Effects of *Ganoderma lucidum* on Obstructive Jaundice-induced Oxidative Stress

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OBJECTIVE: Obstructive jaundice develops after occlusion of the common bile duct. Direct hyperbilirubinaemia, which occurs secondary to the condition, causes various life-threatening pathologies. Cytoprotective effects of *Ganoderma lucidum* (GL) have previously been shown. In this study, the effects of GL on oxidative stress and oxidant DNA damage in experimental obstructive jaundice were evaluated.

METHODS: Sixty Wistar albino adult female rats were randomly divided into six weight-matched equal groups: sham group, bile duct ligated group (BDL); after sham operation 250 mg/kg/d of GL administered group, after sham operation 500 mg/kg/d of GL administered group, after bile duct ligation 250 mg/kg/d of GL administered (GL1BDL) group, and after bile duct ligation 500 mg/kg/d of GL administered (GL2BDL) group. GL polysaccharide was orally administered to the rats via gavage tube once a day for 14 days after bile duct ligation.

RESULTS: The plasma malondialdehyde levels of the GL1BDL and GL2BDL groups were significantly lower than those of the BDL group (p < 0.01). The plasma 8-hydroxy-2′-deoxyguanosine levels of the GL1BDL and GL2BDL groups were significantly lower than those of the BDL group (p < 0.001). The liver tissue Cu-Zn superoxide dismutase level of the GL2BDL group was significantly higher than that of the BDL group (p < 0.05).


Key Words: direct hyperbilirubinaemia, DNA damage, experimental cholestasis, *Ganoderma lucidum*, obstructive jaundice, oxidative stress

Introduction

Obstructive jaundice (OJ) develops after occlusion of the common bile duct as a result of benign or malignant diseases such as choledocholithiasis, bile duct strictures, or cholangiocarcinoma. Direct hyperbilirubinaemia, which occurs secondary to OJ, causes liver dysfunction, gastrointestinal barrier dysfunction, immune dysfunction, coagulation dysfunction, lack of detoxification, and diminished wound healing. Icteric conditions ameliorate the harmful oxidant molecular modifications that causing life-threatening conditions.3,4
Reactive oxygen species (ROS), namely superoxide and hydroxyl free radicals, together with hydrogen peroxide, are believed to be directly toxic, and ROS can initiate free-radical-mediated chain reactions. ROS damage the building structures of the cell membrane, nucleus, and genetic material by causing scission, carbonylation, fragmentation, cross-linking, and oxidation. These structural changes lead to the decrease or loss of protein biological function. Malondialdehyde (MDA) is one of the products of lipid per-oxidation. The compound 8-hydroxy-2′-deoxyguanosine (8-OHdG) is an oxidant of deoxyguanosine and a marker for oxidative DNA damage. Oxidant and antioxidant statuses are vital for regulation of homeostasis. Glutathione (GSH) and superoxide dismutase (SOD) are involved in the antioxidant system and are important for the protection of tissue from oxidative damage. GSH is a tripeptide. Its oxidized form, the dimer GSSG, which is involved in the transport of certain amino acids, is a coenzyme for various enzymes and protects against oxygen radicals and toxic compounds. GSH removes toxic substances from the environment and protects tissue from harmful substances after biotransformation. SOD, which catalyzes the dismutation of superoxide to hydrogen peroxide, catalyzes the conversion of two $O_2$ molecules into $H_2O_2$ and $O_2$. SOD exists in mitochondrial (Mn-SOD) and cytoplasmic (Cu/Zn-SOD) forms. Oxidative stress is also involved in the regulation of almost all cellular processes, including proliferation, differentiation, stress responses, and cell death.

**Ganoderma lucidum** (GL) is a crude drug used in Chinese medicine for the treatment of fatigue, insomnia, cough in elderly people, and some types of cancer. GL peptides are strong antioxidants and have potent free radical-scavenging activities. GL showed protective actions against acute hepatitis in some experimental studies. However, there is no data about the effects of GL therapy on oxidative stress in OJ. In this study, the effects of GL on oxidative stress and oxidative DNA damage in experimental OJ were evaluated.

