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Araştırma Makalesi - Research Article

Metformin-induced and Mitochondrial Stress-mediated Apoptosis in *Schizosaccharomyces pombe*

Schizosaccharomyces pombe'de Metformin-tetiklemeli ve Mitokondriyal Stres Aracılı Apoptoz

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ABSTRACT

Metformin, a widely used first-line medication in the treatment of type II diabetes, has been proposed to have a second indication in the treatment of cancers and aging. However, its accounting mechanisms in cellular physiology were not clearly understood. Therefore, its cytotoxicity and underlying physiological mechanisms should be explained. *Schizosaccharomyces pombe* was evaluated as a single-cell cytotoxicity model and was treated with metformin and grown on YEL media at 30 °C and 180 rpm. 0,1-20 mM metformin caused dose-dependent apoptosis and necrosis demonstrated by using Annexin V-FITC/PI and DAPI staining. Surprisingly, metformin reduced ROS levels with stable antioxidant enzyme levels, but the mitochondrial transmembrane potential was significantly increased indicating a differential regulation by the dual character of metformin. In addition, a possible role can be attributed to *Cnx1* in apoptotic cell death; which showed a dramatic increase in transcription, however, three other potential apoptotic genes, *Rad9*, *Pca1*, and *Aif1* were stable. To conclude, the dual effect of metformin was clarified, and related cellular physiological effects with accompanying mechanisms (particularly *Cnx1*-mediated) were shown using *S. pombe*.

Keywords- S. pombe, Metformin, Mitochondrial Stress, Apoptosis

ÖZ

Tip II diyabetin tedavisinde ilk müdahale olarak yaygın kullanılan metforminin kanser tedavisi ve yaşlanma için sekonder endikasyona sahip olduğu öne sürülmüştür. Ancak hücresel fizyolojik sorumlu mekanizmalar net olarak anlaşılmamıştır. Bu yüzden, sitotoksik ve fizyolojik mekanizmaların açıklığa kavuşturulması gerekmektedir. *Schizosaccharomyces pombe* tek hücreli sitotoksisite modeli olarak değerlendirilmiş, metforminle muamele edilerek 30 °C sıcaklık ve 180 rpm hızda, YEL medyumu içerisinde inkübasyona bırakılmıştır. Annexin V-FITC/PI ve DAPI boyaması ile gösterildiği üzere, 0,1-20 mM metformin doz-bağımlı apoptoz ve nekroza neden olmuştur. Öte yandan, metformin ROS seviyelerini düşürmüş, uygulamada antioksidan enzim seviyeleri sabit kalmış, fakat mitokondri membran potansiyeli önemli derecede yükselmiştir. Bu durum metforminin ikili karakteri ile diferansiyel regülasyona işaret etmektedir. Ek olarak, apoptotik hücre ölümünde *Cnx1*'e olası bir rol biçilebilir; ki transkripsiyonda dramatik bir yükseliş görülmüştür. Ancak diğer üç olası apoptoz geni *Rad9*, *Pca1* ve *Aif1* stabil

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kalmıştır. Sonuç olarak, bu çalışma ile metforminin ikili etkisi net olarak ortaya konmuş ve sorumlu mekanizmalarla birlikte (özellikle *Cnx1*-aracılı), ilişkili hücresel fizyolojik etkiler *S. pombe* maya hücresinde gösterilmiştir.

Anahtar Kelimeler- Schizosaccharomyces pombe, metformin, mitokondriyal stress, apoptoz

I. INTRODUCTION

Metformin, 1,1-dimethylbiguanide hydrochloride, is synthesized from two guanidine moieties and used as a first-line anti-diabetic drug by patients in the early stages of type 2 diabetes [1]. Although its mechanism of action in the therapy of diabetes has not yet been clearly understood, the activation of AMPK -the principal regulator of glucose and lipid metabolism in the cell- and modulation of mitochondrial function were shown as explanations of metformin's anti-diabetic [2, 3], cardiovascular protective, anticancer and antiaging activities [4].

