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Original Research

Combinatorial effects of metformin and glucose on the immune evasion of breast cancer 4T1 cells

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ABSTRACT

Introduction: Metformin is one current medicine used to treat type 2 diabetes. Numerous studies have shown that high metformin concentrations have an anticancer effect. Therefore, this study aimed to examine whether combining various metformin and glucose concentrations affects mouse breast cancer cell proliferation, migration, and expression of immune escape-related genes. **Methods**: This study assessed 12 glucose and metformin combinations: four glucose concentrations (0, 0.5, 1.0, and 4.5 g/L) and three metformin concentrations (0, 2.0, and 5.0 mM). Mouse breast cancer 4T1 cells were cultured in RPMI 1640 media containing these 12 combinations at 37°C with 5% CO_2. The combinatorial effects of metformin and glucose were evaluated based on 4T1 cell proliferation, migration, and expression of immune escape-related genes. **Results**: Combining 2 mM metformin with 4.5 g/L glucose concentration inhibited 4T1 cell proliferation, migration, and expression of immune escape-related genes. **Conclusion**: Our findings provide more information about the anticancer effects of metformin under high glucose conditions, help explain why metformin effectively treats cancer in patients with type 2 diabetes, and suggest combining metformin with glucose in anticancer treatment.

Key words: Anti-cancer, Breast cancer, Glucose, Metformin, Tumorigensis, Type 2 diabetes

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INTRODUCTION

Patients with type 2 diabetes are known to be at a higher risk of tumorigenesis[1]. Therefore, researchers have suggested that glucose levels could affect tumor growth. Glucose is considered an important fuel source because it provides energy to cells in the form of ATP and produces intermediate products, such as lactate, for cell survival and growth[2]. Cancer cell populations have been demonstrated to utilize high glucose levels for proliferation. Glucose participates in many metabolic pathways within cells. Unlike normal cells, cancer cells can reprogram their metabolism, extracting energy from glucose via glycolysis (Warburg effect). Therefore, these reprogramming mechanisms promote cancer cell proliferation, migration, and especially immune escape, making them challenging to kill[3–5]. Furthermore, immune cells use this metabolism to grow and proliferate. However, immune cells compete with cancer cells for glucose sources in the tumor environment. Therefore, immune cell proliferation and survival are impeded, making it more challenging to eradicate cancer cell populations.

The immune system is responsible for protecting the body from harmful agents. Notably, cancer cells interact closely with the immune system. One key step influencing immune escape is blocking the interaction between cancer and immune cells. As is typical of the interaction between programmed death-ligand 1 (PD-L1) on cancer cells and programmed cell death 1 (PD-1) on immune cells, once *PD-L1* is overexpressed, a PD-1/PD-L1 complex forms, causing a signaling cascade that inhibits immune cells[6]. In addition, the metabolism of the immune cells is impacted by the nutritionally uneven tumor microenvironment. Glycolysis dominates in the tumor microenvironment, having multiple effects on immune cell populations, such as decreased activity of CD4 and CD8 T cells and capacity of memory T cells through several intracellular signaling pathways such as the AMP-activated protein kinase (AMPK)/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) pathway[7]. The tumor microenvironment also impacts tumor-associated macrophages. It promotes cancer cell survival and growth, which are stimulated to form the M1 phenotype when the glycolysis pathway dominates. Numerous studies on glucose metabolism have been initiated due to these drugs to ensure efficient cancer treatment. Metformin is one drug utilized in current basic research and clinical studies.

The drug metformin acts on the glucose metabolism pathway and is effective in treating type 2 diabetes.

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Recent studies have demonstrated its impact on the effectiveness of cancer treatment. It was more effective in treating patients with type 2 diabetes and cancer than those with type 2 diabetes alone[8,9]. One of the numerous ways metformin impacts cancer cells is through their relationship with the immune system, which is one process through which they avoid being eliminated by the immune system, called immune escape[10,11]. In order to eliminate "foreign" cells in this area, cancer cells produce enzymes and cytokines through surface markers, immune checkpoints, and immune cells. Metformin acts on cell signals, causing changes in the expression of these immune markers, which help in the effective recognition of cancer cells by immune cells[?]. PD-L1 is one well-known checkpoint in cancer cells. Metformin was found to interact favorably with PD-L1, influence PD-L1 expression, and be a potent anti-PD-L1 agent that limits the immune-escape ability of cancer cells by enhancing the activity of immune cells such as T cells[?]. Cancer killing may affect other cell populations at different metformin concentrations, such as mesenchymal stem cells (MSCs)[?].

Therefore, we wondered whether high glucose levels impact the cancer population through metformin. Combining an appropriate metformin concentration with a suitable glucose concentration could inhibit immune escape and protect beneficial cell populations. This study aimed to investigate the combinatorial effects of metformin and glucose on mouse breast cancer 4T1 cell proliferation, migration, and expression of immune escape-related genes.

MATERIALS AND METHODS

Cell line

The mouse 4T1 cell line (American Type Culture Collection) was thawed and expanded according to the provided guidelines. It was maintained at 37°C in a humidified atmosphere (95%) containing 5% CO_2. It was cultured in growth medium (Roswell Park Memorial Institute [RPMI] 1640 containing 2 mmol/L glutamine [Sigma-Aldrich, Louis St, MO, USA], 10% fetal bovine serum [FBS; Gibco], 1% antibiotic-antimycotic [Sigma-Aldrich]).

