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Investigation of the ameliorative effects of gallic acid against neurotoxicity caused by glutamate in C6 cells

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Abstract

Gallic acid (GA) is present as a phenolic component of various foods and plants. GA is a molecule with broad biological properties such as antioxidant, anti-microbial, and anti-inflammatory activities. As the main excitatory neurotransmitter in the mammalian central nervous system excessive extracellular glutamate can activate the glutamate receptors and neuronal/intracellular calcium (Ca^{2+}) overload, producing neurotoxicity, a common pathway for neuronal injury or death and is associated with neurodegenerative diseases. The present study aimed to investigate the effect of gallic acid on glutamate-induced cytotoxicity in C6 glioma cells. For the study, groups were formed from C6 cells as control, GA (100 µM, 24 h), Glutamate (10 mM, 24 h), and GA+Glutamate. In the study, Total oxidant (TOS), total antioxidant (TAS), MDA, and caspase-3 levels in the cells were determined by ELISA kit. The results showed that glutamate administration increased TOS, MDA, and caspase-3 levels by causing cytotoxicity in C6 cells ($p<0.05$). However, in C6 cells treated with GA before glutamate incubation, TOS, MDA, and caspase-3 levels were decreased, and TAS levels increased compared to the glutamate group ($p<0.05$). As a result, it was determined that GA treatment showed a protective effect in the glutamate-induced cytotoxicity model in C6 cells.

Keywords: Gallic acid, Glutamate, Oxidative stress, caspase-3, C6 glioma cells

Introduction

Gallic acid (3,4,5-trihydroxy benzoic acid, GA) is a potential antioxidant in wheat, hazelnuts, tea leaves, oak bark, green tea, apple peel, grapes, strawberries, pineapple; it is a phenolic compound found in natural products such as bananas and lemons [1, 2]. When these foods are ingested, micromolar concentrations of GA in the free or glucuronidized form are observed in human blood plasma [3]. It is colorless or very light yellow in color, has a crystal structure, and has a wide range of uses in food and pharmaceutical fields. Thanks to the phenolic hydroxyl groups it contains, GA clears ROS and prevents the formation of new free radicals and oxidation of lipids, DNA, and proteins. [4]. Phenolic compounds, which are natural antioxidants, protect against free radicals and become a solution to the problems they bring [5]. Studies have shown that GA reduces oxidative stress. Also, it is known to have anti-cancer, anti-oxidant, anti-inflammatory, antidiabetic, antiviral, antibacterial, antiallergic, and antiulcer effects [6, 7].

Glutamate, a non-essential amino acid, is a broadly directed neurotransmitter used to signal distribution in the central nervous system (CNS) [8, 9]. It is involved in motor and cognitive functions, sensory functions such as taste, smell, and vision integration, and vital functions such as respiration and heart rate. In addition, it plays an important role in many crucial physiological mechanisms, such as thinking, learning, memory, memory, neural plasticity, and neural development [10,11]. Although the amount of glutamate required for the continuation of functions in the CNS is at millimolar levels within the cell, high glutamate levels in the CNS can cause neuronal damage. Glutamate can cross the blood-brain barrier. Likewise, the amount in the CSF is tried to be kept constant by ensuring its passage from the Cerebrospinal Fluid (CSF) to the cells in the nervous system [12]. Glutamate is also used as a precursor molecule in synthesizing gamma-aminobutyric acid (GABA) [13]. Glutamate excitotoxicity causes abnormal Ca^{2+} influx in neuronal cells. This situation causes glutamate derived the mechanism of action of the damage is related to different organs such as brain, testis, liver and induction of oxidative stress. Oxidative stress is an intracellular condition, and in this case, reactive oxygen species levels increase, leading to damage to carbohydrates, lipids, proteins, and nucleic acids, causing damage and disruption of cellular metabolism [14]. Studies show that abnormal glutamate release causes overstimulation of glutamate receptors, cell death, and many neurodegenerative diseases. [15, 16].

