

## PRECONDITIONING OF PLUMULAR APICES EXPLANTS OF PEANUT (*Arachis hypogaeae*) WITH 6-BENZYLAMINOPURINE

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The study presents the *in vitro* potential of preconditioned plumular apices explants of peanut for shoot regeneration. Mature embryo explants were isolated and preconditioned for 10 days on MS medium with 10 and 20 mg/l BAP (6-Benzylaminopurine) followed by postconditioning of isolated plumular apices postconditioned on MS medium enriched with 0.25-2.0 mg/l BAP singly or with 0.25 mg/l NAA (1-Naphthaleneacetic acid). Preconditioning of explants using 20 mg/l BAP was found to be more regenerative to induce 4.38 shoots with shorter shoots (0.75 cm) compared to 3.50 shoots and shoot length of 1.25 cm when preconditioned with 20 mg/l BAP. Number of shoots per explants ranged 2.30-5.36 and 3.17-6.0 respectively on explants preconditioned with 10 and 20 mg/l BAP and postconditioned on 0.25-2.0 mg/l BAP – 0, 0.25 mg/l NAA with shoot length in range of 1.01–2.30 cm and 0.74–1.80 cm, respectively. Maximum number of shoots (6.0) with stunted shoots (0.74 cm) were recorded from 20 mg/l preconditioning followed by culture on medium containing provided with 1.0 mg/l BAP+ 0.25 mg/l NAA. Irrespective of preconditioning treatments, minimum number of shoots per explants but relatively longer shoots were scored on MS medium provided with 0.25 mg/l BAP+0.25 mg/l NAA. Regenerated shoots were successfully rooted using 0.25-1.0 mg/l IBA. They were transferred to pots containing peat moss that resulted in successful acclimatization of *in vitro* regenerated plantlets at room temperature.

**Keywords:** *In vitro*, peanut, plumular apices, preconditioning, postconditioning.

### INTRODUCTION

Peanut (*Arachis hypogaeae*) is an important legume crop (Hassan *et al.*, 2013) of subtropical regions (Venkatachalam and Jayabalan, 1997). The plant oil is rich in different fatty acids is widely used for human and livestock consumption. Furthermore, the plant is an important part of crop rotation systems and used as hay crop (Hill, 2002), an intercrop (Langat *et al.*, 2006) and cover crop (Balkcom *et al.*, 2007) that also increase its importance. Besides that, it is the major source of raw materials to different household or commercial industries. In recent years, the use of plant byproducts as medicine also increases the demand of this plant (Sanders *et al.*, 2000; Blomhoff *et al.*, 2008).

Legumes are generally considered to be recalcitrants (Heatley and Smith, 1996) for plant tissue culture techniques. Therefore, a repeatable regeneration protocol with rooting and adaptation is the prerequisite for genetic improvement and also for the application of molecular or biotechnological techniques (Rey *et al.*, 2000). Different biotic or abiotic factors that adversely affect the growth and yield of peanut provide an opportunity to the researchers to develop new varieties with desired traits (Venkatachalam and Kavipriya, 2012). Plant tissue culture provides an alternate way of plant improvement under *in vitro* conditions by the application of

