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Research Article
Acidified Bile Acid-Induced ROS Facilitates Stemness and Proliferation of Gastric Cancer Cells

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Abstract

Background and Objective: Acidified bile acids facilitate the growth of gastric cancer (GC) by cancer Myelocytomatosis (c-Myc). Reactive oxygen species (ROS) produced during oxidative stress in the body are closely related to tumor development, so the research aims to elucidate whether acidified bile acids regulate GC progression in a ROS-dependent manner. **Materials and Methods:** The GC cells were exposed to bile salts (100 μM chenodeoxycholic acid and deoxycholic acid) in acidic medium (pH = 5.5) for 10 min per day over 60 weeks to mimic the acidified bile acid environment *in vitro*. Cell proliferation and stemness were estimated by 5-ethynyl-2'-deoxyuridine and sphere-forming assays. Some genes were analyzed at the mRNA or protein levels by reverse transcription-quantitative polymerase chain reaction and western blotting. The ROS production and telomerase activity were detected by the fluorescence probe dihydroethidium and telomere repeat amplification protocol. Elimination of ROS was performed with N-acetyl-L-cysteine (NAC) to evaluate the function of ROS. **Results:** Acidified bile acids facilitated GC cell proliferation and stemness, accompanied by increased protein levels of PCNA, Oct4 and CD44. Acidified bile acids induced ROS production, along with elevated mRNA levels of Telomerase Reverse Transcriptase (TERT), protein levels of c-Myc and telomerase activity. The NAC treatment reversed acidified bile acids-mediated effects on the proliferation, stemness and ROS production of GC cells, with accompanying changes in TERT mRNA levels, c-Myc protein levels and telomerase activity. **Conclusion:** Acidified bile acids enhanced telomerase activity through c-Myc in a ROS-dependent manner, thereby contributing to GC cell proliferation and stemness.

Key words: Acidified bile acids, reactive oxygen species, telomerase, stemness, gastric cancer, c-Myc

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

As the fifth most common cancer, gastric cancer (GC) remains a major global health problem and a long-term focus of medical research[1]. The GC may present with asymptomatic or atypical symptoms in the early stages, which often results in delayed diagnosis and poses significant treatment challenges[2]. The occurrence of GC originating from the gastric epithelium is inseparable from the gastric juice environment in the gastric cavity[3]. Bile is a digestive fluid in the intestinal tract, which can reflux into the stomach in pathological conditions such as gastroduodenal motility disorders or post-surgical changes in the anatomy of the gastroduodenal region[4,5]. Although bile reflux is not prominently associated with atrophic gastritis, high concentrations of bile acids in the stomach are associated with a high risk of gastrointestinal epithelial metaplasia which is a precancerous lesion of a type of GC[6].

Bile acids, products of cholesterol metabolism, are involved in food digestion and lipolysis[7]. Also, bile acids work as signaling molecules involved in the regulation of cellular biological functions, such as metabolism, nuclear receptor activation and epigenetic regulation[8]. Bile acids have amphiphilic properties. Regarding hydrophobic bile acids ((such as chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA)), they exhibit cytotoxicity and elicit cancer susceptibility factors[9,10]. Currently, studies on bile acids in GC are more inclined to investigate the mechanisms by which DCA or CDCA affect tumorigenesis[11-13], while fewer studies have been conducted on the mechanisms of GC progression. Interestingly, DCA has been reported to inhibit the growth of GC cells BGC-823 by controlling the p53-mediated pathway[14]. Also, DCA prompted the apoptosis of GC cell SGC-7901 through a mitochondria-dependent pathway[15]. However, CDCA elevated the invasive ability of GC cells by activating protein kinase C signaling and inducing cyclooxygenase-2 expression[16]. Notably, refluxed bile acids in an acidic gastric environment (pH 4-6) can form acidified bile acids, leading to injury of the gastroesophageal mucosa. The previous studies established the promoting effect of acidified bile acids on GC cell invasion and migration[17,18], but the mechanism of GC development promoted by acidified bile acids is not clear.

In response to various harmful stimuli, the body undergoes oxidative stress and causes tissue damage, which is associated with an imbalance between oxidative and anti-oxidative effects caused by excessive production of reactive oxygen species (ROS) in the body[19]. The ROS are the major free radicals generated by oxidative stress in tissues where they participate in lipid peroxidation, DNA damage, protein destruction, immune response, stem cell self-renewal and tumor metastasis[20]. The ROS can cause mitochondrial dysfunction by oxidizing the inner mitochondrial membrane, thereby participating in cancer cell glycolysis[21]. Earlier studies suggested that acidified bile acids-induced pathogenesis in Barrett's esophagus may be associated with oxidative stress and DNA damage in esophageal epithelial cells elicited by it[22-24]. Of concern, prolonged exposure to bile acids can increase ROS levels in GC cells and promote GC progression. The previous studies identified that acidified bile acids could promote GC progression through the cancer myelocytomatosis (c-Myc)/Telomerase Reverse Transcriptase (TERT) pathway[17,18]. However, whether acidified bile acids can mediate gastric carcinogenesis by acting on c-Myc via ROS is not clear. Therefore, the study aimed to investigate whether acidified bile acids are dependent on ROS-mediated c-Myc to regulate GC cell proliferation and stemness, providing new evidence to further elucidate the progression of GC.

MATERIALS AND METHODS

Study area: This study was performed at the Zibo Central Hospital from September, 2022 to February, 2024.

Cell culture: Two human GC cell lines MKN-45 (CAT#iCell-h345, iCell, Shanghai, China) and HGC-27 (CAT#C6365, Beyotime, Shanghai, China) were cultured in RPMI-1640 medium (CAT# iCell-0002, iCell) supplemented with 10% fetal calf serum (CAT# 10091148, Gibco, Thermo, Waltham, Massachusetts, USA) and 1% antibiotics (CAT# iCell-15140-122, iCell) in a humid atmosphere at 37°C with 5% CO_2.

