Combined Effect of N-Acetylcysteine (NAC) and Plasma on Proliferation of HepG2 Cells

S. Zhao, Z. Xiong, X. Mao, X. Lu, G. He, F. Han, and G. Yang

***Abstract*—It is found that proliferation of HepG2 cells is promoted in the N-acetylcysteine (NAC) and plasma cotreatment group while the group without NAC undergoes cell apoptosis or cell death after plasma treatment. Further investigation found that NAC and plasma cotreatment accelerates the G1 to S phase transition of HepG2 cells and cyclinD1, which responds for the proliferation, is shown to be significantly up-regulated in the NAC and plasma cotreatment group. This finding provides a rationale for developing a proliferation-promotion approach and sheds new sights on the potential application of cold plasmas such as large-scale culture of stem cells *in vitro* for stem cell transplantation, enhancing transplanted tissue incorporation.**

***Index Terms*—Cell proliferation, combined effect, N-acetylcysteine (NAC), plasma jet.**

I. Introduction

COLD atmospheric-pressure plasmas (CAPs) can be used in various biomedical applications, such as sterilization, blood coagulation promotion, wound healing, tooth root canal sterilization, and cancer treatment [1]–[11]. Recently, the plasma interaction with cells has become a hot topic. It has been demonstrated by several groups that CAPs are able to induce cell apoptosis [12]–[15]. As is known, when cells are treated by plasmas, the cells are exposed to reactive neutral atoms and molecules (including O, O_3, NO, OH radicals, singlet oxygen, and so on) and maybe also to a significant flux of charged particles (including electrons, positive, and negative ions) when plasma jets are used [16]. These reactive species could promote the antioxidant level inside the cell or damage the antioxidant system of the cell depending on the plasma treatment time. On the other hand, when the treatment time is relatively short, cells will proliferate instead of dying due to the release of growth factors induced by the reactive oxygen species (ROS) [17].

Cell proliferates through controlling the cell cycle progression. The progression of the cell cycle, which comprises of four defined phases, namely, G1, S (DNA synthesis), G2, and M (mitosis), is a tightly controlled process that is governed by several overlapping regulatory mechanisms. Cells respond differently, either promote or inhibit, according to different stimuli. G1/S transition is the main regulatory site of cell growth, and there are checkpoints in G1/S transition, which are regulated by a series of genes. Key to the control of cell proliferation at this G1/S junction is cyclinD1 gene with positive effect of cell cycle regulation. The overexpression of cyclinD1 can accelerate cell cycle from G1 to S and increase cell proliferation. In various mammalian cell lines, overexpressing of cyclinD1 shortens the G1 phase, indicating that cyclinD1 controls a rate-limiting event(s) for the G1/S transition [18]–[21].

As our previous study shows, plasma treatment first increases the NO content in the extracellular cell culture, which penetrates into the cell later, causing NO and ROS content of the intracellular increasing. Then, the cell antioxidant system compromises, which finally causes cell death [22]. Antioxidants have been known to be cytoprotective after exposure to cellular damaging agents such as ROS. N-acetylcysteine (NAC) is a membrane-permeant agent capable of scavenging free radicals such as ROS and is the precursor of intracellular glutathione (GSH) which serves as an important antioxidant to protect cells from oxidative damage. Also, NAC can directly affect the transcriptional activity of transcription factors through the effect of its reducing thiol group [23]. Currently, NAC has been increasingly used as an experimental tool to assess involvement of ROS in cell signaling and is being evaluated as a preventive and therapeutic agent for various diseases associated with oxidative stress [24]–[27]. Although previous studies have shown that NAC can inhibit neural apoptosis, while induce apoptosis in fibroblast cells [28], [29], it remains unclear what effect it will have on HepG2 cells when cotreated with plasma. What would it be if NAC is preadded in the cell culture and then undergo plasma treatment?

Now, in this paper, the combined effect of NAC and CAPs is investigated by pretreatment of the cells with different concentrations of NAC. The experimental results show that NAC

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and plasma cotreatment can promote HepG2 cell proliferation through the up-regulation of cyclinD1 to accelerate the G1–S phase transition.

