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# GRIFOLIN ATTENUATES WHITE MATTER LESION IN OXYGEN/ GLUCOSE DEPRIVATION

## Abstract

The present study evaluates the effect of grifolin (GFL) in oxygen/glucose deprivation (OGD) induced white matter lesion. Injury induced with OGD was found to be significant at the 9th h of OGD induction and the effect of GFL on the proliferation of oligodendrocyte precursor cells (OPCs) was assessed by CCK-8 and Hoechst 33258 assay at GFL 1, 5, 25, 50 and 100  $\mu\text{m}$  concentrations. Whereas immunocytochemistry was performed for the assessment of survival and apoptosis of OPCs, western blot assay and RT-PCR were performed after 8th day of OGD injury for the estimation of expressions of myelin basic protein (MBP) and inhibitor of DNA binding 2 (Id2) in OPCs respectively. Results of the study suggests that treatment with GFL significantly enhances the survival rate and decreases the apoptosis of OPCs in OGD induced injury model. Immunocytochemical staining of Oligodendrocyte transcription factor (Olig2) and Bromodeoxyuridine (BrdU) shows that GFL treatment improves the proliferation of OPCs than OGD group. Moreover data of western blot assay suggested that treatment with GFL significantly enhances the expressions of MBP and Olig2 than OGD. It was observed that expressions of Id2 decreases and Olig2 enhances in GFL treated group than OGD group. Data of our study concludes that GFL enhances the differentiation and proliferation of OPCs in OGD-induced injury by altering the expressions of Id2 and Olig2.

## Keywords

Oligodendrocyte precursor cells • Grifolin • Myelin basic protein • Hypoxia • White matter

## Introduction

In the central nervous system, myelin is formed from oligodendrocytes. Survival of axon and conduction of neuron is rapidly facilitated by oligodendrocytes [1]. Oligodendrocytes mature into myelinated ones, during late embryonic development oligodendrocyte precursor cells migrate and proliferate into oligodendrocytes [2]. Hypoxia and premature delivery results in the injury of white matter of the brain that causes mortality and neurological deficits [3]. Presently, a small number of therapies are available that can prevent white matters injury. Literature suggests that white matter contain oligodendrocyte precursor cells that may help remyelinate the ganglia and thereby repair the injury [4]. Thus it is important to protect the oligodendrocyte precursor cells to attenuate the brain injury, as it matures and proliferates in to myelinated ganglia further.

Previously reported studies suggested that alternative medicine such as herbal extracts

and natural products show promising effect in the management of white matter lesion by promoting proliferation and differentiation of oligodendrocyte precursor cells. Various plants such as garlic, *Ginkgo biloba* and *Nigella sativa* show beneficial effect against cerebral injury in experimental rat model [5, 6]. Grifolin is a phenolic compound isolated from *Albatrellus ovinus* mushroom [7]. Literature reported that grifolin posse's potent anti-inflammatory and anticancer activity [8,9]. A study reports that grifolin inhibits the production of nitric oxide (NO) in LPS stimulated RAW 264.7 cells [10]. In addition, it inhibits the release of histamine from mast cell [11]. It was also found to posse's anti atherosclerotic activity and thereby used for the management of cardiovascular disease [12].

## Material and methods

### Animals


Female Wistar rats were kept individually under observation for delivery in the cage.

All the rats were housed under a controlled condition specified as per the guidelines. All the experiments used in the given study are approved by animal ethical committee of the Tongji Hospital, Huazhong University of Science and Technology, China (HUST/2016/12) and the given study followed the guidelines of Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC) for experimentation and animal use.

### Cell Culture

A previously suggested method by Wang et al., 2011 was used for the proliferation of oligodendrocyte precursor cells. Cells were isolated from the cerebral cortex of pups by placing it in to streptomycin (50  $\mu\text{g}/\text{ml}$ ) and penicillin (50  $\mu\text{g}/\text{ml}$ ) containing ice-cold DMEM/F12 medium. Cell suspension was prepared and filtered it with cell strainer (70  $\mu\text{m}$ ). Later cell were centrifuged for the period of 10 min at 10000 rpm and collected cells

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were resuspended in DMEM/F12 medium and incubated at 37 °C under controlled humid atmosphere for the duration of eight days. After every alternate day, old medium was replaced with fresh medium. Later samples were kept on orbital shaker to isolate the OPCs at 37 °C for the period of 60 min and the medium was replaced with fresh medium to remove the macrophages and microglial cells. Then again flask was shaken at 220 rpm for the period of 18 h by adding fresh DMEM/F12 medium (15 ml). Collected sample of cell suspension was kept in Petri dish and incubated at 37 °C for 30 min. Then the cell suspension was transferred to 50 ml tube by passing it from the sieve of 15 µm size and later centrifuged it for 10 min at 1000 rpm to separate the cells. Medium of DMEM/F12 that contains FGFb (20 ng/ml), PDGF-BB (20 ng/ml) and 2 % B27 was used to resuspend the cells and thereafter placed it into 25 cm<sup>2</sup> flasks at a quantity of 10,000 cells/cm<sup>2</sup>. Collected purified OPCs were differentiated by separating it into control; negative control and grifolin treated group. All the groups were allowed for incubation at 37 °C for 8 days after OGD injury.

