 1. Morbidity and mortality rates of HIV/AIDS in the period of 1999-2009 in China.

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urgently needed in China.

Among the modes of transmission of HIV, sexual transmission has become the fastest mode of transmission in China due to changing sexual behaviors and attitudes since China opened up to the outside world in the late 1970s (*6*). Of the new infections in 2009 in China, about three-fourths were transmitted through sexual contact, either heterosexual or homosexual (*7*). Female sex workers (FSW) and their clients and men who have sex with men (MSM) are the groups most vulnerable to HIV infection. Although condoms were effective in preventing HIV/AIDS and other sexually transmitted diseases, studies indicated that those high-risk populations had a low level of consistent condom use. Specifically, 60% of FSWs and 60-70% of MSMs in China did not use condoms consistently with their clients or sex partners (*8,9*). Common reasons for not using condoms were worries about loss of a partner's trust and reduced sensation (*10*). PrEP has numerous advantages in this regard, potentially allowing people access to treatment while not requiring discussion with one's partner and eliminating problems associated with physical and psychological factors. PrEP may play an important role in combating the prevalence of HIV/AIDS in China if it proves to be effective.

Clinical trials on the efficacy and safety of PrEP in preventing HIV infection have been carried out around the world and some have had positive results (*11*). Oral administration of TDF and FTC daily to HIV-seronegative men or transgender women who had sex with men in Peru, Ecuador, South Africa, Brazil, Thailand, and the United States led to a 44% reduction in the incidence of HIV in those subjects (*12*). Another completed study on sexually active, HIV-uninfected 18- to 40-year-old women in South Africa indicated that a 39% overall reduction in HIV acquisition was achieved by giving tenofovir 1% vaginal gel (*13*). In spite of those inspiring outcomes, some trials were halted due to the obstacles encountered (*14*). The most important issue involved is the ethical dilemma. Currently, most PrEP clinical trials are conducted in poor parts of the world such as Africa and Latin America, whereas funding for those trials is from developed countries in Europe and the US. The concern is that intervention measures are inadequately implemented to safeguard the rights and interests of vulnerable groups.

Capitalizing on the potential for PrEP to prevent HIV transmission, China has actively studied PrEP. Since a PrEP study is a complex and costly project, stable and continuous funding is vital. In this regard, China launched a Major State Science & Technology Special Project entitled "Feasibility Study on Use of PrEP to Reduce New HIV Infections in High-risk Groups of Western China". The project was allocated ¥10 million yuan in funding and was undertaken by Chongqing Medical University, Guangxi Medical University, and Xinjiang Medical University in 2009. Project participants include MSMs, FSWs, and HIV-negative sex partners of HIV-infected individuals. Project researchers are from various fields and have focused on issues associated with ethics, prospects for use of PrEP, and drugs to prevent infection as might arise in the implementation process. In the early phase, a volunteer-based, anonymous, one-on-one survey of 762 subjects in Urumqi and Kelamayi of Xinjiang Uyghur Autonomous Region was conducted to assess the acceptability of PrEP strategy among FSWs in Xinjiang. Of the subjects, 69.29% were willing to undergo PrEP, but the FSWs had concerns about drug safety, effectiveness, and cost (*15*). This study found that a PrEP strategy was acceptable to FSWs in Xinjiang and laid the groundwork for future research.

As a new biomedical approach to HIV prevention, PrEP has the potential to become a powerful tool and may be an important addition to the prevention toolbox. But this prevention strategy is not without challenges. First, principles of human understanding and respect for the human rights of subjects should be adhered to from start to finish during PrEP clinical trials. Fundamental ethics, including respect, justice, non-maleficence, and beneficence, have to be put above study aims. In addition, subjects' rights to life, health, privacy, and free choice should be protected. The second challenge is the safety of long-term use of ARVs by healthy people. Although the safety and tolerance of TDF and FTC has been confirmed studies on HIV-positive animals and humans, more studies should be performed to investigate the toxic or adverse effects of longer exposure to these drugs in HIV-negative individuals. In addition, the resistance of HIV to ARVs should be monitored. Since completely preventing HIV infection through use of the PrEP strategy seems impossible, problems with drug selection should be resolved once individuals undergoing PrEP are accidently infected and drug resistance occurs. The third challenge is the economy's capacity to allow use of PrEP. Because of the high-cost of ARVs, PrEP may be one of the most expensive strategies to prevent HIV. In China, HIV high-risk populations that take drugs or behave promiscuously are mainly on the fringes of society and reside in economically undeveloped districts or rural areas. In the current trial phase, access to ARVs is free. If the PrEP strategy proves effective and its scale is expanded, however, these drugs will not be available to most of the people who need them if they are not free. Given that fact, affordability of medicines might be the decisive factor guiding the adoption of the PrEP strategy. Thus, as clinical trials of this strategy continue the government should be acting as a public health decision-maker to resolve the contradiction between individuals' financial capabilities and their right to health. Issues like ensuring consistent and continuous project funding and allocating limited resources should be thoroughly investigated in order to facilitate the adoption of a PrEP strategy.

Thus far, the verdict is out on whether PrEP will be the most effective strategy for preventing HIV/AIDS. Ongoing and future trials will answer this question. One fact that must not be forgotten, should PrEP prove to be safe and effective, is that such a strategy is only a precautionary measure and is not a substitute of effective prevention strategies currently in practice. Integration of PrEP into an overall care platform may be the right way to go. With scientific data mounting, preparations for the possible roll-out of PrEP must be made should the strategy prove efficacious.

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Review

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Creatinine and HMH (5-hydroxy-1-methylhydantoin, NZ-419) as intrinsic hydroxyl radical scavengers

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ABSTRACT: Creatinine (Crn) is one of the main intrinsic hydroxyl radical (•OH) scavengers and an ideal one for healthy or normal mammals, although this fact has not yet become widely accepted. Our results from urinary data estimated that ca. 0.4-0.6% of Crn is used daily to scavenge •OH in normal mammals [ca. 50 μmole and ca. 400 pmole of •OH in healthy subjects and normal rats, respectively]. In human subjects, Crn reacts non-enzymatically with •OH to form creatol (CTL: 5-hydroxycreatinine) and demethylcreatinine (DMC) in a one to one ratio, and CTL partially decomposes to methylguanidine (MG). And so, the scavenged mole of •OH by Crn is nearly equal to their molar total sum (CTL + MG + DMC) or 2 × (CTL + MG). The molar ratio of (scavenged •OH)/Crn in healthy subjects and normal rats are 4.4 and 6.0 mmole/mole, respectively, *i.e.* almost similar, but in patients with chronic kidney disease (CKD) the ratio increases up to ca. 60 mmole/mole in proportion to the severity of CKD. Since the level of Crn might not be enough to scavenge all •OH, and MG starts accumulating as a uremic toxin, Crn is not really the ideal scavenger. 5-Hydroxy-1-methylhydantoin (HMH, NZ-419), a Crn metabolite, is another antioxidant, having •OH scavenging ability, and has been shown to inhibit the progression of CKD in rats in stead of Crn, if sufficient amounts are given orally.

Keywords: Creatinine, HMH (NZ-419), intrinsic antioxidant, creatol, methylguanidine, demethylcreatinine

1. Introduction

Creatinine (Crn) is one of the main intrinsic hydroxyl radical (•OH) scavengers (*1-8*), but this simple fact has not yet become widely accepted. One main reason might be an incorrect old belief that Crn has been taught to be an end-metabolite in mammals. However, this belief is not true. Therefore, we first introduced a common dual oxidative metabolic pathway of Crn in mammals (Figure 1) (*3,9-11*), *via* creatol (CTL: 5-hydroxycreatinine, 2-amino-4-hydroxy-3-methyl-4*H*-imidazol-5-one, CAS: 133882-98-1) (Figure 1) (*3,9-17*), and 5-hydroxy-1-methylhydantoin (HMH, NZ-419, 5-hydroxy-1-methylimidazolidine-2,4-dione, CAS: 84210-26-4) (Figure 1) (*3,9-12,18,19*). In this review, we add a further bypass venue, from Crn to demethylcreatinine (DMC, glycocyamidine: GA, 2-amino-1,4-dihydroimidazol-5-one, CAS: 503-86-6) (Figure 1) (*20*) in addition to the above recognized pathways.

The chemistry of Crn has advanced dramatically since Ienaga *et al*. isolated HMH from inflamed rabbit skin tissues in 1987 (*21*) thanks to new bioorganic technologies such as high performance liquid chromatography (HPLC) purification, instrumental and spectroscopic analyses and so on. Before that, there were hundreds of mixed right and wrong reports on the chemistry and/or biochemistry of Crn. However, in seeking more knowledge of the properties of the purely synthetic authentic samples of Crn metabolites [HMH (*19*), CTL (*20*), creatone-A (CTO-A, 2-amino-1-methyl-4,5-imidazoledione, CAS: 115012-08-3) (Figure 1) (*13*), creatone-B (CTO-B, *N*-(*N'*-methylamidino)oxamic acid, CAS: 51093-33-5) (Figure 1) (*13*), DMC (*20*) and so on], we looked at their physicochemical properties in order to understand their reactions.

Giovannetti *et al*. indicated that methylguanidine (MG, CAS: 471-29-4) might be one of the uremic toxins (*22,23*), and that the classical analytical values of MG using pre-treatment with activated charcoal were too high. The formation of MG by charcoal-catalyzed oxidation of Crn was significant and gave false readings (*24*). It was recognized that Crn and creatine (Cr) could be oxidized to MG *via* creatone (CTO) (*25,26*), but they did not know the precise mechanisms of the oxidation. It was thought that such oxidations were mere chemical reactions before Aoyagi and Nagase indicated that the non-enzymatic reaction of Crn into MG occurred also *in*

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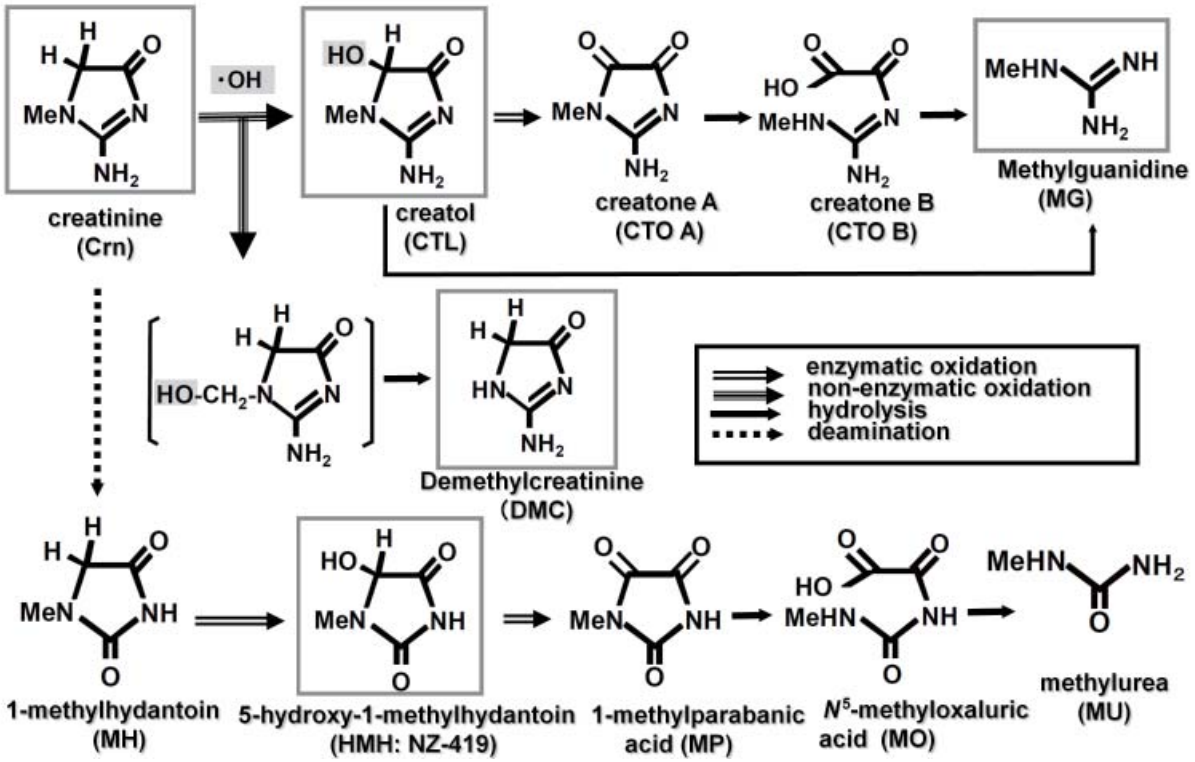


Figure 1. Metabolic pathways of Crn and three key metabolites, HMH, CTL and DMC. The compounds enclosed in boxes were detectable in the sera of CKD patients at stages 3, 4 and 5.

Table 1. Stage of chronic kidney disease

Stage	Description	Clinical GFR*,*** (mL/min/1.73 m^2)	Relative GFR/GFR_0	Rat GFR** (mL/min/kg)
1	Kidney damage with normal or ↑GFR	≥ 90		
2	Kidney damage with mild ↓GFR	60-89		
3	Moderate ↓GFR	30-59	0.30-0.59	1.7-3.3
4	Severe ↓GFR	15-29	0.15-0.29	0.9-1.6
5	Kidney failure	< 15 (or dialysis)	< 0.15	< 0.9

* Clinical GFR_0, GFR of normal subjects, has been reported to be about 100 mL/min/1.73 m^2.
** Rat GFR_0, GFR of normal rats, has been reported to be about ca. 5.7 mL/min/kg.
*** The National Kidney Foundation. K/DQI Clinical practice guidelines on chronic kidney disease (2002).
We classified CKD stages of rats based on rat GFR. Ienaga & Yokozawa (2010).

vivo, and they recognized that the main reactive oxygen species (ROS) in this oxidation was the •OH radical, at least in hepatocytes (*27-29*). Since then Yokozawa *et al*. indicated that the precursor of MG *in vivo* was truly Crn (*30*), and we hypothesized a new pathway *via* CTL which had been just isolated from the urine of creatininemia patients (*12*). We soon proved the hypothesis that a dual metabolic pathway of Crn *via* CTL or HMH was common in mammals by using a ^{13}C-labeling technique (*9*), and began to investigate the precise metabolic pathways of Crn which are discussed below.

For the precise determination of MG in the serum of human subjects, we needed to take special sampling precautions. For example, abstention from sake (rice wine) on the previous day is recommended, because acetylagmatine in sake interferes with the analyses (*31*).

Crn has been taught as being a key compound in monitoring kidney function for chronic kidney disease (CKD) patients. This is even now true, and recently serum Crn (sCrn) is widely used to calculate the estimated glomerular filtration rate (eGFR) (*32-34*). As shown below, up to ca. 10% of Crn is catabolized daily *via* HMH, CTL, and probably DMC in diabetic (DM) and/or CKD patients. The problems of catabolism as well as excretion for CKD patients might be a kind of warning bell to use sCrn. However, the estimation of GFR is based on the correlation between the GFR and the sCrn value itself. Regardless of such problems, eGFR seems to remain important. Since we have classified rats into five stages of CKD as well, we can now use the data in Table 1 to define the extent of kidney damage in human patients and in rats (*6*).

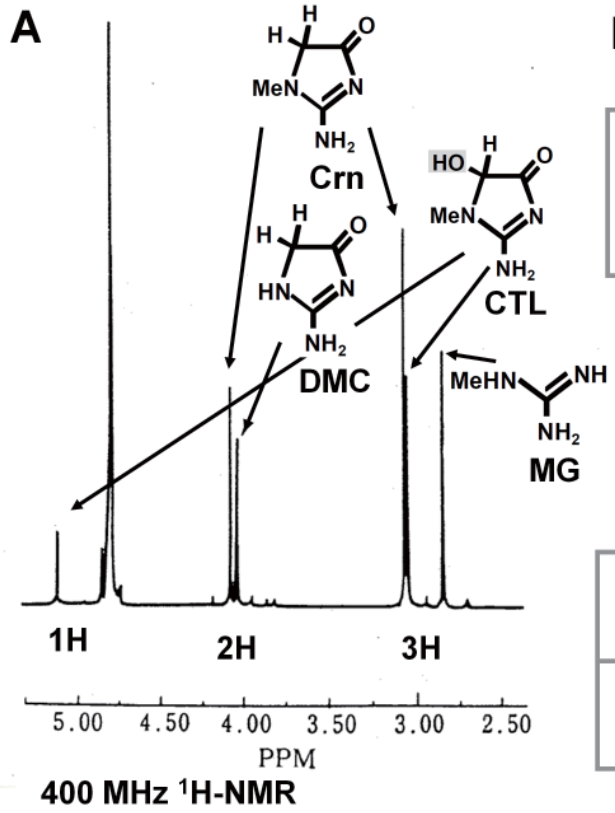
Principally, we explain in this review how Crn reacts with •OH radicals and acts as an •OH radical scavenger. We revised the correct structures of CTO-A and CTO-B (*13*), and proposed the •OH related oxidation mechanism of Crn *via* CTL, CTO-A, CTO-B to MG (Figure 2B). Our *in vivo* Crn pathway, including the pathway to DMC, has been already reproduced in cell-free *in vitro* test tube experiments under Fenton's reaction conditions (*15*). By reviewing clinical data, we not only showed that Crn seems to be a good intrinsic •OH scavenger, but it also allowed us to estimate how many moles of •OH might be scavenged daily in normal rats and healthy human subjects. For CKD patients, however, Crn cannot now be considered as the ideal compound (*35*), because CTL and MG as well as Crn start to accumulate inside bodies. We want to show here that the Crn might not be enough to scavenge all of the •OH radicals present, although up to ca. 3% of Crn it seems to be used to scavenge •OH radicals in CKD patients just before therapy for end-stage renal disease (ESRD) (*7,8*).

Last we want to introduce HMH as another, and possibly, alternative intrinsic antioxidant (*3,10,36-39*). Intrinsic levels of HMH increase in mammals (*8,10*) in DM and CKD patients and orally administered HMH has prevented the progression of CKD at stages 3-5 in rats (*3,5,6,40,41*). We believe that HMH could act as a 'self-defense' substance.

2. Mammalian metabolic pathways of Crn

About twenty years ago, Ienaga *et al*. isolated HMH (*20*) as a novel rice germination regulator together with MH (1-methylhydantoin, 1-methylimidazolidine-2,4-dione, CAS: 616-04-6) (Figure 1) from inflamed rabbit skin tissues but not from normal skin tissues. Others isolated MH as a rice germination regulator from some anaerobic microbes (*42*), but not together with HMH. The Crn metabolic pathway to sarcosine *via* MH in anaerobic microbes had been well clarified (*11*). We soon proved that once MH (formed probably under anaerobic conditions) is absorbed into aerobic mammalian bodies, it is converted by oxidation readily to HMH which is then metabolized to methylurea (MU: CAS: 623-59-6) (Figure 1) (*18,19*).

Analogically-based thinking about chemical structures led us to the isolation of CTL, together with HMH, from urine of creatininemia patients (*12*). Crn reacts non-enzymatically, at first with •OH radicals to give CTL or DMC, and then CTL is further enzymatically oxidized to CTO-A which is hydrolyzed to MG *via* CTO-B (Figures 1 and 2B). Under slow reaction conditions [ex. Crn (20 mM), $K_3Fe(CN)_6$ (0.1 mM), 3% H_2O_2 (100 mL), 25ºC, 2 days], only peaks of Crn, CTL and DMC were detectable in ^{1}H-NMR spectra (Figure 2A) and HPLC chromatograms. In contrast, under faster reaction conditions [ex. Crn



B Formation of CTL, MG & DMC

Crn → CTL → CTO-A → CTO-B → MG
↓ ·OH — oxidation
DMC — hydrolysis

C Oxidation of CTL into MG

	Non-enymatic	Vitamin C synthase	*L*-2-hydroxyl acid oxidase
Rat liver	○	○	
Rat kidney	○		○
Human Liver	○		
Human kidney	○		○

Figure 2. Fenton's reaction of Crn. A: Proton-NMR spectrum of the reaction mixture. **B:** Reaction scheme observed in the reaction mixture. **C:** Enzymatic and non-enzymatic conversion of CTL to MG.

