Revisiting the systemic lipopolysaccharide mediated neuroinflammation: Appraising the effect of L-cysteine mediated hydrogen sulphide on it

Abdulaziz S. Al-Saeedan a,1, Varsha Gautam b,1, Mohd Nazam Ansari a,⇑, Manjari Singh b, Rajnish K. Yadav b, Jitendra K. Rawat b, Uma Devi c, Swetlana Gautam b, Subhadeep Roy b, Gaurav Kaithwas b

a Department of Pharmacology, College of Pharmacy, Prince Sattam Bin Abdulaziz University, Al-Kharj, Saudi Arabia
b Department of Pharmaceutical Sciences, School of Biosciences and Biotechnology, Babasaheb Bhimanrao Ambedkar University (A Central University), Lucknow, UP, India
c Department of Pharmaceutical Sciences, PHMSIASM SHAITS-Deemed University (Formerly Allahabad Agriculture Institute), Allahabad, UP, India

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1. Introduction

Hydrogen sulphide (H₂S) is a colorless gas, with a consideration of having no physiological importance, until recently being renowned as third most important gaseous signaling molecule respectively followed by nitric oxide (NO) and carbon monoxide (CO) (Abe and Kimura, 1996; Wang, 2002; Martelli et al., 2012). The H₂S has gained importance as a bioactive molecule with several regulatory effects on biological systems including brain. In fact, comparatively high concentration of H₂S has been recorded in the brain tissues of mammals and rodents, which further strengthens the importance of H₂S in neuroscience (Goodwin et al., 1989; Savage and Gould, 1990). Indeed, inorganic H₂S donors have been extensively studied in variety of experimental neuronal disorders.

The biosynthetic pathway of H₂S involves the use of L-cysteine and homocysteine as a substrate. The intracellular pools of free cysteine are maintained by diverse mechanisms and in experimental system H₂S/H₂S donors have demonstrated various physiological protections particularly for the cardiovascular, gastrointestinal and central nervous system (Wang, 2012). The H₂S donors are also reported to reduce inflammation through variable mechanisms including inhibition of neutrophil adhesion and tissue infiltration, decrease in production of reactive oxygen species (ROS) and inflammatory cytokines (Gupta et al., 2010).

It is worth to mention that H₂S is also considered being a broad spectrum poison and having the potential to down regulate several systems of the body, among which the central nervous system is most vulnerable. The toxic effects of H₂S are due to its ability to complex with iron in mitochondrial cytochrome enzymes with subsequent curtailment of cellular respiration (Gerasimon et al., 2007).
Considering the above H2S donors are nowadays suggested/evaluated in various pathologies and on the same line l-cysteine has been evaluated in variable preclinical setups. Nonetheless there are variable reports with few citing it is neurotoxic and some favoring it as neuroprotective (Gu et al., 2000; Janaky et al., 2000; Hashimoto et al., 2004)

A diversified range of mechanisms have been proposed for the neurotoxic as well as neuroprotective effects of l-cysteine and therefore, requires a strict reconsideration before further recommendations. While going through most of studies associated with l-cysteine, it was apparent that there was large variability in the dose selection. Therefore, the authors are in opinion that the dose of l-cysteine is the key player for the variable effects of l-cysteine observed by various researchers.

Henceforth, the further use of l-cysteine needs a careful randomization of its dose. Subsequently, the recent research was undertaken to elucidate the consequence of variable dose of l-cysteine contrary to the neuroinflammatory changes in albino rats.

2. Materials and methods

2.1. Drugs and chemicals

l-cysteine was acquired from Himedia Laboratories, Mumbai, India and lipopolysaccharide (LPS) was purchased from Santa Cruz Biotechnology, Texas75220, USA. SGPT and SGOT kits were procured from Erba Diagnostics Inc, Mumbai, India. All other chemicals were of analytical grade and obtained from Himedia Laboratories, Mumbai, India and lipopolysaccharide (LPS) was purchased from Santa Cruz Biotechnology, Texas75220, USA. SGPT and SGOT kits were procured from Erba Diagnostics Inc, Mumbai, India. All other chemicals were of analytical grade and obtained from Himedia Laboratories, Mumbai, India else otherwise specified in the text.

