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Virus-Free Plant Regeneration from Shoot Apical Meristem of *Coccinia grandis* L., an Important Medicinal Plant in Bangladesh

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Abstract
This investigation was undertaken to establish an efficient protocol for virus-free plant regeneration in *Coccinia grandis* L. through shoot apical meristem culture. Murashige and Skoog (MS) basal medium supplemented with different concentrations and combinations of 6-benzyl amino purine (BAP), gibberellic acid (GA_3), naphthalene acetic acid (NAA), and indole-3-butyric acid (IBA) was used for meristem establishment, shoot regeneration, and root induction as well as elongation. MS liquid medium supplemented with 1.0 mg l^{-1} BAP + 0.10 mg l^{-1} NAA was found to be the best medium for the primary establishment of meristems. MS medium containing 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} GA_3 + 0.5 mg l^{-1} NAA was found to be best for shoot regeneration percentage at 100.0 ± 0.0 and multiplication with 10.0 ± 0.8 shoots per meristem as well as shoot elongation (highest 9.0 ± 0.0 cm). *In vitro* grown shoots were subcultured and rooted with 11.0 ± 0.8 roots per shoot subsequently on MS medium containing 0.5 mg l^{-1} IBA. Well-rooted plantlets were gradually acclimatized and successfully established in the field condition with 100% survival rate.

Keywords: *Coccinia grandis*; Shoot apical meristem; Plant regeneration; Acclimatization

1. INTRODUCTION

Coccinia grandis L. is an important medicinal and vegetable crop plant belonging to the family of Cucurbitaceae. *C. grandis* plant contains carbohydrate 12.62%, total protein 15.0%, water-soluble protein 11.2%, lipid 4.0%, total phenol 61.9 mg, vitamin C 25.5 mg, β-carotene 70.0 mg, potassium 3.3 mg, phosphorous 1.1 mg, sodium 0.9 mg, iron 2.2 mg, and calcium 3.7 mg in 100 g of fresh weight [1]. The plant also contains resin, starch, fatty acids, carbonic acid, triterpenoid, saponin, coccinoside, lupeol, taraxerol, steroids, ellagic acid, lignin, and other compounds such as alkaloids, tannins, flavonoids, glycosides, phenols, B-amyrin acetate, taraxerol, B-carotene, lycopene, cryptoxanthin, xyloglucan, carotenoids, and β-sitosterol [2]. The various parts of ivy gourd plant are popularly used in folk medicine for the treatment of diabetic, eye diseases, carminative, hypertension, fever, anti-inflammatory, headache, typhoid, sunstroke, hypnotic, jaundice, stomach pain, antipyretic, mental disease, leucorrhea, alopecia, dermatitis, eczema, emetic, dysentery, scabies, and blood purifier [1, 3, 4]. Leaves and fruits are used as food crops in several countries such as Australia, Asia, Caribbean, the Southern United States, and Pacific Islands [5]. *C. grandis* fruits are eaten immature and green, or mature and deep red [6]. At the advent of plant biotechnology, meristem culture offers a novel tool for the production of virus-free plants. There are many reports on meristem culture obtaining virus-free plants, including *Helicteres isora* [7], snake gourd [8], purple passion fruit [9], pumpkin [10], orchid [11], cowpea [12], and bitter gourd [13]. Plant tissue culture methods have not only the advantage of built-in disease protection but also have many other advantages, such as rapid multiplication of disease-free plant materials. Devendra *et al*. [14] reported that in cucurbits the seed setting and seed germination rate is low, probably due to the presence of a thin nuclear membrane lending impermeability to water and gases that make them dormant for a long time. Rapid *in vitro* multiplication for virus-free plantlets of *C. grandis* is required to overcome its extinction. There are few reports in the literature where a small number of shoot regeneration of *C. grandis* from nodal segments and shoot tips have been done [15, 16] but no report has been published on direct regeneration of virus-free plantlets from meristem culture of *C. grandis*. In this study, we established an *in vitro* virus-free plantlets propagation protocol for *C. grandis* and their successful acclimatization in the field condition.

