Curcumin inhibits NF-kB and Wnt/β-catenin pathways in cervical cancer cells

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ABSTRACT

Curcumin is a natural non-toxic phenol which is isolated from *Curcumin longa* L. Mounting evidence has revealed the anticancer properties of curcumin in various tumors, but the underlying molecular mechanisms of this suppression in cervical cancer is still remained unclear. Here we assessed the antitumor effects of curcumin compared with 5-Fluorouracil in Hella cells in spheroids models and monolayer cell cultures.

The anti-proliferative effects of curcumin and 5-Fluorouracil were as examined in spheroid and monolayer models. The expression levels of Wnt/β-catenin and NF-kB pathways as well as the influence of the cell cycle were evaluated. Curcumin inhibited cell growth in Hella cells through the regulation of NF-kB and Wnt pathways. Also, cells developed a G2/M cell cycle arrest followed by sub-G1 apoptosis with 5-Fluorouracil and curcumin. It was also shown that curcumin either considerably affects the Wnt/β-catenin and NF-kB pathways. We showed that curcumin inhibits invasion and proliferation of cervical cancer cells *via* impairment of NF-kB and Wnt/β-catenin pathways, proposing further studies on the potential impacts of this compound on cancer therapy.

1. Introduction

According to the data released by World Health Organization (WHO) in 2012, cervical cancer is the 4th most prevalent cancer in women and the 7th most common among all cancer types. Cervical cancer, with 275,000 deaths and 528,000 diagnosed cases per year [1], is classified into two subtypes based on the pathology: adenocarcinoma (10–15% of cases) and squamous cell carcinoma (80%) [2]. The primary and secondary sarcomas and lymphomas are the rest [3]. According to the estimated age-standardized rates of cervical cancer incidence and mortality in 2012, Iran have an incident of 2.8 and a mortality of 1.2 which are 14.0 and 6.8 in the world, respectively [4]. Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) [5], the main compound extracted from rhizome of *Curcuma longa* L. and turmeric the, is commonly used as a food additive and dietary pigment. Numerous investigations indicated that curcumin possesses antioxidant, anti-inflammatory, and anticancer properties [6–10]. It is tolerable and safe even at high doses, but its low bioavailability limits its therapeutic application [11]. Curcumin is a potential anti-angiogenic agent and induces the apoptosis process in tumor cells resulting in cancer suppression [12]. Many studies have shown antitumor activities of curcumin on prostate cancer, head and

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neck squamous cell carcinoma, lung cancer, breast cancer, and brain tumors [13]. Anticancer function of curcumin involves the inhibition of the NF-κB and STAT3 pathways, the main molecular mechanisms in cancer progression and development [14]. Curcumin also exerts anti-proliferative activities owning to different pathways, such as forkhead box O3 (FOXO3), β-catenin, cyclooxygenase-2 (COX-2), as well as transcription factors, including cyclin D1, protein kinase B (Akt), and HIF-1a [15,16]. The aim of our investigation was to determine the in-hibitory property of curcumin on Wnt/β-catenin and NF-kB pathway in cervical cancer cell line.

2. Materials and methods

2.1. Provision of reagents

Curcumin (Sami Labs Ltd, Bangalore, India) and 5-Fluorouracil (Biolysis Pharma Company, Canada) were provided. The drugs were dissolved in water and diluted in culture medium without FBS and Antibiotic before use. Penicillin-streptomycin, RPMI-1640 medium, trypsin-EDTA, and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA).

2.2. Provision of human cervical cancers cell line Hella

The human cervical cancer cell line Hella was obtained from the Pasture Institute (Tehran, Iran). Cells were cultured with DMEM medium consisting 1% penicillin-streptomycin (50 mg/L penicillin; 100000U/L streptomycin) and 10% heat-inactivated FBS in 75-cm^3 tissue culture flasks. Cells were incubated in humidified (5% CO$_2$) at-mosphere at 37 °C.

2.3. Curcumin and Paclitaxel effects on the Hella cells livability

We placed the Hella cells in a 1×10^4 cells/well density and they were cultured for 24 h. We added enhanced condensations of curcumin 1000, 500, 100, 50, 10, 0.1, 0.05, 0.001, 0.0001 μM/ml and 5-Fluorouracil 0.15, 0.31, 0.62, 1.25, 2.5, 5, 10 mg/ ml, and cells were grown for 24 h at 37 °C, while untreated cells were considered as con-trol group. The livability of Hella cells was evaluated by MTT assay after 24 h culture and the test was conducted at least three times.