Experimental designs

The RMPI 1640 medium was supplemented with four glucose concentrations (0, 0.5, 1.0, and 4.5 g/L) and three metformin concentrations (0, 2, and 5 mM). Therefore, this study examined 12 glucose and metformin combinations (G1 to G12; **Table 1**).

The 4T1 cells were expanded in the standard growth medium and then split into six-well plates at 5,000 cells/well. After 24 h of culturing, the standard growth medium was replaced with the G1–G12 media. The cells were collected for further experiments after 24, 48, and 72 h of culturing in the G1–G12 media. Each medium was used in three wells, and the experiments were performed in triplicate.

Cell viability

The Alamar blue assay was used to assess cell proliferation. The cells were seeded at a density of 5,000 cells/well in 96-well plates. After 24 hours of culturing, the growth medium was discarded and replaced with G1–G12 media. Then, the cells were stained with Alamar blue (Sigma-Aldrich) at a dye:medium ratio of 1:10. After one hour of staining at 37°C with 5% CO_2, each well's optical density at 595 nm was determined using a DTX880 multimode detector (Beckman Coulter, USA). Data was collected every 24 hours. Triplicates were performed for each group.

Quantitative real-time PCR (qRT-PCR)

The qRT-PCR was performed according to a general protocol. RNA was extracted using the EasyBlue Kit (Thermo Fisher Scientific, USA), and cDNA was synthesized via reverse transcription. The relative mRNA levels were determined using a Luna One-Step RT-qPCR Kit (New England Biolabs). Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was used as the endogenous control. The experiment was performed in triplicate. The following primers were used to amplify the target genes by RT-qPCR: *Gapdh* forward (GCATCTTCTTGTGCAGTGCC) and reverse (TACGGCCAAATCCGTTCACA), solute carrier family 2 (facilitated glucose transporter), member 1 (*Slc2a1*/*Glut1*) forward (ATCGTCGTTGGCATCCTTATT) and reverse (ATCGTCGTTGGCATCCTTATT), *Pd-l1* forward (TCCATCCTGTTGTTCCTCATT) and reverse (TCCATCCTGTTGTTCCTCATT), Fas cell surface death receptor (*Fas*/*Cd95*) forward (TATCAAGGAGGCCCATTTTGC) and reverse (TGTTTCCACTTCTAAACCATGCT), C-X-C motif chemokine ligand 12 (*Cxcl12*) forward (TGCATCAGTGACGGTAAACCA) and reverse (CACAGTTTGGAGTGTTGAGGAT), CD276 antigen (*Cd276*/*B7h3*) forward (AGCACTGTGGTTCTGCCTCACA) and reverse (CACCAGCTGTTTGGTATCTGTCAG), transforming growth factor beta 1 (*Tgfb1*) forward (CGGGTCTACTATGCTAAAGAGGTCAC) and reverse (TTTCTCATAGATGGCGTTGTTGC),

Table 1: The examined glucose and metformin combinations

Groups	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
Glucose (g/L)	0	0	0	0.5	0.5	0.5	1	1	1	4.5	4.5	4.5
Metformin (mM)	0	2	5	0	2	5	0	2	5	0	2	5

and SMAD family member 3 (*Smad3*) forward (GCAGCCGTGGAACTTACAAGGC) and reverse (GGTAGACAGCCTCAAAGCCCTG).

Wound-healing assay

The 4T1 cells were seeded into 24-well plates (SPL, Korea) at 1.5×10^5 cells/well in RPMI 1640 medium supplemented with 10% FBS and cultured at 37°C with 5% CO_2 for 24 h to form a monolayer. When the cells reached 80% confluence, the middle of the monolayer was scratched with a sterile 100 μL pipette tip and washed with phosphate-buffered saline to clear the scratch. Then, fresh G1–G12 medium was added to each well. After 24 h of culturing, wound closure was evaluated in three randomly selected fields in each well using an inverted microscope (Carl Zeiss Microscopy, LLC). The experiment was performed in triplicate for each group.

Cell morphology

The 4T1 cells were imaged at 5× and 10× magnification after treatment with various metformin and glucose concentrations. The 4T1 cells were seeded at a density of 5,000 cells/well in 96-well plates, and images were taken every 24 h for three days. The images were processed with Axio Vision software. All experiments were performed at least three times.

Statistical analysis

The data were analyzed using GraphPad Prism 8.0 software (GraphPad, USA). Data were compared between groups using Student's *t*-test or one-way analysis of variance. The data are presented as mean ± standard deviation (SD). A $P < 0.05$ was considered statistically significant. All experiments were repeated at least three times.

RESULTS

The joint effect of metformin and glucose on 4T1 cell proliferation

The 4T1 mouse breast cancer cells showed considerable proliferation when cultured in G1, G4, G7, and G10 media that lacked metformin, particularly at higher (G10) than lower (G4 and G7) glucose concentrations. The 4T1 cells showed lower proliferation in the G2, G3, G5, G6, G8, G9, G11, and G12 media containing metformin than in the G1, G4, G7, and G10 media lacking metformin. In particular, the proliferation of 4T1 cells was significantly decreased in the G2, G3, G5, and G6 media, which combined metformin with low glucose concentrations (**Figure 1**). These results suggest that metformin inhibits 4T1 cell proliferation at any glucose concentration; the inhibition increased with the metformin concentration.

