Long-Term Metformin Effect on Endometrial Cancer Development Depending on Glucose Environment in vitro

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Article

Long-term metformin effect on endometrial cancer development depending on glucose environment in vitro

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Abstract

Background: The incidence of endometrial cancer has increased worldwide over the past years. Common risk factors include obesity and metabolic disturbances, like hyperinsulinemia and insulin resistance, as well as prolonged and elevated estrogen exposure. Metformin, an anti-hyperglycemic and insulin-sensitizing biguanide, displayed anti-proliferative effects in recent studies. Therefore, metformin may act as a therapeutic and prophylactic anti-cancer agent in several tissues, including endometrium. **Methods:** Two different endometrial cancer cell lines, reflecting type I (Ishikawa) and type II endometrial cancer (HEC-1A) were cultured under normoglycemic (5.5mM) or hyperglycemic (17.0mM) conditions and treated with different concentrations of metformin (0.01–5.0mM). **Results:** Effects of metformin on proliferation, cell viability, clonogenicity and migration were investigated after treatment for 7d. Long-term treatment with metformin showed effects on cellular viability, proliferation and migration of endometrial cancer cells in a concentration-dependent manner *in vitro*. Additionally, glucose levels affected the outcome of the experiments. **Conclusion:** Our *in vitro* findings support the hypothesis that metformin has a direct effect on endometrial tissues and reflects the importance of the local glucose environment, suggesting that metformin may be considered as a potential adjuvant agent in endometrial cancer therapy due to its direct and indirect effects on endometrial development.

Keywords: metformin, endometrial cancer, proliferation, migration, clonogenicity, cell viability

1. Introduction

Endometrial cancer (EC) is one of the most common gynecological malignancies, the 6th most common cancer in women and the 15th most occurring cancer overall. In 2018, over 380,000 new cases and 90,000 deaths related to EC were reported worldwide [1]. EC is classified into an estrogen-dependent type I, accounting for 75–85% of all cases, and an estrogen-independent, more aggressive and invasive type II cancer [2,3].

One known risk factor for EC development is unopposed estrogen, as seen e.g. during perimenopausal years in women [4]. Estrogen stimulates endometrial cell proliferation and inhibits apoptosis induction in the tissue [5,6]. Additionally, hyperglycemia contributes to the growth and progression of EC in women with type II diabetes [7]. Therefore, diabetic women not only have a 2-fold higher risk for EC development [8], but also display an increased cancer mortality rate compared to normoglycemic individuals [9].

Metformin, an anti-hyperglycemic and insulin-sensitizing agent, commonly used in the treatment of type II diabetes, has recently been suggested as a therapeutic agent to inhibit cellular overgrowth and hyperplasia in several tissues, including EC [10,11]. Previous studies indicated that metformin could be effective as an adjuvant in cancer therapy along with its traditional role in the treatment of type II diabetes [12-17]. However, most experimental studies analyzed metformin effects at unphysiologically high concentrations (up to 100mM) during short-term treatment of 24–72h [18,19]. We therefore believe that those effects described in the literature are related to cytotoxicity rather than the desired anti-cancer effects of metformin [11]. Considering that the beneficial impact of metformin in EC remains to be determined, this study investigated the direct effects of low metformin concentrations (0.01–5.0mM) during long-term treatment (7d) on EC cell growth, viability, clonogenicity and motility. Furthermore, cells were exposed to metformin in an environment with normal (5.5mM, equivalent to 100mg/dL) or high (17.0mM, equivalent to 306mg/dL) glucose levels to mimic a diabetic condition, in order to investigate the metformin effect within different metabolic conditions. Furthermore, as increased estrogen levels are considered as an additional risk factor, ß-estradiol was added to the cell culture, a factor that was often omitted in prior studies [18,19].

The underlying aim of this study was to investigate the potential direct effects of long-term exposure of endometrial cancer cells to physiological concentrations of metformin under different metabolic conditions.

2. Materials and Methods

2.1 Cell Culture

The human endometrial adenocarcinoma cell lines HEC-1A (type II EC, post-menopausal model; HTB112, ATCC, Manassas, VA, USA) and Ishikawa (type I EC, pre-menopausal model; 99040201, Sigma-Aldrich, Munich, Germany) were used for the *in vitro* experiments. Ishikawa cells were grown in Eagle's minimal essential medium (MEM; Sigma-Aldrich) supplemented with 5.0 % (v/v) charcoal-tripped fetal bovine serum (FBS; Gibco, Waltham, MA, USA), 1.0% (v/v) penicillin/streptomycin (Gibco) and 1.0% (v/v) non-essential amino acids (Sigma-Aldrich). HEC-1A cells were cultured in the same medium, supplemented with 10% (v/v) FBS. All cells were grown in an incubator at 37°C and 5.0% CO_2 in a humidified environment.

During experiments, cells were maintained in a normoglycemic (NG) environment (5.5mM glucose), representing physiological blood glucose levels of 100mg/dL. For the experiments under hyperglycemic (HG) conditions, the medium was supplemented with glucose (Sigma-Aldrich) to achieve a final concentration of 17.0mM glucose, equivalent to 306mg/dL as seen in diabetic patients. A 100mM stock solution of metformin (Sigma-Aldrich) was freshly prepared on the day of administration in normo- or hyperglycemic culture medium, respectively, and cells were treated with different concentrations of the drug (0.01–5.0mM) during experiments. Furthermore, cell culture media were supplemented with 10nM ß-estradiol (E2; Sigma-Aldrich) during treatments to mimic high estrogen levels, a common risk factor for EC development, in the experimental setting.

2.2 MTT Cell Viability Assay

To evaluate the effect of metformin on cellular viability, HEC-1A and Ishikawa cells were seeded into 96-well plates at a density of 5,000 cells/well and incubated in a normal or high glucose, drug-free medium for 24h. Afterwards, cells were treated with different concentrations of metformin (0.01–20mM) for 7d and the medium was changed every 2–3d. Untreated cells served as the reference control. After treatment, 20µL of a 2.5mg/mL MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Alfa Aesar, Karlsruhe, Germany) was added per well and incubated at 37°C for 4h. Afterwards, the MTT-containing media was substituted by 50µL DMSO (Carl Roth, Karlsruhe, Germany) for the extraction of insoluble formazan crystals. The absorbance was measured at 570nm (OD_{570}) with a microplate reader (Anthos Microsystems, Friesoythe, Germany) and cellular viability was calculated with the following formula (cells with DMSO served as a blank sample): % cell viability = ($OD_{570,\ treatment}$ - $OD_{570,\ blank}$) / ($OD_{570,\ control}$ - $OD_{570,\ blank}$) x 100%. SPSS version 24 (IBM,

Armonk, NY, USA) was used for the establishment of concentration-response curves and the calculation of IC_{50} and IC_{90} values (inhibitory concentrations, where 50% and 90% of the measured effect, i.e. loss of cellular viability, was observed). All experiments were carried out in triplicates and were repeated at least three times.

