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Research Article

Eisenia foetida (Sav.) coelomic fluid protect human umbilical vein endothelial cells against metformin-induced cell toxicity

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Abstract: The coelomic fluid of the red California earthworm *Eisenia foetida* (Sav.) includes a number of bioactive substances with antitumor and protective effects, thus making the fluid to also act as a defensive agent for the organism. *Eisenia foetida* coelomic fluid can be used as an alternative medication, for it is readily available and has few adverse effects. Metformin are widely used for managing type 2 diabetes mellitus by improving insulin sensitivity and reducing hepatic glucose production; however, therapeutic dose-related adverse effects have been reported. The present investigation aims to determine, for the first time, the protective effects of *E. foetida* coelomic fluid against possible metformin toxicity at the molecular and cellular levels. Metformin-induced cell toxicity was conducted following *E. foetida* coelomic fluid pre-treatment in Human Umbilical Vein Endothelial Cells (HUVEC). In addition to cell and nuclear morphology observation, a decrease in reactive oxygen species and apoptotic cell rate was determined. According to the obtained findings, the coelomic fluid of *E. foetida* preserved cell viability and morphology, reduced reactive oxygen species and apoptosis cell death, and enhanced anti-apoptotic mRNA expressions. When treating diseases, medical support and adjunct therapies should be taken into account. In this sense, our present preliminary *in vitro* findings showed that *E. foetida* coelomic fluid might has great additional treatment potential, and further molecular and animal studies to support this effect are needed.

Özet: Kırmızı Kaliforniya solucanı *Eisenia foetida*'nın (Sav.) sölomik sıvısı, antitümör ve koruyucu etkilere sahip bir dizi biyoaktif maddeyi içerdiğinden, bu sıvının aynı zamanda organizma için bir savunma maddesi olarak da işlev görmesi sağlanmaktadır. *Eisenia foetida* sölomik sıvısı, kolayca bulunabilmesi ve yan etkilerinin az olması nedeniyle alternatif bir ilaç olarak kullanılabilir. Metformin, insülin duyarlılığını artırarak ve hepatik glukoz üretimini azaltarak tip 2 diyabetin tedavisinde yaygın olarak kullanılmaktadır; ancak terapötik doza bağlı yan etkiler bildirilmiştir. Bu araştırmanın amacı, *E. foetida* sölomik sıvısının olası metformin toksisitesine karşı moleküler ve hücresel düzeyde koruyucu etkilerini ilk kez belirlemektir. Metformin kaynaklı hücre toksisitesi, *E. foetida* sölomik sıvısı ön muamelesinin ardından gerçekleştirildi. Hücre ve nükleer morfoloji gözlemine ek olarak, reaktif oksijen türleri ve hücre ölümü üzerindeki koruyucu etkisi belirlendi. Elde edilen bulgulara göre, *E. foetida* sölomik sıvısı hücre canlılığını ve morfolojisini korudu, reaktif oksijen türlerini ve apoptotik hücre ölümünü azalttı ve anti-apoptotik mRNA ekspresyonlarını arttırdı. Hastalıkların tedavisi, destekleyici bakım ve yardımcı tedavilerin dikkate alınmasını içermelidir. Bu anlamda, bu *in vitro* ön bulgularımız *E. foetida* sölomik sıvısının büyük yardımcı tedavi potansiyeline sahip olduğunu gösterdi ve bu etkiyi desteklemek için daha fazla moleküler ve hayvan çalışmasına ihtiyaç vardır.

Introduction

Eisenia foetida (Sav.) (the red California worm) is a red earthworm and since they are sensitive to light, they live in the dark and show hermaphrodite characteristics. Their body cavities are filled with a special fluid called coelom and they bear a unique natural defensive mechanism (Heredia Rivera *et al.* 2020). Production of a wide range of bioactive compounds and the coelomic fluid, known to have antioxidant, hemolytic, antitumor, antibacterial, and protective effects, as a defense mechanism when animals are under stress is typical (Grdisa *et al.* 2001, Hua *et al.* 2011, Kilciler *et al.* 2022, Deveci Özkan *et al.* 2023). Although the ecological,

physiological, and other health-related consequences of *E. foetida* coelomic fluid (ECF) have been extensively studied, little is known about its therapeutic potential, pharmacological relevance, and cellular effects (Deng *et al.* 2018, Lin *et al.* 2018). Researchers claim that because ECF is widely accessible, reasonably priced, and has few side effects, it can be used as an alternative treatment agent (Dajem *et al.* 2020). More research on the capacity of ECF to protect against the side effects of various drugs is necessary, given its therapeutic utility, pharmacological relevance, and cellular impacts.

