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3D h9e peptide hydrogel: An advanced three-dimensional cell culture system for anticancer prescreening of chemopreventive phenolic agents

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ABSTRACT

Traditional 2D monolayer cell culture model may overestimate chemopreventive agent's response due to lacking physiological relevance in three-dimensional microenvironment. This study was aimed to apply a novel 3D h9e peptide hydrogel cell culture system to evaluate the anticancer efficacy of chemopreventive phenolic acid on hepatocarcinoma HepG2 and colon adenocarcinoma SW480 cells. Both cell lines grew better in this 3D system with better cell growth and longer exponential phase than that in 2D model. Chlorogenic acid (CGA), known as a chemopreventive phenolic acid, at 0–40 μM for 72 h inhibited cell growth but not viability in both HepG2 and SW480 cells. The inhibition was much less potent in 3D system with an IC_{50} value of 58.0 ± 15.8 or 285.6 ± 75.4 μM when compared with 2D model with IC_{50} of 5.3 ± 0.3 or 12.0 ± 2.5 μM for HepG2 or SW480, respectively. Furthermore, the recovery of cells grown in 3D system after post-CGA appeared faster than 2D model. Taken together, an advanced 3D model has been established with favoring cell growth and less susceptible to inhibitory treatments in contrast to 2D model, thus predict closely to *in vivo* situation and may bridge the gap of *in vitro* to *in vivo* for prescreening chemopreventive agents for cancer prevention.

1. Introduction

Traditional flat 2D cell culture system has been used *in vitro* for many purposes, including prescreening of anticancer agents for cell growth impact and cytotoxicity as well as the underlying mechanisms before *in vivo* animal experiments and human clinical trials. However, a 2D monolayer cell culture cannot provide a three-dimensional physiological microenvironment and thus cannot reflect the complexity of *in vivo* cell to cell interaction. Therefore, 2D monolayer cell culture method often results in an overestimated response in anticancer chemopreventive agent prescreening, misleading to time- and money-consumed *in vivo* experimental studies (Hutmacher, 2010; Kim et al., 2014; Verjans et al., 2018).

To date, most of cancer research *in vitro* is conducted in traditional two-dimensional (2D) monolayer culture which is flat. 2D cell culture has been reported to be a valuable method for cell-based studies (Edmondson et al., 2014). However, the drawbacks of 2D monolayer cell culture have been recognized. 2D cell culture is simplified and unrealistic condition for cell growth due to lacking physiological properties of real tissues. Cells *in vivo* are surrounded by extracellular matrix (ECM) in a three-dimensional (3D) structure, whereas 2D cell culture cannot provide. As a result, tests in 2D cell culture may provide misleading and nonpredictive data for *in vivo* responses (Bhadriraju and Chen, 2002; Birgersdotter et al., 2005). In a long term, it will result in the experiment failure and research fund waste. In contrast to 2D cell culture, 3D cell culture system has been promised more *in vivo* such as physiological relevance in cell viability, morphology, proliferation, differentiation, stability, and lifespan, *etc.* (Antoni et al., 2015; Gurski et al., 2010; Rodrigues et al., 2018; Shield et al., 2009).

Various 3D cell culture platforms have been developed, including hydrogels, scaffolds, decellularized tissues, and cell-derived matrices (Fitzgerald et al., 2015). Hydrogels are the network of hydrophilic polymers composed of over 95% volume as water, but the polymers display semi-solid-gel like attributes and facilitate nutrient, oxygen, and metabolite transport (Sathaye et al., 2015; Verjans et al., 2018). Many natural and synthetic materials have been currently used in hydrogels such as collagen, Matrigel, and synthetic peptide hydrogels. The synthetic peptide hydrogels include EAK16 and RADA16, Fmoc-FF and Fmoc-RDG, and h9e, *etc.* (Huang et al., 2012; Worthington et al., 2015). We previously developed a h9e peptide hydrogel that could be self-

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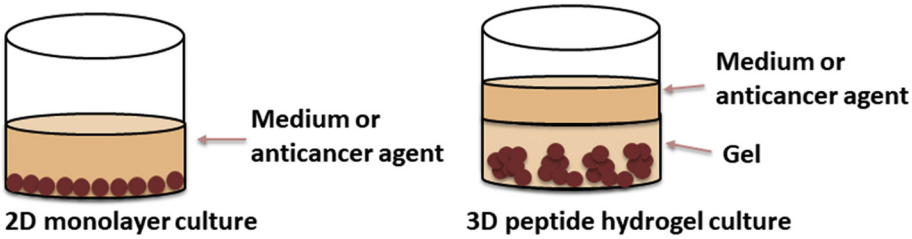

Fig. 1. Schematic diagrams of traditional 2D monolayer cell culture and 3D peptide hydrogel cell culture.

assembling without pH or temperature change and provided a superior physiological property for 3D cell culture in encapsulating and growing breast cancer MCF-7 cells (Huang et al., 2013). We also demonstrated more comparable viability of Hela cells with better anticancer drug diffusion in this 3D h9e peptide hydrogel when compared with 2D cell culture system (Liang et al., 2017). The schematic diagrams of traditional 2D monolayer cell culture and 3D peptide hydrogel cell culture were shown in Fig. 1.

