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RESEARCH ARTICLE

PRENATAL BAICALEIN EXPOSURE INDUCED OXIDATIVE STRESS AND IT'S RECOVERY BY TESTOSTERONE ADMINISTRATION DURING POSTNATAL PERIOD IN MALE WISTAR MICE

Sridevi, V., Naveen, P. and *Ramachandra Reddy, P.

Department of Biochemistry, Yogi Vemana University, Vemanapuram, Kadapa-516003, A.P, India

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ABSTRACT

Excessive baicalein (BC; a flavonoid) intake during pregnancy period has been demonstrated to provoke oxidative stress an associated factor of male infertility in offspring. The role of testosterone depot in the recovery of damaged antioxidative system in prenatal BC exposed male mice was tested. For this female Wistar mice were administrated IP with 30, 60 and 90 mg/kg BW of BC on gestation days 11, 13, 15 and 17. F1 BC exposed males were sacrificed on post natal day (PND) 60 to assess the oxidative stress. The results showed a significant increase ($P<0.0001$) in testicular, liver and kidney malondialdehyde (MDA) levels in prenatal BC exposed males. In contrast to increased lipid peroxidation observed decrease in ($P<0.001$) levels of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) in prenatal BC exposed adult males in a dose dependent manner than controls. Administration of testosterone (4.16 mg/kg body weight) to BC exposed adult mice were normalized the levels of MDA, SOD and catalase in testis, liver and kidney and are comparable to controls. The administration of testosterone refused the antioxidant defence system and is proved in the present study.

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INTRODUCTION

Phytoestrogens are estrogen mimics, similar to estradiol (E_2) of mammals and bind with the estrogen receptors either to activate or to inhibit the functions of estradiol (Mueller *et al*., 2004). and are non-steroidal phytochemicals present in the plants (Navarro, 2005). Consumption of flavonoids through food is increased both in animals and human beings (Rachuonyo *et al*., 2005). Flavonoids are a class of phytoestrogens and are in use as antimicrobial, anti-bacterial, anti-viral and anti-inflammatory, and anti- cancer activities (Yao *et al*., 2004). Soy food products and fruits are rich with flavonoids. However, excessive intake of flavonoids can affect the reproductive system, especially induces the by causing the oxidative stress and decreases the male fertility. The occurrence of oxidative stress is determined by measuring the reactive oxygen species and other enzyme activities involved in antioxidant mechanism like catalase and superoxide dismutase (SOD). Oxidative stress leads to alteration in lipid peroxidation and/or failure in intrinsic antioxidant defence. According to Morales *et al*. (2004) reactive oxygen species (ROS) occurs due to the regular process of oxidative metabolism of cells, resulting from univalent reduction of O_2 can damage most cellular components which eventually lead to cell death. To avoid this, antioxidant defence system plays a key role against tissue damage through free radicals liberation (Mourad and Noor, 2011). In diseased conditions ROS play an important role in defence mechanism but immoderate release of ROS may damage to living tissues (Burchiel and Luster, 2001). Oxidative stress is one of the major contributors which cause reproductive disorders in animals (Turner and Lysiak, 2008). The abnormal levels of ROS harmful and causes sperm abnormalities, whereas the low levels of ROS are essential for normal sperm activity (Aitken, 1995). Exposure of isoflavonoid biochanin-A during prenatal period causes the oxidative stress in offspring which lead to decrease in male fertility at their adulthood (Soujanya *et al*., 2014). Baicalein (BC: 5, 6, 7 trihydroxyflavone) a phytoestrogen belongs to flavonoid family majorly identified in Scutellaria baicalensis. BC is known for decreased blood cholesterol levels (Baowain, 2009) and treatment of chronic hepatitis (Shimizu *et al*., 1999). It also acts proven with anti-inflammatory (Hsieh *et al*., 2007), antithrombic, antidepressant, anticancer and neuroprotective agent (Graham *et al*., 2012). Metabolite of baicalein is baicalin is having the antiviral properties (Moghaddam *et al*., 2014). However the role of BC on reproduction and on antioxidant defence system is not yet reported. Hence, the present investigation undertaken to elucidate the role of BC on oxidative defence system at the adulthood of male mice exposed to BC at prenatal period. Sine oxidative defence system is linked to male fertility, we further tested whether

Corresponding author: **Ramachandra Reddy, P.**
Department of Biochemistry, Yogi Vemana University, Vemanapuram, Kadapa-516003, A.P, India.

male fertility recovery drug testosterone can revert the oxidative damage of BC exposure in mice.

