The Journal of Applied Pharmacology, **12**, 133-137 (2004)

Focus on Anti-Oxidative and Free Radical Scavenging Activity of *Ganoderma lucidum*

Zhi-Bin LIN*

Department of Pharmacology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100083, China

(Received May 5, 2004)

Abstract – Present review is built on base of research work on *Ganoderma lucidum* in our laboratory. A great deal of experimental evidence has suggested that the pharmacological activities of *Ganoderma lucidum* (Lingzhi) are related to anti-oxidative and free radical scavenging activity. The anti-oxidative and free radical scavenging effects of polysaccharides and triterpenoids isolated from *Ganoderma lucidum* in different oxidative injury models including tert-butylhydroperoxide (tBOOH)- damaged mice peritoneal macrophages, alloxan-induced diabetes, experimental liver injury models induced by carbon tetrachloride (CCl4), D-galactosamine (DGal) and Bacillus Calmette-Guerin (BCG) plus lipopolysaccharides (LPS) were investigated. It is also demonstrated that Lugu lingzhi, one of *Ganoderma* product, significantly inhibited LDL oxidation mediated by endothelial cells and decreased monocyte adhesion to endothelial cell (EC) induced by Oxidative low-density lipoprotein (ox-LDL) and advanced glycation endproducts (AGE). Lugulingzhi-treated serum could markedly inhibit the expression of intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) induced by ox-LDL and AGE.

Keywords □ *Ganoderma lucidum*, polysaccharides, triterpenoids, anti-oxidative, free radical scavenging

INTRODUCTION

Ganoderma lucidum (Leyss.ex fr.) Karst. (Lingzhi) is a medicinal fungus with long history in China as a tonic and remedy. Lingzhi was highly ranked as an herbal medicine in *Shen Nong Materia Medica* (*Shen Nung Ben Cao Jing*) which was published in the second century B.C. Li Shi-Zhen, a well known ancient Chinese physician, also described the efficacy and medical uses of Lingzhi in the world renown classic *Compendium of Materia* (*Ben Cao Gang Mu*) in the 16th century. Ancient Chinese medical scholars indicated that Lingzhi could strengthen body resistance and consolidate the constitution of patients i.e., "Fuzheng Guben" which is one of the major principles in the therapeutics of traditional Chinese medicine.

In the Pharmacopoeia of P.R. China (2000 ed., Part 1), both *Ganoderma lucidum* and *Ganoderma sinensis* are listed as Lingzhi. The use of Lingzhi as a drug or health food is on the rise in China.

*Corresponding author
Tel: 86-10-82801686, Fax: 86-10-82801686
E-mail: linzb@public3.bta.net.cn

The main chemical constituents of *Ganoderma lucidum* consist of polysaccharides, triterpenoids, nucleosides, steroids, alkaloids, amino acids and peptides, inorganic elements and fatty acids, etc. Among these ingredients, polysaccharides and triterpenoids appear to be the major components with significant pharmacological effects (Lin, 2001).

Ganoderma polysaccharides were extracted from *Ganoderma lucidum* (Leyss ex Fr) Karst. A great deal of experimental evidence has accumulated in the past several decades, suggesting that *Ganoderma lucidum* polysaccharides had wide pharmacological activities, such as immunomodulating and antitumor, anti-atherosclerosis and lipidelowering, hypoglycemic, liver-protective anti-oxidative and free radical scavenging and anti-aging activities, etc. (Lin, 2001).

The clinical observations indicate that *Ganoderma* preparations can treat bronchitis, arteriosclerosis, diabetes, hepatitis, cancers and aging, etc. (Lin, 2001). The pathological mechanisms of all these diseases are associated with the reactive oxygen species (ROS) (Yang et al, 1998; Hessler et al, 1979; Ceriello et al, 2000; Mecocci et al, 1993.). Present review on the anti-oxidative and free radical scavenging activity of *Ganoderma lucidum* is build on base of our research work.