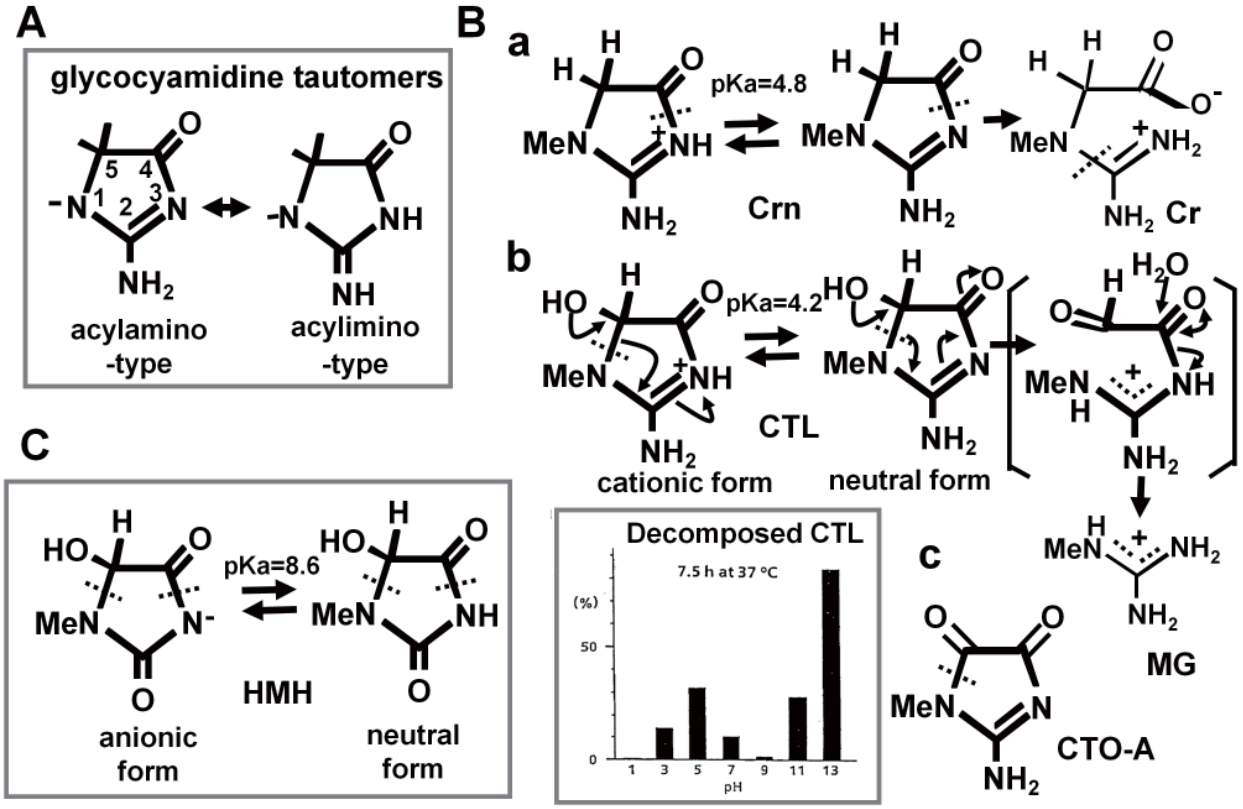


Figure 3. Physicochemical properties of Crn derivatives. A: Glycocyamidine tautomers. **B:** Ring opening reaction: **a**. Crn; **b**. CTL; **c**. CTO-A. **C:** Ionization of HMH.

(20 mM), $FeCl_3$•$6H_2O$ (10 mM), 1% H_2O_2 (100 mL), 25ºC, 3 h], CTO-A and CTO-B were also detectable in addition to Crn, CTL and DMC in ^{1}H-NMR spectra and HPLC chromatograms (*15*). Once CTL is formed, an alternative ring-opening reaction of CTL by hydrolysis can also readily occur to give MG directly (Figures 1, 2B and 3Bb), as mentioned above. Although CTO-A and CTO-B could not be detected in physiological fluids, we thought that enzymatic oxidation can occur even *in vivo*. In fact, we isolated two kinds of MG synthases (enzymes that oxidize CTL to CTO-A) from rat liver and rat kidney (Figure 2C): *viz*, vitamin C synthase (L-gulono-γ-lactone oxidase) (*43*) and L-2-hydroxyl acid oxidase (*44,45*), respectively. The concentrations of MG both in physiological fluids and tissues in rats were higher than those of CTL, whereas CTL was predominant in human sera. We think that the difference might be explained by the presence or absence of vitamin C synthase.

We herein revise our *in vivo* pathways by addition of a pathway from Crn into DMC (Figure 1) even *in vivo*, because we can measure DMC in human sera of CKD patients and find that 1.3 mole of serum DMC (sDMC) was formed with one mole of serum CTL (sCTL) (Figure 4B), *i.e.* molar ratio of DMC to CTL was roughly one. This result indicated that this conversion *in vivo* should be a non-enzymatic oxidation reaction with •OH radicals, too.

3. Physicochemical properties of Crn and Crn metabolites; tautomerism, ionization and hydrolysis

We have found that there is some misunderstanding about the chemistry of Crn. For example, even though Kenyon *et al*. clearly reported tautomeric preferences for Crn in 1971 (*46*), the wrong tautomer (acylimino type: Figure 3A) of Crn is still written in biochemical textbooks and papers instead of the correct one (acylamino type: Figure 3A). Without exact knowledge of the physicochemical properties of Crn, it is hard to fully understand its chemical or biological reactions. Better sense can be made if the acylamino tautomer for Crn is used.

Our CTL structure as the acylamino form (*1,8,12,47*) was recently supported also by Krawczyk (*48*), who detected CTL as the intermediate from Crn to MG in oxidation with activated charcoal.

Another consideration which should be taken into account is the ionization of Crn and its metabolites in order to understand their reactions in solution. Ionization constants give us very valuable information about the ionic species involved. We know the corresponding ionization constants (pKa values) of our substances, *viz* Crn = 4.8 (basic pK), CTL = 4.2 (basic pK), DMC = 4.8 (basic pK), MG = 13.4 (basic pK), HMH = 8.6 (acidic pK). These values indicate that MG is a strong base and exists in an acidic (cation) form at neutral pH (around 7.0). However, glycocyamidine derivatives

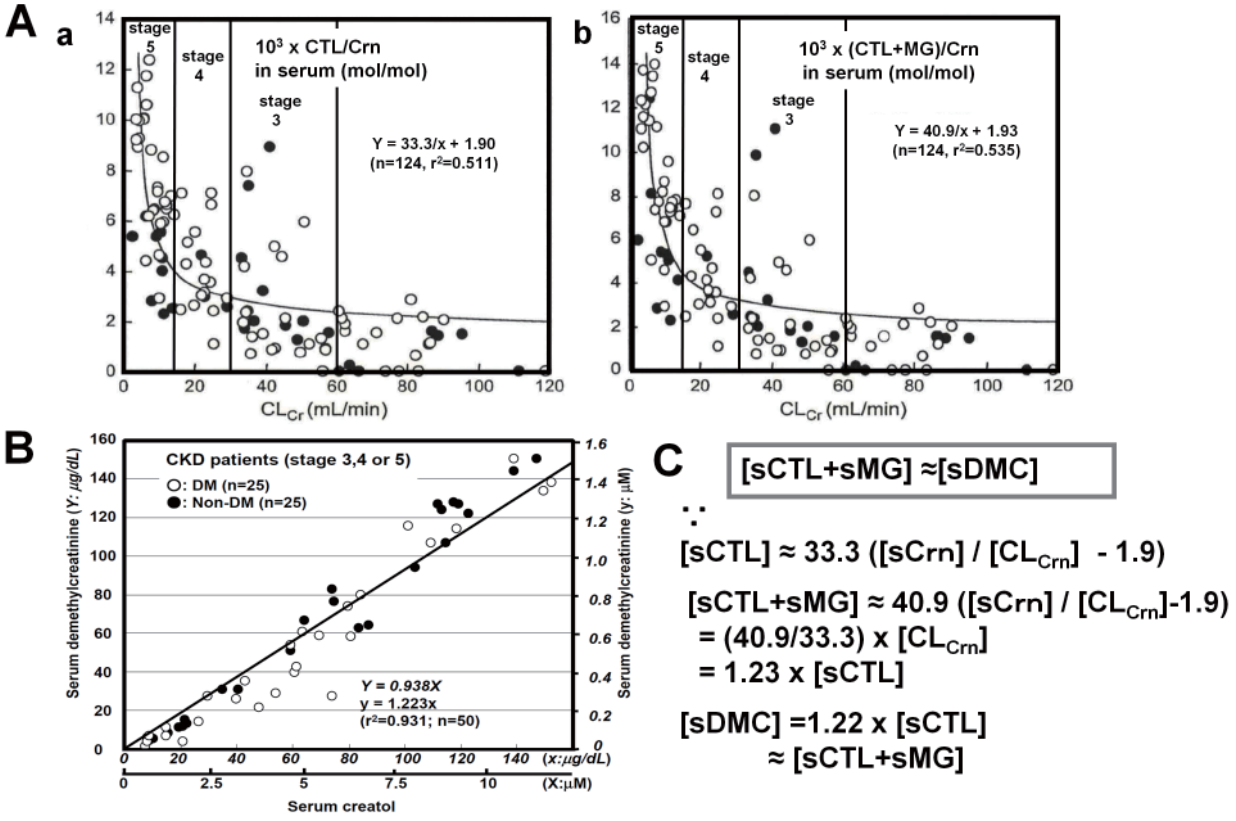


Figure 4. Correlationship between molar ratios: (CTL + MG)/Crn, CTL/Crn and DMC/Crn. A: Relationship between CL_{Cr} and (CTL + MG)/Crn or CTL/Crn in serum. **B:** Relationship between CTL/Crn and DMC/Crn in serum. **C:** Relationship between (CTL + MG)/Crn and DMC/Crn in human subjects.

(Figure 3A), Crn, CTL and DMC, exist as neutral forms (non-ionized species) in blood and tissues. In contrast, in acidic urine a proportion of a corresponding acidic form of glycocyamidine derivatives are observed. Since the ring-opening reaction of CTL by hydrolysis showed a local maximum value at pH 5 (enclosed decomposed CTL in Figure 3Bb) (*47*), a contribution of the acidic form cannot be ignored (Figure 3Bb).

The cleavage bond of Crn is the N3-C4 bond (Figure 3Ba). We can explain that CTL cleaves selectively at the N1-C5 bond (Figure 3Bb) (*3,15,47*), although some people might feel that something may be wrong. However, when we consider the correct acylamino tautomer and the non- or ionized forms together with the effect of a hydroxyl group at position C-5, a cleaving bond other than the N1-C5 bond is unacceptable. The cleavage bond of CTO-A can be explained differently, simply by high steric hinderance around the N1-C5 bond compared with that around the N3-C4 bond (Figure 3Bc).

One mystery which was confusing was that previously no peak was seen for CTL in physiological fluids observed even using sensitive HPLC techniques. The reason is simple: although CTL exists in mammals in a concentration at least equimolar to that of MG, the detectability of cyclic guanidino compounds such as Crn and CTL is very poor (three digits) in comparison with non-cyclic guanidino compounds such as MG in conventional HPLC methods using fluorogenic agents such as 9,10-phenanthrenequinone (PQ) (*49*). A simple solution involved the addition of an alkaline hydrolysis step between column separation and the reaction with PQ (*47,50*): we were thus able to increase the detectability of CTL 50-fold to overcome the problem.

HMH is an acidic substance (pKa is 8.6) (*21*), although fairly weaker than acetic acid (pKa 4.8). It is recognized that the anionic form of acetic acid is predominant under physiological conditions (pH around 7.0). In contrast, the neutral form of HMH should be predominant (97.5%) at pH 7.0 (Figure 3C).

4. Background of our estimation

4.1. *Detection of •OH radicals*

Because the •OH radical is so reactive, its existence cannot be directly monitored and an indirect biomarker of the •OH radical would be useful for patients with various diseases. In order to estimate the amount of •OH radicals, the •OH adducts or reaction products with •OH have to be measured as •OH biomarkers. For example, one of the most frequently used biomarkers for •OH radicals are 8-hydroxyguanine (8-OHG) or 8-hydroxydeoxyguanosine (8-OHdG); •OH

adducts of guanine or deoxyguanosine (dG) are formed, respectively. Their use is limited because they show only the amount of •OH radicals formed inside nuclei and mitochondria. Before determination of 8-OHG and 8-OHdG in urine, several reaction steps with 8-OHdG containing nucleotides must occur. It should be noted that the reported daily excreted 8-OHdG in urine of healthy subject is fairly low: 8-OHdG/Crn: 8.4 ± 2.7 μg/g (n = 25) (*51*); 1.86 ± 1.09 μmole/mole (n = 67) (*52*). If we assume urinary Crn (real range from 500 to 2,000 mg/day) to be roughly one gram, then the 8-OHdG amount would be ca. 10 μg (ca. 35 nmole). This result shows at the same time that dG scavenges ca. 35 nmoles of •OH radicals daily. Therefore, our estimation (see below) that the daily scavenged amount of •OH radicals by Crn is ca. 50-500 μmoles in healthy subjects and severe CKD patients; making it shockingly high.

4.2. *Classification of CKD into 5 stages for human subjects and rats*

The normal glomerular filtration rate (GFR_0) for human healthy subjects and rats had been reported to be ca. 100 mL/min/1.73 m^2 (*53*) and ca. 5.7 mL/min/kg (*54*), respectively, so the relative value of the glomerular filtration rate (GFR), GFR/GFR_0 can be calculated. As reported earlier (*6*), we classified the CKD of rats into 5 stages like those in humans (*32,55*). Table 1 shows a description of the 5 stages of CKD in human subjects and rats (*6*). In the case of rats, if the GFR had not been measured, we can estimate the eGFR by using our correlation equation below (y: GFR/GFR_0 = GFR/5.7; x: sCrn mg/dL) (*6*):

$$y = 0.47/x - 0.035 \ (n = 12: R^2 = 0.97)$$

5. *In vitro* and *in vivo* reaction of Crn with •OH radicals

5.1. *Cell-free in vitro reaction of Crn with •OH radicals*

We can now follow the bio-mimetic reactions of Crn with •OH radicals generated in a cell-free *in vitro* Fenton reaction. In the nuclear magnetic resonance (NMR) tube, we can follow the reactions even without purification (Figures 2A and 2B) (*15*). Both NMR spectra and HPLC charts indicated that the oxidation of Crn with •OH radicals gave at first CTL or DMC and then CTL was further oxidized to CTO-A which is hydrolyzed to MG *via* CTO-B (Figures 1 and 2B). Direct hydrolysis of CTL to MG was also indicated (Figures 1 and 2B). All these reactions also occur *in vivo* except for the step from CTL to CTO-A in which this oxidation is enzymatic instead of non-enzymatic (*43-45*).

5.2. *In vitro reaction of Crn with •OH radicals in cultured cells*

Since CTL is an •OH radical adduct of Crn, and MG is the product of CTL, the molar sum (CTL + MG) equals the corresponding moles of •OH radicals reacted with Crn. In isolated rat hepatocytes, Crn is converted by •OH radicals to CTL and MG, and the conversion is enhanced by stimulation with puromycin aminonucleoside (*39,56,57*). The contribution of protein kinase C activation has been clearly shown, by its translocation from cytosol to membrane, not only in •OH radical generation but also in formation of CTL and MG (*57*).

5.3. *Evidence of the in vivo reaction of Crn with •OH radicals*

Aoyagi and Nagase have already shown that the non-enzymatic reaction of Crn with •OH radicals provided MG (*27-29*). We have shown that •OH radicals play a crucial role in the first *in vivo* step, Crn→CTL, but not in the following next step, CTL→MG (*15-17*). Dimethylthiourea, an •OH radical scavenger, selectively inhibited the first step (*17*).

6. Estimation of scavenged amount of •OH radicals by Crn

Theoretically, the daily scavenged amount of •OH radicals by Crn is nearly equal to the daily urinary molar sum of CTL, MG and DMC, because CTO-A and CTO-B could not be detected in physiological fluids. In this review, the concentration of substances is depicted within square brackets [ ], and s and u before the abbreviation of the names of substances mean the substance in serum and urine, respectively. There is a good correlation between [sDMC] and [sCTL] as shown in Figure 4B (*20*), *i.e.*

$$[sDMC\ (\mu M)] = 1.22 \times [sCTL\ (\mu M)]$$

In Figures 4Aa and 4Ab, both [sCTL (μM)]/[sCrn (μM)] and [sCTL (μM) + sMG (μM)]/[sCrn (μM)] were correlated with Crn clearance [CL_{Crn} (mL/min)]. Therefore,

[sCTL (μM) + sMG (μM)] ≈ 1.23 × [sCTL (μM)] ≈ [sDMC (μM)] (Figure 4C).

In other words, the total sum (sCTL (mole) + sMG (mole) + sDMC (mole)) in an arbitrary amount of serum is likely to be equal to 2 × (sCTL (mole) + sMG (mole)).

Both molar ratios (CTL+MG)/Crn in serum and urine were shown to be almost equal (*4*), although the ratio CTL/Crn would not be steady, because the ratio MG/CTL increased during excretion from blood into urine. Among amounts of urinary CTL, MG and DMC (uCTL, uMG and uDMC), we assumed that uDMC

(mole) might be also nearly equal to (uCTL (mole) + uMG (mole)), although we do not have data on uDMC (mole). Therefore, (uCTL (mole) + uMG (mole) + uDMC (mole)) might be equal to 2 × (uCTL (mole) + uMG (mole)). The double amounts of the obtained absolute molar concentration of (CTL + MG) in 24 h urine were thought to be the estimated scavenged moles of •OH radicals.

6.1. *Estimation of daily •OH radical scavenging capacity of Crn in normal mammals*

The daily urinary molar sum of (uCTL + uMG) was calculated based on reported values (*1,9*). There seemed to be a great difference between values of healthy human subjects and normal rats (ca. 25 μmole/day and ca. 200 pmole/day, respectively) (Figure 5A). However, it is noteworthy that mean values of the corresponding molar ratios (uCTL + uMG)/uCrn were similar: 1.73 mmole/mole and 2.93 mmole/mole, respectively (Figure 5B) (*1*). These values seem to be reproducible, because the control subjects in another clinical report gave almost the same value (1.77 mmole/mole) (*2*). This fact might suggest that if we determined the muscle corrections, then oxidative stress related to •OH radicals in normal animals does not show a crucial species difference, although oxidative stress in rats seems to be on the higher side compared to those in human subjects.

For calculation of the molar ratio (uCTL + uMG)/uCrn, spot urine instead of 24 h urine can also be used (*4*) (Figure 5B). As stated above, (scavenged •OH radicals)/Crn can be calculated to be equal to the molar ratio 2 × (uCTL + uMG)/uCrn.

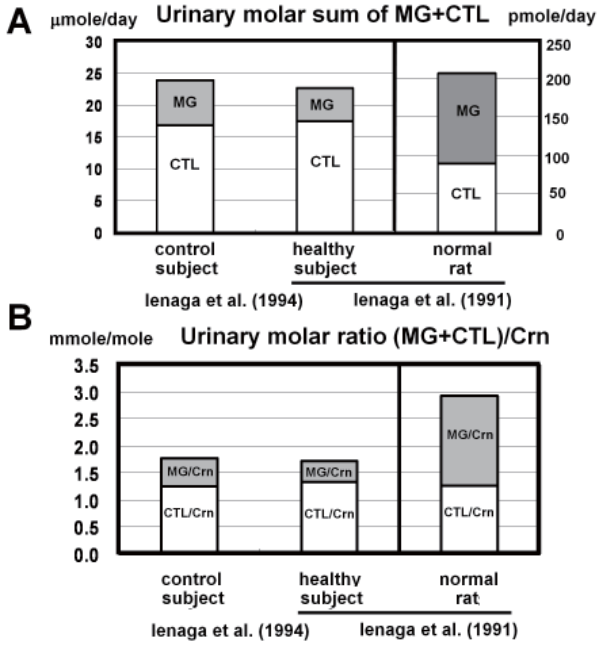


Figure 5. Estimation for amount of •OH radicals scavenged with Crn in mammalian bodies with CKD: based on daily urinary molar sum, MG + CTL (A) and urinary molar ratio, (MG + CTL)/Crn (B). *Ref.* Ienaga *et al*. (1991); Ienaga *et al*. (1994).