It was first suggested in 1969 that glutamate, an excitatory amino acid, could have a toxic effect on neurons, and the concept of excitotoxicity was defined [17]. As a result of in vitro and in vivo experiments, it has been found that excessive amounts of excitatory amino acids such as glutamate and their analogs cause neurodegeneration [18, 19]. When glutamate is released from the presynaptic neuron, it stimulates both NMDA and AMPA receptors. Na^{+} entering the cell causes depolarization. The NMDA receptor is permeable to sodium, potassium, and calcium. The channel closed by Mg^{2+} is released from the blockage by depolarization of the membrane [20]. Na^{+} entry through AMPA receptor-dependent channels causes depolarization in the cell. Thus, Ca^{2+} enters through NMDA channels. Briefly stated activation of glutamate receptors shows its effect through Ca^{2+} passage. After Ca^{2+} entry, excitatory events occur in which receptors are activated by glutamate [21]. In a study conducted to understand the effect pathway of glutamate toxicity in C6 glioma cells, which we used in our study, it was emphasized that the cells went to apoptosis due to the decrease in intracellular antioxidant level after glutamate incubation and the increase in OS [22].

Considering that increased glutamate-dependent cytotoxicity in the CNS causes neuronal damage, it will be important to develop a protective therapeutic approach against glutamate-induced excitotoxicity of neuronal cells. GA is one of the most widely used over-the-counter drugs in the world. These recently discovered effects have been significantly detected in vitro. It is thought that critical therapeutic effects will be seen with the use of this drug in appropriate doses, with new studies to be done [23]. In this study, we investigated the effect of paracetamol against the increase in oxidative stress in the cytotoxicity model we created in glutamate-induced C6 glioma cells. The main hypothesis of this study is that GA reduces glutamate toxicity and oxidative stress on C6 glioma cells. In this way, it will be shown that GA has a protective feature on glutamate-induced cytotoxicity in C6 glioma cells by suppressing oxidative stress. In this study, we investigated the effect of GA against the increase in oxidative stress in the excitotoxicity model we created in glutamate-induced C6 glioma cells. The main hypothesis of this study is that GA reduces glutamate toxicity and oxidative stress on C6 glioma cells. This way, it will be shown that GA has a protective feature on glutamate-induced excitotoxicity in C6 glioma cells by suppressing oxidative stress.

Methods

Cell culture and experimental groups

The C6 glioma cell line used in our study was obtained from ATTC (American Type Culture Collection). The C6 glioma cell line was cultured in DMEM supplied by ATTC and contained 10% Fetal Bovine Serum (FBS), 1%

penicillin/streptomycin, and 1% L-glutamine. Humidified cells were propagated in the incubator (37 °C and 5% CO_2) under appropriate conditions, incubated for the specified groups, and analyzed. GA and glutamate were dissolved in isotonic saline and prepared freshly on the experiment days. The study was conducted in 4 groups.

- **Control group:** no treatment was applied,
- **Glutamate group:** 10 mM glutamate was added and incubated for 24 hours [24],
- **GA group:** 100 µM glutamate was added and incubated for 24 hours [25],
- **GA+Glutamate group:** By the literature, a dose of gallic acid (100 µg/mL) was added, and 1 hour later, it was incubated with 10 mM glutamate for 24 hours.

One study created a glutamate toxicity group with a single glutamate concentration (10 mM) dose to model toxicity [24]. Based on the literature, we used glutamate at this concentration (10 mM) in this study.

Cell viability assay

To investigate the protective effect of gallic acid against glutamate cytotoxicity on C6 glioma cells, the cell viability rate was checked; for this purpose, The CCK-8 (Abbkine, Cat#KTA1020) assay was performed. The cell viability was measured on the BioTek ELx808 ™ instrument at 450 nm with commercial ELISA kits, following the instructions in the kit procedure. Experiments were repeated 3 times to determine the cell viability rate in groups.