As Doll and Peto (1981) reported that diet could alter 35% of human cancer-related mortality (Doll and Peto, 1981), consumption of vegetables and fruits containing anticancer phytochemicals has been estimated to prevent 66–75% of colon cancer and 33–66% of liver cancer (Marmot et al., 2007). Phytochemicals are health-promoting factors which are uniquely presented in plant foods to provide health benefits like antioxidant, anti-cancer, and anti-inflammatory properties (Birt et al., 2001; Liu et al., 2000).

Phenolics, one of the classified groups of phytochemicals, have been intensively studied due to potent antioxidant activity with less cytotoxic effect (Charlton et al., 2002; He et al., 2018; Jafari et al., 2014; Wang et al., 2000, 2007). Phenolics consist of thousands of flavonoids and phenolic acids. Among of which, chlorogenic acid (CGA) is one of the most abundant phenolic acids derived from vegetable or fruits, such as carrots, pears, apples, strawberries, berries, coffee, potatoes, *etc.* (Brown, 2005; Clifford, 2000). The chemical structure of CGA is shown in Fig. 2.

As relevant to digestive system, both liver and colorectal cancers are highly in mortality rate (Sener and Grey, 2005). Liver cancer has been reported as the sixth most common type of cancer worldwide (Marmot et al., 2007). Colorectal cancer is the third most commonly diagnosed cancer in males and the second in females worldwide (Favoriti et al., 2016). It has been reported colorectal cancer was a top leading cause of cancer related death among adults over the age of 50 in U.S. (Carini et al., 2017). Both HepG2 and SW480 cell lines are well used *in vitro* cell culture system for the study of anticancer agents. HepG2 cell line, known as one of the most promising human hepatomas, is easy to handle and operate, and contain several enzymes which are involved in the activation and detoxification of drugs and reflected the metabolism of xenobiotics better than other cells lines used in the routine testing (Salvadori et al., 1993; Serpeloni et al., 2012). In addition, HepG2 cell lines can display the morphological and functional differentiation, and biochemical activities of healthy hepatocytes and therefore have been widely used to evaluate the toxic effects of various toxicants on hepatocytes for many years (Mersch et al., 2004; Li et al., 2018; Ma et al., 2017).

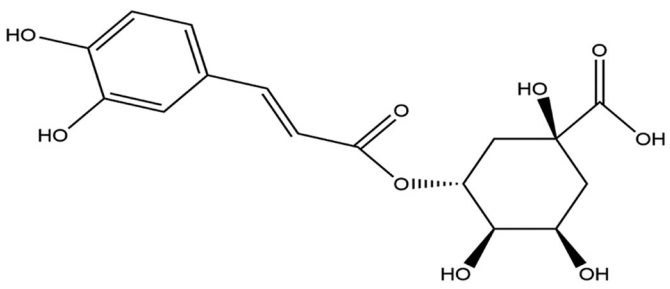

Fig. 2. Chemical structure of chlorogenic acid.

So far, the well-differentiated HepG2 cell line has been widely used in the biochemical and nutritional studies (Ramos, 2007). SW480 cell line is a human colon adenocarcinoma that is well-differentiated and invasive, and commonly used in metastatic studies (Flatmark et al., 2004). SW480 cell line is also well-known as an expression of truncated form of adenomatous polyposis coil (APC) considered the typical 'gatekeeper' tumor-suppressor gene and mutation of which are crucial initiating events in the development of human colorectal cancer (Lamprecht and Lipkin, 2003). In addition, SW480 showed more sensitive to dietary agents than other colon carcinoma cell lines (Owczarek et al., 2017; Wang et al., 2000; Yan et al., 2014). Both cell lines have been routinely used in *in vitro* 2D monolayer cell culture model for anticancer agent research. For example, extensive studies *in vitro* reported that CGA inhibits HepG2 cell growth (Glei et al., 2006; Kulisic-Bilusic et al., 2012; Whiteman et al., 2005; Yan et al., 2017) and SW480 cell growth (García-Gutiérrez et al., 2017; Sadeghi Ekbatan et al., 2018).

