ゲラニルゲラニルアセトンは正常糖濃度でヒト子宮頚癌細胞の増殖を抑制する

Geranylgeranylacetone inhibits proliferation of human cervical cancer cells in a normal glucose environment

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Abstract

Oral geranylgeranylacetone (GGA) administration has been used in Asia, particularly in Japan, as an anti-ulcer drug for more than 20 years. Recently, some studies reported that GGA suppresses cell growth and induces apoptosis in cell models of human leukemia, ovarian carcinoma, and colon cancer in vitro. Therefore, the aim of the present study was to determine whether GGA can have a therapeutic effect on human cervical cancer cell line (HeLa cells) in normal and high glucose environments (NG and HG, respectively). The results showed that GGA dose-dependently inhibited proliferation of HeLa cells with cell damage in an NG environment and inhibited those without cell damage in an HG environment. The extent of cell injury was strongest in an NG/GGA (100 μM) group 3 days after treatment. DNA fragmentation and flow cytometry analyses revealed that the type of cell death was a combination of necrosis and apoptosis, indicating the potential anti-cancer activity of GGA; however, further studies are needed to investigate the optimal GGA administration route and dose.

Introduction

Oral geranylgeranylacetone administration (GGA; Japanese name, Teprenon) has been used in Asia, particularly in Japan, as an anti-ulcer drug for more than 20 years, and no major adverse effects have been reported. Recently, GGA was shown to activate heat shock protein 70 (HSP70) and exert cytoprotective effects against various stressors in a variety of cells and tissues. In contrast, GGA was shown to suppress cell growth and induce differentiation and apoptosis in human leukemia cell lines (1,2), and it also inhibits lysophosphatidic acid-induced invasion of human ovarian carcinoma cell lines by attenuating activation of rhodopsin (*Rho*) and rat-sarcom–mitogen-activated protein kinase (*Ras-MAPK*) pathways (3,4). Furthermore, a recent study reported that GGA inhibits proliferation of human colon cancer cells by inducing apoptosis and cell cycle arrest (5,6). Taken together, these studies indicated that GGA may have therapeutic potential for treatment of various cancers. Therefore, the aim of the present study was to determine whether GGA can have a therapeutic effect on HeLa cells, which is a human cervical cancer cell line.

Materials and Methods

Cell culture and GGA treatment

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 200 μg/ml streptomycin, and 200 U/ml penicillin. Experiments were performed using 3–7-day-old cell cultures in DMEM supplemented with either a high glucose (HG; 25 mM) or a normal glucose (NG; 5.6 mM) concentration. GGA (Teprenon; Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was dissolved in ethanol and added to each culture. Control cultures were treated with the vehicle alone. Staurosporine (STS; 4 μM) was added to the cultures, which were then incubated for 8 h, as a positive control of apoptosis. To activate HSP70 expression, the cells were incubated at 42°C for 2 h.

Cell viability assay

Cell viability was assessed using the trypan blue exclusion test. In total, 2.0×10^5 HeLa cells were seeded in dishes (35 mm in diameter) and treated with various GGA concentrations (3, 10, 30, and 100 μM, respectively). As a control, the vehicle alone was assessed. The trypan blue exclusion test was performed by adding equal volumes of a 0.4% trypan blue solution and cell suspension and then evaluated 3 days after treatment. The number of total and live cells was counted under light microscopy 3 days after GGA treatment.

DNA fragmentation assay

Internucleosomal DNA fragmentation was assessed by a DNA ladder. Briefly, cells were digested in a lysis buffer (5 mM ethylenediaminetetraacetic acid/0.2% sodium dodecyl sulfate/0.2 mg/ml proteinase K/100 mM Tris·HCl; pH 8.5) at 37°C overnight. Chromosomal DNA was analyzed by agarose gel electrophoresis (2%) followed by staining with ethidium bromide, as previously reported (7). DNA fragmentation was also assessed by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling (TUNEL) using the In Situ Cell Death Detection Kit with Tetramethylrhodamine red (Roche diagnostics, Basel, Switzerland) for detecting and quantifying apoptosis. The cells were treated with GGA (100 μM) and then assayed 3 days after treatment.

Flow cytometry

The cells were treated with GGA (100 μM) and then analyzed by flow cytometry 3 days after treatment. During early apoptosis, membrane integrity was assessed by staining with annexin V, which binds to exposed membrane phosphatidylserine, an acidic (anionic) phospholipid and one of the earliest features of apoptosis, using the PE Annexin V Apoptosis Detection Kit (BD Biosciences, Franklin Lakes, NJ, USA). Necrotic change was concurrently assessed using 7-amino-actinomicin (7-AAD) staining solution to exclude viable cells with intact membranes.

