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Examining the Effect of Metformin on Cell Death Mechanisms in Relation to Hippo Signaling in MDA-MB-231 Breast Cancer Cells

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ABSTRACT

Breast cancer is one of the most common cancer types in women in the world and our country. Antitumorigenic activity is achieved with various therapeutic drugs by directly suppressing the constantly active PI3K/Akt/mTOR signaling pathway or enabling AMPK activation. AMPK, a positive regulator of autophagy, ensures the induction of autophagy by suppressing the Akt/mTOR pathway. Metformin, an anti-diabetic drug, achieves its anti-tumorigenic effect by activating AMPK. Deregulation of the Hippo signaling pathway is a new therapeutic target because it causes cancer cells to become aggressive and evade cell death mechanisms. The study aims to reveal the effects of metformin treatment on Hippo signaling pathway activity on apoptosis and autophagy, depending on drug treatment in MDA-MB-231 breast cancer cells. Metformin decreased the cell viability through induction of mitochondria membrane potential loss in dose and time dependent manner in MDA-MB-231 cells. The colony forming potential of the MDA-MB-231 cells were suppressed by 10 mM metformin treatment which was induced apoptotic cell death and autophagy by increasing Bim, Bad, Bak and cleavage of caspase 3, 9, PARP and Beclin1, Atg5 and Atg7. Moreover, Hippo signaling related protein levels showed remarkable increase due to metformin treatment. It was shown that metformin treatment increased the activity of the hippo signaling pathway, resulting in the induction of apoptosis and autophagy.

Keywords: Breast cancer, Metformin, Hippo signaling, Apoptosis, Autophagy.

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Introduction

Breast cancer is one of the most common cancer types in women in the world. Risk factors such as menopausal age, obesity, type 2 diabetes, personal cancer history, familial cancer history, BRCA1-2 mutations, and environmental factors such as smoking and alcohol use are involved in the development of breast cancer. According to 2018 World Health Organization (WHO) data, one in every six deaths is caused by cancer, and breast cancer ranks first in women [1]. According to research by the International Agency for Research on Cancer (IARC), breast cancer, which was seen in 2.26 million women in 2020, is predicted to be seen in 3.19 million women 20 years later. According to 2020 IARC data, 24,175 women were diagnosed with breast cancer in Turkey and 7,161 women died due to breast cancer [2]. New therapeutic agents need to be developed for the treatment of breast cancer, which is a common type of cancer among women and its incidence is increasing.

Metformin is a biguanide-class antidiabetic drug used as first-line treatment in patients with type 2 diabetes. The primary effect of metformin is to inhibit glucose production in the liver, but it also increases the insulin sensitivity of peripheral tissues [3]. In addition to regulating insulin metabolism, metformin provides weight control and is also used in the treatment of insulin resistance. The required daily dose has been determined as 500-2500 mg/day [4]. The main molecular targets of metformin are the mitochondrial electron transport chain (ETC), Adenosine monophosphate-activated protein kinase (AMPK) and mTOR [5]. ETC causes downregulation of AMPK by producing Adenosine triphosphate (ATP). Metformin, on the other hand, inhibits ETC, reducing ATP synthesis, and the high AMP/ATP ratio activates AMPK, which suppresses mTOR. Metformin-mediated inhibition of ATP synthesis provides mTOR inhibition. Many studies in colon cancer cells, breast cancer cells and the model organism *C. elegans* have shown that metformin slows down the growth of cancer cells in various types of cancer [6,7]. Studies have shown that metformin has tumorigenesis-suppressing and antiproliferative effects in various types of cancer. It shows its anticancer effect through AMPK-dependent or AMPK-independent pathways [8]. Signal transmission in cancer cells occurs through the activation of cytoplasmic kinases such as serine/tyrosine kinase and receptor tyrosine kinases (RTK). Uncontrollable and continuous RTK activity is observed during the tumor formation phase. The PI3K/Akt/mTOR signaling pathway is involved in important cellular events such as cell growth, autophagy, apoptosis and lipid metabolism. Antitumorigenic activity is achieved by directly suppressing or AMPK-dependent inhibition of the PI3K/Akt/mTOR signaling pathway using various drugs. AMPK, a positive regulator of autophagy, induces autophagy by suppressing the Akt/mTOR pathway

[9]. AMPK/mTOR signaling induces apoptosis by causing an increase in the expression of caspase-3, which provides DNA fragmentation in apoptosis as well as autophagy [10]. Metformin induced apoptosis through induction of ROS production and the effect of metformin were highly associated with the glucose concentration on MDA-MB-231 aggressive breast cancer cells. But, the relation of these mechanism with Hippo signaling still largely unknown in MDA-MB-231 cells.