2.2. Animals

The male Wistar albino rats (100–120 g) were acquired from central animal house. The animals were kept under standard laboratory conditions of normal temperature (25 ± 1 °C) and with a light/dark cycle of 12 h with free access to commercial pellet diet and water ad libitum. Animals were accustomed to laboratory conditions for seven days before the experiment. The study follows the guidelines laid by committee for the purpose of control and supervision of experiment in animals, Government of India, with approval number (SDCOP & VS/AH/CPCSEA/01/0029).

2.3. Experimental design

Rats were arbitrarily distributed into five groups (n = 6) and subjected to the treatment for 28 days as elaborated afterwards. Group I as control (normal saline, 3 ml/kg, p.o.); group II: toxic control (LPS, 125 μg/kg, i.p.); group III (l-cysteine, 25 mg/kg, p.o. + LPS, 125 μg/kg, i.p.); group IV (l-cysteine, 12.5 mg/kg, p.o. + LPS, 125 μg/kg, i.p.); group V (l-cysteine, 6.5 mg/kg, p.o. + LPS, 125 μg/kg, i.p.). Neurotoxicity was induced through the administration of LPS (125 μg/kg, i.p.) for the four consecutive days, starting four days and last four days.

2.4. Evaluations

2.4.1. Neurobehavioral analysis

The neurobehavioral analysis in Wistar albino rats were analyzed using the elevated plus maze (EPM), rotarod test, and locomotor activity method on 17th, 21st, and 22st day of the study respectively. The detail procedure was followed as described by our laboratory (Yadav et al., 2016; Tiwari et al., 2016).

2.4.2. Biochemical assays

After 28 days of treatment, blood samples were collected in preheparinized tubes through retro-orbital plexus using chloroform anesthesia. The blood samples were centrifuged at 10,000 rpm to collect plasma. Animals were sacrificed through light ether anesthesia pursued by heart perfusion. Brain tissues were evacuated rapidly and sensibly escaping damage, rinsed in ice-cold saline and dried out on filter paper. Brain tissue homogenates (10% ice cold KCl) were prepared and centrifuged at 10,000 rpm for 10 min at 4 °C in cooling centrifuge and separated the supernatant. Tissue supernatant was used for the biochemical estimations of thiobarbituric reactive substances (TBAR’s), superoxide dismutase (SOD), glutathione (GSH), catalase, protein carbonyl (PC) and acetylclohexine-esterase (AchE) by using the procedure previously standardized at our laboratory (Reznick and Packer, 1994; Kaithwas et al., 2011; Kaithwas and Majumdar, 2012).

2.4.3. Estimation of SGOT and SGPT in blood plasma

The activity of hepatic enzymes SGOT, and SGPT was determined in plasma samples as method described by the manufacturer using colorimetric kits.

2.4.4. Estimation of tissue H2S

The zinc precipitation method for sulphide determination was followed with slight modifications. Concisely, 500 μl of tissue supernatant was added to 400 μl of premixed zinc acetate (350 μl, 1% w/v) solution and 50 μl of sodium hydroxide (1.5 M) followed by centrifugation. The collected pellets were re-suspended with 160 μl of Milli-Q water and mixed with 40 μl of dye (20 μl of 20 mM NNDP in 7.2 M HCl and 20 μl of 30 mM FeCl3 in 1.2 M HCl). The mixture was incubated at 37 °C for 10 min and read absorbance at 670 nm using UV spectrophotometrically (Cary60, Agilent Technologies, CA 95051, USA) (Ang et al., 2012).

2.4.5. Estimation of plasma H2S

Methylene Blue method was used for the determination of plasma H2S level with slight modification as reported by our laboratory (Tiwari et al., 2016).

2.4.6. Determination of Nitric Oxide (NO) level

Production of NO in the plasma samples was interceded by quantifying nitrite accumulation, using Griess reagent. The same quantity (500 μl) of plasma and Griess reagent was added followed with incubation at 37 °C for 5 min. Subsequently, the absorbance of resultant mixture was read on UV–Visible spectrophotometer at 540 nm (Cary 60, Agilent technologies, CA95051, USA) (Giustarini et al., 2008).