2. METHOD(S)

2.1. Plant Materials
This study was carried out at Professor Joarder DNA and Chromosome Research Lab, Department of Genetic Engineering and Biotechnology, Rajshahi University, Rajshahi, Bangladesh. Shoot apical meristems were isolated from 15 to 21 days *in vivo* grown shoot tips of ivy gourd and were used as plant materials.

2.2. Other Materials
All the chemical compounds including macro- and micronutrients, sugar, agar, $HgCl_2$, and 70% ethyl alcohol were used in this study, grade product of Sigma Chemical Co., St. Louis, MO, USA. The vitamins, amino acids, and growth regulators were also the products of Sigma, UNI- Chemical Company, China and S.R.L, India.

2.3. Preparation of Culture Media
MS [17] liquid and semisolid media containing sucrose 3% and different concentrations and combinations of BAP and NAA were used for meristem establishment and development. The pH of the medium was adjusted at 5.7. Then, the medium (10 ml) was dispensed into test tubes capped with nonabsorbent cotton plugs and stream sterilized by autoclaving the medium at 121°C for 21 m at 1.0 kg cm^{-2} pressure.

2.4. Preparation of Explants and Meristem Isolation
Shoot tips were cut with the help of forceps and dissecting blade; then, the tips were kept in a conical flask and thoroughly washed under running tap water for 30 m to remove the loose contaminants attached to explants. Then, the explants were washed with distilled water containing 1% savlon (v/v) and four drops of Tween-80 for 20 m to remove gummy substances. That was followed by successive five washing with distilled water to make the material free from savlon. Subsequently, the explants were transferred to the laminar airflow cabinet. Washed shoot tips were treated with 0.05% $HgCl_2$ for 30 s for surface sterilization. Then, the meristems (2.5–3.0 mm in size) with two leaf primordia were isolated from shoot tips under 4× zoom stereomicroscope.

2.5. Meristems Culture
The isolated meristems were inoculated on a paper bridge in liquid medium containing test tubes. After inoculation, cultures were kept in a dark chamber for 2 days and then incubated at 25 ± 2°C under the warm fluorescent light intensity varied from 2000 to 3000 lux in the growth chamber.

2.6. Subculture of Meristems for Shoot Multiplication
After 10 days of culture in paper bridge MS liquid medium, meristems that showed morphogenesis response were removed aseptically from the culture tubes and transferred carefully into semi-solid MS medium supplemented with different growth regulators for shoot multiplication directly. Successful shoots formation became evident when the explants grew into a small, leafy structure with several auxiliary branches; they were rescued aseptically from the culture and again cultured to freshly prepared medium containing different combinations of hormonal supplements for multiplication of shoots. Several passes of subcultures were done for the formation of multiple shoot buds and the elongation of shoots.

2.7. Shoot Culture for Rooting
When the *in vitro* regenerated shoots grew about 4–5 cm in length, they were separated aseptically from the culture vessels and the separated individuals were transferred to freshly prepare in half-strength MS media containing different concentrations of hormonal supplements for root induction.

2.8. Acclimatization and Transfer of Plantlets to Soil
After 7 weeks, the well-rooted plantlets were taken out very carefully from the test tubes, washed gently in running tap water, and planted in small plastic pots containing sterile sand, soil, and humus in the ratio of 1:2:2. They were covered with transparent polythene bags to maintain high humidity and kept in the growth chamber for 7 days. Within 7 days, the plantlets began to form new leaves and resumed fresh growth. Plantlets were subsequently transferred to larger pots and gradually acclimatized to outdoor conditions.

2.9. Data Analysis
The data for the percentage of meristem response and the average number of shoots per meristem and roots per shoot were determined after 5 weeks of subculture. Ten replicates were tested in each treatment, and each experiment was repeated thrice. Means and standard errors ($M \pm SE$) were calculated for each experiment.