The Hippo signaling pathway regulates organ size and maintains tissue stability by managing cell proliferation and apoptosis. The Hippo signaling pathway consists of mammalian STE20-like kinase 1/2 (MST 1/2), large tumor suppressor kinase 1/2 (LATS 1/2) and Yes-associated protein 1 (YAP) and its paralogue WW-domain-containing transcription regulator 1 (TAZ) elements [10]. The basis of the Hippo pathway is based on serine/threonine phosphorylation. Activation of the Hippo pathway results in phosphorylation of YAP at Ser127 *via* LATS 1/2 and consequently YAP inactivation. Phosphorylated YAP is degraded in the cytoplasm as a result of its interaction with 14-3-3, resulting in inhibition of target gene transcription [11,12]. When the Hippo pathway is inactivated, the inactive MST1/2 and LATS1/2 dephosphorylate YAP/TAZ, leading to YAP/TAZ accumulation in the nucleus. YAP/TAZ accumulates in the nucleus and binds with TEAD family transcriptional factors (TEAD1-4) to stimulate gene transcription that regulates various cellular activities such as cell proliferation [13]. Defects in the Hippo signaling pathway encourage breast cancer cells to metastasize. YAP deficiency has been observed to reduce the incidence of lung metastases in a genetically engineered mouse model of breast cancer [14]. A study showed that metformin inhibited the stem structure and epithelial-mesenchymal transition of glioma cells by regulating YAP activity [15]. The Hippo signaling related genes showed variation between various breast cancer cells. So, the oncogenic and tumor suppressor role of Hippo signaling against chemotherapeutics are still needed to be investigated

In line with the information in the literature, this study aims to demonstrate the effects of metformin treatment in breast cancer cells on the Hippo signaling pathway activity in a relation with apoptosis and autophagy, depending on the drug treatment concentrations. In addition, the results obtained are intended to be used as preliminary data in phase studies for breast cancer treatment.

Material and Methods

MTT Cell Viability Test

MDA-MB-231 (ATCC HTB-26) breast cancer cells were seeded in a 96-well/plate with $1x10^4$ cells in each well, and the cells were incubated at 37°C for 24 hours. Then, the cells are incubated with 1-10 mM metformin for 24h and 48h at 37°C. At the end of the incubation, 10 µl of 3-(4,5-dimethyltriazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) agent was added to the medium on the cells and incubated for 4 hours at 37°C. After incubation with the MTT agent, 100 µl Dimethyl sulfoxide (DMSO) was added and kept in the dark for 5 minutes to dissolve the formazan crystals formed by living cells metabolizing the MTT agent. After dissolving Formazan crystals with DMSO, the cell viability rate was measured by absorbance at 570 nm in a microplate ELISA reader (Bio-Rad, California, USA). The results were shown as the average of three experiments with at least four repetitions.

Colony Formation Test

MDA-MB-231 cells were seeded into a 6-well petri dish as $2x10^3$ cells. 10 mM metformin was treated for 24h and 48h. Then, the medium on the cells was replaced with fresh medium. Compared to the control group cells reaching 90% density, the media was removed after approximately 14 days. Cells were washed with 1X PBS and incubated for 15 minutes at RT with a 3:1 ratio of 100% methanol: acetic acid. 500 µl 0.5% crystal violet was added to the cells and incubated at RT for 20 minutes. After 20 minutes, the dye was removed by washing with distilled water. Colonies were drawn and counted.

Trypan Blue Cell Proliferation Assay

A cell survival experiment was performed to determine the effect of metformin on cell proliferation in MDA-MB 231 breast cancer cells. $5x10^4$ cells were seeded in a 6-well/plate. 10 mM metformin was treated for different time points. At the end of every 24, 48 and 72 hours, cells were collected with trypsin and centrifuged at 2000 rpm for 5 minutes. Cells were dissolved with 10 µl media and mixed with 10 µl of trypan blue and counted in a hemocytometer. Cell survival assay was examined as the average of three different experiments with at least duplicates.