### Injury induced with OGD

Injury induced with OGD was performed as per previously described method. OPCs were kept for the duration of 30 min into the anaerobic chamber for the incubation after washing it with phosphate-buffered saline on 3<sup>rd</sup> day. Later for the different time interval like 3, 6, 9, or 12 h at 37 °C cells were placed in the chamber and then placed into a normoxic chamber after resuspending it into OPCs medium.

### CCK-8 Assay

CCK-8 assay was performed for the evaluation of protective effect of grifolin on the viability of OPCs. Cells of each group were poured into 96 well plate and treated with condition medium. Later cells were incubated at 37 °C for the period of 4 h with CCK-8 solution. Viability of OPCs was absorption was observed at a wavelength of 450 nm.

### Hoechst 33258 assay

Hoechst 33258 assay was done for the estimation of apoptosis as per the previously reported method. Paraformaldehyde was used

to fix the treated cells at room temperature for half an hour and later wash it with PBS for three times. Hoechst 33258 dye was used at a concentration of 5 µg/ml for the staining of cells at 37 °C for the period of 15 min and thereafter washes it with PBS solution under fluorescent microscope.

### Immunocytochemistry

Paraformaldehyde was used to fix the treated cells at room temperature for half an hour and later washed with PBS for three times. Primary antibodies such as mouse anti-O4, mouse anti-BrdU, rabbit anti-Olig2 and mouse polyclonal anti MBP were used to incubate with the cells at 4 °C overnight and later for 60 min with secondary antibodies such as goat antirabbit IgG and CY3-conjugated goat anti-mouse at 4 °C. In BrdU staining, Sodium citrate (30 mM), NaCl (280 mM) and formamide (50%) was used to treat cells for the duration of 60 min at 65 °C and later incubated with primary antibody for 20 min at 37 °C. then rinse the same with boric acid (0.1 M) for the duration of 10 min at room temperature. confocal laser scanning microscope system was used to estimate the fluorescence signals.

### Western Blot Assay

Western blot assay was performed for the estimation of protein expressions of Olig2 and MBP. All the cells were centrifuged at 4 °C for the period of 10 min at 1000 rpm and later lysis buffer that contains protease inhibitor, Tris-HCl (40 mM), EDTA (10 mM), dithiothreitol (1 mM) and Nonidet P-40 (0.1 %) was used to suspend cells for the period of half an hour. Thereafter BIO-RAD protein assay kit used for the estimation of concentration of protein. Membrane were incubated with anti-MBP and anti-Olig2 like antibody at 4 °C for overnight and further incubated with secondary antibody for the duration of 90 min after the washing of it. Densitometry was used for the determination of intensity of band in this study.

### RT-PCR

Expressions of Id2 mRNA and Olig2 proteins in OPCs were estimated by RT-PCR after OGD. As per the instruction given by manufacturer total RNAs was extracted with TRIzol from the

cells. cDNA was formed by reverse transcription of total RNA and later PCR was done. RT-PCR was done by using specific primers and gel electrophoresis was used to separate the product of PCR. All the products were visualized by UV trans-illumination and samples were normalized compared to GAPDH band. Image J software was used to analyze the images.

### Statistical analysis

Values of the given data represented as mean ± SD (n=6) and the data of this study statistically analyzed by one-way ANOVA (Dunnnett post hoc test). Level of significance was considered as  $p < 0.05$ .

## Result

### Determination of GFL on OGD-Induced Injury

Survival rate and apoptosis rate of OPCs by CCK-8 assay and Hoechst 33258 nuclear staining respectively in OGD induced injury was shown in (Fig. 1). It was observed that at different time intervals survival rate of OPCs was found to decrease compared to controls in the CCK-8 assay. However, the result of Hoechst 33258 nuclear staining shows that the apoptosis rate increases by the increase of time in OGD induced injury than control.

### Effect of GFL on the survival rate

Effect of grifolin on the survival rate of OPCs after 3 days of treatment by CCK-8 assay was shown in Fig. 2. It was observed that treatment with GFL significantly increases the survival rate of OPCs at a concentration of 5 µm and decreases with 50 and 100 µm concentration of GFL than control. Thus for further study GFL was used at concentration of 1, 5, 25 and 50 µm.