3. RESULTS

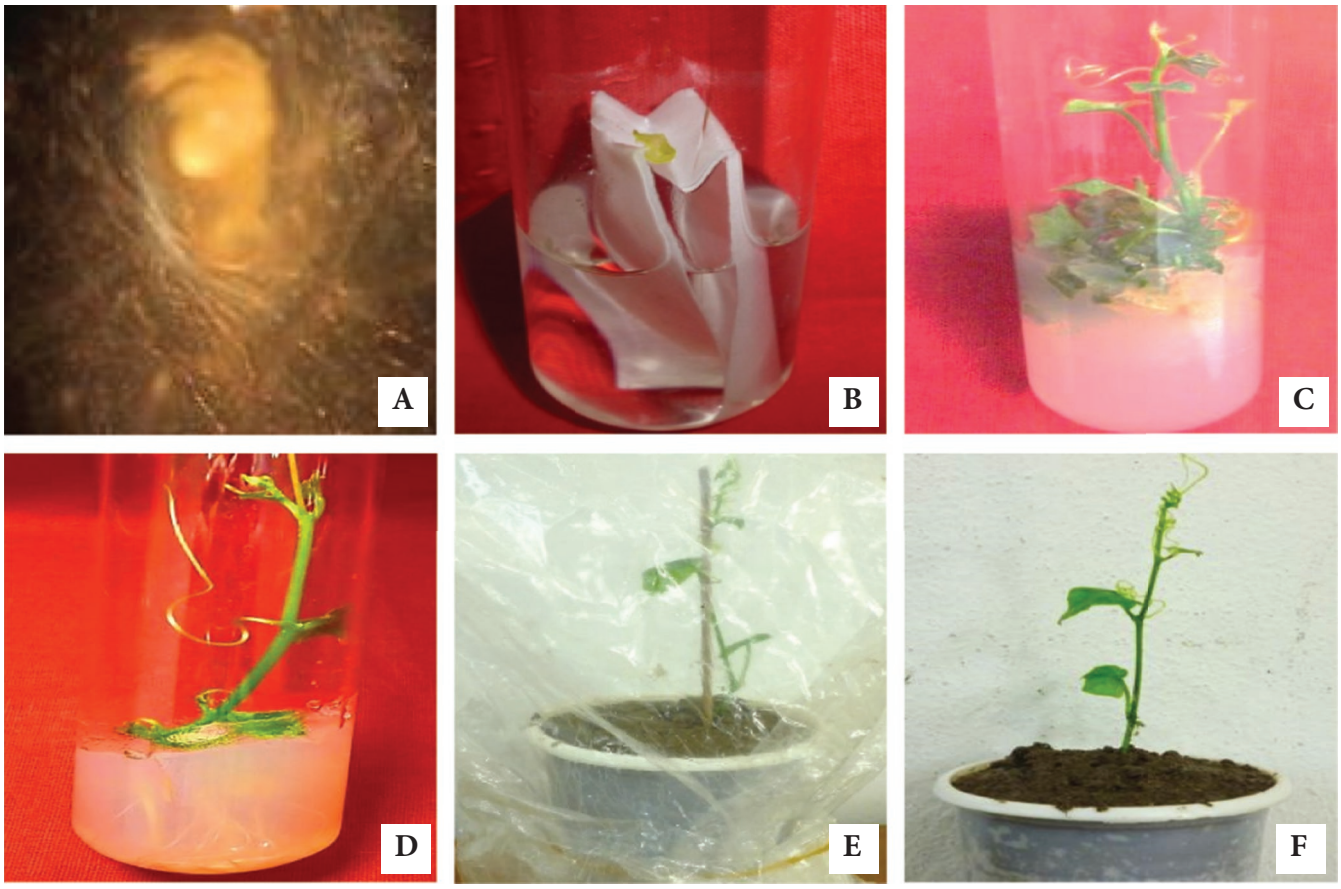
3.1. Primary Establishment of Meristems

Isolated meristems (Fig. 1A) were cultured (Fig. 1B) on paper-bridge used liquid MS medium supplemented with different concentrations and combinations of BAP, NAA and GA_3. After 5 days of inoculation, the meristems started to change in size and color. The highest percentage of meristem culture response was 96.6 ± 0.8 in MS liquid medium containing 1.0 mg l^{-1} BAP + 0.10 mg l^{-1} NAA followed by 90.0 ± 0.8% in the semisolid medium contained the same hormonal supplements. On the other hand, the lowest percentage of meristem culture response was 20.0 ± 0.0% in MS_0 semisolid medium. After 8-10 days of inoculation, meristems become around 0.6 cm. The results of the meristems establishment are given in Table 1.