II. Experimental Setup

A. Cell Culture

Human hepatoma cancer cell lines, HepG2, were purchased from China Center for Type Culture Collection (Wuhan, China). The cells were placed into 25-cm^2 tissue culture flasks and grown in a high-glucose Dulbecco's modified Eagle medium [(DMEM); Gibco BRL, Grand Island, NY, USA] supplemented with 10% (v/v) heat-inactivated fetal bovine serum [(FBS); Sijiqing, Hangzhou, China] in an incubator containing a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Culture medium was replaced every other day. After attaining confluence, the cells were subcultured following trypsinization.

NAC (Beyotime, Jiangsu, China) was dissolved in phosphate-buffered saline [(PBS); calcium free, 7.9 g of NaCl, 1.8 g of K_2HPO_4, 0.24 g of KH_2PO_4, and 0.2 g of KCl per 1 L of distilled water] at a concentration of 100 mM and was stored at −20 °C. The stock was diluted to the required concentration with the medium when needed. Prior to plasma treatment, cells were pretreated with NAC at different concentrations (0–60 mM) for 1 h.

B. In Vitro Nonthermal Plasma Treatment

A single-electrode plasma jet device is used to generate the plasma plume. The high-voltage (HV) electrode, made of copper wire (diameter of 2 mm), is inserted into a 4-cm-long one-end-closed quartz tube. The inner and outer diameters of the quartz tube are 2 and 4 mm, respectively. The quartz tube along with the HV electrode is then inserted into a hollow barrel of a syringe. The diameter of the hollow barrel is about 6 mm, and the diameter of the syringe nozzle is about 1.2 mm. Distance between the tip of the HV electrode and the nozzle is 1 cm. More detailed description of the experimental setup can be found in [30]. He/O_2 (1%) mixture is used as the working gas with the total flow rate of 1 L/min. The pulse frequency, pulse width $t_{\rm pw}$, and applied voltage are fixed at 8 kHz, 1.6 μs, and 8 kV, respectively. Fig. 1 shows the schematic and photograph of the experimental setup. Distance between the end of the syringe nozzle and the cells is fixed at 1 cm.

C. Cell Proliferation Activity Assay

The cell proliferation activity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on the ability of living cells to reduce MTT to formazan that absorbs at 492 nm [31]. The optical absorption of the solution with dissolved formazan correlates with the number of living cells. If the cell growth is inhibited or promoted, the reduction of MTT to formazan will decrease or increase; thus, the optical density (OD) will change accordingly.

For determination of cell proliferation, HepG2 cells were incubated in 96-well plates (Nunc, Roskilde, Denmark) at a density of $\sim 3 \times 10^4$ cells/well. For experiment, cells were pretreated with NAC at the given concentrations (0–60 mM) for 1 h followed by plasma treatment for 15 s. After treatment, cells were cultured for 24 h in fresh medium in incubator at the same condition. Cells without NAC and plasma treatment (untreated well) served as a control for cell viability. Then, the medium was discarded, washed with PBS for two times, and added with 20 μL of MTT (Sigma, USA) dissolved in PBS solution at a concentration of 5 mg/ml to each wellz and incubated in CO_2 incubator for 4 h. Finally, the medium was discarded from each well, and 100-μL solvent of DMSO (Sigma, USA) was added to dissolve formazan crystals. The OD value of each well was obtained using a microplate reader (Sunrise, Tecan) at a test wavelength of 492 nm. All assays were performed at least in triplicate for each concentration, and means ± standard error of the mean (SEM) values were used to estimate the cell proliferation activity.

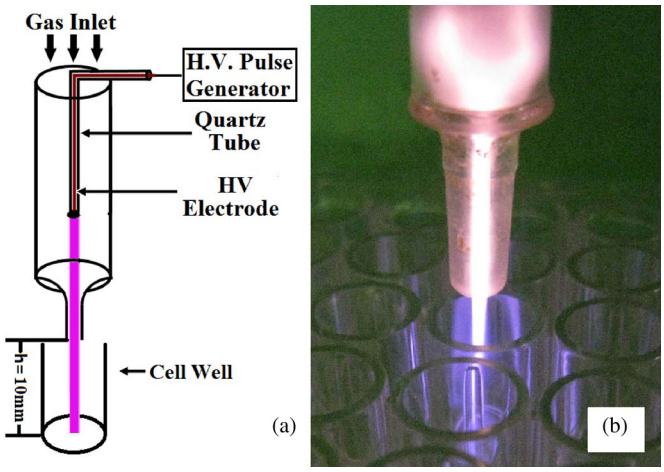

Fig. 1. (a) Schematic of the experimental setup. (b) Photograph of plasma jet acting on the HepG2 cells.

