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Age-related changes in apoptosis in rat hippocampus induced by oxidative stress

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Accepted November 8, 2010

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Abstract

Also known as programmed cell death, apoptosis is a sequence of events that leads to elimination of cells without releasing harmful substances into the surrounding area. Apoptosis may be induced by intracellular or extracellular signals. Certain apoptotic signals activate mitochondrial pro-apoptotic events and increase reactive oxygen species (ROS). Increased ROS production may lead to oxidative stress. The goal of our study was to characterize age-related changes in apoptosis induced by oxidative stress in the hippocampus. Rats 2, 7, 21 and 38 days old, and adult rats were used for our study. Hippocampal CA1, CA2, CA3 and dentate gyrus apoptosis, and hippocampal superoxide dismutase (SOD), glutathione peroxidase (GPx) enzyme activities and thiobarbituric acid reactive substances (TBARS) levels were measured. We found that numbers of hippocampal neurons were low in rats 2, 7 and 21 days old (CA1, $p < 0.001$; CA3, $p < 0.05$; gyrus dentatus, $p < 0.001$). The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive cell count was highest in the CA1 and dentate gyrus of 21-day-old rats. Among 21-day-old rats, the hippocampal TBARS levels and SOD enzyme activity were high, whereas GPx activity was low. These results demonstrate that the hippocampal CA1 and dentate gyrus of 21-day-old rats are more prone to damage by oxidative stress.

Key words: apoptosis, glutathione peroxidase, hippocampus, lipid peroxidation, superoxide dismutase

Apoptosis is the process by which cells are eliminated during proliferation and differentiation without releasing harmful substances into their environment (Kerr et al. 1972). It is a vital process in the growth, development and homeostasis of organ systems, including mammalian brain (Woodle and Kulkarni 1998, Sastry and Rao 2000). Apoptosis may be induced extracellularly or intracellularly. Caspases are cysteine proteases that are activated during early stages of apoptosis. They cleave and eliminate structural proteins that constitute the cytoskeleton, DNA repair enzymes and other key proteins important for normal cellular functions. They also can activate other "destructive" enzymes, such as DNAse, that ultimately culminate in apoptosis (Bratton et al. 2000, Leist and Jaattela 2001).

Superoxide, hydroxyl radicals and hydrogen peroxide are reactive oxygen species (ROS) formed during normal physiological metabolic reactions. ROS are eliminated by anti-oxidant defense systems. The most important anti-oxidants are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). Brain catalase activity is low and limited to peroxisomes. Therefore, GPx is more important in neural tissues and removes excess hydrogen peroxide (Gaunt and De Duve 1991, Sinet et al. 1980). Anti-oxidant enzymes play important protective roles against cellular and histological

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damage produced by ROS. Disequilibrium between anti-oxidant defense and free radicals may lead to oxidative stress (Halliwell 1999).

Some apoptotic stimuli may induce mitochondrial pro-apoptotic events; a cytosolic death signal protein complex may free cytochrome C from mitochondria (Salvesen and Renatus 2002). Mitochondrial dysfunction and increased ROS cause DNA damage and apoptotic death of neurons (Cherian et al. 2008). Increased ROS production results in oxidative stress. Because oxidative stress results in the formation of lipid peroxides, it is an important mediator of apoptosis. Lipid hydroperoxides are highly toxic and cause DNA degradation (Buttke and Sandstrom 1994, Forrest et al. 1994). This leads to mitochondrial damage and apoptotic cell death by cytochrome C release and caspase activation (Leon et al. 2004, 2005). Following caspase activation, cell death signals are amplified and result in cell death (Earnshaw et al. 1999).

The brain is prone to oxidative damage owing to its high oxygen consumption, high unsaturated fat content and low anti-oxidant capacity (Mishra et al. 1989). Environmental factors, therefore, may readily cause brain damage (Lau et al. 2005). Newborns have low anti-oxidant capacity and are prone to oxidative damage by formation of ROS (Rice and Barone 2000). Brain development starts *in utero* in mammalians and continues until the end of adolescence (Rubbo et al. 1994). During brain development, nearly half the cortical neurons are lost through apoptosis (Samson et al. 1958). The role of oxidative stress in the onset of apoptosis at various ages, however, is not known. The goal of our study was to elucidate the role of oxidative stress in apoptosis observed in rat brain at different ages.