Measurement of Biochemical Parameters in C6 Glioma Cells by ELISA Kits

The cells for each group were placed in separate sterile tubes. The tubes were centrifuged at 1000 rpm for 20 min by the kit's procedure. Following the removal of the supernatants, the cell pellets were suspended in PBS (pH 7.4) to produce a cell suspension at a concentration of approximately 1×10^6/ml. Cells were lysed through repeated freeze-thaw cycles to allow internal components to escape. It was centrifuged for 10 min at 4000 rpm at 4 °C. The supernatants were collected for biochemical analysis. The Bradford protein assay kit (Merck Millipore, Darmstadt, Germany) was used to determine the total protein levels in the samples. Glutamate toxicity induction of TAS (BT Lab. Cat. No. E1710Ra), TOS (BT Lab. Cat. No. E1512Ra), MDA (Abbkine, Cat#KTE61683), and caspase-3 (BT Lab. Cat. No. E1648Ra), levels in the supernatants of C6 glioma cells was determined by ELISA kits. These analyses were performed considering the protocols determined by the companies for commercial kits. Samples incubated at 37 °C for 60 minutes were placed in 96-well plates. Standard and supernatant samples were inserted into the plate and incubated for 60 minutes. After the washing step, staining solutions were added and incubated for 15 minutes. A stop solution was inserted, and absorbances were read at 450 nm on an ELISA microplate reader (Thermo Fisher Scientific).

Data analysis

All data were expressed as mean ± standard deviation (SD). Data analyses were performed with the SPSS program. One-way ANOVA was used to evaluate the differences between the groups. Post-hoc Tukey test was used in all data with a statistically significant difference. $p \leq 0.05$ was considered statistically significant.

Ethics approval

The current study has no study with human and human participants. Commercially purchased cell lines are not subject to ethics committee approval.

Results

Effect of gallic acid on C6 glioma cell viability

In this study, cell viability was investigated in study groups using the effect of GA against glutamate toxicity. A CCK-8 cell viability kit was used for this. When cell viability was examined among the groups (% of the control group), the cell viability rate was the lowest in the Glutamate group compared to the other groups. However, the cell viability rate was significantly higher in the GA+Glutamate groups compared to the Glutamate group ($p < 0.05$, Figure. 1).

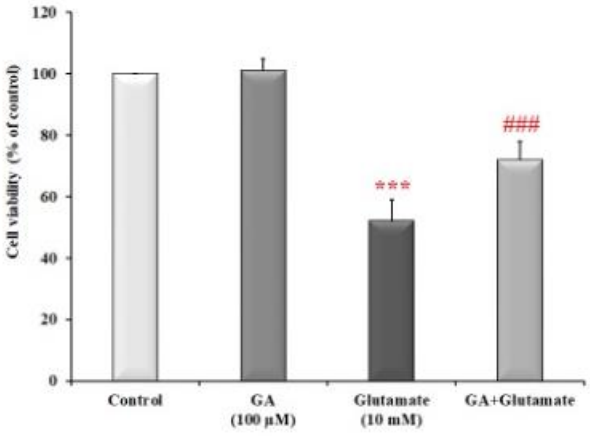

Figure 1. *Effect of gallic acid on cell viability after glutamate-induced excitotoxicity in the C6 cells. ***p≤ 0.05 as compared to control group; ###p≤ 0.05 compared to glutamate-treated group.*