D. Analysis of the Cell Cycle

Cell cycle analysis was detected by flow cytometry. Briefly, HepG2 cells were seeded at a concentration of $\sim 3 \times 10^5$ cells/well in two six-well plates (Nunc, Roskilde, Denmark) in DMEM/10% FBS. After attachment overnight, cells were divided into four groups, i.e., Con group (without any treatment), N+P− group, N−P+ group, and N+P+ group. N+P− group is treated by 15-mM NAC (without plasma treatment). N−P+ group is treated by the plasma for totally 60 s (without NAC treatment). N+P+ group is pretreated by 15-mM NAC followed by totally 60-s plasma treatment. In all the totally 60-s treatment groups, four spots per well, where each spot has 15-s plasma treatment, are taken. Each experiment was performed in three replicates. After the treatment, cells were harvested by trypsinization and fixed by 70% (v/v) ethanol at 4 °C overnight. The fixed cells were rinsed twice with PBS and resuspended in PBS containing 50-μg/mL proliferative index (PI) and 0.1-mg/mL RNaseA (Beyotime, Jiangsu, China). After incubation for 30 min at 37 °C in the dark, cells were immediately analyzed by FACScan flow cytometer (Beckman Coulter, USA). The distribution of cells in the G1, S, and G2/M phases of the

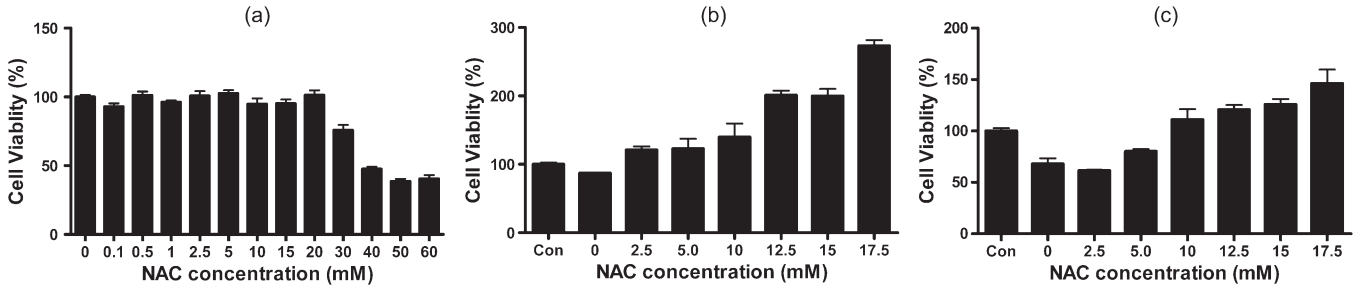

Fig. 2. Relative proliferation levels of HepG2 cells measured using an MTT assay. The y-axis represents the relative rate to the control group. Mean value and SEM are shown ($n = 3$). (a) Effect of NAC treatment on the HepG2 cell proliferation. (b) Effect of NAC (different concentrations) and plasma (15 s) cotreatment on cell proliferation with a lower initial concentration of HepG2 cells. (c) Same as in (b) but with a higher initial concentration of cells. All the results are expressed as mean ± SEM of three separate experiments.

cell cycle was determined. The PIs were calculated as follows: $PI = (S + G2/M)/(G1 + S + G2/M)$.

E. Analysis of CyclinD1 Expression by Western Blot

CyclinD1 protein expression was detected using western blot. The western blot (sometimes called the protein immunoblot) is a widely used analytical technique to detect specific proteins in the given sample of tissue. It uses gel electrophoresis to separate denatured proteins by the length of the polypeptide. The proteins are then transferred to a membrane (typically, nitrocellulose or PVDF), where they are probed using antibodies specific to the target protein. Western blot relies on the primary antibody to detect this protein from the thousands of proteins on the membrane (a cell can contain 30 000 different proteins). A secondary antibody is used to recognize your primary antibody, and then, protein–primary antibody–secondary antibody sandwich is built up. The secondary antibody has a horseradish peroxidase (HRP) enzyme which converts a luminol substrate to a light-releasing substance and can be easily detected as a spot. From the spot, we can analyze the protein expression directly.