Free radical scavenging activity of *Ganoderma lucidum* polysaccharides peptide (GLPP) in the mice peritoneal macrophages

Ganoderma lucidum polysaccharide peptide (GLPP) is isolated from *Ganoderma lucidum*(leyss.ex fr.)karst. (Lin SQ et al, 2003). Primary studies demonstrate that GLPP decreased oxidation of low-density lipoprotein (LDL) and the relative electrophoretic mobility (REM) of oxidative product of LDL, reduced the content of malondialdehyde(MDA) and increased GSHpx enzyme activity in serum and heart of mice (You YH and Lin ZB, 2003)

Recently, we studied whether GLPP could protect the peritoneal macrophages against the injury by ROS in mice. . Tert-butylhydroperoxide (tBOOH), as a membrane-permeant oxidant has been extensively used as a model of oxidative injury in different cells *in vitro*. We used it as an oxidant to produce oxidative damage stress on mice macrophages. The result showed that survival rate of macrophages exposed to tBOOH (0, 0.1, 0.22 mmol/L) for 24 h was increased respectively by GLPP (100, 200 mg/kg, ig) *in vivo*. GLPP (3.125, 12.5, 50, 200 mg/L) also increased the survival rate of macrophages *in vitro*. Under the light microscope, above half of the macrophages appeared asteroid and there was no or little phagocytic granule before treatment with tBOOH. After treated by tBOOH, macrophages in control group became round and the granule increased. The injury of macrophages increased following the increase of the concentration of tBOOH. Cell appeared foam and lipid droplets were found. Necrosis was found in part of the macrophages. Cell debris could be seen and the number of macrophages decreased. The injury of macrophages was decreased by treatment with GLPP. Most of the macrophages stills appeared asteroid and the granule and the lipid droplet of macrophages were reduced. GLPP showed protective effects on macrophages against injury by tBOOH *in vivo* and *in vitro*. The result from scanning electron microscope showed that the microvilli of the macrophages were short and scare, with spot like distribution and part of the membrane even became smooth in tBOOH injured group. The microvilli were injured slightly or kept intact in the GLPP-treated group. Under the transmission electron microscope, injured mitochondria were obviously observed in tBOOH-treated group. There appeared huge mitochondria and some of the mitochondria become stratified. Rough endoplasmic reticulums were reduced in numbers. Many lipid droplets appeared and a lot of macrophages showed foamy change. The injury of mitochondria was decreased in GLPP-treated group,. The cristae of the mitochondria were slightly disorganized but the structure was still intact. Endoplasmic reticulums were tubiform and vesiculiform. There was little lipid droplet and foamy changes in the structure of microphages cytoplasm. The results indicate that GLPP could protect macrophages against injury by tBOOH (You YH and Lin ZB, 2003).

2', 7'-dichlorodihydrofluorescein diacetate (DCHF-DA) is used as a detector of intracellular oxidants as ROS or free radical. DCHF-DA could be used with flow cytometry to detect oxidant formation by activated live cells. It is speculated that the probe diffuses into the cell, intracellular esterases hydrolyze the acetate groups, and the resulting 2', 7'- dichlorofluorescin (DCHF) then reacts with intracellular oxidants resulting in the observed fluorescence. The fluorescence intensity is proportional to the mount of peroxide produced by the cells. We used confocal microscopy with this fluorescent probe to detect the effects of GLPP on the free radical formation in mice peritoneal macrophages. Results showed that DCHF-DA exhibited low fluorescence in cells of the control group and fluorescence apparent increased in cells of oxidant injury group, no matter whether they were oxidant injured by alloxan or by tBOOH. But GLPP given *in vivo* or *in vitro* could decrease the fluorescence in macrophages.

The result of time series scan of conforcal microscope revealed that GLPP lowered the fluorescence in macrophage over time. It took effect immediately after GLPP were given. PMA greatly increased the fluorescence in macrophage. After treatment with GLPP, the increased fluorescence by PMA was lowered over time and GLPP took effect immediately as well. The results showed that DCHF exhibited low fluorescence in the control cells. The injury cells by alloxan or by tBOOH gave a bright signal. But GLPP lowed the fluorescence signal. It indicated that GLPP could decrease free radical in mice peritoneal macrophages (You YH and Lin ZB, 2004).

The results of time series scan with confocal microscopy showed that GLPP could lower the free radical in mice peritoneal macrophages immediately. No matter weather the cells were at rest state or at the respiratory burst state, GLPP took effect immediately. The results indicate that GLPP have the free radical scavenging activity. It could scavenge not only the free radical directly in mice peritoneal macrophages at rest state but also the decreased free radical in mice peritoneal macrophages by pathological cause.