### Patients and methods

This study was performed with approval from the Ethics Committee of the Animal Care Review Board of Istanbul University Experimental Medicine Research Institute. Adult female Wistar albino rats weighing 200–250g were obtained from the Experimental Animal Research Laboratory of Cerrahpasa Medical Faculty. The animals were housed in cages in a regulated environment (23 ± 2°C and 55 ± 15% relative humidity) under a 12-hour light/dark cycle and permitted ad libitum access to standard lab chow and tap water before and after surgery. The experiment was performed in the Cerrahpasa Medical Faculty Experimental Animal Research Laboratory. Sixty rats were randomly divided into six weight-matched equal groups. Group 1: sham group; Group 2: bile duct ligated group (BDL); Group 3: after sham operation GL (250 mg/kg/d) administered (GL1) group; Group 4: after sham operation GL (500 mg/kg/d) administered (GL2) group; Group 5: after bile duct ligation GL (250 mg/kg/d) administered (GLBDL) group; and Group 6: after bile duct ligation GL (500 mg/kg/d) administered (GL2BDL) group. GL polysaccharide (Ling Zhi extract) was purchased from Khao-La-Or Laboratories Ltd. (Samut Prakan, Thailand). GL polysaccharide was diluted with saline solution at daily doses of 250 mg/kg and 500 mg/kg as an aqueous extract. GL polysaccharide was orally administered to the rats via gavage tube once a day for 14 days after bile duct ligation. The sham operated groups and the BDL group received equal amounts of saline solution. The rats were sacrificed on postoperative day 14 by cervical dislocation, and blood and the tissue samples were collected.

### Surgical procedure

Under ketamine (40 mg/kg, intraperitoneal) and xylazine (5 mg/kg, intramuscular) anaesthesia, through a midline abdominal incision, the common bile duct was isolated and double-ligated in its middle third with 0000 silk suture; it was then transected between the two ligatures. In the sham operation, the common bile duct was isolated without ligation or transection after a midline abdominal incision. The abdomen was closed with continuous sutures.

### Biochemical procedures

#### Preparation of the tissue samples
Approximately 190–200 mg of each liver sample was weighed and diluted 20% weight per volume in 20 mM ice-cold Tris-HCl, pH 7.4, and homogenized with a homogenizer (Bosch Scintilla, SA, Switzerland). The homogenate was centrifuged at 5000g for 10 minutes, and various analyte determinations were performed in the supernatant fraction.

#### Assay of protein carbonyl
Plasma and tissue protein carbonyl levels were measured spectrophotometrically using the method of Reznick and Packer. Protein carbonyl groups react with 2,4-dinitrophenylhydrazine (DNPH) to generate chromophoric...
Dinitrophenylhydrazones. DNPH was dissolved in HCl, and following the DNPH reaction, proteins were precipitated with an equal volume of 20% trichloroacetic acid (w/v) and washed three times with 4 mL of an ethanol/ethyl acetate mixture (1:1). Washings were performed by mechanical disruption of pellets in the washing solution using a small spatula and repelleting by centrifugation at 6000 g for 5 minutes. Finally, the precipitates were dissolved in a 6-M guanidine-HCl solution, and the absorbances were measured at 360 nm using the molar extinction coefficient of DNPH (ε = 2.2 × 10^4/M·cm). The protein content was spectrophotometrically determined on the HCl blank pellets using a Folin kit (Sigma Diagnostics, St. Louis, MO, USA). The coefficients of intra-assay and interassay variations for the carbonyl assay were 4.1% and 8.5%, respectively.

Assay of malondialdehyde
Lipid peroxidation levels in plasma and tissue were measured with the thiobarbituric acid reaction. This method was used to obtain a spectrophotometric measurement of the colour produced during the reaction to thiobarbituric acid with MDA at 535 nm. The coefficients of intra- and interassay variations for the MDA assay were 3.6% and 5.3%, respectively.

Assay of total thiol content
Plasma and tissue thiol concentrations were determined using 5,5′-dithio-bis(2-nitrobenzoic acid) as described by Hu. The coefficients of intra-assay and interassay variations were 2.2% and 4.9%, respectively.

Assay of glutathione
GSH concentrations were determined according to the method of Beutler et al using metaphosphoric acid for protein precipitation and 5,5′-dithio-bis (2-nitrobenzoic acid) for colour development.