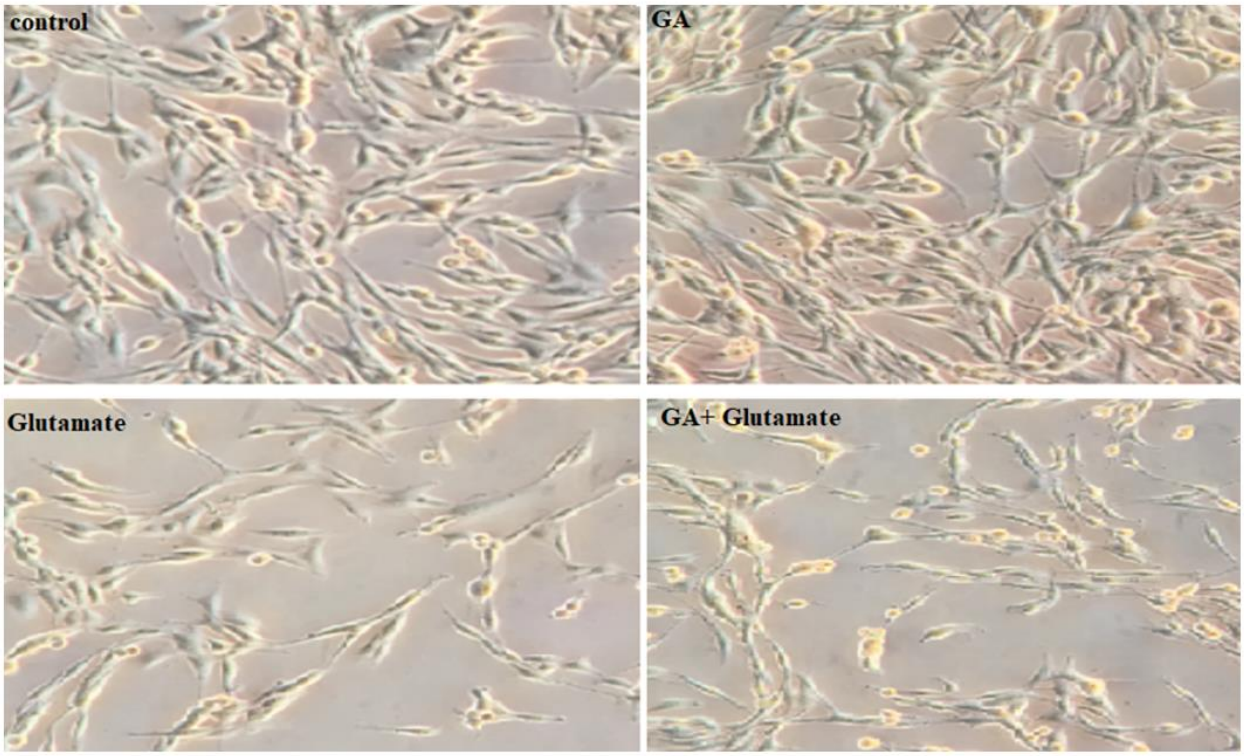

Figure 2. *Morphological image of glial cells in groups.*

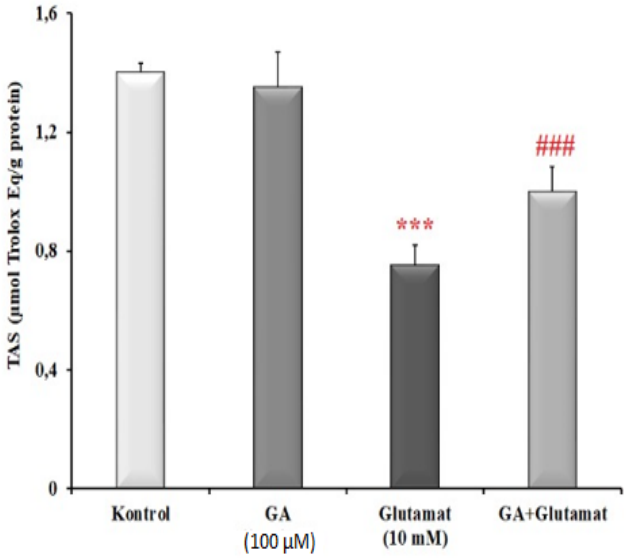

Figure 3. *Effect of gallic acid on TAS level in C6 cells after glutamate-induced cytotoxicity. ***p≤ 0.05 as compared to control group; ###p≤ 0.05 compared to glutamate-treated group.*

Also, the microscopic morphological image of glial cells in groups at 20x magnification is shown in Figure 2. Here, the toxicity effect of glutamate and the protective effect of GA are observed on C6 glial cells.

Effect of gallic acid on TAS and TOS levels in C6 glioma cells

The changes in TAS and TOS levels in cells against glutamate cytotoxicity of GA in the groups formed were measured with the Elisa kits. TAS levels were significantly reduced between the groups when the glutamate-treated group was compared to the control and GA+Glutamate groups ($p<0.001$; Figure 3). There was no statistically significant difference between the control and GA+Glutamate groups (Figure 3).

When the glutamate-treated group was compared to the control and GA+Glutamate groups between the groups, it was observed that the TOS level increased considerably ($p<0.001$; Figure 4). There was no statistically significant difference between the control and GA+Glutamate groups (Figure 4).