Healthy subjects and normal rats daily use ca. 0.4 and 0.6% of Crn, respectively, in order to scavenge •OH radicals. The scavenged amount of •OH radicals can be estimated to be ca. 50 μmole/day and ca. 400 pmole/day, respectively.

Two reports on daily excreted values of CTL and MG in healthy human subjects with normal kidney function gave similar values: the first are 17.4 and 5.3 μmole, respectively, for healthy subjects (*9*); and the second are 16.8 and 7.1 μmole, respectively, for normal subjects (*1*) (Figure 5A). Despite the conversion of CTL into MG during kidney excretion, CTL remained still dominant (Figure 5A).

Daily excreted mean moles of CTL and MG for normal rats were 78 and 118 pmole, respectively, making MG the main metabolite of Crn for normal rats. In the conversion of CTL into MG, there are a few pathways. Although non-enzymatic hydrolysis and MG synthase in kidney (*44,45*) seemed to play a common important role in mammals, the presence of vitamin C synthase in rat liver (*43*) and its absence in human beings is likely to be one reason for the difference between human subjects and rats.

6.2. *Estimation of daily •OH radical scavenging capacity of Crn in CKD mammals*

The molar ratio (CTL + MG)/Crn in serum and urine is correlated well with sCrn values (*1,50,58*). It was thought that sMG increases in proportion to sCrn according to a simple equation, but we have shown that the correlation followed a quadric equation (*1,50,58*). Since the molar ratios, sCTL/sCrn and sMG/sCrn increase in proportion to sCrn (x: M) according to the simple equation, then sCTL and sMG should theoretically increase according to a quadric equation. Based on the reported data (*1,58*), we recalculated the molar equivalents and obtained new equations. Y: (CTL + MG)/Crn mmole/mole; Z: (CTL + MG) μM; X: Crn μM; X': Crn: mg/dL (Figure 6).

For DM patients with CKD:

$$Y = 0.00627X - 0.0142$$
or $Y = 0.71X' - 0.61$ (the 1st equation) (Figure 6A)

$$Z = 0.00627X^2 - 0.0142X$$
or $Y = 0.71X'^2 - 0.61X'$ (the 2nd equation) (Figure 6B)

It should be noted that if we treat the serum specimens from CKD patients at stages 3 and 4 carefully, sMG is frequently not detectable, whereas sCTL can be measured easily since it is relatively quite high (Figure 6C) (*1,57*).

The molar ratio (CTL + MG)/Crn (Z: mmole/mole) in serum and urine is correlated well also to the inverse

of GFR (or Crn clearance: CL_{Crn}) (W: mL/min) (*4*), *i.e.*

$$Z = 40.9/W + 1.93 \text{ (in serum) (Figure 4Ab)}$$

As shown in Figure 7, the molar ratio (CTL + MG)/Crn in urine increased 10 and 17 times more in CKD patients (*1*) and in rats at stage 5 (*9*), respectively: from 3.0 mmole/mole (CTL/Crn = 1.3, MG/Crn = 1.7 mmole/mole) in the control rats up to 31.3 mmole/mole (CTL/Crn = 12.0, MG/Crn = 19.3 mmole/mole) in

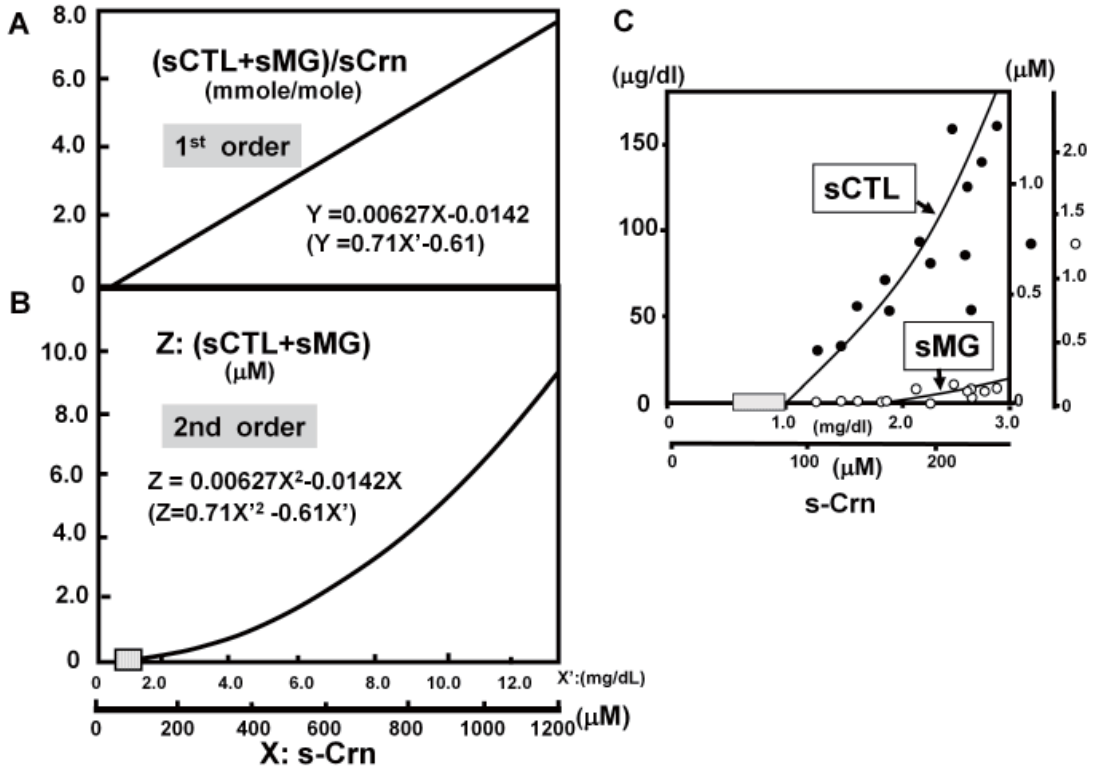


Figure 6. Correlation between Crn and (CTL + MG)/Crn or (CTL + MG). A: 1st order equation of (CTL + MG)/Crn with Crn. **B:** 2nd order equation of (CTL + MG) with Crn. **C:** Correlation between Crn and CTL or MG.

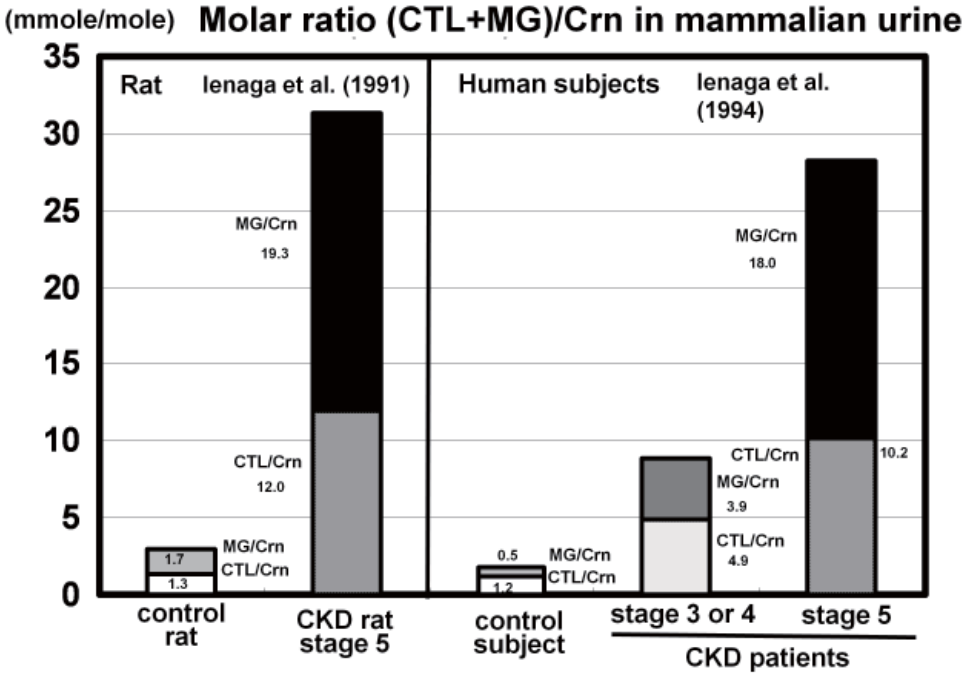


Figure 7. Molar ratio of (CTL + MG)/Crn in mammalian urine with or without CKD.

CKD rats at stage 5; from 1.7 mmole/mole (CTL/Crn = 1.2, MG/Crn = 0.5 mmole/mole) in the control subjects up to 28.2 mmole/mole (CTL/Crn = 10.2, MG/Crn = 18.0 mmole/mole) in CKD patients at stage 5.

We can suppose that molar ratios of (DMC/Crn) and (CTL + MG)/Crn are nearly equal. If so, it should be noted that about up to 6% of Crn was used to scavenge •OH radicals, in other words, one mole of Crn scavenged 60 mmole of •OH radicals in clinical patients or rats with CKD at stage 5, just before ESRD therapy. For CKD patients at stage 3 or 4, the molar ratio (CTL + MG)/Crn had an in-between value, 8.8 mmole/mole (CTL/Crn = 4.9, MG/Crn = 3.9 mmole/mole) when compared with controls (Figure 7).

The GFR values together with MG levels in every 48 h-urine sample in the progression of CKD (produced by adenine-loading) has been reported in previous research (Figure 8) (*54*). If we use the GFR_0 value of 5.65 ± 0.27 mL/min/kg (value at the day 0), each relative renal function GFR/GFR_0 (%) can be calculated and each stage can be estimated (Figure 8A). Thus, a CKD stage is identified for each MG value (Figure 8B). As shown in Figure 8C, urinary MG/Crn values increased inversely with decrease in GFR/GFR_0 (%): from 1.6 mmole/mole (GFR/GFR_0 = 100%) to 27.4 mmole/mole (GFR/GFR_0 = 4%) (Figure 8C). Since, however, we do not have any data about DMC in rats, we dare not estimate the amount of •OH radicals scavenged as DMC.

These data show that at least more than 3% of Crn

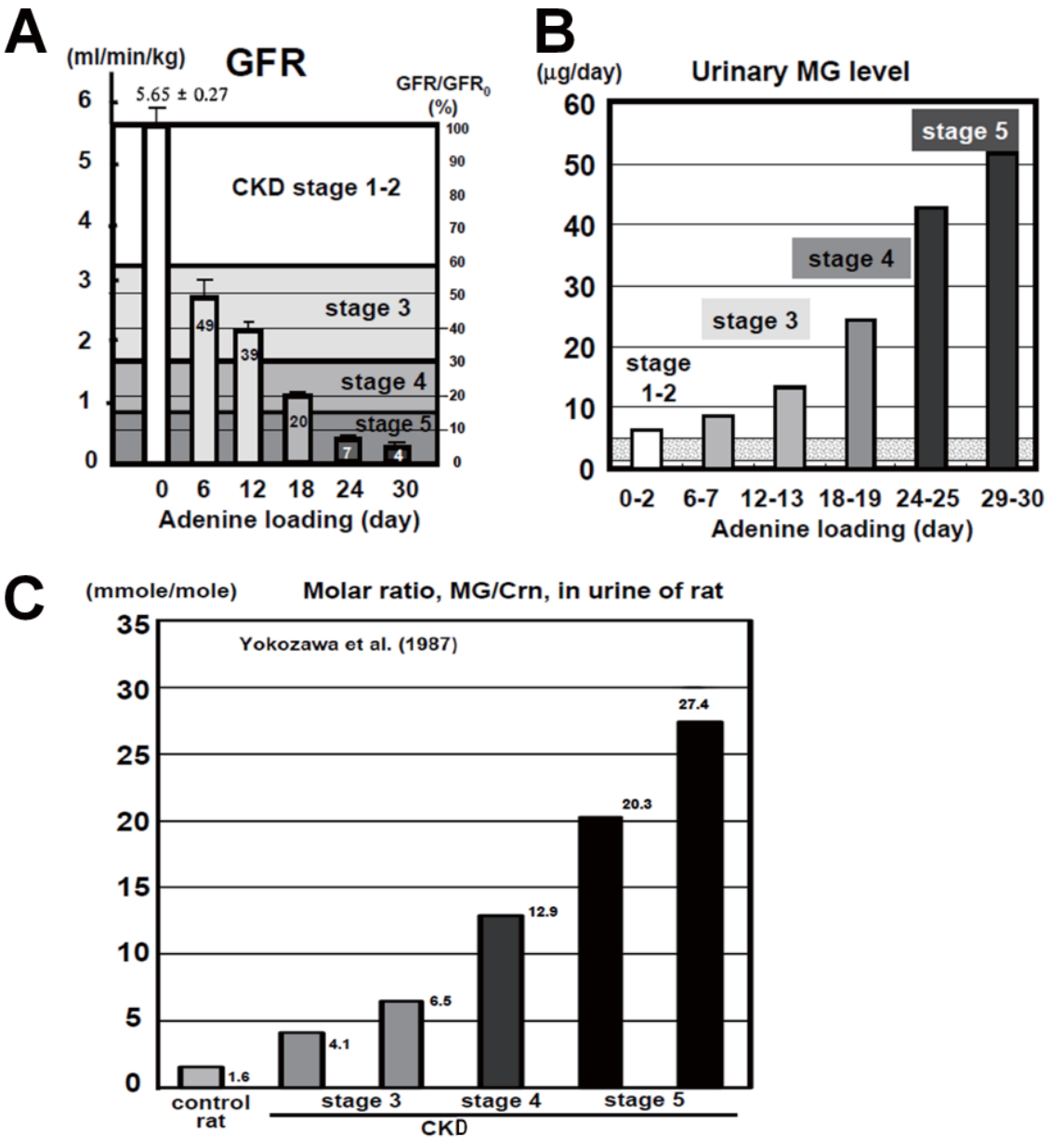


Figure 8. Increase in scavenging amount of •OH radicals with Crn in rats in proportion to the progression of CKD. A: Classification of CKD stage in adenine-loaded rats based on GFR value and its relative GFR percent to normal rats. **B:** Urinary MG level and CKD stage of rats. **C:** Urinary molar ratio, MG/Crn, and CKD stage of rats. *Ref.* Yokozawa *et al*. (1987).

in CKD rats at stage 5 is used in order to scavenge •OH radicals.

7. Presumption of places where Crn scavenges •OH radicals in mammals

7.1. *Places where Crn might scavenge •OH radicals in mammals*

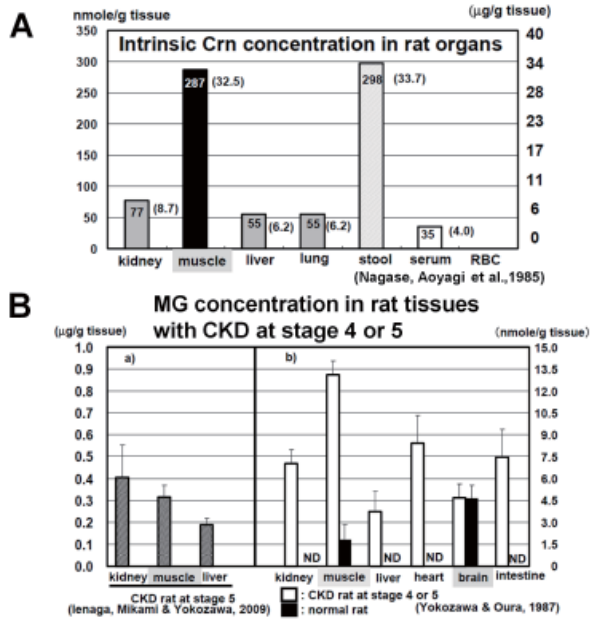


Figure 9. Concentration of Crn and MG in rat organs. A: Intrinsic Crn concentration in organs of normal rats. **B:** MG concentration in organs of rats with and without CKD at stage 4 or 5. *Ref.* Nagase *et al*. (1985); Ienaga *et al*. (2009); Yokozawa and Oura (1987).

Fluids and organs where Crn scavenges •OH radicals in mammals were presumed from the reported Crn levels in rat organs (*28*) and/or the reported MG levels in rat organs induced by Crn injection (*28*) or CKD induction (*5,59*) and from the autoradiogram of ^{14}C-Crn (*60*).

For normal rats, intrinsic Crn levels in kidney, liver and lung were nearly comparable (77, 55, 55 nmole/g tissue, respectively) but in muscle (the main Crn synthesizing organ) (287 nmole/g tissue) it was ca. five times higher (*28*) (Figure 9A).

Autoradiograms of *i.v.* injected ^{14}C-Crn (*60*) had clearly shown that Crn was easily distributed within 5 minutes and rapidly disappeared in kidney, liver, heart and lung. In contrast, ^{14}C-Crn was never absorbed in muscle and brain, where intrinsic Crn is mainly synthesized.

Detectable levels of MG have been observed in normal rat muscle and brain (*59*). In contrast in organs, which receive a fairly high blood supply, such as kidney, liver and heart, MG was readily detectable in rats with CKD at stage 4 or 5 (Figure 9B) (*5,59*). We believe that in those organs where MG was detectable, Crn scavenges •OH radicals.

7.2. *Flow diagrams of Cr and Crn*

We have illustrated the flow of Cr and Crn in mammalian bodies in Figure 10. Cr biosynthesis in mammals is fairly well established (Figure 10A) (*11*), and we illustrate analogously how Crn might be catabolized *via* CTL in mammals (Figure 10B).

In organs, where Crn is mainly synthesized, such as muscle and brain: the concentration of Crn is

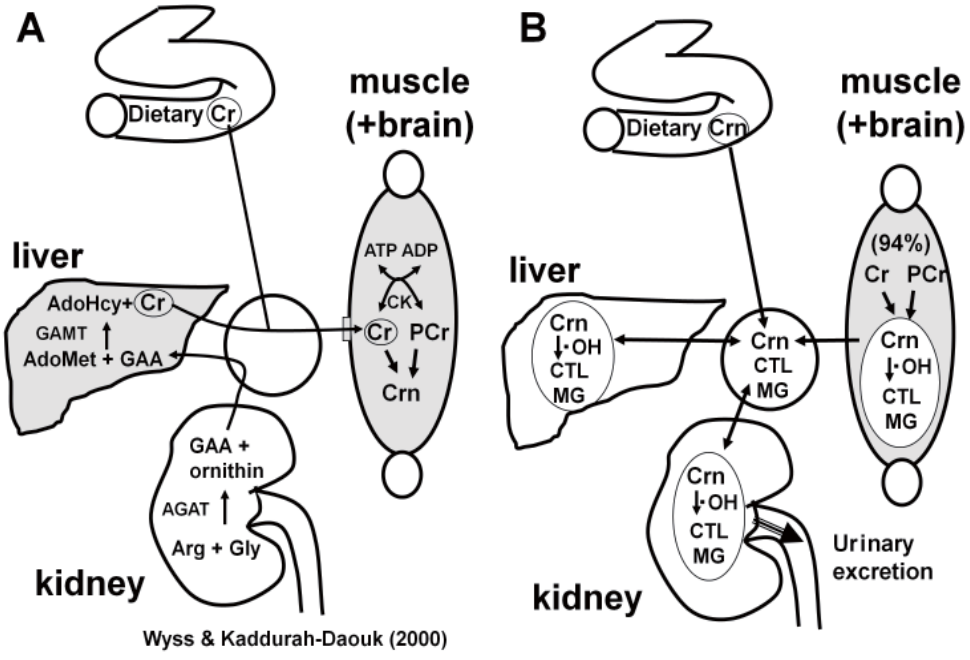


Figure 10. Flow of Cr and Crn in mammalian organs. A: Cr. *Ref.* Wyss and Kaddurah-Daouk (2000). **B:** Crn and its metabolites.