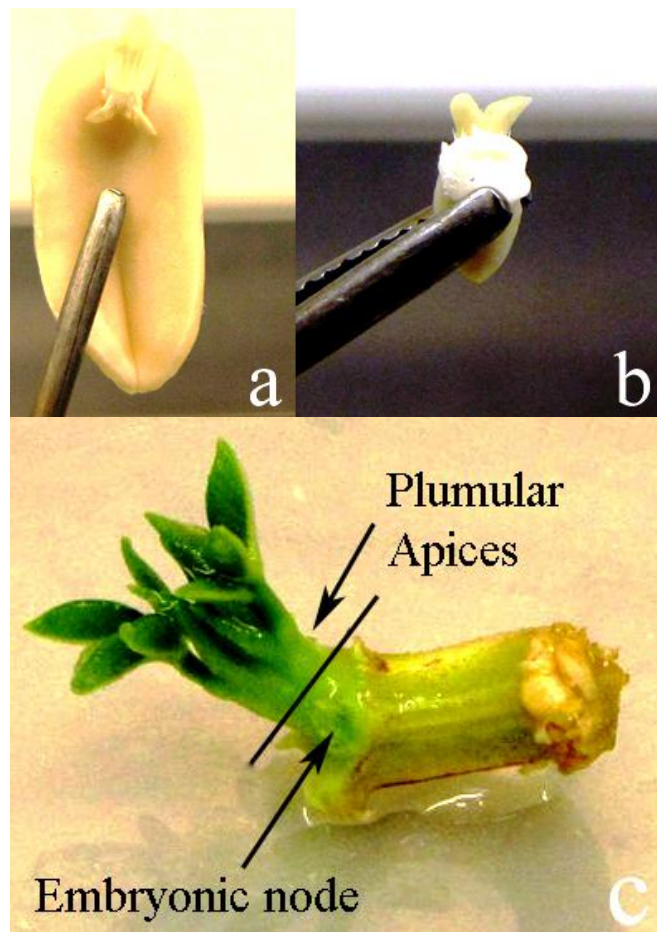
different biotechnological techniques like genetic transformation. However, there is need to develop *in vitro* regeneration protocol which can be employed for different cultivars successfully. In recent years, number of successful regeneration protocol of peanut has been reported using different types of explants. However, variable regeneration frequency or complex regeneration protocols are the major constraints (Matand and Prakash, 2007; Tiwari and Tuli, 2009; Wang *et al.*, 2011; Memon *et al.*, 2013) for successful regeneration which is of immense importance.

Considering the issues related with regeneration, the present study was designed to precondition explants at initial stage for a certain period of time followed by the regeneration on low doses of BAP+NAA.

### MATERIALS AND METHODS

The seeds of cv. Halisbey of peanut were collected from the Department of Field Crops, Cukurova University, Adana, Turkey, These were exposed to commercial bleach (Ace, Turkey, containing 5% NaOCl) for surface sterilization by continuous stirring using a magnetic stirrer for 30 min. It was followed by 3×5 min rinsing with bidistilled sterilized water.

The surface sterilized seeds were separated into two halves for separation of embryos from (Fig 1a, 1b) the seeds under sterile conditions. Half of them were preconditioned on agar solidified MS Murashige and Skoog (1962) medium containing 10 and the other half were preconditioned on 20 mg/l BAP for 10 days. Thereafter, plumular apices (Fig. 1c) were excised from these embryos and postconditioned on agar solidified MS medium enriched with 0.25, 0.50, 1.0 and 2.0 mg/l BAP with 0.25 mg/l NAA or without NAA, supplemented with 3.0% sucrose and 1.0% Polyvinylpyrrolidone (PVP) in Magenta GA<sub>7</sub> vessels.



**Figure 1. Isolation of plumular apices explants from preconditioned mature embryo of peanut (a) mature embryo with cotyledon (b) mature embryo and (c) preconditioned mature embryo with BAP**

For rooting,  $\approx 1\text{--}1.25$  cm long *in vitro* regenerated shoots were carefully cut from explants under sterilized conditions and cultured on MS medium containing 0.25, 0.50, 1.0 and 2.0 mg/l IBA for rooting. Each treatment had 3 replications with 8-10 shoots and were kept at  $24\pm 2^\circ\text{C}$ . Rooting percentage was recorded after 4 weeks of culture. For

transfer of *in vitro* rooted plantlets, they were initially washed with tap water to remove agar followed by submerging in tap water for 10-15 min prior to transplantation in pots filled with peat moss placed in growth rooms for acclimatization. Pots were covered with polythene bags for 7-10 days to keep 80-90% humidity followed by gradual opening of these bags under ambient conditions of temperature.

All types of culture media (preconditioning, postconditioning for regeneration or rooting medium) had pH adjusted between 5.6 - 5.8 prior to autoclaving that was performed at 104 kPa atmospheric pressure and  $120^\circ\text{C}$  for 20 min. The incubation of all culture media was done using Panasonic growth chamber adjusted to 16 h light photoperiod ( $35\ \mu\text{mol photons m}^{-2}\text{ s}^{-1}$ ) with temperature of  $24\pm 2^\circ\text{C}$ . Data pertaining to callus induction (%), shoot regeneration (%), shoots per explant and shoot length were scored after 8 weeks of culture. Each treatment contained 36 explants of 6 replicated groups and contained 6 explants per replication and were repeated twice.

