The Significance of Hydrogen Sulfide for Arabidopsis Seed Germination

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Hydrogen sulfide (H$_2$S) recently emerged as an important gaseous signaling molecule in plants. In this study, we investigated the possible functions of H$_2$S in regulating Arabidopsis seed germination. NaHS treatments delayed seed germination in a dose-dependent manner and were ineffective in releasing seed dormancy. Interestingly, endogenous H$_2$S content was enhanced in germinating seeds. This increase was correlated with higher activity of three enzymes (L-cysteine desulfhydrase, D-cysteine desulfhydrase, and $\beta$-cyanoalanine synthase) known as sources of H$_2$S in plants. The H$_2$S scavenger hypotaurine and the D/L cysteine desulfhydrase inhibitor propargylglycine significantly delayed seed germination. We analyzed the germinative capacity of des1 seeds mutated in Arabidopsis cytosolic L-cysteine desulfhydrase. Although the mutant seeds do not exhibit germination-evoked H$_2$S formation, they retained similar germination capacity as the wild-type seeds. In addition, des1 seeds responded similarly to temperature and were as sensitive to ABA as wild type seeds. Taken together, these data suggest that, although its metabolism is stimulated upon seed imbibition, H$_2$S plays, if any, a marginal role in regulating Arabidopsis seed germination under standard conditions.

Keywords: hydrogen sulfide, seed germination, seed dormancy, Arabidopsis, cysteine desulfhydrase

INTRODUCTION

Hydrogen sulfide (H$_2$S) is a toxic gas found in volcanic emissions or produced in waterlogged wetlands. It is also formed during human activities including agriculture and car use. Plants are therefore subjected to H$_2$S exposure during their whole lifespan. Although acute exposure to H$_2$S has long been known as deleterious for plants (Lisjak et al., 2013), numerous reports also indicate that low doses of H$_2$S might be beneficial for plant fitness. For instance, exogenous treatments with H$_2$S-releasing chemicals such as sodium hydrosulfide (NaHS) improve the tolerance to abiotic stresses such as drought, salinity, heavy metals, or high temperature in a variety of plant species (Jin et al., 2011; Christou et al., 2013, 2014; Shen et al., 2013; Shi et al., 2013). The mechanisms for H$_2$S alleviation of abiotic stress effects unraveled so far are essentially the stimulation of antioxidant defense. For instance, the activities of superoxide dismutase, catalase, and ascorbate peroxidase are enhanced by NaHS treatments and contribute to maintain low levels of H$_2$O$_2$ (for review, Li, 2013). In addition, wheat plants water-stressed in the presence of NaHS display higher ascorbate and glutathione contents, as well as a higher proportion of reduced forms (Shan et al., 2011). Besides stimulating antioxidant defense, exogenous H$_2$S treatments also trigger stomata
movements which might participate in plant adaptive response to water and osmotic stress (García-Mata and Lamattina, 2010; Jin et al., 2013; Honda et al., 2015). Nevertheless, as opposite effects were observed i.e., the promotion of either stomata closure or opening (García-Mata and Lamattina, 2010; Lisjak et al., 2010), the outcome of such exogenous treatments might be conditioned by the overall plant physiological status.

Recent data raised further interest toward H$_2$S in plants by highlighting a signaling function of endogenously produced H$_2$S (for review, Lisjak et al., 2013). Three enzymes have been identified as sources of H$_2$S in plants (Riemenschneider et al., 2005b; Alvarez et al., 2010; García et al., 2010). L- and D-cysteine desulfhydrases (L/D-CDes) produce H$_2$S as a by-product of L/D-cysteine degradation. On the other hand, β-cyanoalanine synthase (β-CAS) catalyzes the conversion of cysteine and cyanide to β-cyanoalanine and H$_2$S. β-CAS activity is located in the mitochondria when desulfhydrases have been reported in mitochondria and cytosol (Romero et al., 2014). Of particular interest, Alvarez et al. (2010) characterized a cytosolic L-CDes designated DES1 that modulates the generation of H$_2$S in the cytosol. The corresponding des1 mutants exhibit a reduced H$_2$S content that is correlated with a premature leaf senescence and the overexpression of senescence associated genes (Alvarez et al., 2012a). Further, investigations also indicated that des1 mutants are more sensitive to drought, in good accordance with their impaired ABA-responsive stomatal closure (Jin et al., 2013). More globally, des1 mutants appear more sensitive to abiotic and biotic stresses and present a lower induction of stress-responsive genes (Shi et al., 2015). Altogether it is now obvious that data obtained by manipulating exogenous or endogenous H$_2$S level converge and plead for an important role of this compound in the regulation of plant response under stress.