MATERIALS AND METHODS

Procurement and maintenance of mice: Healthy sixteen female and five male mice of 40-45 day old (29 ± 2 g body weight) were purchased from an authorised dealer (Sri Venkateswara Enterprises, Bangalore, India). Animals were well maintained by following the guidelines for care and use of laboratory animals (NRC, 1996). The experiment of this study is planned after the approval from Institutional Animal Ethical Committee at Yogi Vemana University, Kadapa, India (Resolution No: 1841/GO/Re/S/15/CPCSEA dt. 18-11-2015) and CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals, Government of India) (CPCSEA, 2003).

Chemicals: Baicalein procured from Sigma Aldrich, USA dissolved in 100% dimethyl sulfoxide (DMSO). Testosterone depot (100 mg/ml German Remedies, Goa, India) purchased from local drug medical stores was used for recovery studies. All other chemicals used in the present study were purchased from Sigma Aldrich, USA.

Experimental plan: Inseminated female mice four of each group were maintained in individual cages. Group 1, 2, 3, 4 injected with DMSO (control), 30, 60 and 90 mg BC/Kg BW respectively on gestation day 11, 13, 15 and 17. All pregnant mice of all groups were delivered pups. Oxidative stress was determined in male pups by dividing them four and six animals in separate cages after weaning period from all groups. Six animals of each group were used for assessment of oxidative stress, on 60^{th} day. Four males from 30, 60 and 90 mg BC/Kg BW prenatally exposed were injected with 4.16 mg /Kg BW testosterone (Harini *et al*., 2009) on PND (Post natal day), 21, 31 and 41 and sacrificed on 60^{th} day to assess any recovery oxidative stress occurred.

Autopsy and collection of tissue samples: After cervical dislocation, the control and experimental F1 males were opened and collected testis, liver and kidney, and stored at -80º C for further analysis.

Lipid peroxidation: Oxidative stress can be assayed by measuring the concentration of malondialdehyde (MDA: A end product of lipid peroxidation) (Hiroshi *et al*., 1979). To test this, tissue (Testis, liver and kidney) 10% w/v were taken and homogenized individually in 1.15% potassium chloride solution. To 2.5 mL of the homogenate, 20% w/v TCA 1.0 mL, saline (0.9% sodium chloride) 0.5 mL were added and kept for centrifugation at 4000 x g for 20 min at 4°C. To the 1.0 ml supernatant added 0.25 ml of thiobarbituric acid (TBA) and this mixture was kept for incubation at 95°C for 1h. Then thoroughly mixed after adding n-butanol and the samples were centrifuged for 15 min at 4000 X g at 4°C. The organic layer was collected carefully and the absorbance was measured at 532 nm. in UV-visible spectrophotometer (Model No. UV 3092, LABINDIA). The u moles of MDA formed/g wet weight of tissue were calculated for each sample.

Assays of antioxidant enzymes

Superoxide Dismutase (SOD) (E.C. 1.15.1.1) Activity: The inhibition of epinephrine auto-oxidation was measured as an activity of SOD by following the method (19). To these testes, liver and kidney of individual samples were homogenized (10% W/V) in 50mM ice-cold sodium phosphate buffer (pH 7.0) with 0.1mM EDTA and centrifuged at 1, 05,000 X g for 60 min. at 4°C. The supernatant was used as enzyme fraction. For reaction, the final volume of 2.0 mL containing 0.05 M carbonate buffer (pH 10.2), 30 mM freshly prepared epinephrine and enzyme fraction was taken. The absorbance of each sample was recorded at 480 nm with 10 seconds intervals for 1.0 min. in a spectrophotometer (Model No. UV 3092, LABINDIA) against reagent blank. The protein content in the enzyme source was estimated by the method of Lowry *et al* (20) and SOD activity was expressed as units/mg protein/min.