Materials and methods

Wistar albino male rats were divided into five groups according to age: 2, 7, 21, and 38 days old, and 6 months old ($n = 16$ for each group). The animals were maintained under standard colony conditions with a 12 h light:12 h dark cycle (lights on 07:00 h) at constant room temperature (23 ± 2° C) and humidity (60%), and food and water *ad libitum* throughout the experiments. Our study was carried out between 09:00 and 12:00 in a sound-attenuated, air-regulated experimental room. All experiments were performed in accordance with the guidelines of the Experimental Animal Laboratory and approved by the Animal Care and Use Committee of the Dokuz Eylul University, School of Medicine.

Half of each group of rats were sacrificed by cervical dislocation under ether anesthesia and perfused transcardially with heparinized saline followed by 10% formalin in phosphate buffer. Brains were removed and fixed in 10% formalin in phosphate buffer for 24 h. The brains were sectioned coronally into sequential 6 μm slices using a rat brain slicer. The number of neurons in each sample was estimated by taking three coronal sections through the hippocampus that corresponded approximately to plates 21, 23, 25 in the rat atlas of Paxinos and Watson (Paxinos and Watson 1982). All sections were stained with cresyl violet. The images were analyzed using a computer-assisted image analyzer system consisting of a microscope (Olympus BH-2 Tokyo, Japan) equipped with a high-resolution video camera (JVC TK-890E, Japan). The numbers of neurons in hippocampal CA1, CA2, CA3 and gyrus dentatus regions were counted using a 6000 μm^2 counting frame viewed through a 20 X Nikon lens at the monitor. The counting frame was placed randomly five times on the image analyzer system monitor, the numbers of neurons were counted (UTHSCA Image Tool for windows version 3.0 software) and the average was determined. Hippocampal neuron density then was calculated. To detect DNA fragmentation in cell nuclei, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) reaction was applied to the paraffin sections using a kit (Promega, Madison, WI). After deparaffinization, the sections were treated with 20 μg/ml proteinase K for 10 min. After treatment with 0.3% H_2O_2 in methanol for 10 min and 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice, the sections were incubated with TUNEL reaction mixture for 60 min at 37° C. Further incubation with peroxidase-conjugated antibody was performed for 30 min at 37° C. The sections were stained with diaminobenzidine solution for 10 min at room temperature, then counterstained with Mayer's hematoxylin.

Rats of the remaining half groups were sacrificed by cervical dislocation under ether anesthesia. Hippocampus tissues were dissected on an ice-cold surface. Tissue homogenates and supernatants were prepared as described by Carrillo et al. (1992). An aliquot of the homogenate and supernatant was stored at −85° C until the determination of thiobarbituric acid reactive substances (TBARS) levels, and SOD and GPx enzyme activities. All measurements were made within 10 days.

SOD and GPx activities were determined from the supernatant using RANSOD and RANSEL kits (Randox Labs., Crumlin, UK). Results were expressed as U/mg protein. TBARS levels were

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measured in the homogenate according to the method of Rehncrona et al. (1980) and expressed as nmol/mg protein. Protein contents of supernatant and homogenate were determined using a Total Protein Kit (Randox Labs.).

Differences in the histopathological parameters (cell number and TUNEL positive cell count), SOD and GPx enzyme activities, and TBARS levels among all groups were tested by one-way analysis of variance followed by the post hoc Bonferroni test. Correlation between apoptosis and cell number, SOD and GPx enzymes activities, and TBARS levels were calculated using Spearman correlation analysis. Results are presented as mean ± S.E.M. A level of $p < 0.05$ was accepted as statistically significant.