The joint effect of metformin and glucose on 4T1 cell migration

The 4T1 mouse breast cancer cells cultured in G1, G4, G7, and G10 media lacking metformin showed strong migration abilities, particularly at higher glucose concentrations. The 4T1 cells showed lower migration when cultured in the G2, G3, G5, G6, G8, G9, and G12 media containing metformin than in the G1, G4, G7, and G10 media lacking metformin. In particular, while 4T1 cells cultured in the G2, G3, and G6 media shed and could not migrate, 4T1 cell migration was significantly decreased when cultured in the G5 medium, which combined metformin with a low glucose concentration. In contrast, 4T1 cells showed better migration when cultured in the G8, G9, G11, and G12 media, which combined high metformin and glucose concentrations. However, these groups migrated less than 4T1 cells cultured in media lacking metformin. These results indicate that metformin inhibits 4T1 cell migration at any glucose concentration; the inhibition increased with the metformin concentration (**Figure 2**).

The joint effect of metformin and glucose on *Pd-l1* gene expression in 4T1 cells

Pd-l1 gene expression was similar in 4T1 cells cultured in G1, G4, G7, and G10 media lacking metformin. Interestingly, the media lacking glucose (G1, G2, and G3) but containing metformin did not affect *Pd-l1* gene expression in 4T1 cells. However, in 4T1 cells cultured in media containing physiological (G7, G8, and G9) or low (G4, G5, and G6) glucose

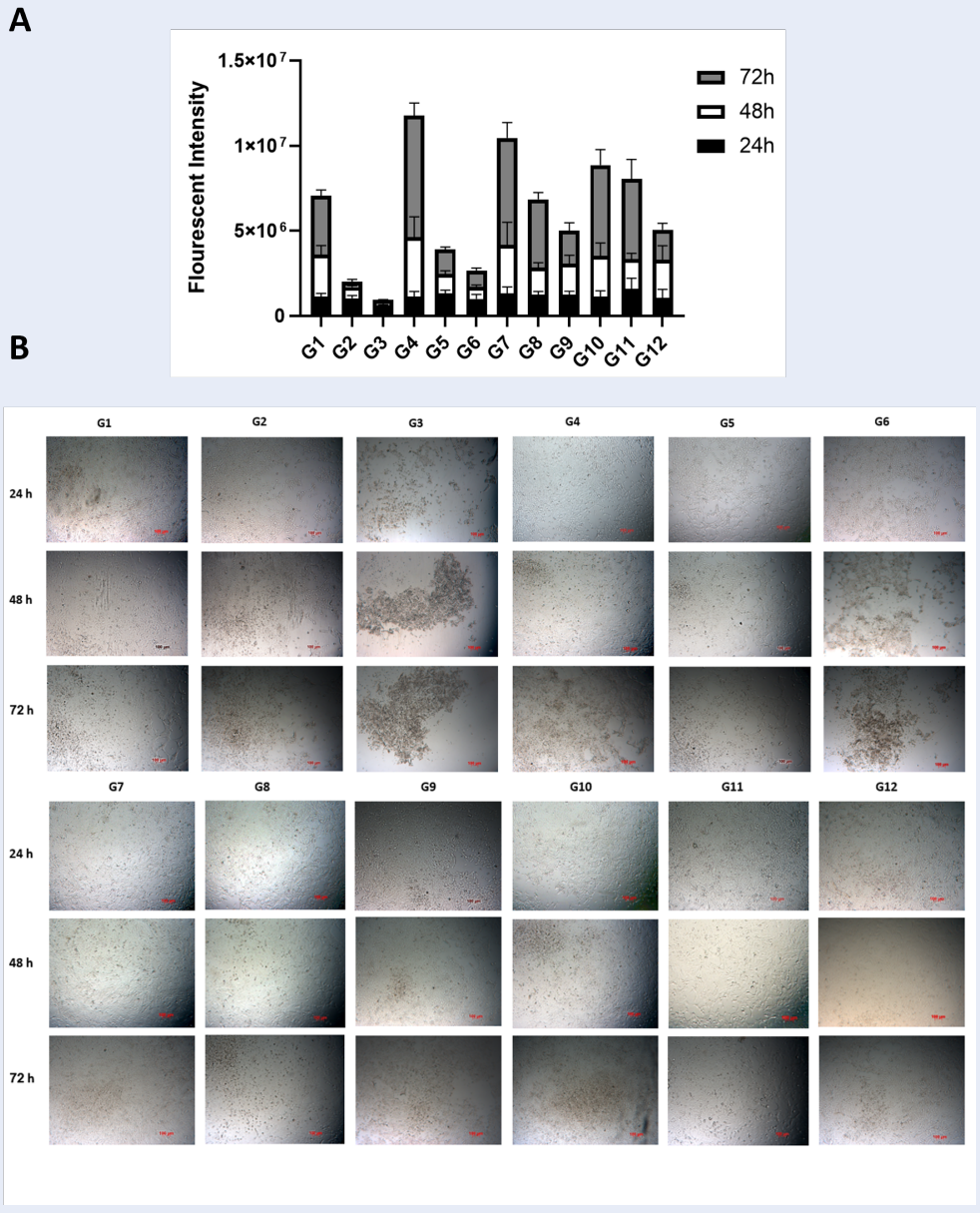

Figure 1: **The proliferation of 4T1 mouse breast cancer cells under different concentrations of metformin and glucose**. (**A**) The proliferation graph exhibited under different concentrations of metformin and glucose at 24, 48 and 72 hours. (**B**) The data exhibited the morphology of 4T1 under different concentrations of metformin and glucose at 24, 48 and 72 hours.