The cytotoxic ability of compounds was assessed with IC50 obtained from the does-response curve in Graph Pad Prism (version.7)

2.4. Cell cycle analysis

We assessed the quantity of cells in sub-G1 phase by flow cytometry. Briefly, before cells were harvested by centrifugation, 1×10^6 Hella cells/10-cm dish were incubated with enhanced condensations of cur-cumin relying on the IC50 for 24 h. After harvest, the cells were washed in PBS then slowly fixed in 70% ethanol. We set the cells in an ice bath overnight and then suspended in PBS (40 μg/ml). We analyzed the cells with flow cytometry (BD FACSCalibur) equipped with an argon laser at 488 nm after incubation for 20 min at 37 °C in the dark. The present relationship between apoptosis and cell cycle was analyzed by using FLOWJO software.

2.5. Effect of curcumin on multicellular spheroids

We suspended Hella cells at a concentration of 5×10^3 cells/ml in serum-free medium containing RMPI and seeded in six agarose-coated well plates. Curcumin and 5-Fluorouracil cytotoxicity on spheroid for-mation was evaluated *via* using inverted phase contrast microscope Leica-DMI300B (Leica, Wetzlar, Germany).

2.6. Quantitative reverse-transcriptase polymerase-chain-reaction (qRT-PCR)

Hella cells were cultured by seeding 1×10^6 cells/well in RMPI in 6 well plates. After adding IC$_{50}$ 5-Fluorouracil and curcumin, the cells incubated at 37 °C for 24 h. We extracted Total RNAs from the cells using the Trizol reagent (Invitrogen, CA, USA), based on the manu-facturers' protocol. Total RNA was used to synthesize from cDNA by the cDNA Synthesis Kit (Fermentas, USA) pursuant to manufacturer's pro-tocol. A quantitative RT-PCR analysis was carried out exploiting spe-cific primers (Macrogene co, Seoul, Korea) that amplify the Wnt/β-ca-tenin and NF-kB pathway. Real-time RT-PCR was conducted in ABI-PRISM StepOne instrument (Applied Biosystems, Foster City, CA) with the SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan). The levels of expression of these genes were normalized to the housekeeping gene (GAPDH)expression levels, using a standard curve of cDNAs get from Quantitative PCR Human Reference RNA (Stratagene, La Jolla, CA), as explained previously.

2.7. Invasion assays

Cell invasion was carried out in BD BioCoat Matrigel invasion chambers (24 wells, 8 μm pore size; BD Biosciences) pursuant to man-ufacturer's protocol. Briefly, the top chamber was seeded with 1×10^6 Hella cells in RPMI without FBS. The bottom chamber was replete with in RPMI with 10% FBS supplemented. Cells were treated with IC$_{50}$ concentration of 5-Fluorouracil and curcumin in the top chamber, fol-lowed by 24 h incubation. Then, cells were fixed with formalin and stained with toluidine blue. Ten random fields/wells were counted for cell invasion.

2.8. Statistical analysis

Information was demonstrated with mean ± standard deviation. The untreated cells were assigned as control group. The distinction between groups was compared with one-way ANOVA and p-values < 0.05 were set a significant difference for all trials. We were utilized SPSS v.20 statistical software (IBM, Chicago) to analyze data.

3. Results

3.1. Curcumin and 5-Fluorouracil inhibit cell growth

The inhibitory effects of curcumin and 5-Fluorouracil on Hella cell line growth are present in (Fig. 1). These analyses showed that cur-cumin inhibited the cell growth in a dose-dependent manner, compared to 5-Fluorouracil. The concentration of IC$_{50}$ was determined for cur-cumin (34.23 μM/ml) and 5-Fluorouracil (0.66 mg/ml) respectively. Analysis of the sub-G1 region of cell cycle showed that curcumin treatment enhanced cell death. It was controlled by flow cytometry analysis after propidium iodide staining of the cellular DNA (Fig. 2).