Statistical analysis

Statistical analysis was performed using SPSS statistical software (ver. 17; SPSS Inc., Chicago, IL, USA). Analysis of variance was used to determine statistical significance. A p-value <0.05 was considered statistically significant.

Results

Cell viability assay

The effects of various GGA concentrations on cell viability were assessed. The cell concentration increased from 2.0×10^5 to 21.1×10^5 in HG/control culture and 11.7×10^5 in NG/control culture. The total cell count decreased in a dose-dependent manner in GGA-treated cultures under both HG and NG conditions (Fig. 1A). The percentage of live cells decreased in the NG/GGA (100 μM) group in a dose-dependent manner, whereas there was no remarkable change in the HG/GGA group. The reduction was remarkable in the NG/GGA (100 μM) group (Fig. 1B), and cell toxicity in that group was observed only 3 days after treatment (Fig. 1C). The percentage of live cells in the NG /GGA (100 μM) group reduced to 44.0% 3 days after treatment, whereas that under other conditions was approximately 90%. Microscopic changes in morphological features are shown in Fig. 2.

DNA fragmentation assay

The DNA fragmentation assay was performed to detect whether cell death was caused by apoptosis or necrosis. As shown in Fig. 3A, DNA laddering was not evoked in the NG/GGA (100 μM) group, but it was evoked with exposure to 4 μM STS for 8 h, the positive control of apoptosis. The TUNEL assay results were positive in the NG/GGA (100 μM) group (Fig. 3B).

Flow cytometry

Late apoptotic or dead cells were measured by PE Annexin V and 7-AAD staining following NG/GGA (100 μM) and control treatments. As shown in Fig. 4, the population of late apoptotic/dead cell increased after GGA treatment in the NG environment. The percentage of late apoptotic/dead cells was 81.4 ± 9.1% in the NG/GGA (100 μM) group and 12.34 ± 4.60% in the NG/control group. There was no significant change in the HG/GGA (100 μM) group, and the percentage of early apoptotic cells in each group did not significantly change (Fig. 4).

DISCUSSION

The anti-ulcer mechanism of GGA can reportedly enhance the mucous and mucosal barrier, thereby protecting the stomach against its own secretions. In addition to this mechanism, in 1996, Hirakawa et al. reported that GGA can nontoxically induce HSP70 expression and protect cultured gastric mucosal cells (8). Several studies revealed that low-dose GGA induced HSP70 expression and had protective effects in glaucoma (9), cerebellar infarction (10), liver transplantation (11), cardiac ischemia/reperfusion injury (12), polyglutamine disease (13), and pulmonary fibrosis (14) in animals.

Recently, inhibitors of isoprenyl compounds such as farnesol, geranylgeraniol, and geranylgeranoic acid were shown to induce apoptotic cell death. Some isoprenoids with strong anti-cancer effects, such as docetaxel and pacritaxel (15-20), are currently available for clinical use. The chemical structure of GGA is similar to that of geranylgeranyl pyrophosphate, which is a component of the Rho metabolic pathway and is essential for Rho geranylgeranylation. Furthermore, GGA has been shown to induce differentiation and apoptosis in human leukemia cell lines through modulation of small G protein activation (1,2) and inhibit ovarian cancer progression through Rho inhibition and Ras-MAPK activity (3,4). More recently, GGA was reported to inhibit human colon cancer cell proliferation through induction of apoptosis and cell cycle arrest (5). Taken together, these studies indicate that GGA has potential as an anti-cancer drug. Therefore, we evaluated the effects of GGA using HeLa cells, as an in vitro model of human cervical cancer.

In this study, we employed a cell viability assay to evaluate the influence of GGA concentration on proliferation of HeLa cells, which are generally cultured in HG DMEM (450mg/dL; 25mM); however, serum NG content in humans is approximately 100 mg/dl (5.6 mM). Therefore, we examined both HG DMEM (25 mM) and NG DMEM (5.6 mM). The medium was changed with GGA treatment. The number of

HeLa cells in the HG and NG control groups increased by approximately 10-fold and 5-fold, respectively, after 3 days. GGA dose dependently inhibited proliferation of HeLa cells in both the HG and NG groups. The total cell count of the HG/GGA and NG/GGA (100 μM) groups 3 days after treatment was approximately half of each control group.

The trypan blue exclusion test was performed to investigate cell damage caused by GGA. The percentage of live cells dose-dependently decreased in the NG group, whereas there was no change in the HG group. The percentage of live cells was clearly decreased in the NG/GGA (100 μM) group 3 days after GGA treatment. These results indicated that GGA dose-dependently inhibited proliferation of damaged HeLa cells in an NG environment and inhibited proliferation of undamaged cell in a HG environment. Notably, cell injury was most pronounced in the NG/GGA (100 μM) group 3 days after treatment.