Cell treatment: Bile acids CDCA (A) (CAT# 700198P, Sigma, St. Louis, Missouri, USA) and DCA (B) (CAT# 30960, Sigma) were dissolved in dimethyl sulfoxide. The N-acetyl-L-cysteine (NAC) (CAT# A0737, Sigma) was dissolved in PBS at 100 mM. To simulate an acidified bile acid environment *in vitro*, MKN-45 and HGC-27 cells were cultured in an acidified growth media (pH = 5.5) containing A (100 μM) and B (100 μM). Following the previously optimized conditions[17], MKN-45 and HGC-27 cells were chronically exposed to acidified bile acids for 60 weeks at 10 min per day. Those cultured in a neutral RPMI-1640 medium (pH = 7.4) were set as control cells. For NAC treatment, MKN-45 cells were pretreated with NAC (5 mM) for 1 hr[25], followed by stimulation with acidified bile acids.

5-ethynyl-2'-deox-yuridine (Edu) incorporation assay: Assessment of the proliferative ability of GC cells was done using the BeyoClick™ EdU-488 kit (CAT# C0071S, Beyotime) following the manufacturer's instructions. In brief, the cultured cells were incubated with the Edu solution (10 mM) for 2 hrs. After fixation with 4% paraformaldehyde (CAT# P0099, Beyotime), the cells were permeabilized with 0.3% Triton X-100 (CAT# P0096, Beyotime) for 15 min, followed by incubation with the click addictive solution (50 µL) for 30 min. Finally, the cells were stained with the Hoechst 33342 (10 µg/mL) and the proliferative cells were observed under inverted fluorescence microscopy (IX71; Olympus, Japan).

Western blotting: Protein samples of GC cells in different groups were isolated with RIPA buffer containing phosphatase and proteinase inhibitors (CAT# K10034, KeyGen, Beijing, China). Determination of protein concentration was carried out using the enhanced BCA protein assay kit (CAT# P0010S, Beyotime). Electrophoresis was performed to separate equal amounts of proteins on sodium dodecyl sulfate polyacrylamide gel and the isolated proteins were transferred to PVDF membranes (CAT# IPVH15150, Millipore, Darmstadt, Germany). Following blocking with 5% skim milk, the PVDF membranes were probed with primary antibodies, including anti Proliferating Cell Nuclear Antigen (PCNA) (CAT# AF1363, 1:1000, Beyotime), anti CD44 (CAT# AG1491, 1:1000, Beyotime), anti octamer-binding transcription factor 4 (Oct4) (CAT# AF2506, 1:1000, Beyotime), anti-c-Myc (CAT# ab32072, 1:1000, Abcam, Cambridge, California USA) and anti Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) (CAT# AF1186, 1:1000, Beyotime). Afterward, HRP-labeled goat anti-rabbit IgG (CAT# A0208, 1:1000, Beyotime) was utilized as a secondary antibody. After signal development with a chemiluminescence detection system (CAT# P0018S, Beyotime), protein blots were visualized and quantified by ImageJ software (version 1.54, NIH, Bethesda, Maryland, USA).

Sphere-forming assay: For the sphere formation assay, about 1×10^3 GC cells with different treatments were cultured in RPMI-1640 medium supplemented with epidermal growth factor (20 ng/mL) (CAT# E9644, Sigma), basic fibroblast growth factor (20 ng/mL) (CAT# GF003AF, Sigma), 1% B27 (CAT# iCell-12587010, iCell) and 1% methyl cellulose (CAT# 9004-67-5, Cytiva, Shanghai, China) in ultra-low attachment 6-well plates (Corning, New York, USA) for 10 days. The number of spheres formed (diameter >100 µm) was counted manually using an inverted light microscope (Olympus).

ROS detection: Intracellular ROS levels were measured using the fluorescence probe dihydroethidium (DHE) (CAT#S0063, Beyotime). The GC cells with different treatments were collected and incubated with 10 µM DHE for 30 min. Stained cells were washed twice in PBS and fixed in 4% paraformaldehyde for 15 minutes. After the final wash by PBS, cells were treated with DAPI (CAT#iCell-01400, iCell) for 10 min. The cells were observed and photographed with a fluorescence microscope (Olympus). Integrated fluorescence density was analyzed using ImageJ software (version 1.54).

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR): Total RNA of GC cells was isolated using TRIeasy total RNA extraction reagent (CAT#10606ES60, Yeasen, Shanghai, China) according to the manufacturer's guidelines, followed by quantification by reading absorbance values at 260 and 280 nm. Synthesis of complementary DNA was performed using Hifair reverse transcriptase (CAT#11111ES92, Yeasen). The qPCR was run using Hieff qPCR SYBR green master mix (CAT#11202ES03, Yeasen). To analyze the output cycle threshold (Ct) values, the reference housekeeping gene (GAPDH) was used to determine Ct values. Fold changes of TERT mRNA levels were quantified by $2^{-\Delta\Delta Ct}$ method[26].

Telomere repeat amplification protocol (TRAP): Measurement of telomerase activity was carried out using human telomerase activity kit (CAT#15-00003, Bingene, Suzhou, China) following the operating instructions provided with the reagent kit. In short, after washing with pre-chilled PBS, GC cells were lysed with 200 µL of pre-chilled TRAP-specific cell lysis buffer. Telomerase-containing cell supernatants were collected and total protein concentration was determined using a BCA protein assay kit. The total volume of the PCR amplification reaction was set to 20 µL: Ultrapure water (4 µL), probe-based PCR MagicMix (10 µL), human TRAP template-primer-probe mix (4 µL) and cell lysis supernatant (2 µL). The standard curve was drawn with the Ct values of the positive control and internal reference as the vertical axis and then the log value of the DNA concentration of the test sample and its concentration were calculated as their Ct values. Telomerase activity was expressed as relative telomerase activity.