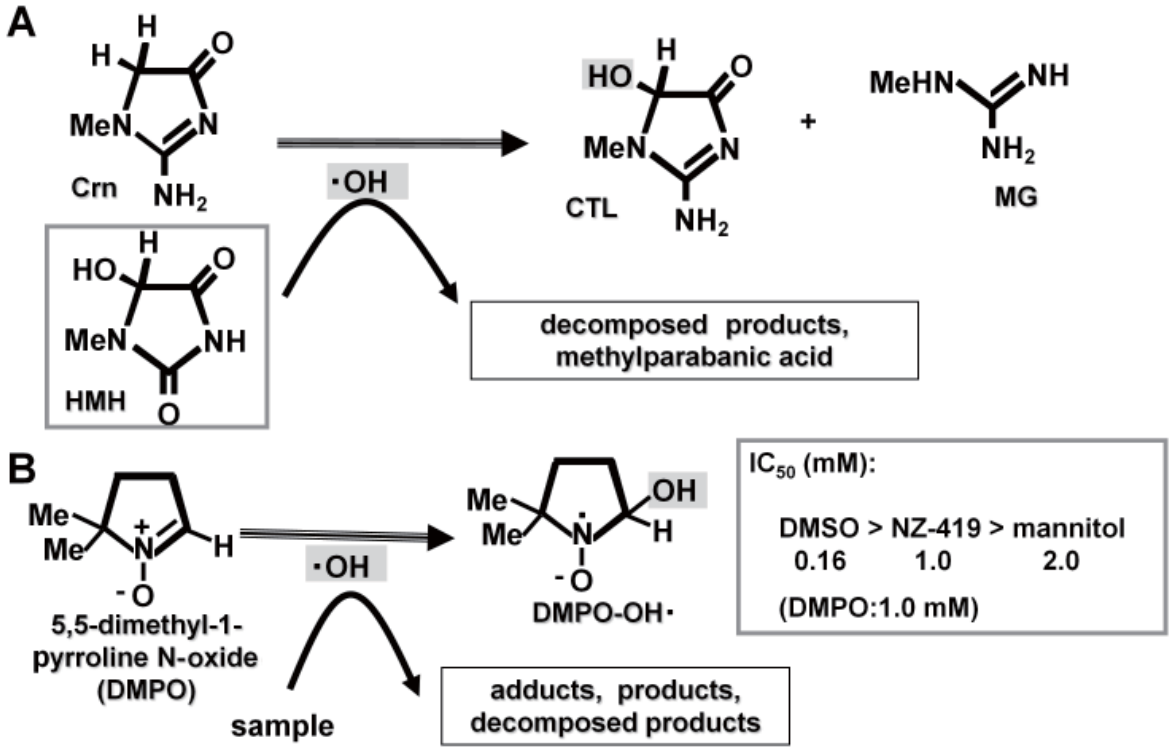


Figure 11. Antioxidant effect of HMH. A: Inhibition of CTL formation from Crn by HMH. **B:** Inhibition of DMPO-OH adduct formation by HMH.

maintained at a high level and MG is detectable not only for rats with CKD at stage 4 or 5, but also for normal rats (Figure 10B). Since the concentration of Crn never increased in those organs on injection of Crn, there seems to be a one-way flow of Crn following the concentration gradient, from muscle to blood flow. Although in contrast, in other organs such as kidney, liver and heart, where Crn is not synthesized, Crn seems to flow both in and out of the organs. In those organs, when Crn levels are elevated and the GFR decreases enough, CTL and MG are accumulated: just like MG in rats with CKD at stage 4 or 5 (Figure 10B).

8. Merits of measurement of 8-OHdG or Crn-related markers such as (CTL + MG)/Crn in mammals

If we want to know the total amount of •OH radicals in mammalian bodies, Crn-related markers are most likely to be more reliable than 8-OHdG markers for urinalysis. The absolute amount of the former is 10^3-fold higher than the latter, and the former markers are measured directly without any further degradation process, whereas the latter is indirect, requiring not only degradation from the nucleotide chain but also excretion from the nucleus or mitochondria in cells into the urine *via* cytoplasm, blood, *etc.*

However, for estimation of DNA damage by •OH radicals inside the nucleus or mitochondria, 8-OHdG is likely to be one of the best markers. Thanks to specific antisera against 8-OHdG we can show the difference in DNA damage between nucleus and mitochondria. Thus, at 8 weeks after the onset of diabetes, the levels of 8-OHdG are significantly increased in mitochondrial DNA (mtDNA) from kidney of diabetic rats but not in nuclear DNA; suggesting the predominant damage of mtDNA (*61*).

If we want to know further •OH radical levels scavenged by Crn in the nucleus and mitochondria as well as in organs, we need specific antibodies against CTL and/or MG. Preliminary results, using such kinds of antisera, have shown specific staining for CTL and MG in brain and kidney.

9. HMH as an alternative intrinsic antioxidant

Since the level of Crn might not be enough to scavenge •OH radicals in mammals with CKD at stages 3, 4 and 5 and MG starts accumulating as a uremic toxin, Crn is no more the ideal scavenger. Alternatively, HMH has been recognized as an another intrinsic antioxidant (*3,10,36-39*). Intrinsic levels of HMH increase in DM and CKD patients (*8,10*). HMH prevents the progression of CKD at stages 3-5 in rats, if enough HMH is given orally (*3,5,6,37-41*) instead of Crn. We believe that HMH might be a kind of 'self-defense' substance.

However, the •OH radical scavenging effect of HMH, tested by the ESR method, is not so very different in comparison to Crn (Figure 11B) (*36-39*): it is stronger than mannitol but weaker than DMSO. The

intrinsic concentration of HMH cannot be higher than Crn, however. Therefore, we think it is hard to explain the mode of action of HMH only by the direct •OH radical scavenging effect. Formation of CTL from Crn both under *in vitro* and *in vivo* conditions is inhibited by HMH (Figure 11A) (*3-7,35,39-41*). We have noticed that its inhibition in cells is more apparent than its effect in a cell-free system and is comparable to DMSO (*39*). We are now investigating further the cellular protective effects of HMH as an anti-oxidant, or some as yet unknown effect, in detail.

When HMH is made to react with •OH radicals, the products are too labile to be detected. Under mild conditions, only methylparabanic acid (Figure 1), an oxidative metabolite of HMH (*18,19*), can be partially monitored. At the same time, a radical of HMH seems much more labile than the radical from Crn. CTL is also an •OH radical scavenger like HMH. However, ESR data suggests that HMH and Crn are stronger than CTL in this respect. These phenomena might explain why HMH seems safe to use as a drug.

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Brief Report

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GW501516 acts as an efficient PPARα activator in the mouse liver

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ABSTRACT: The peroxisome proliferator-activated receptor (PPAR) subtype specificity of GW501516, a well-known PPARδ-specific agonist, was studied by examining its effects on the expression of endogenous genes in primary hepatocytes and the liver of wild-type and PPARα-null mice. GW501516, like the PPARα-specific agonist Wy14,643, induced the expression of several PPAR target genes in a dose-dependent manner but this action was mostly absent in the cells and liver of PPARα-null mice. Results indicated that GW501516 acts as an efficient PPARα activator in the mouse liver.

Keywords: Peroxisome proliferator-activated receptor (PPAR), GW501516, subtype specificity, lipid metabolism

1. Introduction

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated transcription factors that play important roles in lipid and glucose metabolism. The distinct PPAR subtypes PPARα, PPARδ, and PPARγ have been identified. All PPARs bind as heterodimers with the retinoid X receptor (RXR) to well-conserved response elements (PPRE) (*1*). Their functions are believed to overlap but diverge when acting on endogenous genes in various tissues although their expression patterns and ligand specificities also overlap (*2*). Knowledge of the distinct physiological functions of PPARs is based mostly on experimental results obtained using subtype-specific ligands (*3*) and gene-knockout mice (*4*) or gene-knockdown cells (*5*).

GW501516 has been widely used as a specific PPARδ agonist and experimental results have helped broaden understanding of the role of PPARδ (*6,7*). However, the effects of treatment with GW501516 are not always absent in gene-knockout animals, and thus the *in vivo* subtype specificity of the compound has not been confirmed. A previous study of PPAR agonists using primary cultured hepatocytes from wild-type and PPARα-null mice revealed that the effect of GW501516 on target gene expression could not be detected when hepatocytes derived from PPARα-null mice were used. The current study investigated this unexpected finding and concluded that GW501516 acts as an efficient PPARα activator in the mouse liver.

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2. Materials and Methods

2.1. *Compound*

GW501516 was purchased from Calbiochem (San Diego, CA, USA) or synthesized at Nippon Chemiphar (Misato, Japan) and the two compounds produced the same results. Troglitazone was synthesized at Nippon Chemiphar (*8*). (4-Chloro-6-(2,3-xylidino)-2-pyrimidinyl-thio)acetic acid (Wy14,643) and 2-(*p*-chlorophenoxy)isobutyric acid ethyl ester (clofibrate) were purchased from Tokyo-Kasei (Tokyo, Japan). Bezafibrate and gemfibrozil were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. *Animal experiments*

All protocols were approved by the Meiji Pharmaceutical University Committee for Ethics of Experimentation and Animal Care. Male C57BL or PPARα-null mice around 8 weeks in age were used as described (*9*). Mice were fed a control diet or a diet containing 0.01% GW501516 or 0.05% Wy14,643 for 2 days. The mice were sacrificed at the end of each treatment period and total RNA was isolated.

2.3. *Primary culture of mouse hepatocytes*

Mouse hepatocytes were isolated by a two-step *in situ* collagenase perfusion procedure (*10*). The livers of C57BL or PPARα-null mice were first perfused with Krebs-Ringer bicarbonate buffer at 7.2 mL/min and then with the buffer containing 0.3 mg/mL collagenase type IV at

4.6 mL/min. Cells were released from the liver into 20 mL of MEM, filtered through a cell strainer, and purified by sedimentation twice at 50 × g for 1 min. Cells were then plated on collagen-coated 6-well plates at a density of 0.35 × 10^6 cells/well. After 3 h, the medium was replaced to remove non-adherent hepatocytes and the treatment was started.

2.4. *Cell culture and DNA transfection*

CV-1 cells were maintained in DMEM (GIBCO®; Invitrogen, Carlsbad, CA, USA) with 10% (v/v) fetal bovine serum (FBS) as described (*11*). The cells were transiently transfected with plasmid DNA (0.4 μg/well) using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and a reporter assay was performed as described (*11*).

2.5. *Isolation of RNA and real-time PCR analysis*

Total RNA from mouse tissue and primary hepatocytes was prepared using TRI Reagent® (Molecular Research Center, Cincinnati, OH, USA) as described (*9*). Reverse transcription was done with the PrimeScript® RT reagent Kit (Takara Bio, Kyoto, Japan). Real-time PCR was done with a LightCycler 1.5 instrument and SYBR premix ExTaq (Takara Bio) as directed by the manufacturer. The primers for real-time PCR of PPARα-target gene transcripts were as described (*12*) and L32 was used as an internal control (*12*).

3. Results and Discussion

To confirm that primary mouse hepatocytes were suitable for the PPAR agonist assay, the primary cultured hepatocytes were treated with several PPARα agonists and the levels of mRNA expressed by the endogenous PPARα-target genes were measured. Primary hepatocytes are known to maintain liver function and presumably these cells could be used to estimate the *in vivo* response of various agonists at various doses. Quantitative real-time PCR was used to measure the levels of hydratase-dehydrogenase bifunctional enzyme (HD) mRNA in control primary hepatocytes and primary hepatocytes treated with a PPARα agonist (Figure 1A). All of the PPAR-target genes examined, including the genes acyl-CoA oxidase (AOx) and liver-type fatty acid binding protein (L-FABP), responded to an extent to a wide variety of PPARα agonists in a dose-dependent manner (not shown). This response was much stronger in primary cultured mouse hepatocytes than in a standard transactivation assay system using CV-1 cells and the PPARα expression plasmid, in which no response to the weak agonist clofibrate was observed (Figure 1B).

In vitro studies suggest that GW501516 acts strictly as a PPARδ-specific agonist in a dose-dependent manner. To test this assumption, the response of wild-type primary hepatocytes to GW501516 was compared to the response in similar hepatocytes from PPARα-null mice. Using PPARα-null hepatocytes can exclude the possibility of the presence of significant residual activity of the receptor due to insufficient knockdown. Real-time PCR was used to measure changes in the mRNA levels of the most sensitive PPARα-target genes, HD (Figure 2A) and pyruvate dehydrogenase kinase 4 (PDK4) (Figure 2B) (*13*), in primary hepatocytes with various concentrations of PPAR agonists. No response to the PPARα agonist Wy14,643 was noted in PPARα-null hepatocytes and the response to the PPARγ agonist troglitazone was not significantly affected, as expected. In contrast, the dose-dependent response to GW501516 in hepatocytes was greatly reduced or eliminated by PPARα knock-out. The residual response to GW501516 in the PPARα-null hepatocytes was detectable only in the cells treated with the compound at concentrations much higher than those reported for specific activation of PPARδ. These results suggest that the increased levels of the mRNA of PPAR-target genes induced by treatment with GW501516 are achieved by activation of PPARα and that the remaining response was induced by activation of PPARδ or PPARγ or by some other unknown pathway.

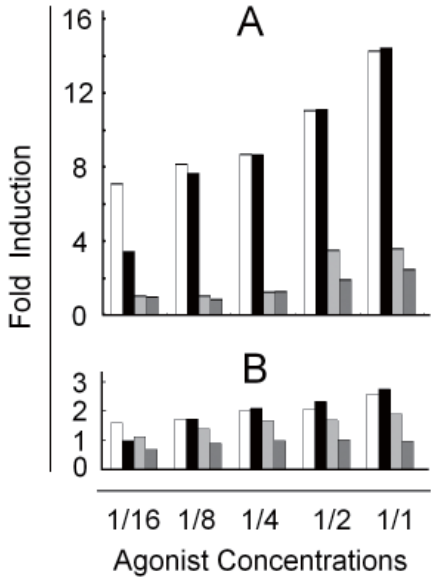

Figure 1. Cell-based PPAR ligand assay. PPARα agonist assays of various compounds were performed using the endogenous response in primary cultured hepatocytes from wild-type mice **(A)** or by reporter gene assay in cultured CV-1 cells **(B)**. **A)** Mouse primary hepatocytes were prepared and cultured with serial 2-fold dilutions of PPARα agonists for 18 h. The starting concentrations of the agonists were: Wy14,643 (open bar), 50 μM; bezafibrate (closed bar), 200 μM; gemfibrozil (lightly shaded bar), 200 μM; and clofibrate (darkly shaded bar), 200 μM. cDNA was synthesized from total RNA and the levels of mRNAs were quantified with real-time PCR. The levels of hydratase-dehydrogenase bifunctional enzyme (HD) mRNA are shown. Each agonist was assayed using independently isolated hepatocytes from at least three mice and a representative result is shown. **B)** A standard reporter gene assay was done with serial dilutions of the agonists in **A)**. Experiments were conducted at least three times and a representative result is shown. Experimental error was less than 30% in all cases.

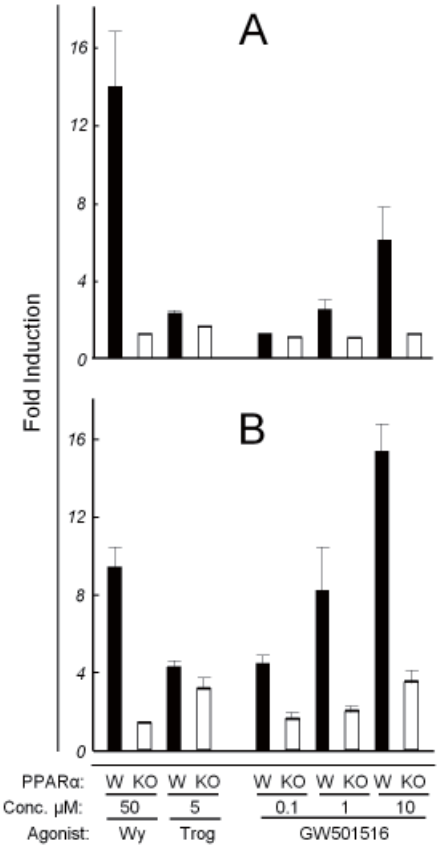

Figure 2. Effect of PPARα knockout. The effect of PPARα-knockout on the response of endogenous target genes in primary hepatocytes to the PPARδ agonist GW501516 was examined. Primary hepatocytes were prepared from wild-type and PPARα-null mice and cultured with various concentrations of GW501516 for 18 h. The PPARα agonist Wy14,643 (at 50 μM) and the PPARγ agonist troglitazone (5 μM) served as controls. cDNA was synthesized from total RNA and the levels of mRNAs of hydratase-dehydrogenase bifunctional enzyme (HD) **(A)** and pyruvate dehydrogenase kinase 4 (PDK4) **(B)** were measured with real-time PCR. Solid bars represent data from hepatocytes isolated from wild-type mice (W) and open bars represent the data from PPARα-null mice (KO). Data represent means (± S.D.) from 4 experiments.

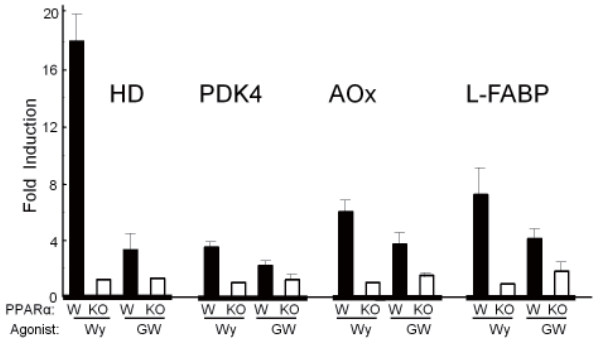

Figure 3. Response in the mouse liver. The effect of PPARα-knockout on the response of endogenous target genes to PPARδ agonist GW501516 in the mouse liver was examined. Wild-type (W) and PPARα-null mice (KO) were fed a control diet or a diet containing 0.01% GW501516 or 0.05% Wy14,643 (as the control) for 2 days. cDNA was synthesized from total RNA isolated from the liver and the levels of four PPAR-regulated mRNAs were measured with real-time PCR. Data represent means (± S.D.) from 3 experiments.

To examine whether the response of primary hepatocytes to PPAR agonists is a true reflection of *in vivo* response, wild-type and PPARα-null mice were fed a control diet or a diet containing the PPAR agonist GW501516 or Wy14,643. The levels of expression of mRNA of the target genes in the liver were then compared. As shown in Figure 3, the levels of HD, PDK4, AOx, and L-FABP mRNA increased with administration of GW501516 and Wy14,643 in the liver of wild-type mice but did not in the liver of PPARα-null mice, suggesting that the effect of GW501516 on the expression of PPAR-target genes in the liver was mostly due to activation of PPARα.

Most of the effects of GW501516 in the mouse liver that have been reported to activate lipid metabolism can be explained by its effects on PPARα instead of on PPARδ. If the subtype specificity of GW501516 is limited to PPARδ (*3*), the two nuclear receptors may play almost identical roles and there may be no physiological significance in having two overlapping receptors in the liver. This was the major impetus for the present study, which found that the effect of GW501516 on several PPAR target genes in primary hepatocytes and the liver depends on the expression of PPARα. The possibility that the effect of GW501516 on PPARα was overestimated because of the much lower level of expression of PPARδ compared to that of PPARα in the liver can be excluded given the results of a recent study by Girroir *et al.* (*14*). Using quantitative Western blotting, Girroir *et al.* found that liver tissue is one type of tissue that expresses the highest levels of PPARδ in mice. In addition, the doses of GW501516 used in the current study were lower than those suggested to be strictly specific to PPARδ. At the very least, the current doses were no higher than those used to specifically activate PPARδ in previously published studies (*5-7*). As the level of expression of PPARδ in the liver was not greatly affected by the absence of PPARα or by treatment with PPAR ligands (not shown), the present results strongly suggest that the effects of GW501516 in the liver are the result of activating PPARα. However, this does not necessarily exclude the possibility that activation of PPARα by GW501516 depends on PPARδ.