Results

Hippocampal CA1, CA1, CA2 and CA3 neuron numbers were decreased in rats 2 and 21 days old (CA1, $p < 0.001$; CA2, $p < 0.05$; CA3, $p < 0.001$). Numbers of neurons in the gyrus dentatus were fewer in 2-day-old rats than at other ages, increased in 7-day-old rats, then remained unchanged up to adulthood ($p < 0.001$) (Figs. 1 and 2). We observed increased numbers of apoptotic cells in the hippocampal CA1 region of 21-day-old rats (Figs. 3 and 4). We also observed that hippocampal TBARS levels were increased and GPx enzyme activity was decreased in 7- and 21-day-old rats. The SOD enzyme activity was increased in 21-day-old and adult rats. GPx enzyme activity remained unchanged at a low level up to postnatal day 21, increased in 38-day-old rats, then decreased again in adult rats. The TBARS level peaked in 2-day-old rats. The TBARS level gradually decreased in 38-day-old rats, but it increased in adult rats. Oxidant and anti-oxidant equilibrium appeared adequate in 38-day-old and adult rats (Table 1). We found a statistically significant and strongly positive correlation between SOD enzyme activity and apoptosis in hippocampal CA1 region ($p = 0.001$, $r = 0.630$) (Fig. 5). There was a strongly negative correlation between the neuronal count in the CA1 region and apoptosis ($p = 0.007$, $r = -0.504$).

Discussion

We sought to determine across different ages the extent of apoptosis induced by oxidative stress in hippocampus where neuronal plasticity continues throughout life. To our knowledge, ours is the first comprehensive report in the literature. We found that oxidative stress increases in the hippocampus of 21-day-old rats, and this is associated with increased apoptosis and decreased numbers of neurons. Other studies have demonstrated that the free radical scavenger enzymatic systems eliminate effectively the superoxide anion, but may have difficulty eliminating the hydrogen peroxide produced by the reaction (Castagne et al. 1999a). If increased SOD enzyme activity is associated with increased GPx enzyme activity, it is called an adaptive response (Ceballos et al. 1988) and is favorable for oxidative defense. If increased SOD enzyme activity is not associated with an increase in GPx, however, it may lead to tissue damage. SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide and GPx converts hydrogen peroxide to water. If the ratio of SOD to GPx favors SOD, however, the hydrogen peroxide that is produced is not converted

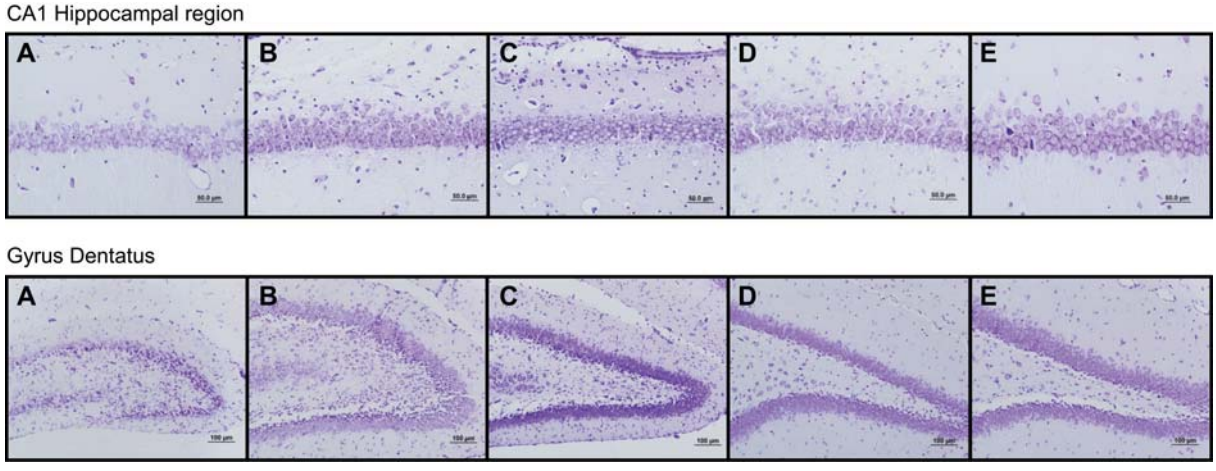


Fig. 1. Representative images of the hippocampal CA1 regions and gyrus dentatus (GD). A) 2 days old. B) 7 days old. C) 21 days old, D) 38 days old. E) 6 months old.

