Telomerase associated apoptotic events by mushroom - Ganoderma lucidum on pre-malignant human urothelial cells.

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ABSTRACT

Relapse of transitional bladder tumors remains a challenge despite advances in immunological therapies. The chemopreventive effects of *Ganoderma. lucidum* on the tumorigenic phase of bladder cancer were tested, using a 4-aminobiphenyl (ABP) tumorigenic transformable human urothelial cell (HUC-PC) model. Our *in vitro* data clearly show that *G. lucidum* inhibited the viability and growth of HUC-PC. This could be explained by a concomitant induction of apoptosis and inhibition of telomerase activity in *G. lucidum* treated HUC-PC culture. Significant exteriorization of phosphatidylserine was detected by Annexin-V on cell surface at 3h upon *G. lucidum* incubation, and the HUC-PC cells subsequently lost the membrane integrity for uptake of DNA-specific 7-AAD dye, reaching 100% apoptotic in 48 hours. Moreover, the presence of *G. lucidum* in HUC-PC culture significantly elevated the levels of hydrogen peroxide and 8-hydroxy-2'-deoxyguanosine (8-OHdG) production. Taken together, physiological doses of *G. lucidum* promote apoptosis and oxidative DNA damage as well as suppress telomerase activity in HUC-PC cultures, which are essential to explain its anti-HUC-PC growth properties. The findings of this study strongly supports that *G. lucidum* is a potential source of chemopreventive agents for bladder cancer, based on its effectiveness on controlling the pre-malignant urothelial cell growth and carcinogen-induced transformation. (199)

INTRODUCTION

The carcinoma of the urinary bladder is the most common urologic malignancy encountered (1,2). The most frequent form (up to 90%) of bladder cancer on clinical presentation is urothelial carcinoma, i.e. transitional cell carcinoma (TCC). Risk factors for TCC include tobacco exposure, chemicals such as N-nitrosamines and aromatic amines (3) and genetic susceptibility (4). Majority of TCC are superficial disease (90%) and standard clinical management calls for transurethral resection with or without intravesical immunotherapy or chemotherapy (5). The most commonly used immunotherapeutic agent - Bacillus Calmette-Guérin (BCG), is based on the urothelial internalization to trigger inflammatory response, which ultimately results in TNF-α-induced apoptosis (6). Despite the positive impact (reduction by 20-30% of recurrence) and historical applications (30 years) of BCG, recurrence rate of bladder cancer remains high with lethal side-effects, as well as with approximately 10-30% of recurrent cancers progressing to invasive muscle disease to threaten survival rate (2). Therefore, powerful chemopreventive agents are demanded for prevention bladder cancer recurrence and progression.

Ganoderma lucidum, called “Red Lingzhi” in China has been used for longevity and tonicity in the East for over two thousand years, and because of its perceived health benefits, the mushroom are nowadays widely consumed as a supplement for different kinds of diseases and health maintenance (7). *G. lucidum* is commonly used for immune boosting and cancer prevention, whereas the evidence of anticancer properties comes from experimental studies *in vitro* and animals to humans’ *in vivo* (8). Triterpenes contain skeletons of lanostane and are responsible for the bitter taste of *G. lucidum*, exerting cytotoxic-based carcinostatic activities (9,10). Cytoxicity and

growth inhibition on various cancer cells have been well documented, and most of them are based on controlling the cell cycle and signaling pathways (11-13). Apoptosis has also been demonstrated by *G. lucidum* in malignant cell lines, such as leukemia (14-17), and skin (18). Recently, it was reported that *G. lucidum* inhibits the growth of a "chemically tumorigenic transformable" human urothelial cell line (HUC-PC) (19). However, the toxic mechanisms of *G. lucidum* on urothelial cells remain virtually unknown. The HUC-PC cell line is a clonal line derived from the simian virus 40 (SV40) - immortalized urothelial cell line "SV-HUC" cells (20), thus providing a relevant model, akin to the intravesical contact exposure of carcinogens and candidate therapeutic agents, for understanding the cause-and-effects between carcinogenesis and chemoprevention.

Unlimited replicative potential is an essential element for carcinogenesis. The enzyme telomerase plays a vital role controlling cell proliferation (21,22), and telomerase expression is found in over 85% human cancers, including 95% of all advanced malignancies (23). Up-regulation of telomerase can be detected in transformed bladder cancer cells (24-26), voided urine (27) and bladder-wash specimens (28) of most patients with urothelial cancer. Telomerase has been proposed as a urine-based marker for bladder cancer with 70-85% sensitivity and 80-95% specificity (29). Emerging evidences show the protective effects of telomerase on cell growth and survival (30). In this report concentrated extract of *G. lucidum* (GLE) at physiological doses were found to reduce viability and growth of HUC-PC cells, which could be explained by a concomitant suppression of telomerase activity and increase of apoptosis under oxidative stress (elevated hydrogen peroxide (H_2O_2) and oxidative DNA damage).

MATERIALS AND METHODS

Preparation of concentrated extract from *G. lucidum*

A proprietary extract composed of *G. lucidum* fruiting bodies and cracked spores, branded ReishiMax GLp™, was purchased from Pharmanex Inc. (Hong Kong, China). The active ingredients of the product was standardized as 13.5% polysaccharides (β-1,3-glucans) and 6% triterpenes (ganoderic acids and other), which is the highest level of extractable activities, whereas the remaining 80% composed of nucleosides, fatty acids, and amino acids, according to the manufacturer's technical bulletin. The powdered extract was sonicated with 95% ethanol for 30 minutes, and the supernatant was further extracted by successive sonication using absolute ethanol (19). The water-insoluble brown powder, i.e. GLE was retrieved from the filtrate (through 0.45 μm polypropylene filter) under reduced pressure.

Cell culture

SV-HUC-PC cell line originates from Department of Human Oncology, University of Wisconsin Medical School, gifted by Dr. Rao from the University of California, Los Angeles. The cell line was cultured in F12 Ham enriched Dulbecco's Modified Eagle's Medium (Sigma, St. Louis, MO) with 1% penicillin and streptomycin (10,000 μg/ml penicillin, 10 mg/ml streptomycin) and 10% Fetal Bovine Serum (GIBCO BRL Island, New York, U.S.A.). Logarithmically growing cells were harvested and seeded at concentration of 1 x 10^6 cells per 100-mm culture dish for assays. Cultures were maintained at 37^{o}C in a water-saturated atmosphere containing 5% CO_2. For assays, various concentrations (0, 40, 80 and 100 μM) of GLE (in assay media containing at most 0.01% absolute ethanol) were inhibited with HUC-PC cells, in the presence or

absence of 100 µM ABP (Sigma, St. Louis, MO), which dissolved in DMSO (Sigma, St. Louis, MO) with final concentration at not more than 0.02% in assay media.

