Effect of Exogenous pH on Cell Growth of Breast Cancer Cells

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Research Article

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Abstract

Breast cancer is the most common cancer in women and the most life-threatening cancer in women worldwide. One key feature of cancer cells including breast cancer cells is a reversed pH gradient, and extracellular pH (pHe) of cancer cells is more acidic than normal cells. Cancer cells have lower pHe of ~ 6.7–7.1 and higher intracellular pH (pHi) of 7.4, while normal cells have pHe of 7.4 and lower pHi of 7.2. Here, we investigated how exogenous pH affected breast cancer cells. MDA-MB-231 cell lines were cultured in five different pHs, pH 6.0, pH 6.7, pH 7.4, pH 8.4, and pH 9.2 of medium. The cells were growing in pH 6.0 and pH 9.2 however, not as fast as in other pHs. Especially they were floating in more acidic conditions than pH 6.3. In alkaline pH (pH 8.4 and pH 9.2), more cells were early apoptotic and they were in S phase. In acidic pH (pH 6.0), more cells were late apoptotic or necrotic and more cells were at G2/M phase in acidic pH (pH 6.0 and pH 6.7). The results suggested that MDA-MB-231 cells experienced different cell growth and cell metabolism in different pHs.

Introduction

Breast cancer is the most common cancer in women and the leading cause of cancer death[1,2]. Like other cancer cells, breast cancer cells have the ability to invade nearby tissues and spread to distant regions of the body. Breast cancer most commonly originates from the inner lining of milk ducts or lobules that supply the ducts with milk[3]. Breast cancer cells differ from normal cells in many ways. Human breast carcinomas express more receptors such as receptor tyrosine kinases[4,5]. Three important receptors of breast cancer cells are estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)[6]. Approximately 70–80 percent breast cancers are hormone receptor-positive, which has ER or PR[7]. Hormone receptor-positive breast cancers can be treated with hormone therapy drugs. Breast cancer cells may or may not have the receptors. Cells that do not have any of these three receptor types are called triple-negative[8]. MDA-MB-231, a highly aggressive and invasive cancer cell line, is one of triple-negative breast cancer cell lines[8]. Another characteristic of most malignant tumors is a reduction in extracellular pH (pH_e).

Extracellular and intracellular pHs in tissues affect the function of the cells and play an important role in cancer development[9]. Normal cells regulate intracellular pH (pH_i) to near neutral values tightly by ion transport proteins[10]. Normal differentiated adult cells have pH_i of ~ 7.2 and extracellular pH (pH_e) of ~ 7.4. However, cancer cells create a reversed pH gradient and pH_e of cancer cells is more acidic than normal cells. Cancer cells have a higher pHi of > 7.4 and a lower pH_e of ~ 6.7–7.1[9,11]. Otto Warburg *et al.* first reported the abnormal anaerobic glycolysis and the metabolic alterations in tumor cells[12]. In tumor cells, pyruvate, last product of glycolysis, is converted into lactate, which is same as the glycolysis process in anaerobic conditions[13]. Extracellular acidification is due to lactate secretion from tumor cells[14,15]. Cancer cells increase expression and activation of transporters and pumps such as the Na^+/H^+ exchanger (NHE-1), the H^+-lactate co-transporter, and the proton pump (H^+-ATPase), which contributes to

H^+ secretion[16,17]. Therefore, cancer cells have a reversed pH gradient with a slightly elevated intracellular pH despite an acidic microenvironment[18,19]. Low extracellular tumor pH plays a critical role in drug resistance and it also enhances invasive growth and metastases[20,21]. Tumor cells in acidic pH_e increase expression of several genes including genes encoding matrix degrading enzymes and proangiogenic factors *in vitro*. Metastatic potential in acidic pH_e has been demonstrated *in vivo* as well. Tumor cells cultured *in vitro* at pH_e 6.8 was inoculated into the tail vein of BALB/c nu/nu mice. The tumor cells increased pulmonary metastases with time of *in vitro* pre-treatment[22]. Acidic pH_e upregulated activity of metastatic effectors such as serine proteases and angiogenic factors.[22] Modulation of tumor pH_e has also been shown to reduce metastasis and improve survival in a breast cancer mouse model[23].

Based on the reverse pH gradient of cancer cells, some research suggests that alkaline treatment might be an effective complementary treatment for people who have cancer[24]. The basic idea of alkaline treatment is that the reverse pH gradient of breast cancer can be reduced or eliminated by systemic treatment with alkaline compound such as sodium bicarbonate ($NaHCO_3$)[25]. However, there is no strong evidence to prove that diet of alkaline food can manipulate whole body pH or that it has an impact on cancer. Sodium bicarbonate treatment reduced the colonization of lymph nodes; however, it couldn't significantly affect the levels of circulating tumor cells[26]. In order to apply alkaline treatment to cancer cells more efficiently, it is critical to understand what the effect of pH to cancer cells is.

In this research, we investigate the effect of exogenous pH on the growth of MDA-MB-231 cells, cell death via either apoptosis or necrosis, and cell cycle.

Materials And Methods

Cell culture

MDA-MB-231 (ATCC® HTB-26™) cell lines from the American Type Culture Collection (ATCC) (Manassas, VA) were maintained at 37°C under a humidified atmosphere of 5 % CO_2. MDA-MB-231 cell, a metastatic human breast cancer cell line, were maintained in either Dulbecco's Modified Eagle Medium (DMEM) or Rosewell Park Memorial Institute (RPMI-1640) medium containing 10 % (v/v) fetal bovine serum, supplemented with 1 % penicillin (100 U/mL) and streptomycin (100 µg/mL).