2.4.7. Fatty acid methyl ester (FAME) analysis of brain tissue

Brain tissue homogenate (0.05%) was prepared in the mixture of chloroform: methanol (2:1). The tissue homogenate was subsequently filtered with a Whatman filter paper. A 4 ml of the double distilled water was added to the filtrate to remove the non-lipid contaminates. The mixture was allowed to settle down for 30 min followed by centrifugation. The upper phase thrown out and lower phase carried the brain lipid used for preparation of methyl esters.

The lower phase (0.5 g) mix well in hexane (2 ml) and subsequently, 2 N methanolic KOH (0.2 ml) was added to the above mixture and vortexed for 15 min. The phases were allowed to settle down and the upper layer containing the FAME was collected. Furthermore, the FAME analysis was performed as reported by our laboratory (Folch et al., 1957; Kaithwas et al., 2011).
2.4.8. Enzymatic activity of COX and LOX

The enzymatic activity of COX and LOX was determined by using 10% brain tissue homogenate in (50 mM) tris buffer and plasma detail procedure was follow as reported by (Tiwari et al., 2016; Cullen et al., 1998; Riendeau et al., 2001; Lu et al., 2013).

2.4.9. Western blot analysis

Brain tissue of control and treated rats was used for the quantification of protein by the Bradford reagent (Ahmad and Sharma, 2009). Concisely, quantified protein samples were mixed with sample buffer (125 Mm Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.05% bromphenol blue, 10% 2-mecaptoethanol). A 30 µg of protein sample of brain tissue was allowed to resolve through 12% polyacrylamide gel over SDS-PAGE (GX-SCZ2+, Genetix Biotech Asia Pvt. Ltd, New Delhi). The resolved proteins through SDS-PAGE were relocated on a PVDF membrane (IPVH 00010 Millipore, Bradford MA USA) with semidy transfer (GX-ZY3, Genetix Biotech Asia Pvt. Ltd, New Delhi). Consequently, the transferred protein on membrane was blocked using 5% skimmed milk and 5% bovine serum albumin (BSA) for 2 h followed by incubated overnight with primary antibody against NF-jBP65 (MA5-1616, 1:2000 dilutions), and β-actin (MA5-15739-HRP, 1:3000dilutions). The PVDF membrane was washed away with TBST thrice and incubated with HRP conjugated rat anti-mouse secondary antibody (31430, 1:5000dilutions) at room temperature for 2 h. The signals were distinguished using chemiluminescence substrate (Western Bright ECL substrate, Advansta, Melanopark, California, US). The quantification of the individual protein was accomplished through densiometeric digital analysis of protein bands using Image J software (Laemmli, 1970; Towbin et al., 1979).

2.4.10. Histopathological changes

Brain tissues of albino Wistar rats were evaluated for their morphological modifications using haematoxyline and eosin (H & E) staining. The brain tissues were preserved in paraformaldehyde succeeded by 70% isopropanol overnight and exposed to increased isopropanol concentration (70%, 90%, and 100%) followed by dehydration with 100% xylene. The tissue samples were embedded in paraffin wax and 5 μm sections were primed using microtome succeeded by haematoxyline and eosin staining. The sections were visualized and photographed at 40X using digital biological microscope (N120, BR-Biochem Life Sciences, New Delhi, India) (Belur et al., 1990).

2.4.11. Statistical analysis

All data were presented as mean ± SEM and evaluated by one-way ANOVA followed by Student Newmans Keuls test and for the possible significance between the various groups. *p < 0.05, **p < 0.01, ***p < 0.001, were considered as statistically significant. Statistical analysis was performed using Graph Pad Prism software (5.02), CA 92037, USA.

3. Results

The animals were analyzed for the behavioral models on the EPM, rotarod and locomotor activity. No significant variations were recorded, in the treatment groups either in the open arm and closed arm entries. It would be worth to mention that total time spent in the EPM on the open arm was noticeably reduced in the LPS as well as L-cysteine treated groups with vice versa observations for the time spent in closed arm (Fig. 1A, B). When evaluated on the rotarod, LPS treatment afforded significant decline in the time to fall (Fig. 1C). Subsequently, in the locomotor activity test, a non-significant decline in the locomotor activity was available in all treatment groups (Fig. 1D).