Statistical analysis: The data were expressed as Mean±Standard Error of the Mean (SEM) from triplicates. Values were measured statistically significant at $p<0.05$ by GraphPad Prism (version 9.00, GraphPad, Software, La Jolla, California, USA). Comparisons between two groups were analyzed by an independent two-tailed Student's t-test. Multiple group comparisons were performed by One-way Analysis of Variance (ANOVA) followed by Tukey's *post hoc* test.

RESULTS

Long-time exposure to acidified bile acids enhanced the proliferative ability of GC cells: To elucidate the influence of acidified bile acids on GC cells, GC cells were co-stimulated with bile salts (A+B) under low pH conditions (pH = 5.5) for 60 weeks, per day for 10 min. A schematic representation of GC cells stimulated by acidified bile acids *in vitro* was shown in Fig. 1a. Regarding cell proliferation, long-time stimulation with acidified bile acids markedly strengthened the proliferative ability of GC cells (Fig. 1b). Moreover, an increase in PCNA protein levels accompanied the promotion of GC cell proliferation mediated by acidified bile acids (Fig. 1c). These results suggested that stimulation with acidified bile acids for a long period of time could boost the proliferative capacity of GC cells.

Stimulation with persistent acidified bile acids intensified the stemness of GC cells: To interpret the association between acidified bile acids and GC cell stemness, tumor sphere-forming experiments were then performed. As displayed in Fig. 2a, the sphere-forming capacity of GC cells was prominently boosted post-acidified bile acid stimulation versus the control group. Furthermore, a significant upregulation of the protein levels of the stem cell markers CD44 and Oct4 was detected in GC cells stimulated with acidified bile acids in contrast to the control group (Fig. 2b). Collectively, continuous stimulation with acidified bile acids increased GC cell stemness.

Exposure to acidified bile acids for long periods of time raised ROS levels, c-Myc and TERT expression levels and telomerase activity in GC cells: As a next step, the DHE fluorescent probe was utilized to detect ROS levels for interpreting the relationship between acidified bile acids and ROS in GC cells. The outcomes exhibited that GC cells in the control group kept very low levels of ROS. Differently, acidified bile acids significantly increased ROS levels in GC cells initially, but ROS levels in GC cells were maintained at an intermediate level up to 30 and 60 weeks with acidified bile acid stimulation (Fig. 3a). The reason for this is that the sudden stimulation of GC cells by acidified bile acids generates large amounts of ROS and causes cellular damage, but these cells are able to adapt to acidified bile acids after a series of intracellular adjustments, thus releasing stable and moderate levels of ROS. A previous study showed that acidified bile acids promoted GC invasion and migration through the c-Myc/TERT pathway, so the expression levels of c-Myc and TERT were detected. As expected, c-Myc protein levels in GC cells did not differ from the control group at the beginning of acidified bile acid stimulation, but c-Myc protein levels gradually increased with the duration of stimulation (Fig. 3b). Similarly, there were no differences in TERT mRNA levels in GC cells stimulated with acidified bile acids suddenly compared with control cells, but TERT mRNA levels were progressively elevated with longer stimulation times (Fig. 3c). In addition, the variation tendency of telomerase activity was consistent with the changes in TERT mRNA levels (Fig. 3d). Long-term exposure to acidified bile acids increased ROS levels, c-Myc and TERT expression levels and telomerase activity in GC cells.

Long-time exposure to acidified bile acids induced ROS to confer proliferation and stemness for MKN-45 cells: Considering that long-time exposure to acidified bile acids can raise ROS levels accompanied by increased levels of c-Myc and TERT in GC cells, whether acidified bile acids mediate the proliferation and stemness of GC cells through ROS-mediated the c-Myc/TERT pathway was further evaluated. Since acidified bile acid stimulation-induced ROS overproduction in GC cells, the role of ROS was further elucidated by preincubating MKN-45 cells with NAC (a ROS scavenger that blocks ROS generation) prior to acidified bile acid stimulation, as shown in Fig. 4a. Functional analyses showed that the increase in c-Myc protein levels, TERT mRNA levels and telomerase activity in MKN-45 cells induced by acidified bile acids was significantly impaired by ROS elimination (Fig. 4b-d). Moreover, acidified bile acids-mediated promoting effects on cell proliferation and PCNA protein levels in MKN-45 cells were significantly inhibited upon ROS elimination (Fig. 4e-f). In addition, acidified bile acids-mediated intensification of stemness on MKN-45 cells and upregulation on CD44 and Oct4 protein levels in MKN-45 cells were significantly inhibited in response to ROS abatement (Fig. 4g-h). Together, these results suggested that sustained stimulation with acidified bile acids evoked ROS to confer proliferation and stemness to MKN-45 cells by mediating the c-Myc/TERT pathway.