LDH cytotoxicity assay and Cell viability

The cytotoxicity of test substances, i.e. 4-aminobiphenyl (ABP, CAS. No. 92-67-1) and GLE, were tested on the cell lines by using LDH Cytotoxicity Detection kit (TaKaRa Bio Inc., Shiga, Japan), preformed on a 96-well microplate. In addition, no interference and cytotoxicity was observed from any test substances and solvent concentrations used for assays. Cell viability was assessed by using an automated Beckman Coulter Vi-CELL^{TM} XR Cell Viability Analyzer with its reagent pack (Miami, FL). Percentage cell growth inhibition (%GI) was calculated using the following formulae:

$$\%GI = \frac{\left(\begin{array}{l}\text{Mean control}\\ \text{viable cell number}\end{array} - \begin{array}{l}\text{Experimental viable}\\ \text{cell number}\end{array}\right)}{\left(\begin{array}{l}\text{Mean control}\\ \text{Viable cell number}\end{array} - \begin{array}{l}\text{Initial seeded cell}\\ \text{number}\end{array}\right)} X\ 100$$

All cell counting results were verified between the manual and automated methods.

Apoptosis assay

The Beckman Coulter annexin V-FITC /7-AAD kit (Immunotech, France) was used to measure apoptosis. Briefly, cells were treated with various assay media and assayed periodically (at 1, 2, 3, 4, 5, 6, 8, 12 and 48 hours) during the 48-hour incubation. Cell analysis was performed on Beckman Coulter COULTER® EPICS® XLTH (Miami, FL) equipped with the Elite software version 5. A minimum of 10,000 events were collected and measured at FL1 (525nm) and FL4 (675nm). Cells incubated with 3% formaldehyde- containing PBS for 30 minutes on ice was used as positive control.

Untreated cells without staining were used as negative control, and those with staining were used as background control.

Real Time Quantitative –Telomeric Repeat Amplification Protocol (RTQ-TRAP)

Cells (1-5 x 10^6) were lysed in 1x CHAPS buffer containing RNase inhibitor, and incubated on ice for 30 minutes. The lysate was then centrifuged at 12,000*g* for 40 minutes at 4^{o}C, and the supernatant was collected (31). Total protein concentration of the cell extract was determined using a Bio-Rad Bradford protein assay kit (Hercules, CA). The protein level of each extract was adjusted to 10 ng/µl for telomerase activity analysis. The telomeric repeat amplification protocol (TRAP) is a landmark method for measuring telomerase activity, and a real-time PCR technique has been incorporated to allow rapid and precise quantitation (31). TS and ACX Primers were purchased from Molecular Information Laboratory (South Korea). 10X TRAP buffer containing 200 mM Tris, 630 mM KCl, 35 mM $MgCl_2$, 10 mM EGTA, 1 mg/ml BSA, and 0.05% Tween 20 was prepared and stored at -20^{o}C until use. The total volume of the reaction mixture was 25 µl, containing 40X SYBR Green (Invitrogen), 1X Fluorescein (Bio-Rad), 10mM dNTP (Promega), 0.25 µl Hot Star Taq polymerase (Qiagen), 14.75 µl RNase/DNase free distilled water, 0.1 µg each of TS (5'-AATCCGTCGAGCAGAGTTAG-3') and ACX (5'-GCGCGG(CTTACC)$_4$-3') primers, 2 µl 10X TRAP buffer, and 5 µl protein extract. The PCR was performed in a 96-well microtiter plate on a MyiQ Single-Color Real-Time PCR Detection System. The reaction mixture was first incubated at 25^{o}C for 30 minutes to elongate the TS primer. The PCR was started at 95^{o}C for 15 minutes to activate the Hot Star Taq polymerase, followed by 30-cycle amplification (95^{o}C for 30 seconds, 60^{o}C for 30 seconds, and 72^{o}C for 60 seconds). Fluorescence signal generated from SYBR green was collected

and analyzed with iCycler iQ Detector software (Ver. 3.0a; Bio-Rad), where measurable threshold intensity (threshold cycle; C_t) was achieved or at maximum 30 cycles. Relative telomerase activity was calculated using the derived equation $2^{-\Delta Ct}$ (1).

Extracellular H_2O_2 assay in culture media

Culture media from each study group was collected after 48-hour incubation, and filtered using Centricon YM-10 microconcentrator (Millipore Co., Bedford, MA) with 10nm pore size for the H_2O_2 assay. The method was based on the FOX assay as published elsewhere (32), with minor modifications. Briefly, 100 µl of sample was added to 170 µl of working reagent in a 96 microplate well, mixed and left for 20 minutes at room temperature, and the absorbance at 590 nm was read against a complete media blank, using microplate reader (TECAN, Austria). The H_2O_2 concentration of each sample was read from a standard curve, with 0, 0.5, 1.0, 2.5 and 5.0 µM working standards freshly prepared from a 30% H_2O_2 stock solution (RDH).

8-OHdG ELISA assay

Oxidative DNA damage was analyzed by measuring free 8-OHdG present in the culture media. Very low quantity of 8-OHdG is expected in the culture media. Filtered culture media were assayed immediately using the Highly Sensitive 8-OHdG Check Kit (Japan Institute for Control of Aging, Shizuoka, Japan). The manufacturer's instructions are strictly followed. Duplicated samples of each study group were run in triplicate. The outer most wells of the ELISA plate were not used to avoid edge effect, and phosphate buffer was added to the unused wells to maintain the uniform temperature within the wells.

Statistical Analysis

All experiments were performed in duplicate for reproducibility. Descriptive statistics, such as mean and standard error, were used to summarize the results. GraphPad Prism (GraphPad software, version 3.0 for windows, U.S.A.) was used to perform Student's t test for statistical comparisons. Pearson's correlation test was carried out to measure the relationship between measured parameters. Statistical significance was sought at two-tailed P-value ≤ 0.05.

RESULTS

Effects on HUC-PC cell growth

Cell growth was enhanced by 16%(±4%) with ABP incubation for 48 hours (Table 1). 22%(±6%) more of the cell growth was shown on ABP-pretreated cells (24 hours) followed by 48 a hour-culture in complete media, as compared with control (Table 2). The GLE re-extracted from the capsule powder is water-insoluble. As determined by LDH release from cells, no direct cytotoxicity was observed from GLE at 80 µg/ml or below after 24 hour-incubation, but 14%(±2%) and 100%(±6%) of the cells were killed with 100 µg/ml and 200 µg/ml GLE, respectively. Growth inhibition was demonstrated at 100% by 80 µg/ml GLE, as initial cell seeding number (i.e. 1 x 10^6 cell / 100-mm dish) was maintained after 48 hour-incubation, regardless of the presence or absence of ABP (Table 1). Table 1 and 2 also summarize the growth inhibitory effects of GLE at different concentrations, with ABP treatments, in a dose-dependent manner.