In the experiment, HepG2 cells treated as described earlier were washed with cold PBS and lysed with lysis buffer (Beyotime, Jiangsu, China) in the presence of protease inhibitor PMSF (Beyotime, Jiangsu, China). Lysates were centrifuged at 12 000 g at 4 °C for 5 min, and the supernatants were used as total cell lysates. The protein concentrations were determined by BSA assay following the manufacturer's protocols (Beyotime, Jiangsu, China). A quantity of 20 μg of total cellular proteins per lane was separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. The membrane was then blocked using 5% nonfat dried milk in Tris-buffered solution plus 0.1% Tween-20 (TBS-T) overnight at 4 °C. Blocked membranes were then incubated at room temperature for 2 h with the antibodies against β-actin (Boster, Wuhan, China) and cyclinD1 (Santa Cruz Biotechnology, USA) diluted with TBS-T containing 5% (w/v) BSA (Beyotime, Jiangsu, China). After being washed in TBS-T, membranes were incubated with HRP-conjugated goat antimouse or goat antirabbit IgG (Boster, Wuhan, China) diluted in TBS-T/5% BSA for 1 h at room temperature. Detection of transferred proteins (antigens) was performed with an enhanced chemiluminescence detection kits (Millipore, USA) using ChemiDoc XRS+ (Bio-Rad, USA). The abundance of protein was evaluated by the intensity of the corresponding band. The experiments were also repeated three times.

III. Experimental Results

A. Effect of NAC and Plasma Cotreatment on HepG2 Cell Proliferation

To evaluate the proper nontoxic concentration of NAC to HepG2 cells, we first investigated the effect of different concentrations of NAC on the proliferation of the cells. The MTT assay detects mitochondrial dehydrogenase activity which produces the resulting formazan in living cells. Therefore, OD represents the living cell numbers, and the cell viability was calculated as follows: $Cell\ viability$ (in percent) $= (ODt/ODc) \times 100\%$. ODt and ODc represented the mean values of treated group and control group, respectively. As shown in Fig. 2(a), the cell viability remains nearly unchanged compared to the nontreated control group when the NAC concentration varies from 0.1 to 20 mM. However, when cells are treated with escalating concentration more than 20 mM, the cell viability reduces markedly. These findings suggest that the NAC itself (concentration lower than 20 mM) has no effect on cell growth.

Next, we examined the combined effect of NAC and plasma on HepG2 cells. The cells planted in 96-well plates were pretreated with different concentrations of NAC (from 0 to 17.5 mM) for 1 h and then exposed to plasma for 15 s. After being cultured for 24 h, MTT assay was used to detect the rate of cell proliferation. It was found out that the cells treated with plasma only have reduced viability (not shown here), but when the cells were pretreated with NAC, the rate of cell proliferation increased with the increase of NAC concentration as shown in Fig. 2(b) and (c). The proliferation of cells cotreated by NAC and 15-s plasma is promoted when the NAC concentration is higher than 10 mM, and cells in the 17.5-mM group become almost 2.5 times compared to the control group as seen in Fig. 2(b). In addition, it is found that the difference of the cell proliferation rate depends on the initial concentrations of cells. It shows that lower cell proliferation rate appears when the initial concentration of cells is higher. This might be due to the contact inhibition of cells which is the natural process of arresting cell growth when two or more cells come into contact with each other. Thus, it can be concluded that NAC and plasma cotreatment are able to promote HepG2 cell proliferation rate