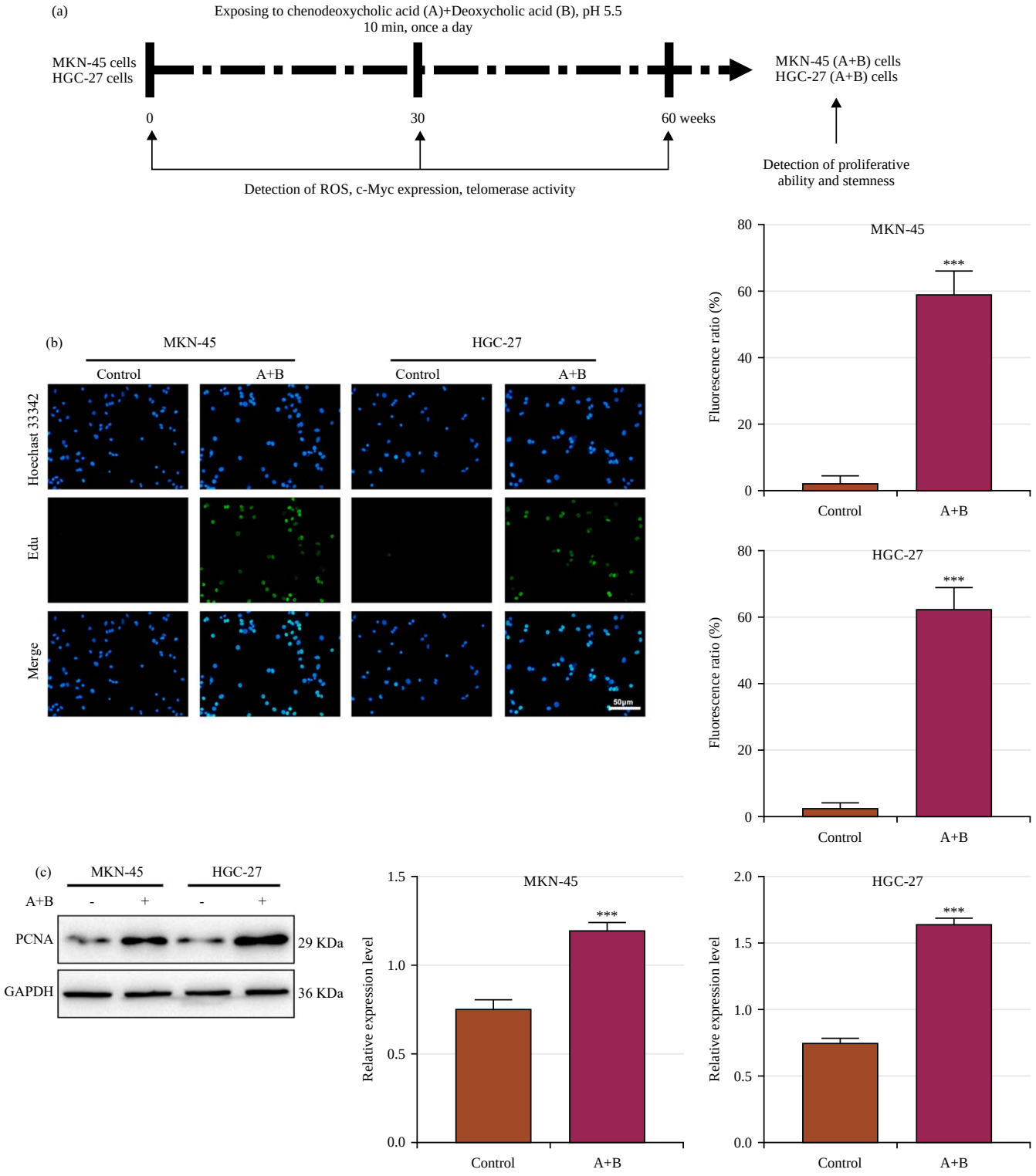

Fig. 1(a-c): Proliferative capability of GC cells was strengthened by stimulation with acidified bile acids for a long time, (a) Schematic representation of an *in vitro* simulation of GC cells subjected to long-term stimulation with acidified bile acids, (b) Edu assays estimated the proliferative capability of GC cells stimulated with acidified bile acids and (c) Relative protein levels of PCNA in GC cells were detected by western blotting

Scale bar: 50 μm. Error bars indicated the Mean±SEM. Statistical significances were assessed using unpaired t-test (***p<0.001 vs control)