Apoptotic effects of GLE on HUC-PC cells

As shown in Figure 1a, cell shrinkage, elongation and blebs formation appeared in 48 hours for GLE-treated cells. A time-dependent progressive apoptotic effect of GLE on the cells is shown in Figure 2a & b. When incubated with 80 μg/ml of GLE, about 30% of the cells were apoptotic at 3 hours, and gradually progressed to reach 70% at 8 hours, predominantly Annexin-V positive but 7-AAD negative phenotype. At 12 hours, the apoptotic cell population was maintained at 70%, however, the phenotype became both Annexin-V and 7-AAD positive. The cell culture was continued and reached 100% apoptotic with almost 60% Annexin-V + 7-AAD positive phenotype. 10-20% of control cells were shown apoptotic (data not shown). Similar trend of the apoptotic process was also shown on the cells treated simultaneously with GLE and ABP, which was in a dose-dependent response (Figure 3).

Inhibitory effects of GLE on HUC-PC telomerase activity

Relative telomerase activity was enhanced significantly ($P<0.001$) by 33%(±4%) for ABP-pretreated cells (Figure 4a). When incubated with GLE, 30% of relative telomerase activity of HUC-PC was inhibited significantly ($P < 0.001$), and 40% was inhibited for the ABP-pretreated cells ($P<0.001$), and this inhibitory effect was in a dose-dependent manner (Figure 4a & b). However, the relative telomerase activity of the cells was inversely proportional to the growth inhibition exerted by GLE (Fig. 4c).

Oxidative stress in apoptotic HUC-PC cells

In the presence of GLE, there was significant increase of H_2O_2 ($P<0.05$) and 8-OHdG ($P<0.01$), by 20% and 22% respectively, in the ABP-cultured media (Fig. 5a). The 8-OHdG formation induced by GLE was in a clear dose-dependent manner (Fig. 5b). In

the absence of ABP, 43% of H_2O_2 in culture media was significantly increased ($P<0.05$) by TRE, but no increment of 8-OHdG was found (data not shown).

DISCUSSION

Findings of the present study support *G. lucidum* as a source of chemopreventive candidate for urothelial cancer. Apoptosis, as measured by morphological and cytometric changes, is evident to be one of the main cause contributing for HUC-PC growth inhibition. In the early phase of apoptosis, the cells bind positively with annexin V, because of the lost of the asymmetry of cell membrane that causes the exteriorization of negatively charged phospholipids - phosphatidylserine (PS) from the inner leaflet. As shown in the results, the process induced by GLE progressed to late apoptosis, irrespective of the presence or absence of ABP, in dose- and time-dependent manner such that the cells were continuously losing the cell membrane integrity and stained positive with DNA specific viability dye –7-amino-actinomycin D (7-AAD). This is the first report that *G. lucidum* suppress pre-malignant urothelial cells by promoting apoptosis. These results are consistent with other reports, that complex extract or pure *Ganoderma* triterpenes induce apoptosis on cancer cell lines, including the hematopoietic cell lines (14), the highly metastic 95-D lung cancer cells (17), the human hepatoma HuH-7 cells (33), and pure triterpene compounds, such as ganoderic acid T and X (17,33), reported to be active in causing apoptosis. Over hundreds of triterpene molecules have been identified in *G. lucidum* (8). In fact, many commercially available *G. lucidum* products contain complex mixtures of triterpenes and polysaccharides as their active ingredients. We, therefore, accentuate the importance of studying a re-extracted fraction of active ingredient rich rather than a selected pure compound for apoptotic activity.

The inhibitory effect of *G. lucidum* on telomerase activity is also demonstrated in the present study. Both the basal and ABP-induced telomerase activities were significantly reduced to similar levels, in dose-dependent manner. The GLE-induced telomerase inhibition is shown to be directly proportional to the growth inhibitory effect after 48 hour-incubation. Inhibition of telomerase has been shown to limit human cancer cell growth by disrupting telomere maintenance (34). Human tumor cells with shortened telomeres are effectively and rapidly killed after inhibiting the telomerase enzyme (35). Interestingly, according to the Fenton's reaction, H_2O_2 is being dissociated to generate hydroxyl radicals that are able to oxidize C-8 position of G bases to form 8-OHdG (36). In the present study, the increment of H_2O_2 concentration could be a possible mechanism that attacks the telomere repeats (TTAAGGG) of the telomeres, to generate 8-OHdG (dose-dependent) and accelerate telomere shortening, which is unlikely to be compensated when telomerase activity is being suppressed.

Although oxidative DNA damage, in particular 8-OHdG is widely accepted to be mutagenic that promotes carcinogenesis, it has been also proposed as a therapeutic strategy through induction of cell cycle arrests (37-39). It is well-known that G_2-M transition of the cell cycle is a critical checkpoint to verify genomic instability, by blocking DNA-damaged cells to enter mitosis (40). Some chemotherapeutic agents are genotoxic to the DNA during the synthesis (S) phase of cell cycle to cause the G_2-M arrest (41,42). For *G. lucidum*, the regulation of G_2-M related proteins have been uncovered recently to suppress cell growth of prostate cancer (13) and hepatoma (43). In the study of Lu et al, only 6-10% of the HUC-PC cells undergoing G_2-M phase cell

cycle arrest were induced by *G. lucidum*, which was insufficient to explain the exhibited growth inhibition (19). A combination of apoptosis and oxidative DNA damage associated with telomerase inhibition as demonstrated in this study are useful to explain significant growth inhibition by GLE on pre-tumorigenic HUC-PC cells and other cancer cells.

The worthiness of using cells in pre-cancerous stage is particularly important for chemoprevention. Nothing is better than eliminating the adverse cells before it turns malignant, especially looking at bladder carcinoma where rate of recurrences remain exceptionally high even after complete transurethral resection. The non-tumorigenic but ABP-transformable characteristic of HUC-PC cell line provides an excellent model for studying the chemoprevention of bladder cancer, since majority of bladder cancer are diagnosed as TCC at presentation and residual cells after resection are regarded as the key stimulus factor for recurrence. The superficial mucosal urothelial lining is targeted for attack by carcinogens. The HUC-PC cell line has been proven to be sensitive to ABP for tumorigenic transformation (20). It was demonstrated by several investigators that the exposure of ABP and its metabolites to HUC-PC cells caused changes in proteomic profile (44), alteration of F/G-actin ratio (45), formation of DNA adducts (46), and instability of the genome (47). In addition, Hahn and associates (26) have shown that direct tumorigenic conversion of normal human epithelial cells can be achieved by combining ectopic expression of human telomerase catalytic subunit with SV40 large-T oncoprotein. Therefore, the enhancement of telomerase activity and cell proliferation demonstrated in the present study, provides evidence that the carcinogenic transformation of HUC-PC cells requires telomerase activation.