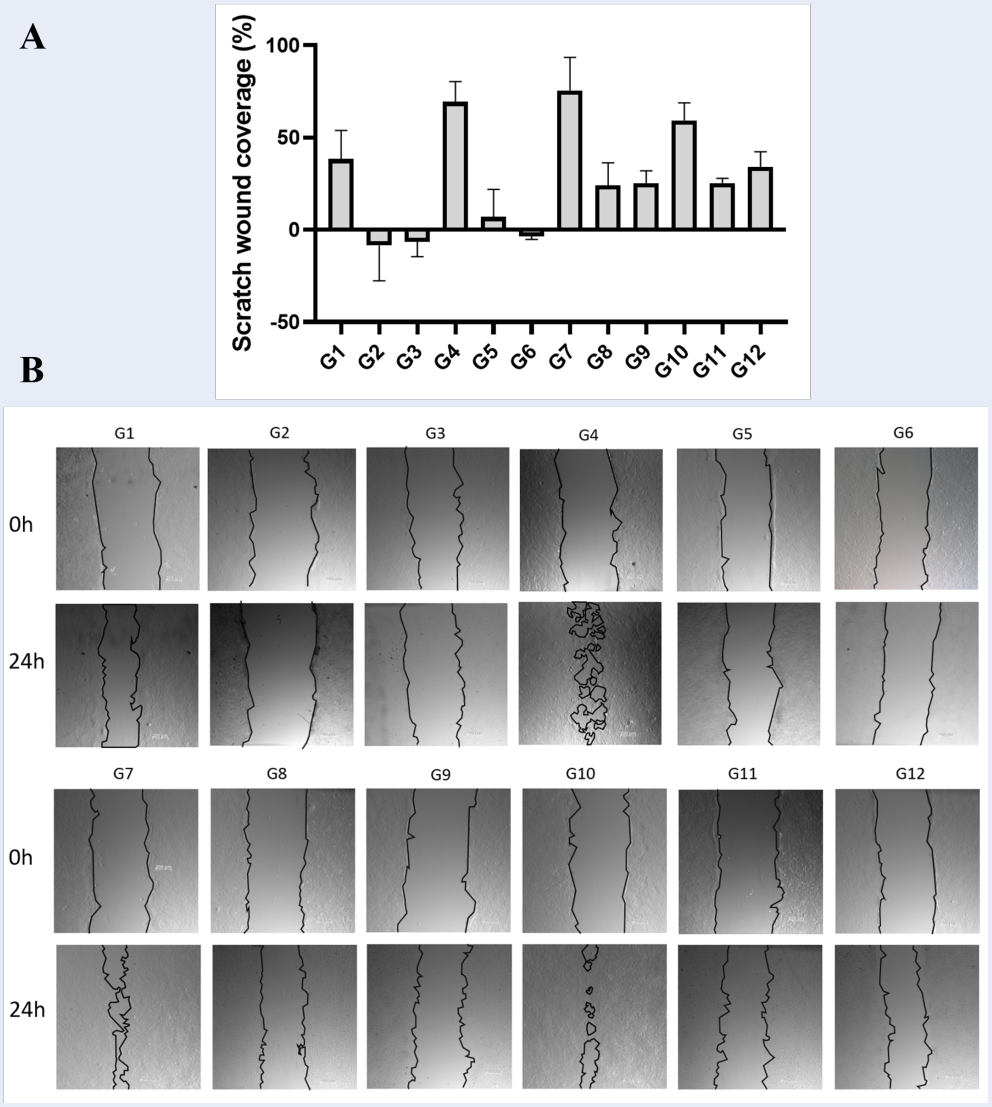

Figure 2: **The migration of 4T1 mouse breast cancer cells under different concentrations of metformin and glucose**. (**A**) The graph depicted the migratory capability of the 4T1 cell line under different concentrations of metformin and glucose at 24 hours. (**B**) The data exhibited the morphology of 4T1 under different concentrations of metformin and glucose at 0 hours and 24 hours.

concentrations, *Pd-l1* gene expression tended to increase with the metformin concentration. Interestingly, when considering only the media with the highest glucose concentration (G10, G11, and G12), *Pd-l1* gene expression was significantly lower in 4T1 cells cultured in media containing metformin (G11 and G12) than lacking metformin (G10) (**Figure 3**). These results showed that metformin inhibits *Pd-l1* gene expression in 4T1 cells at any glucose concentration; the inhibition increased with the metformin concentration.

Combining high glucose concentrations with metformin reduced the immune escape capacity of 4T1 cells

Based on the above findings, particularly for *Pd-l1* gene expression, a high glucose concentration (4.5 g/L) appeared to impede the immune escape capacity of mouse 4T1 breast cancer cells (**Figure 3**). Therefore, we investigated the migration of 4T1 cells cultured at a high glucose concentration and treated with various metformin concentrations. When cultured with a high glucose concentration (4.5 g/L), the migration of 4T1 cells decreased significantly with in-