Catalase (E.C. 1.11.1.6) Activity: Using method of Chance and Machly (Chance and Machly, 1955) activity of catalase was measured. The tissues (testis, liver and kidney) were homogenized in 50 mM phosphate buffer (pH 7.0) and centrifuged at 1, 05,000 X g for 60 minutes at 4°C. The supernatant was used as catalase source. The final volume of 2.5 mL containing 0.05 M phosphate buffer (pH 7.0), appropriate amount of enzyme source and 19.0 mM hydrogen peroxide (H_2O_2) was taken as reaction mixture. Immediately after addition of H_2O_2 the absorbance was measured at 240 nm against reagent blank 1 min. often equal intervals in UV-VIS spectrophotometer (Model No. UV 3092, LABINDIA). The protein content was estimated in the enzyme source by the method of Lowry *et al* (Lowry *et al*., 1951), and catalase activity was expressed as μ moles of H_2O_2 metabolized/mg protein/min.

Statistical analysis: The two tailed ANOVA followed by Bonferroni post-test to compare replicate means by row were used to analyze data statistically by using GraphPad.Prism.v5.0.3.477. The results were expressed as mean ± SEM and considered $P<0.05$ as statistically significant.

RESULTS

Toxicity: Observed no clinical signs of toxicity, behaviour (circling, walking backwards, head flicking), respiration and urination in all the animals, and no animal are excluded from the study.

Lipid peroxidation: The level of malondialdehyde was significantly increased ($P <0.0001$) in the testis, liver and kidney of prenatal BC exposed males when compared to the controls (Table 1). Administration of testosterone to the prenatal BC exposed males reduced MDA levels was normalized compared to corresponding prenatal BC exposed males and with controls (Table1).

Stress defence enzyme activity

Superoxide dismutase: The mean average values of SOD in F1control mice is 4.44 ± 0.47, 5.11 ± 0.46 and 2.64 ± 0.23 in testis, liver and kidney respectively whereas the mean values for 30, 60 and 90 mg BC exposed mice are 3.57 ± 0.3, 3.10 ± 0.16, 1.51 ± 0.29 for testis, 4.23 ± 0.42, 3.62 ± 0.33, 1.96 ± 0.20 for liver and 2.27 ± 0.17, 2.07 ± 0.28, 1.69 ± 0.10 for kidney respectively. The testicular and liver levels of SOD was decreased significantly ($P<0.001$) in prenatal BC exposed males when compared to control mice. Whereas no significance ($P=0.0759$) in SOD activity of kidney was recorded (Figure 2A). In testosterone treated animals the mean

Table 1. Administration of testosterone on lipid peroxidation (MDA) levels of adult male mice exposed to baicalein (BC) prenatally

Tissue	Group	Control/ BC exposed groups	4.16 mg T/ Kg BW (BC +T) #
Testis	Control	5.91±0.12	5.95±0.103
	30 mg	6.19±0.16 (4.92), *P*=0.074	5.87±0.191 (-0.61), *P*=0.547
	60 mg	11.11±0.29(88.11), *P*<0.0001	6.50±0.370 (9.95), *P*=0.146
	90 mg	15.70±0.66(165.84), *P*<0.0001	6.87±0.432 (16.23), *P*=0.141
		P<0.0001, F=161.1	*P*=0.0741, F=3.248
Liver	Control	6.97±0.19	6.80±0.079
	30 mg	7.24±0.32 (3.87), *P*=0.544	7.03±0.04 (3.44), *P*=0.059
	60 mg	11.15±0.15 (59.88), *P*<0.0001	7.66±0.278 (12.72), *P*=0.0629
	90 mg	14.50±0.43(107.90), *P*<0.0001	7.65±0.362 (12.5), *P*=0.1195
		P<0.0001, F=138.9	*P*=0.0471, F=3.961
Kidney	Control	9.22±0.07	9.271±0.040
	30 mg	9.33±0.16 (1.18), *P*=0.593	9.29±0.146 (0.21), *P*=0.913
	60 mg	13.29±0.10 (44.02), *P*<0.0001	9.72±0.246 (4.42), *P*=0.213
	90 mg	17.15±1.25 (85.90), *P*<0.0001	9.87±0.340 (6.42), *P*=0.1472
		P<0.0001, F=38.48	*P*=0.2557, F=1.605