Assay of Cu-Zn SOD activity
Plasma and tissue Cu-Zn SOD activity was determined by the method of Sun et al. The assay involved inhibition of nitroblue tetrazolium (Sigma Chemical Co., St. Louis, MO, USA) and reduction with xanthine-xanthine oxidase (Sigma Chemical Co.), which is used as a superoxide generator. One unit of SOD is defined as the amount of protein that inhibits the nitroblue tetrazolium reduction rate by 50%. The coefficients of intra-assay and interassay variations were 1.8% and 3.2%, respectively.

Assay of 8-OHdG
The plasma 8-OHdG levels were determined using an enzyme-linked immunosorbent assay detection kit (Bioxytech 8-OHdG-EIA; OXIS Health Products, Portland, OR, USA). The coefficients of intra-assay and interassay variations for the 8-OHdG assay were 6.4% and 7.4%, respectively.

Histopathological evaluation
Liver biopsies obtained from the same lobe were fixed overnight in 10% buffered formalin and paraffin-embedded. The sections were stained with haematoxylin and eosin (H&E) for histological evaluation and examined under a light microscope. In brief, 4-μm-thick sections of paraffin-embedded rat liver were dewaxed in xylene, rehydrated in graded alcohol series, and washed with distilled water for 2 minutes. The sections were then stained with haematoxylin for 5 minutes at room temperature. After 15 minutes, the sections were counterstained with eosin for 2 minutes, dehydrated in graded alcohol series, washed with xylene, and blocked by rosin.

H&E-stained slides were evaluated under a microscope at 40× magnification by an experienced pathologist (Figure 1). The pathologist did not know which specimen belonged to which group. A method similar to that of Lichtman et al was used to score liver histology. Parenchymal injury (cell lining, regenerative activity, hepatocyte necrosis, deposition of bile pigments, hydropic degeneration, and polymorphonuclear cell infiltration), sinusoid injury (ductal proliferation, sinusoidal thrombosis, and Kupffer cell proliferation), and portal area injury (ductal proliferation, cholangitis, acute inflammation, chronic inflammation, bile retention, portal vascular congestion, and portal vascular thrombosis) were numbered from 0 to 3 (0 = none, 1 = slight, 2 = moderate, and 3 = severe). Fibrosis was graded according to the previously described method: Grade 0 = normal liver; Grade 1 = increase in collagen without formation of septa; Grade 2 = formation of incomplete septa from portal tract to central vein (septa that do not interconnect with each other); Grade 3 = complete but thin septa interconnecting with each other, dividing the parenchyma into separate fragments; and Grade 4 = as Grade 3, but with thick septa (complete cirrhosis).

Statistical analysis
All data are expressed as means and SD and 95% confidence intervals. For biochemical parameters, one-way
analysis of variance and Tukey’s post hoc test were used for statistical analysis and \( p < 0.05 \) was considered significant. For histological parameters, the Bonferroni-adjusted Mann-Whitney test was used for statistical analysis and \( p < 0.0083 \) was considered significant.

**Results**

The results are summarized in Tables 1 and 2. No rats died during the experimental procedure. Bilirubin levels of the BDL, GL1BDL, and GL2BDL groups were significantly higher than those of the sham group \( (p < 0.001) \). There were no significant differences between the sham, GL1, and GL2 groups in the bilirubin levels. The oxidative stress parameters of the BDL group were significantly higher than those of the sham group \( (p < 0.05) \). The plasma MDA, 8-OHdG, and protein carbonyl levels of the BDL group were significantly higher than those of the sham group \( (p < 0.001) \). The plasma Cu-Zn SOD, thiol, and GSH levels of the BDL group were significantly lower than those of the sham group \( (p < 0.001) \). There were no differences between the GL1, GL2, and sham groups in the plasma oxidative stress parameters. The plasma MDA levels of the GL1BDL and GL2BDL groups were significantly lower than those of the BDL group \( (p < 0.01) \) (Figure 2). The plasma 8-OHdG levels of the GL1BDL and GL2BDL groups were significantly lower than those of the BDL group \( (p < 0.001) \) (Figure 3). The plasma protein carbonyl level of the GL2BDL group was significantly lower than that of the BDL group \( (p < 0.01) \).