Fluorescence Microscopy

$1x10^4$ cells were seeded into 6 wells/plates and then treated with various concentrations (1, 2, 5 and 10 mM) of metformin for 24h and 48h. The effect of metformin on mitochondrial membrane potential loss was examined by 3,3'-dihexyloxacarbocyanine iodide (DiOC6) (4 nM; Calbiochem, La Jolla, CA, USA) fluorescent staining and visualized by fluorescence microscopy (Ex./Em.: 488/525 nm) [16]. Propidium iodide (PI) staining was performed to observe the cell death ratio after metformin treatment (Ex./Em.: 536/617 nm) [17].

Immunoblotting

MDA-MB-231 cells were treated with the 10 mM metformin for 24h and 48 h. As was proceeded in our previous study; first, cells were washed with ice-cold 1x PBS and lysed mPER extraction buffer in the presence of a protease inhibitor cocktail (Complete, Roche)[18]. Following lysis of the cells, total protein lysate was obtained after centrifugation for 15 min at 13,200 rpm. Bradford protein assay was used to determine the protein concentrations (Bio-Rad, California, USA). Samples were kept in the -80° freezer until use. Separation of total protein lysates proceeded with a 10-12% SDS-PAGE and

proteins were transferred onto PVDF membranes (Roche, Basel, Switzerland). The membranes were then blocked with a 5% milk-blocking solution (prepared with Tris buffer saline-Tween 20) and incubated with appropriate primary and HRP-conjugated secondary antibodies (CST) in antibody. Then membranes were washed

with 1X TBS-Tween 20, the proteins were analyzed using an enhanced chemiluminescence detection system (Chemidoc Bio-Rad, California, USA).

Statistical Analysis

The results obtained are the average of three different experiments with at least two repetitions. All numerical results obtained from the experiments were converted into graphs using GraphPad Prism 6 and statistical analysis was carried out using the Two-way ANOVA method. The p value for significant changes is *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001.

Results and Discussion

Metformin Reduced Cell Viability in a Time and Dose-dependent Manner in MDA-MB-231 Breast Cancer Cell

Metformin is a first-line anti-diabetic drug used to treat type 2 *diabetes mellitus* and regulate blood sugar in overweight people. The risk of breast cancer in women with diabetes increases compared to healthy people, especially the risk of breast, pancreas, colon, liver and uterine cancer increases in people with diabetes. A meta-analysis has shown that long-term metformin treatment will reduce the risk of T2D-related breast cancer [19]. In the study conducted by Phoenix et al. in 2010, long-term treatment with metformin was shown to be effective in primary tumor growth [20]. In light of this information, the mechanisms of action and signaling pathways of metformin on the MDA-MB-231 triple-negative breast cancer cell line were investigated and the research was supported by *in-vitro* experiments. MTT survival test was performed to determine the effect of metformin on cell viability. Metformin was treated to MDA-MB-231 breast cancer cells depending on dose (1-10 mM) and time (24-48 hours) (Figure 1A). Metformin caused a dose-dependent decrease in cell viability. While a 40% loss of cell viability was observed in a 24h application of 10 mM metformin, a 50% cell viability was observed in a 48h application of 10 mM metformin. The effect of 10 mM metformin on cell proliferation depending on time was examined by trypan blue assay (Figure 1B). Compared to the control group, 10 mM metformin showed a cytotoxic effect and suppressed the cell proliferation rate in a time-dependent manner in cell viability. The study on the xenograft model of breast cancer showed the anti-proliferative role of metformin at the concentration of 150 mg/kg/day led to significant reduction of tumor growth [21].