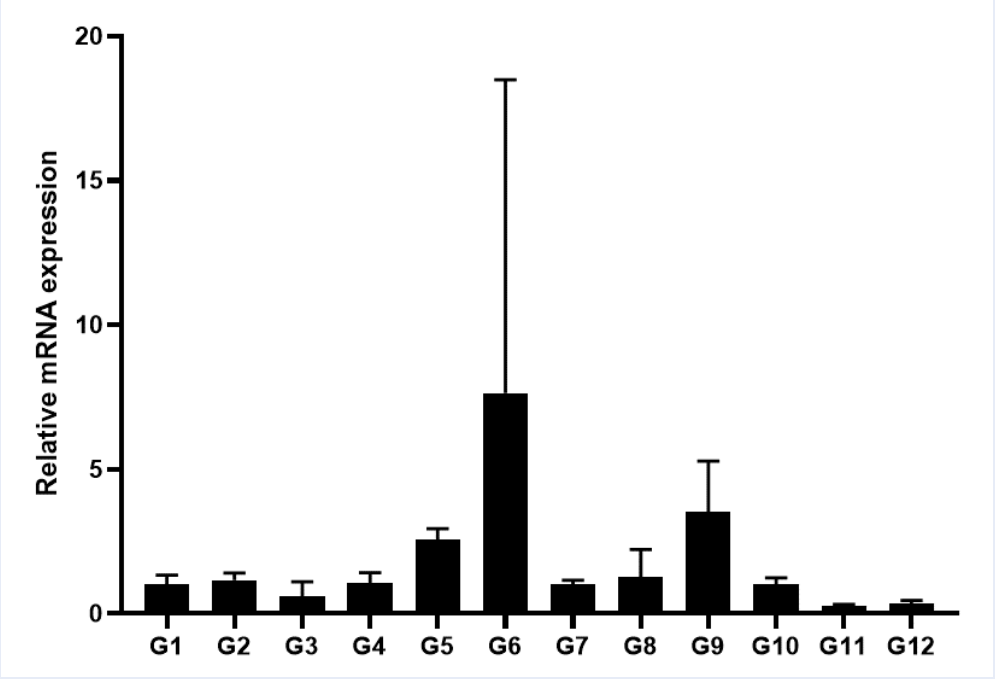

Figure 3: **The expression of *PD-L1* was evaluated in response to varying concentrations of glucose and metformin**. Data were normalized to GAPDH levels. Experiments were repeated three times with similar data.

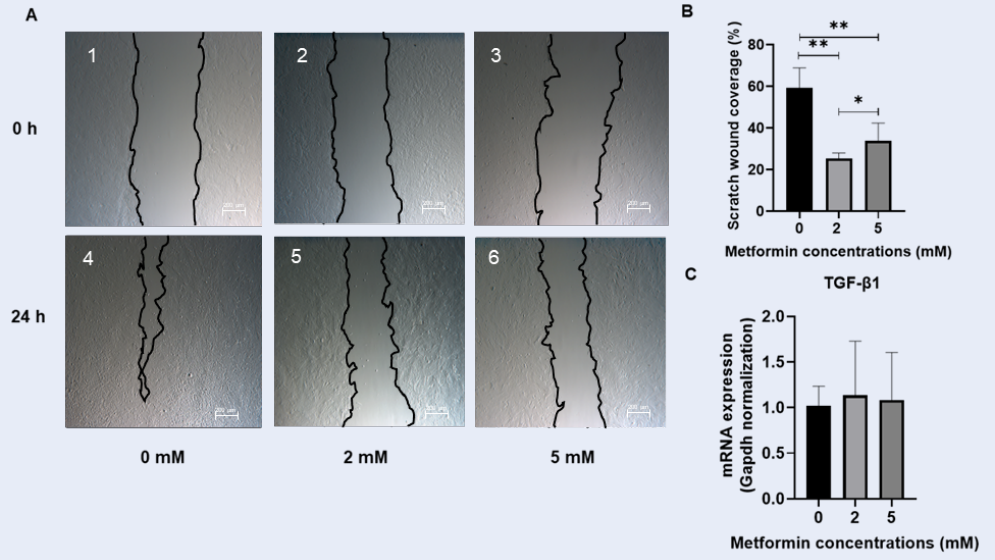

Figure 4: **The migration of 4T1 mouse breast cancer cells at 4.5 g/L glucose concentrations in 3 metformin concentrations including 0mM, 2mM and 5mM**. (**A**) The data exhibited the morphology of 4T1 at 3 metformin concentrations under 4.5g/L glucose at 0 hours (1-3) and 24 hours (3-6). (**B**) The graph depicted the migratory capability of the 4T1 cell line under 3 different metformin concentrations at 24 hours (*P < 0.05, **P < 0.01). (**C**) The expression of TGF-β1 was also assessed at various metformin concentrations, including 0 mM, 2 mM, 5 mM.