Whereas the involvement of H$_2$S in plants under stress is well established, its function in plant development has been far less investigated. In that sense, seed germination represents an interesting study case. Indeed germination being the first step of plant life cycle is crucial for the establishment of a robust plantlet. Under natural conditions, it also determines the efficiency of wild species to proliferate in ecosystems. The ability to germinate is therefore tightly controlled to occur only when environmental conditions get favorable for the successful development of the plantlet. In most species, mature seeds are dormant i.e., are unable to germinate under favorable conditions, and germination capacity is acquired only after seed dormancy has been released. Seed dormancy release and germination are therefore the two integral faces of a same coin when considering freshly harvested seeds. Dormancy release is controlled by a complex set of environmental, e.g., temperature or light, and internal, e.g., hormones, signals (Shu et al., 2016). When successful, germination is completed within a short window that starts with seed imbibition and ends with radicle protrusion.

Several studies have addressed the effect of H$_2$S treatment on seed germination (Zhang et al., 2008, 2010a,b; Li et al., 2012; Dooley et al., 2013). Nevertheless, in most of the cases, only combined effects of H$_2$S and stress (e.g., osmotic, oxidative, or metal stress) have been reported. When studied alone (Zhang et al., 2010a; Dooley et al., 2013), contrasting effects of H$_2$S were observed. Zhang et al. (2010a) observed no differences of wheat germination in the absence or presence of up to 1.5 mM NaHS. On the other hand, Dooley et al. (2013) reported a stimulation of germination in the four species analyzed (i.e., wheat, corn, pea, and bean). Noteworthy, in most of the cases (species, NaHS concentration), NaHS treatment only accelerated germination but hardly modify final germination efficiency (Dooley et al., 2013). Although these data suggest that modifications of H$_2$S availability during seed imbibition could affect seed germination, no information on a possible role for endogenously evolved H$_2$S have been reported. In the present paper, we further investigated the metabolism and possible involvement of H$_2$S in Arabidopsis seeds germinated under standard conditions.

**MATERIALS AND METHODS**

**Plant Material and Germination Assays**

Arabidopsis (Arabidopsis thaliana) seeds of Columbia-0 (Col-0) ecotype were used throughout the study. The des1-1 mutant (SALK_103855) presents a T-DNA insertion in the second exon of DES1 gene (At5g28030) leading to a knockout mutation (Alvarez et al., 2010). Col-0 and des1-1 seeds were propagated simultaneously as previously described (Basboss-Serhal et al., 2015). Plants were grown in a climate chamber (20/22°C under long day photoperiod (16 h light/8 h dark)) on a soil/perlite (2:1) mixture. Floral stems were cut at silique maturity (about 3 months after sowing) and dried for 4 days at room temperature in paper envelopes before seeds were collected. Freshly harvested seeds were stored at −20°C to preserve their dormancy.

Seed germination was tested in 9-cm Petri dishes (50 seeds per dish, three replicates) on a filter paper on the top of a layer of cotton wool moistened with 20 mL of deionized water or indicated solutions. Plated seeds were subsequently kept in the dark in growth chambers at the required temperature. Germination was scored over time, a seed being considered as germinated when the radicle protruded through the testa. All the chemicals assayed during germination tests were directly dissolved in the deionized water used for seed imbibition.

**Chemicals**

D-cysteine (C8005), hypotaurine (H1384), and DL-propargylglycine (P7888) were purchased from Sigma-Aldrich (L’Isle d’Abeau Chesnes, France).

**Enzyme Activity Measurements**

Seed material (30 mg of dry seeds per condition before imbibition) consisting of dry seeds or seeds imbibed for 6, 16, or 24 h was collected by rapid freezing in liquid nitrogen. After grinding in liquid nitrogen, soluble proteins were extracted from seed powder by the addition of 1 mL of 50 mM Tris-HCl (pH 8) containing 2.5 mM DTT and 10 µL of protease inhibitor cocktail (P9599, Sigma-Aldrich, L’Isle d’Abeau Chesnes, France). Following centrifugation (14,000 g, 4°C, 45 min), protein content was determined and was adjusted to 1 mg mL$^{-1}$.