The liver tissue thiol, GSH, and Cu-Zn SOD levels of the BDL group were significantly lower than those of the sham group \( (p < 0.01) \). The values of the parameters in the liver tissue of the GL1 and GL2 groups were similar to those of the sham group. The liver tissue Cu-Zn SOD level of the GL2BDL group was significantly higher than that of the BDL group \( (p < 0.05) \). The liver tissue protein carbonyl levels of the GL1BDL and GL2BDL groups were

![Figure 1.](image-url)
Table 1. Plasma levels of the biochemical parameters

<table>
<thead>
<tr>
<th></th>
<th>Cu-Zn SOD (U/mL)</th>
<th>MDA (nmol/mL)</th>
<th>8-OHdG (ng/mL)</th>
<th>PCO (μM/mg)</th>
<th>Thiol (μM/gHb)</th>
<th>GSH (mg/dL)</th>
<th>Total Bilirubin (μM/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>24.71 ± 2.26</td>
<td>2.98 ± 0.27</td>
<td>5.81 ± 0.99</td>
<td>0.73 ± 0.2</td>
<td>319.56 ± 20.31</td>
<td>4.04 ± 0.21</td>
<td>0.18 ± 0.09</td>
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<tr>
<td>BDL</td>
<td>18.46 ± 1.57</td>
<td>4.65 ± 0.47</td>
<td>11.23 ± 0.95</td>
<td>1.19 ± 0.2</td>
<td>246.73 ± 22.4*</td>
<td>2.88 ± 0.14*</td>
<td>9.48 ± 0.2*</td>
</tr>
<tr>
<td>GL1</td>
<td>24.73 ± 3.16</td>
<td>2.70 ± 0.18</td>
<td>5.75 ± 1</td>
<td>0.73 ± 0.1</td>
<td>300.44 ± 23.25</td>
<td>3.76 ± 0.34</td>
<td>0.19 ± 0.07</td>
</tr>
<tr>
<td>GL2</td>
<td>23.12 ± 3.1</td>
<td>2.76 ± 0.25</td>
<td>5.41 ± 1.04</td>
<td>0.72 ± 0.12</td>
<td>329.25 ± 20.74</td>
<td>4 ± 0.3</td>
<td>0.17 ± 0.1</td>
</tr>
<tr>
<td>GL1BDL</td>
<td>19.99 ± 1.41</td>
<td>3.92 ± 0.46†</td>
<td>6.88 ± 1.09†</td>
<td>1.03 ± 0.16*</td>
<td>253 ± 22.86</td>
<td>2.99 ± 0.41*</td>
<td>9.03 ± 0.79*</td>
</tr>
<tr>
<td>GL2BDL</td>
<td>21.21 ± 1.59§</td>
<td>3.92 ± 0.45†</td>
<td>7.13 ± 0.85†</td>
<td>0.92 ± 0.12</td>
<td>269.38 ± 19.74</td>
<td>3.24 ± 0.26</td>
<td>9.28 ± 0.38*</td>
</tr>
</tbody>
</table>

*p < 0.001 between the sham group and the other groups; †p < 0.01 between the BDL group and the other groups; ‡p < 0.001 between the sham group and the other groups. BDL = bile duct ligated group; GL1 = after sham operation Ganoderma lucidum (GL; 250 mg/kg/d) administered group; GL2 = after sham operation GL (500 mg/kg/d) administered group; GLBDL1 = after bile duct ligation GL (250 mg/kg/d) administered group; GLBDL2 = after bile duct ligation GL (500 mg/kg/d) administered group; SOD = superoxide dismutase; MDA = malondialdehyde; 8-OHdG = 8-hydroxy-2′-deoxyguanosine; PCO = protein carbonyl; GSH = glutathione.