Data taken after eight weeks were analyzed statistically using One Way ANOVA (analysis of variance IBM SPSS 20 for Windows, SPSS Inc., Chicago, IL, USA). Means were subjected to t-test or Duncan's multiple range test (DMRT) for comparison at 0.05 significance level. The treatments were arranged in a completely randomized design. Arcsin ( $\sqrt{X}$ ) transformation (Snedecor and Cochran, 1967) was employed to transform all data given in percentages prior to statistical analysis.

## RESULTS

The study aimed to optimize the plant regeneration efficiency of plumular apices explants after preconditioning with 10 or 20 mg/l BAP followed by postconditioning at different concentrations of BAP and constant dose of NAA. Preconditioning of mature embryo with 10 or 20 mg/l BAP for 10 days sufficiently increased the size and clearly differentiated the plumular apices from cotyledon nodal segments (Fig. 1c). Thereafter, isolated plumular apices were postconditioned on different concentrations and combinations of BAP-NAA. Multiple shoot buds initiation from plumular apices was observed within 7-10 days with callus initiation on basal end of plumular apices explants. All explants induced 100% callus and shoot regeneration (Fig. 2a, 2b). Analysis of data reflected the significant effects of preconditioning or postconditioning concentrations on number of shoots per explant and their shoot length.

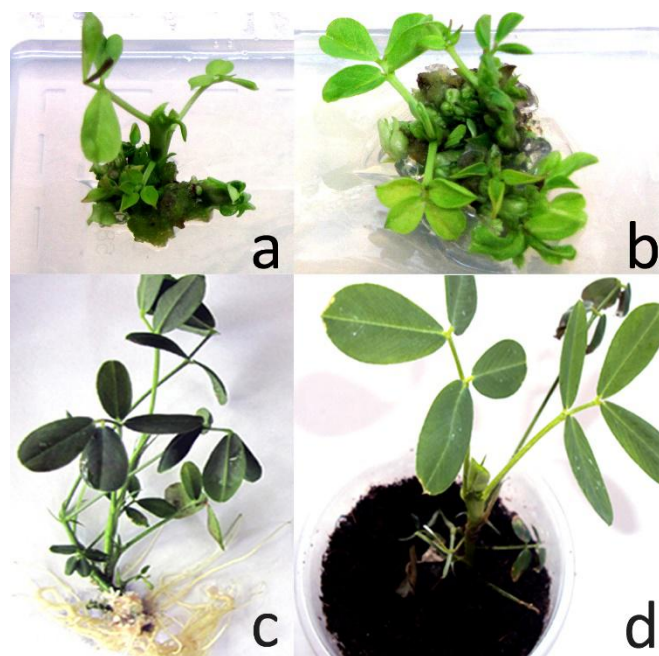
Results on preconditioning with 10 and 20 mg/l of BAP showed clear bearings on shoots proliferation and shoot length. Preconditioning of embryos with 20 mg/l BAP proliferated more number of maximum shoots (4.38) per explant compared to 3.50 shoots regenerated on 10 mg/l BAP preconditioning. Contrarily, preconditioning with 20

mg/l BAP was inhibitory resulting in relatively stunted and smaller shoots (0.75 cm) compared to 1.25 cm long shoots on preconditioning with 10 mg/l BAP (Table 1).

**Table 1. Effects of preconditioning dosage on number of shoots per explant and shoot length of plumular apices of peanut.**

Preconditioning Dosage	Shoots per explant	Shoot length (cm)
10 mg/l	3.50 <sup>b</sup>	1.25 <sup>a</sup>
20 mg/l	4.38 <sup>a</sup>	0.75 <sup>b</sup>

Means followed by different small letters within columns are significantly different using t-test at  $P < 0.005$



**Figure 2. Multiple shoot regeneration, rooting and acclimatization of peanut under *in vitro* conditions (a,b) multiple shoot regeneration with callus; (c) rooted plantlet and (d) acclimatized plant in pot**

Comparing effects of postconditioning, response of explants; it varied with concentration of BAP-NAA concentration used for postconditioning. Shoots per explants ranged 3.67-5.36 and 4.67-5.83 on different concentrations of BAP postconditioning irrespective of their preconditioning with 10 or 20 mg/l BAP respectively (Table 2). Postconditioning with 1.0 mg/l BAP generated maximum number of shoots per explant on both preconditioning treatments. The results further indicated that shoots per explants increased progressively with each increase of BAP concentration + NAA during postconditioning. However, in general terms, postconditioning with any concentration of BAP+NAA was inhibitory compared to any concentration of BAP used singly.