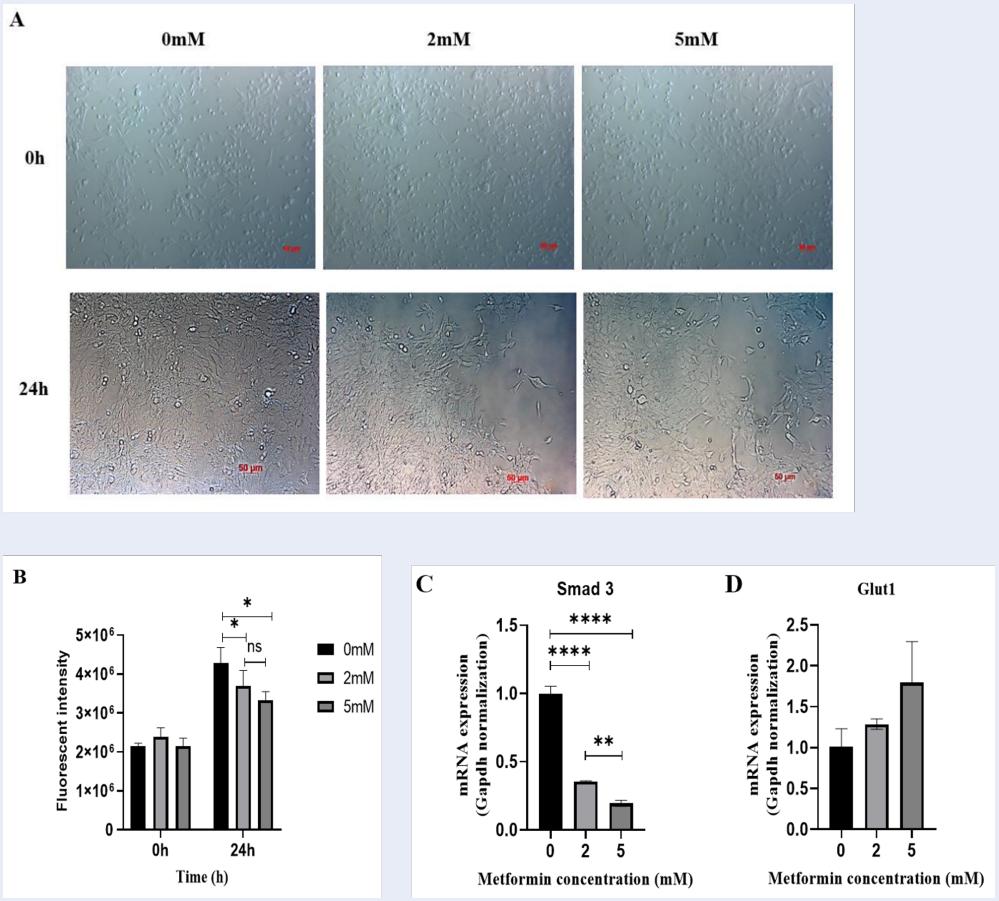

Figure 5: **The proliferation of 4T1 cells under 3 metformin concentrations 0 mM, 2 mM, 5 mM at 4.5 g/L glucose concentration**. (**A**) The 4T1 morphology during proliferation at 0 hours (1-3) and 24 hours (4-6). (**B**) The graph depicted the proliferation capability of the 4T1 cell line under 3 different metformin concentrations at 24 hours (*P < 0.05). (**C**) *Smad3*, (**D**) *Glut1* gene expression at 0 mM, 2 mM and 5 mM metformin (**P < 0.01, ****P < 0.001).

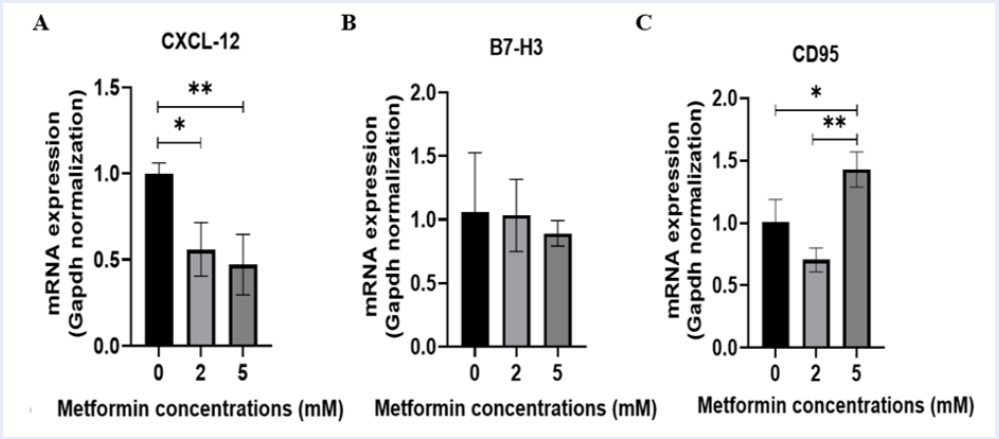

Figure 6: **The immune escape expression gene of 4T1 mouse breast cancer cells under 3 metformin concentrations at 4.5 g/L glucose**. Gene express (**A**) CXCL-12; (**B**) B7-H3; (**C**) CD95 (*P < 0.05, **P < 0.01).