Free radical such as superoxide anions and hydroxyl radicals were associated with carcinogenesis and other pathological conditions. Therefore, elimination or inactivation of free radical

or inhibition their excess generation may be beneficial in terms of reducing the risk for cancer and other disease. GLPP was found to have antioxidant effects. It could take effect by enhancing the antioxidant enzymes activities such as GSHpx or SOD. Now our results showed that it could scavenge the free radical directly as well.

Protective effect of *Ganoderma lucidum* polysaccharide in alloxan-induced diabetes *in vivo and in vitro*

Ganoderma lucidum Polysaccharides is also reported to have hypoglycemic activity by increasing plasma insulin and by affecting the hepatic key enzymes in the carbohydrate metabolism (Hikino, 1985, 1989). Alloxan is a prompt and potent inducer of diabetes in experimental animals because of its damaging effect on insulin-producing β cells. It has been generally accepted that alloxan-induced hyperglycemia is mainly due to its ability to induce oxygen free radicals, which damage the pancreas (Heikkila & Cohen 1974; Grankvist & Marklaud 1986, Winterbourn & Munday 1989). Recently, it has been reported that *Ganoderma lucidum* polysaccharides has the ability to scavenge the reactive oxygen species (Gui XF, 1996; You YH, 2002) and significantly inhibited iron-induced lipid peroxidation in rat brain homogenates and to inactivate hydroxyl radicals and superoxide anions (Lee et al, 1999). Recently, we investigated the hypoglycemic effects of *Ganoderm lucidum* polysaccharides (*Gl*-PS) in alloxan-induced damage *in vivo and in vitro*. The results showed that pretreatment with *Gl*-PS (50-200 mg/kg) significantly decreased serum glucose levels and increased insulin levels, as compared to the group receiving alloxan alone Pretreatment with *Gl*-PS 200 mg/kg decreased the pancreas MDA significantly as compared to the diabetic control .

The effect of *Gl*-PS on the aldehyde fuchsin staining of the pancreas was studied. In the normal pancreas, the β cells occupied about 80% of each islet; the average number of β cells per islet was 34. Alloxan caused nearly all the β cells to be absent in the Langerhans islets, 0~3 β cells commonly appeared in one islet, which led to the reduction in the number and size of the islets. Pretreatment with *Gl*-PS (50 mg/kg, 100 mg/kg and 200 mg/kg) partly protected the β cells from necrosis, the number of β cells were increased, the average number of β cells per islet was 5, 10 and 19 respectively, especially with the dose of 200 mg/kg.

Alloxan administration to the isolated pancreatic islets for 30 min caused dose-dependent toxicity. Pretreatment of *Gl*-PS for 12h partly protected the islets toxicity induced by alloxan at the concentration of 100 μg/ml. Prolong the incubation time to 24h, *Gl*-PS 25 μg/ml and 100 μg/ml partly protected the islets from being destroyed by alloxan. The findings that pretreatment with *Gl*-PS can reduce the lipid peroxidation product MDA content of pancreas, decrease serum glucose levels and increase serum insulin significantly in alloxan-induced hyperglycemia agree with the idea that it is able to alleviate alloxan toxicity. The results reveal that *Gl*-PS can protect the animals from being damaged by free radicals directly; this in turn may contribute to the regulation of blood glucose. One possible hypoglycemic mechanism of *Gl*-PS may be relating to protect the pancreatic islets from free radicals damage. Further mechanism may involve that *Gl*-PS could inhibit NF-κB activation in the pancreas(Zhang HN et al, 2003).

Liver-protective effects of *Gannoderma lucidum* on experimental liver injury animal models