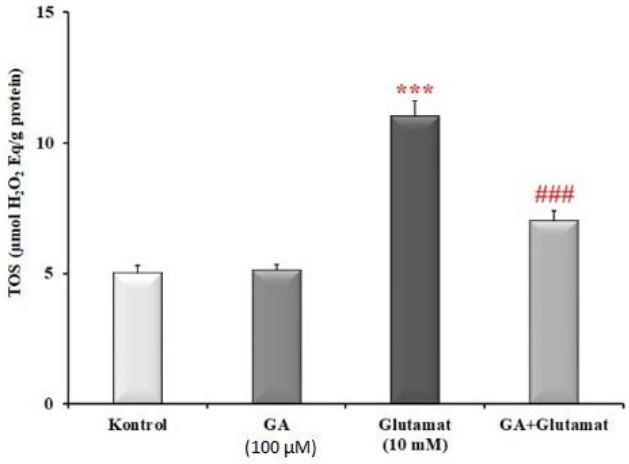

Figure 4. *Effect of gallic acid on TOS level in C6 cells after glutamate-induced cytotoxicity. ***p≤ 0.05 as compared to control group; ###p≤ 0.05 compared to glutamate-treated group.*

TOS levels increased in cells in the high-dose glutamate group compared to the control and Gallic acid groups ($p<0.05$). It was observed that the TAS level increased in the cells in the glutamate + Gallic acid group compared to the glutamate group, and the TOS level decreased significantly ($p<0.05$).

Effect of gallic acid on MDA and Caspas-3 levels in C6 glioma cells

ELISA commercial kits were used to investigate the effect of gallic acid on MDA and caspase-3 levels in glutamate-induced C6 cells. MDA levels were substantially increased between the groups when the glutamate-treated group was compared to the control and GA+Glutamate groups ($p<0.001$; Figure 5). There was no statistically considerable difference between the control and GA+Glutamate groups ($p>0.05$; Figure 5).

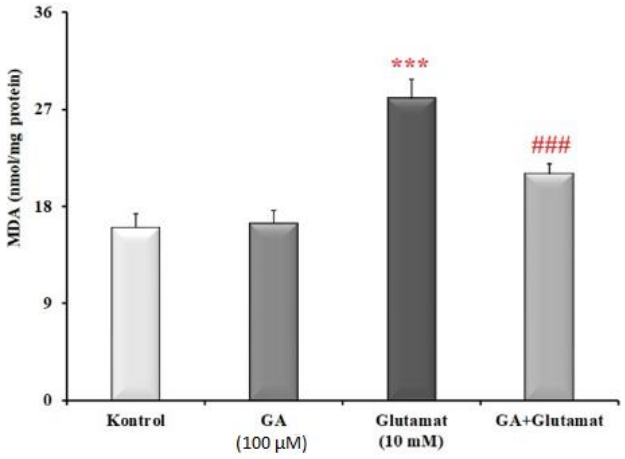

Figure 5. *Effect of gallic acid on MDA level in C6 cells after glutamate-induced cytotoxicity. ***p≤ 0.05 as compared to control group; ###p≤ 0.05 compared to glutamate-treated group.*

Treatment of cells with glutamate increased expression of activated caspase-3. Caspase-3 levels increased in cells in the high-dose glutamate group compared to the control and gallic acid groups ($p<0.05$; Figure 6). It was observed that the caspase-3 level was significantly reduced in the cells in the GA+Glutamate group compared to the glutamate group ($p<0.05$, Figure 6).

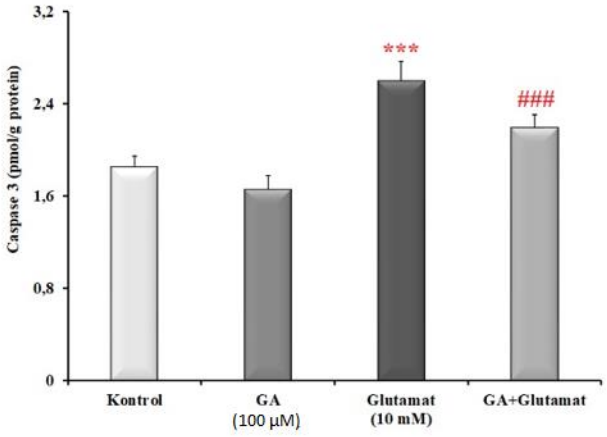

Figure 6. *Effect of gallic acid on caspase-3 level in C6 cells after glutamate-induced cytotoxicity. ***p≤ 0.05 as compared to control group; ###p≤ 0.05 compared to glutamate-treated group*