To our best knowledge, however, there is no study of culturing HepG2 and SW480 cells in 3D h9e peptide hydrogel cell culture system yet. Additionally, little is known about the efficacy of CGA treatment on inhibiting cell growth in 3D *versus* 2D cell culture model. Hence, the objective of this study is to develop a special 3D h9e peptide hydrogel cell culture model and then test the efficacy of CGA's treatment on both HepG2 and SW480 cell growth and cytotoxicity between 3D stereotomic and 2D monolayer cell culture system.

2. Materials and methods

2.1. Materials

The reagents of the Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Dulbecco's Phosphate Buffered Saline (DPBS), penicillin/streptomycin, and 0.5% trypsin-EDTA were purchased from Fisher Scientific Co. L.L.C (Pittsburgh, PA). Chlorogenic acid (CGA) was purchased from Sigma Aldrich (St. Louis, MO). Both HepG2 and SW480 cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, VA). The h9e peptide was synthesized according to our previously published protocol (Huang et al., 2013). Briefly, peptides were synthesized on an automated CEM Liberty microwave peptide synthesizer (CEM Corporation, Matthews, NC) according to the base-labile 9-fluorenylmethoxycarbonyl (Fmoc) strategy with Rink amide resin and Fmoc-protected amino acids. After final N-terminal Fmoc group deprotection, the resin-bound peptides were side-chain-deprotected and cleaved using TFA/TIS/water (95/2.5/2.5 v/v). Molecular weight and purity of the synthesized peptides were confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy and high-performance liquid chromatography. A PGmatrix-Pure kit containing both h9e peptide solution and hydrogelation trigger was commercially available by our PepGel LLC (Manhattan, KS).

2.2. 2D cell culture model

HepG2 or SW480 cells at 2×10^5/well were cultured in a 6-well plate with DMEM media supplemented by 10% of FBS, 100 μg/mL of streptomycin and penicillin. Passage time was determined by cell confluence up to 80–90%. To harvest HepG2 or SW480 cells from 2D monolayer cell culture model, cells were enzymatically detached by using 0.05% trypsin-EDTA at 1 mL/well and incubated at 37 °C for 8 min. Fresh medium of 1 mL/well was then added to stop the trypsinization and cell suspension was then collected after centrifuge at 600 *g* for 6 min (Eppendorf 5702, New York City).

2.3. 3D h9e peptide hydrogel cell culture system

HepG2 or SW480 cells in DMEM media were mixed with h9e peptide solution and hydrogelation trigger at ratios of 48:50:2 (v/v). The mixture of 500 μL was then transferred into each well of 24-well plate and incubated at 37 °C for 30 min to form hydrogel. After hydrogel formation, 1 mL/well of DMEM media supplemented by 10% of FBS, 100 μg/mL of streptomycin, and 100 units/mL of penicillin was added gently on the top of the hydrogel. The final cell number per well in 3D hydrogel was equivalent to 2×10^5 and the peptide concentration was 0.5%.

To isolate cells from 3D hydrogel matrix, 1 mL DMEM media was added to each well. The hydrogel with embedded cells was mixed well by pipetting and transferred to a conical tube. 1 ml/well of DPBS was added to rinse the well and combined into the conical tube. Additional DPBS was added to dilute the hydrogel by 20 folds of total volume of gel and cell mixtures. Then the diluted mixture was centrifuged at 800*g* for 10 min. To separate cell colonies, 1 ml/well of 0.05% trypsin-EDTA was added to the cell pellet and gently pipetted to re-suspend the cells. The sample was placed in the incubator at 37 °C for 8 mins, then 1 mL/well of medium was added to stop the trypsinization. The cell suspension was centrifuged at 600*g* for 6 min and re-suspend the cell pellet in cell medium for further cell counting or passage.

2.4. Cell morphology

The morphological characters were determined by using an inverted light microscope (Nicon Eclipse TE2000-u, Kanagawa, Japan). Morphologies of HepG2 or SW480 cells cultured in 2D monolayer or 3D hydrogel system were observed daily.

2.5. Cell treatment and analyses of cell growth and cytotoxicity

2.5.1. Cell treatment in 2D cell culture model

A healthy group of 10 subjects consumed 200 mL coffee and the average maximal plasma concentration of CGA was reported around 0.26 μmol/L after 1 h (Manach et al., 2005). Another study *in vivo* in mice model demonstrated that the maximal concentration of plasma of CGA was reported as 11.2 μmol/L (Feng et al., 2016). In our study, HepG2 or SW480 cells at 2×10^5/well were cultured with CGA at 0–40 μM up to 72 h. Then CGA was removed and fresh media without CGA were added for post-treatment up to another 72 h.