2.3 Cell Proliferation Assay by Crystal Violet Staining

HEC-1A and Ishikawa cells were seeded into 24-well plates at a density of 5,000 cells/well and incubated in a normal or high glucose, drug-free medium for 24h. Afterwards, cells were treated with different concentrations of metformin (0.01–5.0mM) for 7d with renewed treatments by regular medium changes. Untreated cells served as the reference control. The relative cell proliferation was measured by crystal violet (CV) staining according to an adapted protocol [20]. Briefly, cells were rinsed twice with phosphate-buffered saline (PBS; Sigma-Aldrich) and stained with 0.5% (w/v) CV (Carl Roth) for 20 min. After repeated washing with PBS, the CV stain was extracted by incubation with 33% (v/v) acetic acid for 20 min. From each well, 100µL of the extract were transferred into 96-well plates in duplicates and the absorbance was measured at 570nm (OD_{570}) with a microplate reader (Anthos Microsystems). The absorbance of the untreated control group was considered as 100% and the effect of different treatments was compared to the control. Each experiment was performed in duplicates and repeated at least three times in independent experiments.

2.4 Colony Formation by Clonogenic Assay

HEC-1A and Ishikawa cells were seeded into 6-well plates at a density of 5,000 cells/well and incubated in a normal or high glucose, drug-free medium for 24h. Afterwards, cells were treated with different concentrations of metformin (0.01–5.0mM) for 7d with regular medium changes. Untreated cells served as the reference control. Subsequently, cells were stained with 0.5% (w/v) CV, washed three times with PBS and air-dried before image acquisition. The colony formation was quantified as % area with the ImageJ software [21] and the ColonyArea plugin [22]. All experiments were repeated at least three times.

2.5 Cell Migration by Wound Healing Assay

Cell migration was assessed by quantifying the % wound closure in the wound healing assay. The cells were seeded into 6-well plates at a density of 50,000 cells/well in a normal or high glucose, drug-free medium for 24h. Afterwards, cells were treated with selected concentrations of metformin (0.5 or 5.0mM) for 7d with medium changes every 2–3d. On day 7, confluent monolayers were wounded using a sterile pipette tip and the medium was replaced by fresh medium to remove cellular debris. Representative images were taken with an inverse light microscope (Leica, Munich, Germany) at 40× magnification directly and 24h after wounding of the monolayer and the migration area A was measured using the ImageJ software [21]. The % wound closure was calculated as follows: $(A_{t=0h} - A_{t=24h}) / A_{t=0h} \times 100\%$, where $A_{t=0h}$ is the area of the wound measured immediately after scratching and $A_{t=24h}$ is the area of the wound measured 24h after the scratch was performed. Measurements were taken after 24h in order to limit the observations to migration rather than cellular proliferation [23]. HEC-1A and Ishikawa cells displayed doubling times of 27–29h during routine culture, and thus the effect of proliferation in the wound healing assay was minimized. All experiments were repeated at least three times in duplicates.

2.6 Statistical Analysis

Quantitative data are represented as the mean ± standard error of the mean (SEM) of at least three independent experiments. SPSS version 24 (IBM, Armonk, NY, USA) was used to perform a generalized estimating equation test or a paired t-test, as appropriate. A p value ≤ 0.05 was considered statistically significant.

3. Results

3.1 Long-term Metformin Treatment Decreased Endometrial Cancer Cell Viability and Proliferation Rate

Firstly, the effects of metformin on cellular viability were analyzed after long-term treatment with metformin for 7d with the MTT cell viability assay and IC_{50} and IC_{90} values were calculated (Fig. 1A–B). The IC_{50} values for Ishikawa cells were 5.1 ± 2.7mM (IC_{90}: 44.4mM) under normal glucose conditions and 3.9 ± 1.2mM (IC_{90}: 33.7mM) in a high glucose environment (Fig. 1B). For HEC-1A cells, an IC_{50} value of 0.75 ± 0.25mM (IC_{90}: 6.8mM) was established under normal glucose conditions (Fig. 1A). No IC_{50} and IC_{90} values could be calculated for HEC-1A cells in high glucose medium due to repeated overgrowth of the substrate surface by the cells. At a concentration of ≤ 0.1mM, cellular viability for both cell lines was ≥ 80% compared to an untreated control, irrespective of glucose levels in the media. Cellular viability dropped to ≤ 10% at concentrations higher than 5.0mM for HEC-1A and higher than 10mM for Ishikawa cells under any tested metabolic condition in a concentration-dependent manner.

Additionally, to evaluate the effects of metformin and the role of glucose on endometrial cancer proliferation during long-term treatment, the present study examined changes in growth after treatment with low concentrations of metformin after 7d (Fig. 1C–D). Independent of the glucose concentration in the medium, the lowest metformin concentrations (0.01 and 0.1mM) did not show any effect on the growth potential of HEC-1A or Ishikawa cells. Nevertheless, metformin concentrations of 0.5, 1.0 and 5.0mM led to a significant decrease in proliferation of HEC-1A cells by 32% ($p = 0.05$), 38% ($p = 0.02$) and 55% ($p < 0.01$), respectively (Fig. 1C). In Ishikawa cells, proliferation rates significantly dropped by 12% ($p < 0.01$), 25% ($p = 0.04$) and 55% ($p = 0.02$) compared to the control group, when cultured in a normoglycemic environment (Fig. 1D). However, in the presence of high glucose levels, only the highest metformin concentration (5.0mM) was able to decrease the proliferation rate of HEC-1A cells (40%; $p < 0.01$), equal to a 15% ($p < 0.01$) lower effect compared to results under normoglycemic conditions (Fig. 1C). Yet, this resistance to metformin was not observed in Ishikawa cells at high glucose concentrations, where 0.5, 1.0 and 5.0mM metformin decreased the proliferation rate of cells also significantly by 18% ($p < 0.01$), 37% ($p < 0.01$) and 62% ($p < 0.01$). Therefore, Ishikawa cells did not show a glucose-dependent resistance in response to metformin treatment (Fig. 1D).