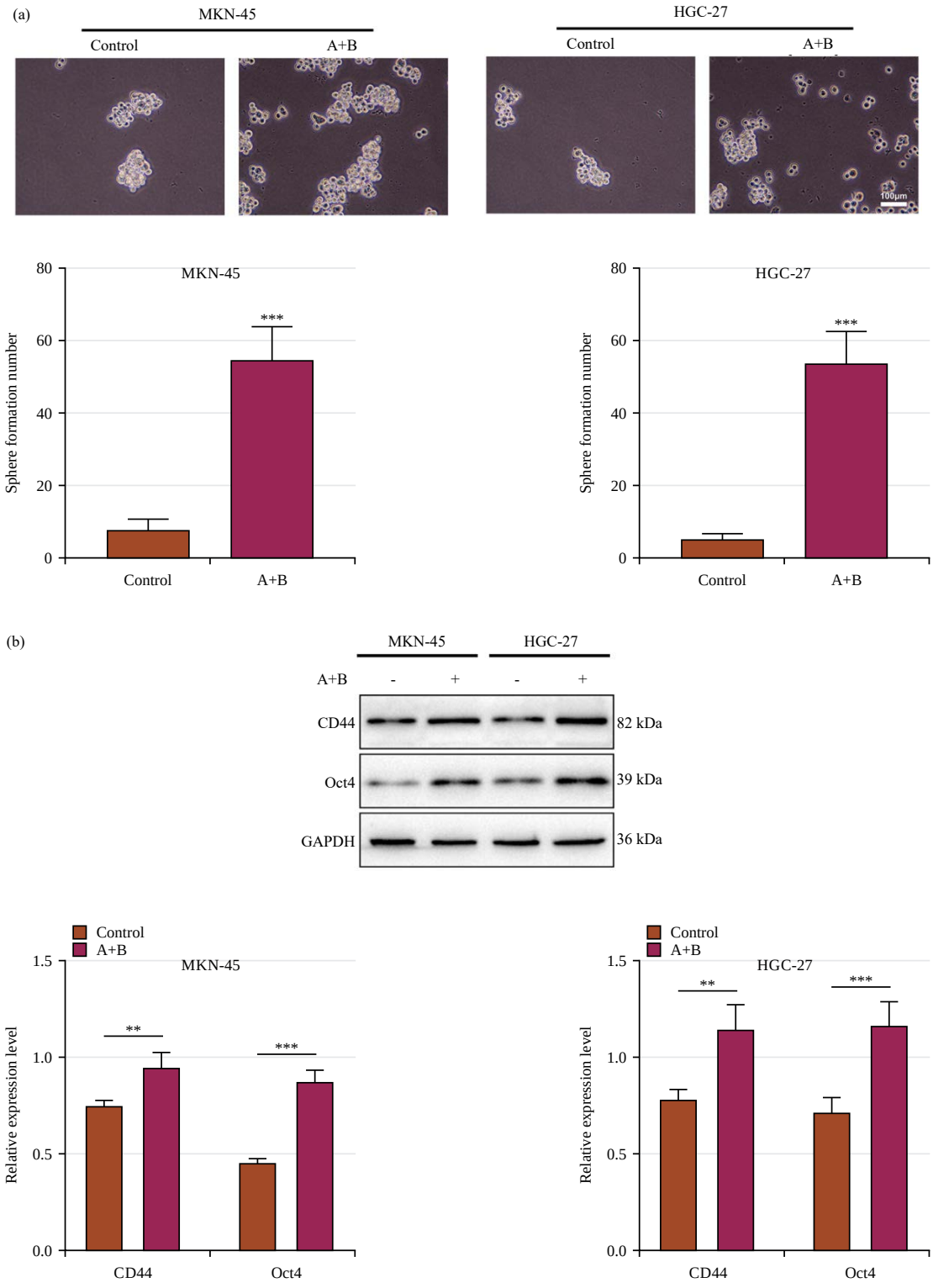

Fig. 2(a-b): Enhancement of GC cell stemness was elicited by sustained stimulation with acidified bile acids, (a) Comparison of the sphere-forming ability of GC cells in control and acidified bile acid groups was done by sphere-forming assays and (b) Comparison of protein levels of stem cell markers CD44 and Oct4 in GC cells was performed by western blotting
Scale bar: 100 μm. Error bars indicated the Mean±SEM. Statistical significances were assessed using unpaired t-test (**p<0.01 and ***p<0.001 vs control)