3.2. Curcumin reduces the capability of cervical cancer cell invasion

The number of invasive Hella cells treated with curcumin sig-nificantly decreased in transwell invasion assays compared to untreated cells. The experiments were carried out three times and represented comparisons between treated and control cells using the Student's *t*-test (Fig.3).

3.3. Curcumin causes tumor shrinkage in Hella cells

Three-dimensional (3D) culture models have been shown to be further radio-/chemo-resistant, compared to two-dimensional mono-layer cell cultures, supporting the utilization of 3-D models for drug screening/discovery. We revealed shrinkage of tumor in the spheroids
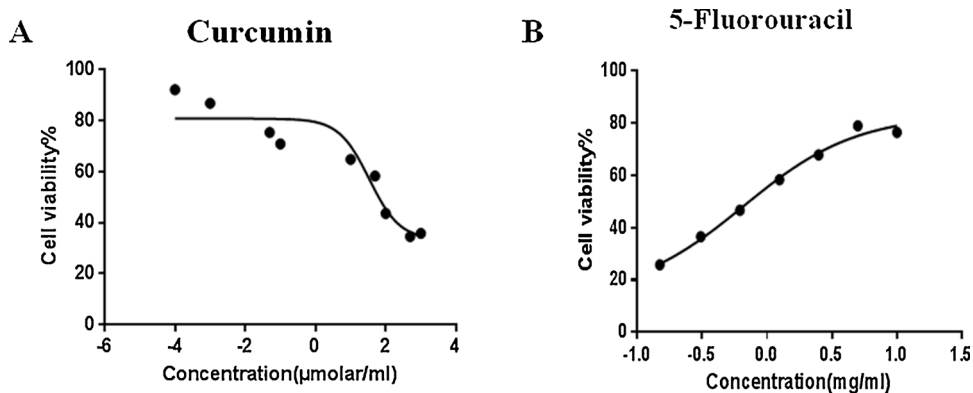
Fig. 1. Inhibition of cell proliferation in Hella cells. Growth inhibitory effects after 24 h' exposure to A) Curcumin, B) 5-Fluorouracil.

after 7 days, in comparison to untreated spheroids (Fig. 4).

3.4. Curcumin enhances cell death and inhibits NF-kB and Wnt signaling pathways

The mRNA expression levels of NF-κB and Wnt/β-catenin signaling pathways in Hella cells treated with curcumin were examined by quantitative Real-time PCR. To determine the related mechanism of anti-proliferative activities of curcumin, we assessed the expression pattern of genes implicated in NF-Kβ and Wnt/β-catenin (*WNT*) signaling pathways after treatment of these cells with $5 \times IC_{50}$ of curcumin for 48 h compare with control group (Fig. 5).