Cell Proliferation Assay and pH Monitoring

MDA-MB-231 cells were trypsinized and resuspended in five different pHs, pH 6.0, pH 6.7, pH 7.4, pH 8.4, and pH 9.2 of DMEM. They were seeded on 12-well plates at 2×10^5 cells/well in 1 mL of medium. The pHs of DMEM were measured by a pH meter every day. The cells were resuspended with PBS containing 0.4% trypan blue for viable cell counts with a haemacytometer. Total viable cell counts were also performed every day without changing media for up to 5 days.

Analysis of apoptosis and necrosis with flow cytometry

MDA-MB-231 cells were incubated at five different pHs, pH 6.0, pH 6.7, pH 7.4, pH 8.4, and pH 9.2 of DMEM for 5 days. Cells were harvested and washed with 1 mL PBS. For detection of apoptotic and necrotic MDA-MB-231 cells, cells were labeled by adding 20 µL of binding buffer and 5 µL of Annexin V-FITC to each sample. Propidium iodide (2 µL of 1 mg/mL) were also added to each sample. Samples were mixed gently and incubated for 15 min at room temperature in the dark. After centrifugation at 500 ×*g* for 5 min at 4°C, cells were resuspended in 100 µL PBS. A minimum of 10,000 cells were analyzed immediately.

Measurement of Hydrogen Peroxide and Superoxide

MDA-MB-231 cells were incubated at five different pHs, pH 6.0, pH 6.7, pH 7.4, pH 8.4, and pH 9.2 of DMEM for 5 days. The cells were washed with PBS and stained by either 5 µM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) (Invitrogen, Carlsbad, CA) for measuring H_2O_2 or 5 µM dihydroethidium (hydroethidine) (DHE) (Invitrogen, Carlsbad, CA) for measuring superoxide. After 20 minutes, cells were washed 3 times with ice cold PBS. Fluorescence was measured by an Infinite® 200 Pro microplate reader (Tecan Trading AG, Switzerland) using a laser for either CM-H_2DCFDA ($\lambda_{ex}/\lambda_{em}$ = 488/530 nm) or DHE ($\lambda_{ex}/\lambda_{em}$ = 518/605 nm).

Cell cycle analysis with flow cytometry

For the cell cycle analysis, MDA-MB-231 cells in different pHs were collected and washed 2–3 times in PBS. Cells were fixed in cold 70 % ethanol for 30 min at 4°C. They were washed 2–3 times in PBS again and harvested by centrifugation at 850 x *g*. Cells were treated with 50 µL of a 100 µg/mL ribonuclease to ensure that only DNA, not RNA, is stained. For staining DNA, 200 µL of 50 µg/mL propidium iodide (PI) was added to cells and fluorescent cell population was measured by a flow cytometer (BD Accuri™ *C6* cytometer, BD Bioscience) (San Jose, CA) using a laser for PI ($\lambda_{ex}/\lambda_{em}$ = 488/605 nm)

Statistics

Each bar graph represents a mean ± standard deviation of at least three independent experiments. Statistical analysis was performed using Student's t-test, comparing each treatment to cells only unless otherwise mentioned. In all statistical analysis $p < 0.05$ (*) was considered significant.

Results And Discussion

Cancer cells including breast cancer cells has a reversed pH gradient. Cancer cells have lower extracellular pH (pH_e) of ~ 6.7–7.1 and higher intracellular pH (pH_i) of 7.4, while normal cells have pH_e of

7.4 and lower pH_i of 7.2[9,11]. The acidic pH_e or increased pH_i is necessary for directed cell migration.[27] Low pH_e also plays an important role in drug resistance, invasive growth and metastases.[20,21] In this study, breast cancer cells were grown in various pH media, and we monitored the cell growth profile with time and extracellular pH changes.

In Fig. 1A, MDA-MB-231 cell, a metastatic human breast cancer cell line, were cultured in 5 different pHs of Dulbecco's Modified Eagle Medium (DMEM), pH 6.0, pH 6.7, pH 7.4, pH 8.4, and pH 9.2. Initial cell density was 2×10^5 cells/well in 1 mL of medium of 5 different pH. MDA-MB-231 in pH 6.7, pH 7.4, and pH 8.4 were confluent in 5 days. Cells in pH 6.0 and pH 9.2 were growing; however, not as fast as in other pHs. Especially pH 6 prevented MDA-MB-231 cells from attaching to the surface of a 12 well plate. In Fig. 1B, all cell numbers at designated time points in 5 different pH were counted.

In order to specify the acidic pH conditions that prevented MDA-MB-231 cells from attaching to the surface, cells were incubated at five different pHs, pH 6.00, pH 6.15, pH 6.30, pH 6.45, and pH 6.60 of DMEM. In Fig. 2, cells stayed at the surface of plates with pHs higher than 6.30; however, they were floating in more acidic conditions than pH 6.30.

Cancer cells lower the extracellular pH, which promotes tumor growth, metastasis, and drug resistance. An acidic extracellular pH induces the expression of certain genes associated with tumor metastasis[28]. Metastasis begins with detachment of metastatic cells from the primary tumor and tumor cells travel to different sites via blood/lymphatic vessels and settle at a distal site[29]. MDA-MB-231 cell line is an epithelial, human breast cancer cell line. MDA-MB-231 cancer cells metastatic mammary adenocarcinoma and they are highly aggressive and invasive[30]. In the acidic environment of Fig. 1 and Fig. 2, MDA-MB-231 cancer cells were detached from the surface of a tissue culture flask and they were floating, which was not observed in the alkaline pH. This result suggested that metastatic process was activated in the acidic environment, not in the alkaline pH.