Activation of PPARα by GW501516 can be explained by two mechanisms: either by direct activation by binding of GW501516 to PPARα or by indirect activation through direct binding and activation of PPARδ. The first mechanism raises two important points; the *in vivo* subtype specificity of PPAR ligands might differ from that predicted by *in vitro* studies (*3*), and earlier experimental data using GW501516 as a PPARδ-specific ligand, and especially data from whole animal studies, may need to be re-interpreted. The subtype specificity of GW501516 for human PPARδ is reported to be > 500-fold higher than that for PPARα and PPARγ. This conclusion is based on the results

obtained using a cell-based transient transactivation assay and a binding assay using recombinant proteins. In such instances, only the ligand-binding domains (LBDs) of PPARs usually represent whole receptors. However, the LBD domains do not exist alone in the cell but interact with other domains of the receptors and with many other transcription factors, all of which affect the overall structures of the ligand-binding pockets (*15*). The results of GW501516 and PPAR-LBDs docking studies by I. Nakagome (Kitasato University, personal communication) suggest that the binding pocket of PPARα may be distorted or widened by such interactions for GW501516 to efficiently bind. Thus, the subtype specificity of a chemical compound for PPARs may differ somewhat between the subtype specificity according to an oversimplified assay system and that determined with an *in vivo* system. In a recent study of a physiologically relevant endogenous ligand for PPARα in the liver, Chakravarthy *et al*. reported a similar discrepancy in ligand specificity for *in vitro* estimations using LBD and the *in vivo* response of full-length receptors (*16*).

Indirect activation of PPARα by GW501516 could be achieved by increasing the level of expression of PPARα and its co-activators and/or by activating production of an endogenous ligand of PPARα. Since the level of PPARα expression in the mouse liver is high enough for a large response (*17*), increased production of an endogenous ligand may play a crucial role. Subsequent to the current study, Barroso *et al*. (*18*) suggested the possibility of a PPARδ-dependent effect of GW501516 on increased production of an endogenous PPARα ligand, 1-palmitoyl-2-oleoyl-phosphatidilcholine, although their suggestion is based solely on the assumption that GW501516 is a specific PPARδ ligand. They examined the long-term effect of GW501516 on high-fat diet-induced hyperglyceridemia and hepatic fatty acid oxidation in wild-type mice. Thus, increased production of an endogenous PPARα ligand was one of many changes observed, and evidence of a cause and effect relationship was not provided. PPARδ-null mice will need to be used to clarify the mechanism of PPARα activation by GW501516.

The results of the current study do not conflict with the position that PPARδ has an important physiological role in the liver. Shan *et al*. (*19*) showed that PPARδ protects against liver toxicity induced by environmental chemicals in PPARδ-null mice, so PPARδ could have a unique role in the liver. Sanderson *et al*. (*20*) suggested a unique function of PPARδ in the liver.

Preliminary results suggested that the response to GW501516 in skeletal muscle was only partially eliminated in PPARα-null mice. GW501516 appears to activate both PPARδ and PPARα in skeletal muscle. A primary cultured muscle cell system is needed for further analysis, but the effect of GW501516 is likely to differ somewhat between tissues.

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Brief Report

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Effects of Gosha-jinki-gan (Chinese herbal medicine: Niu-Che-Sen-Qi-Wan) on hyperinsulinemia induced in rats fed a sucrose-rich diet

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ABSTRACT: We investigated the effects of a Chinese herbal medicine, Gosha-jinki-gan (GJG), on the regulation of insulin levels in rats fed a sucrose-rich diet (SRD). Normal Wistar rats in the SRD group were fed an SRD for 4 weeks. Increased dietary sucrose did not alter plasma glucose levels but it increased plasma insulin levels at 2 and 4 weeks in the SRD-fed rats relative to control rats that were fed standard chow. GJG treatment significantly suppressed the SRD-induced elevation in plasma insulin levels. These results suggest that GJG improves hyperinsulinemia caused by an SRD.

Keywords: Herbal medicine, Gosha-jinki-gan, sucrose-rich diet, hyperinsulinemia, rats

1. Introduction

Insulin dysfunction, including hyperinsulinemia, is a major metabolic abnormality in populations with non-insulin-dependent diabetes mellitus (type 2 diabetes). Studies in rats have shown that a sucrose-rich diet (SRD) leads to abnormal insulin sensitivity in the liver and peripheral tissues, resulting in hypertriglyceridemia (*1*,*2*). Hypertriglyceridemia is an important risk factor for coronary heart disease, especially in populations with type 2 diabetes. In rats, hyperinsulinemia and subsequent dyslipidemia are known to occur after administration of an SRD (*2*,*3*). Hence, the relationship between hyperinsulinemia and hypertriglyceridemia is explained by the following correlations: *i*) insulin resistance and compensatory hyperinsulinemia, *ii*) hyperinsulinemia and hepatic synthesis and secretion of very low-density lipoprotein-triglyceride (VLDL-TG), and *iii*) the hepatic VLDL-TG secretion rate and plasma triglyceride concentrations (*4*).

Gosha-jinki-gan (GJG) (Niu-Che-Sen-Qi-Wan), a traditional Chinese herbal complex of 10 medical herbs, has been widely used to treat patients with melalgia, lower back pain, and numbness. Recently, GJG was reported to effectively attenuate the subjective symptoms of diabetic neuropathy (*5*,*6*). In addition, Suzuki *et al*. reported that the antinociceptive activity of GJG was significantly greater in diabetic mice than in non-diabetic mice as gauged by nitrous oxide (NO) production (*7*). Further, homeostasis model assessment of the insulin resistance (HOMA-R) index in patients with type 2 diabetes found that the index decreased significantly after GJG treatment (*8*). However, few reports have described the effects of GJG on hyperinsulinemia induced by sucrose in the diet.

The present study sought to investigate the effects of GJG on hyperinsulinemia in normal rats that were given an SRD for a period of 4 weeks.

2. Materials and Methods

2.1. *Animals*

Male Wistar rats (Japan SLC Inc., Shizuoka, Japan) weighing 180-190 g were used in this study. The rats were maintained on a standard powder diet (MF® diet; Oriental Yeast, Tokyo, Japan) for 1 week. They were allowed free access to rat chow and water and were kept in a room maintained at 22 ± 2°C with a 12-h/12-h light/dark cycle (light cycle begun at 8:00 AM). All experimental procedures were conducted according to the Osaka Ohtani University Guidelines for the Care and Use of Laboratory Animals, and the study protocol was approved by the local Animal Ethics Committee.

2.2. *Drugs*

Spray-dried GJG powder was manufactured and supplied by Tsumura & Co. Ltd. (Tokyo, Japan). The composition of GJG is as follows: 5 g of Rehmanniae Radix (*Rehmannia glutinosa* Liboschitz); 3 g each of

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Achyranthis Radix (*Achyranthes bidentata* Blume), Corni Fructus (*Cornus officinalis* Sieb. et Zucc), Dioscoreae Rhizoma (*Dioscorea batatas* Decaisne), Plantaginis Semen (*Plantago asiatica*), Alismatis Rhizoma (*Alisma orientale* Juzep), Hoelen (*Poria cocos* Wolf), and Moutan Cortex (*Paeonia suffruticosa* Andrews); and 1 g each of Cinnamomi Cortex (*Cinnamomum cassia* Blume) and Aconiti Tuber (*Aconitum carmichaelii* Debeaux). The three-dimensional high-performance liquid chromatography (HPLC) profile of the GJG extract powder provided by Tsumura Inc. is shown in Figure 1.

2.3. *Animal treatments and collection and preparation of blood samples*

The rats were randomly divided into 3 groups consisting of 5 rats each. Rats in the control group were maintained on standard chow. The rats in the SRD group were maintained on standard chow supplemented with 50% sucrose (370 kcal/100 g chow) without GJG, whereas those in the SRD + GJG group were fed chow containing 50% sucrose and 1% powdered GJG extract. The rats had access to the chow and tap water *ad libitum*. Body weights of the rats and the food and water intake per cage were measured on a weekly basis. For 4 weeks, non-fasting blood samples were collected daily from the jugular vein at 10:00 AM, and the samples were stored in chilled tubes with 30 mM EDTA (final concentration).

2.4. *Assays to determine plasma glucose, triglyceride, cholesterol, and insulin levels*

Plasma glucose levels were determined using a commercial assay kit (Glucose CII-Test Wako; Wako Pure Chemical Industries Ltd., Osaka, Japan). Plasma triglyceride and cholesterol levels were determined using the commercial lipid assay kits Triglyceride E-Test Wako and Cholesterol E-Test Wako, respectively (Wako Pure Chemical Industries Ltd.). Plasma immunoreactive insulin levels were measured using a commercial radioimmunoassay kit (Insulin Eiken RIA kit; Eiken Chemical Co. Ltd., Tokyo, Japan).

2.5. *Data analysis*

Experimental data are expressed as mean values with standard deviations (S.D.). Statistical analysis of the differences between the mean values obtained was performed using Tukey's multiple comparison test and an unpaired Student's *t* test with a significance level of $p < 0.05$.

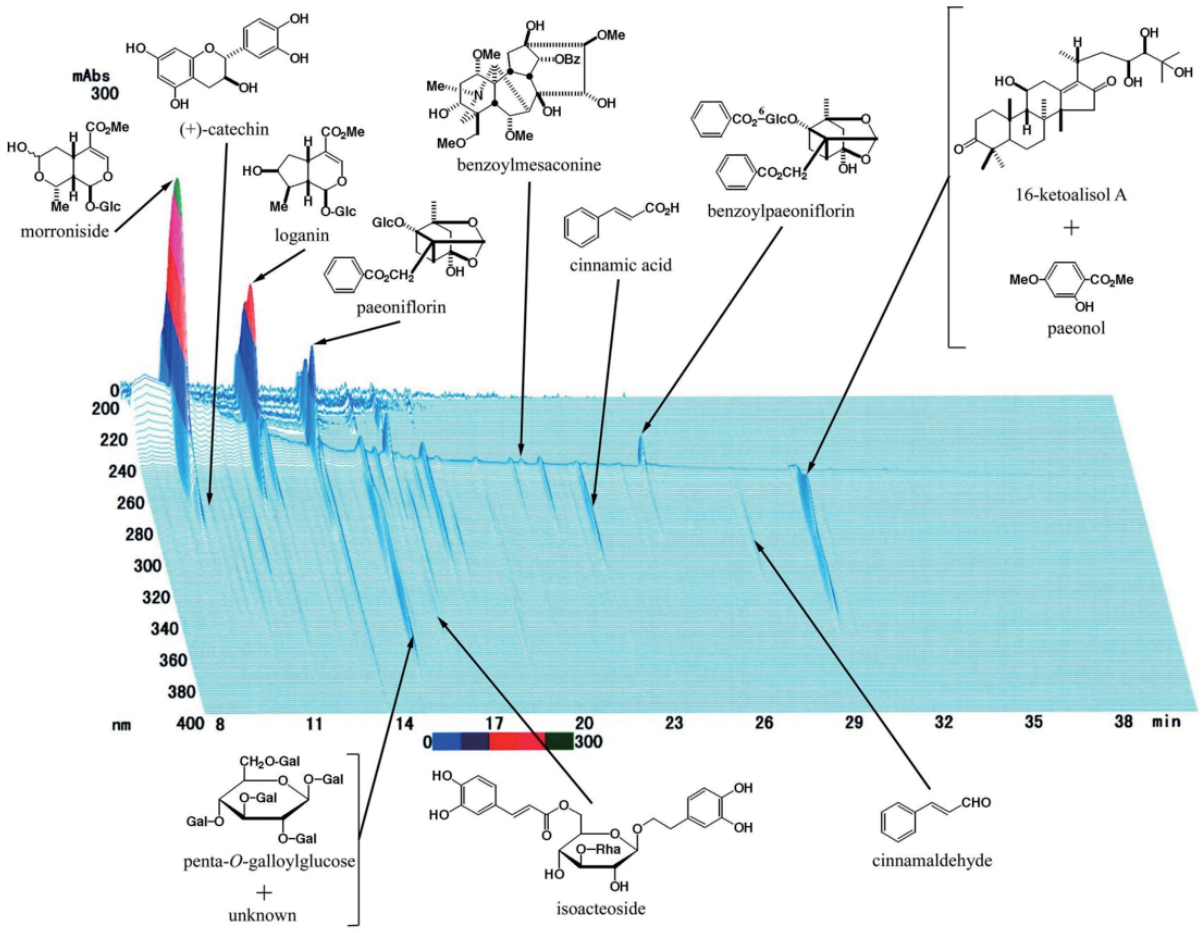

Figure 1. Three-dimensional HPLC profile of GJG.

3. Results and Discussion

Sucrose is a major ingredient of many processed foods and has been implicated in the development of obesity and dyslipidemia in humans (*9,10*). Further, diets rich in sucrose are known to induce hyperinsulinemia and hypertriglyceridemia in rodents (*11,12*). Rodent models of diet-induced hyperinsulinemia and hypertriglyceridemia are used to assess the therapeutic efficacy of drugs and nutrients that are likely to affect insulin sensitivity and lipid concentration in the blood (*12,13*). However, the effects of GJG in ameliorating the metabolic dysregulation induced by high sucrose intake have not been previously reported.

The three-dimensional HPLC profile of the GJG extract is shown in Figure 1. Standard compounds that have been isolated, purified, and identified (*via* mass spectroscopy, infrared spectroscopy, and nuclear magnetic resonance) from botanical raw materials in GJG are analyzed under the same conditions, and data from the UV spectra and column retention times are used to create a chromatogram library. Subjecting the library to a peak detector (an auxiliary function of HPLC) allows evaluation of the degree of similarity of peaks and peak purity. Morroniside, loganin, and paeoniflorin were the major components of GJG; (+)-catechin, penta-*O*-galloylglucose, isoacteoside, benzoylmesaconine, cinnamic acid, benzoylpaeoniflorin, cinnamaldehyde and 16-ketoalisol A were also detected.

The changes in the body weights of the rats are shown in Figure 2. These changes were significantly lower in the SRD-fed rats than in the control rats ($p < 0.05$ or $p < 0.01$), except in week 1. The changes in body weight in the rats administered SRD + GJG were similar to those in the control rats. The food and water intake by the SRD and SRD + GJG groups was similar to that by the control group (data not shown). An SRD alters energy partitioning in a way that is conducive to body-weight gain (*4,14*). However, the body weights of the SRD-fed rats significantly decreased in the current study (Figure 2). Sucrose has been reported to increase body weight when administered in solution form but not when added to chow (*4*). Addition of sucrose to the drinking water of rats induces obesity with intraabdominal fat deposition. Hence, the decrease in the body weights of rats in the present study was probably due to a decrease in food intake as a result of the high sucrose content in the chow. Notably, food and water intake did not vary among the 3 groups. These results indicate that GJG restored the body weights of the SRD-fed rats.

No significant changes were detected in the plasma glucose levels of any of the 3 groups throughout the study period, except in the SRD group in week 4 (Figure 3A). Figure 3B shows the changes in the non-fasting plasma insulin levels during the study period. In the SRD group, the non-fasting plasma insulin levels were significantly higher than those in the control group ($p < 0.05$). Further, the SRD + GJG group had significantly decreased plasma insulin levels in weeks 2 and 4 ($p < 0.05$) compared to the SRD group. In the present study, administration of SRD alone was followed by a significant increase in the plasma insulin levels; as a consequence, the plasma glucose levels tended to decrease. SRD + GJG administration significantly prevented the development of hyperinsulinemia in weeks 2 and 4 ($p < 0.05$) (Figure 3B); therefore, GJG normalized the plasma glucose and insulin levels of the rats fed SRD. An important point is that GJG alone does not reduce plasma glucose and insulin levels in normal rats (*15*). The nitric oxide pathway has been reported to potentially mediate the effects of GJG on insulin action in insulin-sensitive tissues (*15*). Cinnamomi cortex, a component of GJG, has been shown to improve insulin action by enhancing the insulin-signaling pathway in skeletal muscles (*16*). Qin *et al*. reported that GJG administration improved impaired insulin sensitivity in rats with streptozotocin (STZ)-induced diabetes (*17*). Since GJG is a complex medical preparation containing individual ingredients with different pharmacological actions, further studies are required to ascertain the molecular mechanism by which GJG affects insulin sensitivity.

No significant changes in the plasma triglyceride levels were detected in the SRD and SRD + GJG group

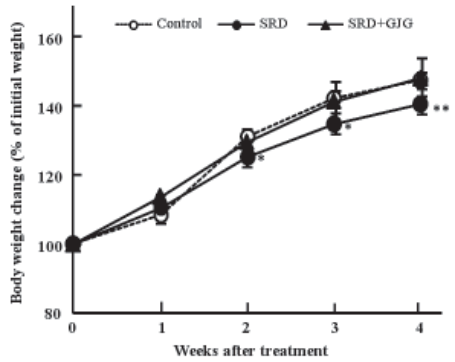

Figure 2. Effects of GJG on the post-treatment body weight of SRD-fed rats. * $p < 0.05$, ** $p < 0.01$ compared to the control group. Data are mean ± S.D. ($n = 5$).

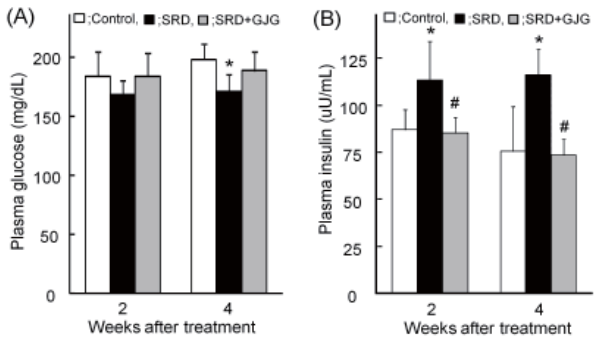

Figure 3. Effects of GJG on post-treatment plasma glucose (A) and insulin (B) levels in SRD-fed rats. * $p < 0.05$ compared to the control group. # $p < 0.05$ compared to the SRD group. Data are mean ± S.D. ($n = 5$).

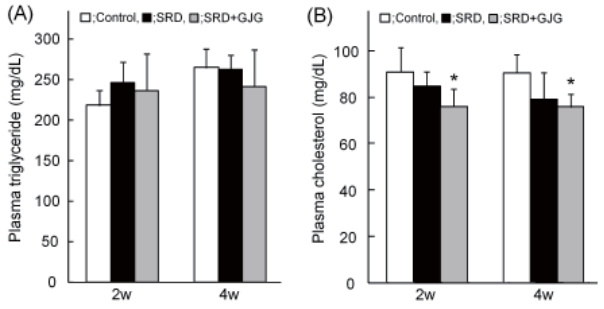

Figure 4. Effects of GJG on post-treatment plasma triglyceride (A) and cholesterol (B) levels in SRD-fed rats. * $p < 0.05$ compared to the control group. Data are mean ± S.D. ($n = 5$).

rats in weeks 2 and 4, compared to levels in the control group (Figure 4A). The non-fasting plasma cholesterol levels in the SRD group did not significantly differ from those in the control group, whereas those in the SRD + GJG group were significantly lower than the levels in the control group in weeks 2 and 4 ($p < 0.05$; Figure 4B). Chemical compounds such as alisol A and cinnamon in GJG components might decrease plasma cholesterol levels (*18,19*). That said, short-term administration of an SRD was not found to alter plasma triglyceride and cholesterol levels. However, long-term administration of GJG has been found to reduce elevated plasma triglyceride levels induced by SRD treatment (*20*).

In conclusion, the data in the present study suggest that GJG may prove useful in the treatment and/or prevention of hyperinsulinemia in non-diabetic subjects. However, further investigation is needed to elucidate the molecular mechanisms associated with the effects of GJG.

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Brief Report

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Sedative and anxiolytic effects of the methanolic extract of *Leea indica* (Burm. f.) Merr. leaf

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ABSTRACT: The sedative and anxiolytic potential of *Leea indica* (Burm. f.) Merr., a Bangladeshi tribal medicinal plant was studied for the first time. The crude methanol extract of *L. indica* leaves was evaluated for its central nervous system (CNS) depressant effect using rodent behavioral models, such as hole cross, open field and thiopental sodium induced sleeping time tests for its sedative properties and an elevated plus-maze (EPM) test for its anxiolytic potential, respectively. The methanol extract of *L. indica* at doses of 200 mg/kg, *p.o.* and 400 mg/kg, *p.o.*, displayed a dose dependent suppression of motor activity, exploratory behavior (in hole cross and open field tests) and prolongation of thiopental induced sleeping time in mice; the highest CNS depressant effect was shown at a dose of 400 mg/kg, *p.o.* In the EPM test, both doses of methanol extract significantly ($p < 0.01$) increased exploration to and time spent by the treated mice in EPM open arms in a dose dependent manner. These results provide *in vivo* evidence that leaves of *L. indica* in general have significant sedative and anxiolytic effects. However, these results may rationalize the scientific basis for use of this plant in traditional medicine for treatment of anxiety and related disorders.