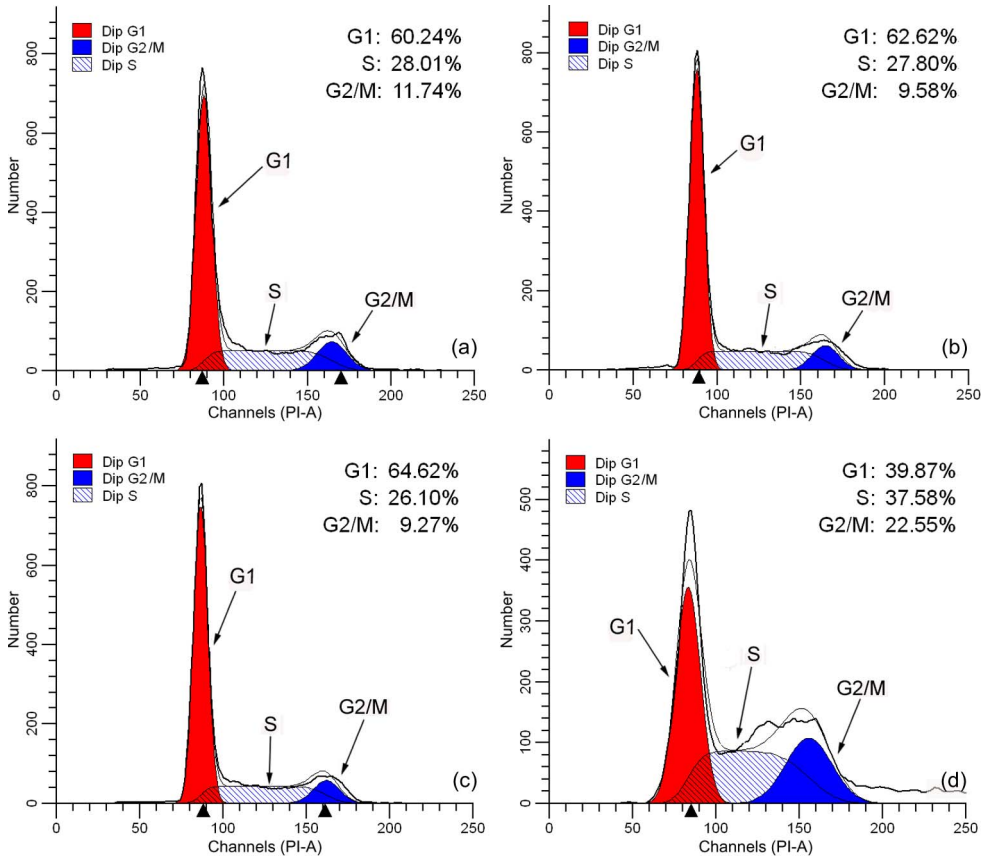

Fig. 3. Flow cytometry analysis for cell cycle distribution of HepG2 cells. (a) Cells without any treatment (Con). (b) Cells with 15-mM NAC treatment only (N+P−). (c) Cells with 60-s plasma treatment only (N−P+). (d) Cells with 15-mM NAC and 60-s plasma cotreatment (N+P+). Cells harvested were fixed and stained with propidium iodide, and their DNA contents were analyzed by flow cytometry. The result of one representative assay from three similar independent experiments is shown. x- and y-axes denote DNA content and cell number, respectively. Each phase was calculated by using the cell ModFit software. The percentages of cells in G1, S, and G2/M were also shown as indicated.

TABLE I
CELL CYCLE DISTRIBUTION OF HEPG2 CELLS

Treatment	Phase			PI
	G1	S	G2/M	
Con	60.24±0.24	28.01±8.01	11.74±1.74	39.76±9.76
N+P-	62.63±2.63	27.80±7.80	9.5900.07	37.37±7.37
N-P+	64.62±4.62	26.10±6.10	9.2700.07	35.38±5.38
N+P+	39.87±9.87	37.58±7.58	22.55±2.55	60.13±0.13

Values are means ±SD of three independent experiments (each conducted in triplicate).

significantly. However, the proliferation rates depend on the initial concentrations of cells.

B. Phase Changes in Cell Cycle Cotreated by NAC and Plasma

To explore the possible mechanism of the increase of the cell proliferation rates, the cell cycles of HepG2 cells were examined. Cells cultured in two six-well plates were divided into four groups, the same as mentioned earlier in Section II-D. The experimental results show that NAC and plasma cotreatment significantly accelerated the cell cycle progression [Fig. 3(d)]. The percentage of cells entering into the S and G2/M phases is significantly higher than control [Fig. 3(a)], whereas the N−P+ and N+P− groups showed little differences on the cell cycle distribution [Fig. 3(b) and (c)].