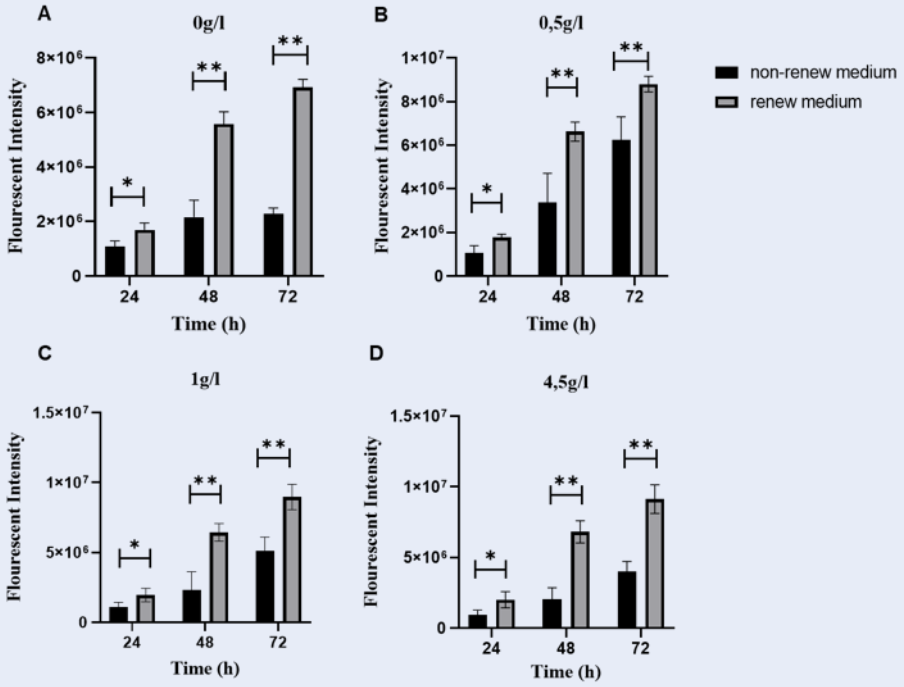

Figure 7: **The proliferation of 4T1 mouse breast cancer cells at four different glucose concentrations containing (A) 0 g/L; (B) 0.5 g/L; (C) 1 g/L; (D) 4.5 g/L which non-renew medium and re-renew medium** (*P < 0.05, **P < 0.01).

creasing metformin concentration. At 2 and 5 mM metformin, the migration of 4T1 cells was decreased by around 2 ($p < 0.01$) and 1.5 ($p < 0.01$) times, respectively, compared to 0 mM metformin (control). In addition, the migration of 4T1 cells differed significantly between 2 and 5 mM metformin, with migration 1.3 times higher with 5 mM metformin than with 2 mM metformin ($p < 0.05$; **Figure 4**). In contrast, *Tgfb1* gene expression did not change significantly with metformin concentration ($p > 0.05$; **Figure 4**). These results indicate that high glucose concentrations influence the immune escape abilities of 4T1 cells via *Pd-l1* gene expression and migration. However, they do not indicate that high glucose concentrations influence the expression of the migration-related gene *Tgfb1*.

We next investigated the effects of a high glucose concentration (4.5 g/L) and various metformin concentrations on mouse 4T1 breast cancer cells using various approaches, including assessing cell density using inverted microscopy, proliferation ability using Alamar blue staining, and the expression of proliferation-related gene *Smad3* and metabolism-associated gene *Glut1*. The cell density assessments showed that 4T1 cells grew normally and without apoptosis (**Figure 5**), consistent with the earlier findings (**Figure 1**). In addition, 4T1 cells showed lower proliferation with than without metformin after 24 h. Specifically, 2 and 5 mM metformin decreased the growth of 4T1 cells by 1.58 times ($p < 0.05$) and 1.64 times ($p < 0.05$), respectively, compared to 4T1 cells not treated with metformin. However, 4T1 cell proliferation did not differ significantly between 2 and 5 mM metformin ($p > 0.05$; **Figure 5**).

Smad3 gene expression was closely associated with the proliferation of 4T1 mouse breast cancer cells, decreasing as the metformin concentration increased (**Figure 5C**). *Smad3* gene expression was significantly lower in 4T1 cells treated with 2 mM (2.86-fold; $p < 0.0001$) and 5 mM (5.26-fold; $p < 0.0001$) metformin than in cells not treated with metformin (**Figure 5 C**). In addition, *Glut1* gene expression increased with the glucose concentration, although it did not change significantly ($p > 0.05$; **Figure 5 D**).

The immune escape capacity of mouse 4T1 breast cancer cells was then explored by examining the expression of some associated genes: *Cxcl12, B7h3*, and *Cd95* (**Figure 6**). The expression of immune escape-related genes decreased significantly with increasing metformin concentration. *Cxcl12* gene expression

was significantly lower at 2 mM metformin (1.6-fold; $p < 0.05$) and 5 mM metformin (2.0-fold; $p < 0.05$) than at 0 mM metformin (**Figure 6A**). In contrast, *Cd95* gene expression was significantly lower at 2 mM metformin than at 0 mM metformin (1.25-fold; $p < 0.05$) and 5 mM metformin (1.8-fold; $p < 0.05$; **Figure 6 C**). However, while *B7h3* gene expression decreased as the metformin concentration increased, the changes were nonsignificant ($p > 0.05$; **Figure 6 B**).

Then, we compared the proliferation of mouse 4T1 breast cancer cells cultured with fresh medium every 24 h to those cultured without media exchange over three days. This experiment was conducted to ensure that the daily change of the medium maintained the desired glucose concentration, replacing the glucose used by the cells, which could lead to inaccurate assessments for each glucose concentration (0, 0.5, 1.0, and 4.5 g/L). The growth rate differed significantly between changing the medium daily and not changing the medium. The growth rate was significantly higher with than without daily medium changes at 24 h ($p < 0.05$), 48 h ($p < 0.01$), and 72 h ($p < 0.01$; **Figure 7**). Therefore, replacing the medium every 24 h ensured a constant glucose concentration that helped stabilize the environment for cancer cells.

Overall, these results showed that a high glucose concentration (4.5 g/L) affected the expression of immune escape-related genes in mouse 4T1 breast cancer cells when combined with different metformin concentrations.

DISCUSSION

The combined effects of glucose and metformin on mouse 4T1 breast cancer cells revealed that glucose influenced cell proliferation, although proliferation did not differ significantly between concentrations. When combined with metformin, mouse 4T1 breast cancer cell proliferation varied depending on glucose levels. Mouse 4T1 breast cancer cell growth was clearly inhibited by 2 and 5 mM metformin. Notably, 2 mM metformin showed lower inhibition of 4T1 cell proliferation than 5 mM metformin, and there was a difference in glucose concentrations over 72 h. A wound healing experiment demonstrated that 4T1 cells showed reduced proliferation and migration with a high glucose concentration (4.5 g/L) and 2 mM metformin after 24 h. In addition, the scratch images after 24 h were consistent with the hypothesis that a 2 mM metformin concentration inhibited migration but did not induce cell death, as seen at lower glucose concentrations.