Discussion

Glutamate has important functions as a major excitatory neurotransmitter in the mammalian brain [8, 9]. However, excessive release of glutamate results in excitotoxicity. Excitotoxicity is an important factor in a variety of pathologies, from ischemic brain injury to neurodegenerations associated with many acute and chronic diseases [24]. Many studies have been conducted to understand the pathogenesis of these diseases. The basic mechanism is excitotoxicity caused by excessive glutamate stimulation. Various studies have been conducted to prevent pathologies caused by glutamate-induced excitotoxicity. Although many substances with anti-inflammatory, antioxidant, and antiproliferative properties have been used in studies to prevent excitotoxicity through various mechanisms, no study has been found in the literature on the effect of gallic acid on glutamate excitotoxicity. Studies have shown that GA exhibits a wide range of bioactivities, such as antitumor, antiulcer, antimicrobial, and antiproliferative effects on cancer cells [1, 2, 3]. One of the anticancer, antiulcerative, and antiproliferative effects of GA is that it increases oxidative stress, leading the cell to apoptosis. Our study investigated the effect of pre-treatment of gallic acid using glutamate-dependent toxicity, which we induced in vitro.

It was observed that pretreatment with gallic acid increased cell viability and reduced cell death against glutamate-

dependent cytotoxicity in C6 cells (Figure 1).

Many indicators of oxidative stress and antioxidant status have parameters. One of these is TOS, which measures the oxidative level, and TAS, which measures the antioxidant level. A study on glutamate toxicity in C6 glial cells found that paracetamol increased the TAS level while the TOS level decreased [19]. A recent study on TAS and TOS levels concluded that gallic acid increased the TAS level and decreased the TOS level in C6 cells. Our study observed that the TOS level increased considerably in the Glutamate group compared to the control group, whereas the TAS level decreased significantly (Figures 2 and 3).

Measurement of MDA is used as an indicator of lipid peroxidation. In the study conducted by Naglaa M. El-Lakkany and her colleagues on hepatic stellate cells, they observed that gallic acid reduced the MDA level [26]. In another study conducted by Ergul and Taşkıran, examining the protective effect of Thiamine against cytotoxicity caused by glutamate in the C6 cell line, an increase in MDA levels was observed after glutamate incubation, while a decrease in SOD and CAT levels was detected [27]. In our study, which aimed to determine the mechanisms that trigger neurotoxicity due to stimulation with glutamate, we also examined the parameter of MDA. We observe that glutamate increases the MDA level in C6 glial cells and decreases the MDA level in the treatment group (Figure 4).

Caspases are a group of enzymes that control processes such as cell death. Caspase-3 is one of them. In a study, the effect of gallic acid on rats created as an Alzheimer's model with aluminum chloride was examined. According to the study, the protective role of gallic acid was demonstrated by the decrease in caspase-3 level [28]. In a study, the effect of quipazine against glutamate-induced cytotoxicity was investigated in the HT-22 cell line. It was observed that glutamate-induced toxicity caused an increase in caspase-3 levels in cells [29]. In our study, gallic acid had neuroprotective effects on C6 glioma cells by preventing cells from undergoing apoptosis due to glutamate excitotoxicity.

In summary, it was observed that pretreatment with gallic acid increased cell viability and reduced cell death against glutamate-dependent cytotoxicity in C6 cells. Also, gallic acid pretreatment decreased TOS levels and increased TAS levels in C6 cells. In addition, gallic acid pretreatment was also observed to suppress MDA and caspase-3 levels.

Conclusion

The findings of this research showed that the use of an appropriate dose of gallic acid increased cellular damage resistance to glutamate-induced cytotoxicity in C6 glioma cells. We think that this damaging impact of quipazine occurs with the activation of oxidative stress pathways. Therefore, the use of appropriate doses of gallic acid may be protective against glutamate toxicity that may occur in CNS disorders and can be used as a beneficial therapeutic agent in neuronal damage-related damage. However, further in vitro and in vivo studies are needed to answer questions about the possible mechanisms of action of gallic acid in glutamate toxicity.

Ethical Declarations

The current study has no study with human and human participants. The study is not subject to ethics committee approval.

Conflict of Interest Statement

The authors disclosed no conflict of interest during the preparation or publication of this manuscript.

Financial Disclosure

The author disclosed that they did not receive any grant during the conduction or writing of this study.

Author Contributions

All of the authors declare that they have all participated in the design, execution and analysis of the paper and that they have approved the final version.

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