In Fig. 3, we monitored pH changes of medium during the incubation of cells in a 37°C and humidified 5 % CO_2 incubator. In five different pHs, pH 6.0, pH 6.7, pH 7.4, pH 8.4, and pH 9.2, MDA-MB-231 cells lowered extracellular pH with time, and cells were in more acidic environment than in the initial pH conditions. Figure 3F demonstrated that the medium changed the color and all samples lost pink color with time. DMEM includes phenol red as a pH indicator. The color of phenol red is yellow below pH 6.8, and it is orange-red around pH 7.4 that is the physiological pH, and it turns bright pink over pH 8.2. DMEM also includes sodium bicarbonate ($NaHCO_3$) that is a buffer used to stabilize pH and it requires a 5–10 % CO_2 environment to maintain physiological pH.

$$NaHCO_3 \leftrightarrow Na^+ + HCO_3^-$$

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

Sodium bicarbonate ($NaHCO_3$) dissociates into sodium ion (Na^+) and bicarbonate ion (HCO_3^-). In the bicarbonate buffering system, pH is maintained via Le Chatelier's principle. When the pH of the system decreases, the increased H^+ ions drives the equation to the left. Similarly, a decrease in H^+ ions will move the equation to the right.

Partial pressure of carbon dioxide (pCO_2) in arterial blood is between 35 mmHg and 45 mmHg. When mammalian cells are cultured, CO_2 concentration (pCO_2) in a cell culture incubator is usually set at 5% (v/v) or 38 mmHg that is 5% of 760 mmHg.

When CO_2 is dissolved in water, it forms carbonic acid (H_2CO_3) and H_2CO_3 then freely dissociates into ions.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

Buffering system in the medium can resist pH changes, and medium pH should remain stable. However, pH disturbances are inevitable in the live-cell culture system due to the cell metabolism.[31] When the disturbance exceeds the buffering capacity, the medium pH will change[31]. The fluctuation of pH usually leads to the acidification of growth medium. Medium in five pHs, pH 6.0, pH 6.7, pH 7.4, pH 8.4, and pH 9.2, tended to be acidified in an incubator with time; however, the acidification was accelerated by MDA-MB-231 cells in all pHs. Cancer cells induce extracellular acidification by lactate secretion. Cancer cells increase expression of transporters such as the Na^+/H^+ exchanger (NHE-1) and the H^+-lactate co-transporter, which results in H^+ secretion. As a result, the acidification was accelerated by MDA-MB-231 cells.

MDA-MB-231 cells grow faster in pH 7.4 than in pH 9.2. In order to understand if the pre-treatment of pH affected the cell growth, MDA-MB-231 were cultured in pH 7.4 and 9.2. When they were stable in each pH, cells in each pH were transferred into two pH groups, pH 7.4 and pH 9.2 respectively. As a result, two samples in pH 7.4 and pH 9.2 were still in the same pHs, pH 7.4 and pH 9.2, and two other samples switched the pH either from pH 7.4 to pH 9.2 or from pH 9.2 to pH 7.4. In Fig. 4, pH transition from pH 7.4 to pH 9.2 demonstrated the same cell growth profile as continuous pH 9.2. When cells experienced the pH change from pH 9.2 to pH 7.4, the cell growth at day 1 was slow; however, the cell growth rate after day 1 would be same as cells in continuous pH 7.4. The results demonstrated that current pH of medium was more important for the cell growth. The pH history that MDA-MB-231 experienced did not affect the cell growth too much. If we could control or maintain the unfavorable pH for cancer cells *in vivo*, then we could retard the growth of cancer cells or treat the cancer.

Cell death in five different pHs was measured by double staining of Annexin V and propidium iodide (PI), and the fluorescence of both Annexin V and PI was detected by a flow cytometer. Apoptotic cells expose phosphatidylserine on the outer leaflet of the plasma membrane and Annexin V specifically binds to phosphatidylserine in apoptotic cells[32]. PI penetrates cell membranes of dead cells, not healthy cells, and

it has fluorescence when it binds to DNA of dead cells[33]. Therefore, Annexin V is a very sensitive method for detecting cellular apoptosis and propidium iodide (PI) is used to detect necrotic or late apoptotic cells.

The flow cytometry plots can be divided in four regions. In Fig. 5, healthy cells were negative to both Annexin V and PI and they were labeled as Q1 in the flow cytometry plot. Early apoptotic cells have the fluorescence signal with Annexin V only and they were labeled as Q2. Q3 demonstrated necrotic cells that have the fluorescence with PI only and Q4 were apoptotic cells that have the fluorescence with both Annexin V and PI.

MDA-MB-231 cells are regarded as a healthy condition if there is no sign of apoptosis or necrosis. Healthy cell population of MDA-MB-231 cells in pH 6.7 and pH 7.4 was over 90 %; however, healthy cells in Q1 window of pH 6.0, pH 8.4, and pH 9.2 were 48.7 %, 70.2 %, and 55.7 % respectively. In alkaline pH (pH 8.4 and pH 9.2), more cells were early apoptotic than in acidic pH (pH 6.0). In acidic pH (pH 6.0), more cells were late apoptotic or necrotic than in alkaline pH (pH 8.4 and pH 9.2). Cell death process of MDA-MB-231 cells was accelerated when they were in acidic pH (pH 6.0) compared to alkaline pH (pH 8.4 and pH 9.2).