Recently, the effects of *Ganoderma lucidum* polysaccharides (GLP) isolated from mycelium of *Ganoderma lucidum* on the liver weight and the activity of serum alanine transaminase (ALT) were studied in BCG-induced immune hepatic injury in mice. The results shown that under the presence of BCG stimuli conditions, administration of GLP could decrease the liver weight within the range of 50 mg/kg to 200 mg/kg, simultaneously, serum ALT release were significantly decreased by GLP treatment in a dose-dependent manner with the similar range of doses. On the other hand, the histological examination shown that GLP (100mg/kg) alleviated hepatic damage in BCG induced acute inflammation, such as markedly decrease of infiltration within liver lobules by inflammatory cells, nuclear narrow, etc. in the observed liver section. Moreover, granulomas formation was also decreased by GLP treatment at doses range from 100-200 mg/kg. Further study indicated that in the presence of inflammatory cytokines (IL-1, TNFα, IFNγ plus LPS) BCG prestimuli condition, ALT activity and NO production were markedly inhibited by treatment of GLP in primary cultured mice hepatocytes *in vitro*. The results of immunohistochemistry shown that GLP significantly inhibited the expression of inducible nitric oxide synthase (iNOS) protein stimulated by BCG.. Furthermore, the mechanism of protective role by GLP for BCG-induced immune liver injury in mice may be due to influence NO production (Zhang GL, et al, 2002).

Early research found that the extract isolated from *Ganoderma lucidum* and *Ganoderma sinense* by ethanol could reduce liver injury induced by carbon tetrachloride (CCl_4) in mice (Department of Pharmacology, Beijing Medical College, 1974; Liu et al., 1979), and total triterpenoids isolated from *G. tsugae* Murr. could decrease alanine aminotransferase (ALT)

activity of serum in CCl_4-intoxicated mice (Su et al., 1993). In our laboratory, two major components, GT and GT_2 were isolated from *Ganoderma lucidum* and they were determined as total triterpenoids by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). The effect of GT and GT_2 on three different experimental liver injury models induced by carbon tetrachloride (CCl_4), D-galactosamine (DGal) and Bacillus Calmette-Guerin(BCG) plus lipopolysaccharides(LPS) were studied in mice. The results indicate that both the activity of serum alanine aminotransferase (ALT) and the content of liver triglycerides (TG) were increased significantly in CCl_4- damaged mice GT (80 mg/kg), and GT_2 (10, 20, 40 mg/kg) successfully decreased ALT and TG with different degrees. The histopathological results indicate that liver structures of the control group were basically normal. The livers of the CCl_4-injured group were hurt severely and showed large necrotic changes. However, the histopathological changes of liver in the animals treated with GT and GT2 were improved significantly. The results also demonstrate that GT given as 32.5, 65, 130 mg/kg could antagonize the decrease of activity of superoxide dismutase (SOD) and glutathione (GSH)content of liver and inhibit the increase of liver malondialdehyde (MDA) content in CCl_4-injured nice (Wang MY *et al.*, 2000, 2002). Further experiment reveals that the serum ALT and liver TG increased significantly in the D-Gal group. GT 80 mg/kg and GT_2 10, 20, 40 mg/kg reduced ALT and TG significantly. GT 32.5, 65, 130 mg/kg, can antagonize the decrease of activity of SOD and GSH content of liver and inhibit the increase of liver MDA content in the liver-injury mice induced by D-Gal. In immunological liver injury model induced by BCG plus LPS, GT (80 mg/kg) and GT_2 (10, 20, 40 mg/kg) significantly inhibited the increase of serum ALT, NO and Liver TG levels and improved the liver injury in different degree. GT (0.5, 5, 50, 100 μg/ml) and GT_2 (0.5, 2, 10, 50 μg/ml also decreased ALT and NO level in primary cultured hepatocytes *in vitro* (Wang MY, Lin ZB, 2000).

Results mentioned above suggest that both polysaccharides and triterpenoids isolated from *Ganoderma lucidum* have liver-protective effect in experimental animal models and the mechanism may be relating to anti-oxidative and scavenging activities of *Ganoderma lucidum*.

Effect of Lugu Lingzhi on Monocyte Adhesion to Endothelium and Adhesion Molecule Expression