In vitro and *in vivo* studies reported dose-dependent genotoxicity (DNA damage, chromosome aberration) at 100-600 µg/ml chronic metformin exposure, which was shown by comet assay and micronucleus assay [5]. Metformin arrested the cell cycle and induced apoptotic cell death in Large and Small duct-type primary iCCA (intrahepatic cholangiocarcinoma) cells and their xenograft mouse models with intracellular changes in nuclear morphology and tumor volume diminution measured by histological observation [6]. Besides, studies with ovarian cancer [7], bursal lymphoma [8], cervical cancer [9], hepatocellular carcinoma [9], colon cancer [10], and myeloid leukemia cell lines [11] showed that metformin has a high cytotoxicity potential and may be used with anti-cancer drugs as a combination against chemoresistant cancers including many solid and liquid tumors [12, 13]. Nevertheless, metformin was found to play a critical role in CD19 CAR-T cell therapy and negatively affect T-cell proliferation reprogrammed artificially and recruited for fighting against cancer cells by inducing T-cell apoptosis following AMPK phosphorylation- and mTOR suppression-related cytotoxic cell death [14]. Therefore, a complete explanation of metformin toxicity and (de)regulation of controlled (or programmed) cell death in *Schizosaccharomyces pombe* (fission yeast) are by now unknown and should be cleared.

Schizosaccharomyces pombe represents a promising unicellular model to be used in genotoxicity, cell physiology, and molecular biological studies, in which similar "mitochondria-effect" on cellular energy metabolism and cell death [15], and controlled cell death mechanism create opportunities in human cancer and physiological disease research [16]. Accordingly, as metformin constitutes an anti-cancer candidate therapeutic reported by a broad range of cancer cell line studies, metformin's accounting mechanisms in cellular physiology can be unraveled by employing *S. pombe* as a eukaryotic cell death model.

In this study, we aimed to understand the cytotoxic effects of metformin and its mechanism and to screen the ability of *S. pombe* as a drug-screening model. Accounting mechanisms, i.e. autophagy, apoptosis, excessive ROS levels and oxidative stress, necrosis, mitochondrial impairment, and nuclear damage, were assessed.

II. MATERIALS AND METHODS

A. Reagents

Yeast extract, dextrose, methylene blue, arsenic(III) oxide, metformin (1,1-dimethylbiguanide hydrochloride), for staining, DAPI, Rhodamine 123, and DCFDA were ordered from Sigma. Annexin V-FITC/PI kit was from Clonetech Laboratories, Inc.

B. Yeast, Media, and Conditions

S. pombe ED666 and mutant yeast cells (*pca1Δ*, *aif1Δ*, and *rad9Δ*) were grown in yeast extract liquid media (1% yeast-extract, 2% dextrose) at 150 r.p.m. at 30 ^{0}C. Cultures were adjusted to 1x10^6 cells per 1 mL concentration in the treatment.

C. Metformin Treatment and the Determination of Cytotoxicity

Overnight yeast culture ($OD_{600}\approx1$) was adjusted to 1x10^6 cells/mL. Metformin was prepared with sterile distilled water and applied to cells at 0-20 mM concentrations for 24 h. Cells were solved in 1xPBS and ready-to-use methylene blue solution (0.1 mg/mL) was used for the evaluation of mortality. At least 200 cells were analyzed under a light microscope in biological replicates (n=5). Cells were also spread on the YE agar plate after 1:10 dilution in PBS and incubated for 3 days at 30 ^{0}C. Viability was evaluated as the ratio of metformin and control groups.