### Effect of grifolin on the Proliferation of OPCs

Effect of grifolin on the Proliferation of OPCs in OGD induced injured model was shown in Fig. 3. Proliferation rate of OPCs in OGD group was found to be lower. There was introduction of oxygen/glucose for the duration of 3 days to the cell and grifolin (5 µm) treated group significantly ( $p < 0.01$ ) enhances the proliferation rate of OPCs than OGD group.

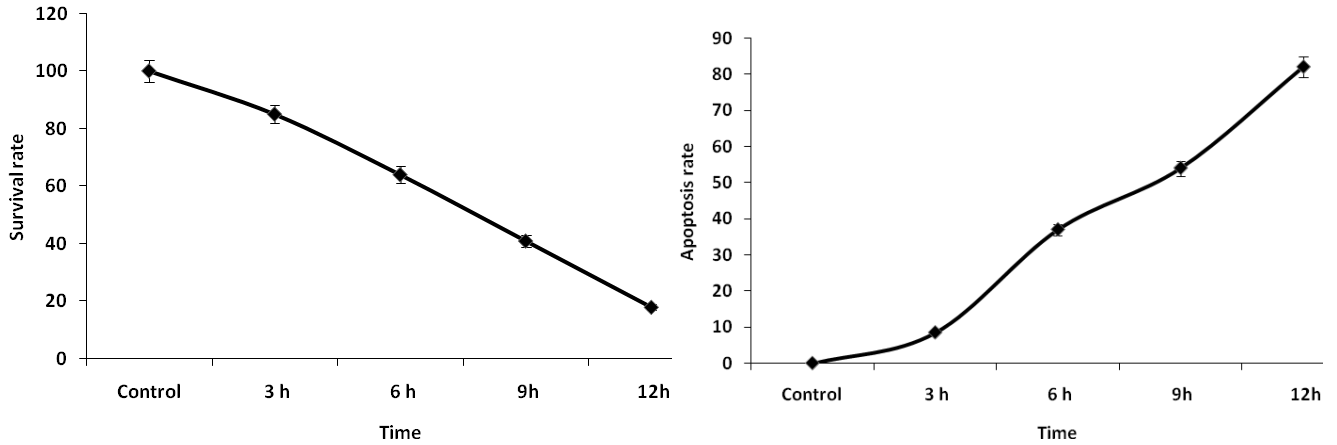


Fig. 1. Survival rate and apoptosis rate of OPCs in OGD induced injury

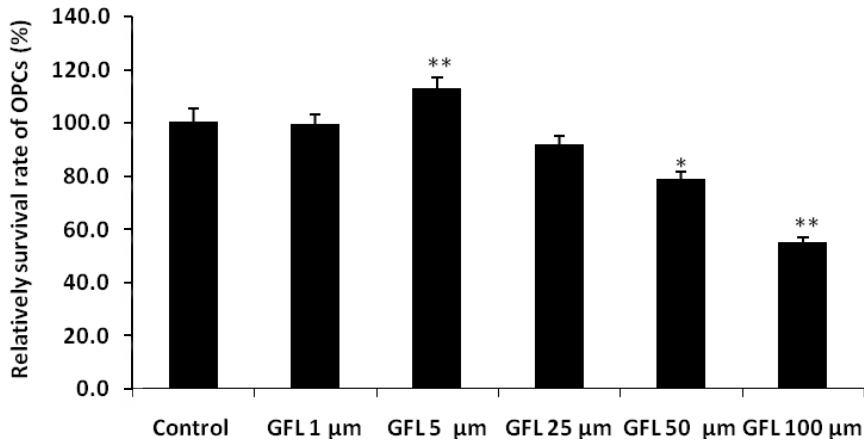


Fig. 2. Effect of grifolin on the survival rate of OPCs by CCK-8 assay  
Means  $\pm$  SD (n = 6), \*\*p < 0.01 than control

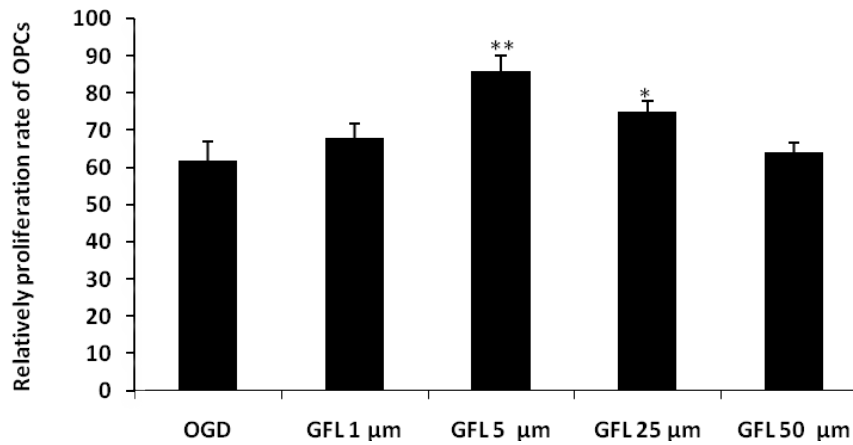


Fig. 3. Effect of grifolin on the Proliferation of OPCs in OGD induced injured model  
Means  $\pm$  SD (n = 6), \*\*p < 0.01 than control