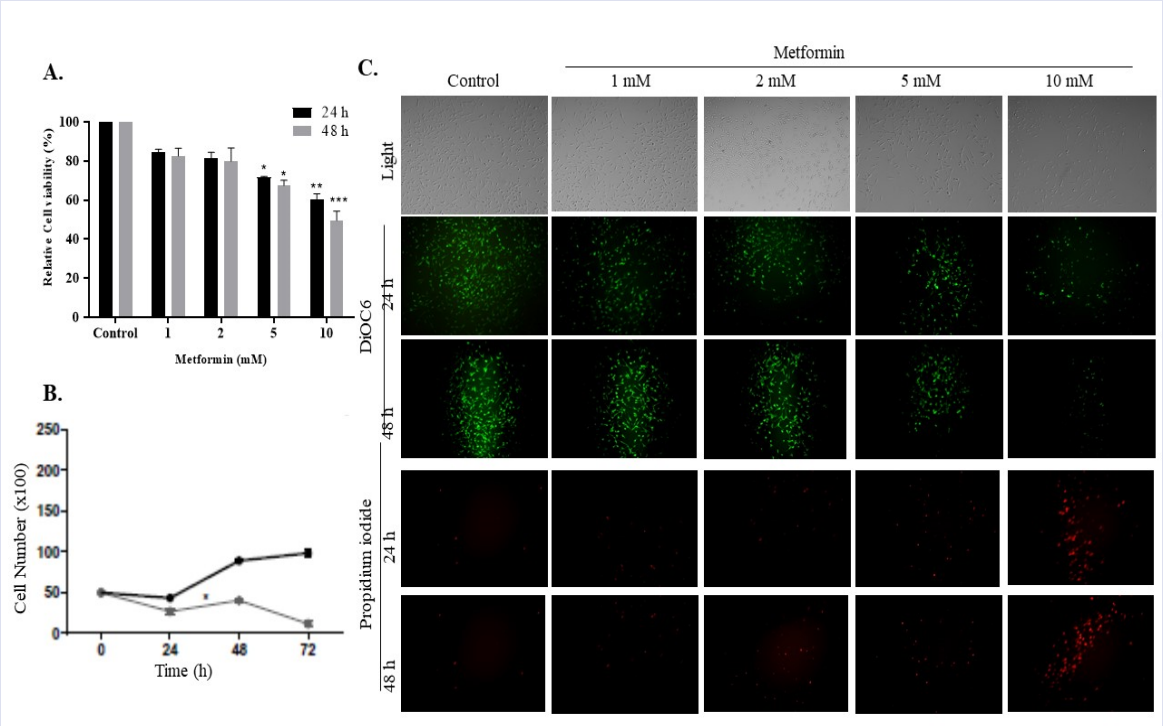

Figure 1. Metformin increased cell viability loss in MD-MB-231 breast cancer cells. A. MTT cell survival assay was performed after metformin treatment in MDA-MB-231 breast cancer cells in a dose- and time-dependent manner (* p<0.05; ** p<0.01, *** p<0.001). B. Cell survival assay (Trypan blue assay) was proceeded after metformin treatment to MDA-MB-231 cells (*p<0,05). C. The effect of metformin on cell viability was examined by DiOC6 and PI staining by fluorescence microscopy. Scale bar: 50 µm

It was examined by DiOC6 staining to observe the effect of metformin on cell viability depending on mitochondrial membrane potential (MMP) (Figure 1C). Live cells stained with DiOC6 were observed in green fluorescent color. Treatment of 1 mM and 2 mM metformin for both 24h and 48h did not cause a significant decrease in cell viability. However, although 5 mM metformin was more effective in 48h metformin treatment, it also reduced cell viability by 50% in the 24h treatment period. By increasing MMP loss, 10 mM metformin significantly reduced MDA-MB-231 breast cancer cell viability. Propidium iodide is a fluorescent intercalating agent that can bind to DNA insertion with little or no sequence preference between bases. When 10 mM metformin was treated, cells with impaired membrane integrity were observed in red color (Figure 1C). In the study with ovarian cancer, metformin enhanced the cytotoxic effect of cisplatin [22]. It was known that metformin as an anti-diabetic drug reduces the glucose levels. The cell death mechanisms under the low glucose level especially in MDA-MB-231 breast cancer cells still under investigation. The high glucose concentration (25 mM) prevent the anti-proliferative mechanism of metformin in MDA-MB-231 cells [23]. Therefore, the mechanism under induction of cell death is further investigated.

Time-dependent Metformin Treatment Increased Apoptotic Cell Death of MDA-MB-231 Triple-negative Breast Cancer Cells