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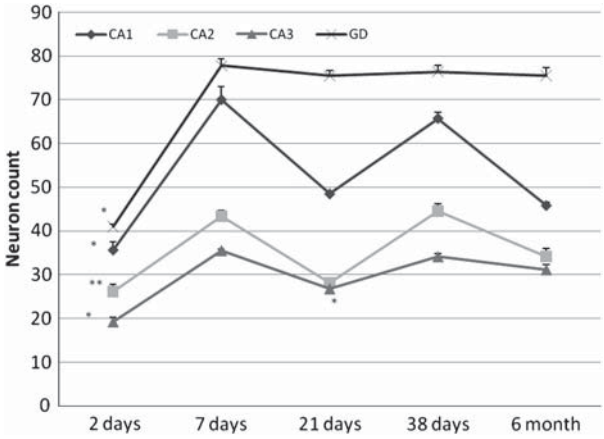


Fig. 2. Quantitative evaluation of age related alteration of the neuron numbers in hippocampus. $^{*}p < 0.05$, compared to the other groups. $^{**}P < 0.05$ compared to 2-, 7-, 38-day-old groups.

into water and accumulates. Hydrogen peroxide crosses the membranes, forms hydroxyl radicals and damages cells (Halliwell 1992). Consequently, the lack of increase in GPx despite increased SOD enzyme activity and TBARS levels in 21-day-old rats is damaging to the hippocampus. This result is consistent with the insufficient adaptive response of anti-oxidative defense in 21-day-old rats; thus the hippocampus of 21-day-old rats may be affected readily by external and internal factors.

We also found that in the 21-day-old group, apoptosis was increased in the hippocampus CA1 region and dentate gyrus, and there was a strongly positive correlation between SOD enzyme activity and CA1 apoptosis. Hippocampal CA1 pyramidal cells are generated during the embryonic period (Bayer 1980) and they continue to grow during the first postnatal month (Pokorny et al. 1981). The pyramidal cells grow rapidly during postnatal weeks 3 and 4 (Groc et al. 2002). The apical and basal dendrites of the pyramidal cells branch and grow (Steward and Falk 1991). Axons of pyramidal cells also display intensive growth and branching (Aniksztejn et al. 2001). Rapid growth of tissues requires more oxygen and greater oxygen use results in generation of more free radicals (Groc et al. 2001).

Immature brain may be damaged easily by oxidative stress, because the number of mitochondria in the cells and the number of cristae in the mitochondria where anti-oxidant enzymes are located is low (Samson et al. 1960). At the same time, during the period of maximum synapse formation, nitric oxide synthesis activity and nitric oxide levels also are increased (Lizasoain et al.