4. Discussion

Cervical cancer, as a leading cause of mortality and morbidity, is one of the most frequent malignancies among females [17]. Despite all developments in discovering the underlying mechanisms implicated in the progression and generation of this cancer, its therapy and prognosis has remained poor. Curcumin has some distinguished chemical attributes leading to its interaction with multiple molecules inside and outside the cell which are actively involved in the onset and progression of cancer, this interaction results in inhibiting the progression of cancer [13,18–20]. Numerous studies have demonstrated that deregulated inflammatory pathways have a crucial role in many chronic diseases, including cancer [21]. Chronic inflammation leads to onset and progression of cancer through increment of pro-inflammatory agents, including chemokines, cytokines, overexpression of oncogenes, cyclooxygenase (COX-2), reactive oxygen species (ROS), matrix metalloproteinase (MMPs), intracellular signaling pathway mediators, activator protein 1 (AP1) and signal transducer and activator of transcription 3 (STAT3) that motivate tumor cell proliferation, invasion,
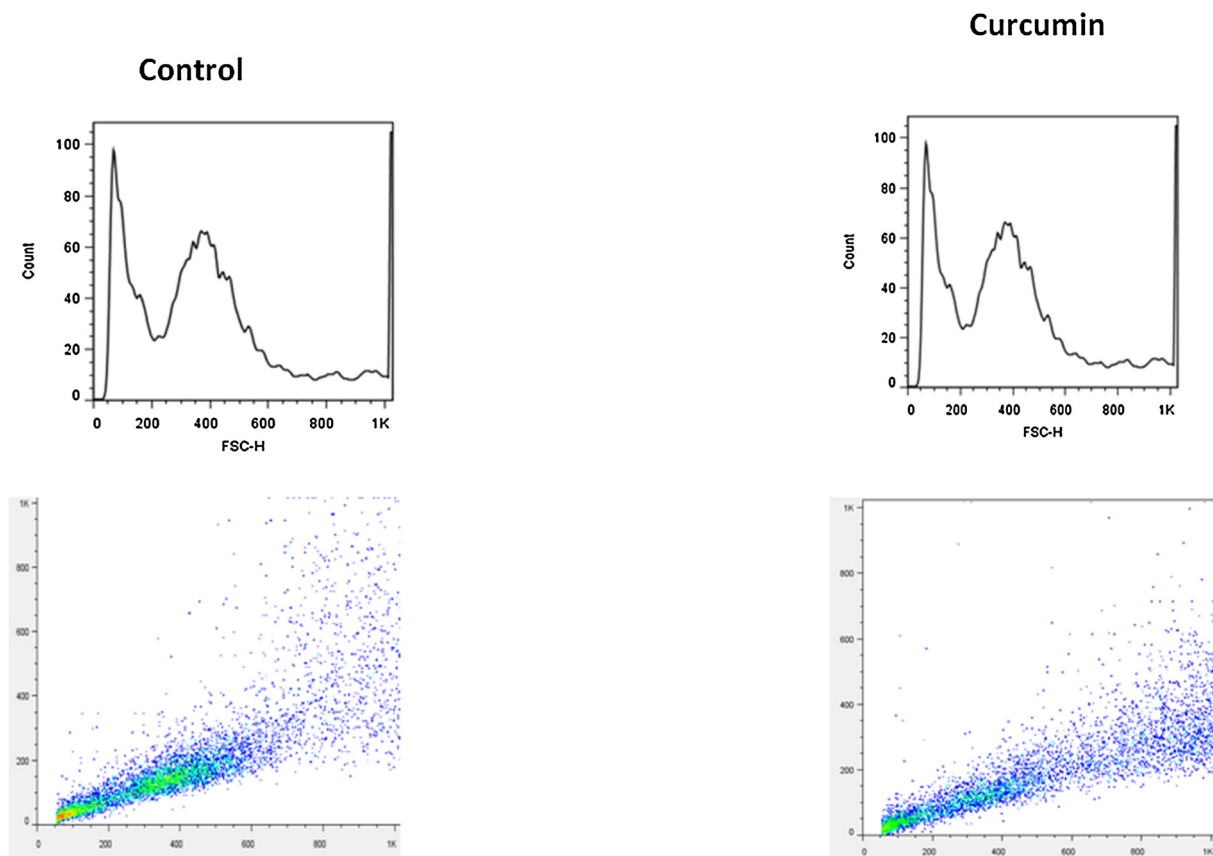

Fig. 2. The cell growth inhibitory effects of curcumin and 5-Fluorouracil in Hella cell line.
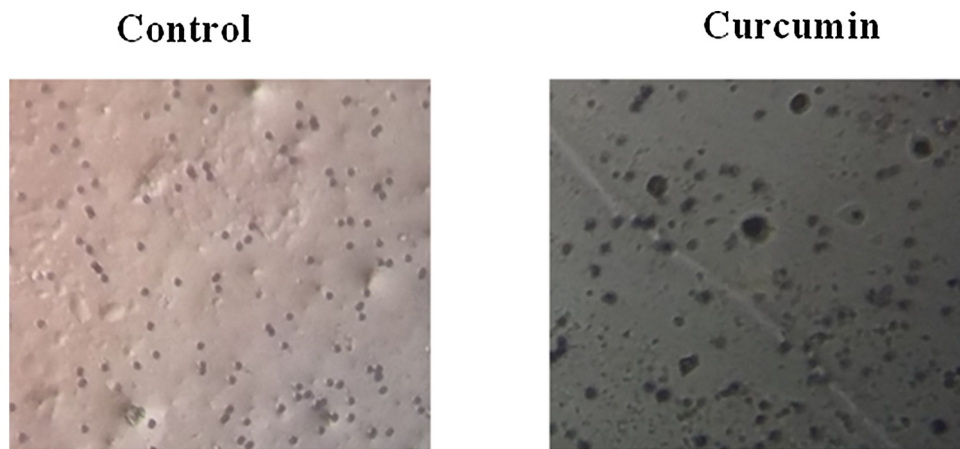
Control

Curcumin

Fig. 3. Effects of Curcumin in cancer cell invasion. Effects of Curcumin on Hella cells compare with control group in transwell invasion assays.