Conclusively, data presented in the current study provide mechanistic insight on the chemopreventive effects of *G. lucidum* on pre-cancerous human urothelial cells. Growth inhibition induced by GLE is mediated via apoptosis associated with suppression of telomerase activity and oxidative DNA damage. Our findings support *G. lucidum* is a source of chemopreventive candidate for bladder cancer. Detailed evaluations are essential before positioning it into the category of chemoprevention. Last but not the least, studies of immunological events including chemotaxis and phagocytes are ongoing to define the efficacy and effectiveness of *G. lucidum* on pre-malignant cell clearance.

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REFERENCES

1. Kassouf W, Kamat AM: Current state of immunotherapy for bladder cancer. *Expert Rev Anticancer Ther* **4,** 1037-1046, 2004.

2. Schenkman E, Lamm DL: Superficial bladder cancer therapy. *ScientificWorldJournal* **28**, 387-399, 2004.

3. Strickland PT, Kensler TW: Environmental factors. In: *Clinical Oncology*, 3 ed. Abeloff MD, Armitage JO, Niederhuber JE, Kastan MB, Mckenna WG (eds). USA: Elsevier: Churchill Livingstone, 2004, pp173-189..

4. Marcus PM, Hayes RB, Vineis P, Garcia-Closas M, Caporaso NE, et al: Cigarette smoking, N-acetyltransferase 2 acetylation status, and bladder cancer risk: A case-series meta-analysis of a gene-environment interaction. *Cancer Epidemiol Biomarkers Prevention* **9**, 461-7, 2000.

5. Oosterlinck W: The management of superficial bladder cancer. *BJU Int* **87**, 135-140, 2001.

6. Ludwig AT, Moore JM, Luo Y, Chen X, Saltsgaver NA, et al: Tumor necrosis factor-related apoptosis-inducing ligand: a novel mechanism for Bacillus Calmette-Guerin-induced antitumor activity. *Cancer Res* **64**, 3386-3390, 2004.

7. Chen AW, Miles PG: Biomedical research and the application of the mushroom nutriceuticals from Ganoderma lucidum. In *Mushroom Biology and Mushroom Products*, Royse DJ (eds). Hong Kong: Chinese University Press, 1996, pp 161-75.

8. Yuen JWM, Gohel MDI: Anticancer effects of Ganoderma lucidum: A review of scientific evidence. *Nutr Cancer* **53**, 11-17, 2005.

9. Sliva D: Ganoderma lucidum (Reishi) in cancer treatment. *Integr Cancer Ther* **2**, 358-364, 2003.

10. Min BS, Gao JJ, Nakamura N, Hattori M: Triterpenes from the spores of Ganoderma lucidum and their cytotoxicity against Meth-A and LLC tumor cells. *Chem Pharma Bull* **48**, 1026-1033, 2000.

11. Xie JT, Wang CZ, Wicks S, Yin JJ, Kong J: Ganoderma lucidum extract inhibits proliferation of SW 480 human colorectal cancer cells. *Exp Oncol* **28**, 25-29, 2006.

12. Hu H, Ahn NS, Yang X, Lee YS, Kang KS: Ganoderma lucidum extract induces cell cycle arrest and apoptosis in MCF-7 human breast cancer cell. *Int J Cancer* **102**, 250-253, 2002.

13. Jiang JH, Slivova V, Harvey K, Valachovicova T, Sliva D: Ganoderma lucidum suppresses growth of breast cancer cells through the inhibition of Akt/NF-kappa B signaling. *Nutrn Cancer* **49**, 209-216, 2004.

14. Muller CI, Kumagai T, Kelly JO, Seeram NP, Heber D: Ganoderma lucidum causes apoptosis in leukemia, lymphoma and multiple myeloma cells. *Leukemia Res* **30**, 841-848, 2006.

15. Kim KC, Kim JS, Son JK, Kim IG: Enhanced induction of mitochondrial damage and apoptosis in human leukemia HL-60 cells by the Ganoderma lucidum and Duchesnea chrysantha extracts. *Cancer Lett* **246**, 210-217, 2006.

16. Cao QZ, Lin ZB: Ganoderma lucidum polysaccharides peptide inhibits the growth of vascular endothelial cell and the induction of VEGF in human lung cancer cell. *Life Sci* **78**, 1457-1463, 2006.

17. Tang W, Liu JW, Zhao WM, Wei DZ, Zhong JJ: Ganoderic acid T from Ganoderma lucidum mycelia induces mitochondria mediated apoptosis in lung cancer cells. *Life Sci* **80**, 205-211, 2006.

18. Gu YH, Belury MA: Selective induction of apoptosis in murine skin carcinoma cells (CH72) by an ethanol extract of Lentinula edodes. *Cancer Lett* **220**, 21-28, 2005.

19. Lu QY, Jin YS, Heber D, Rao JY: Ganoderma lucidum extracts inhibit growth and induce actin polymerization in bladder cancer cells in vitro. *FASEB J* 18, A216, 2004.

20. Bookland EA, Swaminathan S, Oyasu R, Gilchrist KW, Lindstrom M: Tumorigenic transformation and neoplastic progression of human uroepithelial cells after exposure in vitro to 4-aminobiphenyl or its metabolites. *Cancer Res* **52**, 1606-1614, 1992.

21. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP: Extension of life-span by introduction of telomerase into normal human cells. *Science* **279**, 349-352, 1998.

22 Greider CW: Telomerase activity, cell proliferation, and cancer. *Proc Natl Acad Sci* **95**, 90-92, 1998.

23. Holt SE, Shay JW: Role of telomerase in cellular proliferation and cancer. *J Cell Physiol* **180**, 10-18, 1999.

24. Shay JW: Senescence and immortalization: Relationships to telomeres and telomerase. *FASEB J* **20**, A890-A891, 2006.

25. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD: Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011-2015, 1994.

26. Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW: Creation of human tumour cells with defined genetic elements. *Nature* **29**, 464-468, 1999.

27. Sanchini MA, Gunelli R, Nanni O, Bravaccini S, Fabbri C, et al: Relevance of urine telomerase in the diagnosis of bladder cancer. *JAMA* **294**, 2052-2056, 2005.