Variable response was noted on shoot length from regenerated explants preconditioned with 10 or 20 mg/l BAP postconditioned on all concentrations of BAP used singly or in combination with 0.25 mg/l NAA. such that 20 mg/l preconditioning was inhibitory to shoot length on all BAP-NAA postconditioning treatments compared to those explants that were preconditioned with 10 mg/l. Shoot length range was recorded as 1.01-2.30 cm and 0.74-1.80 cm (Table 2) respectively on different concentrations of BAP-NAA preconditioned with BAP (10 or 20 mg/l) respectively. Shoot length showed declining pattern with increase of BAP concentration used singly or BAP with NAA (0.25 mg/l). Results also showed that low concentrations of 0.25 mg/l BAP alone or 0.25 mg/l BAP-0.25 mg/l NAA was found most suitable for gaining longer shoots of 2.30 cm and 1.80 cm respectively (Table 2).

**Table 2. Effects of postconditioning dosage of BA-NAA on number of shoots per explant and shoot length of preconditioned plumular apices of peanut.**

BA	NAA	Shoots per explant		Shoot length (cm)	
		10 mg/l BAP	20 mg/l BAP	10 mg/l BAP	20 mg/l BAP
0.25	-	4.33 <sup>fg</sup>	4.67 <sup>ef</sup>	2.04 <sup>b</sup>	1.49 <sup>c</sup>
0.50	-	3.67 <sup>i</sup>	4.83 <sup>de</sup>	1.55 <sup>d</sup>	1.35 <sup>f</sup>
1.00	-	5.36 <sup>bc</sup>	5.83 <sup>a</sup>	1.01 <sup>i</sup>	1.02 <sup>i</sup>
2.00	-	4.15 <sup>gh</sup>	5.17 <sup>cd</sup>	1.14 <sup>g</sup>	1.01 <sup>i</sup>
0.25	0.25	2.30 <sup>k</sup>	3.17 <sup>j</sup>	2.30 <sup>a</sup>	1.80 <sup>c</sup>
0.50	0.25	3.50 <sup>ij</sup>	3.83 <sup>hi</sup>	1.53 <sup>d</sup>	1.35 <sup>f</sup>
1.00	0.25	3.83 <sup>hi</sup>	6.00 <sup>a</sup>	1.06 <sup>h</sup>	0.74 <sup>j</sup>
2.00	0.25	4.33 <sup>fg</sup>	5.67 <sup>ab</sup>	1.01 <sup>i</sup>	0.74 <sup>j</sup>

Means followed by different small letters within columns are significantly different using DMRT test at  $P < 0.005$

Rooting medium provided with 0.25, 0.50, 1.00 and 2.00 mg/l IBA was used for induction of adventitious rooting. The rooting started within 7-15 days at the cut end of shoot bases irrespective of the rooting medium. Whereas, shoots without white mass at the cut end of shoots failed to induce rooting. Rooting frequency ranged 56.67-75.67% (Table 3) after four weeks of culture along with callusing at the basal end.

**Table 3. Effects of different IBA and sucrose concentrations on rooting of *in vitro* regenerated shoots from preconditioned plumular apices of peanut**

IBA mg/l	Frequency (%) of rooting
0.25	56.67 <sup>c</sup>
0.50	63.33 <sup>b</sup>
1.00	78.67 <sup>a</sup>
2.00	75.67 <sup>a</sup>

Means followed by different small letters within columns are significantly different using DMRT test at  $P < 0.005$

Although, 1.0 or 2.0 mg/l IBA induced statistically insignificant rooting percentage, it was 1.0 mg/l IBA that resulted in low callusing from the basal end which resulted in more acclimatization frequency. For acclimatization, rooted plantlets (Fig. 2e) were shifted to pots filled with different rooting substrates and covered with polyethylene bags and kept at ambient conditions of temperature undergrowth room conditions. Polyethylene bags were removed after 10-12 days after rooted plantlets showed high survival rate (Fig. 2f). The plants set seed under these conditions.