Effect of grifolin on the proliferation of OPCs in OGD induced injury model by Brdu immunofluorescence staining was shown in Fig. 4. There was significant decrease in the proliferation of cells in OGD group; whereas treatment with GFL significantly ( $p < 0.01$ ,  $p < 0.05$ ) improves the proliferation of cells than OGD group. Thus these results suggest that GFL improves that survival rate of cells than control group.

There was significant decrease in the proliferation of cells in OGD group compared to control group. While treatment with GFL was found to enhance the number of positive cells with Olig2/DAPI staining at a concentration of GFL  $5\mu\text{m}$ , thus GFL improves the proliferation of cells. Staining of Olig 2 indicates that GFL inhibit the OGD induced apoptosis and proliferate the cells as shown in Fig. 5.

**Effect of grifolin on the maturation of OPCs**

Effect of grifolin on the maturation of OPCs to oligodendrocytes in OGD induced injury model was observed on 8<sup>th</sup> day of OGD injury as shown in Fig. 6. In this study Olig2, O4 and MBP were used for the markers of OPCs, immature oligodendrocytes and mature oligodendrocytes respectively. It was observed that treatment with GFL significantly improves

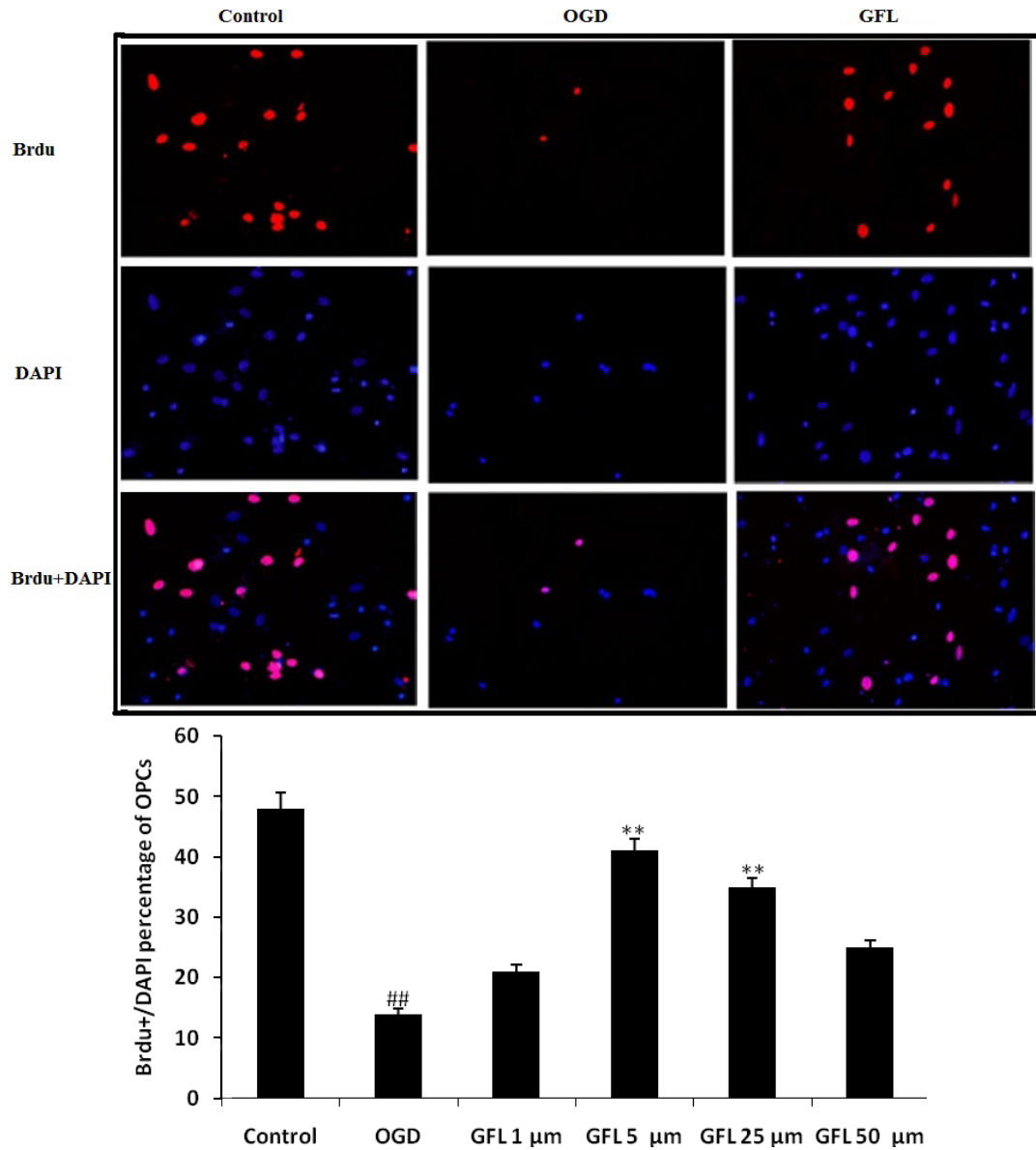


Fig. 4. Effect of grifolin on the proliferation of OPCs in OGD induced injury model by DAPI and Brdu immunofluorescence staining Means ± SD (n = 6), ## p < 0.01 than control, \*\* p < 0.01 than OGD

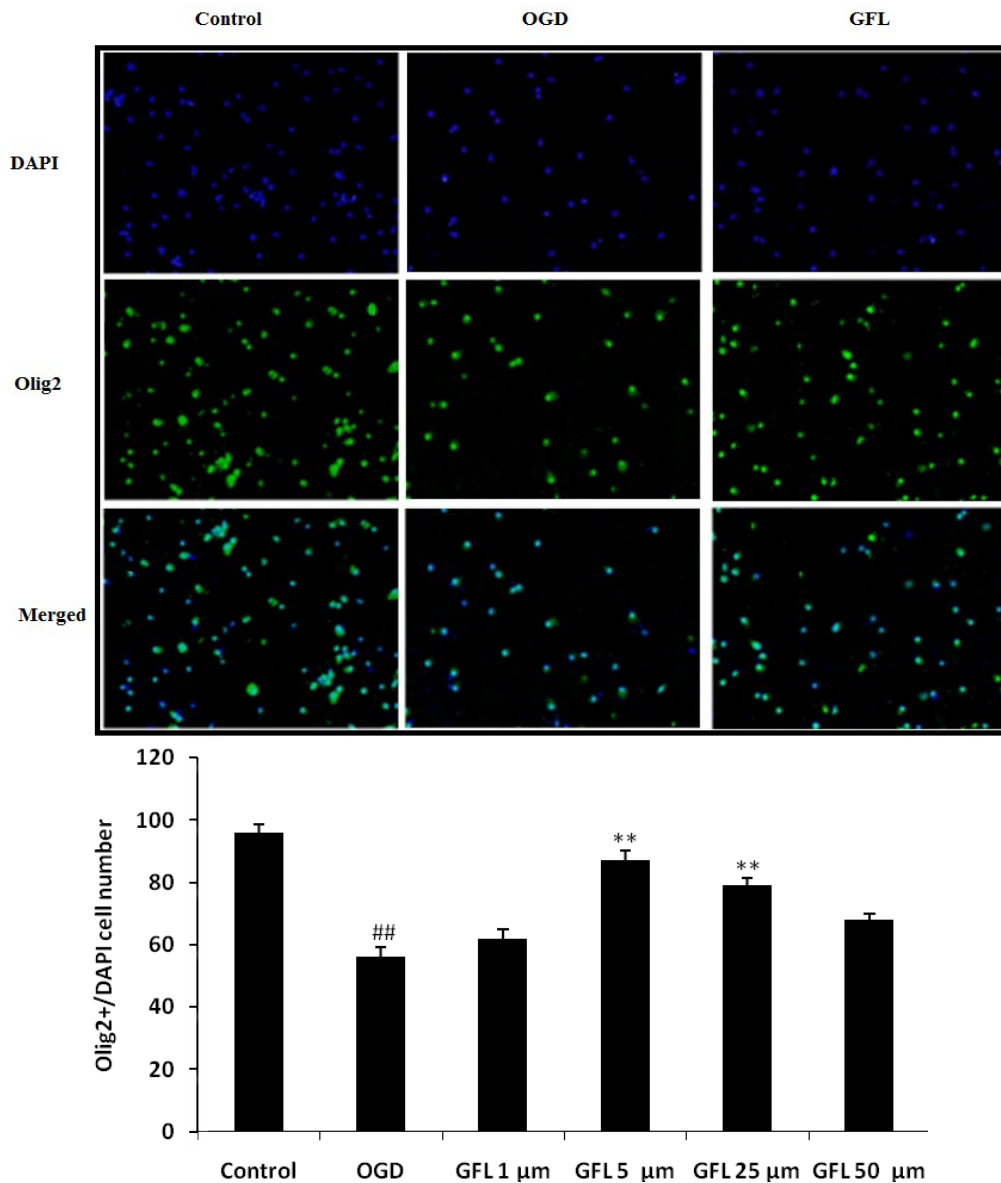


Fig. 5. Effect of grifolin on the proliferation of OPCs in OGD induced injury model by Olig-2 immunocytochemical staining Means ± SD (n = 6), ## p < 0.01 than control, \*\* p < 0.01 than OGD