D. Evaluation of Apoptosis by Annexin V-FITC/PI and DAPI

DAPI solution at 1 µg/mL concentration was prepared to screen the nuclear DNA as declared earlier [17]. A fluorescent microscope (Carl-Zeiss, Axio Observer 3) was used at two different wavelengths, 358 and 461 nm. For apoptosis evaluation, yeast was stained with Annexin V-FITC/PI dual stain as mentioned earlier [18], and observed at 500 and 530 nm for Annexin V-FITC, and 510 and 595 nm for PI. Briefly, yeast was washed with sorbitol buffer (0.5 mM $MgCl_2$, 1.2 M sorbitol, and 25 mM potassium phosphate, pH: 6.8), incubated with 300 U/mL Zymolyase (Zymo, USA) for 30 minutes at 30 ^{0}C for the lysis of the cell wall, and washed with sorbitol buffer again followed by suspension with sorbitol-containing binding buffer (10 mM Hepes/NaOH, pH: 7.4, 2.5 mM $CaCl_2$, 140 mM NaCl). Afterward, 2 µL Annexin V-FITC and 2 µL propidium iodide were mixed with 38 µL cell suspension and incubated at room temperature for 20 minutes. After washing with binding buffer, cells were observed under the fluorescent microscope given above, and more than 250 cells were screened and counted in each replicate (n = 5).

E. ROS detection by DCFDA staining

DCFDA was used to measure ROS levels as mentioned previously [19, 20] and examined under a fluorescent microscope at 495 and 529 nm.

F. Determination of ΔΨm (Mitochondrial Transmembrane Potential) by Rhodamine 123 stain

Mitochondria were visualized with Rhodamine 123 solution prepared with sodium citrate and glucose for 15 min as indicated previously [21]. Cells were screened under fluorescent microscope at 505 and 534 nm.

G. Real-time PCR Experiments

Alteration in pro-apoptotic gene expressions (*aif1*, *pca1*, *rad9*, and *cnx1*), autophagy-related gene expressions (*atg6* and *atg8*), and antioxidant gene expressions (*sod1* and *gpx1*) were calculated with real-time PCR following total RNA isolation, and protocols were given in ESI.† PCR primers were designed with Primer3Plus (see Table in Supplementary File†).

H. Statistical calculations

Data were demonstrated as SEM (mean ± standard error of the mean). One-way ANOVA and Tukey's test (GraphPad, USA) were selected to analyze the difference.

III. RESULTS

A. Metformin induces cytotoxicity and cell death

Metformin-induced cellular death in *S. pombe* was potentially related to its apoptosis-inducing activity. We firstly counted dead cells with the help of methylene blue's permeation through dead cell membranes, which were highly different to the control group ($p < 0.01$; Figure 1A), whereas viability shown by colony-forming assay was dramatically decreased ($p < 0.01$; Figure 1B). The IC_{25} value was 20 mM.