The plant *Galega officinalis* L. is the source of a class of chemicals called biguanides, which are based on the biguanidine molecule. The primary component of *G. officinalis*, guanidine, was shown to reduce blood glucose levels and used to make a number of anti-diabetic medications, including metformin (MET) (1,1 dimethylbiguanide) (Wang & Hoyte 2019, LaMoia & Shulman 2021). Although MET's label states that it treats type 2 diabetes mellitus, it is also used to treat other conditions such hyperinsular obesity, polycystic ovarian syndrome, and weight gain caused by antipsychotic drugs (Pfeiffer & Klein 2014, DeFronzo *et al.* 2016, Houston *et al.* 2021). Although MET is a widely used drug in many different conditions, therapeutic dose-related adverse events have been reported. Abdominal discomfort, nausea, diarrhea, and other gastrointestinal symptoms, and infrequently acute hepatitis and cholestasis are among the side effects connected to the therapeutic use of MET (Nammour *et al.* 2003, Kutoh 2005, Biyyani *et al.* 2009, Cone *et al.* 2010, Stanton 2015, Abutaleb & Kottilil 2020). Patients who experience hyperlactatemia and metabolic acidosis when taking MET are susceptible to two primary types of toxicity: metformin-associated lactic acidosis (MALA) and metformin-induced lactic acidosis (MILA) (Blough *et al.* 2015). There is no specific treatment for MET toxicity. The foundation of its therapy is a supportive care, which includes the control of fluids, electrolytes, acid-base, respiratory, metabolic, renal, and hemodynamic abnormalities. Examples of adjunct therapy include metabolic rescue, extracorporeal techniques to reduce the body load of MET, serum alkalinization, glucose and insulin, and intestinal decontamination (Abad *et al.* 2020).

It is thus important to search for different treatments and supportive options for MET's side effects, which is commonly used and even has a very tiring treatment for reducing side effects. In this study, we investigated the protective effects of ECF as a natural active component with a potential for protective effect, whose content was previously determined (Ozkan *et al.* 2022), on potential MET-toxicity.

Materials and Methods

Cell Culture Conditions

We investigated the protective effect of ECF against MET-induced cell toxicity *in vitro* using Uuman Umbilical Vein Endothelial Cells (HUVEC). HUVECs come from the umbilical cord's venous endothelium and are used as a model system for the function and pathophysiology of endothelial cells (Park *et al.* 2006). Commercial HUVECs were purchased from the American Type Culture Collection (ATCC) and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 0.1% penicillin and streptomycin. The cells were grown at 37°C with 5% CO_2. The details in Ozkan *et al.* (2022) was followed to obtain ECF of *E. foetida* (Ozkan *et al.* 2022).

Cell Viability Assay

The cytotoxic effects of ECF and MET on HUVECs were determined by Water-Soluble Tetrazolium-1 (WST-1) cell viability assay. For this purpose, the cells were seeded in 96 well plates (2×10^4 cells/well) and incubated for 24 h. After incubation, the cells were treated with different concentrations of ECF (2, 4, 8, 16, 32, and 64 µg/mL) and MET (1.25, 2.5, 5, 10, and 20 mM) for 24 and 48 h at 37°C with 5% CO_2. After the incubation times, WST-1 reagent (Biovision) was added to each well and optic density (OD) values were measured at 450 nm with a microplate reader (Thermo Fisher Scientific). For the MET-induced toxicity model, HUVECs were pre-treated with ECF and followed by exposure to MET. After treatments, cell viability was measured by the cell viability assay described below.