Keywords: Neuropharmacology, open field, elevated plus-maze (EPM), medicinal plant, *L. indica*

1. Introduction

Anxiety disorders are the most common emotional disorders affecting people in all countries worldwide. It is reported that more than 20% of the adult population suffer from these conditions at some stage during their life (*1,2*). Anxiety is a natural emotion but becomes a problem when it occurs too often. According to the U.S. National Institute of Mental Health (NIMH), anxiety disorders can be related to other mental/emotional disorders, including depression and traumatic events. They can also be related to other physical illnesses. These illnesses include endocrine disorders such as thyroid disorders and problems with glucose regulation in the body, including diabetes and hypoglycemia. Although medications can not fully cure anxiety disorders they can, to a great degree, relieve the symptoms and reduce their occurrences. Prescription drugs which are commonly used in treating anxiety disorders may include Benzodiazepines (commonly known as anxiolytics) and several types of antidepressants, especially those from the group of selective serotonin reuptake inhibitors (SSRI). β-Adrenergic blocking drugs, to be more precise, could also be prescribed for reducing the peripheral symptoms such as palpitations and tremors.

Leea indica (Burm. f.) Merr. (Family Leeaceae) is a large evergreen shrub or small tree (*3-5*) indigenous to tropical Asia, Australasia, and the Pacific and grown mostly in Bangladesh, India, China, Bhutan, and Malaysia. Plant pacifies vitiated pitta, diarrhea, dysentery, colic, ulcers, skin diseases, vertigo, and headache. Marma of Chittagong Hill Tracts, Bangladesh, prescribes combined root paste of this plant along with the root of *Oreocnide integrifolia* and *Cissus repens* for bubo and boils (*6,7*). The plant leaf is also used as a folk medicine in the treatment of insomnia, although it is not reported yet in the literature.

The present study dealt with various psycho-pharmacological effects of the methanol extract from the leaves of *L. indica* on some neuropharmacological experimental models. Previous biological studies have shown that it possessed strong antioxidant and antimicrobial activities but still there is no report showing the sedative and anxiolytic effect of *L. indica* leaves on an animal behavioral model. In view of that, it was thus necessary to expand the present study for sedative anxiolytic potential and possible mechanisms underlying action.

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2. Materials and Methods

2.1. *Drugs and chemicals*

The following drugs and chemicals were used in this study: diazepam (Square Pharmaceutical Ltd., Bangladesh), thiopental sodium (Gonoshastho Pharmaceuticals Ltd., Bangladesh), methanol (Sigma Chemicals Co., USA).

2.2. *Plant material*

The plant was collected from Forests of Chittagong Hill Tracts in October 2010 when leaves were in their maximum densities. The plant leaves were thoroughly washed with water and were dried in a hot air oven at room temperature for 7 days and at 40°C for the next 2 days.

2.3. *Preparation of plant extract*

The dried leaves were coarsely powdered and about 1,000 g of powdered material was macerated with 99% methanol at room temperature for a period of 7 days with occasional shaking and stirring. The whole mixture was filtered and the filtrate thus obtained was concentrated using a rotary evaporator (Bibby RE200, Sterlin Ltd., UK) to get a viscous mass. The viscous mass was kept at room temperature under a ceiling fan to get a dried extract (about 10%). The extract prepared was used for pharmacological screening.

2.4. *Animals*

White female albino mice (Swiss-webstar strain, 25-35 g body weight) were collected from the animal research branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDRB). The animals were provided with standard laboratory food and tap water *ad libitum* and maintained on a natural day night cycle. All experiments were conducted under isolated and noiseless conditions. Test animals were divided into two groups at doses of 200 and 400 mg/kg body weight. The animals were acclimatized to laboratory conditions for one week prior to experimentation.

2.5. *Hole cross test*

The method was carried out as described by Takagi *et al.* (*8*). A steel partition was fixed in the middle of a cage of 30 × 20 × 14 cm. A hole of 3 cm diameter was made at a height of 7.5 cm in the center of the cage. The animals were divided into control, positive control, and test groups containing five mice each. The test groups received methanol extract of *L. indica* at doses of 200 and 400 mg/kg body weight orally whereas the control group received vehicle (1% Tween 80 in water). The number of passages of a mouse through the hole from one chamber to the other was counted for a period of 3 min at 0, 30, 60, 90, and 120 min after oral administration of both doses of the test drug.

2.6. *Open field test*

In the open field test, the animals were divided into control, positive control, and test groups containing five mice each. The test groups received methanol extract of the leaves of *L. indica* at doses of 200 and 400 mg/kg body weight orally whereas the control group received vehicle (1% Tween 80 in water). The floor of a half square meter open field (*9,10*) was divided into a series of squares each alternatively colored black and white. The apparatus had a 40 cm height wall. The number of squares visited by the animals was counted for 3 min at 0, 30, 60, 90, and 120 min after oral administration of both doses of the test drug.

2.7. *Thiopental sodium induced sleeping time test*

The animals were randomly divided into five groups consisting of five mice each. The test groups received methanol extract from the leaves of *L. indica* at doses of 200 and 400 mg/kg body weight while the positive control was treated with diazepam (1 mg/kg) and control vehicle (1% Tween 80 in water). Thirty minutes later, thiopental sodium (40 mg/kg) was administered to each mouse to induce sleep. The animals were observed for the latent period (time between thiopental administrations to loss of righting reflex) and duration of sleep *i.e.* time between the loss and recovery of righting reflex (*11*).

2.8. *EPM test*

The method initially suggested by Handley and Mithani was employed with minor modifications (*12*). The apparatus consists of two open arms (5 × 10 cm) and two closed arms (5 × 10 × l5 cm) radiating from a platform (5 × 5 cm) to form a plus-sign figure. The apparatus was situated 40 cm above the floor. The open arms edges were 0.5 cm in height to keep the mice from falling and the closed-arms edges were 15 cm in height. Sixty minutes after administration of the test drugs, each animal was individually placed in the center of the EPM and were allowed 5 min for free exploration. Next, the number of open and enclosed arm entries, and time spent on open arms were manually registered (*13*). Entry into an arm was defined as the point when the animal placed all four paws onto the arm. The percentage of open arm entries (100 × open/total entries) and the percentage of time spent in the open arms (100 × open/(open + enclosed)) were calculated for each animal. Observations made from an adjacent corner produced significant ($p < 0.001$, $p < 0.05$) decreases of locomotion from its initial value during the period of the experiment (Table 1). Maximum suppression of locomotor activity was displayed at the dose of 400 mg/kg body weight, which

was comparable to the reference drug diazepam.

2.9. *Statistical analysis*

Data were expressed as mean ± standard error of mean (S.E.M.). Statistical comparisons were performed using one way ANOVA followed by Dunnett's multiple comparison test (DMCT). The values obtained were compared with the vehicle control group and were considered statistically significant when $p < 0.05$.

3. Results

3.1. *Thiopental sodium induced sleeping time test*

In the thiopental induced hypnosis test, methanol extract of leaves of *L. indica* at the dose of 400 mg/kg, induced the sleep at an earlier stage while the same extract at the dose of 200 mg/kg was also found to have a dose dependent effect on the onset of thiopental induced sleep. In addition, both doses dependently prolonged the duration of sleeping time in test animals compared to controls (Figure 1).

Table 1. EPM test of *L. indica*

Animal group	% number of entry into open arm	% time (in seconds) spent in open arm
I (Control)	55.88 ± 2.133	51.93 ± 8.243
II (Diazepam)	76.28 ± 1.847**	79.39 ± 5.749*
III (*L. indica* 400)	80.27 ± 2.524**	86.13 ± 5.355*
IV (*L. indica* 200)	65.71 ± 2.607*	68.33 ± 4.201

Effect of methanolic extract from *L. indica* on the percentage number of entries and the time in seconds spent in open arms of the EPM during the 5-min test session. Values are mean ± S.E.M., ($n = 5$); * $p < 0.05$, ** $p < 0.01$, Dunnet test as compared to control (Vehicle = 0.4 mL/mouse).

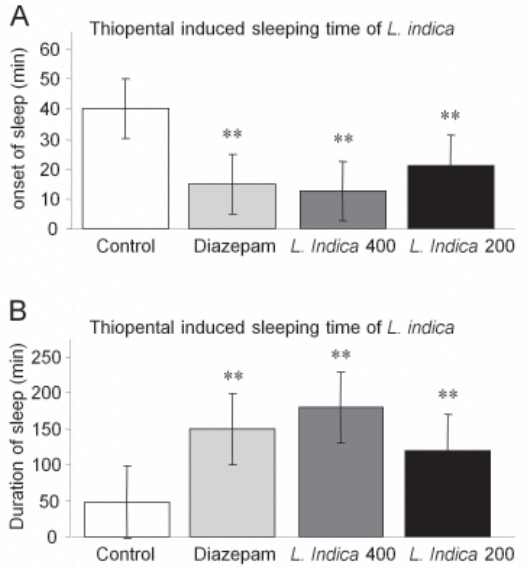

Figure 1. Effect of methanol extract from leaves of *L. indica* on thiopental induced sleeping time in mice. Values are mean ± S.E.M., ($n = 5$); * $p < 0.05$, ** $p < 0.01$. Dunnet test as compared to control (Vehicle = 0.4 mL/mouse).

3.2. *Hole cross test*

Both doses, at 400 mg/kg and 200 mg/kg body weight produced a significant ($p < 0.01$) decrease of locomotion from its initial value during the period of the experiment (Figure 2). Maximum suppression of locomotor activity was displayed at the dose of 400 mg/kg body weight, which was comparable to the reference drug diazepam.

3.3. *Open field test*

The number of squares traveled by the mice was suppressed significantly in the second observation period at both dose levels (200 and 400 mg/kg body weight) of the methanol extract from the leaves of *L. indica.* The results were dose dependent and statistically significant (Figure 3).

3.4. *EPM test*

The methanol extract of *L. indica* at the dose of 400 mg/kg body weight, significantly increased the percentage of entries (Table 1) of mice into the open arms, and the percentage of time spent (Table 1) in the open arms of the EPM. The effects of treatment of mice at the dose of 200 mg/kg body weight, on open arm entries and time spent in open arms were dose dependent.

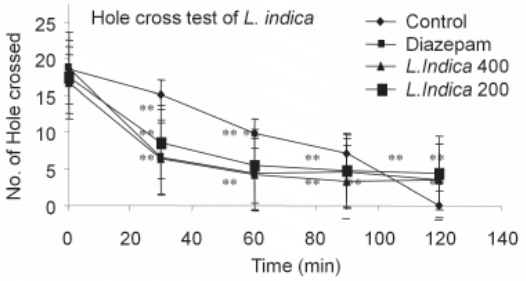

Figure 2. Effect of methanol extract from leaves of *L. indica* on hole cross test in mice. Values are mean ± S.E.M., ($n = 5$); * $p < 0.05$, ** $p < 0.01$. Dunnet test as compared to control (Vehicle = 0.4 mL/mouse).

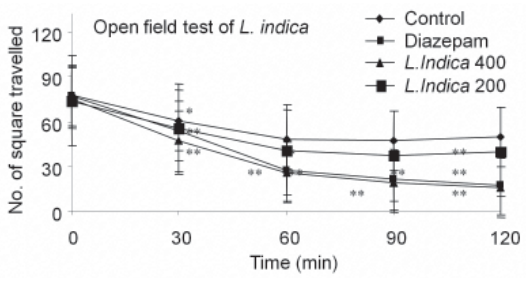

Figure 3. Effect of methanolic extract from leaves of *L. indica* on open field test in mice. Values are mean ± S.E.M., ($n = 5$); * $p < 0.05$, ** $p < 0.01$, Dunnet test as compared to control (Vehicle = 0.4 mL/mouse).

4. Discussion

The present study demonstrated that the administration of different doses (200 and 400 mg/kg body weight) of methanol extract from *L. indica* leaves shows strong sedative and antianxiety properties. Both doses dependently potentiated sleep induced by thiopental suggesting that the leaves of the plant possesses a sleep inducing property. "Thiopental" basically a hypnotic agent, given at appropriate dose, induced hypnosis by potentiating GABA mediated postsynaptic inhibition through allosteric modification of $GABA_A$ receptors. Substances which possess CNS depressant activity either decrease the time for onset of sleep or prolong the duration of sleep or both (*14,15*). In addition, the study on locomotor activity, as measured by hole cross and open field tests, showed that both doses of methanol extract from the leaves of *L. indica* decreased the frequency and the amplitude of movements. Since locomotor activity is a measure of the level of excitability of the CNS (*16*), this decrease in spontaneous motor activity could be attributed to the sedative effect of the plant extracts (*17*). Both doses significantly decreased locomotion in mice. The locomotor activity lowering effect was evident at the 2nd observation (30 min) and continued up to the 5th observation period (120 min). The results were also dose dependent and statistically significant (Figures 2 and 3).

However, the anxiolytic effect was evidenced by the EPM test that has been recognized as a valuable model able to predict anxiolytic effects of drugs in rodents (*18*). The anxiolytic effect is observed when the experimental drug increases open arms entries without altering the total number of arm entries (*19*). Although the methanol extract at 200 mg/kg body weight, in mice, did not display a significant increase in the percentage of entries into open arms, the same extract at the dose of 400 mg/kg body weight showed a significant increase in the percentage of time spent in the open arms of the maze. This was slightly larger than the effects observed following treatment with the reference anxiolytic drug diazepam, in a dose dependent manner. These results could indicate an anxiolytic-like activity of the methanol extract from the leaves of *L. indica*.

$GABA_A$-benzodiazepine receptors are the most abundant inhibitory receptor (*20*) system in the CNS and binding of a benzodiazepine agonist to its recognition site results in increased chloride ion flux (*21*) which in turn hyperpolarizes the postsynaptic membrane at a level below that at which spike generation is possible and for this reason some $GABA_A$ agonists are frequently used for their hypnotic effects. The compounds identified from the leaves of *L. indica* (*22,23*) contain ursolic acid and several other compounds like eicosanol, farnesol and β-sitosterol, act as $GABA_A$ agonists and this agonistic property could be attributed to the CNS depressant effect of *L. indica* leaves although there is no consensus about which substances are exactly responsible for these effects. However, further studies are necessary to evaluate the contribution of other substances that are isolated for the activity observed, because it still remains to be determined which components exactly were responsible for these effects.

5. Conclusion

The results from the experiments confirmed that the methanol extract from *L. indica* leaves possesses a strong sedative and anxiolytic potential. Therefore, we advance the suggestion that this extract may fulfill the therapeutic need for the treatment of anxiety and related neuropsychiatric disorders. However, further studies would be necessary to evaluate the contribution of other substances for the activity showed as it still remains to be determined which components were exactly responsible for these effects.

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Original Article

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Hyaluronic acid in combination with chondroitin sulfate and hyaluronic acid improved the degeneration of synovium and cartilage equally in rabbits with osteoarthritis

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ABSTRACT: The purpose of this study was to compare the chondroprotective effects of chondroitin sulfate (CS)-hyaluronic acid (HA) (CS-HA) injection and HA injection in an experimental model of osteoarthritis. After induction of osteoarthritis in rabbits, 28 rabbits were randomized into four groups: control group, 'HA' group, 'CS' group, and 'CS-HA' group. After 7 days, rabbits in the control group, 'HA' group, 'CS' group and 'CS-HA' group were respectively treated with normal saline, HA, CS, or CS-HA injection in the knees. All animals were treated once weekly. The animals were treated continuously for 5 weeks. Histological and biochemical evaluations were performed. As shown by histological observation, CS-HA injection treatment showed a chondroprotective effect on osteoarthritis. However, the histological scores of 'HA' group and 'CS-HA' group were not significantly different ($p > 0.05$). The results of biochemical evaluation showed that the expression levels of IL-1β, TNF-α, TIMP-1 and NO in synovial fluid of treated groups were all different from the control group ($p < 0.05$). However, the expression levels of these biochemical molecules in three treated groups were not significantly different ($p > 0.05$). In conclusion, CS-HA injection showed no obvious advantage over HA injection in osteoarthritis treatment.

Keywords: Osteoarthritis, histological evaluation, Mankin score, biochemical evaluation

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1. Introduction

Osteoarthritis is among the most frequent and symptomatic medical problems for the middle-aged and elderly. The main features of osteoarthritis include slow-developing joint pain, stiffness, and hypertrophy accompanied by limitation of motion (*1,2*). Osteoarthritis is also referred to as osteoarthrosis, degenerative arthropathy, hypertrophic arthritis or senile arthritis (*3*). In clinical practice, osteoarthritis of the knee is most common. The exact etiology, pathogenesis, and progression of this disease have yet to be determined (*4*). Studies have indicated that inflammation of the synovium might play an important role in its pathogenesis (*5,6*).

Chondroitin sulfate (CS) is a natural complex polysaccharide belonging to glycosaminoglycans (GAGs) composed of alternate disaccharide sequences of differently sulfated residues of D-glucuronic acid (GlcA) and of D-*N*-acetyl-galactosamine (GalNAc) linked by (1→3) bonds (*7*). CS is currently recommended by the European League Against Rheumatism (EULAR) as a symptomatic slow acting drug for osteoarthritis (SYSADOA) in Europe in the treatment of knee and hand osteoarthritis based on research evidence and meta-analysis of numerous clinical studies (*7-10*). Furthermore, recent clinical trials demonstrated its possible structure-modifying effects (*10,11*). CS prevents joint space narrowing and reduces joint swelling and effusion. To produce these effects, CS elicits an anti-inflammatory effect at the chondral and synovial levels (*12*).

Hyaluronic acid (HA) is a glycosaminoglycan composed of D-glucuronic acid and D-*N*-acetyl-glucosamine, with versatile biological activities. High molecular weight HA has been used in the treatment of human and animal osteoarthritis. Intra-articular HA treatment of the knee of patients with osteoarthritis has been verified to reduce painful symptoms and improve joint mobility. The purpose of intra-articular HA therapy is to make up for the loss of viscoelasticity of synovial fluid induced by inflammation and to protect against the

degradation of cartilage (*13,14*).

The aim of this study was to evaluate the effect of intra-articular injection of the combination of CS and HA on osteoarthritis to probe its feasibility in treating osteoarthritis. Meanwhile, the levels of biochemical molecules such as IL-1β, TNF-α, TIMP-1, iNOS and NO were also monitored to evaluate the effect of the combination of CS and HA on inflammation.

2. Materials and Methods

2.1. *Preparation of injections*

HA (Injection Grade) with M_r of 1.5~2.0 × 10^6 was obtained from a bacterial strain of *Streptococcus zooepidemicus* and was provided by Shandong Freda Biopharm Co., Ltd. (Ji'nan, Shandong, China). CS purified from porcine cartilage (M_w 35~50 kDa, Injection Grade) was purchased from DongCheng Biochemicals Co., Ltd. (Yantai, Shandong, China). The ratio of CS-A to CS-C was 5.6:1. An injection of CS and HA (CS-HA injection) was prepared according to the following steps: 2.0 g of CS was dissolved in 100 mL of phosphate buffer (pH 7.4); the solution was adjusted to pH 7.30 with 0.05% NaOH; then the solution was heated and kept at 100°C for 30 min; after cooling, the solution was filtered using a 0.45 μm filtration membrane; then 1.0 g of sodium hyaluronate was added to the solution and dissolved sufficiently; the compound solution was sterilized twice with flowing steam, for 30 min each; CS-HA injections were filled under aseptic environments and each injection contained 0.3 mL of compound solution.

An injection of CS (CS injection) was prepared: 2.0 g of CS was dissolved in 100 mL of phosphate buffer (pH 7.4); the solution was adjusted to pH 7.30 and was heated and kept at 100°C for 30 min; after cooling, the solution was filtered using a 0.45 μm filtration membrane; then the solution was sterilized with flowing steam and each injection contained 0.3 mL of solution. An injection of HA was also prepared. Briefly, 1.0 g of HA was added into 100 mL of phosphate buffer (pH 7.4) and dissolved sufficiently. Then the sterilization and aseptic filling were accomplished. Each injection contained 0.3 mL of solution.