Recent studies have shown that metformin may suppress tumor growth in many types of cancer, reduce cancer risk, and be associated with improved prognosis in patients with cancer [24]. When the long-term effect of metformin was examined, the effects of 24h and 48h treatment on colony formation potential were determined as 265 and 72 colonies, respectively, compared to the control group (410 colonies) (Figure 2A). Considering the number of colonies and colony diameters, it was shown that the treatment of 10 mM metformin for 48h significantly suppressed cell proliferation. A study in MCF-7 and MDA-MB-231 cells showed that the anticarcinogenic effect of metformin was induced by increasing glucose uptake [25]. Moreover, metformin treatment in these cells caused an increase in oxidative stress generation and DNA damage [26]. Therefore, in our study, we investigated the effect of metformin on the apoptotic cell death mechanism. Cells were treated with the selected concentration of 10 mM metformin for 24 and 48 h, followed by total protein isolation. Changes in the expression of apoptosis-related proteins (BIM, BAD, BAK, cleaved caspase 3, cleaved caspase 9, and PARP) were examined by immunoblotting (Figure 2A-B). Both pro-apoptotic and anti-apoptotic proteins regulate apoptotic cell death. BIM, a pro-apoptotic protein, works with pro-apoptotic proteins such as BAD to regulate cell death and survival necessary for normal tissue homeostasis. Decreased BIM expression in cancer cells induces tumor formation. As a result, a 4-fold increase in Bad and Bak protein expressions was observed and a 2-fold increase in Bim protein expression was observed compared to the control group. As consistent with our study, metformin exerted pro-apoptotic effect on HT-29 colon cancer cells by inhibiting nuclear factor-kappa B (NF-κB) signaling which activated caspases [27]. The high concentration of metformin (25 mM) induced apoptotic cell death *via* upregulation of p53 but decreasing in cyclin D1 expression. Additionally, the AMPK expression level of the breast cancer cells also effect the anti-proliferative role metformin [28]. Other proteins that are effective in apoptotic cell death are caspases. Caspases, which remain inactive until the appropriate signal arrives, take part in the apoptotic pathway after being activated. Activated caspase 9 is cleaved and acts as the initiator caspase, enabling the activation of caspase 3, which is the effector caspase, and thus causing cell death through the intrinsic apoptotic pathway [29].

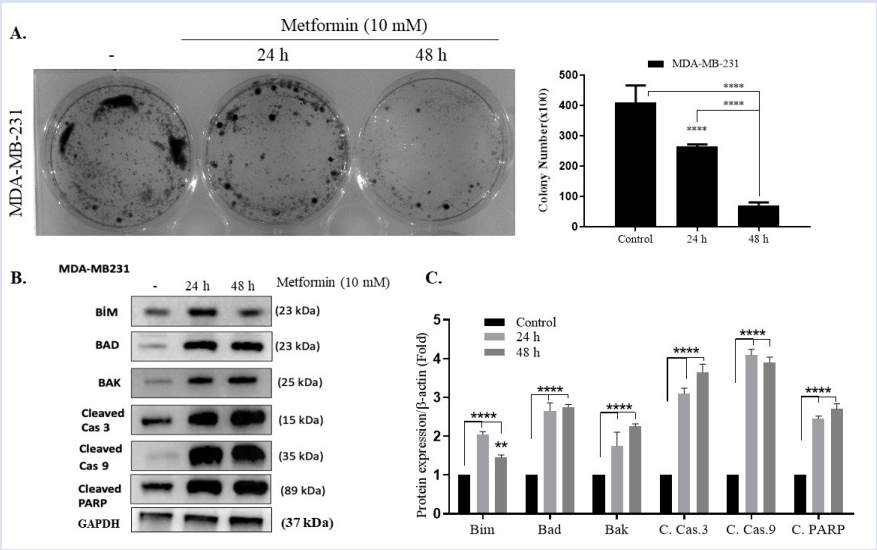

Figure 2. A. Examination of the effect of metformin on the colony formation potential of MDA-MB-231 breast cancer cells. B. Display the number of colonies formed as a result of time-dependent metformin treatment using the Image J program (****p<0.0001). C. Apoptotic marker protein expressions (Bim, Bad, Bak, Cle. Caspase 3, Cle. Caspase 9 and Cle. PARP) were analyzed by immunoblotting flowing 24h and 48h metformin treatment in MDA-MB-231 cells. D. Graphical representation of the changes in the expression of apoptotic marker proteins by using the Image J program (****p<0,0001).

A 4-fold increase was observed in cleaved caspase 9 and therefore cleaved caspase 3 protein expressions compared to the control group, depending on time, after metformin treatment. Caspase-mediated apoptotic cell death occurs through the cleavage of PARP. Due to the increase in caspase 3 expression, an increase in the expression of cleaved PARP is also observed (Figure 2A-B). A study in A498 renal cell carcinoma cells also showed the apoptotic effect of metformin which induced cleavage of caspase 3 and PARP in a dose-dependent manner. In the same study it was concluded that the metformin-induced apoptosis was modulated by degradation of caspase 8 (FLICE)-like inhibitory protein (c-FLIP$_L$) which has an inhibitory role on caspase-8 activation [30]. As a result, it has been shown that metformin treatment causes a 4-fold decrease in colony formation in MDA-MB-231 cells depending on the treatment time, proliferation is suppressed. Similar to the study conducted by Jang et al. on T4 bladder cancer cells, a positive effect on caspase-dependent apoptosis was observed in our study after metformin treatment [31]. Treatment with metformin caused an increase in the expression of intrinsic apoptosis pathway markers in breast cancer cells, causing the cell to undergo programmed cell death.