The plasma NO and H2S levels were abolished after the LPS treatment (Fig. 2). The LPS (i.p.) could not afford any modulation in the enzymatic activity of COX and LOX in the brain tissue. However, the plasma COX, LOX activity was marked up by the LPS (i.p.) and the same confronted well by L-cysteine (Fig. 3). When
appraised for the markers of liver toxicity the LPS accorded upregulated levels of SGOT and SGPT which were further appraised after the L-cysteine treatment (Fig. 4).

The brain tissues were scrutinized against the markers of oxidative stress (Table 1). The LPS administration could not afford any significant change in the TBARs and protein carbonyl levels. Similar pattern of non-significant variability was available in the SOD and catalase level as well. Neither LPS nor L-cysteine could accorded any significant variability in the animals. The tissue GSH and AchE levels were not modified either by the LPS or L-cysteine administration. The brain tissues were scrutinized against the markers of oxidative stress (Table 1). The LPS administration could not afford any significant change in the TBARs and protein carbonyl levels. Similar pattern of non-significant variability was available in the SOD and catalase level as well. Neither LPS nor L-cysteine could accorded any significant variability in the animals. The tissue GSH and AchE levels were not modified either by the LPS or L-cysteine administration.

No significant variability in the total brain fatty acids was accorded either through the LPS and L-cysteine administration (Table 2). Inline to the biochemical and inflammatory markers, we could not perceive any changes in terms of neuronal losses or degeneration in the LPS treated animals. On the contrary, persistent damage was evident in the brain tissues of LPS treated animals. Concomitant, L-cysteine administration demarcated significant protection of brain tissue, when scrutinized through H&E staining (Fig. 5). When contemplated through the NF-kBp65 expression in the brain tissue, authors perceived no noteworthy variations in the NF-kBp65 expression in either of the treatment. Interestingly, the L-cysteine 12.5 mg/kg was evident for decrease in NF-kBp65 expression (Fig. 6). Furthermore, After the FAME analysis of the brain tissue there were not any significant changes in the lipid was observed after the LPS administration in the rat (Table 2, Supplementary Fig. 1).
which was restored by L-cysteine following an inverse relationship strated skeletal muscle relaxant/depressive nature in the animals rotarod test and locomotor activity, administration of LPS demon-
produced by LPS.

Fatty acid profile of the brain tissue of the animals treated with L-cysteine.

Table 1
Effect of L-cysteine on markers of oxidative stress in brain tissue.

| Group | Treatment | TBARs (nM of MDA/mg of protein) | GSH (mg%) | SOD (Units of SOD/mg of protein) | Catalase (nM of H2O2/ min/mg of protein) | Protein carbonyl (nM/ml unit) | AchE (nM/ml) |
|-------|-----------|--------------------------------|-----------|---------------------------------|------------------------------------------|-------------------------------|---------------|
| I     | Control (normal saline, 3 ml/kg,) | 1.4 ± 0.12 | 0.09 ± 0.03 | 1.05 ± 0.12 | 31.36 ± 4.94 | 63.15 ± 6.94 | 0.06 ± 0.02 |
| II    | Toxic (LPS, 125 µg/kg) | 1.39 ± 0.17 | 0.09 ± 0.01 | 1.22 ± 0.07 | 27.68 ± 5.67 | 52.01 ± 2.67 | 0.06 ± 0.02 |
| III   | L-cysteine + LPS (25 mg/kg + 125 µg/kg) | 1.85 ± 0.06 | 0.10 ± 0.01 | 1.42 ± 0.13 | 20.30 ± 5.87 | 63.03 ± 4.60 | 0.12 ± 0.06 |
| IV    | L-cysteine + LPS (12.5 mg/kg + 125 µg/kg), L-cysteine + LPS (6.25 mg/kg + 125 µg/kg) | 1.74 ± 0.25 | 0.06 ± 0.01 | 1.27 ± 0.10 | 16.46 ± 2.44 | 66.53 ± 5.75 | 0.01 ± 0.012 |
| V     | L-cysteine + LPS (6.25 mg/kg + 125 µg/kg) | 1.86 ± 0.10 | 0.05 ± 0.01 | 1.08 ± 0.14 | 5.29 ± 1.57* | 55.58 ± 3.16 | 0.17 ± 0.09 |

Table 2
Fatty acid profile of the brain tissue of the animals treated with L-cysteine.