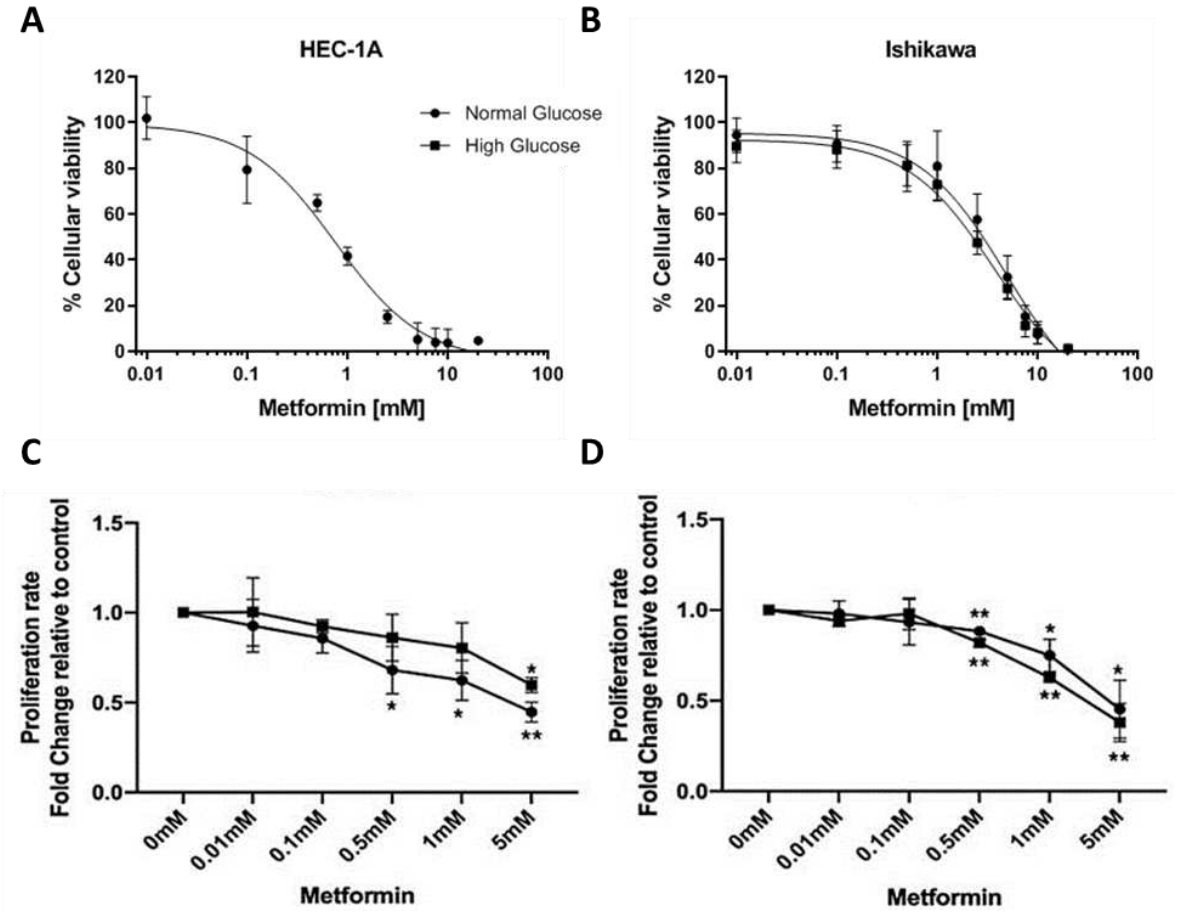


Figure 1: Effects of metformin on proliferation and cellular viability of EC cells as assessed by crystal violet (CV) and MTT assay. HEC-1A (**A**, **C**) and Ishikawa cells (**B**, **D**) were treated with 0.01–20mM (MTT; **A**, **B**) or 0.01–5.0mM (CV; **C**, **D**) metformin in a normoglycemic (●; 5.5mM glucose) or hyperglycemic (■; 17.5mM glucose) environment for 7d. Untreated cells served as reference controls in the assays. Results are presented as mean ± SEM from at least three independent experiments; * $p \leq 0.05$, ** $p < 0.01$.

3.2 Inhibition of Endometrial Cancer Colony Formation by Metformin Treatment

Considering the excellent indication of long-term tumor cell survival of the colony formation assay *in vitro*, the effects of metformin on clonogenicity of HEC-1A and Ishikawa cells were assessed when exposed in an environment with normal or high glucose levels (Fig. 2). Metformin treatment caused concentration-dependent effects on the colony formation of EC cells and decreased the number and size of the colonies. In HEC-1A cells, 0.5mM metformin reduced the colony formation by 44% under normoglycemic ($p = 0.02$), but only by 29% under hyperglycemic conditions ($p = 0.04$), followed by a further reduction with increasing metformin concentrations (Fig. 2A). Treatment at 1.0 and 5.0mM metformin inhibited clonogenicity by 59% ($p = 0.01$) and 80% ($p < 0.01$) at 5.5mM glucose as well as 43% ($p = 0.02$) and 81% ($p < 0.01$) at 17.0mM glucose, respectively. In Ishikawa cells, however, substantial decreases in colony formation under normo- and hyperglycemic conditions were only observed at 1.0mM (35%, $p = 0.2$ and 35%, $p = 0.01$) and 5.0mM metformin (86%, $p < 0.01$ and 88%, $p < 0.01$), respectively (Fig. 2B).