Cell death can be distinguished by apoptotic and necrotic events. In this study, the type of cell death was examined by flow cytometry. HeLa cells that were treated by HG/GGA and NG /GGA (100 μM) for 3 days were stained with PE Annexin V and 7-AAD to detect early apoptosis and necrosis, respectively. The percentage of late apoptotic/dead cells was significantly increased in the NG/GGA (100 μM) group compared with other groups. The percentage of early apoptotic cells did not significantly increase. These results were in accordance with those of the cell viability assay. The DNA fragmentation assay was also used to examine the type of cell death induced by GGA. Our results showed no DNA fragmentation in NG/GGA (100 μM) group 3 days after treatment, in which the most significant cell injury was observed in the cell viability assay. In the STS group, a clear DNA fragmentation was observed, which was used as a positive control of apoptosis. In contrast, apoptotic change was detected in the NG/GGA (100 μM) group by the TUNEL assay. These results indicated that cell death and apoptotic changes were induced in HeLa cells after NG/GGA (100 μM) treatment for 3 days, but typical apoptosis was not induced by STS. GGA treatment remarkably disrupted cellular adhesion, but it was maintained in the STS group. Previous studies have shown that effects of in vitro GGA concentrations on cancer cells vary approximately from 1 μM (2,21) to 100 μM (1,4,22), which were supported by our results.

An HG dose combined with GGA has cytostatic and cytotoxic effects on HeLa cells in an NG environment, but it remains unknown whether an HG dose combined with GGA induced HSP70 expression in HeLa cells. Western blot analysis revealed that HSP70 was induced in HeLa cells incubated in NG/GGA (100 μM) for 1 day (data not shown). HSP induction is upregulated in cancer cells and can be a factor of thermotolerance and

resistance to thermotherapy by cancer cells (23). HSP70 was upregulated in HeLa cells following HG/GGA treatment, but it was ineffective against cytotoxicity and GGA-induced cell death.

Our results showed that HG/GGA treatment was cytotoxic to HeLa cells in an NG environment, indicating the potential of GGA as an anti-cancer drug. Because of hypoxia, hypoglycemia, and acidemia in the hypovascular environment of solid cancers, cells become resistant to anti-cancer drugs such as etoposide, doxorubicin, and camptothecin (24-26). However, further studies are required to examine the effects of hypoxic and hypoglycemic environments on cancer cell proliferation. Furthermore, a pharmacokinetic study revealed that the serum concentration of a usual therapeutic dosage after GGA administration was 1–10 μM (27). Therefore, the route of administration should be considered because serum GGA concentrations cannot increase to 100 μM by oral administration. Therefore, studies of actual GAA therapeutic concentrations in cancer tissue should be examined using animal models. We examined the effect of GGA on HeLa cells in vitro and found that GGA exerted a cytostatic effect in a dose-dependent manner in NG and HG environments, and it had cytotoxic effects in an NG environment. Apoptosis was induced in the damaged cells. The results of the present study indicate that GGA is a potentially useful anti-cancer drug, but further studies are required to validate these results.

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Figure legends

Fig. 1

(A) Total cell count and (B) Percentage of live cells 3 days after treatment with Geranylgeranylacetone (GGA) (3, 10, 30, and 100 μM). (C) Changes in the percentage of live cells after 3 days in control and high glucose (HG) and normal glucose (NG) GGA (100 μM)-treated groups. All data are presented as means ± SEM (vertical bar) of 5 observations. $*p < 0.05$ vs. corresponding control

Fig. 2

Microscopic changes in the morphological features of HeLa cells 2 days after Geranylgeranylacetone treatment day 3 (magnification, ×400) (A) High glucose (HG)/control; (B) HG/(GGA) (100 μM); (C) Normal glucose (NG)/control; (D) NG/GGA (100 μM); and (E) Staurosporine (STS)

Fig.3

(A) DNA laddering and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick and labeling (TUNEL) 3 days after GGA (100 μM) treatment. (B) High glucose (HG)/control; (C), HG/GGA (100 μM); (D), Normal glucose (NG)/control; (E), Normal glucose (NG)/GGA (100 μM); and (F) Staurosporine (STS)

Fig. 4

Cell apoptosis was detected by annexin staining (x-axis), and late apoptosis or necrosis was determined by 7-AAD staining (y-axis)

(A) Representative results (B) The percentage of early apoptotic cells and late apoptotic or necrotic cells are shown. All data are presented as means ± SDM (vertical bar) of 3 observations. $*p < 0.05$ vs. corresponding control