Table 2. Liver tissue levels of the biochemical parameters

<table>
<thead>
<tr>
<th></th>
<th>Cu-Zn SOD (U/mg)</th>
<th>MDA (nmol/mg)</th>
<th>PCO (nmol/mg)</th>
<th>Thiol (nmol/mg)</th>
<th>GSH (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.62 ± 0.11</td>
<td>0.93 ± 0.19</td>
<td>0.22 ± 0.03</td>
<td>109.22 ± 12.59</td>
<td>31.33 ± 2.6</td>
</tr>
<tr>
<td>BDL</td>
<td>0.35 ± 0.09*</td>
<td>1.47 ± 0.16</td>
<td>0.72 ± 0.11*</td>
<td>83.6 ± 9.31†</td>
<td>23 ± 3.16*</td>
</tr>
<tr>
<td>GL1</td>
<td>0.63 ± 0.07</td>
<td>0.9 ± 0.18</td>
<td>0.21 ± 0.07</td>
<td>100 ± 15.05</td>
<td>29.44 ± 2.5</td>
</tr>
<tr>
<td>GL2</td>
<td>0.66 ± 0.11</td>
<td>0.83 ± 0.19</td>
<td>0.2 ± 0.05</td>
<td>115 ± 14.2</td>
<td>31.13 ± 2.26</td>
</tr>
<tr>
<td>GL1BDL</td>
<td>0.45 ± 0.06†</td>
<td>1.12 ± 0.14</td>
<td>0.5 ± 0.07**</td>
<td>88.67 ± 8.31†</td>
<td>24.67 ± 3.5*</td>
</tr>
<tr>
<td>GL2BDL</td>
<td>0.5 ± 0.04§</td>
<td>1.35 ± 0.9</td>
<td>0.38 ± 0.11†</td>
<td>93.25 ± 15.87</td>
<td>26.63 ± 3.35§</td>
</tr>
</tbody>
</table>

*p < 0.001 between the sham group and other groups; †p < 0.01 between the sham group and other groups; ‡p < 0.001 between the BDL group and the other groups; †p < 0.01 between the BDL group and the other groups. BDL = bile duct ligated group; GL1 = after sham operation Ganoderma lucidum (GL; 250 mg/kg/d) administered group; GL2 = after sham operation GL (500 mg/kg/d) administered group; GLBDL1 = after bile duct ligation GL (250 mg/kg/d) administered group; GLBDL2 = after bile duct ligation GL (500 mg/kg/d) administered group; SOD = superoxide dismutase; MDA = malondialdehyde; PCO = protein carbonyl; GSH = glutathione.

Figure 2. The plasma malondialdehyde (MDA) levels of the GL1BDL and GL2BDL groups were significantly lower than those of the BDL group. *p < 0.01. GLBDL1 = after bile duct ligation Ganoderma lucidum (GL; 250 mg/kg/d) administered group; GL2BDL = after bile duct ligation GL (500 mg/kg/d) administered group; BDL = bile duct ligated group.

Figure 3. The plasma 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels of the GL1BDL and GL2BDL groups were significantly lower than those of the BDL group. *p < 0.001. GLBDL1 = after bile duct ligation Ganoderma lucidum (GL; 250 mg/kg/d) administered group; GL2BDL = after bile duct ligation GL (500 mg/kg/d) administered group; BDL = bile duct ligated group.
significantly lower than those of the BDL group \((p < 0.001)\) (Figure 4).

In the histological evaluation (Table 3), the cell lining, hepatocyte necrosis, deposition of bile pigments, hydropic degeneration, polymorphonuclear cell infiltration, ductal proliferation, sinusoidal thrombosis, Kupffer cell proliferation, cholangitis, acute inflammation, chronic inflammation, bile retention, portal vascular congestion, portal vascular thrombosis, and fibrosis scores of the BDL group were significantly higher than those of the sham group \((p < 0.000)\). There were no significant histological differences between the sham, GL1, and GL2 groups. Lower ductal proliferation and sinusoidal thrombosis scores were observed in both the GL1BDL \((p < 0.001)\) and GL2BDL \((p < 0.007)\) groups according to the BDL group. Kupffer cell proliferation \((p < 0.002)\), cholangitis \((p < 0.001)\), acute inflammation \((p < 0.000)\), chronic inflammation \((p < 0.004)\), and bile retention \((p < 0.002)\) scores of the GL1BDL group were significantly lower than those of the BDL group.