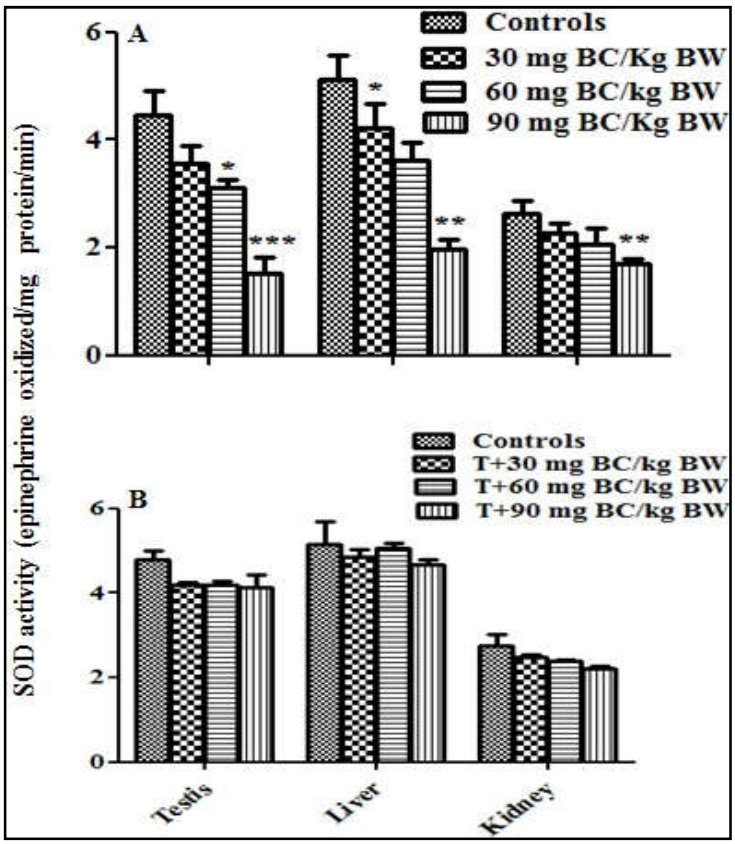
Units of lipid peroxidation unit: µ moles of malondialdehyde formed/g wet weight
Values are mean ± SEM of six individuals; # represents n=4 animals
Values in the parentheses are percent changes from control. Significance was checked from controls to BC exposed and testosterone groups.
P *, ** and *** represents *P*<0.05, < 0.01 and <0.001 respectively.

average values for F1control mice is 4.80 ± 0.20, 5.13 ± 0.54 and 2.76 ± 0.26 in testis, liver and kidney respectively. The catalase mean values for 30, 60 and 90 mg BC exposed mice treated with testosterone are 4.20 ± 0.05, 4.20 ± 0.09, 4.14 ± 0.301 for testis, 4.84 ± 0.176, 5.07 ± 0.11, 4.67 ± 0.117 for liver and 2.48 ± 0.05, 2.38 ± 0.04 2.20 ± 0.05 for kidney respectively. The administration of testosterone in prenatal BC exposed adult males was reverted the levels of SOD and are normalized with controls (Figure 2B).

Catalase: The mean average values of catalase in F1control mice is 20.28 ± 1.28, 29.17 ± 1.16 and 18.46 ± 0.61 in testis, liver and kidney respectively whereas the mean values for 30, 60 and 90 mg BC exposed mice are 14.88 ± 0.85, 10.74 ± 0.80, 5.62 ± 0.83 in testis, 26.97 ± 0.40, 19.95 ± 0.59, 12.82 ± 0.67 in liver and 15.91 ± 0.40, 12.74 ± 0.67, 7.73 ± 0.45 in kidney respectively. The testicular and kidney levels of catalase in prenatal BC exposed males showed significant decrease (P<0.0001) when compared to controls (Figure 3A). In testosterone treated animals the mean average values of catalase in F1control mice is 20.27 ± 1.05, 29.18 ± 1.21 and 18.70 ± 0.96 in testes, liver and kidney respectively. The values for 30, 60 and 90 mg BC exposed adult mice treated with testosterone are18.47 ± 0.795, 17.28 ± 0.541, 18.00 ± 0.686 for testis, 27.21 ± 0.504, 26.67 ± 0.545, 24.92 ± 0.700 for liver and 16.18 ± 0.45, 15.99 ± 0.69 and 15.69 ± 1.070 for kidney respectively. Administration of testosterone to prenatal BC exposed mice restored the tissue catalase levels and normalized with controls except in the testis and kidney of prenatal 60 mg BC/Kg BW exposed males treated with testosterone (Figure 3B).