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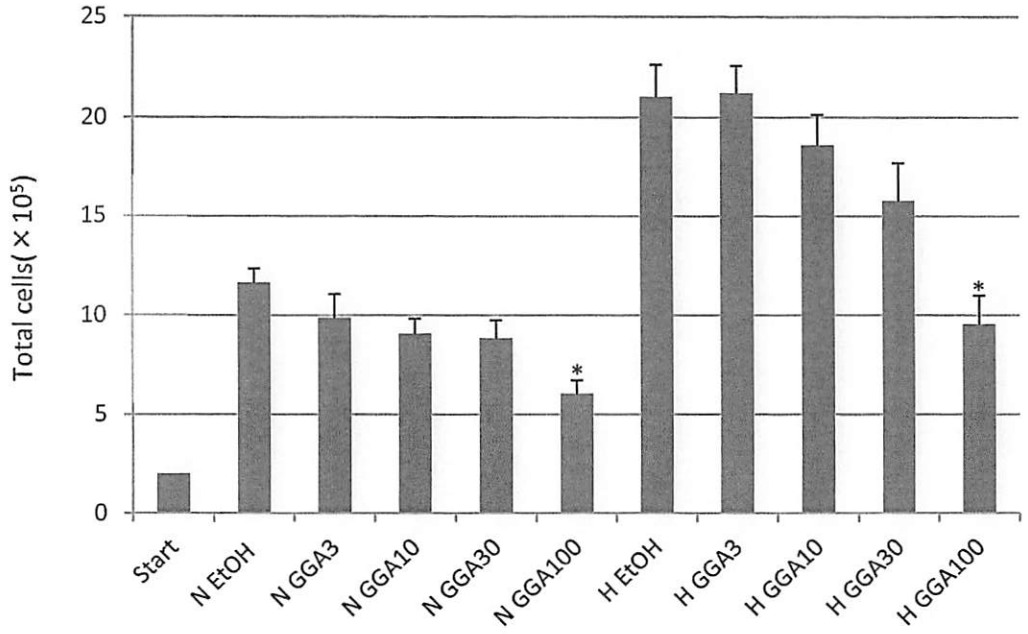
Fig. 1 (A)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*
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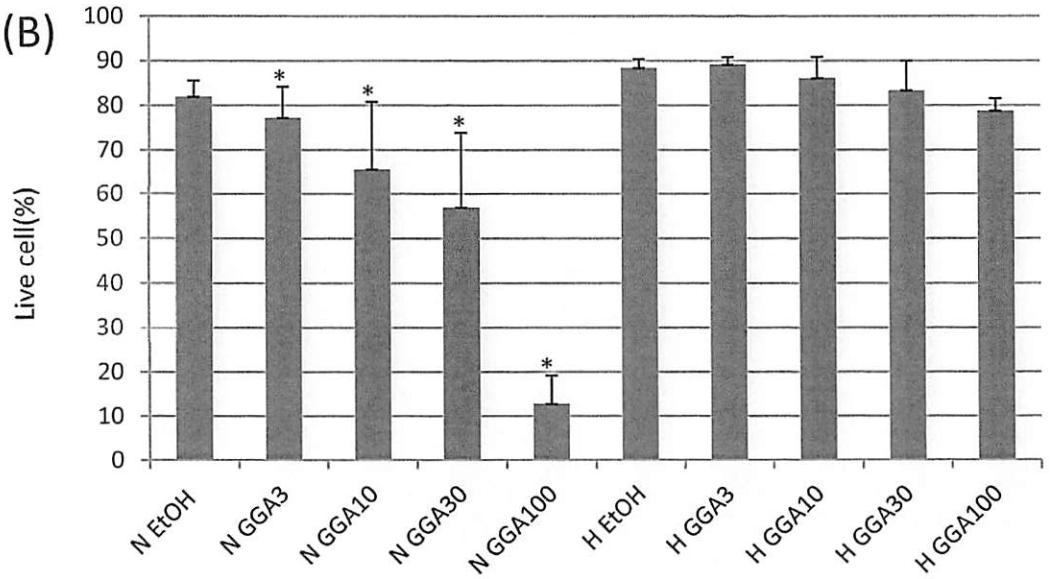
H GGA10
H GGA30
H GGA100