2.5.2. CGA diffusion assay

To assure a well-distributed amount of CGA in 3D hydrogel matrix, the diffusion of CGA was determined by measuring the fraction of CGA diffused into the hydrogel. Total volume of 0.5 mL hydrogel alone without cells at a concentration of 0.5% was prepared into the 24-well plate following the same procedure mentioned above in Section 2.3. After hydrogel formation, 1 mL of CGA at 0–40 μM was added gently to the top of hydrogel and incubated at 37 °C. Fifty μL of top solution above the hydrogel was taken at various times and CGA was evaluated by UV absorbance to obtain the CGA remained. The CGA diffused into the hydrogel matrix was then calculated by the difference between total CGA added and CGA remained. UV absorbance measurements were conducted on a UV-1650PC spectrophotometer (SHIMADZU, Kyoto, Japan) based upon a standard curve of CGA at 323.5 nm.

2.5.3. Cell treatment in 3D hydrogel system

After the hydrogel with embedded HepG2 or SW480 cells was formed, the top media (1 mL) containing CGA at 0, 15, 30, and 60 μM (which was corresponsive to 0, 10, 20, and 40 μM CGA in the gel) was applied up to 72 h. Then these cells were continuously cultured with FRESH medium without CGA for another 72 h to study the post-treatment of CGA.

2.5.4. Cell growth and viability assay

After treatment, viable cell number and cell viability were measured using acridine orange/propidium iodide (AO/PI) assay from Nexcelom Bioscience and counted by Cellometer Auto 2000 (Nexcelom Bioscience LLC, Lawrence, MA).

2.6. Statistical analysis

All data was analyzed by the SAS statistical system, version 9.4. The cell number and viability within 2D or 3D were analyzed by two-way ANOVA, while impact of cell growth compared between 2D and 3D was analyzed by three-way ANOVA. A general linear model procedure followed by Tukey's multiple comparison was applied. All samples were assayed in triplicate in two to three different experiments. The results are present as means ± SD and a probability at ≤ 0.05 is considered significantly.

3. Results and discussion

3.1. Cell growth of HepG2 or SW480 cells in 3D hydrogel system versus 2D monolayer model

Either HepG2 or SW480 cells were encapsulated in the 3D hydrogel cell culture system as similar as MCF-7 or Hela cells according to our previous reports (Huang et al., 2013; Liang et al., 2017). As shown in Fig. 3, the morphologies of cells grown in 3D system for 72 h started to form more clusters than that in 2D model due to more space and favorable interaction. As incubation continued, either cell line continued growing in the 3D culture system up to 10 days, but that gown in 2D model was begun to plateau as early as 8 or 9 days (Fig. 4). According to the growth curves of HepG2 or SW480 cells in Fig. 4, 3D hydrogel culture system provided more viable cells and extended the exponential phase without significant difference in cell viability, indicating a favorable growth environment in contrast to 2D model.

3.2. Inhibiting Impact of CGA on HepG2 or SW480 cell growth in 3D hydrogel system versus 2D cell culture model

3.2.1. Diffusion of CGA into 3D hydrogel matrix

As shown in Table 1, the CGA concentration in 3D hydrogel matrix reached to the concentration equilibrium after 30 min, indicating CGA diffused through the nanofiber porous scaffold unobstructed well in the range of 10–40 μM tested, and also assuring the amount of CGA applied to cells in 3D was similar as that in 2D. Therefore, the experimental design for CGA inhibitory treatment should be comparable between 3D hydrogel system and 2D cell culture model.

3.3. Inhibiting Impact of CGA treatment on HepG2 cell growth in 3D hydrogel system versus 2D cell culture model

Effect of CGA on HepG2 cell growth and cell viability in both 3D and 2D culture was shown in Fig. 5. CGA treatment at 0–40 μM up to 72 h significantly reduced HepG2 cell growth in both 3D system and 2D model with some dose- and/or time-dependent effects. However, more