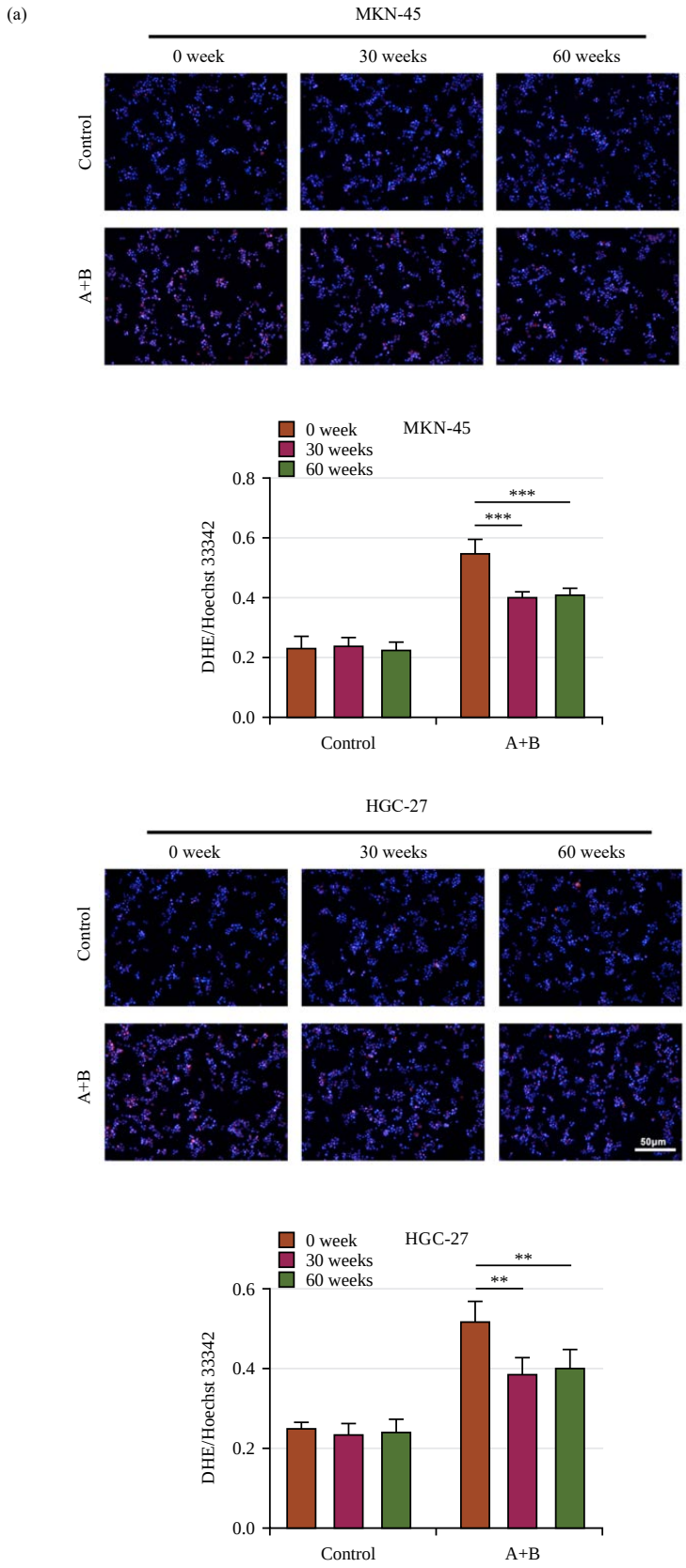

Fig. 3(a-d): Continue

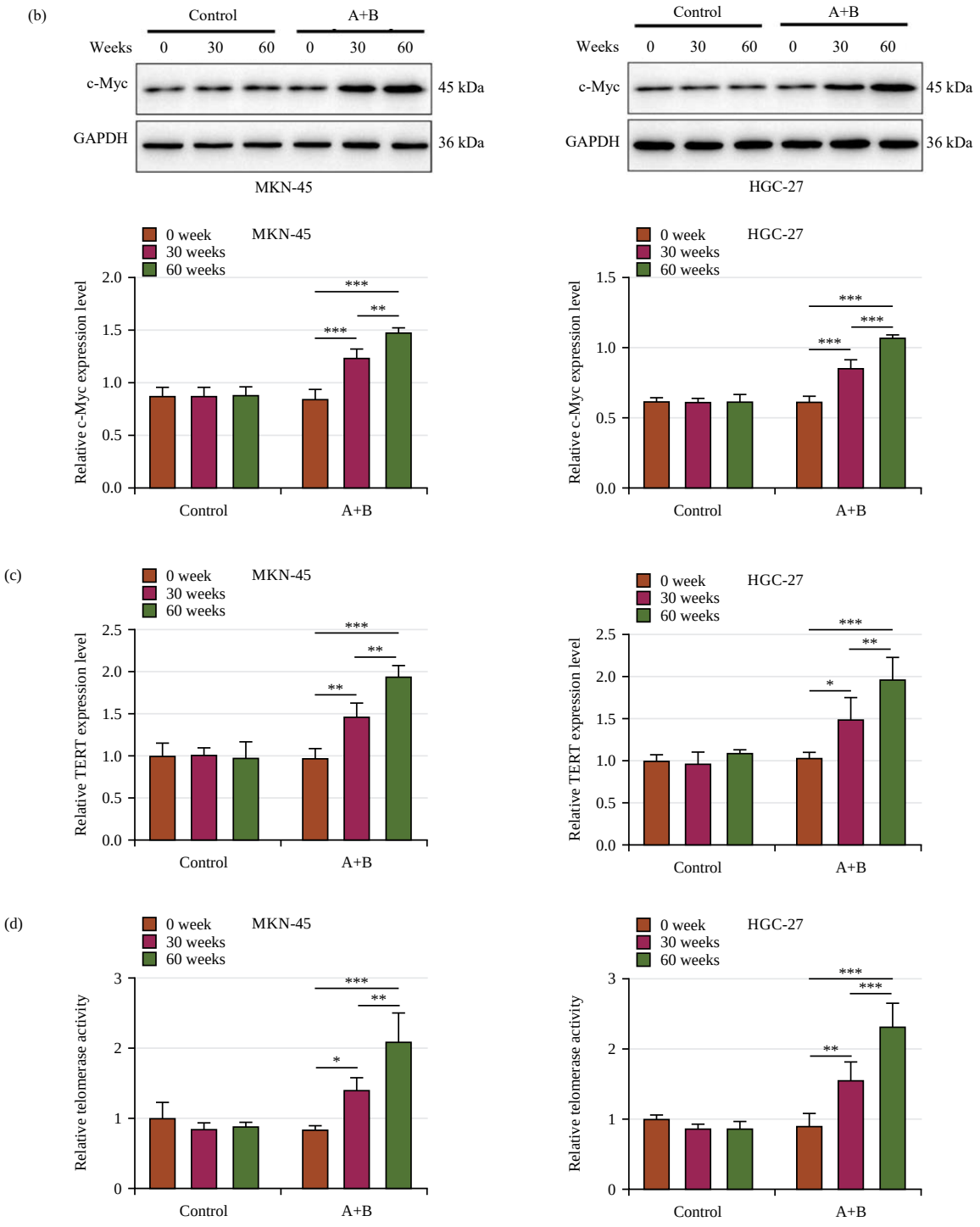

Fig. 3(a-d): Long-term stimulation with acidified bile acids increased ROS levels, c-Myc and TERT expression levels and telomerase activity in GC cells, (a) Detection of ROS levels in GC cells from control and acidified bile acids groups was conducted with DHE fluorescent probes, (b) Assessment of c-Myc protein levels in GC cells was done by western blotting, (c) Measurement of TERT mRNA levels in GC cells was carried out using RT-qPCR and (d) Analysis of telomerase activity in GC cells was performed with TRAP

Scale bar: 50 μm. Error bars indicated the Mean±SEM, Statistical significances were assessed using ANOVA (*p<0.05, **p< 0.01 and ***p<0.001 vs 0 or 30 weeks)