(B)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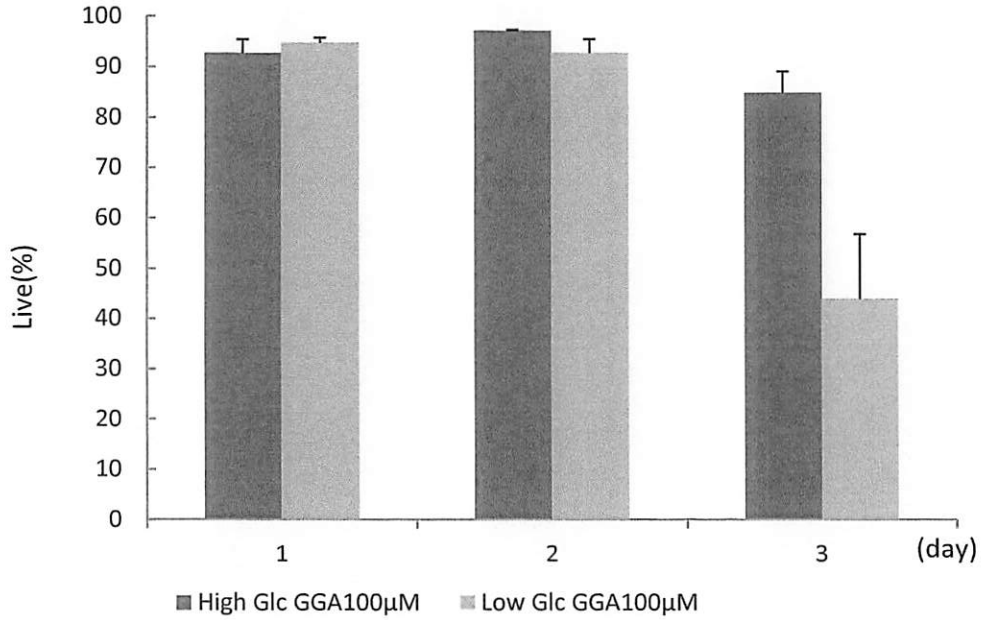
(C)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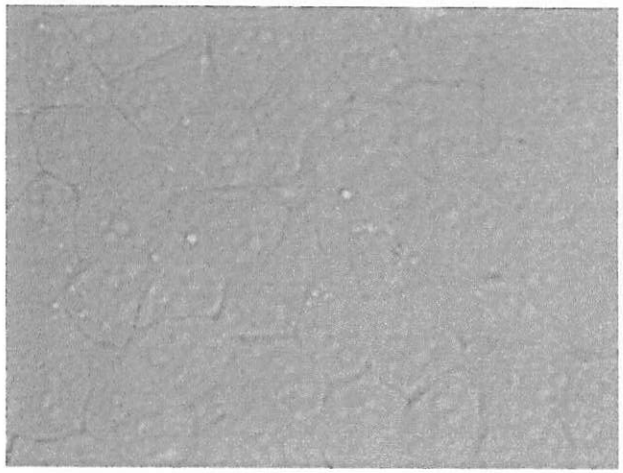

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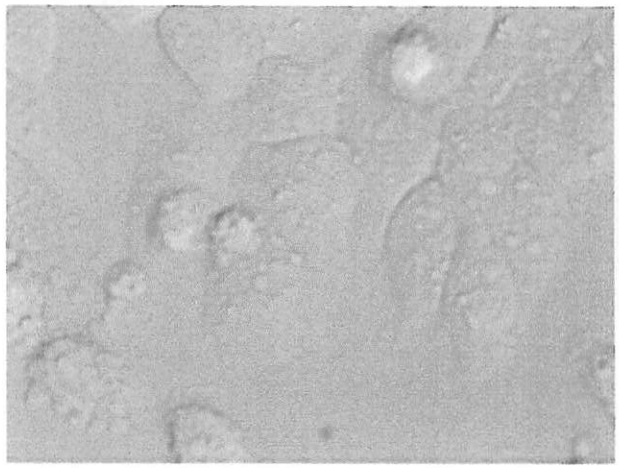

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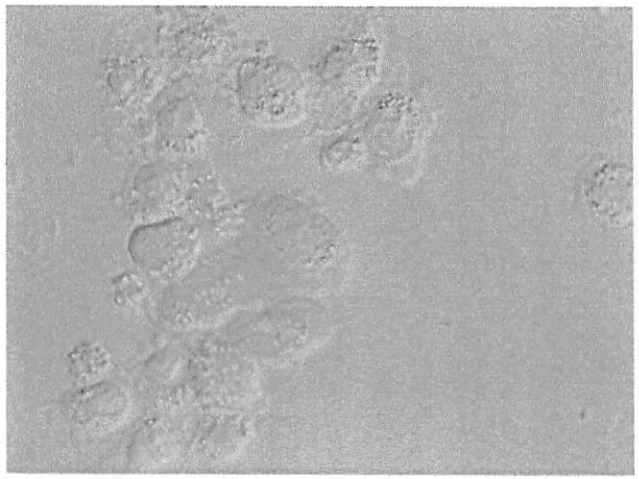