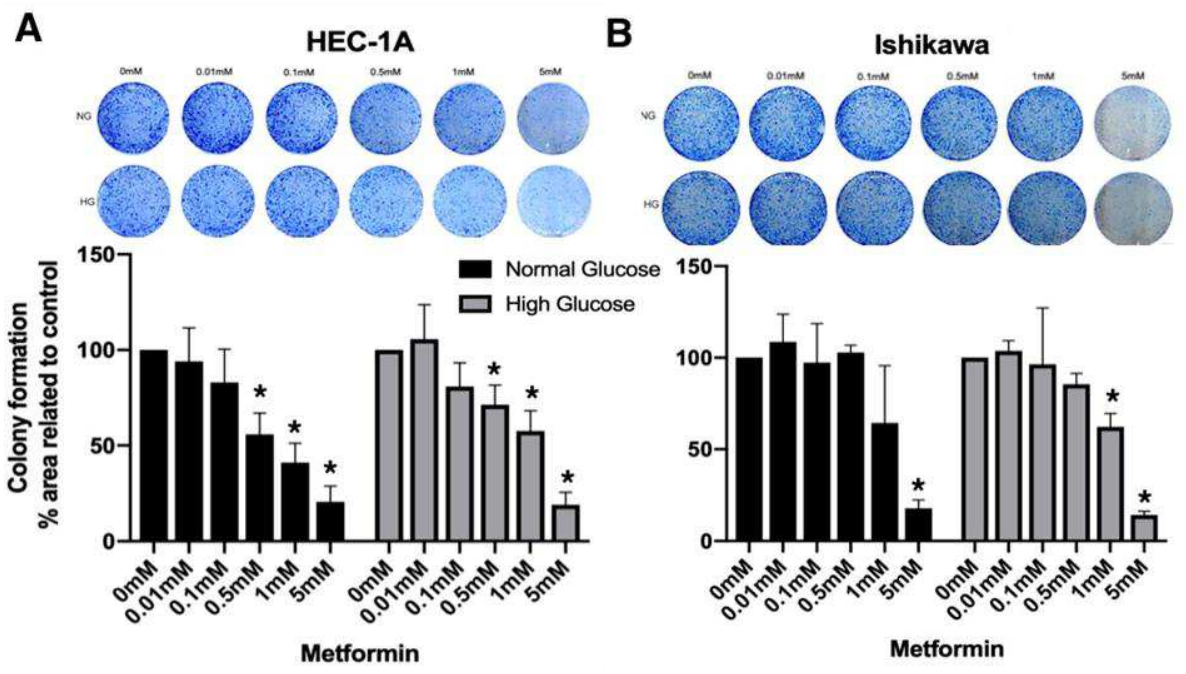
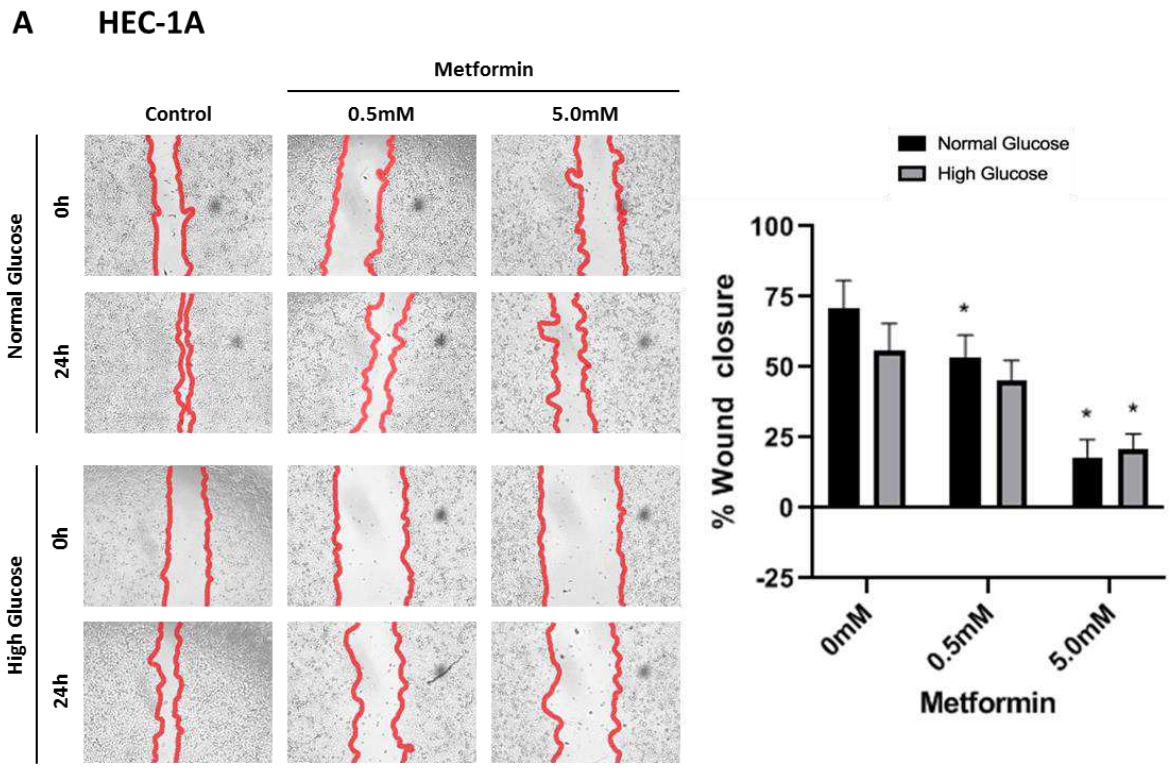


Figure 2: Effects of metformin on clonogenicity of EC cells as detected in the colony formation assay. HEC-1A (**A**) and Ishikawa cells (**B**) were treated with 0.01–5.0 mM metformin in a normoglycemic (■; 5.5 mM glucose, NG) or hyperglycemic (■; 17.5 mM glucose, HG) environment for 7d. Untreated cells served as a reference control in the assay. The colony formation was quantified as % area with the ImageJ software and the ColonyArea plugin. Data are presented as mean ± SEM from at least three independent experiments; * $p \leq 0.05$.

3.3 Metformin Long-term Treatment Reduced Endometrial Cancer Motility

Considering the doubling time of HEC-1A and Ishikawa cells of 27–29h, motility of EC cells after long-term metformin treatment was evaluated by a wound healing assay after 24h to minimize the influence of proliferation on the outcome of the assay (Fig. 3). At 24h after scratching, untreated HEC-1A control cells covered the scratch up to 71% and 56% at normal and high glucose levels (Fig. 3A), respectively, while Ishikawa control cells migrated into the scratch wound by 16 % and 22 % (Fig. 3B). Metformin treatment at 0.5 mM significantly reduced the migration ability of HEC-1A cells by 18% ($p = 0.03$) in a normoglycemic environment. However, in a high glucose environment, the migration was reduced by only 11 % ($p = 0.07$). Under these conditions, Ishikawa cells showed a minor reduction of motility by only 6–7% with 0.5mM metformin at both glucose concentrations, which was below the result obtained under high glucose conditions (hyperglycemia: p=0.02, normoglycemia: $p = 0.46$). The highest effect of metformin was detected at 5.0mM metformin, where the treatment significantly inhibited the motility of HEC-1A cells by 53% ($p < 0.01$) and 35% ($p = 0.02$) at 5.5 and 17.0mM glucose, respectively. In Ishikawa cells, a drop of the motility rate by 25% ($p = 0.02$) was observed at high glucose levels with 5.0mM metformin, resulting in a complete inhibition of cellular motility. The effect of metformin at 5.0mM on the motility of Ishikawa cells could not be evaluated under normoglycemic conditions due to fact that cells were not able to build up the required monolayer over a period of 7d. Nevertheless, this finding highlights the inhibitory effect of high metformin concentrations on the migration capacity of Ishikawa cells at physiological glucose levels.