28. Heine B, Hummel M, Muller M, Heicappell R, Miller K: Non-radioactive measurement of telomerase activity in human bladder cancer, bladder washings, and in urine. *J Pathol* **184**, 71-76, 1998.

29. Steinberg JR, Dinney CPN, Grossman HB: Screening, early detection, and prevention of bladder cancer. In *Comprehensive Textbook of Genitourinary Oncology*, Vogelzang NJ, Scardino PT, Shipley WU, Debruyne FMJ, Linehan WM (eds), 3 ed, Philadelphia, Baltimore, New York, London, Buenos Aires, Hong Kong, Sydney, Tokyo: Lippincott Williams & Wilkins, 2006, pp403-410.

30. Smith LL, Coller HA, Roberts JM: Telomerase modulates expression of growth-controlling genes and enhances cell proliferation. *Nat Cell Biol* 5, 474-479, 2003.

31. Hou M, Xu D, Bjorkholm M, Gruber A: Real-time quantitative telomeric repeat amplification protocol assay for the detection of telomerase activity. *Clin Chem* **47**, 519-524, 2001.

32. Yuen JWM, Benzie IFF: Hydrogen peroxide in urine as a potential biomarker of whole body oxidative stress. *Free Rad Res* **37**, 1209-1213, 2003.

33. Li CH, Chen PY, Chang UM, Kan LS, Fang WH, Tsai KS, Lin SB. Ganoderic acid X, a lanostanoid triterpene, inhibits topoisomerases and induces apoptosis of cancer cells. *Life Sci* **77**, 252-265, 2005.

34. Hahn WC, Stewart SA, Brooks MW, York SG, Eaton E: Inhibition of telomerase limits the growth of human cancer cells. *Nat Med* **5**, 1164-1170, 1999.

35. Zhang X, Mar V, Zhou W, Harrington L, Robinson MO:Telomere shortening and apoptosis in telomerase-inhibited human tumor cells. *Genes Dev* **13**, 2388-2399, 1999.

36. Aust AE, Eveleigh J: Mechanisms of DNA oxidation. *Proc Soc Exp Biol Med* **222**, 246-252, 1999.

37. Kamata H, Hirata H: Redox regulation of cellular signalling. *Cell Signal* **11**, 1-14, 1999.

38. Duan J, Duan J, Zhang Z, Tong T: Irreversible cellular senescence induced by prolonged exposure to H_2O_2 involves DNA-damage-and-repair genes and telomere shortening. *Int J Biochem Cell Biol* **37**, 1407-1420, 2005.

39. Valko M, Izakovic M, Mazur M, Rhodes CJ, Telser J: Role of oxygen radicals in DNA damage and cancer incidence. *Mol Cell Biochem* **266**, 37-56, 2004.

40 Foijer F, Te RH. Check: double check: the G2 barrier to cancer. *Cell Cycl*e **5**, 831-836, 2006.

41. Pan J, She M, Xu ZX, Sun L, Yeung SC: Farnesyltransferase inhibitors induce DNA damage via reactive oxygen species in human cancer cells. *Cancer Res* **65**, 3671-3681, 2005.

42. Preisler VK, Wuck D, Stopper H: Combination of paclitaxel and radiation: genotoxicity in vitro in four mammalian cell lines. *Cancer Lett* **145**, 29-33, 1999.

43. Lin SB, Li CH, Lee SS, Kan LS: Triterpene-enriched extracts from Ganoderma lucidum inhibit growth of hepatoma cells via suppressing protein kinase C, activating mitogen-activated protein kinases and G2-phase cell cycle arrest. *Life Sci* **72**, 2381-2390, 2003.

44. Kanitz MH, Swaminathan S, Savage RE, Jr: Two-dimensional electrophoretic protein profile analysis following exposure of human uroepithelial cells to occupational bladder carcinogens. *Cancer Lett* 205, 121-131, 2004.

45. Rao JY, Hurst RE, Bales WD, Jones PL, Bass RA: Cellular F-actin levels as a marker for cellular transformation: relationship to cell division and differentiation. *Cancer Res* **50**, 2215-2220, 1990.

46. Swaminathan S, Frederickson SM, Hatcher JF, Reznikoff CA, Butler MA, et al: Neoplastic transformation and DNA-binding of 4,4'-methylenebis(2-chloroaniline) in SV40-immortalized human uroepithelial cell lines. *Carcinogenesis* **17**, 857-864, 1996.

47. Kao C, Wu SQ, DeVries S, Reznikoff WS, Waldman FM, et al: Carcinogen-induced amplification of SV40 DNA inserted at 9q12-21.1 associated with chromosome breakage, deletions, and translocations in human uroepithelial cell transformation in vitro. *Genes Chromosomes Cancer* 8, 155-166, 1993.

Table 1. Total viable cell count and growth inhibition measured after 48 hours incubation (n=3) with GLE together with ABP.

	Exposure (48 hrs)		Mean ± SEM	
	ABP (μM)	GLE (μg/ml)	Viable cell number (x 10^6) ¶	%GI
I	0	0	1.95 ± 0.07	-
II	0	80	0.97 ± 0.05	104 ± 5 † ***
III	100	0	2.26 ± 0.12	-
IV	100	40	1.56 ± 0.07	56 ± 5 ‡ ***
V	100	80	1.06 ± 0.06	96 ± 5 ‡ ***
VI	100	100	0.48 ± 0.04	141 ± 3 ‡ ***

¶ Initial cell seeding concentration at 1 x 10^6 cells / 100-mm dish. † (I) was used as control for calculation. ‡ (II) was used as control for calculation. *** $P<0.001$.

Table 2. Total viable cell count and growth inhibition after 48 hours incubation on ABP-pretreated cells (n=3).

	Exposure		Mean ± SEM (n=3) Viable cell number (x 10^6)	
	24 hrs #	48 hrs ##	¶	%GI
I	Media	Media	3.40 ± 0.20	-
II	Media	80 µg/ml GLE	2.45 ± 0.05	40 ± 2 † *
III	ABP	Media	4.15 ± 0.22	-
IV	ABP	40 µg/ml GLE	2.96 ± 0.23	38 ± 7 ‡ **
V	ABP	80 µg/ml GLE	2.40 ± 0.12	56 ± 4 ‡ ***
VI	ABP	100 µg/ml GLE	0.61 ± 0.04	113 ± 1 ‡ ***

¶ Initial cell seeding concentration at 1 x 10^6 cells / 100-mm dish. # Pre-treatment of either 100 µM ABP or complete medium. ## Post-treatment for 48 hours. † (I) was used as control for calculation. ‡ (II) was used as control for calculation. * P<0.05; ** P<0.01; ***P<0.001.

Figure 1.