| S. No. | Type of fatty acid | Group-I (%) | Group-II (%) | Group-III (%) | Group-IV (%) | Group-V (%) |
|--------|-------------------|-------------|-------------|-------------|-------------|-------------|
| 1      | C4:0 Butanoic acid | –           | –           | –           | –           | –           |
| 2      | C6:0 Hexanoic acid | –           | –           | –           | –           | –           |
| 3      | C8:0 Octanoic acid | –           | –           | –           | –           | –           |
| 4      | C11:0 Undecanoic acid | –           | –           | –           | –           | –           |
| 5      | C12:0 Dodecanoic acid | –           | –           | –           | –           | –           |
| 6      | C14:0 Tetradecanoic acid | –           | –           | –           | –           | –           |
| 7      | C14:1 Myristoleic (ω-5) | –           | –           | –           | –           | –           |
| 8      | C15:0 Pentadecanoic acid | –           | –           | –           | –           | –           |
| 9      | C17:1 Heptadecanoic acid | –           | –           | –           | –           | –           |
| 10     | C18:1 trans Vaccenic acid (ω-7) | –           | –           | –           | –           | –           |
| 11     | C20:2 Eicosanoic acid | –           | –           | –           | –           | –           |
| 12     | C20:3n6 Dihomo-gamma-linolenic acid (ω-3) | –           | –           | –           | –           | –           |
| 13     | C21:0 Heneicosanoic acid | 0.16        | 0.02        | –           | 0.08        | 0.01        |
| 14     | C23:0 Tricosanoic acid | 0.00        | –           | –           | –           | –           |

4. Discussion
A diversified set of information is available towards the effect of L-cysteine on neurotoxicity, with some suggesting it to be a neurotoxic while others elaborating it as neuroprotective (Janaky et al., 2000; Simintzi et al., 2008; Dhanda et al., 2013; Jia et al., 2013). Hence, the current study was ventured upon, to study the consequence of L-cysteine against the neuro-inflammation produced by LPS.

When examined on the basis of behavioral paradigms of EPM, rotarod test and locomotor activity, administration of LPS demonstrated skeletal muscle relaxant/depressive nature in the animals which was restored by L-cysteine following an inverse relationship with the dose. It is to be noted, that the peripheral LPS (i.p.) could not demonstrate any neurobehavioral abnormality rather a skeletal muscle relaxant effect on the periphery and the same could be accredited to the systemic peripheral inflammation induced by LPS.

When perceived for the oxidative stress paradigms in the brain tissue no significant alteration was available after the peripheral LPS (i.p.) administration.

The GSH/SOD/catalase constitutes a triplet team of defense against oxidative stress. The administration of L-cysteine dissemi- 
ated non-significant diminution in the physiological antioxidant protection of GSH/SOD/catalase which authors are in opinion could be the effect of oxidative stress/neurotoxicity accumulated by the L-cysteine administration.

Oxidative stress is characterized by the cell membrane degra-
dation, which is represented in term of lipid and protein peroxi-
dation. The free radical mediated destruction of proteins and lipids is characterized through the formation of malondialdehyde (TBARs) and Protein carbonyl (Kaithwas and Majumdar, 2012; Kumar et al., 2014). The L-cysteine afforded non-significant upregulation of the TBARs (MDA) generation with non-
significant upregulation of the protein carbonyl content at the dose 12.5 mg/kg. In corroboration to other biochemical markers of oxidative stress the peripheral LPS could not afford any change in the AchE activity. From the first line of indications, authors are in opinion that the peripheral injection of LPS has no influence on the brain and concomitant L-cysteine can precipitate neurotoxic effects.