Apoptosis or necrosis can be induced by many factors such as reactive oxygen species (ROS), cytochrome c, Fas, and/or tumor necrosis factor (TNF) family.[34] The factors are involved in the caspase-mediated apoptosis[35]. ROS are oxygen-based free radicals such as hydrogen peroxide (H_2O_2), superoxide ($\cdot O_2^-$), hydroxyl radical (·OH), and singlet oxygen ($^1O_2$). Overexpressed ROS can cause cell death by damaging DNA, RNA, and proteins[36]. ROS levels of MDA-MB-231 cells in different pH were measured by 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) and dihydroethidium (DHE). CM-H_2DCFDA can detect ROS including H_2O_2 and DHE is a widely used probe for intracellular superoxide. In Fig. 6, pH 6 induced more ROS inside MDA-MB-231 cells and ROS levels of intracellular MDA-MB-231 cells in pH 8.4 and pH 9.2 were higher than those of cells in pH 6.7 and pH 7.4. The results demonstrated that higher level of intracellular ROS or H_2O_2 induced apoptosis and more ROS or H_2O_2 level could cause necrotic cell death. In pH 7.4, ROS levels were more than 10 times higher in MDA-MB-231 cells than in RAW 264.7 cells, one of normal cells[37]. MDA-MB-231 cells in acidic or basic conditions increased the ROS levels to cause more apoptosis and necrosis.

Cancer is a disease of uncontrolled cell division and inappropriate cell proliferation, which is associated with the cell division cycle. Cell cycle has five phases including G0 (resting phase) G1 (gap phase 1), S (DNA synthesis), G2 (gap phase 2), and M (mitosis) [38]. Figure 7 demonstrated that more MDA-MB-231 cells were at G0/G1 phase in pH 7.4. Cell population in S phase increased in alkaline pH (pH 8.4 and pH 9.2) and more cells were at G2/M phase in acidic pH (pH 6.0 and pH 6.7). In the cell culture experiment, S phase of MDA-MB-231 cells in alkaline pH (pH 8.4 and pH 9.2) or G2/M phase in acidic pH (pH 6.0 and pH 6.7) did not increase the cell number due to increased cell death.

In summary, we investigated how exogenous pH affected breast cancer cells. MDA-MB-231 cell lines were cultured in five different pHs, pH 6.0, pH 6.7, pH 7.4, pH 8.4, and pH 9.2. Cells were floating in more acidic conditions than pH 6.3 and the cell growth rate is lower than other pH conditions. Acidic pH (pH 6.0) induced late apoptosis or necrosis via higher levels of ROS. In alkaline pH (pH 8.4 and pH 9.2), more cells were early apoptotic and they were in S phase; however, it did not increase the cell number due to increased cell death. The results enhanced the understanding of exogenous pH effect on MDA-MB-231 cancer cells.

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Figures

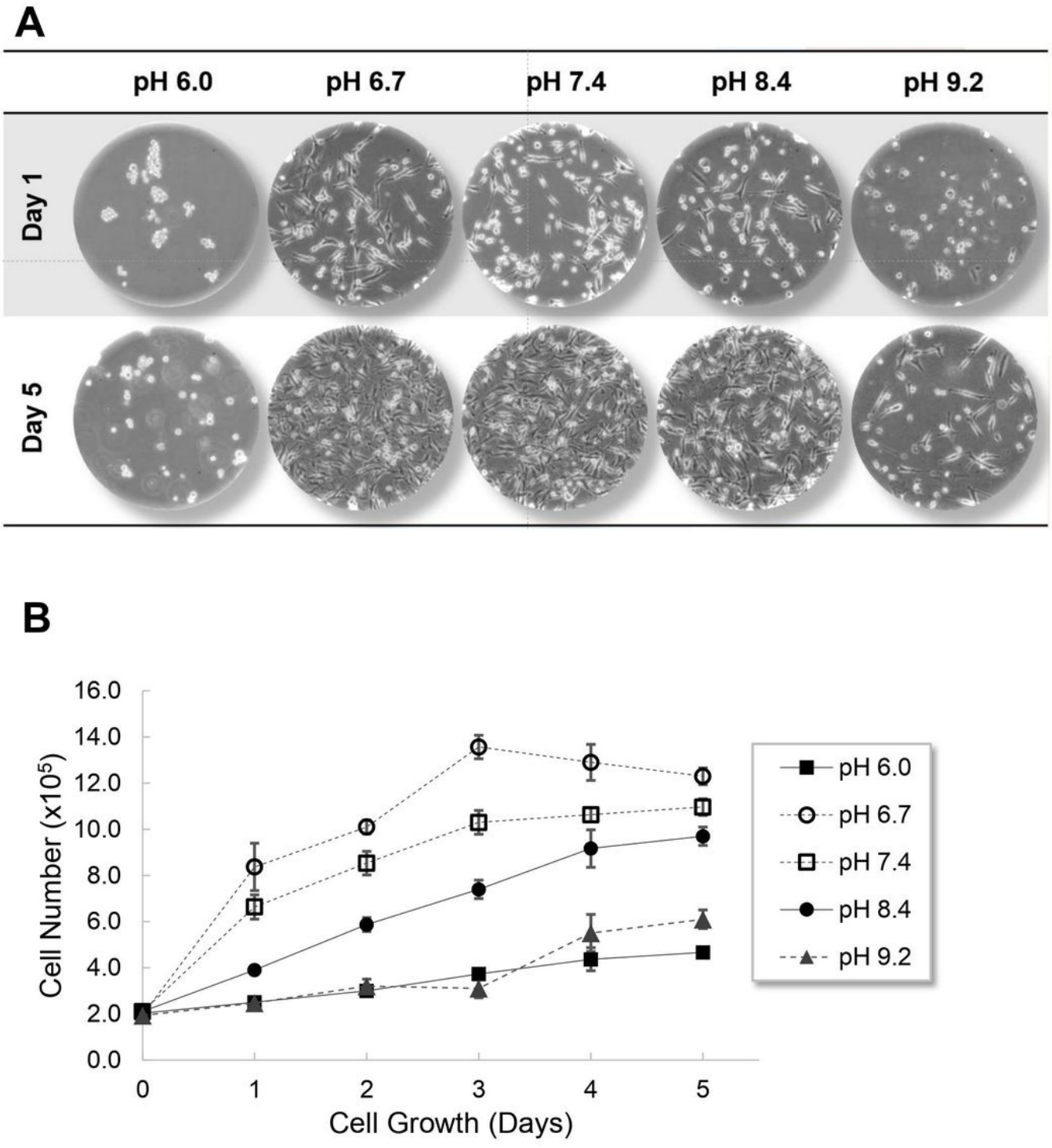

Figure 1

Effect of external pH on the growth of MDA-MB-231 cells, a metastatic human breast cancer cell line. MDA-MB-231 cells were seeded in a 12 well plate and they were incubated at five different pHs, pH 6.0, pH 6.7, pH 7.4, pH 8.4, and pH 9.2 of DMEM. A. Cells were imaged by a light microscope on day 1 and day 5, and B. the number of cells were also counted everyday by a hemocytometer.