## DISCUSSION

Selection of right explant, its appropriate size and its age are very important factors for successful and efficient micropropagation. Use of new type of explants and their culture on novel plant growth regulator combinations are necessary for their use in breeding studies (Wang *et al.*, 2011). Plumule explant or plumular apices explants are reported rarely for regeneration studies irrespective of high regeneration potential that has been reported for pea (Molnar *et al.*, 1999), pigeon pea (Surekha *et al.*, 2005), cowpea (Aasim *et al.*, 2009), lentil (Aasim *et al.*, 2012) and chickpea (Aasim *et al.*, 2013) and Peanut (Day *et al.*, 2016). Although these reports showed the efficiency of these explants for shoot regeneration, yet the main problem associated with explant is the isolation due to variably small size of embryo. To overcome this problem, preconditioning of explants with higher concentration of cytokinin has proved very efficient for isolation of plumule explants under aseptic conditions (Aasim *et al.*, 2009, 2012 and 2013). Preconditioning of mature/immature embryo at initial stage for few days at variable (higher) concentrations of cytokinins enables to separate plumular apices from embryonic axis. Preconditioning of explants using higher cytokinins concentration for few hours to few days may lead to rapid cell division. Successful shoot regeneration with precondition of explants with different cytokinins and concentrations has been employed successfully for *Picea abies* (Von Arnold and Tillberg, 1987), banana (Madhulatha *et al.*, 2004), cowpea (Brar *et al.*, 1999); (Aasim *et al.*, 2009 and 2010), chickpea (Aasim *et al.*, 2011 and 2013), dwarf chicklings (Saglam, 2012), lentil (Aasim *et al.*, 2012), and grass pea (Barpete *et al.*, 2014).

Results of postconditioning concentrations and combinations revealed the insignificant effects on callus induction (%) and shoot regeneration (%) which showed the supremacy of explant compared to reported previously in peanut. Previous results on peanut showed that both callus induction and shoot regeneration frequency rate is subjected to growth regulators (Tiwari and Tuli, 2008), type of cultivar (Banerjee *et al.*, 2007; Li *et al.*, 2008) and explant (Tiwari

and Tuli, 2009). Our results revealed the efficiency of explant and induced 100% shoot regeneration on all BAP concentrations with or without NAA. Whereas, relatively low and variable shoot regeneration (%) frequency using different explants of peanut has been reported (Sharma and Anjaiah, 2000; Burns *et al.*, 2012). Contrarily, Venkatachalam and Kavipriya (2012) reported 33.33-100% shoot regeneration from BAP and 100% from BAP-NAA. Whereas, Shan *et al.* (2009) reported 40-80.7% shoot regeneration from epicotyl explant of peanut on different BA-NAA concentrations.

Optimum postconditioning conditions (growth regulator(s) type or combination of cytokinin-auxin) is very important for successful shoot regeneration after preconditioning. Results revealed the supreme efficiency of explant type and preconditioning effects which resulted in multiple shoot buds initiation in very short time. Aasim *et al.* (2009) obtained multiple shoots with shoot length above 1 cm within 25 days after preconditioning with 10 mg/l BAP of plumular apices of cowpea. It was also noted that callus induction started at the basal end of the explants with late shoot regeneration from callus. This phenomenon of shoot regeneration from callus is not reported earlier by other researchers worked on preconditioning followed by postconditioning of explants (Aasim *et al.*, 2009, 2011, 2012 and 2013; Barpete *et al.*, 2014).

Analysis of variance showed statistically insignificant effects of preconditioning dosage on callus and 100% shoot regeneration frequency in agreement with (Aasim *et al.*, 2009) and embryonic axis (Aasim *et al.*, 2010) of cowpea, mature embryo and embryonic axis (Aasim *et al.*, 2011) and plumular apices (Aasim *et al.*, 2013) of chickpea, plumular apices of lentil (Aasim *et al.*, 2012) and (Barpete *et al.*, 2014) from embryonic node of lathyrus preconditioned with 20 mg/l TDZ. Contrarily, variable response of cotyledonary node of dwarf chickling pulse treated with cytokinin on callus and shoot induction has been reported by Saglam (2012). Matand *et al.* (2013) exposed different cotyledon explants of peanut to higher concentrations of 5, 10, 20 and 30 mg/l Kinetin, BA and TDZ for 55 days. They recorded 10-14%, 5-12% and 75-95% shoot regeneration frequency from whole cotyledon; 5-22%, 12-58% and 82-98% from mono/side cut cotyledon cultured on Kin, BA and TDZ respectively. Akasaka *et al.* (2000) cultured leaf explants of peanut on 5-50 mg/l BA and obtained very low shoot regeneration frequency 7.9-10.5% after two months of culture.