Effect of Time-dependent Metformin Treatment on the Autophagy Mechanism in MDA-MB-231 Breast Cancer Cells

Although autophagy, a process that regulates cell homeostasis that occurs under various stressful conditions such as organelle damage, the presence of abnormal proteins and nutrient deprivation, is associated with non-apoptotic cell death, it is considered a survival mechanism because it maintains intracellular balance. In the study conducted by Gözüaçık and Kimchi in 2006, p53 activation stimulates autophagy by inhibiting mTOR activity, which is effective in an antiapoptotic signaling pathway [32]. To demonstrate the effect of time-dependent metformin treatment on autophagy-induced cell death, immunoblotting with autophagy-related proteins (Atg5, Atg7, Beclin-1) was examined after 24 and 48 hours of metformin treatment (Figure 3A-B). Beclin-1 protein is the central regulator of autophagy. It is also a tumor suppressor whose expression is decreased in many types of cancer such as breast and ovarian [33,34]. Therefore, decreased Beclin-1 expression has been associated with tumorigenesis. As a result of the treatment of 10 mM metformin, which is the effective dose, for 24 and 48 h, a 2-fold and 2.3-fold increase in Beclin-1 expression was observed in MDA-MB-231 cells, respectively. Atg5, another autophagy regulator, is a central regulator required for autophagy in terms of its participation in autophagosome elongation. In studies, inhibiting Atg5 caused partial resistance to chemotherapy [35,36]. Deficiency of Atg7, which is involved in the regulation of autophagy, causes a phenotype lacking autophagy and disruption of cell homeostasis [37]. As a result of immunoblotting, no significant difference was observed in the expression of Atg5 with 24-hour metformin treatment, while 48-hour metformin caused a 2-fold increase in Atg5 expression. A 2-fold increase in Atg7 expression was observed compared to the control group, depending on both 24 h and 48 h metformin treatment duration (Figure 3A-B).

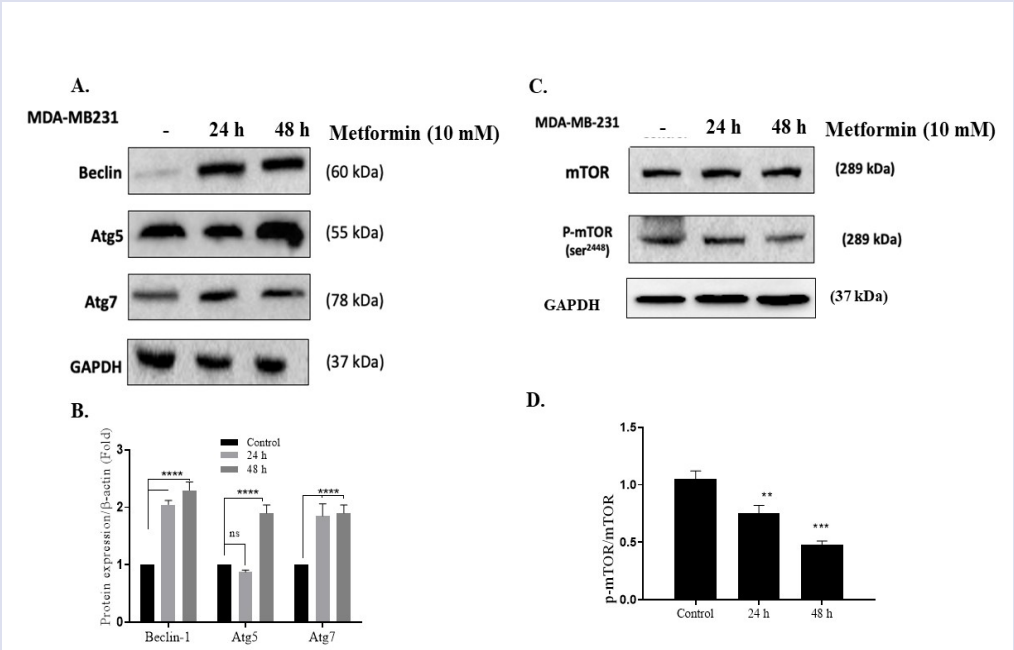

Figure 3. Demonstration of autophagy marker protein expressions (A. Beclin-1, Atg-5, Atg7) in MDA-MB-231 cells following metformin administration using the ChemiDoc MP Imaging System. B. Graphical representation of the changes in the expression of autophagy marker proteins as a result of time-dependent treatment with 10 mM Metformin in the MDA-MB-231 breast cancer cell line, using the Image J program. (****p<0.0001) C. Demonstration of mTOR and p-mTOR protein expressions in MDA-MB-231 cells following metformin treatment and analyse with the ChemiDoc MP Imaging System device. D. Graphical expression of mTOR and p-mTOR protein expression rates by using the Image J program. (** p<0.01, *** p<0.001)