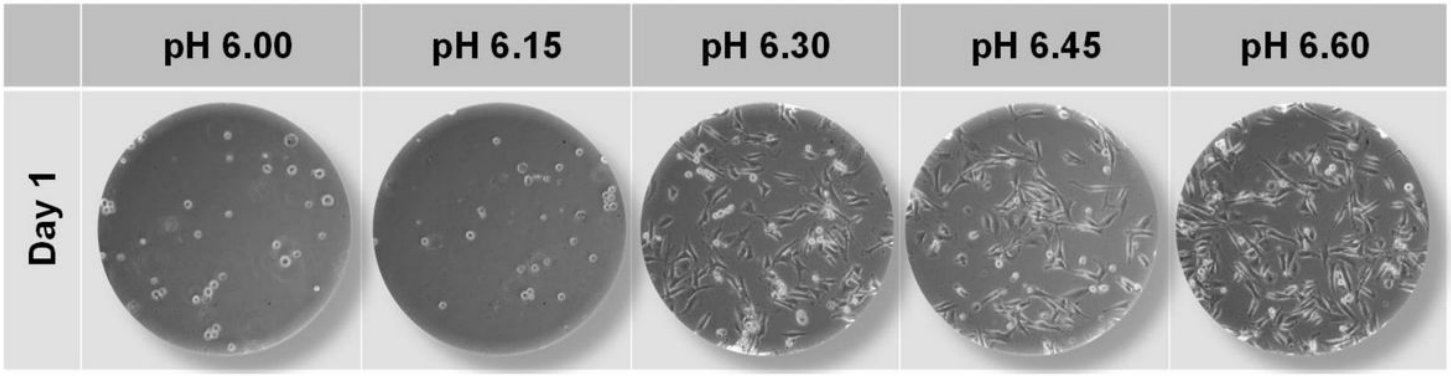

Figure 2

Effect of acid pHs on the growth of MDA-MB-231 cells. MDA-MB-231 cells were seeded in a 12 well plate and they were incubated at five different pHs, pH 6.00, pH 6.15, pH 6.30, pH 6.45, and pH 6.60 of DMEM. Cells were imaged by an Olympus IX70 microscope using a 10× objective on day 1.