The mean PI (percentage of the S+G2/M phases), an index reflecting the proliferation characteristic of cells, was also evaluated. The PI of the N+P+ group (60.13 ± 0.15) was significantly higher than those of the control group (39.76 ± 0.16), N+P− group (37.37 ± 0.13), and N−P+ group (35.38 ± 0.05) (Table I). These data suggest that NAC and plasma cotreatment shortened the G1 phase of the cell cycle by accelerating cell transition from the G1 to S phases.

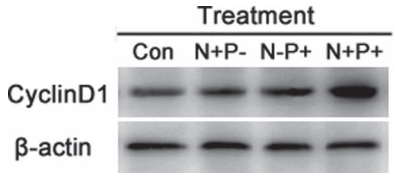

Fig. 4. CyclinD1 expression of HepG2 cells in different treatment groups. (Con) Cells without any treatment. (N+P−) Cells with 15-mM NAC treatment only. (N−P+) Cells with 60-s plasma treatment only. (N+P+) Cells with 15-mM NAC and 60-s plasma cotreatment. The total proteins were extracted from the harvested HepG2 cells with different treatments, and then, the expression of cyclinD1 was synthesized using western blot. The abundance of protein was evaluated by the intensity of the corresponding band. β-actin was used as the internal control.

C. CyclinD1 Level Changes in HepG2 Cells by NAC and Plasma Cotreatment

To identify molecules responsible for the cell proliferation effect induced by NAC and plasma cotreatment, the expression of cyclinD1 was detected using western blot. β-actin was used as the internal control to represent the same loading protein dose in each lane. As shown in Fig. 4, no significantly different levels of cyclinD1 could be seen in N−P+ and N+P− groups compared with control group, while cyclinD1 protein level is higher in N+P+ group than in the other three groups. This result suggests that the shortened G1 phase has probably resulted from the increased expression of cyclinD1 protein in HepG2 cells.

IV. Discussion

Nonthermal plasma could generate various short- and long-living active particles such as ROS and reactive nitrogen species, which can change the redox state of the cells [12], [22]. Researchers find that cell behavior after plasma treatment depends on the plasma dose. High level dose of plasma treatment may cause cell apoptosis or direct cell death with the cracked cell structure [32]. OH and O probably could easily penetrate into the cell, causing a series of oxidative stress reaction, and act with DNA which may change the cell cycle. Also, NO generated by plasma has been proved to affect intracellular NO production. Low dose of these reactive species may contribute to the cell proliferation [33], [34]. Kalghatgi *et al.* [17], [35] found that low-power plasma treatment could induce fibroblast growth factor-2 release in endothelial cells due to the ROS. NAC is a known thiolic antioxidant that acts as a precursor of the natural antioxidant glutathione (GSH). By direct reaction between its reducing thiol group and oxygen-free radicals, it may also protect against oxidant damage *in vitro* and *in vivo* [36]–[38].

Moreover, there is a growing consensus that the redox state of a cell plays a pivotal role in regulating proliferation. Both the plasma and NAC can influence the cellular redox state. Thus, an alternative mechanism is that NAC and plasma cotreatment might alter the cellular redox state which, in turn, alters the activity of specific transcription factors. Thus, we can assume that the cotreatment of NAC and plasma might alter transcription to promote cell proliferation from apoptosis, maybe via reduction and activation receptors for growth factor, which has appeared in the medium or is released by HepG2 cells induced by plasma treatment. Growth factor stimulation and activation of growth factor receptors induce an increase of specific transcription factors that, in turn, induces cyclinD1 level up-regulation [39]–[45].

V. Conclusion

In conclusion, this study has demonstrated that NAC and plasma cotreatment can promote the proliferation of HepG2 cells at least partially through the up-regulation of cyclinD1 to accelerate the G1/S phase transition. Since all of the mammalian cells share nearly the same mechanism of cell growth, this study may provide a rationale for developing a proliferation-promotion approach and sheds new sights on the potential application of CAPs such as large-scale culture of stem cells *in vitro* for stem cell transplantation, enhancing transplanted tissue incorporation, angiogenesis, accelerating wound healing, and repairing damaged skin. All these can be achieved through the regulation of redox state in cells via regulating the working gas of plasma or coregulation supplemented with antioxidants (or other species).

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Authors S. Zhao, Z. Xiong, X. Mao, X. Lu, and G. He contributed equally to this paper.

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Authors' photographs and biographies not available at the time of publication.