Photographs of HUC-PC taken after 48-hour culture with (a) complete media; (b) 100 μM ABP in complete media; (c) 80 μg/ml GLE in complete media; and (d) 100 μM ABP + 80 μg/ml GLE in complete media. Magnification: 100x

Yuen Fig. 1

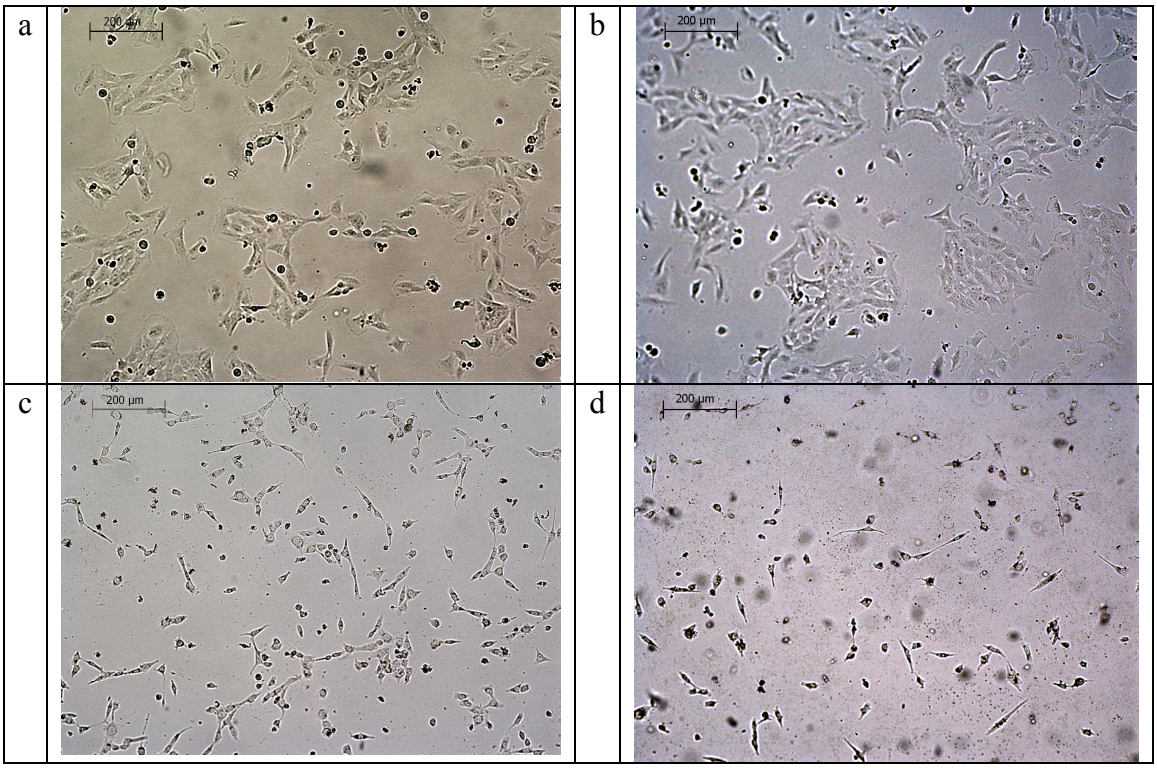
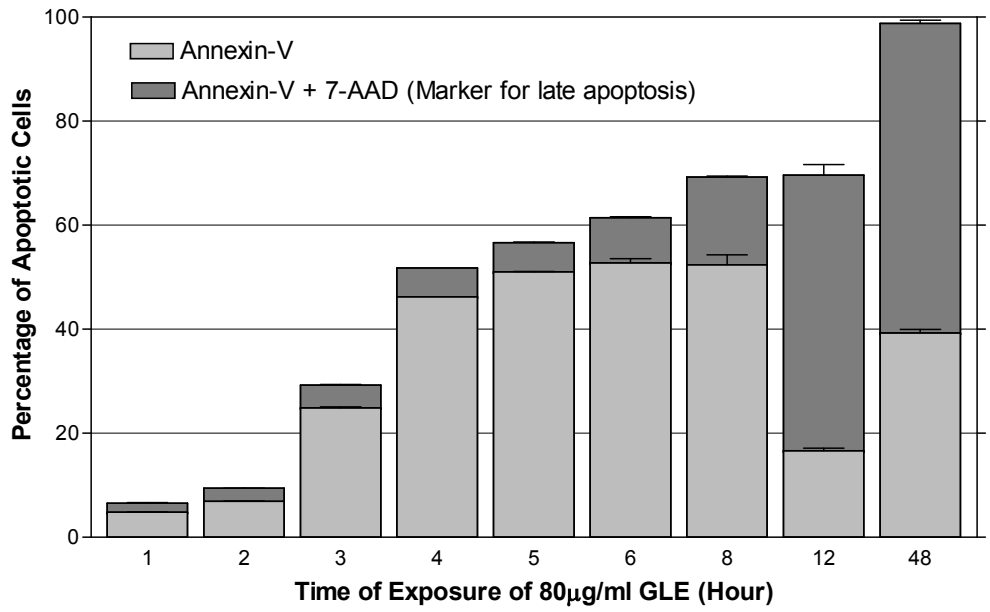


Figure 2.

(a) The progress of apoptosis induced by 80 µg/ml GLE. Annexin-V binds specially to phosphatidylserine on the cell surface when the asymmetry of membrane phospholipids was lost at early apoptosis. 7-AAD is a DNA specific viability dye binds DNA guanine-cytosine base pair when membrane integrity of the cells were lost at late apoptosis. **(b)** Biparametric histogram LOG PMT2 (525nm) vs LOG PMT4 (675nm) shows the progression of apoptosis at important time points.

Yuen Fig. 2

a


Annexin-V
Annexin-V + 7-AAD (Marker for late apoptosis)
Percentage of Apoptotic Cells
0
20
40
60
80
100
1
2
3
4
5
6
8
12
48
Time of Exposure of 80μg/ml GLE (Hour)