2.2. *Induction and treatment of osteoarthritis in rabbits (animal experimentation)*

Papain was from Sigma-Aldrich (St Louis, MO, USA). Adult skeletally mature New Zealand White rabbits (body weight 2.5~3.0 kg) provided by Centre for Drug Safety Evaluation of Shandong Province (Ji'nan, Shandong, China) were housed individually in cages. Osteoarthritis was induced according to the method described in our previous report (*14*): 0.3 mL of sterile papain solution was injected into the both knees of the rabbits (1 mL of the solution containing 4.0 mg of papain and 50 mg of cysteine hydrochloride) under general anesthesia. After osteoarthritis induction, 28 rabbits were randomized into four groups: control group, 'HA' group, 'CS' group and 'CS-HA' group. After 7 days, rabbits in the control group (n = 7) were injected with 0.3 mL of normal saline in the knees. Rabbits in the 'HA' group (n = 7), 'CS' group (n = 7), and 'CS-HA' group (n = 7) were treated with HA, CS, and CS-HA injection in the knees, respectively. All animals were treated once weekly for 5 weeks.

2.3. *Histological evaluation*

On day 7 after the last treatment, the animals were sacrificed and the articular cartilage and synovium were collected. Synovial fluid was also collected. Routine histological methods, involving fixation in 10% formaldehyde, were followed by decalcification in 10% nitric acid. Standard hematoxylin-eosin (HE) staining was performed, and the specimens were assessed by an independent pathologist who was experienced in the examination of osteoarthritis specimens. The articular cartilage injuries found in the rabbits' knees were evaluated and recorded using the Mankin score (*15*).

2.4. *Biochemical evaluation*

0.3 mL of the collected synovial fluid was centrifuged at 5,000 rpm for 30 min. The levels of IL-1β, TNF-α, and TIMP-1 were determined using enzyme linked immunosorbent assay kits (Xuanhao Science and Technology Development Co., Ltd., Shanghai, China). The levels of iNOS and NO in synovial fluid were tested using detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

2.5. *Statistical analysis*

A t-test was used to analyze data. $p < 0.05$ was considered significant.

3. Results

Histological analysis using the Mankin score is shown in Table 1. The results showed different levels of degenerative changes in controls and the three treated groups. The histological scores of 'HA' group and 'CS-HA' group were not significantly different ($p >$

Table 1. Evaluation by the Mankin score

Groups	Mankin score
Control	8.25 ± 2.22
HA	5.50 ± 0.49*
CS	6.33 ± 1.52
CS-HA	5.17 ± 0.24*

* Compared with control group, $p < 0.05$.

0.05), but were both higher than the control group ($p < 0.01$). Histological score of the 'CS' group was not significantly higher than control group ($p > 0.05$).

As shown in Figure 1A, the structure of normal synovial membrane was intact and epithelial cells were regular and flat. As shown in Figure 1B, lamination of the synovial membrane of animals in the control group disappeared. Some of the epithelial cells swelled and displayed hyaline-like degeneration or shedding. There was mild capillary proliferation in the synovial membrane and focal ischemic necrosis in the synovial cavity. The fibrous tissue swelled and large amounts of capillaries expanded, and inflammation was obvious. As shown in Figure 1C, proliferation of synovial cells of animals in the 'CS' group was obvious. The thickening of synovial membrane was alleviated compared to the control group. There was local hyperemia, edema, infiltration of large amounts of plasmocytes and small amounts of lymphocytes. As shown in Figure 1D, the proliferation of synovial cells and synovial thickening in the 'HA' group was obvious. The proliferation level of the 'HA' group is higher than that of the 'CS' group. Furthermore, local edema and congestion were lighter than those of the 'CS' group. The blood vessels showed mild hyperplasia and the blood circulation was better recovered than that of the 'CS' group. As shown in Figure 1E, in the 'CS-HA' group, epithelial cells proliferated in patches, similar to normal tissue. Recovery of synovial membrane was better than other groups. On the whole, the recovery of the synovial membrane of animals in the 'CS-HA' group was best among all groups.

As shown in Figure 2A, the normal chondrocytes were vacuolar and regularly aligned. The sclerotin was intact and the tissue structure was clear. As shown in Figure 2B, in the control group, the chondrocytes were aligned intensively and the thickness of the fibrocartilage increased. There was obvious karyopyknosis. The gap between the lacunas was enlarged and some cells were broken and dissolved. The cartilage matrix was torn as small gaps along the direction at which the collagen fibers were spread. As shown in Figure 2C, the amount of chondrocytes increased in the 'CS' group. Some of the chondrocyte nuclei shrank and the gap between the lacunas was enlarged. The sclerotin was intact, the tissue structure was distinct and the structure layers were obvious. As shown in Figure 2D, in the 'HA' group, the structure of the cartilage was distinct. There was obvious proliferation of chondrocytes. Some of the chondrocyte nuclei shrank and the gap between the lacunas was enlarged. As shown in Figure 2E, in the 'CS-HA' group, the amount of chondrocytes increased. Some of the chondrocyte nuclei shrank and the gaps between the lacunas were enlarged. The sclerotin was intact, the tissue structure was distinct and the structure layers were obvious. In a word, the recovery of the cartilage of animals in the drug-treated groups was better than that of control animals, but there was no obvious difference among the three treated groups.

The results of biochemical evaluation are shown in Table 2. The results showed that the expression levels of IL-1β, TNF-α, TIMP-1 and NO in synovial fluid of

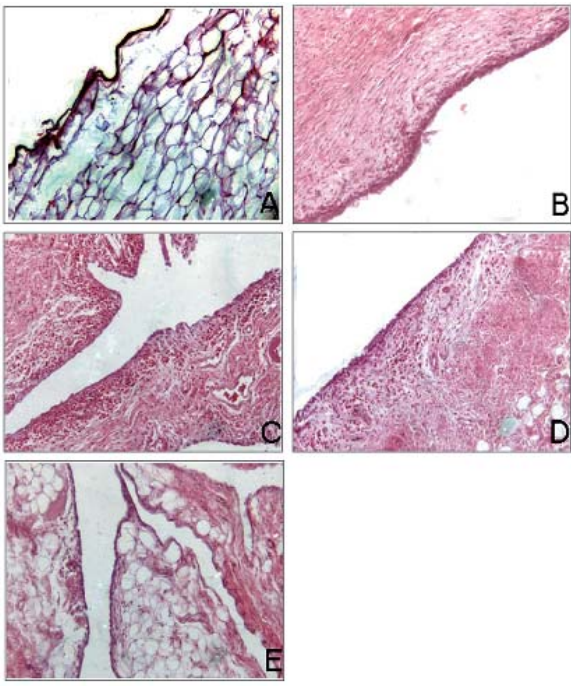

Figure 1. The pathological sections of synovial membrane stained with HE in different groups. A, normal synovial membrane (×200); **B**, control group (×200); **C**, 'CS' group (×200); **D**, 'HA' group (×200); **E**, 'CS-HA' group (×200).

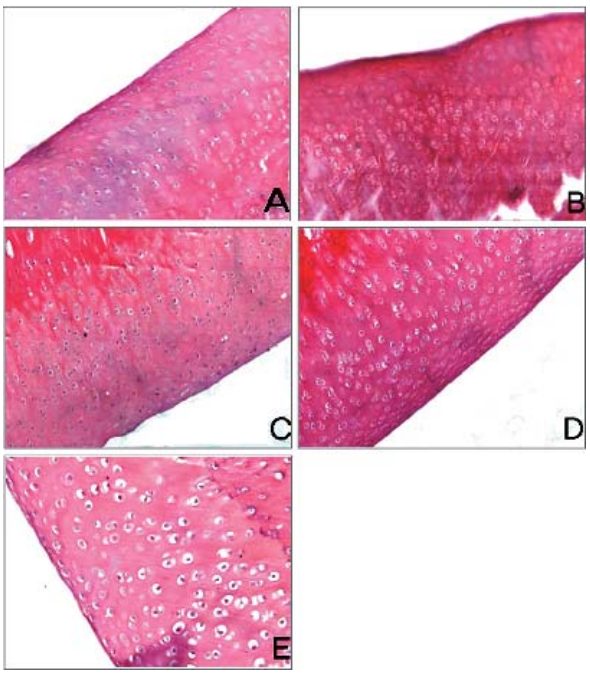

Figure 2. The pathological sections of cartilage stained with HE in different groups. A, normal cartilage (×200); **B**, control group (×200); **C**, 'CS' group (×200); **D**, 'HA' group (×200); **E**, 'CS-HA' group (×200).

Table 2. Expression levels of IL-1β, TNF-α, TIMP-1 and NO in different groups

Groups	IL-1β (pg/mL)	TNF-α (pg/mL)	TIMP-1 (ng/mL)	iNOS (U/mL)	NO (μmol/L)
Control	73.89 ± 5.29	66.20 ± 6.79	4.59 ± 0.38	22.85 ± 3.46	229.40 ± 43.86
CS-HA	45.37 ± 5.09**	51.35 ± 5.49*	9.43 ± 1.20*	16.58 ± 1.53*	125.69 ± 22.00*
CS	52.23 ± 2.68*	48.92 ± 4.72*	7.12 ± 0.97*	16.63 ± 2.24*	106.70 ± 10.29*
HA	58.46 ± 5.50*	47.54 ± 3.20*	7.40 ± 0.63*	21.76 ± 2.88	116.63 ± 18.37*

* Compared to the control group, $p < 0.05$; ** Compared to the control group, $p < 0.01$.

the treated groups were different from control group ($p < 0.05$). iNOS expression levels in synovial fluid of the 'CS-HA' and 'CS' groups were lower than control group, indicating that CS could inhibit the expression of iNOS. IL-1β level in synovial fluid of the 'CS-HA' group was much lower than that of control group. However, there was no obvious difference in the expression levels of the biochemical molecules among the three treated groups.

4. Discussion

SYSADOA are compounds which have been prescribed as drugs in European countries for many years. In Europe, the publication of the EULAR Recommendations for the treatment of Knee osteoarthritis in 2003 listed oral CS as evidence 1A and strength of recommendation A which represents the highest level for a therapeutic strategy (*16*). The benefits of CS for the treatment of osteoarthritis occurs through three main mechanisms: *i*) stimulation of extracellular matrix (ECM) (proteoglycan, CS, hyaluronan) production of chondrocytes; *ii*) suppression of inflammatory mediators (myeloperoxidase, *N*-acetyl glucosaminidase, collagenase, hyaluronidase, elastase), and *iii*) inhibition of cartilage degeneration (*17*).

Nevertheless, the benefit of CS is not accepted by all guidelines, and there is continuing controversy as to the efficacy of these agents as modifying drugs (*18*). A meta-analysis of five placebo-controlled RCTs yielded results that CS might have smaller beneficial effects than expected (*19*).

In this study, we intended to explore the effects of CS injection and CS-HA compound injection on osteoarthritis in rabbits. We found that after 5 intra-articular injections, the recovery of the synovial membrane of animals in the 'CS-HA' group was best among the animals treated with injections. However, the Mankin score of CS-HA treated animals had no obvious difference compared with that of HA treated animals, and the Mankin score of the CS treated group was not different than control. Although the recovery of the cartilage of animals in the drug-treated groups was better than that of control group, there was no obvious difference among the three treated groups. These results suggest that the intra-articular application of CS-HA injection shows no obvious advantage over routine intra-articular HA therapy.

As shown in Table 2, the expression levels of inflammatory factors such as IL-1β, TNF-α and TIMP-1 in synovial fluid of animals in three treated groups were all different from the control group, indicating intra-articular treatment of osteoarthritis with HA, CS or CS-HA injections inhibited joint inflammation. However, the inflammation inhibitory effects of CS and CS-HA were not better than HA. Overexpression of iNOS might increase the level of NO and damage the cartilage of osteoarthritis patients (*20*). In our study, CS and CS-HA injections showed inhibitory effects on the expression of iNOS. However, the NO levels of 'CS', 'HA' and 'CS-HA' groups were not significantly different.

In conclusion, CS-HA injection showed no obvious advantage over HA injection in osteoarthritis treatment.

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Original Article

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Prophylactic effect of *Withania somnifera* on inflammation in a non-autoimmune prone murine model of lupus

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ABSTRACT: The immunosuppressive properties of an aqueous suspension of *Withania somnifera* (WS) root powder were investigated in a pristane induced female Balb/c model of a systemic lupus erythematosus (SLE) like disease. The course of disease is initiated by peritoneal inflammation caused by pristane which results in development of SLE like symptoms, *i.e.* autoantibody production, proteinuria, and nephritis within a period of five to six months. The model of SLE was established by injecting 0.5 mL of pristane intraperitoneally into female Balb/c mice (12-18 weeks old). WS root powder (500 mg and 1,000 mg per kg body weight) was administered orally from one month prior to disease induction and for the following 6 months. Parameters of inflammation like nitric oxide (NO), Interleukin 6 and tumour necrosis factor-α and reactive oxygen species (ROS) in serum and/or ascitic fluid were measured. Prophylactic administration of WS root powder (500 mg and 1,000 mg per kg body weight) potently inhibits the pro-inflammatory cytokines, NO, and ROS in the ascitic fluid as well as in serum. Therefore, our results indicate a preventive effect of WS root powder on the mouse model of lupus.

Keywords: Systemic lupus erythematosus, *Withania somnifera*, pristane, Balb/c, prophylactic effect

1. Introduction

Withania somnifera (WS, Ashwagandha) (L.) Dunal (Solanaceae) is one of the most precious medicinal herbs in Ayurveda. It is generally regarded as health food and is consumed as a general tonic for rejuvenation, vitality, enhancing longevity, endurance and prevention of diseases. Therefore, for thousands of years, WS has been used to treat a wide range of conditions, for example, dyspepsia, emaciation, syphilis, anxiety, insomnia, nervous disorders, gynaecological disorders, musculoskeletal disorders, infections, asthma, male and female infertility *etc*. (*1,2*). The roots of WS are believed to be most potent for therapeutic purposes (*3*).

Studies have been conducted to investigate the immunomodulatory properties of WS. Singh *et al*. (*4*) have reported *in vitro* inhibition of nuclear factor-kappa B (NF-κB) and activator protein transcription factors (which play important roles in stimulating many genes involved in the inflammatory response) using a crude ethanol extract of WS (*4*). Suppressive effects of WS root powder on gouty arthritis and experimentally induced arthritis have also been reported (*5-8*). Withaferin A, a chemical constituent of WS was found to inhibit NF-κB in a cellular model of cystic fibrosis inflammation (*9*). Withanolide sulphoxide, another active constituent from WS roots also inhibits NF-κB along with cyclooxygenase 2 (COX-2) enzyme, which is selectively induced by proinflammatory cytokines at the site of inflammation, to restrain tumor cell progression (*10*).

Systemic lupus erythematosus (SLE) is an autoimmune disorder with inflammation as a main hallmark along with production of autoantibodies. Treatment is symptomatic and non-steroidal anti-inflammatory drugs are given for the initial stages of the disease. Pertaining to side effects of these drugs, herbal product(s) having the same properties would be beneficial to patients. Anti-inflammatory properties of WS root powder have been investigated in animal models of arthritis (*11-14*) in order to authenticate its use as a treatment drug. In a clinical study, WS in herbal formulation proved to be effective against rheumatoid arthritis, proposing its role in this autoimmune disorder (*15*). In order to study the prophylactic properties of WS in lupus, a model in Balb/c mice was generated by intraperitoneal injection of pristane (2,6,10,14-tetra-methylpentadeccane). This hydrocarbon oil induces a

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strong immune response in the peritoneum of female Balb/c mice which leads to development of SLE like symptoms such as autoantibody production, arthritis, proteinuria, and glomerulonephritis (*16*). In view of several reports on anti-inflammatory properties of WS monitoring the prophylactic effect of this candidate drug (WS root powder) on the Balb/c model of SLE-like disease appears to be important. The present study was therefore carried out to understand this effect.

2. Materials and Methods

2.1. *Animal groups*

Eight female Balb/c mice (obtained from Central Animal House facility, Panjab University, Chandigarh, Inida) were used in the following groups: PT group, mice given 0.5 mL of pristane intraperitoneally; WST1000 group, PT mice given WS at 1,000 mg/kg body weight one month prior to pristane treatment; WST500 group, PT mice given WS at 500 mg/kg body weight one month prior to pristane treatment; and PBST group, mice given 0.5 mL sterile PBS. The present study was cleared by the institutional ethical committee, Panjab University, Chandigarh, India.

2.2. *Induction of SLE-like disease (PT)*

Female Balb/c mice (2-3 months old) were acclimatized for one month in a conventional animal house facility (Central Animal House Facility, Panjab University, Chandigarh, India). The mice were injected with 0.5 mL of pristane (Sigma-Aldrich, St Louis, MO, USA) intraperitoneally (*17*). After six months, sera and ascitic fluid were collected for further studies.

2.3. *Drug regimen*

Commercially available WS root powder (Dabur India Limited, Ghaziabad, India) and its aqueous suspension in 2% gum acacia was orally given at different dose levels (500 and 1,000 mg/kg body weight) one month prior to pristane injection and was continued for 6 months.

2.4. *Preparation of sample for analysis*

Peritoneum of mice from each group was aspirated with 1 mL of PBS. The fluid was collected and centrifuged at 1,000 × g for 10 min. The supernatant was used for detection of nitric oxide (NO), interleukin 6 (IL-6), and tumour necrosis factor-α (TNF-α). Cells in the pellet were used for measurement of reactive oxygen species (ROS).

2.5. *Reactive intermediates*

Parameters such as NO and ROS were assessed in ascitic fluid/serum of all the above mentioned groups at the end of the study. NO levels were assessed by measuring nitrite levels in the ascitic fluid and serum using Griess reagent (0.1% naphthylenediamine-HCl/1% sulfanilamide in 5% phosphoric acid; 1:1, v/v) (*18*). The pink color produced by nitrite (the stable end product of NO metabolism) with Griess reagent was read at 540 nm.

ROS generation was analysed in intraperitoneal macrophages by using dichlorofluorocein diacetate (DCFH-DA, Sigma-Aldrich) (*19*). Ten µL of 1 µM DCFH-DA was added to the suspension of 1×10^6 cells in 2 mL PBS and the mixture was incubated at 37ºC for 30 min in the dark. The suspension was pelleted by centrifuging (4ºC) at 1,000 × g for 10 min. After washing three times with PBS, the suspension was kept on ice until flow cytometric detection (BD Biosciences, San Jose, CA, USA). Data acquired was analyzed using the CELLQuest program.

2.6. *Cytokine analysis*

Cytokines (IL-6 and TNF-α) were measured in ascitic fluid as well as serum using ELISA kits (GEN-PROBE, Diaclone, France) according to manufacturer's instructions. Briefly, wells of the microtiter strips coated with monoclonal antibodies specific for mIL-6 and mTNF-α were incubated with samples and biotinylated polyclonal antibodies specific for mIL-6 and mTNF-α simultaneously. Streptavidin-horseradish peroxidase and 3,3',5,5'-tetramethylbenzidene were used as the chromogen. Absorbance was measured at 450 nm as the primary wavelength and optionally at 620 nm as a reference wavelength. Data analysis was carried out using a standard curve for both the cytokines.

2.7. *Autoantibody screening*

Autoantibodies were detected in serum by indirect fluorescence using a kit (The Binding Site, Birmingham, UK). In brief, samples (diluted 1/20) were incubated with the substrate slides containing mouse liver, kidney, and stomach sections. After washing, fluoresceinisothiocyanate (FITC)-labelled sheep anti-mouse IgG (H + L) conjugate was added. After washing again, slides were viewed with a fluorescent microscope (LSM 510 Meta; Carl Zeiss, Oberkochen, Germany).

2.8. *Histopathology*

Kidney, spleen, liver, and lung tissues were fixed in 10% formalin and paraffin sections were stained with hematoxylin and eosin (H&E) dye before viewing them under a light microscope (Eclipse 80i; Nikon, Tokyo, Japan).

2.9. *Statistical analysis*

The results were expressed as mean ± S.D. and statistical significance of the data was determined by using one way ANOVA followed by a LSD test. $p < 0.05$ was taken as significant difference.