Higher concentrations of preconditioning (20 mg/l) clearly enhanced the number of shoots compared to preconditioning with 10 mg/l. It is supposed that preconditioning of explants with higher concentrations promoted the cell division in meristematic region of plumular apices explants which ultimately resulted in more shoots. Superiority of preconditioning effects on shoot regeneration from plumular

apices of lentil (Aasim *et al.*, 2012), chickpea (Aasim *et al.*, 2013) and embryonic node of grass pea (Barpete *et al.*, 2014) compared to unconditioned explants has been confirmed. Similarly, Madhulatha *et al.* (2004) found 50 mg/l preconditioning of BAP-Kin (1:1) for 60 min for optimum shoot proliferation in banana.

Results further revealed that response of shoots per explants were concentration specific and behaved variably irrespective of availability of non-availability of NAA in line with findings of Venkatachalam and Kavipriya (2012). Similarly, variable response to BAP by preconditioned embryonic axis of cowpea (Aasim *et al.*, 2010) and preconditioned plumular apices of lentil (Aasim *et al.*, 2012) has been reported already. Results also showed that shoots per explants increased with increased BAP concentration plus NAA in the culture medium. Shan *et al.* (2009) also reported increased number of shoots with increased concentration of BAP with NAA. Similarly, Zeng *et al.* (2009) reported similar response of epicotyl explants of ponkan mandarin to BAP-NAA concentrations. These findings are contrarily to the previous reports given by Aasim *et al.* (2012, 2013) which might be due to late shoot induction from callus in this study which was not observed in chickpea previously. Similarly, negative effects of increased BAP concentration with NAA has been reported for preconditioned embryonic node of grass pea (Barpete *et al.*, 2014).

The results also confirmed the clear effects of postconditioning concentrations on shoot length and in line with Aasim *et al.* (2009, 2010) in cowpea and chickpea (Aasim *et al.*, 2011 and 2013). Results further revealed prominent inhibition of shoot length in the presence of NAA with BAP are in line with Aasim *et al.* (2011) in chickpea and Aasim *et al.* (2012) in lentil. However, longer shoots from preconditioned plumular apices (Aasim *et al.* 2009) and embryonic axis (Aasim *et al.*, 2010) explants of cowpea cultured on MS medium containing BAP-NAA has been reported. It was supposed that it was primarily due to negative carry-over effects of exposing explants to higher concentrations of BAP during preconditioning that hindered the shoot length.

*In vitro* rooting of regenerated shoots is important step for acclimatization under external conditions of greenhouse or field conditions. Auxins are generally employed for *in vitro* rooting and previous reports on *in vitro* rooting reports low rooting percentage. Hassan *et al.* (2013) reported that rooting of *in vitro* regenerated shoots of peanut is difficult under *in vitro* conditions. In this study, we successfully achieved upto 75.67% rooting using different IBA concentrations irrespective of preconditioning concentrations. Successful rooting of preconditioned explants has been reported in cowpea, grasspea by Aasim *et al.* (2009, 2010), and Barpete *et al.* (2014). Low rooting percentages after preconditioning has been reported in chickpea (Aasim *et al.*, 2011) and lentil (Aasim *et al.*, 2012).

All plants acclimatized well in pots under growth room conditions (Demirbag *et al.*, 2008).

**Conclusion:** The present work presents the first ever report on efficient, reliable and repeatable protocol using pre and postconditioned ground pea plumular apices as explants that offers new strategy for inducing multiple shoot regeneration overcoming problem of recalcitrance. This protocol can be easily employed for improving peanut cultivars for easy screening to different biotic and abiotic stresses or genetic transformation under *in vitro* conditions.

**Author's Contribution:** Sibel Day and Muhammad Aasim contributed equally to research work. Muhammad Aasim and Nazim Hussain contributed for statistical analysis and article writing.

**Conflict of Interests:** The authors declare no conflict of interest regarding the publication of this article.

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