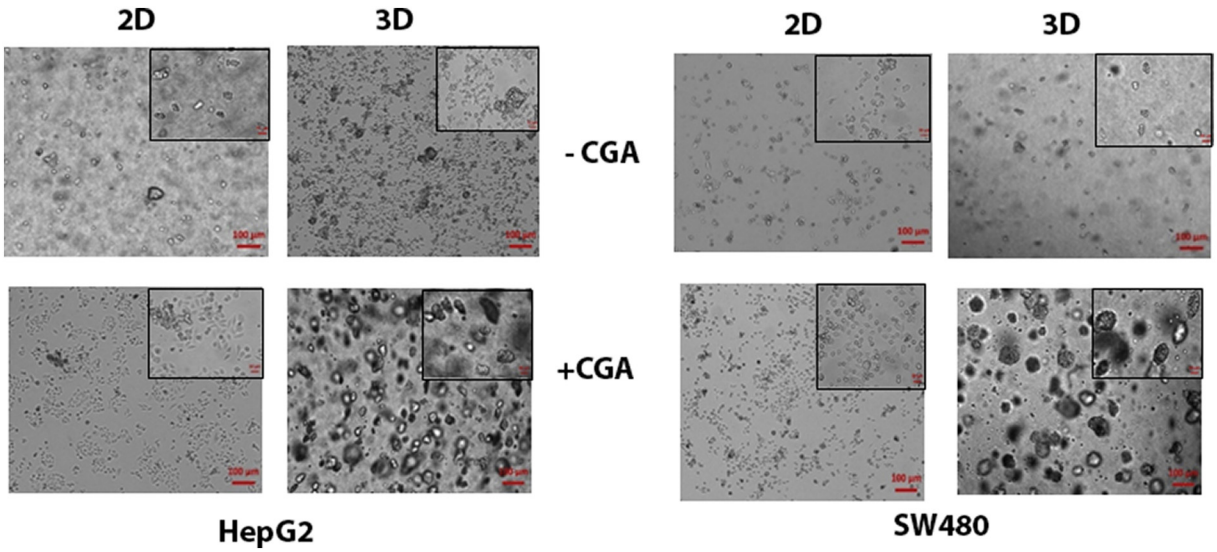

Fig. 3. Representative morphologies of HepG2 or SW480 cells cultured in 3D hydrogel system *versus* 2D model without CGA treatment at culturing day 9. Each image was shown by 5× magnification, with the scale bar of 100 μm.

interestingly, the dose-dependent impact of CGA on inhibiting HepG2 cell growth was clearly observed from the cell growth inhibition after removal of CGA treatment. The results showed the inhibitory effect of CGA on HepG2 cell growth lasted during post-CGA treatment. Cell viability was not significantly affected by CGA treatment (data not shown), indicating CGA was not toxic to HepG2 cells. This was in an agreement with Yan et al. (2017) that reported CGA concentration below 250 μmol/mL would not influence HepG2 cell viability (Yan