b

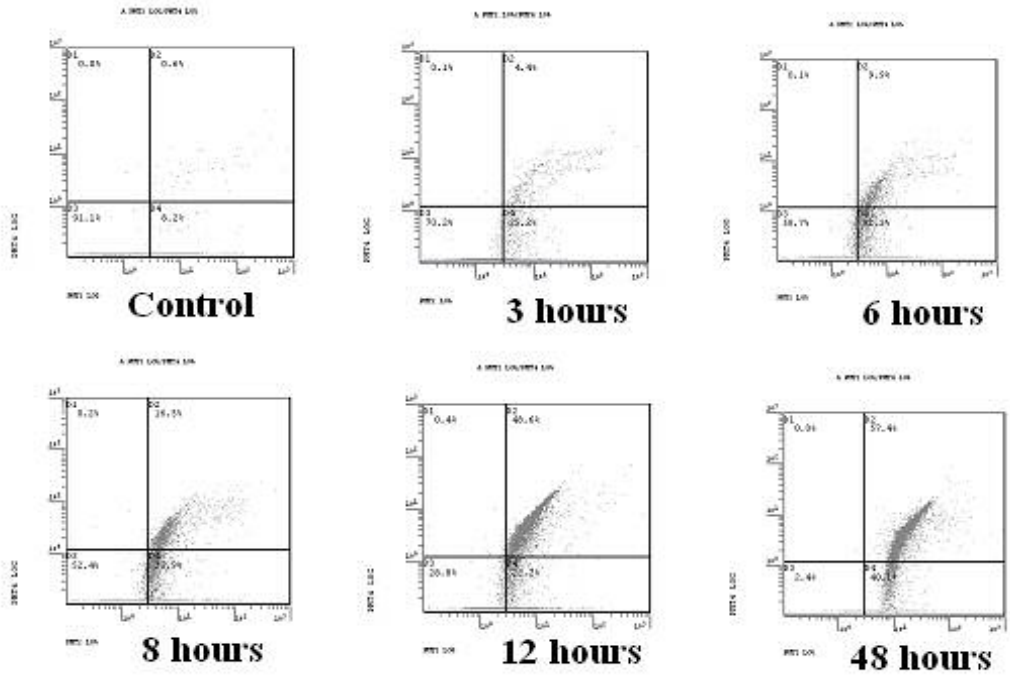

Control
3 hours
6 hours
8 hours
12 hours
48 hours

Figure 3.

Apoptotic events induced by various concentrations of GLE on SV-HUC-PC treated simultaneously with 100 μM of ABP for 48 hours. For all concentrations, almost all cells turned into apoptotic, with small dose-dependent effects, after 48-hour incubation. >60% of cells treated with 40 μg/ml of GLE were at early stage of apoptosis (Annexin-V +ve & 7-AAD –ve phenotype). However, about 60% and >60% of the cells were at late apoptosis (Annexin-V & 7-AAD +ve phenotype) when treated with 80 μg/ml and 100 μg/ml GLE, respectively.

Yuen Fig. 3

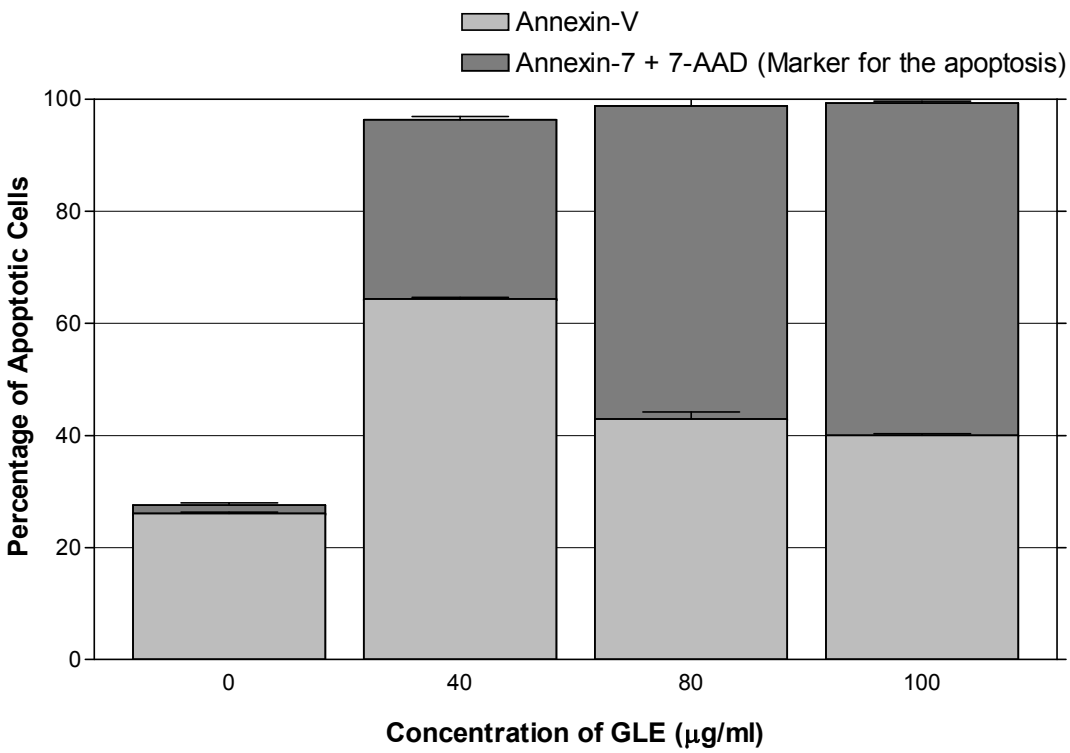

Annexin-V
Annexin-7 + 7-AAD (Marker for the apoptosis)
Percentage of Apoptotic Cells
100
80
60
40
20
0
0
40
80
100
Concentration of GLE (μg/ml)

Figure 4.

(a) Relative telomerase activity was compared, media control vs 80 μg/ml GLE; ***P<0.001, and 100 μM ABP vs 100 μM ABP + 80 μg/ml GLE; ***P<0.001. **(b)** Showing the relative telomerase activity inhibited by GLE (at 0, 40, 80, 100 μg/ml), *** P<0.001, n=2. **(c)** Relative telomerase activity plotted against cell growth inhibition percentage induced by GLE of Reishi (Pearson's correlation r = -0.99, P<0.01).