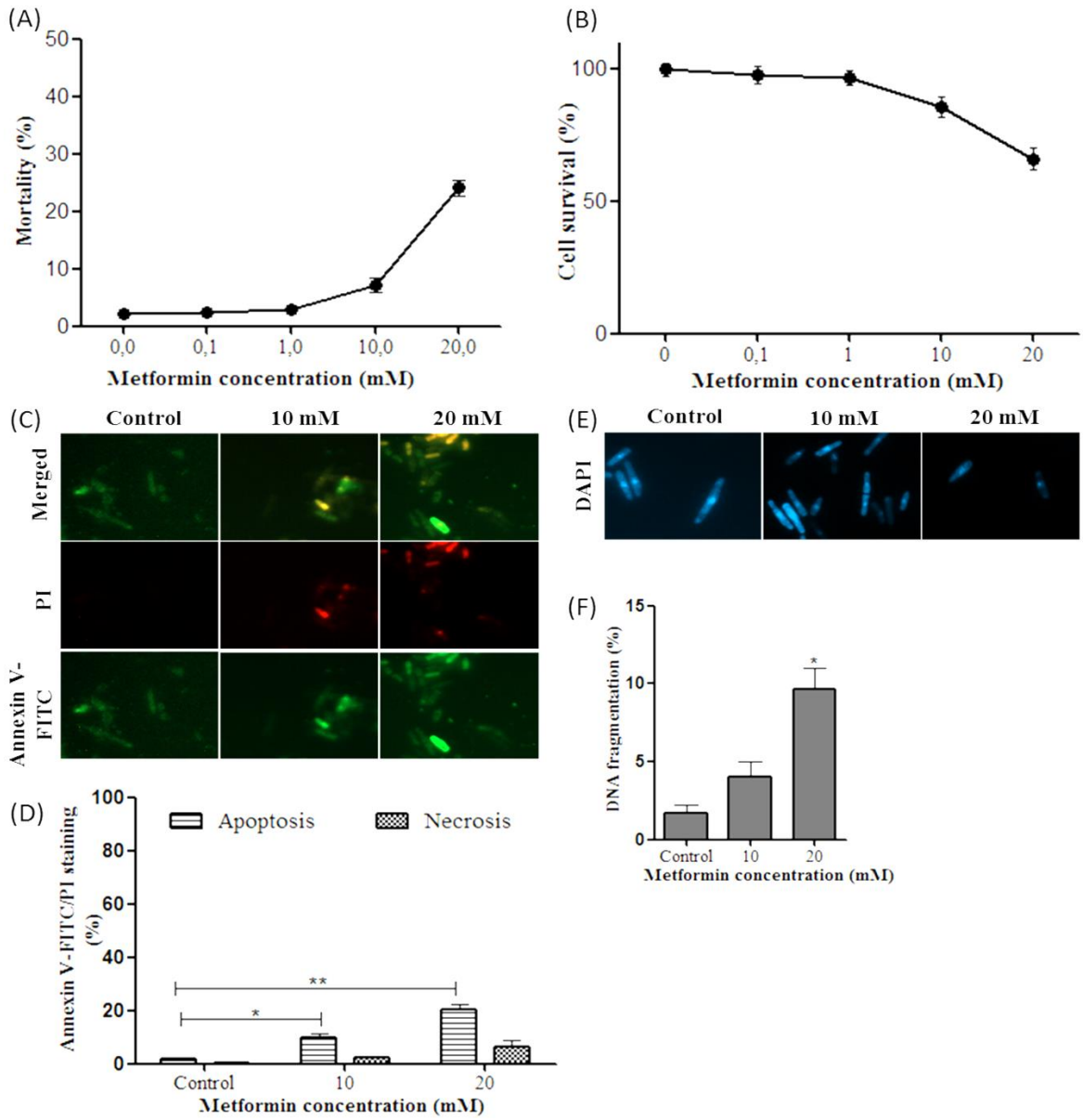

Figure 1. Mortality, cell survival, apoptosis, and DNA fragmentation after exposure to metformin: **A-B.** Mortality and cell survival were assessed by (MB) methylene blue staining and colony-forming (CFA) assay to compare with sterilized distilled water (vehicle control). **C.** Apoptotic and necrotic cells were shown using Annexin V-FITC/PI dual staining after treatment with 10-20 mM metformin. **D.** The graph shows the percentage of apoptotic and necrotic cells treated with 10-20 mM metformin (n=5). **E-F.** DAPI assay was performed after metformin treatment and the graph shows the percentage of DNA fragmentation. Asterisks were used to show statistically significance levels (*p<0.05, ** p<0.01).

B. Apoptosis is Primary Responsible for Cell Death

While Annexin V-FITC stains only phosphatidyl serine residues translocated to the outer face of the cellular membrane indicating apoptotic cells, PI diffuses only dead cells and stains the nucleus, as shown in Figure 1C. Orange cells are apoptotic cells and red cells are necrosis. Cells were exposed to 10 mM and 20 mM metformin. The counted number of apoptotic cells was statistically significant in both doses ($p < 0.05$ and $p < 0.01$) as demonstrated in Figure 1D. Apoptosis moderately and dose-dependently increased (from 10.05% to 20.49%) compared to control (1.80%). Apoptosis rates were found similar to mortality rates (7.95% and 24.14%) at 10-20 mM concentrations.

Apoptosis was also monitored using DAPI staining assay. After overnight fixation with formaldehyde, yeast cells were stained with DAPI-methanol solution. Counted cell nuclei with DNA fragmentation (typical apoptotic marker) [22, 23] in 10-20 mM metformin groups (4.1% and 9.87%) were consistent with apoptosis rates shown in Figure 1E-F.