3.2. Direct Shoot Regeneration

For *in vitro* shoot regeneration, the primary established meristems were cultured on MS semisolid medium supplied with different concentrations and combinations of BAP, NAA, and GA_3. MS medium supplemented with 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} GA_3 + 0.5 mg l^{-1} NAA was found to be the best formulation for shoot initiation as well as elongation from the meristem. The highest number of shoots per meristem was 10.0 ± 0.8 (Figure 1C) in the MS medium supplemented with 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} GA_3 + 0.5 mg l^{-1} NAA followed by 9.0 ± 0.0 shoots per meristem in MS + 1.5 mg L^{-1} BAP + 0.5 mg l^{-1} GA_3+0.1 mg l^{-1} NAA. The lowest number of shoots per meristem was 2.0 ± 0.8 in the MS medium supplied with 0.1 mg l^{-1} BAP. The length of regenerated shoots ranged from 1.3 ± 0.4 to 9.0 ± 0.0 cm. The maximum shoot length of 9.0 ± 0.0 cm (Figure 1C) was recorded on MS containing 1.5 mg l^{-1} BAP + 0.5 mg l^{-1} GA_3 + 0.5 mg l^{-1} NAA, and the minimum shoot length of 1.3 ± 0.4 cm was recorded on MS containing 1.0 mg l^{-1} BAP. All the shoots were vigorous and green. The results of shoots regeneration are presented in **Table 2**.

Figure 1: *In vitro* plant regeneration protocol from shoot apical meristems of ivy gourd



(**A**) Isolated meristem from shoot tip, (**B**) Establishment of meristems on paper bridge in MS liquid medium containing 1.0 mg l^{-1} BAP + 0.10 mg l^{-1} NAA, (**C**) Initiation and elongation of shoots in MS medium containing 1.5 mg l^{-1} BAP + 0.5 mg $l^{-1}GA_3$ + 0.5 mg l^{-1} NAA, (**D**) Initiation and elongation of roots in ½ MS + 0.5 mg l^{-1} IBA, and (**E**, **F**) Acclimatization and transfer of plantlets in natural condition.

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Table 1: The effect of different concentrations and combinations of GA_3, BAP, and NAA on the primary establishment of shoot apical meristem from the ivy gourd plant.

Growth regulators (mg l^{-1})		Response (%) of culture meristems	
		Liquid medium	Semi-solid medium
MS	0.0	30.0 ± 0.0	20.0 ± 0.0
GA_3	0.50	73.3 ± 0.4	66.6 ± 0.8
	1.00	50.0 ± 0.0	53.3 ± 0.4
	2.00	40.0 ± 0.0	20.0 ± 0.8
BAP	0.50	70.0 ± 0.0	60.0 ± 0.0
	1.00	80.0 ± 0.0	76.6 ± 0.8
	1.50	66.6 ± 0.8	50.0 ± 0.8
BAP + GA_3	1.0 + 0.1	50.0 ± 0.0	50.0 ± 0.0
	1.0 + 0.5	86.6 ± 0.8	73.3 ± 0.4
	1.0 + 1.0	70.0 ± 0.0	60.0 ± 0.8
BAP+ NAA	1.0 + 0.01	70.0 ± 0.0	60.0 ± 0.0
	1.0 + 0.10	96.6 ± 0.8	90.0 ± 0.8
	1.0 + 0.5	73.3 ± 0.4	83.3 ± 0.4

Legends: Results are *M* ± SE of three experiments, *M* = mean, SE = standard error, BAP = 6-benzyl amino purine, GA_3 = gibberellic acid, NAA = naphthalene acetic acid.

Table 2: The effect of different concentrations and combinations of BAP, GA_3, and NAA on shoot initiation and elongation from shoot apical meristem of the ivy gourd plant.