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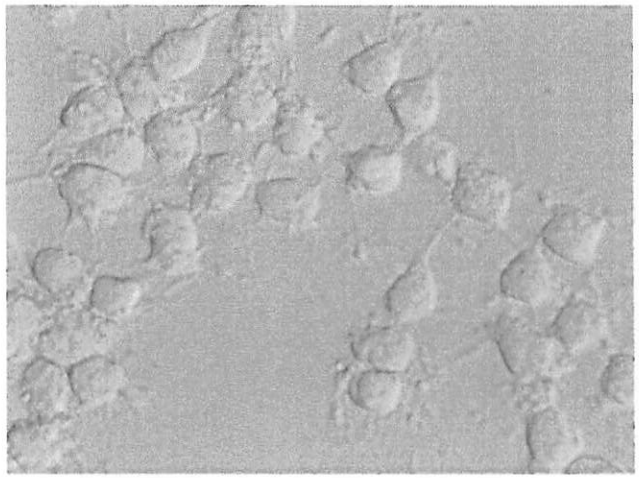

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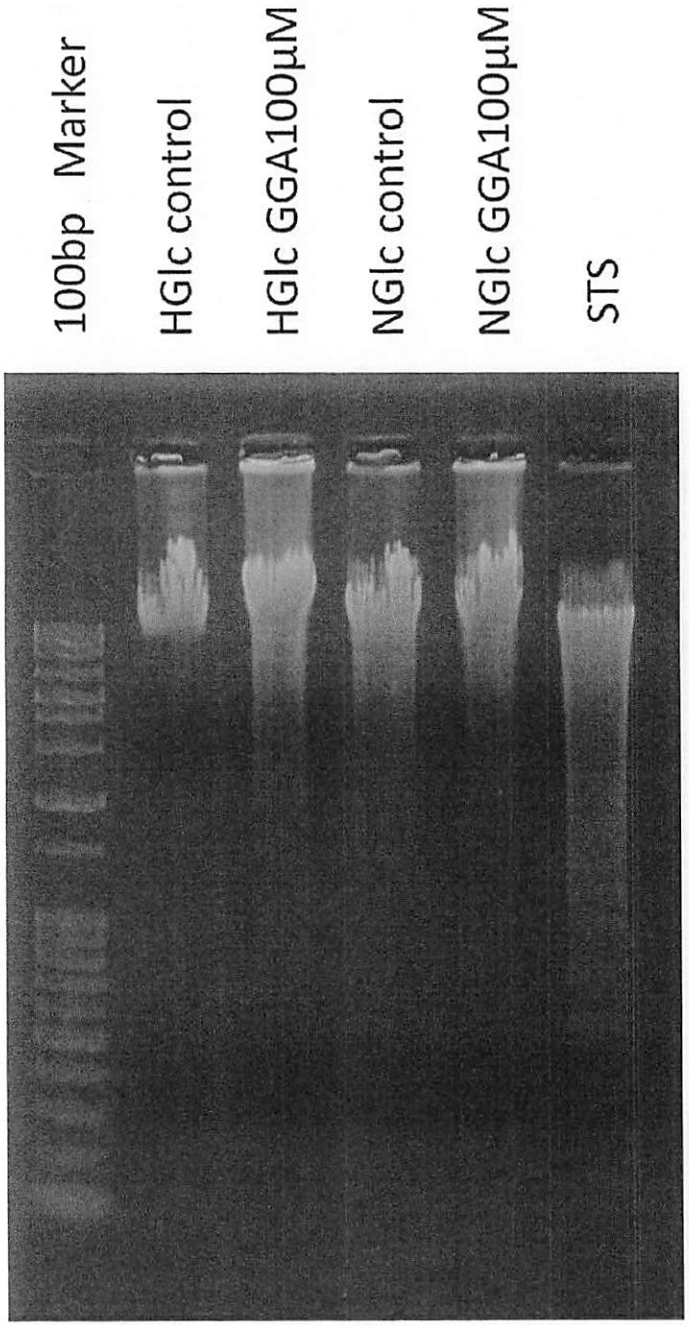
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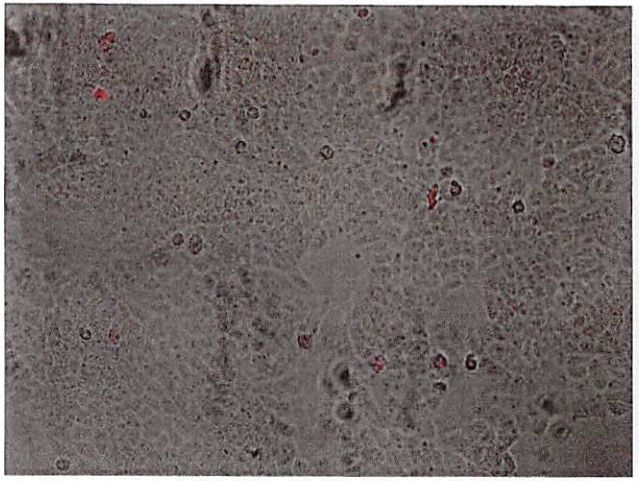
(B)