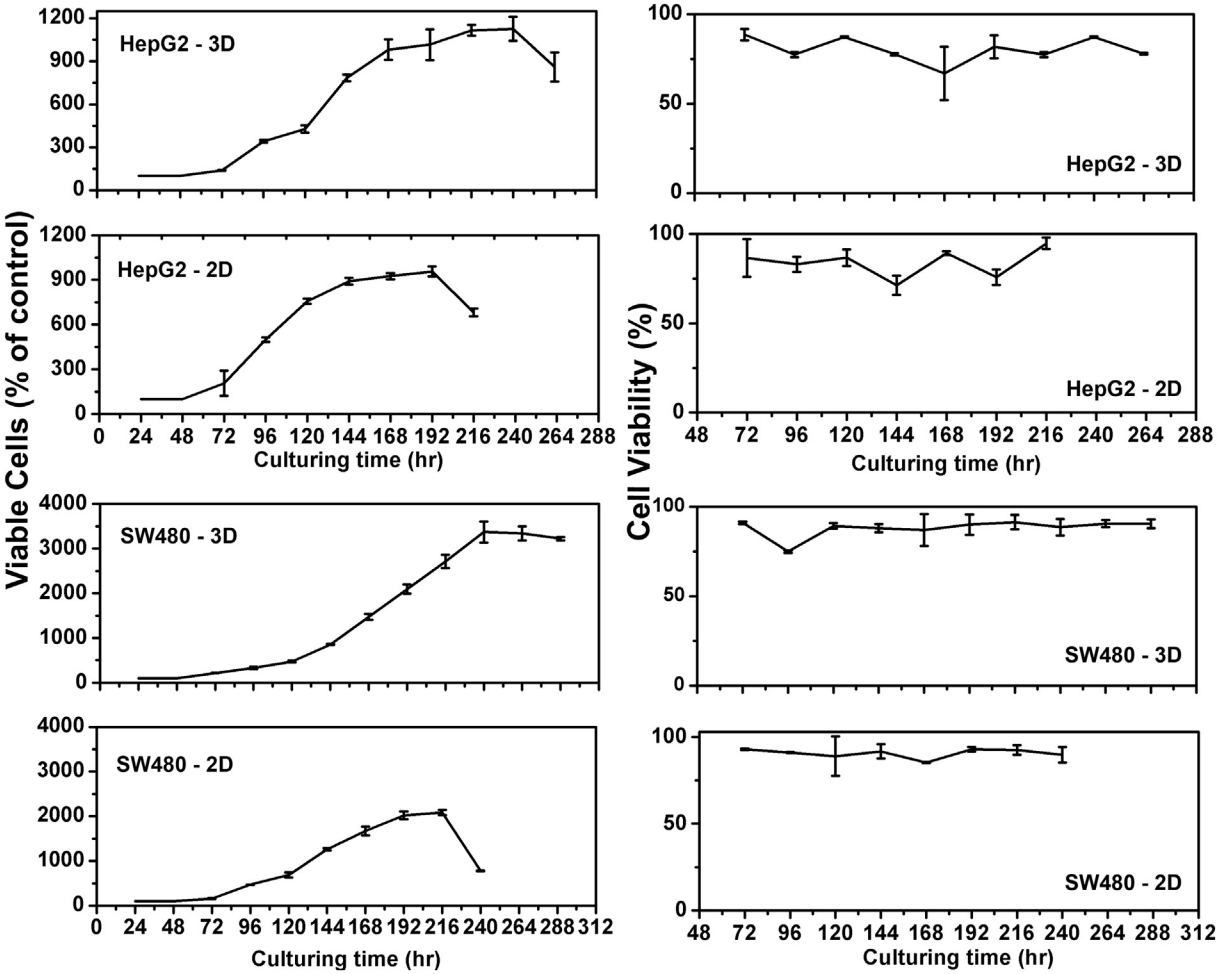

Fig. 4. Cell growth (left) and viability (right) of HepG2 or SW480 cells cultured in 3D *versus* 2D cell culture system. Values are Mean ± SD of two independent experiments.

Table 1
Diffusion of CGA into 3D hydrogel matrix[a].

CGA expected (μM)	30 min	**1 h**	2 h	3 h
10	8.7 ± 0.04	**9.9** ± 0.02	10.0 ± 0.07	10.0 ± 0.03
20	19.7 ± 0.33	**20.3** ± 0.43	20.2 ± 0.22	20.2 ± 0.20
40	38.8 ± 0.110	**39.3** ± 0.36	40.2 ± 0.33	40.1 ± 0.19

[a] Values are Mean ± SD from 3 independent experiments.

et al., 2017). Other studies showed that CGA could induce HepG2 cell death in 2D culture model at low dose of 0.00423 to 0.14 μM/mL (Barahuie et al., 2017; Kan et al., 2014) or a high dose of 60 to 1000 μM (Yan et al., 2017).

When the reduction of cell growth was compared at the same time or same CGA concentration between 3D and 2D, it should be noted that a greater reduction of cell growth was observed in 2D in contrast to 3D, indicating 3D system provided a favor environment for cell growth that was more resistant against CGA treatment. During post-CGA treatment, the inhibitory effect of CGA on HepG2 cell growth was observed in both 2D and 3D, but more reduction of cell growth was observed in 2D model in contrast to 3D system. Such difference further implied cells in 3D system were more resistant against chemopreventive agent than that in 2D model. These results suggest that cells cultured in 3D be closer to *in vivo* situation in terms of physiological growth and response than that in 2D model, leading more accurate evaluation of inhibitory efficacy using 3D culture model.

3.4. Inhibitory Effect of CGA on SW480 cell growth in 3D hydrogel system versus 2D cell culture model

Effect of CGA during CGA treatment and post-treatment on SW480 cell growth in both 3D and 2D culture was shown in Fig. 6. Results showed a similar reduction of CGA treatment as shown in HepG2 cells and the effect on cell viability was not statistically (data not shown). Our results were in accordance with the published studies that CGA effectively decreased the proliferation of colon cancer cells (Caco-2 and SW480) at an effective concentration varied from low dose of 0.5 to 2 μM/mL (Shin et al., 2015) or high dose of 50 to 1000 μM (Sadeghi Ekbatan et al., 2018).

When compared with the reduction of cell growth at the same time or same CGA concentration between 3D and 2D, a greater reduction of cell growth in 2D was observed than that in 3D. Treatment of CGA inhibited both HepG2 and SW480 cell growth in 3D system and 2D model, but the inhibitory potency appeared to be different. Gurski et al. (2010) demonstrated that the difference in morphological properties of cells in 3D and 2D cultures contributed to different responses to anticancer agent (Gurski et al., 2010). As shown in Table 2, IC_{50} values of HepG2 and SW480 cells treated with CGA at 72 h in both 3D and 2D culture were calculated. IC_{50} value of CGA-induced inhibition on HepG2 cells in 3D culture was almost eleven times higher than that in 2D culture, further suggesting that 3D culture model was in favor of cell growth and less susceptible to CGA treatment. For SW480 cells, IC_{50} value in 3D culture was around twenty-four times higher than 2D culture. This result was in accordance with previous studies that cells in 3D culture were more resistant to anticancer agents in contrast to 2D culture (Karlsson et al., 2012; Loessner et al., 2010; Silva et al., 2018). Previously, research has been reported that either nutrient or anticancer drug can be easily exposed to cells in 2D culture, but cannot be