Growth regulators (mg l^{-1})		% of culture responded	Shoots no. per meristem	Shoot length (cm)
BAP	1.0	53.3 ± 0.4	2.0 ± 0.8	1.3 ± 0.4
	1.5	73.3 ± 0.4	5.3 ± 0.4	2.3 ± 0.4
	2.0	50.0 ± 0.0	4.0 ± 0.8	3.0 ± 0.0
BAP + GA_3	1.5 + 0.1	80.0 ± 0.0	4.3 ± 0.4	3.3 ± 0.4
	1.5 + 0.5	93.3 ± 0.4	7.0 ± 0.0	8.0 ± 0.6
	1.5 + 1.0	60.0 ± 0.8	5.0 ± 0.0	5.6 ± 0.8
BAP + GA_3 + NAA	1.5 + 0.5 + 0.1	90.0 ± 0.8	7.6 ± 0.4	6.6 ± 0.8
	1.5 + 0.5 + 0.5	100.0 ± 0.0	10.0 ± 0.8	9.0 ± 0.0
	1.5 + 0.5 + 1.0	83.3 ± 0.4	9.0 ± 0.0	8.3 ± 0.4

Legends: Results are *M* ± SE of three experiments, *M* = mean, SE = standard error, BAP = 6-benzyl amino purine, GA_3 = gibberellic acid, NAA = naphthalene acetic acid.

3.3. Effect of NAA and IBA on Root Induction

For root induction, elongated shoots were isolated and cultured on MS medium supplemented with different concentrations of NAA and IBA. The roots were initiated at the base of single shoots without callus formation. Half-strength MS medium supplied with 0.5 mg l^{-1} IBA showed the best response with 93.3 ± 0.4%, and the highest number of roots per shoot was 11.0 ± 0.8 (Figure 1D) followed by 9.0 ± 0.0 roots per shoot in ½ MS + 0.5 mg l^{-1} NAA (Table 3). On the other hand, the lowest number of roots per shoot was 3.3 ± 0.4 in ½ MS + 0.1 mg l^{-1} NAA. All the shoots were more vigorous compared to the mother shoot and rooted easily in the rooting medium. The maximum root length of 5.0 ± 0.6 cm (Figure 1D) was recorded on MS containing 0.5 mg l^{-1} IBA. All the roots were thin in nature.

3.4. Acclimatization and Transfer of Plantlets to Nature

The well-rooted plantlets were taken out from test tubes, washed gently, and transplanted to the plastic pot containing sterilized soil. They were covered with transparent polythene bags to maintain high humidity and kept in the growth chamber for several days. Within 7 days, the plantlets began to form new leaves and resumed fresh growth. Plantlets were subsequently transferred to larger pots and gradually acclimatized to outdoor conditions where the survival rate of the transferred plantlets to nature was 100% and vigorous (Figure 1E and F).

Table 3: The effect of different concentrations of NAA and IBA on root induction and elongation from *in vitro* grown shoot of ivy gourd.

Growth regulators (mg L^{-1})		% of culture responded	No. of roots per shoot	Root length (cm)
NAA	0.1	63.3 ± 0.4	3.3 ± 0.4	2.3 ± 0.4
	0.5	83.3 ± 0.4	9.0 ± 0.0	4.3 ± 0.4
	1.0	70.0 ± 0.0	5.0 ± 0.8	2.0 ± 0.6
IBA	0.1	73.3 ± 0.4	4.3 ± 0.4	2.3 ± 0.4
	0.5	93.3 ± 0.4	11.0 ± 0.8	5.0 ± 0.6
	1.0	80.0 ± 0.0	6.0 ± 0.0	3.0 ± 0.0

Legends: Results are $M \pm$ SE of three experiments, M = mean, SE = standard error, NAA = naphthalene acetic acid, IBA = indole-3-butyric acid