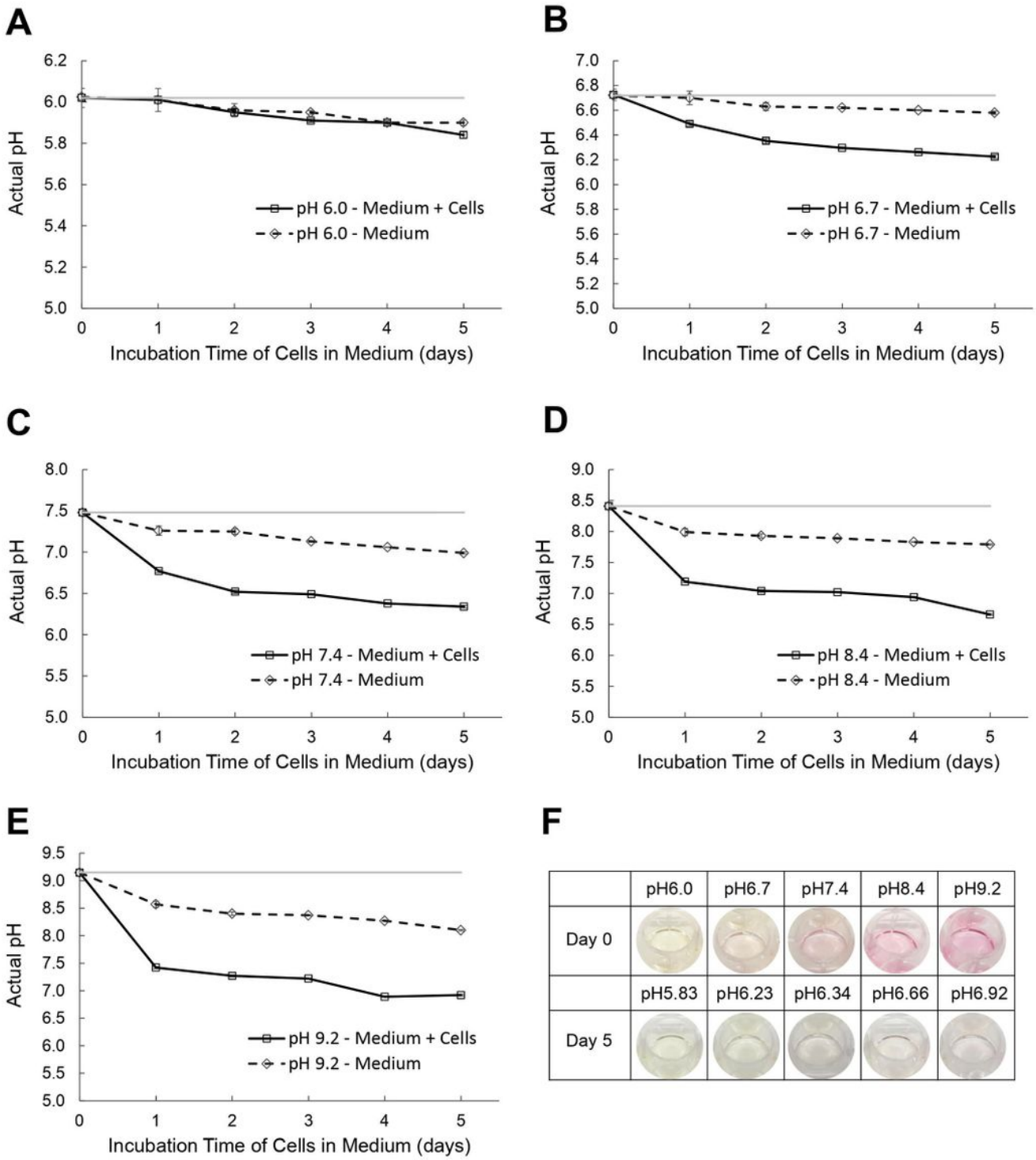

Figure 3

External pH profiles of MDA-MB-231 cells with time. MDA-MB-231 cells were seeded in a 12 well plate and they were incubated at five different pHs, A. pH 6.0, B. pH 6.7, C. pH 7.4, D. pH 8.4, and E. pH 9.2 of DMEM. They were maintained at a 37 °C incubator and pHs were measured every day. Medium only (dotted line), MDA-MB-231 cells in different pH medium (solid black line), and reference line (solid gray line). F. Medium color of MDA-MB-231 cells in 12 well plates at day 0 and day 5. 5

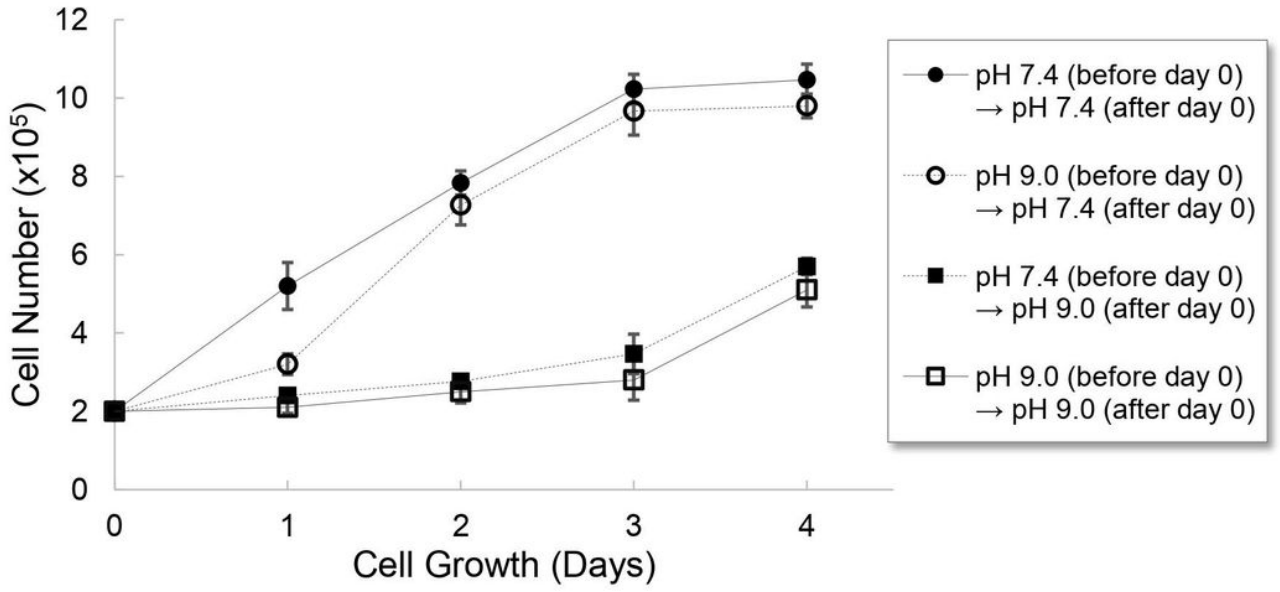

Figure 4

Effect of a medium change on the cell growth of MDA-MB-231 cells. MDA-MB-231 cells were grown in pH 7.4 or pH 9.0 DMEM. The cells in each pH were trypsinized and splitted in two different pHs, pH 7.4 and pH 9.0 respectively at day 0. Total viable cell counts were performed every day without changing media for up to 4 days. pH 7.4 → pH 7.4 (black circles), pH 9.0 → pH 7.4 (white circles), pH 7.4 → pH 9.0 (black squares), and pH 9.0 → pH 9.0 (white squares)