C. Metformin Decreases ROS Production

ROS, including superoxide anions, react with DCFDA as soon as it diffuses to the cytosol. DCFDA is oxidized to green-fluorescing DCF (fluorescent form of DCFDA) and helps to measure intracellular oxidative stress levels. Figure 2A-B shows significant decreases (1.19- and 1.58-fold) in DCF fluorescence in the experimental group at both doses ($p < 0.05$ and $p < 0.001$) reflecting a reduction in oxidation levels. As demonstrated in Figure 2C, SOD and GPx mRNA expressions were also stabilized at all concentrations of metformin.

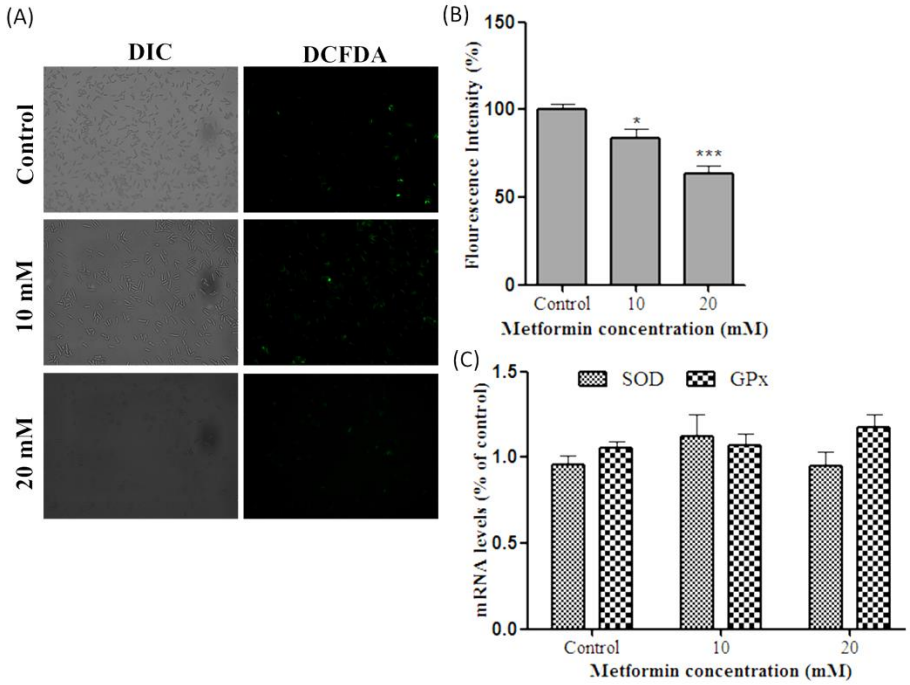

Figure 2. DCF fluorescence (ROS) levels with SOD and GPx (antioxidant enzymes) expressions: A-B. ROS generation in *S. pombe* exposed to 10-20 mM metformin was monitored and fluorescence intensity was measured. **C.** SOD and GPx expressions were calculated by RT-PCR. Significance is shown with asterisks (*p<0.05, ***p<0.001; n = 5).

D. De-regulation of MTP correlates with apoptotic cell death

Alterations in mitochondrial transmembrane potential (ΔΨm) can be used as apoptotic markers [24]. Mitochondrial fluorescence stain Rhodamine 123 is transported and sequestered in the active mitochondrion by proactive membrane ATPases and fluoresces green [25]. Changes in fluorescence intensity were shown in Figure 3A-B. A dramatic increase in fluorescence intensity in the metformin group was dose-dependent (1.38- and 1.51-fold).