After 48 hours of metformin treatment, a 2-fold decrease in phosphorylated mTOR (Ser2448) expression level was observed compared to the control group, but there was no significant change in total mTOR expression due to metformin treatment (Figure 3C-D). mTOR activity supports cell survival and energy metabolism, a mechanism that works opposite to the autophagy mechanism. In a study conducted to demonstrate the effect of metformin on Beclin-dependent autophagy in gastric cancer cells, it was shown that autophagy was induced in gastric cancer cells by decreasing the p-mTOR protein level and resulting in an increase in Beclin protein level [38]. As a result, it has been observed that Metformin induces autophagy through inhibition of mTOR activity in addition to apoptotic death in MDA-MB-231 breast cancer cells and has an inhibitory effect on tumor growth caused by autophagy-dependent cell death.

Metformin Suppressed the Proliferation and Invasion of MDA-MB-231 Breast Cancer Cells through Activation of the Hippo Signaling Pathway.

The Hippo signaling pathway, which modulates cell death, cell proliferation, and cell differentiation, plays a role in cell homeostasis by regulating cell number. YAP and TAZ, two interrelated transcriptional factors, have an important place in tissue repair and organ size control in cancer treatment [39]. In this study, to observe the effect of metformin on the Hippo signaling pathway in MDA-MB-231 triple-negative breast cancer cells, changes in Hippo signaling pathway markers YAP, TAZ and p-YAP protein expressions following 24h and 48h of metformin treatment were examined by immunoblot analysis (Figure 4A-C). It was observed that YAP protein expression, which has an anti-apoptotic, cell migration and invasion-promoting effect, decreased to 0.3-fold and 0.4-fold levels, respectively, after 24h and 48h of metformin treatment, compared to the control group. Metformin treatment for 24h and 48h caused a 2.6- and 2.7-fold increase in the level of p-YAP, which increases apoptotic activity by ensuring YAP phosphorylation, respectively, compared to the control group. When p-YAP expression is compared to total Yap expression, 24h metformin increased 3-fold and 48-h metformin increased 3.5-fold (Figure 4C). Studies have suggested that the Hippo signaling pathway can control apoptosis through the YAP protein, based on the response of the effector YAP protein regulated by the Hippo signaling pathway after DNA damage. Recent studies have suggested that YAP activation is associated with drug resistance in cancer treatment, and as a result of the experiments, it has been observed that there is more YAP expression in drug-sensitive breast cancer cells compared to non-resistant ones. Accordingly, *in vivo* experiments showed that the YAP level in the nucleus was downregulated after treatment with metformin [40].