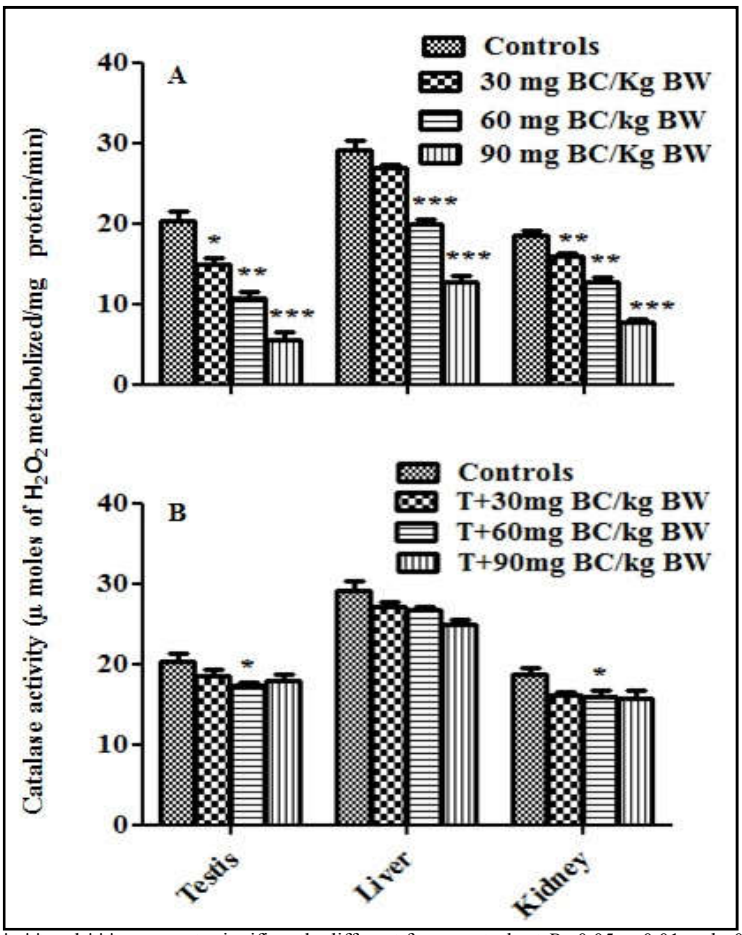
DISCUSSION

The oxidative stress causes deterioration of sperm membrane and it is an important etiological event in the sperm DNA damage, which results in male infertility. Among many reasons for oxidative stress, consumption of high content of phytoestrogenic foods play a remarkable role. To test this, BC was exposed at prenatal period with different dosages (30, 60 and 90mg/kg BW) in mice. Further, the role of antioxidant defence system was tested in the adult F1 males with or without testosterone administration. In the present study, prenatal BC exposure showed significant increase (P<0.001) in the MDA levels in testes, liver and kidney tissues is indicating the elevation of lipid peroxidation a primary indicator for oxidative stress. In contrast to this, SOD and CAT levels in testes, liver and kidney tissues were decreased significantly (P<0.001) by prenatal BC exposure compared to controls. Similar reports were observed by Soujanya *et al.* (2014) after prenatal exposure to biochanin-A in rats. They reported increased MDA levels and decrease in the antioxidant defence system in connection with induced male infertility. Pushpalatha *et al*. (2006) reported the embryonic exposure of hydroxyprogesterone induced elevated lipid peroxidation in liver of adult F1 rats. Epididymal lipidperoxidation elevation and decrease in SOD and catalase after exposure to benzo (a) pyrene was reported in rats (Pratap Reddy *et al*., 2015). Oxidative stress takes up due to disproportion between reactive oxygen species and antioxidants in the body, which results in sperm abnormalities and infertility in men. Reactive oxygen species (ROS) are the byproducts of oxidative phosphorylation. ROS play an important role in physiological intracellular processes and in male reproductive system. Low amount of ROS has a great impact in the spermatozoa activation and fusion of spermatozoa with oocyte (Griveau and Le Lannou, 1997). Elevation in ROS levels causes detrimental effects on cells and damages the tissue membrane. To reduce and normalize ROS, intra and extracellular scavenge system called enzymatic antioxidants get activated. Lipid peroxidation (LP) destroys the lipid membrane there by produces peroxides of polyunsaturated fatty acids and aldehydes. The levels of MDA in tissues are proportional to level of LP and MDA also act as biomarker for the lipid peroxidation of the membrane (Jamil, 2001). Though testis, kidney and liver are diverged tissue but are rich in long-chain-polyunsaturated fatty acids and susceptible to ROS which can damage the tissue by oxidative stress (Emim ozbek, 2012). Earlier studies reported that increase in ROS and oxidative stress leads to the apoptosis and damages of cells (Kannan and Jain, 2000). Progressively increased free radicals are removed by the intracellular antioxidant enzymes, primarily this enzymatic activity is increased but later due to the reduction in antioxidant enzymes, free radicles are not removed so this causes interruption in cell integrity and may lead to cell death (Fang *et al*., 1975). Naturally SOD break down the superoxide radical ($O_2^{-\cdot}$) to hydrogen peroxide (H_2O_2) thereby protects the cells/organs from the oxidative damage. Catalase is another important antioxidant metallic enzyme converts H_2O_2 to water.