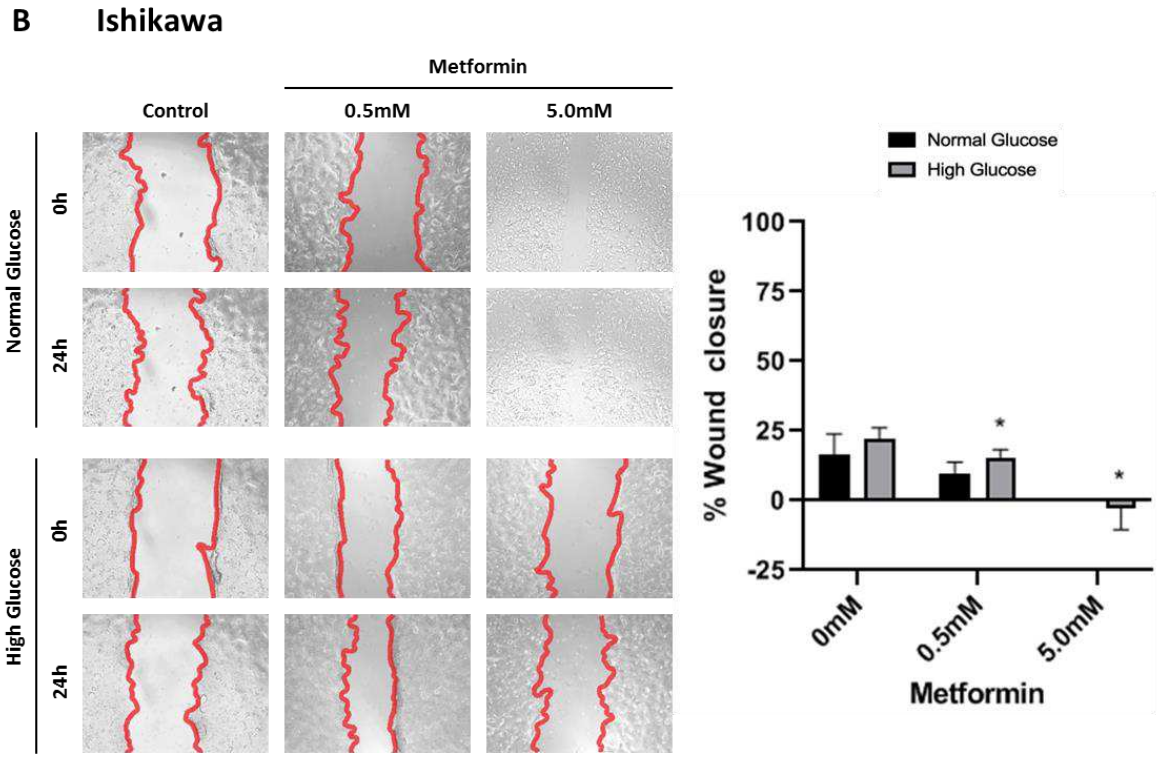


Figure 3: Effects of metformin on migration of EC cells as observed in the wound healing assay. HEC-1A (**A**) and Ishikawa cells (**B**) were treated with 0.5 and 5.0mM metformin in a normoglycemic (■; 5.5 mM glucose) or hyperglycemic (■; 17.5 mM glucose) environment for 7d. Afterwards, wounds were created by scratching the cell monolayer and migration area was measured with the help of ImageJ software after 24h. The migration area A was measured using the ImageJ software and % wound closure was calculated with the following formula: $(A_{t=0h} - A_{t=24h}) / A_{t=0h} \times 100\%$. Untreated cells served as a reference control in the assay. Results are presented as mean ± SEM from at least three independent experiments; * $p \leq 0.05$.

4. Discussion

Cellular behavior is modulated by its environment, which can generate molecular changes in non-physiological conditions, disrupting cellular homeostasis. The understanding of cellular mechanisms involved in EC pathophysiology is crucial for the development of therapeutic strategies targeting cellular growth, proliferation, survival, and motility pathways. Over the last years, metformin has been suggested as a promising drug for both cancer prevention and treatment with a considerably increased number of experimental and epidemiologic studies [10-14]. However, despite the fact that an overall 27% reduction of the risk for developing any type of cancer in metformin-treated patients with type II diabetes has been observed in a meta-analysis of 18 observational studies [24], the drug so far has not been established as an anti-cancer drug, most likely due to methodological weaknesses and insufficient data of currently available studies.

We would like to point out, that the anti-proliferative, pro-apoptotic and motility-suppressing effects of metformin on EC, described in the literature, were analyzed using a short-term exposure of metformin (24–72h) at supra-pharmacological concentrations (up to 100mM). Moreover, many of these studies were performed in the absence of ß-estradiol, a known risk factor in endometrial proliferative disorders [18,19,25]. Our group previously reported that a low concentration of 0.1mM metformin was not able to decrease the proliferation potential of the Ishikawa EC cell line after short-term treatment of 72h *in vitro*, while concentrations of 1.0–5.0mM metformin affected the proliferation rate [26]. However, metformin at a concentration of 0.1mM was able to inhibit the migration ability of EC cells in a normoglycemic environment during short-term treatment and also reduced the metastatic effect of insulin under hyperglycemic conditions. Also, metformin showed a greater effect on EC cells in the presence of physiological glucose levels [26]. These results encouraged us to examine the direct anti-tumor effects of low, therapeutically relevant concentrations of metformin on two different types of EC cells in a long-term setting of 7d under the addition of ß-estradiol, as a common risk factor for the development of EC, which, to the best of our knowledge, has never been done before *in vitro*.

The present study used different glucose concentrations to mimic the physiological setting associated with EC development and showed that long-term treatment with 0.01 and 0.1mM metformin did not have any effects on EC proliferation, growth and motility *in vitro*, independent of glucose levels in the culture medium. In a study of Mitsuhashi *et al.* [25], metformin levels in plasma and EC tissues of patients were monitored after administration of 2,250mg/d metformin for 4–8 weeks. Plasma concentrations of 0.01mM and endometrial tissue levels of 2.0µM metformin were reported, which amounts to 20% of the plasma concentration.