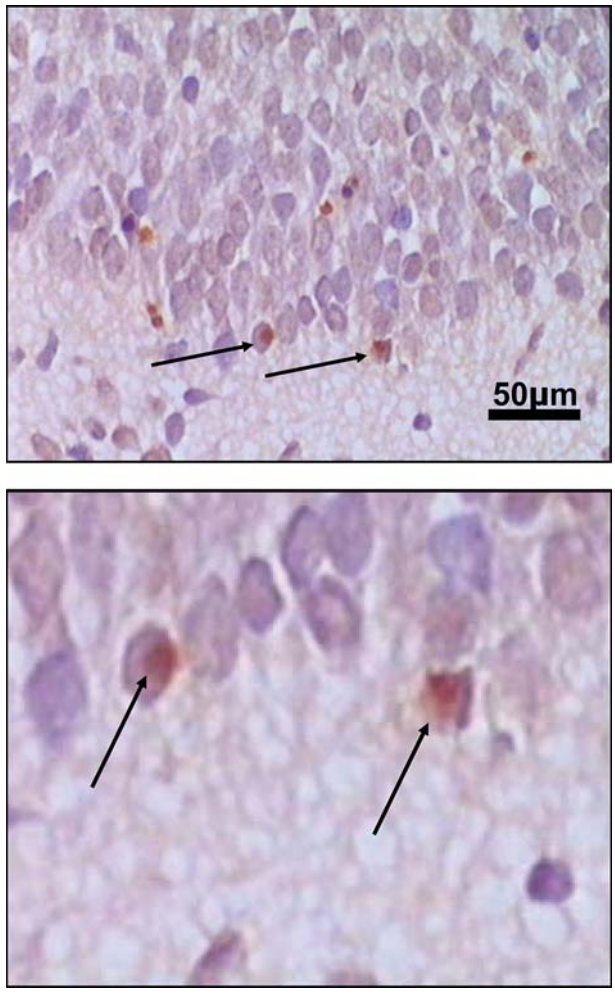


Fig. 3. Comparison of apoptosis in the hippocampal CA1 subfield. The number of TUNEL+neurons was increased in 21-day-old group.

1996). It is known that increased nitric oxide may lead to lipid peroxidation. We found increased TBARS levels in the 2-, 7- and 21-day-old rat hippocampi, which is a sign of lipid peroxidation. It also has been reported that iron content is increased in the developing brain (Connor et al. 1995), defense mechanisms are immature (Baud et al. 2004), and there is an imbalance between SOD, catalase and GPx enzyme activities (Folkerth et al. 2004). These factors lead to vulnerability of the developing brain to ROS. It is not known, however, it at what age this damage is most prominent. Our results demonstrate that sensitivity to ROS damage is greatest in 21-day-old rats.

Oxidative stress in cells is an indicator of an insufficient anti-oxidant defense system. As a result of oxidative stress, cellular contents are disrupted, less resistant to free radical reactions and subject

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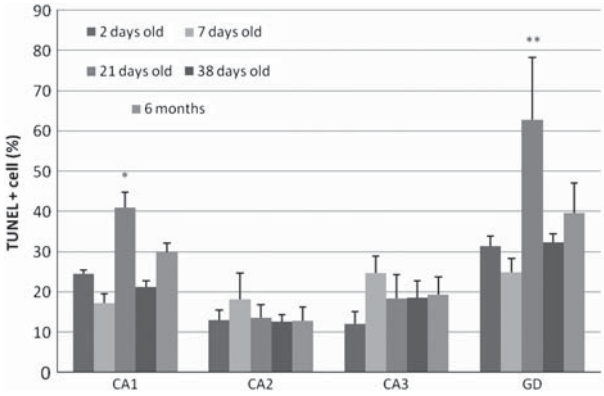


Fig. 4. The percentage of TUNEL+cells in the hippocampus. $^{*}p < 0.05$ compared with the other groups. $^{**}p < 0.05$ compared to 2-, 7-, 38-day-old groups.

to oxidative damage. Free radicals are not always dangerous, however. They may be useful if their intracellular concentrations are maintained within physiological levels (Castagne et al. 1999b). The physiological effects of free radicals may involve modulation of gene expression (Cimino et al. 1997). Furthermore, free radicals act as intracellular second messengers (Suzuki et al. 1997). A balance between intracellular oxidants and anti-oxidants is required for cell survival. The equilibrium may influence cell functions. This equilibrium is impaired in many disorders, e.g., the cortical neurons of human embryos affected by Down syndrome are associated with increased generation of ROS compared to normal neurons (Busciglio and Yankner 1995). Also, various anti-oxidants are diminished in pre-apoptotic neurons (Castagne et al. 1999a). Moreover, some trophic factors may up-regulate neuronal anti-oxidant capacities (Jackson et al. 1994). For example, nerve growth factor increases the activities of glutathione peroxidase and catalase (Sampath et al. 1994). Production of ROS is increased in pre-apoptotic cells deprived of nerve growth factor (Greenlund et al. 1995).

Table 1. Age-related alteration of SOD and GPx enzymes activities and TBARS levels in the rat hippocampus.