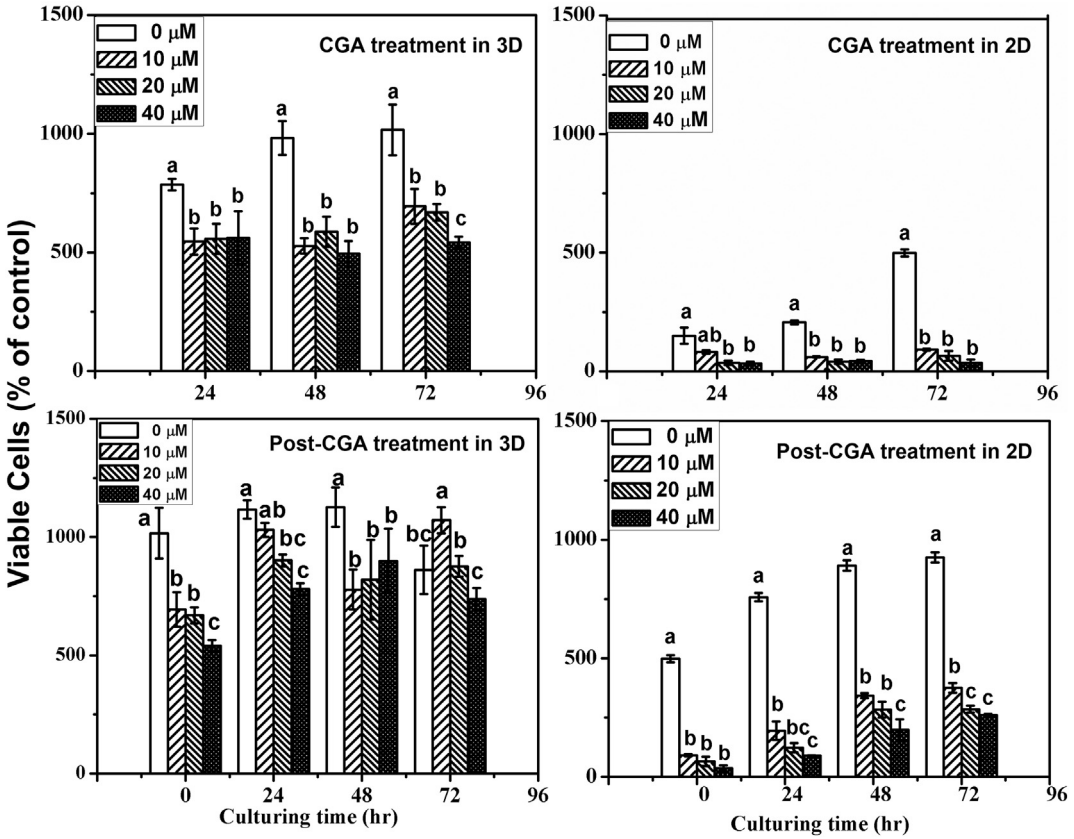

Fig. 5. Inhibitory effect of CGA at 0–40 μM up to 72-h for treatment and another 72-h for post-treatment on HepG2 cells cultured in 3D hydrogel system *versus* 2D monolayer model. Comparison was made within the same culturing time. Values are Mean ± SD of two independent experiments. Means with different superscripts within same culture time are significantly different, $P \leq .05$. Control is defined as the seeding number of cells.