The effects of unphysiologically high concentrations of metformin (0.5–5.0mM) – still considered as low concentrations for *in vitro* experiments – were also evaluated in the present study. Results of the MTT cell viability assay revealed that metformin concentrations of ≥ 1.0mM led to a decreased cellular viability of both EC cell lines. Thus, it cannot be excluded that changes in proliferation rates are at least to some extent related to a loss of cellular viability. Furthermore, the results of the present study have shown that HEC-1A and Ishikawa cells react differently to metformin, as i.e. resistance to metformin was only observed in HEC-1A cells at high glucose levels. In addition, HEC-1A cells in particular showed a high proliferation rate and cellular viability at high glucose levels (Fig. 1), which may explains their more aggressive behavior, especially in a high glucose environment. According to our results, the two cell lines behave very differently to metformin within the distinct metabolic conditions. It is important to take into consideration that the HEC-1A and Ishikawa cell lines differ in their gene expression profile, such as the gene encoding for estrogen receptors (ERs) and metabolic enzymes and therefore represent different *in vitro* models. Ishikawa cells represent a model for estrogen-dependent, pre-menopausal EC expressing various ERs to different extents, whereas HEC-1A cells represent a model for post-menopausal EC with lower sensitivity to E2, and are lacking estrogen receptor 1 (ESR1) expression [27]. Therefore, resistance to metformin effects exhibited by cells cultured in an environment with 17.0mM glucose was potentially driven by differences in ER expression.

Mitsuhashi and colleagues compared metformin concentrations *in vivo* with *in vitro* results and the analysis revealed that the concentration of metformin required for growth suppression *in vivo* was 400-fold lower than the respective concentration that inhibited cellular growth *in vitro* (1.0mM for Ishikawa and HEC-1B cells after 72h) [25]. This effect may be attributed to the fact that metformin has an additional peripheral effect on the reduction of the hyperglycemia due to its known benefit on insulin-sensitizing of the tissues, leading to normalization of glucose levels [10,25]. This hypothesis was supported by our results, as the estrogen-independent HEC-1A cells were resistant to the direct inhibiting effect of metformin on proliferation at very high metformin concentrations of 5.0mM only in the hyperglycemic environment, while an anti-proliferative effect was already detected at 0.5mM in a more physiological glucose environment (Fig. 1). Additionally, HEC-1A cells overgrew the substrate surface in the high glucose environment, making it impossible to evaluate cellular viability in the MTT assay after 7d. Similar effects on HEC-1A cells were observed in the colony formation assay as well as the migration analysis, where the direct metformin effects were substantially lower in a hyperglycemic microenvironment (Fig. 2 and 3). As this effect was not noted in Ishikawa cells, the more aggressive type II HEC-1A cell line may be fueled more in growth and tumorigenesis by elevated glucose levels. However, further experiments are necessary to confirm these findings and to define the molecular effects involved.

Moreover, hyperglycemia is associated with obesity and insulin resistance, leading to hyperinsulinemia that stimulated cellular growth and hyperplasia in different tissues [28-30]. Tumorigenesis of obesity-associated EC was linked to enhanced cellular glucose uptake and increased metabolism [31], but can also be related to increased proliferation *in vitro*, as seen in the present study between untreated control cells. Consequently, agents like metformin, that decrease glucose and insulin levels, might be a strategy to prevent EC development and progression, while it shows less effect in type I EC according to our *in vitro* results. Accumulating evidence from *in vitro* and *in vivo* studies suggests that metformin acts as an anti-tumor agent directly and indirectly [10], which was also supported by our findings regarding to the direct effects. Prior studies indicated several indirect effects by metformin, including the systemic reduction of blood glucose and insulin levels, whereas the activation of 5′adenosine monophosphate-activated protein kinase (AMPK) served as an example for direct effects on cancer cells [6,10,18,26,32,33]. However, multiple other direct mechanisms have been demonstrated, e.g. an upregulation of markers for cell cycle arrest, apoptosis, and autophagy, the inhibition of cell migration and proliferation, as well as a downregulation of markers associated with cellular senescence [18,26,32,33].

Although studies evaluating the relationship between metformin and EC incidence revealed conflicting results [34,35], the findings of the present *in vitro* study supported the hypothesis that metformin not only displays indirect effects on cancer metabolism via changes of the metabolic environment, but also direct effects on endometrial cells that may prevent hyperplasia and EC development. This accounted particularly for the very aggressive type II cancer, as type II HEC-1A cells proliferated more under hyperglycemic conditions and cells were less sensitive to the direct metformin effects at elevated glucose levels. On the other hand, Ishikawa cells, that reflect the more common and less aggressive type I EC, only showed minor differences regarding the direct metformin effects under normo- and hyperglycemic conditions, suggesting that these effects were independent of the glucose state.

5. Conclusions

In conclusion, the present study highlighted the importance of the metabolic environment in EC development and progression, and potential actions of metformin as a therapeutic agent in the treatment for EC subtypes. Furthermore, it was shown that metformin acts on endometrial tissue via direct effects, in addition to its well-known indirect effects, i.e. lowering serum glucose levels. These findings suggest that the drug might be a potential adjuvant agent in EC therapy. However, further studies are required to elucidate the role of metformin in EC prevention and treatment in more detail.

Abbreviations

AMPK	5´adenosine monophosphate-activated protein kinase
CV	crystal violet
E2	ß-estradiol
EC	endometrial cancer
ER	estrogen receptor
ESR1	estrogen receptor 1
FBS	fetal bovine serum
HEC-1A	human endometrial cancer cell line 1A
IC_{50}/IC_{90}	inhibitory concentrations at 50 /90% of the measured effect
MEM	Eagle's minimal essential medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NG/HG	normo-/hyperglycemic
PBS	phosphate-buffered saline

Declarations

Ethical Approval and consent to participate: Not Applicable.

Consent for Publication: Not Applicable.

Availability of data and materials: The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare no conflict of interest.

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Authors' contributions: Conceptualization: A.M.W., A.G.; methodology: A.M.W., C.L., A.G.; software: A.M.W., C.L.; validation: A.M.W., J.J.; formal analysis: A.M.W., J.J., C.L.; investigation: A.M.W., C.L.; resources: A.G., T.S.; data curation: A.M.W., C.L.; writing—original draft preparation: A.M.W., A.G., C.L.; writing—review and editing: A.M.W., A.G., C.L.; visualization: A.M.W., C.L.; supervision: A.G.; project administration: A.M.W., A.G.; funding acquisition: A.G.. All authors have read and agreed to the published version of the manuscript.