L-cysteine desulphydrase (L-CDes) activity was assayed essentially as previously described (Riemenschneider et al., 2005a). Reactions were performed in 1 mL final volume of 0.1 M HEPES buffer (pH 7.5) containing 0.5 mM L-cysteine (C8005), 0.5 mM propargylglycine (P7888), 0.1 mM cysteine (C8005), 50 mM Tris-HCl (pH 7.5), 2.5 mM DTT, and 10 µL of protease inhibitor cocktail (P9599, Sigma-Aldrich, L’Isle d’Abeau Chesnes, France). Reactions were performed at 37°C for 15 min. Reactions were terminated by the addition of 0.5 mL of 0.5 M NaOH. Reactions were performed in triplicate. Results were expressed as nmol minute$^{-1}$ per mg protein.
M Tris-HCl (pH 9), 2.5 mM DTT, 0.8 mM L-cysteine, and 50 µg of soluble proteins. Following incubation at 37°C for 15 min, reactions were stopped by the addition of 100 µL of 20 mM N,N-dimethyl-p-phenylenediamine (in 7.2 N HCl) and 100 µL of 30 mM FeCl3 (in 1.2 N HCl). After 10 min incubation at room temperature and centrifugation (14,000 g, 5 min), the formation of methylene blue was quantified at 670 nm. For the calculation of H2S formation, known quantities of NaHS were assayed in the same conditions, and used for standard curve determination.

D-cysteine desulghydrylase (D-CDes) was assayed in the conditions described for L-CDes activity except that Tris-HCl buffer was pH 8 and L-cysteine was replaced by D-Cysteine.

β-cyanoalanine synthase (β-CAS) activity was assayed as described in Meyer et al. (2003). Reactions were performed in 1 mL final volume of 0.1 M Tris-HCl (pH 9), 0.8 mM L-cysteine, 10 mM KCN and 100 µg of soluble proteins. Following incubation at 30°C for 15 min, reactions were stopped and subsequently processed as described above for L-CDes activity.

Hydrogen Sulfide Quantification
Seed endogenous hydrogen quantification was adapted from Christou et al. (2013). Dry or imbibed seeds (30 mg) were ground in liquid nitrogen and seed powder was resuspended in 500 µL of 100 mM potassium phosphate buffer (pH 7) containing 10 mM 3-M Tris-HCl (pH 9), 2.5 mM DTT, 0.8 mM L-cysteine, and 50 µM Tris-HCl (pH 9), 0.8 mM L-cysteine, and 50 mM KCN and 100 µg of soluble proteins. Following incubation at 30°C for 15 min, reactions were stopped and subsequently processed as described above for L-CDes activity.

RESULTS
Exogenous NaHS Treatments Delay Arabidopsis Seed Germination
Recent data suggested that treating seeds with H2S-releasing chemicals such as sodium hydrosulfide (NaHS) might stimulate seed germination in different legume and cereal species (Dooley et al., 2013). Using freshly harvested Arabidopsis seeds, germination was compared in the absence or presence of various concentrations of NaHS. As shown on Figure 1, germination was not affected when up to 100 µM NaHS was applied, but was delayed by higher concentrations, in a dose-dependent manner. When imbibed at 25°C, seeds did not germinate whatever the concentration of NaHS was, indicating that seed dormancy cannot be alleviated by NaHS treatment (data not shown). These data suggest that exogenously applied H2S, when efficient, negatively impacts Arabidopsis germination.