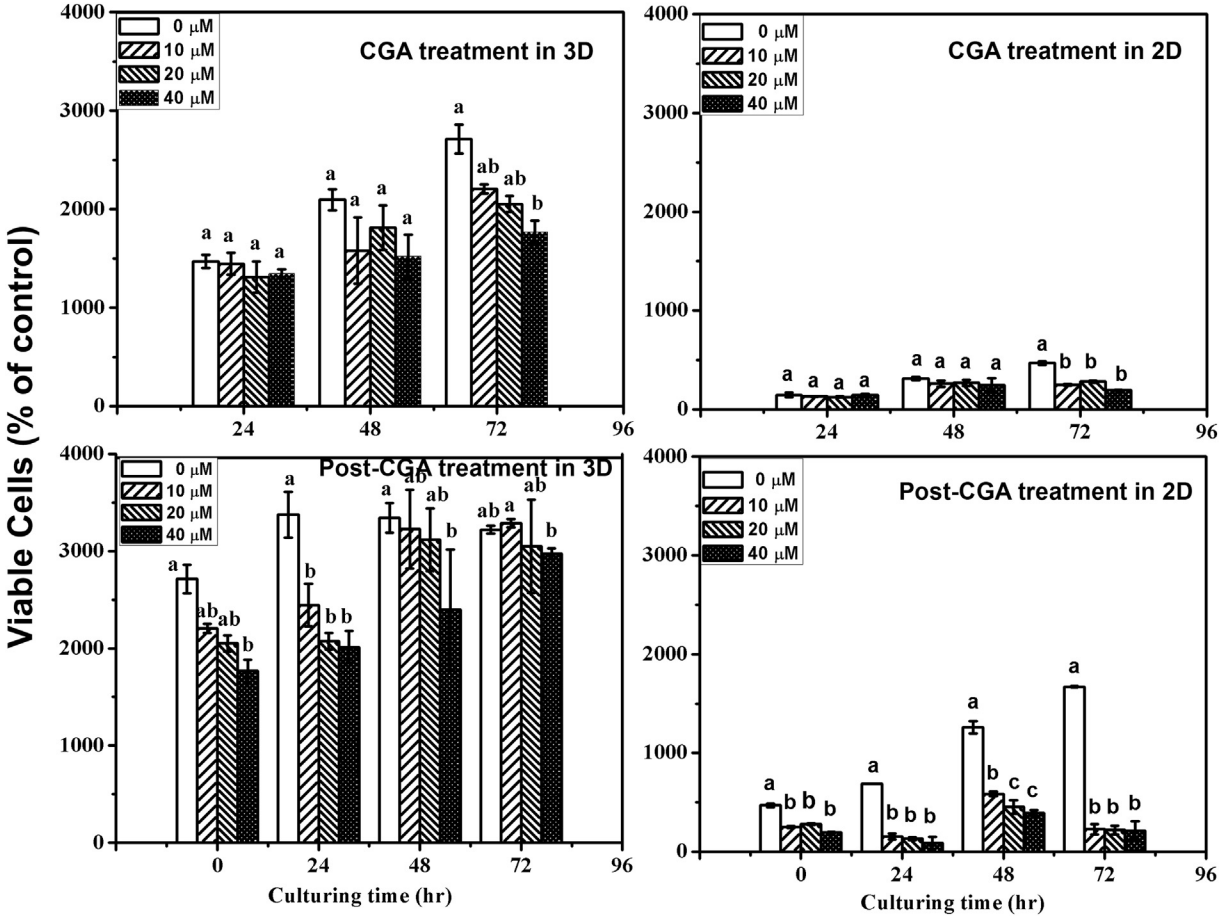

Fig. 6. Inhibitory effect of CGA at 0–40 μM up to 72-h for treatment and another 72-h for post-treatment on SW480 cells cultured in 3D hydrogel system *versus* 2D monolayer model. Comparison was made within the same culturing time. Values are Mean ± SD of two independent experiments. Means with different superscripts within same culture time are significantly different, $P \leq .05$. Control is defined as the seeding number of cells.

Table 2
IC_{50} values of CGA treatment in HepG2 and SW480 cells in 3D hydrogel cell culture system *versus* 2D monolayer model[a].

Cells	IC_{50} (μM)	
	2D	3D
HepG2	5.2	56.3
SW480	17.4	251

[a] Values are Mean of 2 independent experiments.

easily exposed to the core of the cells in 3D culture (Kim, 2005). In addition, research has been demonstrated that the effective inhibition of cell growth in 2D cell culture, but not in 3D cell culture, thus predicting *in vivo* responses (Gurski et al., 2010; Silva et al., 2018). Lower IC_{50} value of CGA on HepG2 cells was observed in contrast to SW480 cells, indicating SW480 cells were more resistant to CGA than HepG2 cells. That was in an agreement with previous studies wherein CGA resulted in reduction of cell growth of human colon cells (HT290) and HepG2 cells, but HepG2 cells were more susceptible than HT290 cells (Glei et al., 2006), and Caco-2 cells were more resistant to CGA treatment than HepG2 cells (Wang et al., 2011).

4. Conclusions

In conclusion, a novel 3D h9e hydrogel system was applied for cell culture of both hepatocarcinoma HepG2 and colon adenocarcinoma SW480 cells. More cell growth and extended exponential phase of HepG2 or SW480 cells were observed in 3D h39 hydrogel model in contrast to 2D monolayer cell culture model. CGA treatment suppressed both HepG2 and SW480 cell growth and showed some dose- and/or time-dependent inhibition in HepG2 or SW480 cells, but the inhibition was less susceptible to CGA treatment and easier recovery during post-CGA treatment in this 3D system when compared with 2D model. Both HepG2 and SW480 cell were much more resistant to CGA treatment in 3D than in 2D by 11- to 23-times higher IC_{50} values, suggesting that the 3D h9e hydrogel system more closely modeled the *in vivo* situation that 2D monolayer model. It might be of particular significance in bridging the gap of *in vitro* to *in vivo* for prescreening of chemopreventive agents for cancer prevention.

Author contributions

Conceptualization: J.X., S.X., W.W.; Data curation: J.X., G.Q., C.S.; Formal analysis: J.X., G.Q., C.S.; Methodology: J.X., G.Q., C.S., S.X.; Supervision: X.S., W.W.; Writing-original draft: J.X.; Writing-review & editing: J.X., G.Q., X.S., W.W.

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Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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