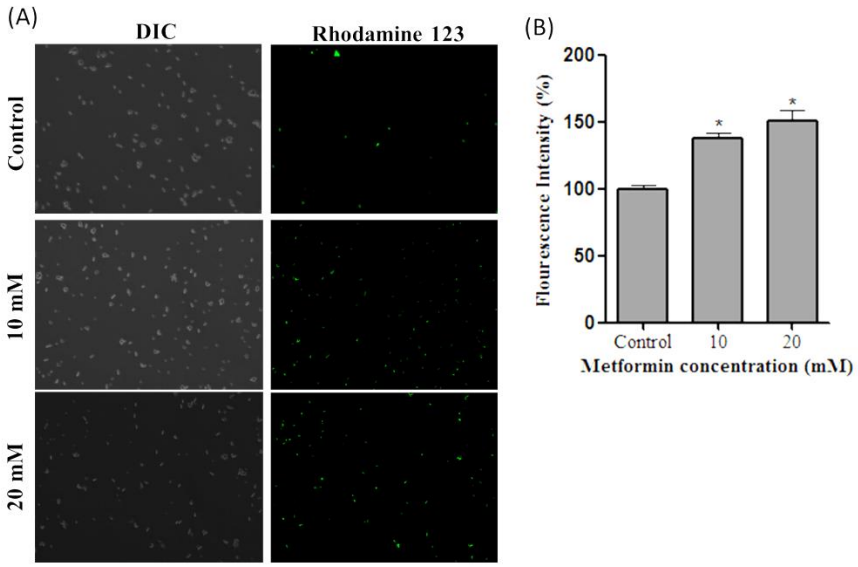

Figure 3. (MTP) Mitochondrial transmembrane potential was shown with Rhodamine123 assay: A. MTPs exposed to 10-20 mM metformin were monitored and measured. **B.** Increase of fluorescence intensity in *S. pombe* exposed to metformin (10-20 mM) was expressed as a percentage of control. Significant differences were indicated by asterisks (*p<0.05; n=5).

E. Cnx1 is potentially related to apoptosis

Rad9, *Pca1*, *Aif1*, and *Cnx1* were hypothesized that they regulate the apoptotic process in *S. pombe* [26–28]. While mutant cells (*Rad9Δ, Pca1Δ*, *Aif1Δ*) did not show different death rates demonstrated by colony-forming assay (Figure 4A), however, *Cnx1* mRNA levels, having potentially a critical role in the regulation of ER-stress-related cell death [26], increased dramatically (1.9-fold, $p < 0.05$; and 1.4-fold, $p > 0.05$) in 10 mM and 20 mM metformin groups, whereas other pro-apoptotic genes did not show any alteration in mRNA expression (see Figure 4B).

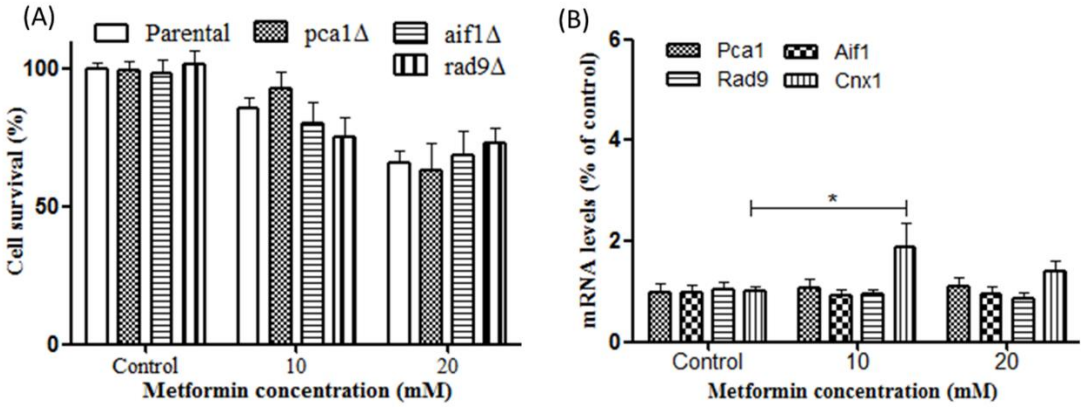

Figure 4. Colony-forming assay and mRNA levels of pro-apoptotic genes in yeast exposed to 10-20 mM metformin: A. *Rad9Δ*, *Pca1Δ*, *Aif1Δ* mutant cells examined by colony-forming assay for their role in metformin-induced apoptosis. B. Pca1, Aif1, Rad9, and Cnx1 mRNA levels were measured by RT-PCR. Significant differences are indicated by asterisks (*p<0.05; n = 3).