Considering the facts that LPS can exacerbate peripheral inflammation and to have a further insight, we scrutinized the enzymatic activity of COX and LOX in the plasma (to study the peripheral effects of LPS) and brain tissue (to upraise the neurological effects of peripheral LPS). The enzymatic activity of COX and LOX in the tissue were not affected after the LPS administration which further strengthens of the previous observation that systemic LPS has no effects on neurological circuits. The concomitant L-cysteine administration afforded a significant upregulation in the AchE activity. From the above observations authors are in strong opinion that peripheral LPS has no effect at the neuro- 
ological circuits, however LPS can precipitate peripheral inflammatory changes which could be dose independently regulated by L-cysteine administration.
In the view of the above observations, it was considered worth to study the systemic NO and H$_2$S signaling in the experimental animals. We observed momentous curtailment in the plasma NO and H$_2$S level after the LPS administration, which is in line with the above outcomes and are in corroboration with the previous reports (Shi et al., 2005; Doujaji and Al-Tawfiq, 2010). Both the H$_2$S and NO, regulate the same physiological functions and affect each other’s signaling through an interplay/crosstalk in between them (Faro et al., 2014). The concomitant administration of l-cysteine improved the plasma NO and H$_2$S levels, thereby reflecting the protective effects of l-cysteine against the systemic inflammatory reactions elicited by LPS (Green et al., 1994; Szabó, 2007; Shibuya and Kimura, 2013). When scrutinized for the tissue H$_2$S levels we could not observe any change after the peripheral LPS administration. Subsequent l-cysteine administration was also devoid of any significant changes in the tissue H$_2$S levels.

Considering the fact that the reports derived from the current experiment are not in affirmation with a large set of previous findings. To put a final affirmation, authors considered it worth to perform the fatty acid profiling and histopathological studies of the brain tissues. In consonance to the previous findings, no significant variability in the fatty acid profile was available in the brain tissues after LPS administration. Similar, pattern of outcomes were contemplated from the histopathological results as recorded with no neuronal loss or neuronal deterioration after LPS treatment. It would be commendable to remark that low dose of l-cysteine was evident with the slight neuronal degeneration in comparison to control, LPS and other doses of l-cysteine.

**Fig. 5.** Histopathological alterations in the brain tissue of the rats treated with LPS and l-cysteine.
LPS has been accredited to employ inflammatory effects through NF-κB (Andreakos et al., 2004) and to put an absolute assertion, we scrutinized the expression of NF-κB in the brain tissues of the treated rats. No momentous variability was available in the expression of NF-κB either in the LPS or i-cysteine treatment in brain tissues. The present observation is in line with the consequences of the behavioral, biochemical and other indicators as evaluated prior. With all above findings, authors are in strict affirmation that LPS cannot pledge neuro-inflammatory cascade, which is in contrast to numerous outcomes, demanding neurotoxic properties associated with systemic LPS administration. The findings from the current study also points that i-cysteine (low dose) can impart neurological toxicity, which is in line with the preceding reports and can be attributed to the alteration in the monoamine levels and abnormality in the purkinje cells (Hannah and Roth, 1991; Skrajny et al., 1992; Xiao et al., 2001; Wang et al., 2008; Banks and Robison, 2010).

The outcomes from our study also rules out the perception of short or long term neuro-inflammatory effects of systemic LPS, considering the fact that LPS was administered twice during the course of study. It would be needless to remark that the dose of LPS selected was in a strict consonance to the previous literature (Semmler et al., 2005; Qin et al., 2007; Abdel-Salam et al., 2012; Fan et al., 2014; Fu et al., 2014). One of the viable elucidations for the above reflection could be that LPS is a polysaccharide endotoxin (large chained hydrophilic molecule) and cannot pass across the brain endothelial cells (hydrophobic nature) or through tight junctions (large molecules). It would be appropriate to note that only marginal movement of LPS through the blood brain barrier (BBB) has been conveyed using radioactive iodine LPS, whereby the authors have evidently demonstrated that the brain uptake of peripherally circulating LPS is low and most of the effects of a peripherally administered LPS are probable to be mediated through the LPS receptor positioned external the BBB (Banks and Robison, 2010).

Considering the non-toxic status of neural tissue (due to least penetration of LPS across BBB), no significant changes were accorded after the i-cysteine administration. However, i-cysteine was able to provide peripheral protection against the LPS toxicity All in all, our study presents two major findings; firstly, peripherally administered LPS (either short or long term) cannot activate neuro-inflammatory cascade; Secondly, the study also evidenced that i-cysteine can afford peripheral protection against LPS through modifying H2S and NO signaling.

At the end, the question remained unelucidated that whether i-cysteine can afford neuroprotective effects which can be taken as a research question for future endeavors.

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Conflict of interest
The authors declare no conflict of interest.

Appendix A. Supplementary material
Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jsps.2018.02.004.

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