*, ** and *** represents significantly different from controls at $P<0.05$, < 0.01 and <0.001 and respectively.

Figure 1. Testosterone induced recovery (B 4.16 mg Testosterone/Kg BW) of lowered SOD levels by prenatal BC exposure (A 30, 60, 90 mg/Kg BW) in testis, liver and kidney of mice



*, ** and *** represents significantly different from controls at P <0.05, < 0.01 and <0.001 and respectively.

Figure 2. Testosterone induced recovery (B 4.16 mg Testosterone/Kg BW) of lowered catalase levels by prenatal BC exposure (A 30, 60, 90 mg/Kg BW) in testis, liver and kidney of mice

In the preset study prenatal BC exposure induced oxidative stress exemplified by elevated LP and decreased SOD and catalase in the testis, liver and kidney resulted in damage of the tissues especially reduced male fertility (results are not presented). The increased ROS may decrease the SOD activity which in turn lowers catalase activity due to low H_2O_2 levels. So the elevated ROS damages the tissue cell membrane by inducing the failure of antioxidant defence system. The administration of testosterone to prenatal BC exposed males during the maturation were reverted the oxidative damage thereby normalized the LP, SOD and catalase in testis, liver and kidney. Recovery with testosterone administration in adult male rat and mice exposed in utero to hydroxyprogesterone and progesterone respectively was reported (Pushpalatha *et al*., 2006; Harini *et al*., 2009). Zhang *et al*. (2011) reported the reduced oxidative stress by testosterone through androgen-receptor (AR)-independent pathway in murine cardiomyocytes. Testosterone therapy reduced the MDA levels and increased SOD levels (Zhang *et al*., 2011).

The modulation of oxidative stress by testosterone in prostate cancer cells is mediated via AR-dependent pathway (Lin *et al*., 2010). The mechanism of restoration of oxidative tissue damage by testosterone in the present study is it by AR-independent or AR-dependent or both is a question to answer. This has to be addressed by further studies.

Conclusion

It is concluded from the present study that prenatal baicalein exposure causes oxidative damage in the testicular, liver and kidney tissues in adult wistar mice and is dose dependent. Maternal exposure of BC induced failure in oxidative defence system is restored by the administration of testosterone at their adult stage (during maturation) thereby fertility. The administration of testosterone after the completion of maturation and its recovery on male health is not tested yet. The delay in administration of testosterone in BC exposed males may permanently cause damage in oxidative defence system and irreversible infertility and is need to be addressed.

Conflict of interest: The authors declare no competing financial interest.

Author contributions: Ms. Sridevi performed the experiments and data compilation. Dr. Ramachandra Reddy designed and supervised the study. Mr. Naveen contributed to complete part of the study and all authors contributed in the writing and have read and approved the final version of the manuscript.

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