Yuen Fig. 4

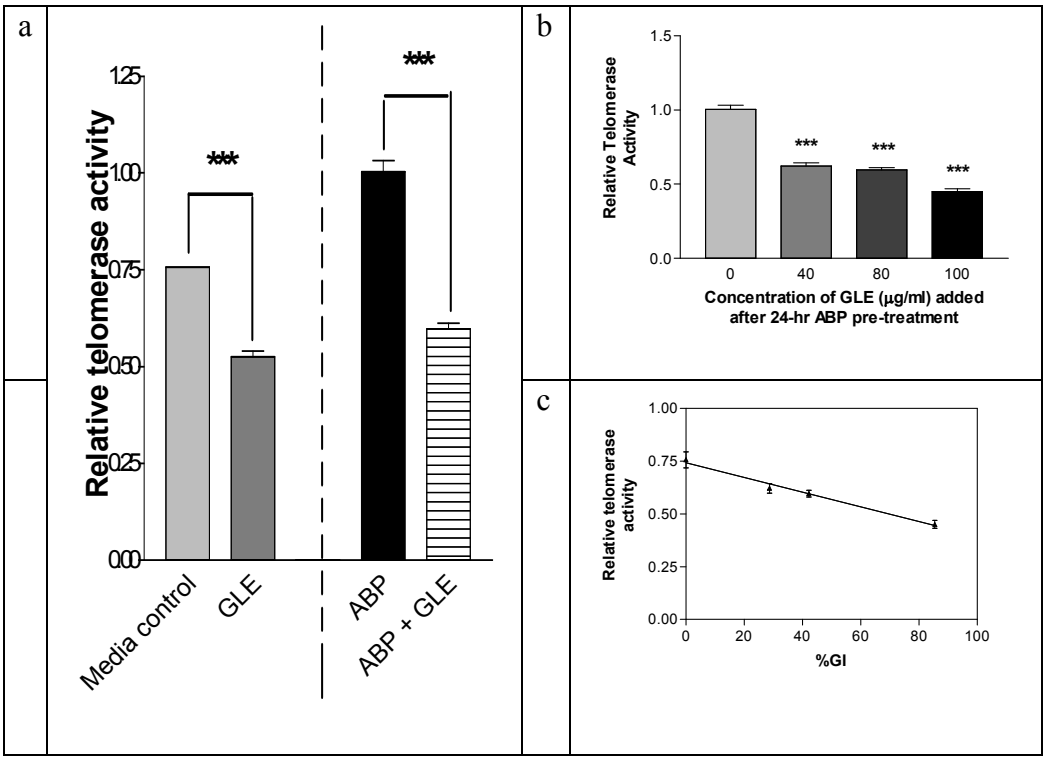
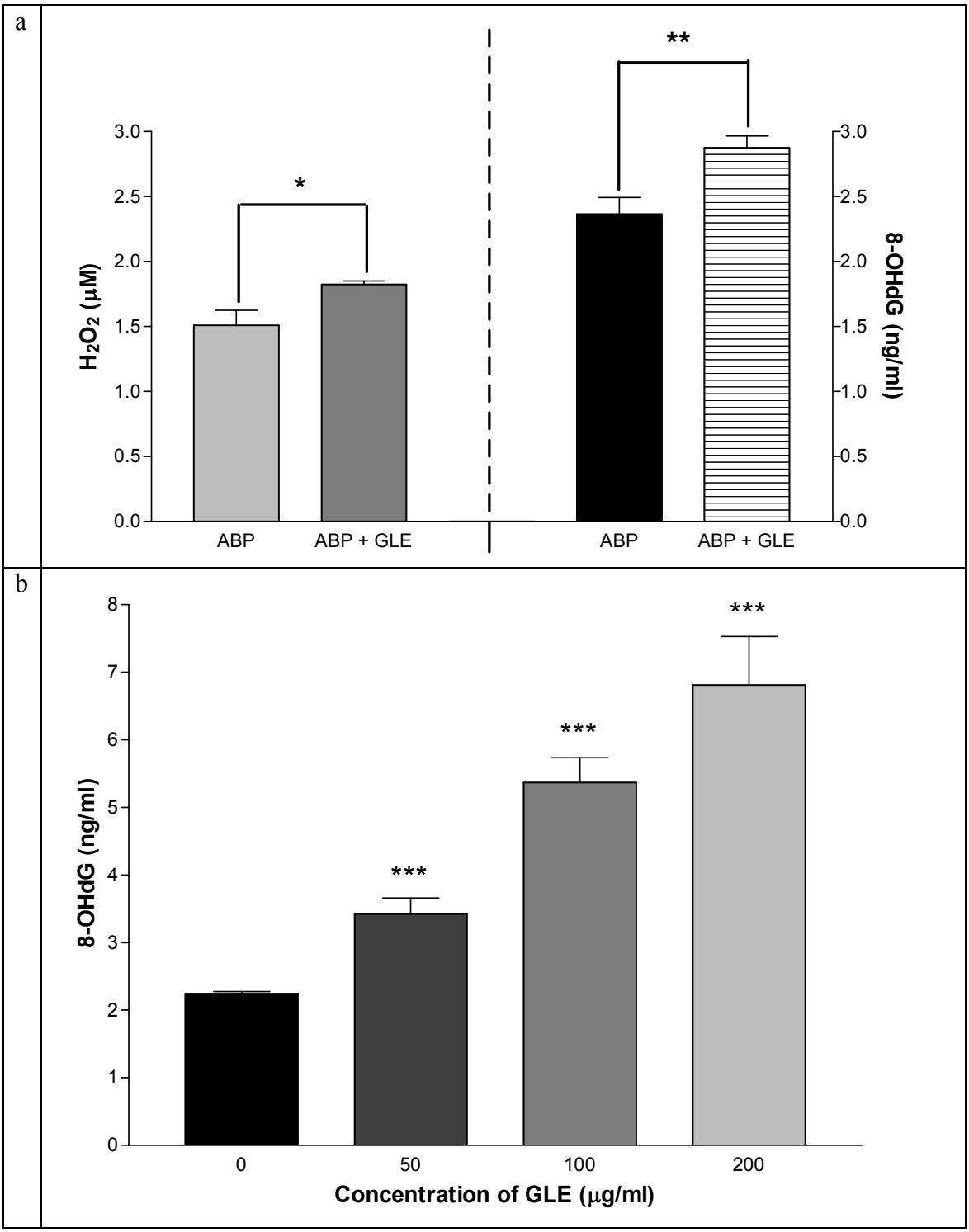

a
Relative telomerase activity
1.25
1.00
0.75
0.50
0.25
0.00
***
***
Media control
GLE
ABP
ABP + GLE
b
Relative Telomerase Activity
1.5
1.0
0.5
0.0
***
***
***
0
40
80
100
Concentration of GLE (µg/ml) added after 24-hr ABP pre-treatment
c
Relative telomerase activity
1.00
0.75
0.50
0.25
0.00
0
20
40
60
80
100
%GI

Figure 5.

H_2O_2 and 8-OHdG formation in culture media were compared, ABP(100 μM) vs ABP(100 μM)+ GLE(80 μg/ml), * P<0.05; ** P<0.01, n=6. **(b)** The dose-dependent trend as shown for the 8-OHdG formation induced by GLE (at 0, 50, 100, 200 μg/ml), *** P<0.001, n=6.

Yuen Fig. 5


a
**
*
3.0
2.5
2.0
1.5
1.0
0.5
0.0
H2O2 (μM)
8-OHdG (ng/ml)
ABP
ABP + GLE
ABP
ABP + GLE
b
***
***
***
8
7
6
5
4
3
2
1
0
8-OHdG (ng/ml)
0
50
100
200
Concentration of GLE (μg/ml)