Day

1   3   7
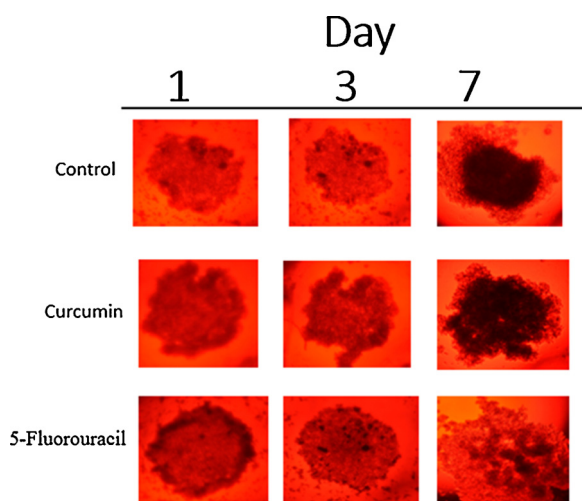

Control

Curcumin

5-Fluorouracil

Fig. 4. Effect of Curcumin on the Hella spheroids.

angiogenesis, transformation, metastasis, chemo resistance, and radio resistance [18,19,21–29]. Effect of curcumin on these factors is assessed in numerous studies. AP-1 as a transcription factor express the genes which pertaining to cancer and activate mitogenic, pro-angiogenic, and anti-apoptotic signals [30–33]. MAPK family members, including ERK1/2 activate and phosphorylate AP-1 leading to CCND1 up-regulation which encodes cyclin D1 [34]. AP-1 is often related to tumor progression and the high expression level of AP-1 and NF-κB seen in gliomas associated with enhanced radio-resistance and chemo-resistance [35]. Curcumin (20 μM) suppressed TPA-stimulated PKC activity in human astroglioma cells and down-regulated pro-angiogenic MMP9 and AP-1 [36]. Curcumin inhibited PKC activation by blocking $Ca2+$ release from the endoplasmic reticulum in human HCT-116 colon cancer cells [37,38]. Another research suggests that curcumin repress JNK activation which is induced by carcinogens. Curcumin suppressed AP-1 activity and led to inhibition of LnCap prostate cancer cells proliferation induced by hydrogen peroxide [39]. It has been demonstrated that curcumin down-regulates the AP-1 expression in cervical cancer cells [40]. Thus, curcumin can suppress tumor neovascularization by inhibiting PKC activity through impairing angiogenesis via the ERK-AP-1-MMP-9 pathway [41]. According to available data
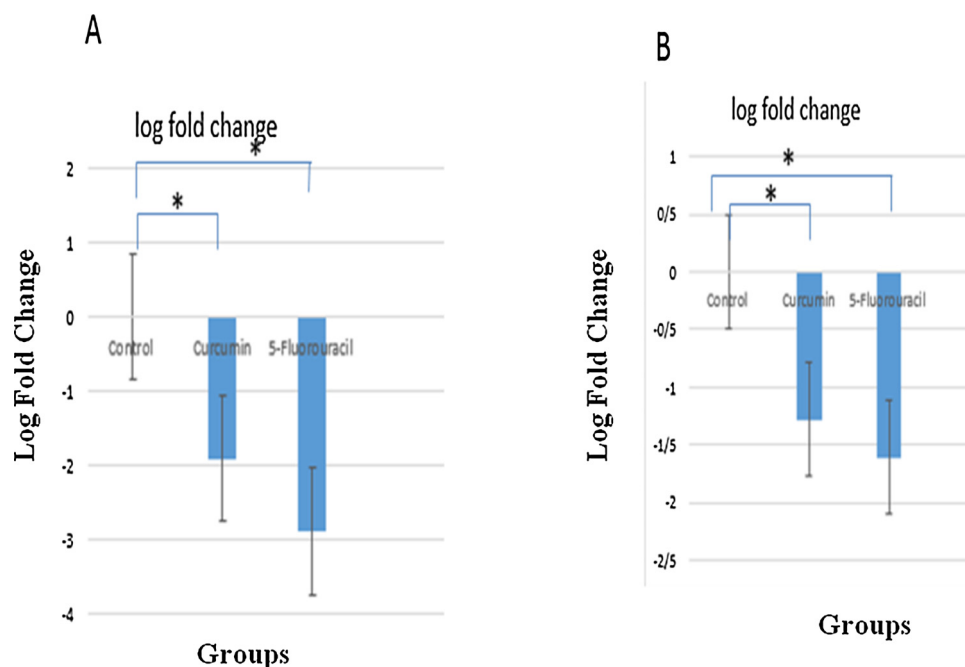
A