IV. DISCUSSION

Apoptosis and necrosis are two different types of cellular death, whereas necrosis is accepted as a random and rapid but frequently overdose-dependent death in response to chemicals and physical effects. Programmed cell death subroutines in *S. pombe*, i.e. apoptosis, and related genetic and biochemical mechanisms are partially unknown [29]. This study focused on metformin cytotoxicity, oxidative damage, apoptotic, necrotic, and related physiological mechanisms including genetic and biochemical factors. Though metformin was widely used as a first-line medication in the treatment of type II diabetes, reports from cell death studies created a new vision and researchers suggested a second indication, an anti-cancer effect primarily connected to apoptosis and autophagy [30]. Metformin caused dose-dependent cytotoxicity following apoptosis in the moderate dose group, but a strong necrotic cell death also contributed to total cell death rates in the high dose group. The primary responsible

mechanism for cell death was apoptosis in both doses shown by similar rates for apoptosis and cell death. In humans, both apoptotic and autophagic cell death subroutines were triggered by metformin treatment of osteosarcoma [31] and esophageal squamous cells [32]. However, this double-ended drug molecule promoted apoptosis in a glucose-starved hepatocellular carcinoma cell line (H4IIE), but surprisingly autophagy was suppressed by reducing expression levels of autophagy-related proteins Beclin-1, LC-3B, and a series of Atg proteins [33].

Pca1, Aif1, and Rad9 were suggested to positively affect *S. pombe* apoptosis progression shown by our early studies [34] which means apoptotic marker genes in *S. pombe* should be investigated for their roles in each chemical drug or natural substance as our current results with metformin were not consistent with the former study that tested different chemicals. In addition to mutant colony-forming assay results, Pca1, Aif1, and Rad9 mRNA levels after metformin treatment in both doses were stable in this study; however, the Cnx1 mRNA level in the 10 mM dose group was exceptionally altered. Although the precise role of Cnx1 was not clearly understood in *S. pombe* and early reports contributed to an assumption that *S. pombe* ER stress-activated unfolded protein toxicity was overcome by Cnx1 [26], our studies with Cnx1 in this and another previous study supported its role in *S. pombe* apoptosis and its transcriptional regulation [35].

The general assumption was that oxidative stress is contemporaneous with organelle impairments in the initiation of apoptosis and necrosis [36], but surprisingly, the DCFDA fluorescence assay showed us a dramatic and dose-dependent reduction after metformin treatment which means that metformin-induced apoptosis is not linked to excessive ROS and oxidative stress and changes our vision on metformin effect in *S. pombe*, whereas oxidative and lipolytic effects of metformin [37] along with, in contrast, antioxidative effect were also reported previously [38] in mammals. Our study also showed a dramatic and gradual increase in mitochondrial transmembrane potential after metformin administration, which is consistent with the results that reported an increased glucose consumption after metformin in *S. pombe* [39]. However, low-dose metformin administration (75-1000 μM) to hepatocytes (Hepa1-6 cells) did not cause mitochondrial accumulation, whereas metabolic parameters, i.e. complex I activity and relative mitochondrial density, were improved in mouse liver treated with oral metformin administration [40].

V. CONCLUSION

To summarize, these findings helped us to understand metformin-induced cell death in the unicellular physiological model *S. pombe* and improved our vision both in model organism physiology and metformin treatment, particularly in cell death research. We showed that mitochondrial reaction and antioxidant response physiology can be differentially affected and regulated when administered with a dual-action chemical drug, metformin. This study also contributes to enlightening the metformin's dual effect: Cytotoxic and/or life-saving. This is also the first study declaring the roles of "death genes" in metformin-induced apoptosis in which we found that the Cnx1 gene was the potential candidate for this role. These results need further characterization of pro-apoptotic factors that may have contributed to cell death signaling, potentially in unfolded protein response as it was consistent with Cnx1 activation.

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