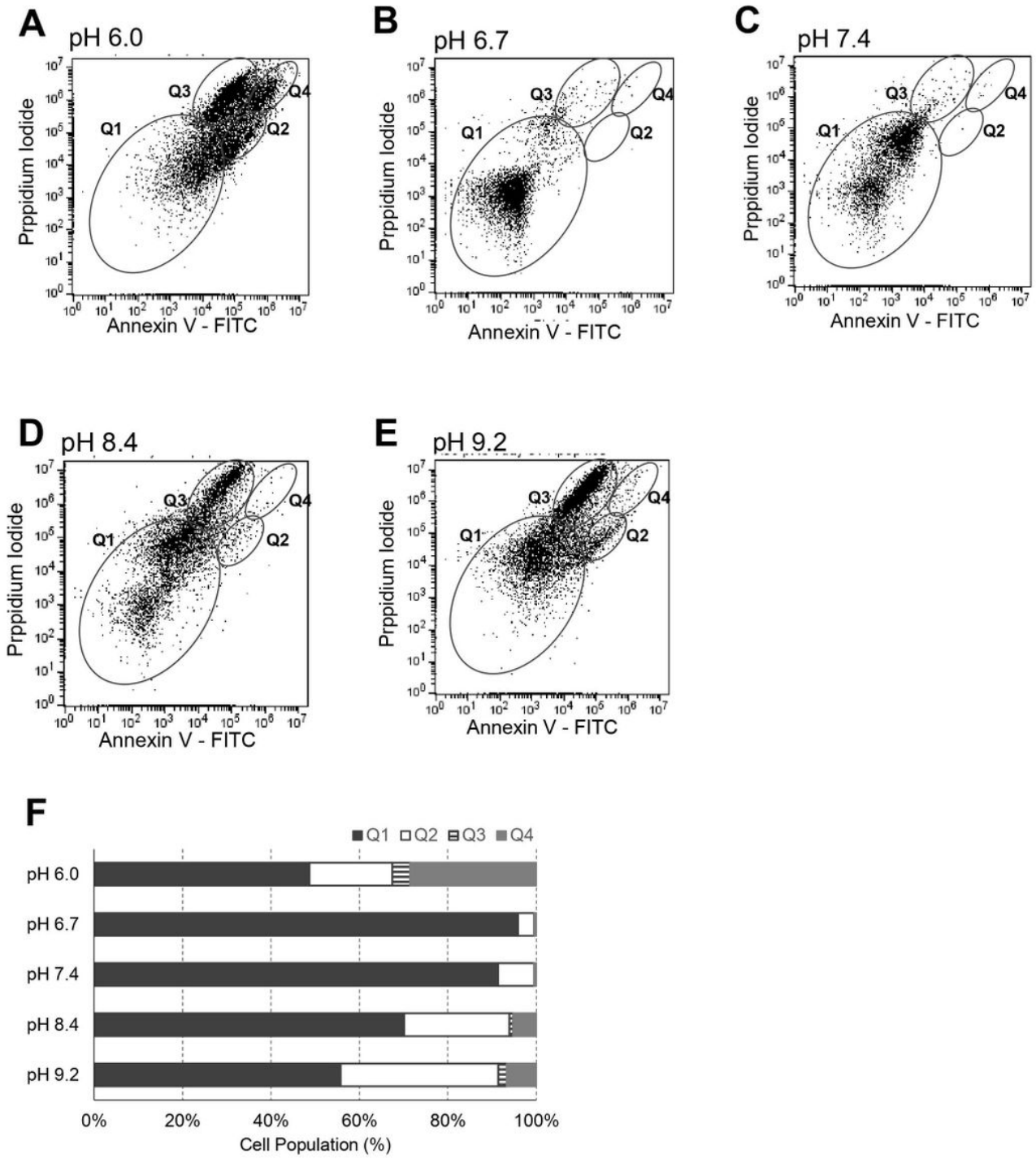

Figure 5

Apoptosis or necrosis analysis of MDA-MB-231 cells in different pHs. MDA-MB-231 cells were seeded in a 12 well plate and they were incubated at five different pHs, A. pH 6.0, B. pH 6.7, C. pH 7.4, D. pH 8.4, and E. pH 9.2 of DMEM. Cells were stained with annexin V-FITC and PI, and apoptosis or necrosis was measured by flow cytometric assay. F. Data in the dot plots were analyzed by Q1, Q2, Q3, and Q4. A