Discussion

Decompression with surgery or minimally invasive techniques is the principle treatment of OJ in clinical practice. However, sometimes these interventional therapies are not sufficient to prevent the development of life-threatening complications of hyperbilirubinaemia.\(^23,24\) In previous experimental studies, it has been shown that bile duct obstruction induces oxidative stress and reduces the production of different enzymes with antioxidant properties. Additionally, increased lipid peroxidation is observed in patients with cholestatic liver disease.\(^25,26\) Increased levels of MDA result in oxidative damage to lipids, and increased levels of protein carbonyl is a marker of oxidative protein modification.\(^27,28\) Decreased GSH levels caused by impaired biliary secretion are seen in prolonged cholestatic liver diseases.\(^23\) Augmented oxidant activity that is mediated by ROS damages DNA.\(^29\) We observed the harmful effect of OJ on the biochemical and histological parameters in the plasma and liver tissue. The level of MDA did not change in the cholestatic liver tissue, but a significant upswing of lipid peroxidation was seen in the plasma. Increased protein oxidation and depleted antioxidant activity were the biochemical evidence of oxidant damage in the liver tissue after bile duct ligation. In our opinion, the uncorrelated liver tissue and plasma MDA levels have two possible explanations. First, hyperbilirubinaemia is a systemic pathology and could cause additional oxidant damage to other organs, and the rank of the oxidized lipid molecules could be higher in plasma according to liver tissue. Second, protein oxidation could occur before lipid peroxidation in hyperbilirubinaemic conditions.

The effects of many different chemicals on oxidative stress were evaluated in experimental OJ. Recently, the ameliorative effects of methylene blue, fluvastatin, and sphingosylphosphorylcholine against cholestatic liver injury via their antioxidant and anti-inflammatory actions have been documented.\(^3,4,30\) We did not observe any toxic effects of GL, which is a popular mushroom-derived drug of traditional Chinese medicine.\(^12\) As a consequence of GL therapy, DNA damage and oxidative stress parameters of plasma were reduced in OJ. Histological findings showed that GL treatment ameliorated the harmful effects of hyperbilirubinaemia. Reduced bile retention and reduced inflammation were noticed in the liver tissue of the GL-treated, OJ-induced groups. GL could be cytoprotective via suppression of damaging factors and support of cell proliferation.\(^31\) Beneficial effects of GL were shown in acute and chronic hepatic inflammation induced by BCG-immunostimuli in mice.\(^15\) Triterpene extract of GL inhibits NF-kappa B and AP-1 signalling pathways, which are main mediators of the inflammatory response.\(^32\) Although the active components responsible for the antioxidant activity of GL are still not well documented, the efficacious effects of GL via its antioxidant properties have been presented.\(^12,13\) GL resulted in reduced liver fibrosis induced by CCL\(_4\. Lin and Lin\(^33\) suggested that oral administration of GL could

![Figure 4. Liver tissue protein carbonyl (PCO) levels of the GL1BDL and GL2BDL groups were significantly lower than those of the BDL group. *p < 0.001. GLBDL1 = after bile duct ligation Ganoderma lucidum (GL; 250 mg/kg/d) administered group; GL2BDL = after bile duct ligation GL (500 mg/kg/d) administered group; BDL = bile duct ligated group.](image)
reduce chronic liver injury via a protective effect against hepatocellular necrosis by its free-radical scavenging ability. Park et al\textsuperscript{34} reported that the polysaccharide of GL reduced serum aspartate transaminase, alanine transaminase, alkaline phosphatase, and total bilirubin; reduced the collagen content in liver; and improved liver morphology. They claimed that GL polysaccharides could act as antifibrotic agents. Wu et al\textsuperscript{35} suggested that GL extracts enhance collagenolytic activity. We noticed increased fibrosis on the cholestatic liver histology, but no fibrolytic activity of GL was observed among the experimental groups. The discrepancy of the results of the studies could be related to the duration and design of the experimental procedures, sequence and dosage of the GL administration, and type of experimental animal. Further analysis must be performed to evaluate the activity of GL against liver fibrosis. In our study, increased total bilirubin levels were not changed after GL treatment because the rats underwent an acute, mechanical, total, and unrelieved biliary obstruction lasting throughout the study period, as previously described.\textsuperscript{36}

In conclusion, GL has antioxidant activity in direct hyperbilirubinaemic conditions and may protect DNA structure and liver tissue by reducing oxidative damage in OJ as a palliative agent. However, further studies are needed to clarify the protective mechanisms of GL.

\textbf{References}