	SOD U/mg protein	GPx U/mg protein	MDA nmol/mg protein
3 days old	1.05 ± 0.03	0.008 ± 0.0002	2.30 ± 0.04*
7 days old	1.04 ± 0.02	0.004 ± 0.0001	1.61 ± 0.03*
21 days old	1.83 ± 0.02*	0.004 ± 0.0001	1.05 ± 0.02*
38 days old	1.26 ± 0.03	0.032 ± 0.0010*	0.36 ± 0.07*
6 months old	3.16 ± 0.08*	0.014 ± 0.0016*	0.73 ± 0.02*

$^{*}p < 0.001$ compared with the other groups.

Pro-apoptotic proteins must be released from mitochondria to activate apoptotic mechanisms in the immature brain. Caspase 3 and Bcl-2 are up-regulated (Blomgren et al. 2001, Merry et al. 1994). Many apoptotic pathways converge on caspase 3, which is critical for neuronal apoptosis during brain development. It has been shown that rats lacking caspase 3 have hyperplastic and disorganized brains, while other organ systems are normal (Kuida et al. 1994). It has been demonstrated that cytochrome C release from mitochondria and caspase 3 and caspase 9 activation are more important in immature brains than in adolescent and adult brains (Zhu et al. 2005). This also is consistent with our findings of increased importance of mitochondria-mediated apoptosis in immature rat brain.

Consistent with the literature (Zhu et al. 2005), we found decreased GPx activity on days 2, 7 and 21 after birth. On the other hand, GPx activity was increased and TBARS levels were decreased in the brains of 38-day-old rats. Xu et al. (2007) reported increased GPx activity in 42-day-old rat brains. As a result, we conclude that in the brain of 38-day-old rats, GPx is the enzyme primarily responsible for anti-oxidant defense. At the same time, decreased apoptosis and increased neuron numbers were observed in the hippocampus of 38-day-old rats. We also found that anti-oxidant defense mechanisms matured during adulthood and appropriate SOD activity was established. It has been shown that GPx activity in whole brain increases until adulthood (Sahoo and Chainy 1997). Another study showed that SOD activity is increased in adult rat brain (Tsay et al. 2000).

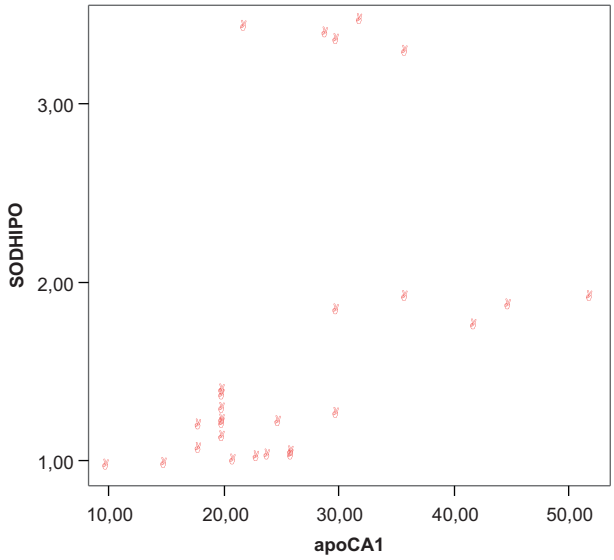


Fig. 5. Scattergrams of apoptosis of CA1 regions. Positive significance for superoxide dismutase (SOD) enzyme activity, $p = 0.000$.

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The release of pro-apoptotic proteins from mitochondria during oxidative stress is important for normal apoptosis in immature brain. Mitochondria are vital to the activation of apoptotic mechanisms, which are required for normal development. The importance of the anti-oxidant mechanism in adult brain is well known; however, ROS related damage seems to be important in immature brain also, especially in 21-day-old rats. Because anti-oxidants, iron chelators, and lipid peroxidation inhibitors may be protective, anti-oxidant enriched nutrition may be more important for in this age group. More studies are needed to elucidate the effects of ROS-related apoptosis in the hippocampi of 21-day-old rats and especially on the spatial memory that is related to hippocampus.

Acknowledgment

The authors thank Dr. Erkan Büyük, Division of Reproductive Endocrinology & Infertility Department of Obstetrics, Gynecology & Women's Health Albert Einstein College of Medicine/Montefiore Medical Center, for his valuable assistance.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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