the Olig2, O4 and MBP positive cells than OGD group on 8<sup>th</sup> day of OGD injury. Hence result of the study suggested that treatment of GFL enhances the maturation of OPCs.

#### Effect of grifolin on the expressions of MBP and Olig-2 in OPCs

Effect of grifolin on the expressions of MBP and Olig-2 in OPCs in OGD induced injury model by Western blot assay was shown in Fig. 7. There were significant increases (p < 0.01, p < 0.001)

in expressions of MBP and Olig2 proteins than the control and OGD groups. Results suggest that the expressions of MBP and Olig2 proteins enhances with the increase in the concentration of GFL (1, 5, 25 and 50 μm).

#### Effect of grifolin on the expressions of Id3 and Olig2 in OPCs

The effect of grifolin on the expressions of Id3 and Olig2 in OPCs in OGD induced injury model was observed by using RT-PCR as shown in Fig.

8. It was observed that OGD injury significantly reduces the expressions of Olig2 and enhances the expressions of Id2 than control group. Whereas, treatment with GFL significantly attenuates the altered expressions of Id2 and Olig2 protein in ODG injured model.

### Discussion

OPCs are developing into the oligodendrocytes at the time of CNS development and later help

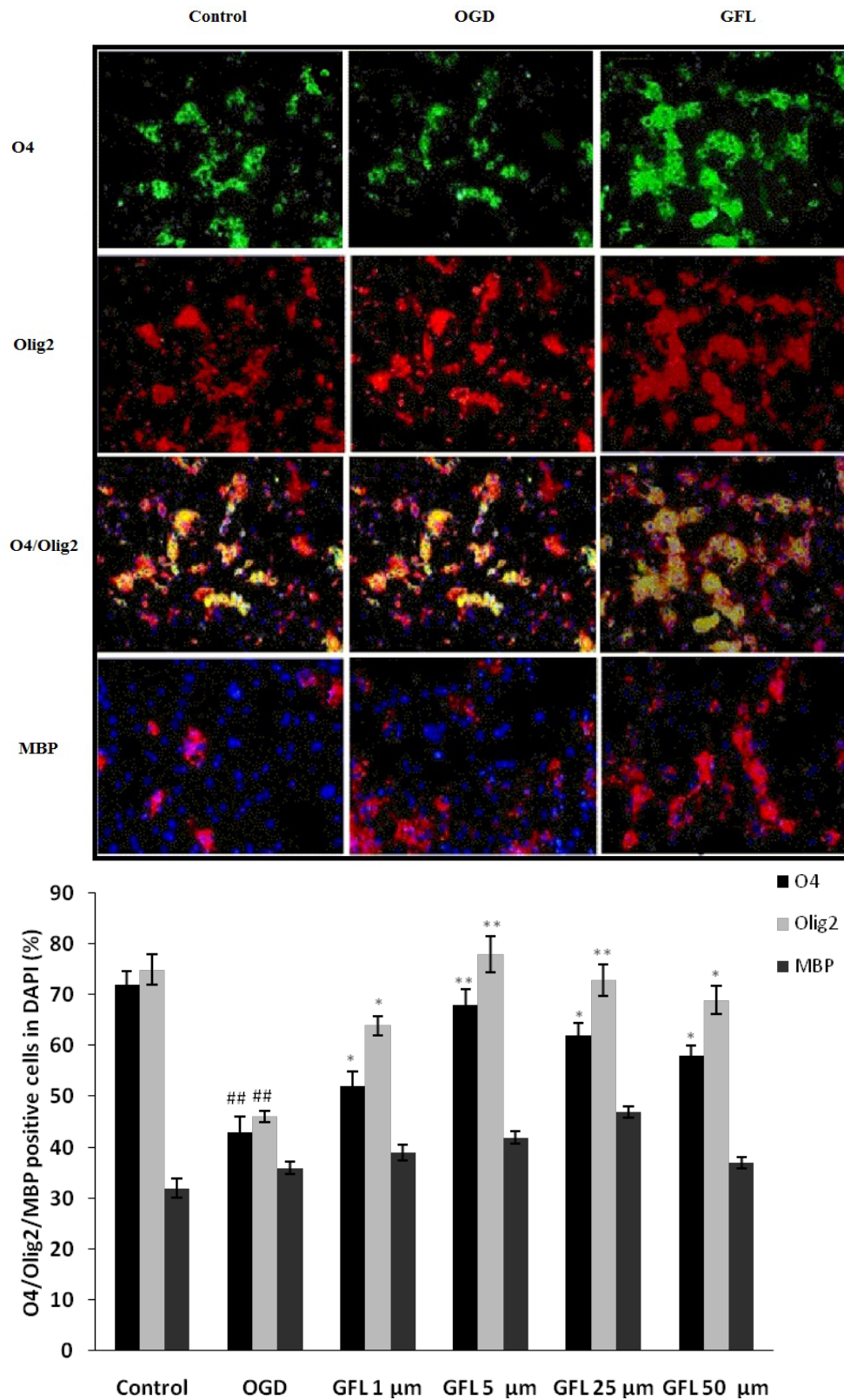


Fig. 6. Effect of grifolin on the maturation of OPCs to oligodentrocytes in OGD induced injury model  
 Means ± SD (n = 6), <sup>##</sup>p < 0.01 than control; <sup>\*</sup>p < 0.05, <sup>\*\*</sup>p < 0.01 than OGD

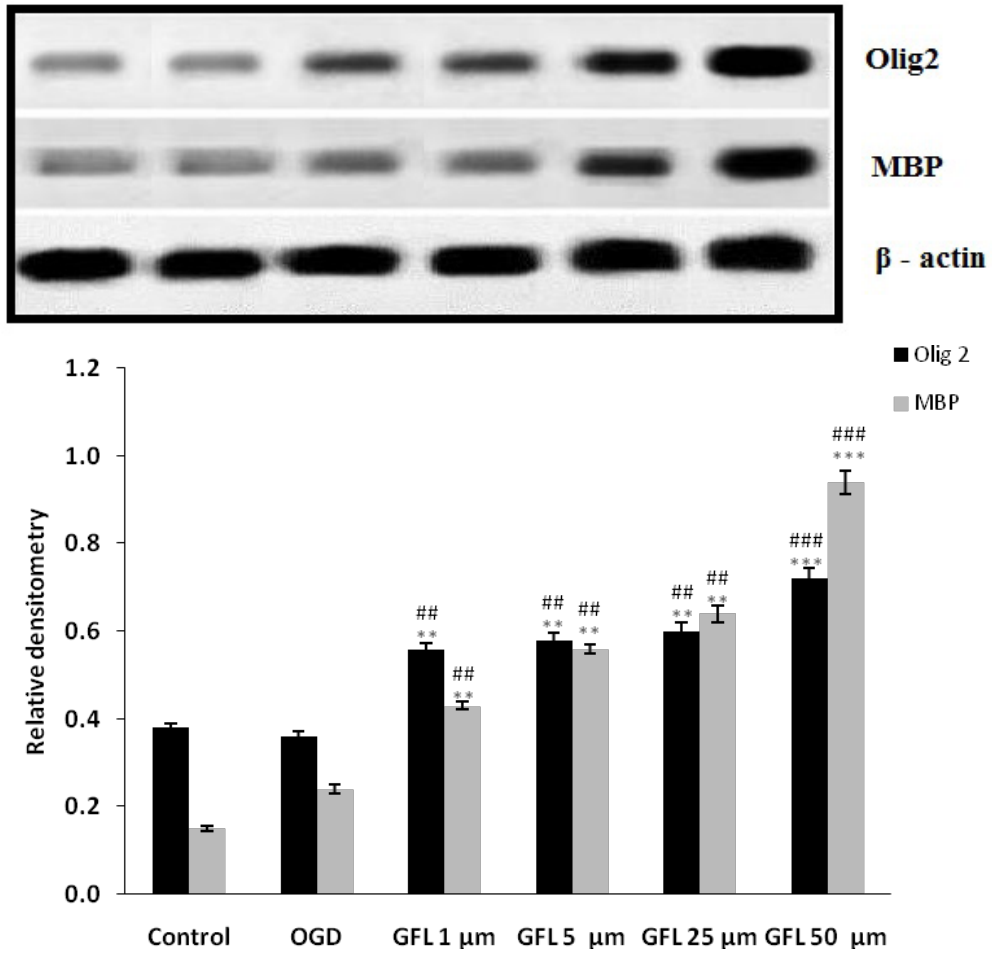


Fig. 7. Effect of grifolin on the expressions of MBP and Olig-2 in OPCs in OGD induced injury model by Western blot assay  
Means ± SD (n = 6), <sup>##</sup>p < 0.01, <sup>###</sup>p < 0.001 than control; <sup>\*\*</sup>p < 0.01, <sup>\*\*\*</sup>p < 0.001 than OGD