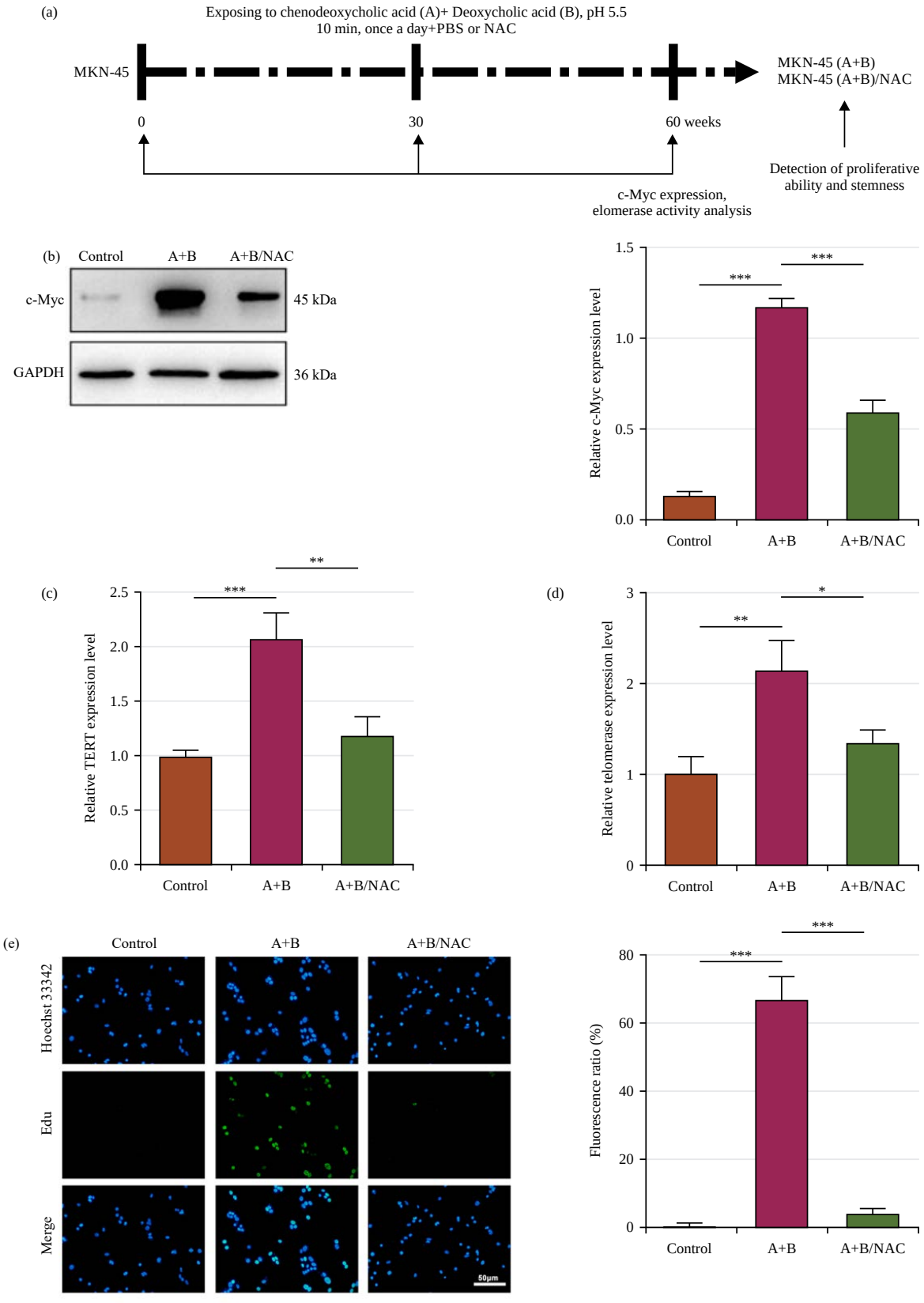

Fig. 4(a-h): Continue

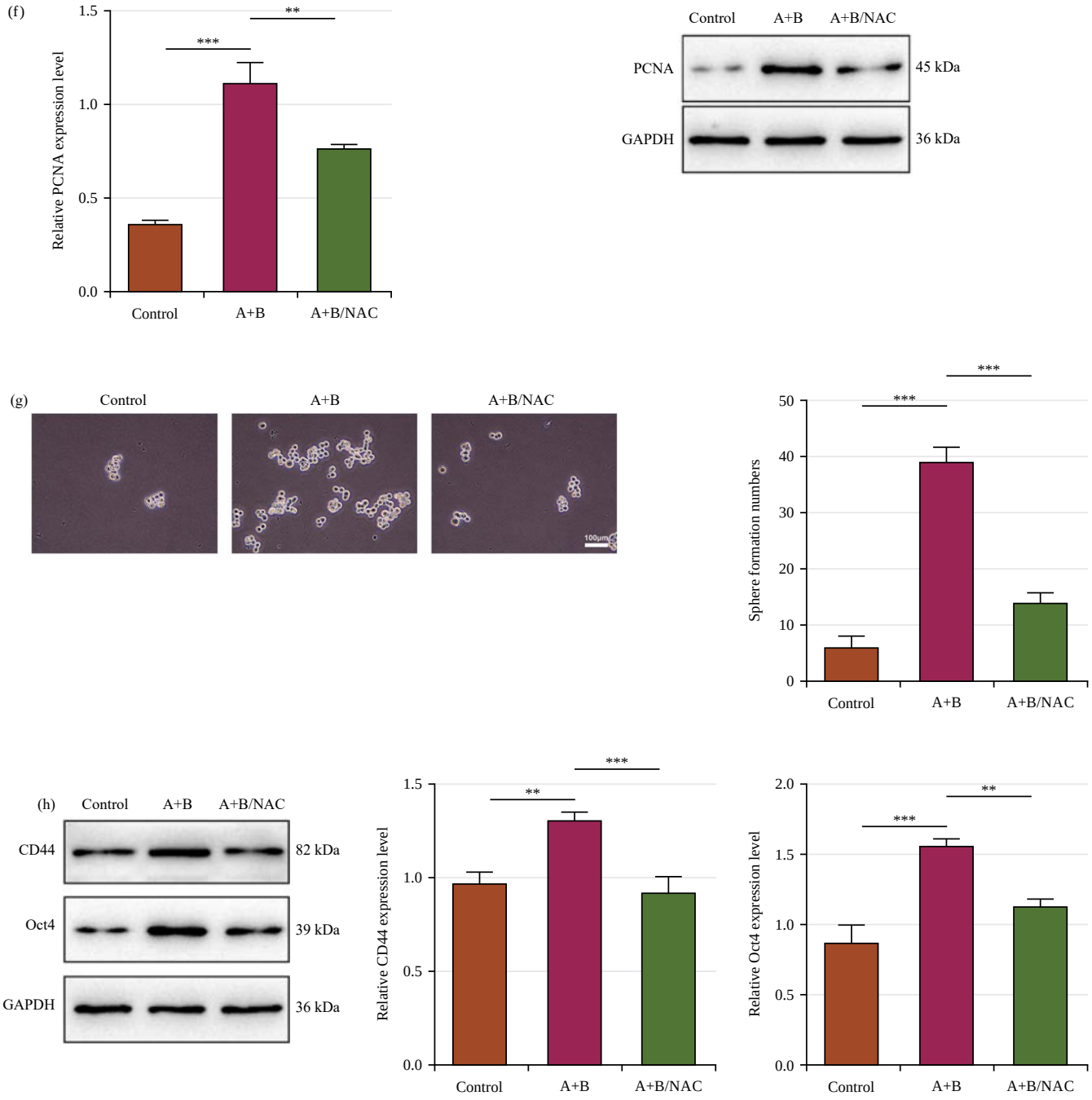

Fig. 4(a-h): Continuous stimulation with acidified bile acids induced ROS to bestow proliferation and stemness to MKN-45 cells, (a) Schematic representation of MKN-45 cells administrated with acidified bile acid stimulation and NAC, (b-d) Effects of NAC addition on c-Myc protein levels, TERT mRNA levels and telomerase activity in MKN-45 cells stimulated with acidified bile acids were estimated by western blotting, RT-qPCR and TRAP, (e) Analysis of the influence of NAC addition on the proliferation of MKN-45 cells stimulated with acidified bile acids was done by Edu assays. Scale bar: 50 μm, (f) Protein levels of PCNA in NAC-preincubated MKN-45 cells under acidified bile acid stimulation were measured by western blotting and (g-h) Effects of NAC addition on the stemness of MKN-45 cells by assessing the sphere-forming capacity and protein levels of the stem cell markers CD44 and Oct4