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Figures

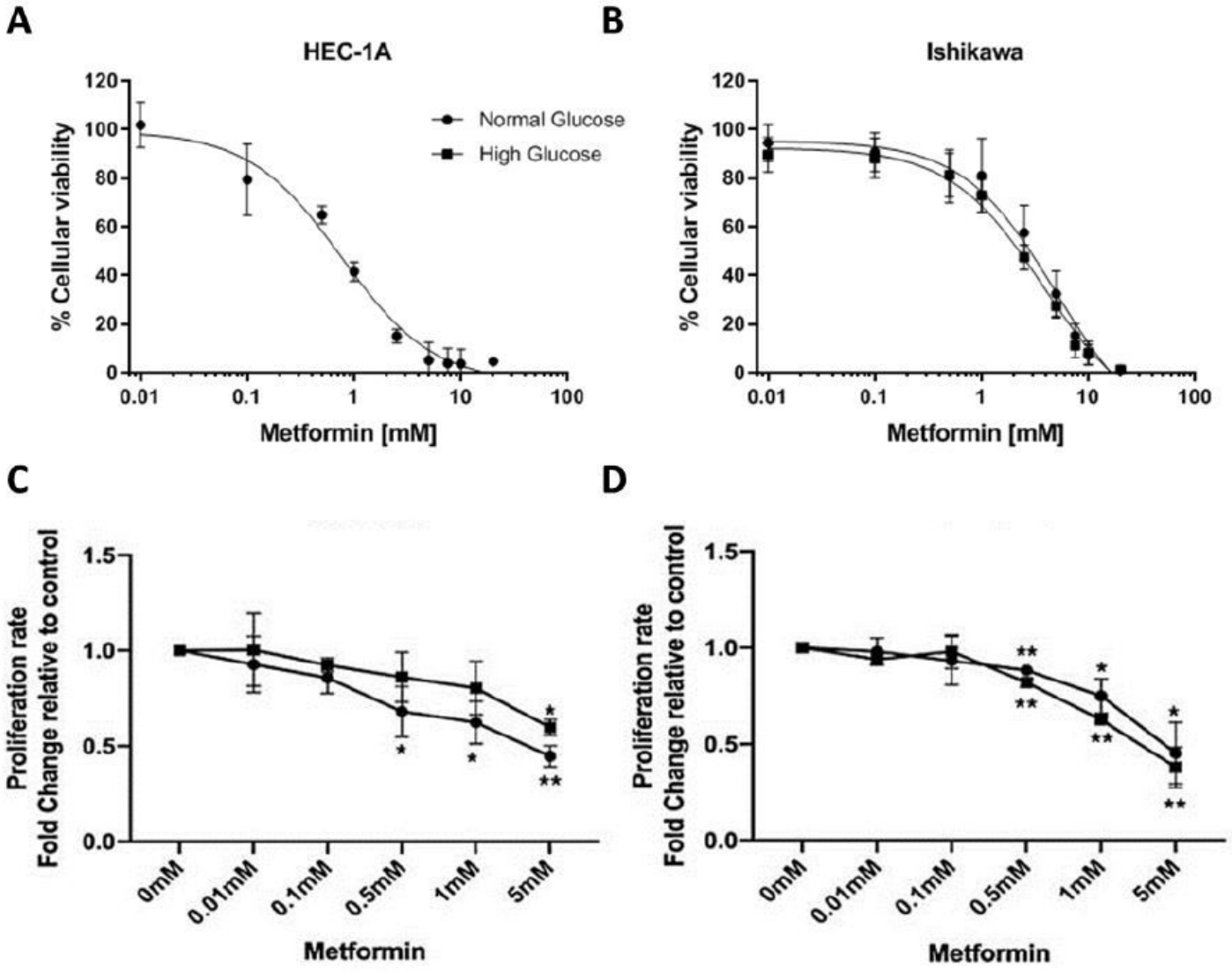

Figure 1

Effects of metformin on proliferation and cellular viability of EC cells as assessed by crystal violet (CV) and MTT assay. HEC-1A (A, C) and Ishikawa cells (B, D) were treated with 0.01–20mM (MTT; A, B) or 0.01–5.0mM (CV; C, D) metformin in a normoglycemic (□; 5.5mM glucose) or hyperglycemic (□; 17.5mM glucose) environment for 7d. Untreated cells served as reference controls in the assays. Results are presented as mean ± SEM from at least three independent experiments; * p <= 0.05, ** p < 0.01.