Primary investigation shows that *Ganoderma* extract (commercial name: Lugu lingzhi) could decrease the serum cholesterol, triglycerides (TG) and increase high-density lipoprotein (HDL) in experimental hyperlipidemia rats. Lugu lingzhi also could reduce the content of malondialdehyde (MDA) and increase the activity of superoxide dismutase (SOD) in normal mice. In recent years, serum pharmacological method is widely used to investigate traditional Chinese medicine. Serum pharmacology means to make *in vitro* experiment using an animal serum with drug when the animal has been taken this drug. The drug treated-serum may contain drug, metabolites of the drug, or endogenous active substances induced by drug *in vivo*, thus the serum with drug produces pharmacological effect. Lately, we using serum-pharmacological method to study the effect of sera containing Lugu lingzhi on the low-density lipoprotein (LDL) oxidation, monocyte adhesion to the human endothelial cells and adhesion molecules expression induced by Oxidative low-density lipoprotein (ox-LDL) and advanced glycation end-products (AGE) (Zhang HM et al, 2002a, 2002b). At the dose of 0.12, 0.24 and 0.72 g (crude materials)/kg, Lugu lingzhi were administered by stomach tube, once a day for 10 days. The rats were killed 10 days later, and serums were taken and stored at -80°C for use. In the experiment Lugulingzhi-treated serum was added to human umbilical cord vascular endothelial cell culture media *in vitro*. Results reveal that serum treated with Lugu lingzhi(0.12, 0.24 and 0.72 g/kg) significantly inhibited LDL oxidation mediated by endothelial cells and decreased monocyte adhesion to endothelial cell(EC) induced by ox-LDL and AGE. Further experiments indicate that Lugulingzhi-treated serum could markedly inhibit the expression of intercellular cell adhesion molecule-1 (ICAM-1) induced by ox-LDL and AGE. Lugu lingzhi-treated serum also significantly inhibit the expression of vascular cell adhesion molecule-1 (VCAM-1) induced by AGE. The inhibition of expression on adhesion molecule induced by ox-LDL and AGE might be contributed to the effects of Lugu lingzhi in preventing the development of vascular complications of diabetes and other cardiovascular disease.

CONCLUSION

Anti-oxidative effect and free radical scavenging activity of *Ganoderma lucidum* on different animal models *in vivo* and *in vitro* is reviewed in present article. This important property of *Ganoderma lucidum* may participate its pharmacological mechanism of immunomodulating, hypoglycemic, liver-protective, cardiovascular and anti-aging effects. Through these available results, it is attempted to explain the "treating different diseases with

the same therapeutic principle (yi bing tong zhi)" which is one of the major principle in the therapeutics of traditional Chinese Medicine. Why *Ganoderma* preparation can treat different diseases? It is considered that oxidative and free radical injury involves pathological mechanism of different diseases, which can treat with *Ganoderma* preparation by its anti-oxidative effect and free radical scavenging activity. However, further study is needed to understand the exact mechanism of the anti-oxidative effect and free radical scavenging activity of *Ganoderma lucidum.*

REFERENCES

Ceriello A., Morocutti A., Mercuri F., Quagliaro L., Moro M. Damante G. (2000). Defective intracellular antioxidant enzyme production in type I diabetic patients with nephropathy. *Diabetes.* **49**, 2170-7.

Department of pharmacology, Beijing Medical College. (1974). Study on *Ganoderma.* I. Pharmacological effect of fruiting body of *Ganoderma lucidum. J Beijing Med College*, (4): 246-254.

Gui, X.F.,Wang, L., Yang, Y.H.(1996). The study of protecting kidney cortical from radical with the use of *Ganoderma lucidum* injection. *Chin. J. Biochem. Pharm.* **17**, 188-190.

Heikklia, R.E., Winsyon, B., Cohen, G., Barden, H.(1976). Alloxan-induced diabetes-evidence for hydroxyl radical as a cytotoxic intermediate. *Biochem. Pharmacol* **25**, 1085-1092.

Hes sler R., Robertson A.L. Jr, Chissoml G.M. (1979). LDL-induced cytotoxicity and its inhibition by HDL in human vas cular smooth muscle and endothelial cell in culture. Atherosclerosis. **32**, 213-29.

Hikino, H., Ishiyama, M., Suzuki, Y., Konno, C. (1989). Mechanisms of hypoglycemic activity of ganoderan B: a glycan of *Ganoderma lucidum* fruit bodies. *Planta Med.* **55**, 423-428.

Hikino, H., Kinno, C., Mirin, Y., Hayashi, T. (1985). Isolation and hypoglyc*emic activity of Ganoderans A and B, glycans of* Ganoderma lucidum fruit bodies. *Planta Med.* **4**, 339-340.