3. Results

3.1. *Inflammatory response caused by pristane in the peritoneum of PT mice*

Intraperitoneal injection of pristane resulted in an inflammatory response and this lead to formation of lipogranulomas. Figure 1b clearly shows serosal membranes full of lipogranulomas. Effusion of fluid in the peritoneal cavity (ascites) resulted in abdominal swelling. A significant elevation ($p < 0.0001$) of NO was observed in ascitic fluid of the PT group (34.7 ± 8.5 μM) as compared to the PBST group (1.36 ± 0.15 μM) (Figure 2a). IL-6 levels were also raised in PT (1,560 ± 401 pg/mL) as compared to PBST groups (0.65 ± 0.08 pg/mL) ($p < 0.0001$) (Figure 3a). A similar result was detected when TNF-α levels were analyzed in PT (30.2 ± 9.0 pg/mL) as compared to PBST groups (0.8 ± 0.1 pg/mL) ($p < 0.0001$) (Figure 3b). ROS levels (expressed as mean fluorescence value) were also significantly increased ($p < 0.0001$) in PT (648 ± 101) as compared to PBST groups (79.0 ± 7.8) (Figure 4).

3.2. *Inflammatory response caused by pristane in the serum of PT mice*

Serum nitrite levels were significantly raised ($p < 0.0002$) in the PT group (12.5 ± 4.8 μM) compared to the PBST group (2.03 ± 0.19 μM) (Figure 2b). Similar results were found when IL-6 (68.2 ± 25.5 pg/mL in PT group as compared to 8.76 ± 2.34 pg/mL in PBST group; $p < 0.0001$) and TNF-α (24.2 ± 11.0 pg/mL in PT group as compared to 6.36 ± 1.87 pg/mL in PBST group; $p < 0.0005$) levels were detected (Figures 3c and 3d). Animals from the PT group showed presence of autoantibodies in serum (Figure 5b) as compared to the PBST group (Figure 5a). Figure 5b demonstrates peripheral and diffused staining of the nuclei of mouse kidney tubular cells depicting autoantibodies against ds-DNA and histones.

3.3. *Anti-inflammatory effect of WS treatment in peritoneal cavity*

After seven months treatment the peritoneal cavity of groups WST1000 and WST500 was found to be clear with little or no lipogranulomas (Figures 1c and 1d), however, 62.5% of the animals still showed some small round calcified material in the gut. Ascitic fluid was virtually absent in both the WST1000 and WST500 groups. A significant reduction in peritoneal nitrite levels of the WST1000 group (2.40 ± 1.04 μM) and the WST500 group (2.35 ± 1.02 μM) (Figure 2a) compared to the PT group ($p < 0.0001$) were observed. A similar trend was observed when IL-6 and TNF-α levels were checked in WST1000 (86.9 ± 29.6 pg/mL and 7.87 ± 2.86 pg/mL, respectively) and WST500 (80.8 ± 30.4 pg/mL and 9.76 ± 3.32 pg/mL, respectively) groups compared to the PT group ($p < 0.0001$) (Figures 3a and 3b). A significant reduction in ROS levels was observed

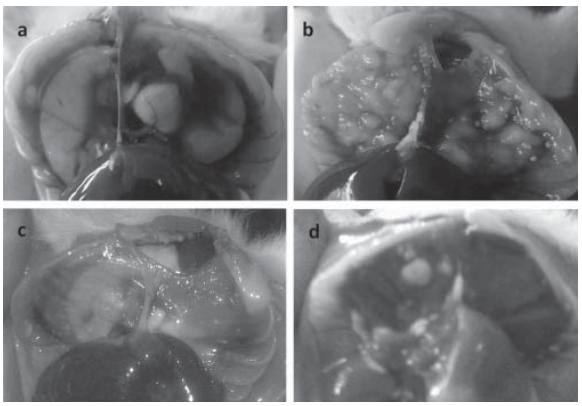

Figure 1. Lipogranulomas on serosal membrane separating gut organs from lungs and heart. (a) PBST, **(b)** PT, **(c)** WST1000, **(d)** WST500.

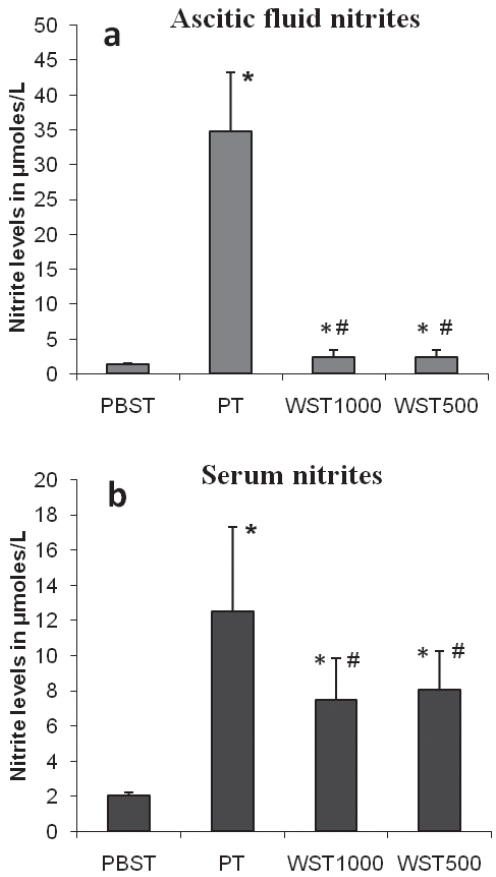

Figure 2. Nitrite levels of ascitic fluid (a) and serum (b) in PBST, PT, WST1000, and WST500. Values are expressed as mean ± S.D. ($n = 8$). * Significant difference from PBST. # Significant difference from PT group.

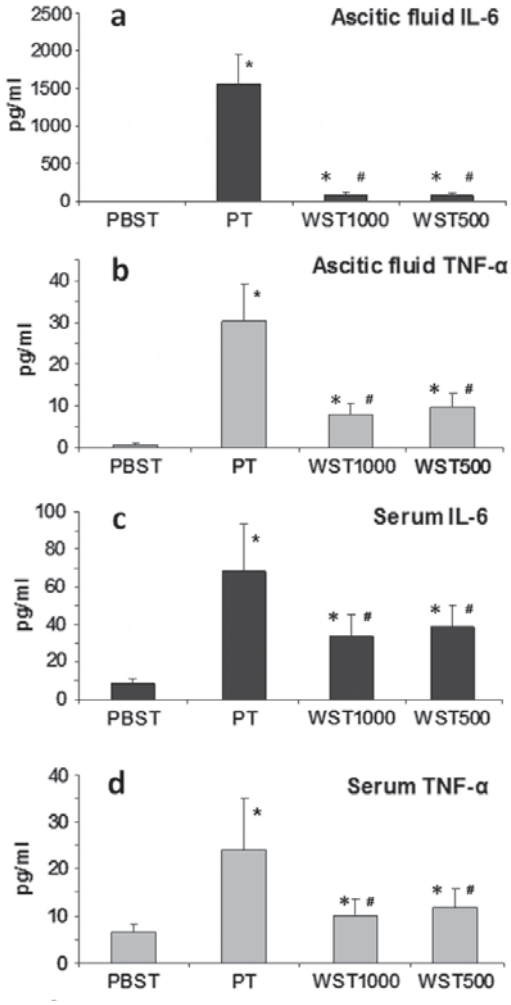

Figure 3. IL-6 and TNF-α levels in ascitic fluid and serum of various animal groups. Mice in PBST, PT, WST1000, and WST500 groups are treated as described in 'Materials and Methods'. Values are expressed as mean ± S.D. (n = 8). * Significant difference from PBST. # Significant difference from PT group.

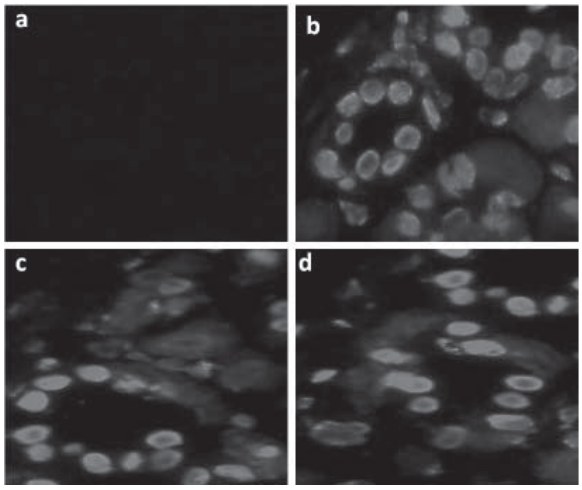

Figure 4. Fluorescence microscopic images of mouse cells stained with anti-mouse IgG-FITC for autoantibodies detection in serum of various animal groups. (a) PBST group, **(b)** PT group, **(c)** WST1000 group, **(d)** WST500 group. Original magnification, 200×.

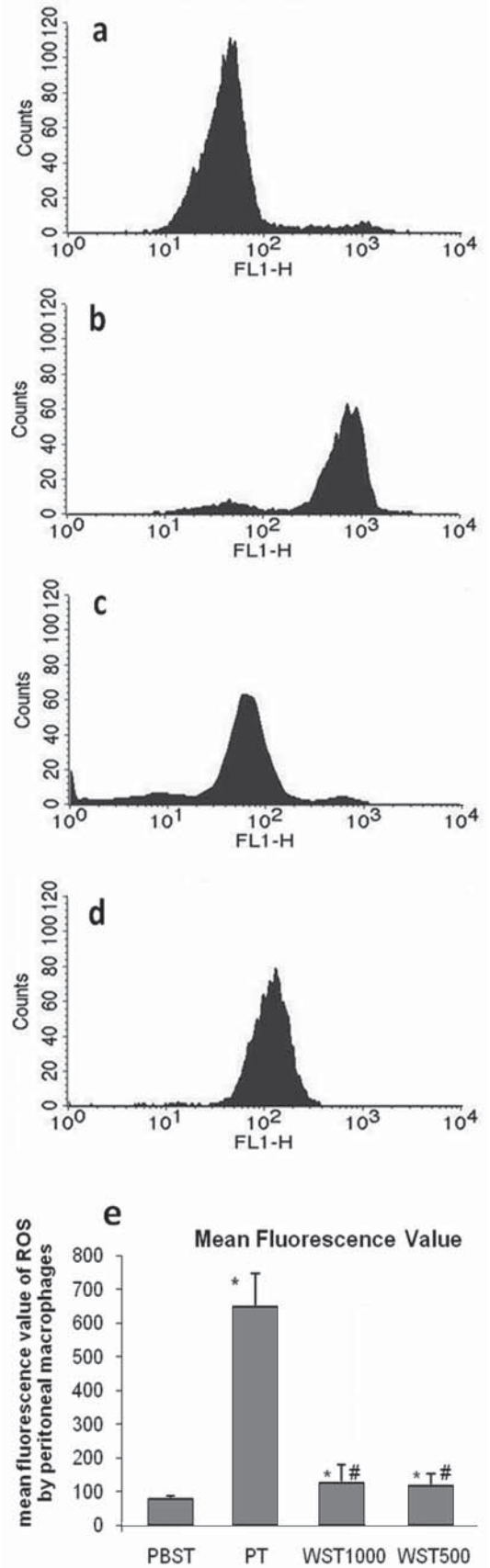

Figure 5. ROS levels in the peritoneal macrophages of various animal groups. (a-d) Flow cytometric profiles for PBST **(a),** PT **(b),** WST1000 **(c),** and WST500 **(d). (e)** Graphical representation of mean fluorescence obtained using DCFH-DA stained peritoneal macrophages. Values are expressed as mean ± S.D. (n = 8). * Significant difference from PBST. # Significant difference from PT group.

in both WST1000 (125 ± 54) and WST500 (118 ± 35) groups when compared to the PT group (648 ± 101) ($p < 0.0001$) (Figures 4c and 4d). Nitrite, IL-6, TNF- α, and ROS levels were still significantly elevated in WST1000 and WST500 groups as compared to the PBST group ($p < 0.05$).

3.4. *Anti-inflammatory effect of WS in serum*

Serum nitrite level was reduced significantly in WST1000 (7.46 ± 2.40 μM) and WST500 (8.04 ± 2.23 μM) groups as compared to the PT group ($p < 0.02$ and $p < 0.03$, respectively) (Figure 2b). IL-6 and TNF-α levels were also decreased significantly ($p < 0.05$) in both groups WST1000 (34.1 ± 10.9 pg/mL and 7.87 ± 2.86 pg/mL, respectively) and WST500 (38.5 ± 11.7 pg/mL and 9.76 ± 3.32 pg/mL, respectively) as compared to the PT group (Figures 3c and 3d). However, autoantibodies were still detected in both WST1000 and WST500 groups (Figures 5c and 5d, respectively). As compared to the PBST group, the levels of nitrites, IL-6, and TNF-α levels were significantly elevated in WST1000 and WST500 groups ($p < 0.05$).

3.5. *Effect of WS on histopathological alterations caused due to pristane*

Histology of kidney, spleen, liver, and lung was found to be altered in PT mice. The treatment groups (WST1000 and WST 500) showed a radical effect on the pristane induced histopathologic alterations. Figure 6 demonstrates histomicrographs of the above tissues in PT, WST1000 and WST 500 groups. Kidneys of the PT group showed focal inflammation in the interstitium (Figure 6a). The inflammatory infiltrate is shown with an arrow. Figure 6b shows normal kidney pathology as observed in WST1000 and WST500 groups. The spleen of PT group animals depicts reactive enlargement of follicles in the lower half of the spleen (marked with arrows; Figure 6c) while normal histology of spleen was observed in WST1000 and WST500 groups (Figure 6d). Figure 6e shows one of the necrotic areas in liver of PT mice depicting inflammatory cells along with oil droplets. Surrounding the necrotic area, Kupffer cell (KC) hyperplasia along with reactive enlargement of liver nuclei (NC) was observed (as shown with arrows). Figure 6f depicts normal liver without any focus of inflammation as found in WST1000 and WST500 groups. Figure 6g illustrates a micrograph of lung with infiltration of mononuclear cells which are predominantly plasma cells in the PT group. Figure 6h shows normal histology of lung as observed in WST1000 and WST500 groups.

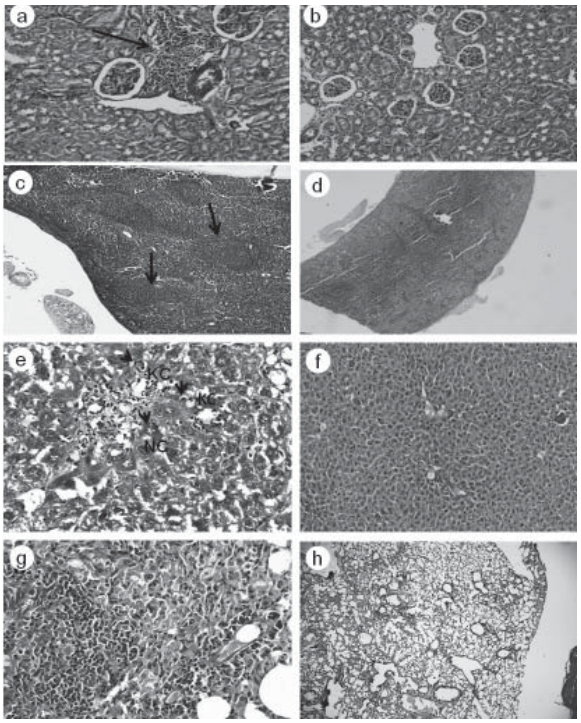

Figure 6. Histological observations by light microscopy. (a) Histomicrograph of kidney from PT group (200×). **(b)** Micrograph of kidney showing normal morphology as found in WST1000 and WST500 groups (40×). **(c)** Section of spleen from PT group (40×). **(d)** Micrograph of spleen depicting normal morphology as discovered in WST1000 and WST500 groups (40×). **(e)** Section of liver from PT group (200×). **(f)** Micrograph of liver with normal pathology as ascertained in WST1000 and WST500 groups (40×). **(g)** Section of lung from PT group (200×). **(h)** Micrograph of lung showing normal pathology as observed in WST1000 and WST500 groups (40×).

4. Discussion

Anti inflammatory properties of WS root powder have been widely investigated in animal models (*5-7,11-14*), however its role in SLE still needs attention. The data observed in the present study revealed anti-inflammatory activity of the WS root powder in the mice model of this complex autoimmune disorder.

When introduced in peritoneum pristane induces a strong immune response leading to formation of lipogranulomas which remain adherent to the serosal surface of the peritoneal cavity. Interestingly, after seven months of oral WS root powder treatment, the lipogranulomas were reduced or disappeared from the peritoneal cavity of mice at both dose levels. As suggested by Shaheen *et al*. (*20*), the macrophages in the lipogranuloma are the likely source of several cytokines that are critical for the development of autoimmunity in the pristane induced lupus model (particularly IL-6). It has been reported earlier that IL-6 deficient Balb/c mice when injected with pristane could not develop plasmacytomas (*21*), highlighting its significant role in disease development. Pro-inflammatory cytokines such as IL-6 and TNF-α were analyzed as they are known to be responsible for the production of autoantibodies in lupus (*22*). IL-6 plays a significant role not only in antibody generation (*23*), but also in triggering helper and cytotoxic T cells (*24*).

The synergic effects of IL-6 and TNF-α further worsen the situation by elevating inflammation, far from the site of action. The current study depicts this picture in the PT group of animals. The WS1000 and WS500 treated groups however showed a significant lowering of the above mentioned cytokines. It has therefore been suggested that any molecule/drug than can suppress or block these cytokines can be used as a means of therapy (*25*).

In the present study, WS root powder in both 500 as well as 1,000 mg/kg body weight doses could bring about reduction in the levels of IL-6 as well as TNF-α whereas untreated the PT group continued to exhibit increased IL-6 and TNF-α. This observation suggests that reduction of these pro-inflammatory cytokines points towards the anti-inflammatory effect brought about by WS mediating inhibition of cytokine synthesis. The WS root powder treatment led to impassive autoantibody production in the PT model of lupus. This is supported by work carried out by Rasool and Varalakshmi (*6*) on experimentally induced (adjuvant induced arthritis) inflammation *in vitro* and *in vivo* where complement activity, lymphocyte proliferation and delayed type hypersensitivity responses were suppressed but humoral antibody response was unaltered. Other than autoantibody production, renal pathology is another hallmark of the pristane induced lupus model. WS, in the present study has clearly shown its protective effect on renal pathology as well as on histopathology of different tissues such as spleen, liver, and lung.

In line with these studies, our report also deals with the role of WS root powder on NO and ROS levels. The overproduction of NO, an important signalling molecule involved in many physiological processes, has been speculated to cause abnormal lymphocyte function contributing to pathogenesis of autoimmunity (*26,27*). During chronic inflammation there is sustained production of ROS which is speculated to be involved in the pathogenesis of many autoimmune diseases (*28*). Elevated NO levels in the ascitic fluid and ROS production by peritoneal macrophages in the PT group were found to be decreased in WS1000 and WS500 groups. Sumantran *et al*. (*29*) using an explant model of *in vitro* cartilage damage study on patients with osteoarthritis have also shown marked reduction in NO release when treated with WS root extract.

Ascitic fluid formation and abdominal swelling were also markedly reduced in both treatment groups, further confirming its action against inflammation caused by pristane.

Anti-inflammatory properties of WS root powder have been widely investigated in animal models of arthritis. Clinical trials using herbal formulation of WS proved to be effective for osteoarthritis (*30*). Results in the present study suggest that one month pretreatment of animals with WS root powder prior to induction of the disease and subsequent treatment for another six months, had inhibitory effects on peritoneal inflammation and the resultant elevation of inflammatory parameters in serum of this model of autoimmune disorders.

Concluding the current work, pristane induced lupus depicts a model of SLE like disease in mice where NO, cytokines and ROS levels are disturbed. We have studied the prophylactic effect of WS root powder on the above mentioned model and found it to be effective against inflammatory response caused by pristane. *W. somnifera* was also found to be effective against the renal pathology occurring in this model. Since, autoantibody production was unaltered; we cannot articulate that *W. somnifera* root powder at 500 and 1,000 mg/kg body weight was able to hinder the progression of disease completely. Future studies at the molecular level are necessary to understand the biochemical processes going on in the presence or absence of this natural product.

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