Endogenous H2S Level Increases during Seed Imbibition
To get further information on the possible involvement of H2S in regulating germinative capacity, we assessed whether seeds processing to germination endogenously produced H2S during imbibition. As shown on Figure 2A, a slight increase (~40% compared to dry seeds) of H2S content was observed after 6 h imbibition and was maintained at 24 h. To evaluate the origin(s) of the H2S produced during seed imbibition, the activity of three enzymes reported to generate H2S in plants, i.e., L-cysteine desulghydrylase (L-CDes; E.C. 4.4.1.28), D-cysteine desulghydrylase (D-CDes; E.C. 4.4.1.15), and β-cyanoalanine synthase (β-CAS; E.C. 4.4.1.9), was compared in dry and imbibed seeds (Figure 2B). The three activities could be detected in protein extracts from dry seeds. All three activities were stimulated in imbibed seeds and reached a maximum after 24 h. At this time point, activity was 3.7, 5.25, and 1.7 fold higher than in dry seeds, for L-CDes, D-CDes, and β-CAS, respectively. Nevertheless, the level of β-CAS activity remained ~10 fold lower than that of CDes activities suggesting that L- and D-CDes were likely the major sources of H2S during seed germination. Based on these observations, we investigated the impact of seed treatments with NaHS on L-CDes, D-CDes, and β-CAS activities. As shown on Figure 2C, the activities of the three enzymes were similar for seeds imbibed 24 h in the absence or presence of NaHS suggesting that the delay of seed germination triggered by high NaHS concentrations is not achieved via the modification of endogenous H2S metabolism.

Impairment of H2S Accumulation during Imbibition Delays Seed Germination
To assess whether endogenous H2S might participate in regulating germination, seeds were germinated in the presence of hypotaurine (HT, a H2S scavenger) or DL-propargylglycine (PG, an inhibitor of CDes). After 3 days, germination was reduced by 25 and 40% by HT and PG, respectively (Figure 3). Nevertheless, HT and PG did not block, but only delayed germination, as comparable final rates of germination were reached after 7 days. These data indicate that impairing endogenous H2S formation impacts seed germination capacity.
FIGURE 2 | H$_2$S synthesis during Arabidopsis seed imbibition. (A) Evolution of Arabidopsis seed H$_2$S content during imbibition. Seeds were imbibed on distilled water in the dark at 15$^\circ$C. Values are mean ± S.E. of three independent experiments. Asterisks represent statistical differences relative to H$_2$S content in dry seeds (Student’s test; *P < 0.05; **P < 0.01); (B) Evolution of the activities of H$_2$S-generating enzymes in Arabidopsis seeds imbibed on water or 1 mM NaHS. Seeds were imbibed on distilled water or 1 mM NaHS in the dark at 15$^\circ$C for 24 h. Total proteins were subsequently extracted and used for the measurement of L-cysteine desulphhydrase (L-CDes), D-cysteine desulphhydrase (D-CDes), and $\beta$-cyanoalanine synthase ($\beta$-CAS) activities. Activities were compared with those of dry seeds. Values are mean ± S.E. of three independent experiments. (Continued)

FIGURE 2 | Continued

D-Cdes Mutant Seeds Do Not Accumulate H$_2$S during Imbibition but Exhibit Unmodified Germination

The impairment of germination by PG treatment together with the strong increase of CDes activities during seed imbibition suggested the involvement of CDes in H$_2$S generation in imbibed seeds. A mutant line for DES1 gene (des1-1) deficient for the sole cytosolic L-CDes characterized to date (Alvarez et al., 2010) was therefore analyzed for H$_2$S production in imbibed seeds. The H$_2$S content of freshly harvested des1 seeds was compared with that of WT seeds at the dry state or after different durations of imbibition at 15$^\circ$C (Figure 4). Comparable H$_2$S contents were measured in WT and des1 dry seeds. In contrast, whereas H$_2$S content raised in WT seeds after imbibition, it remained unmodified over 24 h of imbibition in des1 seeds, therefore implicating DES1 as a major source for H$_2$S in germinating Arabidopsis seeds.