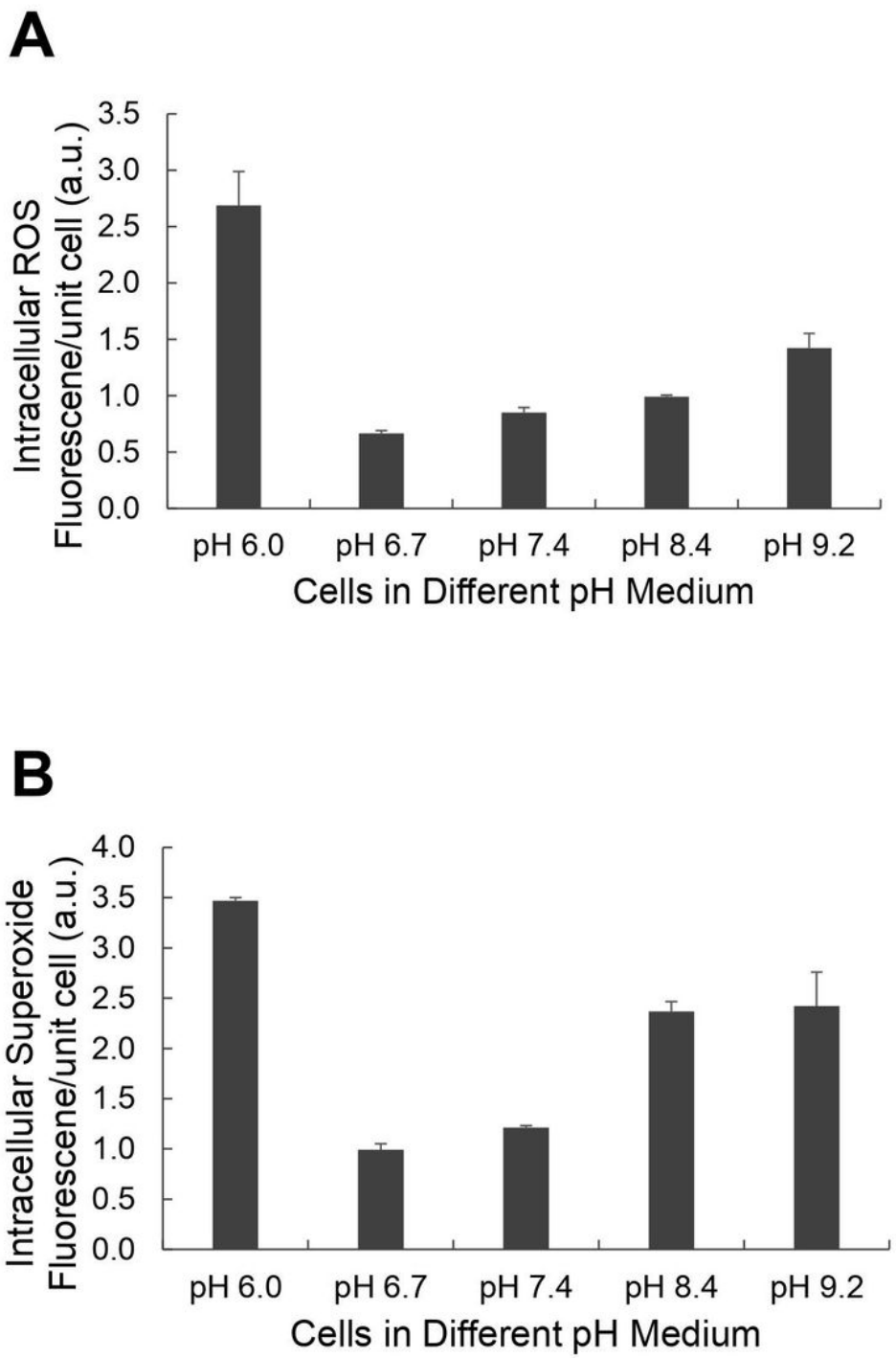

Figure 6

Effect of pH on the intracellular reactive oxygen species (ROS) of MDAMB-231 cells. MDA-MB-231 cells were seeded in a 12 well plate and they were incubated at five different pHs, pH 6.0, pH 6.7, pH 7.4, pH 8.4, and pH 9.2 of DMEM. A. Intracellular ROS or H2O2 are measured by CM-H2DCFDA. B. The level of intracellular superoxide is also obtained by DHE with $p<0.05$ (*).

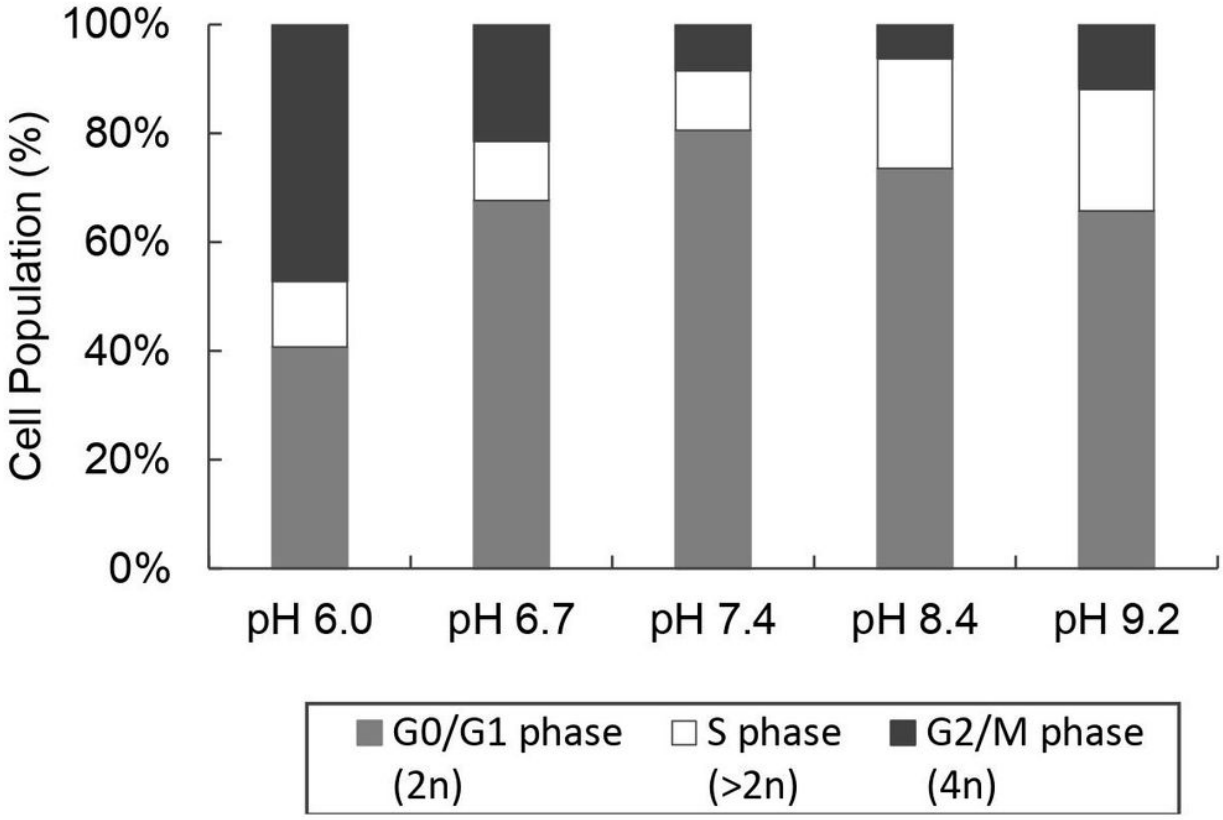

Figure 7

Cell cycle analysis of MDA-MB-231 cells in different pHs. MDA-MB-231 cells were seeded in a 12 well plate and they were incubated at five different pHs, pH 6.0, pH 6.7, pH 7.4, pH 8.4, and pH 9.2 of DMEM for 5 days. Cell cycle, distribution of cells in G0/G1, S and G2/M phase, was measured by a flow cytometer after the staining DNA using propidium iodide.