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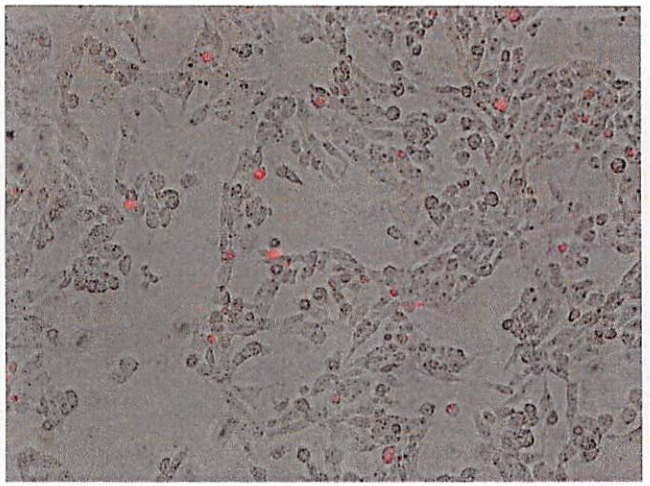

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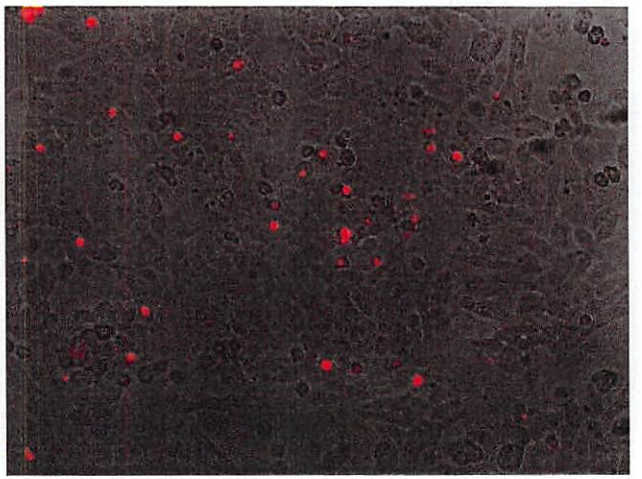

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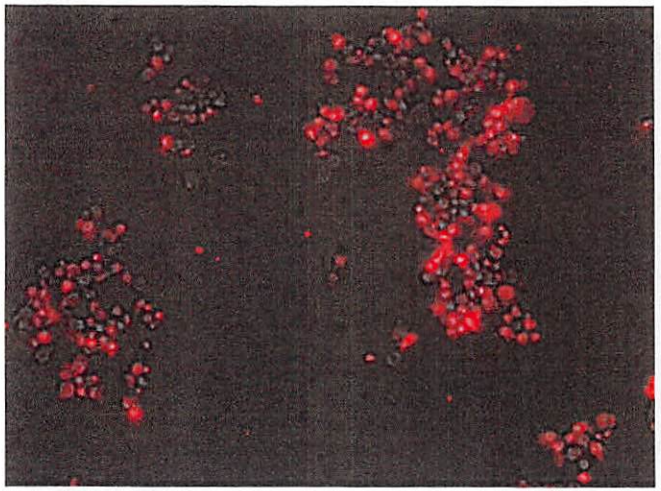

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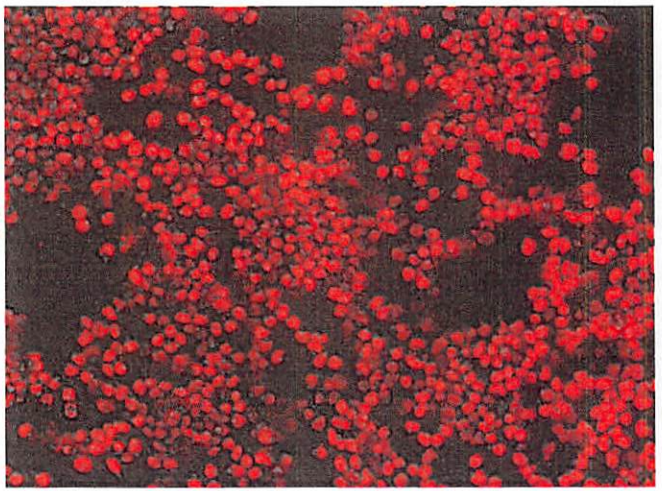