We further compared WT and des1 mutant seeds under conditions known to influence germinative capacity. We first monitored the effect of temperature on the germination of both lines (Figure 5). When imbibed at 25$^\circ$C, freshly harvested WT and des1 seeds presented a low (∼20%) percentage of germination, indicating that both seed lines were essentially dormant. At 15$^\circ$C, the same lines fully germinated after 4 days. Imbibition at 20$^\circ$C also led to full germination, with
Recent years have provided an array of studies evidencing the biological effects of H$_2$S in plants (Lisjak et al., 2013; Jin and Pei, 2015). Among them several reports suggested that H$_2$S stimulated seed germination (Zhang et al., 2008, 2010a,b; Li et al., 2012; Dooley et al., 2013). Nevertheless, the observations essentially evidenced the capacity of H$_2$S to alleviate the negative effects of stresses on germination, but were poorly informative on the effect of H$_2$S on germination per se. Using Arabidopsis seeds as a model, we investigated the involvement of H$_2$S in regulating germination under standard conditions. As reported in several physiological contexts including plant response to auxin (Fang et al., 2014), salt, and osmotic stress (Christou et al., 2013) or hypoxia (Cheng et al., 2013), we observed a significant increase of H$_2$S content in seeds during imbibition. This increase occurred after 6 h and was maintained over 24 h. Increased production of H$_2$S had been observed previously in seeds submitted to Al and osmotic stress (Zhang et al., 2010a,b). Our data indicate that it also occurs under standard germination conditions in the absence of stressing factors. It therefore suggested that endogenously evoked H$_2$S might function as a signal during germination.

Different sources for H$_2$S production have been reported in plants (Romero et al., 2014). In relation with seed physiology, Xie et al. (2014) correlated the variations of H$_2$S content in wheat aleurone layers with the activity of L-cysteine desulphhydrase (L-CDes), the major source of H$_2$S in plants (Alvarez et al., 2010). We could measure a L-CDes activity (27 ± 2 nmol H$_2$S.min$^{-1}$.mg prot$^{-1}$) in dry Arabidopsis seeds in the range of those reported for plant tissues (10–150 nmol H$_2$S.min$^{-1}$.mg prot$^{-1}$) (Riemenschneider et al., 2005a; Alvarez et al., 2010; Hou et al., 2013; Fang et al., 2014; Xie et al., 2014). L-CDes activity was strongly enhanced during imbibition, reaching a maximum after 24 h. It could therefore afford for the higher H$_2$S contents found in imibed seeds. We also observed a concomitant increase of the activity of D-cysteine desulphhydrase (D-CDes) and β-cyanoalanine synthase (β-CAS), two other enzymes generating H$_2$S in plants (Riemenschneider et al., 2005a; García et al., 2010).
Because of the toxicity of H\textsubscript{2}S for cytochrome c oxidase, it is unlikely that H\textsubscript{2}S generated by the mitochondrial β-CAS could accumulate to significant levels in planta, and the higher β-CAS activity is therefore likely related to cyanide detoxification (Álvarez et al., 2012b). D-CDes that exhibits a strong activity in imbibed seeds could generate part of the H\textsubscript{2}S measured in seeds, assuming that D-cysteine levels are sufficient (Riemenschneider et al., 2005b). Nevertheless, our study indicates that L-CDes is critical for H\textsubscript{2}S production in imbibed seeds. Indeed, knock-out mutant seeds for the DES1 gene that encodes the cytosolic L-CDes (Álvarez et al., 2010) presented similar H\textsubscript{2}S contents in dry and imbibed seeds. The stimulation of DES1 activity is therefore the main route for the enhanced H\textsubscript{2}S production observed in imbibed seeds. On the other hand, the basal H\textsubscript{2}S level found in dry seeds and detected in imbibed des1 mutant seeds might be related to β-CAS and/or D-CDes activities. It might also be due to unidentified L-CDes enzymes as suggested by the significant L-CDes activity retained by the des1 mutant (Álvarez et al., 2010).

To decipher the requirement and possible function of H\textsubscript{2}S during seed germination, we used chemicals either artificially releasing H\textsubscript{2}S (NaHS) or inhibiting endogenous H\textsubscript{2}S formation (hypotaurine and propargylglycine). No effect of NaHS was observed at low concentrations (≤100 μM), whereas germination was significantly delayed at higher concentrations. This effect is likely due to a massive and rapid release of H\textsubscript{2}S from NaHS toxic for the plant material. Indeed no such retardation was observed with GYY4137, that releases lower H\textsubscript{2}S doses (Lisjak et al., 2010), and GYY4137 did not affect Arabidopsis seed germination at any concentrations tested up to 1 mM (data not shown). Our data further illustrate contrasted effects of H\textsubscript{2}S on seed germination observed under standard conditions. Indeed, no effect was found on wheat seed germination in the presence of up to 1.5 mM NaHS (Zhang et al., 2010a). On the other hand, Dooley et al. (2013) reported a stimulation of seed germination by H\textsubscript{2}S in diverse plant species including corn, pea, bean, and wheat. Different sensitivities toward stimulators/inhibitors of germination are frequently observed between species and might afford for the different response to H\textsubscript{2}S treatment in different species (Finch-Savage and Leubner-Metzger, 2006). In addition the response might depend on the conditions used for treatment and germination (light status, temperature...) that are varying between studies.