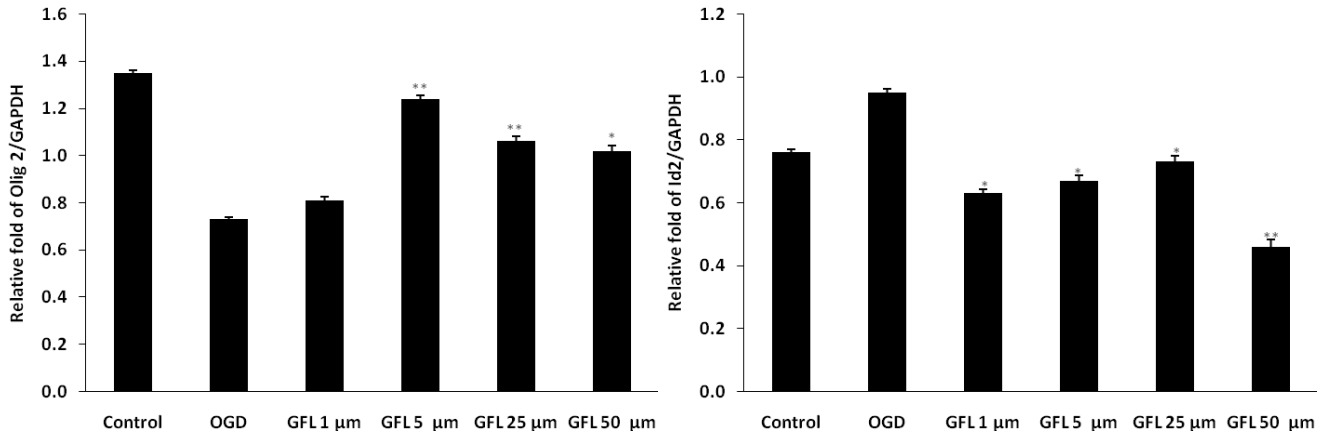


Fig. 8. Effect of grifolin on the expressions of Id3 and Olig2 in OPCs in OGD induced injury model  
Means ± SD (n = 6), <sup>\*</sup>p < 0.05, <sup>\*\*</sup>p < 0.01 than OGD

in the formation of myelin of the neurons [2]. Hypoxia results in the formation of ROS and thereby damages OPCs [13]. Moreover reported study suggested that OPC injury propagates by accumulation of glutamate and also alters the function of mitochondria in OGD model [14]. GFL shows neuroprotective effect in cerebral ischemia induced rat model as it inhibit the process of apoptosis on the basis of its antioxidant and anti-inflammatory property [15]. Thus present investigation reports neuroprotective effect of grifolin against oxidative stress and neurotoxicity induced by ODG.

Culture of OPCs was found to be grown well after 3days and density of cell was found to be suitable for the further experiment. Hoechst 33258 and CCK-8 assay was done to assess protective effect of GFL against OGD induced injury in OPCs. However treatment with GFL blunts OGD's cellular havoc in a

concentration dependent manner. At higher (not exceedingly high) GFL concentrations cell viability levels approached those observed in the control group. Our work had shown that Qu at 1  $\mu\text{m}$  has no protective effect on OGD-induced injury of OPCs; while GFL at 100  $\mu\text{M}$  has a noxious effect on OPCs. The present study's concentrations of GFL ranged from 5 to 50  $\mu\text{M}$ . In order to investigate whether GFL increases the proliferation of OPCs after OGD, the proliferation of OPCs were measured by BrdU uptake and the number of Olig2. Cells was counted by immunocytochemical staining. Results of this study suggest that treatment with GFL significantly enhances the proliferation and survival of OPCs by improving the number of Olig2 and uptake of BrdU stain in OGD induced injury model. This study also evaluated the effect of GFT treatment on the maturation of OPCs in to oligodendrocytes on

8<sup>th</sup> day of OGD injury. Literature suggested that several factor contributes in the development of oligodendrocyte, Oligo2 and Id2 are those factor that suggest about the maturation of OPCs [16, 17]. Development of oligodendrocyte from OPCs helps in the myelination of neurons and neuronal development [18]. Results of our study suggest that treatment with GFL attenuates the expressions of Olig2 and Id2 protein in OPCs of OGD injury model.

## Conclusion

Data of our study concludes that GFL enhances the differentiation and proliferation of OPCs in OGD-induced injury by altering the expressions of Id2 and Olig2. These results show that GFL has a potential to prevent hypoxia related disorders and can be used as a potential therapeutic agent clinically.

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