Scale bar: 100 μm, Error bars indicated the Mean±SEM. Statistical significances were assessed using ANOVA (*p<0.05, **p<0.01 and ***p<0.001 vs control or A+B)

DISCUSSION

Bile and acidic environment are the two main components in the stomach following bile reflux, which synergistically damage gastric mucosal cells and facilitate gastric carcinogenesis, but studies on the mechanism by which these two components jointly promote GC progression are lacking[27]. The present study demonstrated that acidified bile acids induced ROS production, which was consistent with Dvorak *et al*.[28]. Wang *et al*.[17,18] have reported that acidified bile acid promotes GC progression by increasing telomerase activity through activation of c-Myc. Interestingly, the study validated that acidified bile acid-induced ROS enhanced telomerase activity via c-Myc in GC cells, thereby facilitating cancer cell stemness and proliferation. The study provides a mechanism to gain a deeper understanding of GC progression and highlights the possibility of targeting ROS as a strategy for GC treatment.

Bile reflux is an intermittent event in nature, so this property may be critical for biological responses. Unlike continuous exposure, chronic short-duration exposure to acidified bile acids leads to different outcomes, so this situation was simulated *in vitro*. In previous studies, chronic exposure of MGC-803 cells to CDCA (100 μM) and DCA (100 μM) in a low pH environment (pH = 5.5) for up to 120 min had been uncovered to promote cell proliferation, inhibited cell apoptosis and enhanced subcutaneous xenograft formation[17]. In the present study, two other GC cells (MKN-45 and HGC-27) were stimulated under the above conditions to investigate the effects of acidified bile acids on GC cell proliferation and stemness. The outcomes exhibited that acidified bile acids could promote the proliferation and stemness of GC cells.

The DCA and CDCA are the main components of bile acids, which are found in the esophagus of patients with gastroesophageal reflux and Barrett's esophagus[29,30]. Acidified bile acids have been shown to cause greater damage to the esophageal epithelium than either acid or bile acids alone[31]. Furthermore, those with Barrett's esophagus presented a higher rate of exposure to acidified bile acids, accompanied by higher levels of ROS[28]. Moreover, exposure of gastric cancer cells (St23123) to DCA caused oxidative stress and increased ROS production[32]. Here, acidified bile acids could elevate ROS levels in GC cells, but pre-treatment with the ROS scavenger NAC attenuated acidified bile acid-induced ROS production, manifesting that acidified bile acids might promote GC cell proliferation and stemness in a ROS-dependent manner. The possible mechanism associated with this is that acidified bile acids induce mitochondrial damage and generate ROS through oxidative stress[23]. Bile acids disrupt cell membranes and activate phospholipase A2, leading to the release of arachidonic acid, which in turn affects damage to mitochondria[33]. An alternative mechanism by which acid and bile acids synergistically induce oxidative stress is through the activation of NADPH oxidase[34]. In addition, acidic conditions can enhance bile acid-generated ROS via an iron-mediated Fenton reaction[35]. Above proposed mechanisms by which acidified bile acids may induce ROS need to be verified in future experiments.

Telomerase, an enzyme in cells responsible for telomere lengthening, determines cell longevity and proliferative activity[36]. A previous study showed that acidified bile acids increased telomerase activity through activation of c-Myc, thus promoting GC progression[17,18]. Moreover, ROS promoted the phosphorylation of c-Myc via ERK, causing activation of downstream glutathione GSH expression, thereby alleviating cell damage caused by oxidative stress[37]. Additionally, H_2O_2 stimulation increased c-Myc expression in rabbit tracheal epithelial cells, while ROS levels showed a positive correlation with c-Myc expression[38]. In the research, acidified bile acid-induced the production of ROS in conjunction with increased c-Myc protein levels, TERT mRNA levels and TERT activity. However, acidified bile acid-mediated changes in ROS levels, c-Myc protein levels, TERT mRNA levels, TERT activity, as well as proliferation and stemness of GC cells were weakened following the addition of the ROS scavenger NAC. All outcomes urged us to conclude that acidified bile acid-induced ROS boosted telomerase activity via c-Myc, thereby fostering the stemness and proliferation of GC cells. Unfortunately, these results were not validated in animal models in the current study, which is one of the directions for future research. It was demonstrated that acidic bile salt-exposed tumor cell-derived exosomes promote macrophage M2 polarization, thus facilitating esophageal adenocarcinoma progression[39]. In the tumor microenvironment of GC, whether acidified bile acids attacked GC cell-derived exosomes to promote tumor growth by mediating macrophage M2 polarization is a subject for future investigation.

CONCLUSION

The ROS induced by acidified bile acids increased telomerase activity via c-Myc, leading to the promotion of GC cell stemness and proliferation, suggesting that the promoting effect of acidified bile acid on GC is at least in part dependent on ROS-mediated mechanisms. Further studies will be executed in the future to explore whether acidified bile acids attacking GC cell-derived exosomes promote tumor growth by mediating macrophage M2 polarization.

SIGNIFICANCE STATEMENT

Reflux of bile acids in an acidic gastric environment (pH 4-6) can form acidified bile acids that may promote GC cell invasion and migration through the c-Myc/TERT pathway. Long-term exposure to bile acids increases ROS levels and promotes GC progression. Therefore, the present study aims to investigate whether acidified bile acids mediate the proliferation and stemness of GC cells depending on ROS-mediated c-Myc. The results exhibited that acidified bile acids enhanced telomerase activity via c-Myc in a ROS-dependent manner, thereby promoting proliferation and stemness of GC cells. This study highlights the important role of acidified bile acid-induced ROS in GC development and implies the possibility of ROS as a target for the treatment of GC progression.

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