To get further insights in the function of endogenously evoked H\textsubscript{2}S, we used hypotaurine and propargylglycine to modulate H\textsubscript{2}S content in seeds. Hypotaurine (HT) is a potent H\textsubscript{2}S scavenger (Ortega et al., 2008) and has been used in plants to lower intracellular H\textsubscript{2}S content (Li et al., 2014). Propargylglycine (PG) inhibits CDes activities (Steegborn et al., 1999) and blocks H\textsubscript{2}S production in plants (Li et al., 2014). When applied during seed imbibition both compounds strongly delayed germination. As their modes of action are different this effect is certainly reached through the blocking of H\textsubscript{2}S formation. It therefore supports that endogenous H\textsubscript{2}S formation is required for optimal germination. Noteworthy the effect of HT and PG was similar to that of high NaHS treatments, which questioned on the possible impact of NaHS application on seed H\textsubscript{2}S metabolism. Although the mechanisms by which high NaHS concentrations delay germination are unknown, our data indicate that they do not rely on modifications of endogenous H\textsubscript{2}S metabolism. Indeed seeds imbibed in the presence or absence of NaHS exhibit similar L-CDes, D-CDes, and β-CAS activities. Moreover, the germination of des1 seeds was as sensitive as WT seeds to NaHS. These observations are consistent with previous data by Riemenschneider et al. (2005a) indicating that L-CDes and D-CDes activities were not affected in H\textsubscript{2}S-fumigated Arabidopsis plants. On the other hand optimal germination might require a particular level of endogenous H\textsubscript{2}S that would be unbalanced by exogenous NaHS, HT, and PG treatments. This phenomenon has been proposed for H\textsubscript{2}O\textsubscript{2} for which too low or too high intracellular levels both lead to germination impairment (Bailly et al., 2008). In any case, only a delay of germination, and not a full inhibition, has been observed for the different treatments. As long-term effects of HT and PG have been reported with lower concentrations than the one used in our study (Li et al., 2014), it is unlikely that HT and PG loose their efficiency over time.

It more likely reflects that endogenously evoked H\textsubscript{2}S, although fastening germination, is not a requisite. This hypothesis is strengthened by the fact that des1 mutant seeds germinate at similar rates compared to WT seeds. Experiments run at 15°C indicate that DES1 activity, and more generally endogenously evoked H\textsubscript{2}S, is dispensable for germination. Similarly, because both des1 and WT seeds hardly germinate at 25°C, DES1-dependent H\textsubscript{2}S production is not required for seed dormancy. In good accordance with this, des1 and WT seeds exhibited comparable sensitivity toward ABA, which is the major regulator of seed dormancy. Contrarily to stomata that exhibit an altered response to ABA in des1 mutant and in which DES1-dependent H\textsubscript{2}S formation is an intermediate of ABA signaling (Scuffi et al., 2014), our data indicate that ABA signaling in seeds is independent of DES1. More globally, as no altered development has been observed for des1 seedlings (Álvarez et al., 2010; Jin et al., 2013), our data further highlight that DES1 is dispensable during the early stages of plant development under optimal growth conditions.

Taken together our data highlight that although H\textsubscript{2}S is produced at the early stages of seed imbibition essentially via the activity of L-CDes, its importance, if any, for germination under standard conditions is limited in Arabidopsis. This contrasts with other reactive species such as hydrogen peroxide or nitric oxide that are now considered as major regulators of seed germination and seed dormancy release (Arc et al., 2013; Diaz-Vivancos et al., 2013). As treatments with H\textsubscript{2}S donors efficiently alleviate inhibition of germination by abiotic stresses, future works should be focused on deciphering whether endogenously evoked H\textsubscript{2}S participates in abiotic stress tolerance during seed germination and could therefore constitute a trait for variety selection and improvement.

**AUTHOR CONTRIBUTIONS**

EB, JP, and CB designed the research. EB, AP, NI, and FC carried out the experiments and analyzed the data. EB, JP, and CB...
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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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