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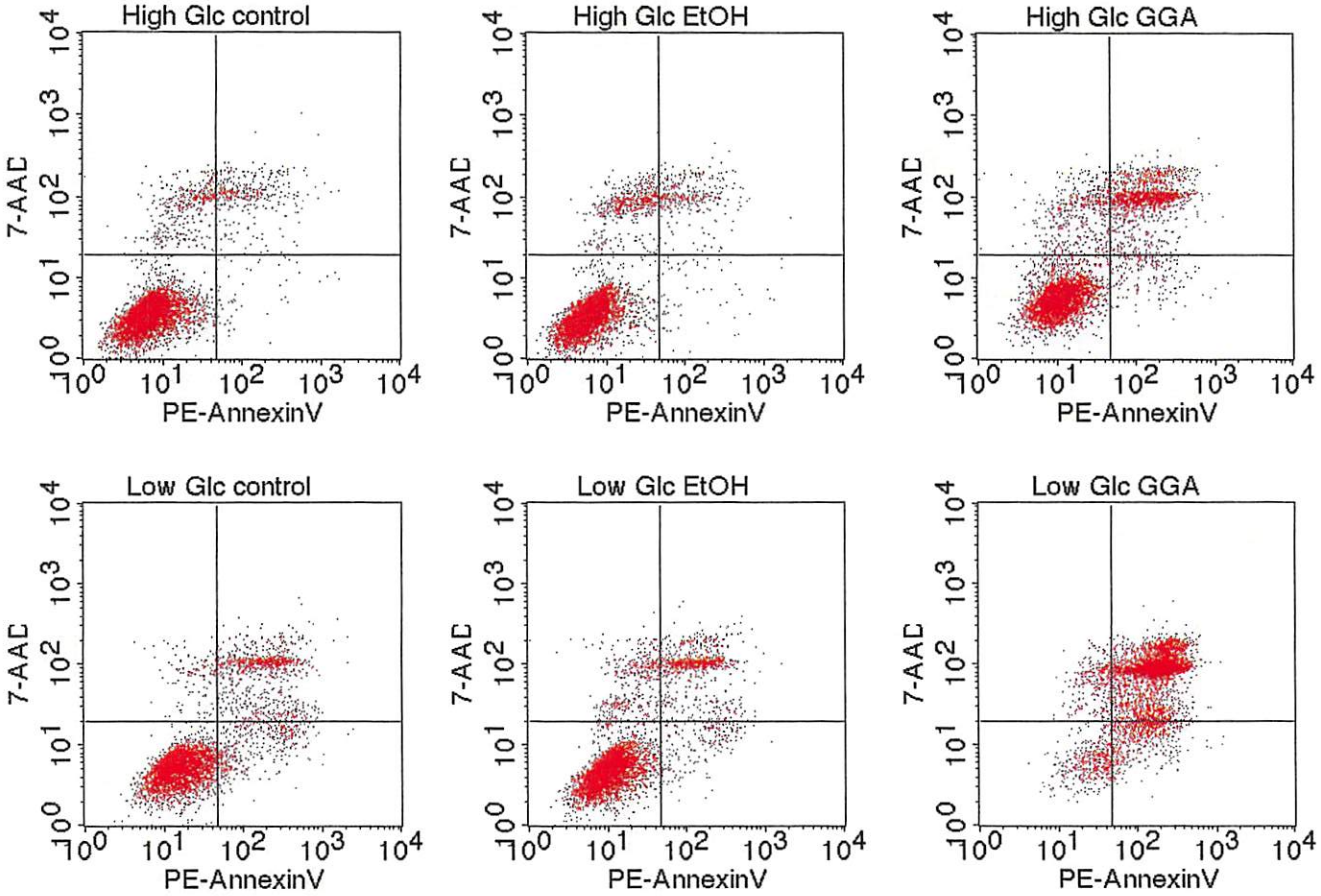
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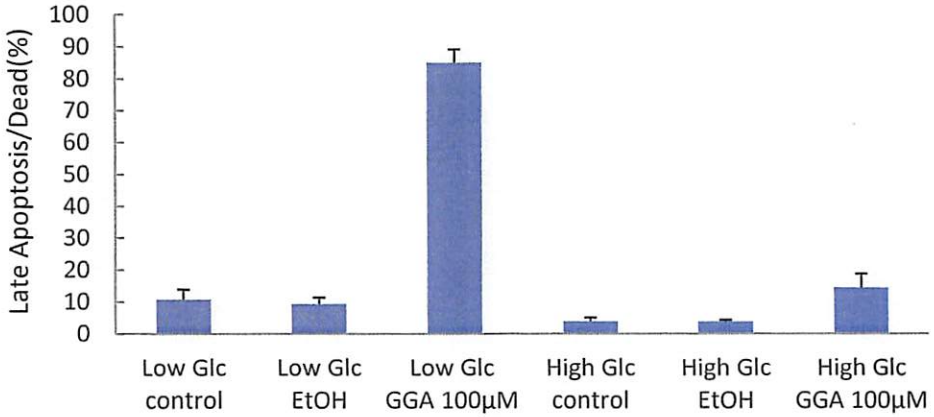
(B)


Late Apoptosis/Dead(%)
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High Glc control
High Glc EtOH
High Glc GGA 100μM