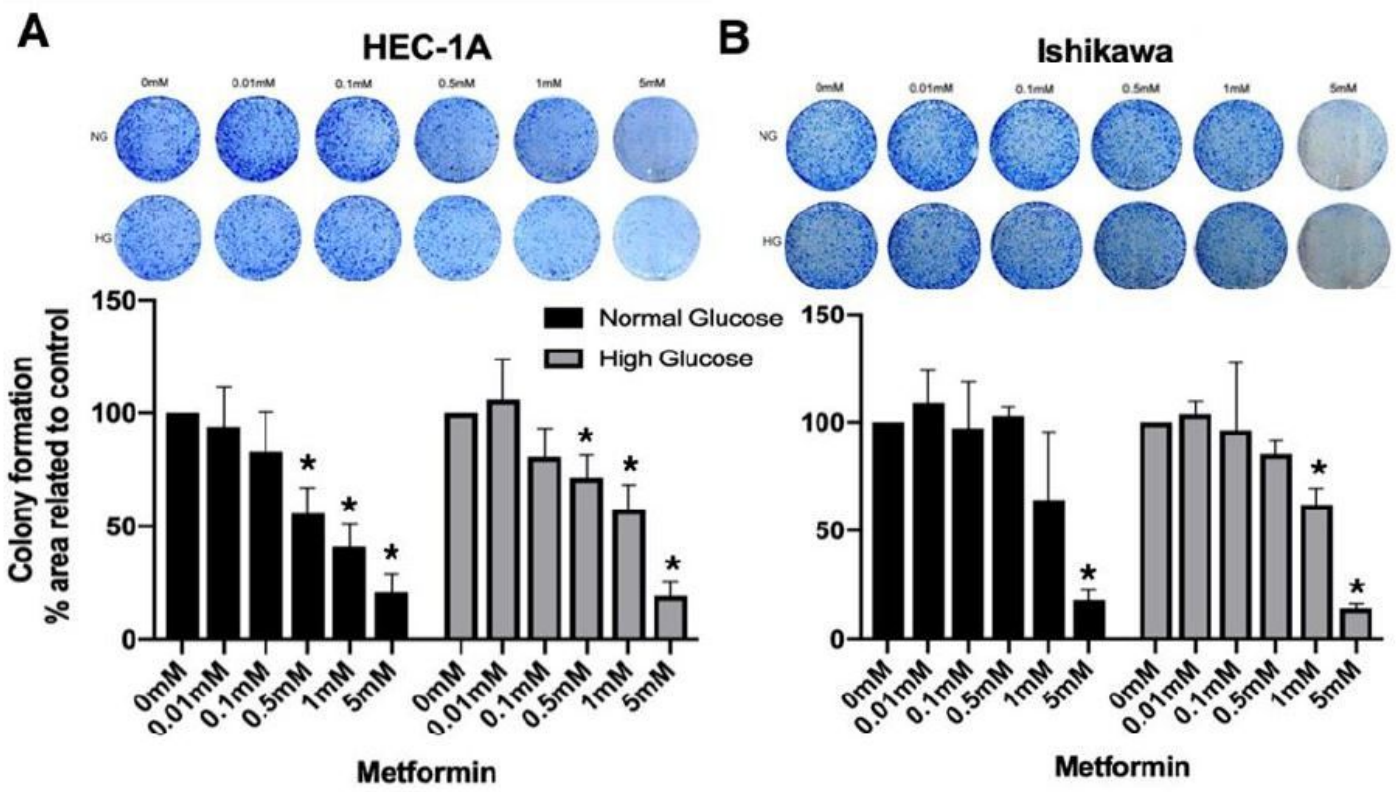

Figure 2

Effects of metformin on clonogenicity of EC cells as detected in the colony formation assay. HEC-1A (A) and Ishikawa cells (B) were treated with 0.01–5.0 mM metformin in a normoglycemic (□; 5.5 mM glucose, NG) or hyperglycemic (□; 17.5 mM glucose, HG) environment for 7d. Untreated cells served as a reference control in the assay. The colony formation was quantified as % area with the ImageJ software and the Colony Area plugin. Data are presented as mean ± SEM from at least three independent experiments; * p <=0.05.

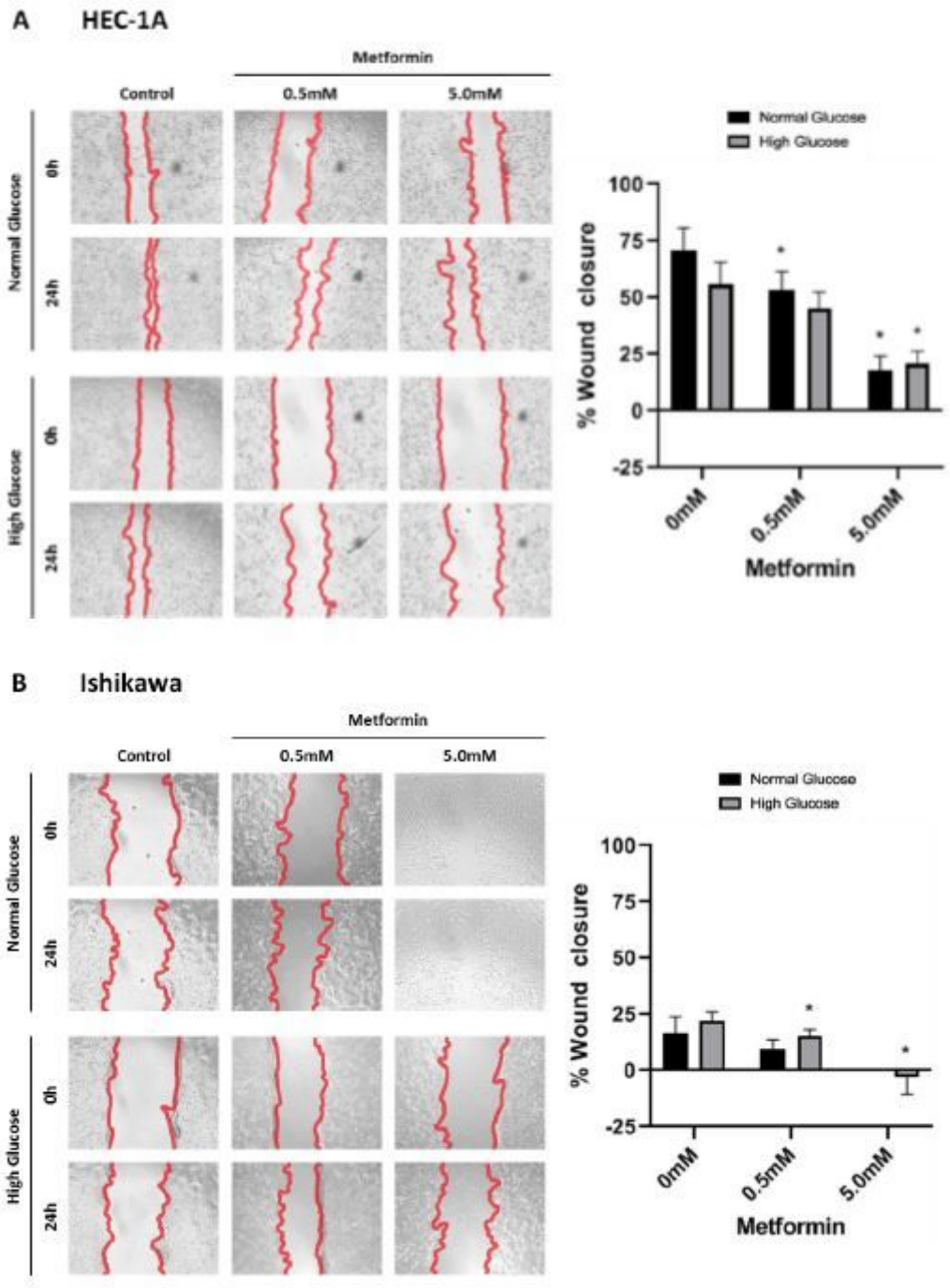

Figure 3

Effects of metformin on migration of EC cells as observed in the wound healing assay. HEC- 1A (A) and Ishikawa cells (B) were treated with 0.5 and 5.0mM metformin in a normoglycemic (□; 5.5 mM glucose) or hyperglycemic (□; 17.5 mM glucose) environment for 7d. Afterwards, wounds were created by scratching the cell monolayer and migration area was measured with the help of ImageJ software after 24h. The migration area A was measured using the ImageJ software and % wound closure was calculated with the following formula: (At=0h - At=24h) / At=0h x 100%. Untreated cells served as a reference control in the assay. Results are presented as mean ± SEM from at least three independent experiments; * p <= 0.05.