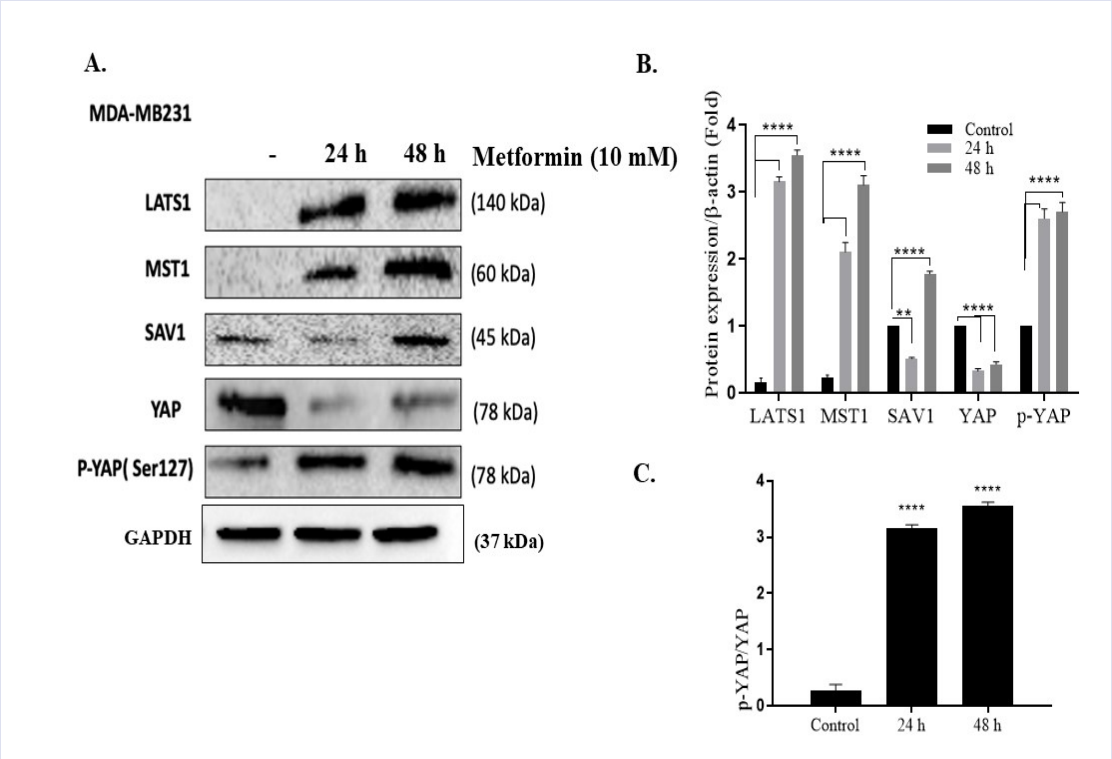

Figure 4. A. Demonstration of Hippo signaling pathway marker protein expressions (LATS1, MTS1, SAV1, YAP and p-YAP (S127) in MDA-MB-231 cells following metformin treatment. GAPDH was used as a loading control. B. Graphical expression of the changes in protein expression using the Image J program. C. Graphical representation of YAP and p-YAP protein expression rates in MDA-MB-231 breast cancer cell line as a result of 10 mM Metformin treatment (24-48 h) using the Image J program. (**p<0.01, ****p<0.0001)

Increased expression of TAZ, a Hippo signaling pathway regulator with anti-apoptotic effect that is highly expressed in many types of cancer, is associated with cancer metabolism [41]. Previous studies have observed that treatment with metformin has a dependent or independent effect on YAP inhibition, depending on the increase in MST1 and LATS1 interaction and expression [42]. In this study, metformin treatment for 24h and 48 h increased MST1 expression by 3.2 and 3.6-fold, respectively, and LATS1 expression level by 2.1 and 3.1-fold, respectively. Accordingly, it caused suppression of YAP expression. The expression of SAV1 protein, which has a tumor suppressor effect in many types of cancer, decreased by 0.5-fold in MDA-MB-231 breast cancer cells after metformin treatment within 24h while it increased by 1.8-fold with 48h metformin (Figure 4A-B). In the study conducted to demonstrate the role and mechanism of SAV1 on the development of pancreatic cancer; overexpression of SAV1 was shown to suppress cell migration and invasion and promote apoptosis [43]. Recent studies have reported that drug-sensitive and resistant cells are affected by different mechanisms of metformin treatment. *In vivo* and *in vitro* experiments indicated that metformin suppressed breast cancer by an AMPK-independent pathway to decrease YAP nuclear localization. In drug-sensitive cells, metformin activated the Hippo pathway by increasing KIBRA and FRMD6 expression, but this did not occur in drug-resistant cells [44].

As a result, following the 48h treatment of metformin, an increase in apoptotic cell death was observed due to activation of the Hippo signaling pathway.

Conclusion

As a result, in the light of the experiments performed, it has been shown that metformin treatment induced Hippo signaling pathway-dependent apoptosis in MDA-MB-231 triple negative breast cancer cells. Moreover, beside the downregulation of Hippo signaling, metformin inhibited cell proliferation and survival through upregulation of autophagy mechanism due to mTOR inhibition in breast cancer cells. The current study has some limitations, albeit the promising results. Firstly, glucose concentration is a limiting factor of metformin therefore the molecular mechanism of metformin as an anti-cancer drug in nondiabetic subjects should be investigated. Secondly, in the healthy cells, the adequate drug concentration of metformin should be determined, and thirdly, metformin-induced hippo signaling pathway in MDA-MD-231 cells should be investigated in various glucose concentration.

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Conflict of Interest

There is not any conflict of interest or common interest with an institution/organization or a person that may affect the review process of the paper.

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