While the physiological level of glucose in the body is 1 g/L, there were similarities in the inhibition of migration to higher glucose concentrations (e.g., 4.5 g/L). However, the cell coverage ratio at 4.5 g/L glucose was proven to correlate with the expression of specific immune escape-related genes, including *Pd-l1* and *Cd95*. The expression levels of proliferative gene *Smad3*, glucose metabolism gene *Glut1*, and immune escape genes *Pd-l1, B7h3, Cxcl12*, and *Cd95* were evaluated in mouse 4T1 breast cancer cells with a glucose concentration (4.5 g/L) and various metformin concentrations using RT-qPCR. Proliferation-associated *Smad3* gene expression was proportionately reduced and positively correlated with 4T1 cell proliferation. Metformin did not significantly affect the expression of the glucose transporter *Glut1*. Similarly, metformin did not significantly affect the expression of migration-associated gene *Tgfb1* in 4T1 cells. However, the expression of immune escape-related genes *B7h3* and *Cxcl12* tended to decrease as the metformin concentration increased. *Pd-l1* showed decreased expression at 2 mM metformin but increased expression at 5 mM metformin. Interestingly, this change in *Pd-l1* gene expression was similar to the change in 4T1 cell migration in the wound healing experiment when using a glucose concentration of 4.5 g/L and different metformin concentrations. No correlation was identified between *Pd-l1* gene expression and 4T1 cell migration at different glucose concentrations without metformin.

These results indicate that combining 2 mM metformin with 4.5 g/L glucose is suitable for inhibiting the growth of mouse 4T1 breast cancer cells but does not completely kill these cells, unlike at the higher 5 mM concentration. In contrast, higher metformin concentrations may harm other populations of beneficial cells that support the body, such as MSCs[?]. Surprisingly, Guo *et al.* showed that low-dose metformin has protective effects on immune cell populations, such as stimulating CD8 and CD4 T cells[?]. Additionally, He *et al.* showed that simultaneously administering 2 mM metformin and a high glucose concentration (30 mM) decreased apoptosis in MSC populations[?]. Our findings show that combining 2 mM metformin with 4.5 g/L glucose efficiently inhibited cancer cell proliferation.

As stated above, we believe that metformin represents a novel approach to cancer support, prevention, or even treatment. However, it is important to account for metformin's body-wide effects because increasing evidence suggests that it can also have adverse effects, particularly regarding potential nutritional abnormalities in various patients that may restrict its ability to treat cancer. Therefore, our study

initially evaluated using metformin with different glucose concentrations. Our findings suggest that future studies should evaluate combining a low metformin concentration (2 mM) with a high glucose concentration (4.5 g/L) in patients with different cancers and on cell populations directly related to cancer, such as immune or stem cells. These findings provide crucial information for determining how metformin and glucose concentrations work together to enhance the effectiveness of treatments to kill cancer cells.

CONCLUSION

This study investigated the joint effects of metformin and glucose on mouse 4T1 breast cancer cell proliferation, migration, and expression of immune escape-related genes. It demonstrated that the combinatorial effects of metformin and glucose differed by their concentration. Metformin could effectively kill mouse 4T1 breast cancer cells at 2 mM when combined with 4.5 g/L glucose, significantly reducing their proliferation, migration, and especially expression of *Cxcl12* and *Cd 95*. These findings explain why metformin can effectively treat cancer in patients with type 2 diabetes and suggest the combined use of metformin and glucose in cancer treatment.

ABBREVIATIONS

AKT: Protein Kinase B, **AMPK**: AMP-activated protein kinase, **ATP**: Adenosin triphosphat, **B7-H3**: B7 Homolog 3 (B7-H3) or Cluster of Differentiation 276 (CD276), **CD4**: Cluster of differentiation 4, **CD8**: Cluster of differentiation 8; **CD95**:
Fas or apoptosis antigen 1, **CXCL12**: C-X-C Motif Chemokine Ligand 12, **DNA**: Deoxyribonucleic acid, **FBS**: Fetal Bovine Serum, **Gapdh**: Glyceraldehyde-3-phosphate dehydrogenase, **Glut**: Glucose transporter, **MSC**: Mesenchymal stem cells, **mTOR**: Mechanistic target of rapamycin, **p-AMPK**: Phospho-AMP-activated protein kinase, **PCR**: Polymerase chain reaction, **PD-1**: Programmed death- 1, **PD-L1**: Programmed death-ligand 1, **RT-qPCR**: Real-time reverse transcription PCR, **RNA**: Ribonucleic acid, **RPMI**: Roswell Park Memorial Institute (RPMI) medium, **SMAD**: Suppressor of Mother Against Decapentaplegic, **Smad 3**: Suppressor of Mother Against Decapentaplegic Homolog 3, **TGF-β**: Transforming growth factor beta, **TGF-β1**: Transforming growth factor beta 1.

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AUTHOR'S CONTRIBUTIONS

Pham Duy Khuong took the main responsibility for layout and content of the manuscript. Le Hoai Nam, Nguyen Anh Nhu, Truong Chau Nhat equally contributed to this work. Pham Van Phuc suggested the idea, corrected results, revised the manuscript, and supervised for the study. All authors significantly contributed to this work. All authors read and approve the final version of the manuscript.

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AVAILABILITY OF DATA AND MATERIALS

Data and materials used and/or analyzed during the current study are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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