4. DISCUSSION

Shoot apical meristem culture has recently become an important technique for virus elimination in plant tissue culture technology. During direct shoot regeneration from the shoot apical meristems inhibit callus induction that establish genetic instability and reduce the probability of somaclonal variations [18]. For surface sterilization, shoot tips were treated with 0.05% $HgCl_2$ for 30 s. Hasan and Sikdar [19] used 0.1% (w/v) $HgCl_2$ for surface sterilization of *Polygonum hydropipper* nodal explants. Sarker *et al*. [20] also reported similar results for citrus that supports our present findings. In this study, shoot apical meristems were excised and cultured on MS liquid and semisolid medium supplied with different concentrations and combinations of plant growth regulators. First, the true meristem culture of an isolated angiosperm meristem into a complete plant was accomplished by Smith and Murashige [21]. Ahmed *et al*. [22] reported the same result in tomato plant regeneration. There are many reports on meristem culture for direct regeneration in many plants [10, 13, 23]. Ahmad *et al*. [24] reported that the combination of Kin with GA_3 was the most effective for the establishment of meristem culture in *Cucurbita pepo*. These results support our present findings for the primary establishment of meristems. For meristem establishment, MS liquid medium containing paper bridge was better than a semisolid medium. For shoot regeneration, meristems were cultured on MS semisolid medium supplied with different concentrations and combinations of BAP, NAA, and GA_3. Among them, the combination of BAP, NAA, and GA_3 showed the best result for direct shoot regeneration. Sarker *et al*. [20] reported similar results for shoot initiation and multiplication from meristem in citrus. However, Altaf *et al.* [25] showed that 2 mg l^{-1} BAP with 0.5 mg l^{-1} NAA induced multiple shoots from *Citrus jambhiri*. Shekhawat *et al*. [15] achieved a maximum number of shoot regeneration on MS medium supplemented with 1.0 mg l^{-1} each of BAP and Kn in *Coccinia indica*. Aasim *et al.* [12] also recorded the maximum number and length of shoots per explant of cowpea on MS containing 0.5 mg l^{-1} BAP with 0.1 mg l^{-1} NAA. In contrast, Bekele *et al*. [26] reported the highest number of shoot regeneration in *Coccinia abyssinica* on MS containing 2.0 mg l^{-1} BAP alone and Al Khateeb *et al.* [27] reported in *Lavandula coronopifolia* on MS containing 0.5 mg l^{-1} BAP. Different concentrations of NAA and IBA were used in ½ MS medium for rooting from excised shoots. The highest number of roots was produced with 0.5 mg l^{-1} IBA. Similar results were reported by Aasim *et al*. [12] in cowpea. A study with *Citrus grandis* [28] and *Citrus aurantifolia* [20] showed similar results with a maximum number of root induction at 0.5 mg l^{-1} IBA. Kaushal *et al*. [23] reported the best root induction of *Gentiana kurroo* meristem culture in ½ MS supplemented with 0.5 mg l^{-1} IBA, which is similar to our present findings. Haque *et al.* [29] reported that IBA gave a better response than NAA in the root formation of pumpkin. These results support our findings. Shekhawat *et al.* [15] reported the best root induction and elongation of *Coccinia indica* on ½ MS+2.0 mg l^{-1}IBA. Al Khateeb *et al.* [27] observed the maximum number of root induction in *Lavandula coronopifolia* at half-strength MS with 1.0 mg l^{-1} IBA. In contrast, Hassan *et al*. [30] reported the highest number of root induction in *Phlogacanthus thyrsiflorus* on half-strength MS with 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} IAA together. Cüce and Sökmen [31] observed the number of root induction in bog bilberry on MS with IBA, IAA, and NAA individually or together. The well-rooted plantlets were hardening and transfer to field conditions with a 100% survival rate. The same results were found by Sarker *et al.* [20] in citrus plant and Hasan and Sikdar [19] in *Polygonum hydropipper.* Kaushal *et al.* [23], Deshpande and Bhalsing [7], and Haque *et al.* [8] also reported a similar survival rate of hardening for different plantlets that support our present findings. Hassan *et al.* [30] also reported the highest 85% survival rate after the acclimatization of *Phlogacanthus thyrsiflorus* plantlets in outdoor conditions.

5. CONCLUSION

In vitro plant tissue culture technology, especially meristem culture is very popular to produce a large number of disease-free and uniform plants of medicinally important species. It is very helpful for the conservation of endangered plant species. *In vitro* grown plants can be used for the extraction of medicinally important compounds or for pharmacological studies. Pathogen-free

plants maintained under *in vitro* conditions can also be used for the safe exchange of germplasm across national borders. The present efficient protocol will be helpful for disease-free rapid multiplication of this valuable medicinal plant.

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Authors' Contributions
MFH, MFJ, RZ, and BS designed the experiments, developed the methodology, and prepared the manuscript. MFH and MFJ collected the data and carried out the analysis. RZ and MAI assisted with data analysis and manuscript preparation.

Conflict of Interest
The authors declare that they have no conflict of interest and there has been no significant financial support for this work that could have influenced its outcome.

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