B

Fig. 5. Curcumin enhances cell death and inhibits NF-kB (A) and Wnt (B) signaling pathways. (P-value, * P < 0.05).

Table 1
The list of primers and their sequence.

Gene		Primer Sequence (5' - > 3')
NF-kB	Forward	GAAATTCCTGATCCAGACAAAAAC
	Reverse	ATCACTTCAATGGCCTCTGTGTAG
WNT	Forward	GTACGCCATCTCTTCGGCAG
	Reverse	GCGATGTTGTCAGAGCATCCT
GAPDH	Forward	AACAAGAGGCCACACAAATAGG
	Reverse	CAGATGTACAGGAATAGCCTCCG

constitutive form of NF-κB can be found in virtually all malignancies, and inhibitory capability of curcumin on NF-κB has made it an potential compound in cancer treatment [42,43]. Curcumin can inhibit NF-κB pathway in numerous cancer cells [44], such as breast cancer [45,46], adenoid cystic carcinoma [47], human oral squamous carcinoma [48], head and neck squamous cell carcinoma [49], cutaneous T-cell lymphoma [50], gastric cancer [51], ovarian cancer [52], medulloblastoma [53], rhabdomyosarcoma [54], human tongue squamous cell carcinoma [55], glioblastoma [56], colorectal cancer [57], Myeloid-derived suppressor cells [58], human biliary cancer [59], Hodgkin's lymphoma [60], prostate cancer [61], T-cell and NFAT activation [62], esophageal adenocarcinoma [63], pancreatic cancer [64], esophageal squamous cell carcinoma [65], human bladder cancer [66], human epidermoid carcinoma [67], non-Hodgkin's lymphoma [68], thyroid carcinoma [69] and lymphoma [70]. Other researches demonstrated that curcumin can repress the activation of upstream kinases of NF-κB pathway, IKKβ and IKKα. It has also been indicated that curcumin suppresses NF-κB activation induced by cigarette smoke in lung epithelial cells [71], and inhibits constitutive activation of NF-κB in mantle cell lymphoma, head and neck cancer and multiple myeloma. NF-κB down-regulation results in inhibited expression of COX-2, cyclin D1, pro-MMP2, and MMP9 [35]. Curcumin also suppresses NF-κB -induced production of CXCL1 and CXCL2 in breast cancer cells [72]. It has been found that the Notch signaling pathway can be repressed by curcumin in pancreatic cancer cells [73]. Moreover, curcumin is also a powerful proteasome inhibitor [74], which suppress the 20S proteasome activation and induce demolition of IκBα in colon cancer [75]. Peroxisome proliferator-associated receptor gamma (PPAR-γ) is known to be an appropriate inducer of separation as well as proliferation suppressor of tumor cells. Recent studies have shown that curcumin can activate PPAR-γ and inhibit Moser cell growth by inhibiting the cyclin D1 and EGFR gene expression [19]. Overall, we showed that curcumin efficiently inhibits the proliferation and invasion of cervical cancer cells through impairing Wnt/β-catenin and NF-kB pathways. Thus, targeting mentioned proliferative pathways *via* curcumin could have valuable clinical impacts on the therapy of cervical cancer and increase chemotherapy effects. But, to determine the therapeutic applications and exact underlying mechanisms of different formulation of curcumin in cervical cancer, more investigations are required (Table 1).

Declaration of Competing Interest

The authors have indicated no potential conflicts of interest in this study.

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