Lee, J.M., Kwon, H., Jeong, H., Lee, J.W. (1999). Inhibition of lipid peroxidation and oxidative DNA damage by *Ganoderma lucidum. Phytother Res.* **13**, 529-531.

Lin SQ, Wang SZ, Lin ZB, Lin YX (2003). Isolation and identification of active components of *Ganoderma lucidum* cultivated with grass and wood log I. Extraction, purification and characterization of glycopeptide. *Chinese Traditional and Herbal Drugs.* **34**, 872-874.

Lin Z.B. (2001). Modern research on *Ganoderma.* Beijing Medical University Press, Beijing.

Liu G.T., Bao T.T., Wei H.L., Song Z.Y.. (1979). The pharmacological effect of alcohol extract from Zi-Zhi and Chi-Zhi on liver of mice. *Acta. Pharm. Sin.* **14**, 284-287.

Mecocci P., MacGarvey U., Kaufman A.E., Koontz D., Shoffner J.M., Wallace D.C. (1993) Oxidative damage to mitochondrial DNA shows marked age-dependent increas e in human brain. *Ann. Neurol.* **34**, 609-16.

Su CH, Lai MN, Chan MH. (1993). Hapatoprotective triterpenoids from *Ganoderma tsugae* Murrill. In: Mushroom biology and mushroom products, Chang ST et al, eds. The Chinese University Press, Hong Kong, 275-283.

Wang M.Y., Lin Z.B. (2000). Effects of triterpenoids from *Ganoderma lucidum* on immunological liver injury in vivo and in vitro. *Chin Pharm J.* **35**, 809-812.

Wang M.Y., Liu Q., Che Q.M., Lin Z.B. (2002). Effects of triterpenoids extract from *Ganoderma lucidum*(Curt:Fr.) P. Kast.(Reishi mushroom) on experimental liver injury models induced by carbon tetrachloride or d-galactosamine in mice. International Journal Medicinal *Mushrooms.* **4**, 337-342.

Wang M.Y., Liu Q., Che Q.M., Lin Z.B. (2000). Effects of triterpenoids from *Ganoderma lucidum* on three models of liver injury in mice. Acta Pharm Sin. **35**, 326-329.

Winterbourn, C., Munday, R.(1989). Glutathione mediated redox cycling of alloxan. Mechanism of superoxide inhibition and metal catalysed OH formation. *Biochem. Phamacol.* **38**, 271-277.

Yang H.L. (1998). Free radical and tumor. In: Yang HL, Pan XX, WuYK, eds. Advanced pathophysiology. Beijing: Science Press, p 433-51.

You Y.H., Lin Z.B. (2002). Protective effects of *Ganoderma lucidum* Polysaccharides peptide on injury of macrophages induced by reactive oxygen species1. *Acta Pharmacol Sin.* **23**: 787-791.

You Y.H., Lin Z.B. (2003). Antioxidant effect of *Ganoderma* polysaccharide peptide. Acta Pharm Sin. **38**, 85-88

You Y.H., Lin Z.B. (2004). Free radical scavenging activity by *Ganoderma lucidum* polysaccharides peptide on peritoneal macrophages in mice. *Chin. J. Clin. Pharmacol. Ther.*, **9(1)**, 52-55.

Zhang G.L., Wang Y.H., Ni W., Teng H.L., Lin Z.B. (2002). Hepatoprotective role of *ganoderma lucidum* polysaccharide against BCG-induced immune liver injury in mice. *World J Gastroenterol.* **8**, 728-733.

Zhang H.M., Tian H.K., Yao X.H., Lin Z.B., Li X.J. (2002a). The effects of *ganoderma lucidum* on expression of adhesion molecules ICAM-1 and VCAM-1 of HUVEC. *Chin. Pharmacol. Bull.* **18**, 460-464.

Zhang H.M., Yao W.J., Tian H.K., Lin Z.B., Li X.J. (2002b) Effect of Lugu *Ganoderma lucidum* on low-density lipoprotein oxidation and adhesion to endothelium. *Chinese Journal of Integrated Traditional and Western Medicine.* **22**, 534-537.

Zhang H.N., He J.H., Yua L., Lin Z.B. (2003). In vitro and in vivo protective effect of *Ganoderma lucidum* polysaccharides on alloxan-induced pancreatic islets damage. *Life Sciences,* **73**, 2307-2319.