Enzyme-Linked Immunosorbent Assay (ELISA)

To determine the cell death situation of cells after ECF pre-treatment against MET-induced cell toxicity, the free Annexin V (ANXA5) protein level in HUVECs were analyzed by the ELISA assay. For this purpose, the cells were seeded in 96 well plates (4×10^6 cells/well) and incubated for 24 h. Then, the cells were pre-treated with determined concentrations of ECF (2, 8, and 32 µg/mL) for 24h followed by exposure to MET (5 mM) for another 24h incubation. At the end of the incubation time, the cell culture media of each treatment group was collected and the free ANXA5 protein level was determined according to the Human ANXA5 ELISA kit (Abcam) procedure. The absorbance of each well was measured at 450 nm using a microplate reader (Benchmark Plus), and the ANXA5 levels of each group were calculated according to the standard curve.

Reactive Oxygen Species (ROS) Microplate Assay

To determine the oxidative stress situation of the cells after ECF pre-treatment against MET-induced cell toxicity, the ROS generation levels of HUVECs were measured by the ROS microplate assay. For this purpose, HUVECs were seeded in 96 well plates (25×10^3 cells/well) and incubated for 24 h. Then, the cells were pre-treated with determined concentrations of ECF (2, 8, and 32 µg/mL) for 24h followed by exposure to MET (5 mM) for another 24 h incubation. After the incubation time, ROS level was determined according to the DCFDA/H2DCFDA-Cellular ROS Assay Kit (Abcam) procedure. The absorbance of each well was measured

using a microplate reader (Biotek), and ROS generation levels of each group were calculated as fold change.

Real-Time Polymerase Chain Reaction (RT-PCR) Analysis

To determine the gene expression level status of cells after ECF pre-treatment against MET-induced cell toxicity, the expression of *Bax* and *Bcl-2* mRNA levels were detected by RT-PCR in HUVECs. For this purpose, HUVECs were seeded in T_{25} flasks (1×10^6 cells/flask) and incubated for 24 h. Then, the cells were pre-treated with determined concentrations of ECF (2, 8, and 32 µg/mL) for 24h followed by exposure to MET (5 mM) for another 24 h incubation. After the incubation, total RNA was isolated from the cells using the Trizol Reagent (Thermo Fisher) and the concentration of total RNA was measured by a Qubit 3 Fluorometer (Thermo Fisher Scientific). After RNA isolation, cDNA was synthesized with a cDNA Reverse Transcription Kit (Thermo Fisher Scientific) and the relative gene expression levels of *Bax* and *Bcl-2* were analyzed by using the CFX Connect Real-Time PCR Detection System from Bio-Rad. Additionally, *β-Actin* was used as an endogenous reference gene.

Acridine Orange (AO) and 4',6-diamidino-2-fenilindol (DAPI) Staining

To determine the morphological changes of cells after ECF pre-treatment against MET-induced cell toxicity, the changes in HUVECs and nucleus morphology were observed by AO and DAPI stainings, respectively. For this purpose, HUVECs were seeded in 6 well plates (1×105 cells/well) with slides and incubated for 24 h. Then, the cells were pre-treated with determined concentrations of ECF (2, 8, and 32 µg/mL) for 24h followed by exposure to MET (5 mM) for another 24 h incubation. After incubation, the cells were fixed with 4% paraformaldehyde solution for 30 min. After fixation, the cells were washed twice and stained with AO and DAPI dyes (100 mg/mL) for 30 min, respectively. The slides were captured and analyzed with a fluorescence microscope (Olympus IX73, Japan).

Statistical Analysis

GraphPad Prism version 6.0 (La Jolla, CA) was used for statistical analyses, and a $p < 0.05$ was recognized as a statistically significant threshold. Two-way ANOVA analysis with Dunnett's and Sidak's multiple comparisons test was employed for multiple comparisons. The Qiagen software (https://www.qiagen.com/tr/shop/genes-andpathways/data-analysis-center-overviewpage/other-real-time-pcrprobes-orprimersdataanalysis-center/) was used to analyze the gene expression results.

Results

The Cytotoxic Effect of ECF and MET in HUVECs

The cytotoxic effects of ECF and MET on HUVECs were assessed using the WST-1 viability assay. The results indicated that ECF treatment reduced HUVEC viability in a dose- and time-dependent manner compared to the control group (Fig. 1, Table 1, $p < 0.0001$). Specifically, all tested ECF concentrations, except for 2 µg/mL, significantly decreased cell viability after 48 hours compared to the control (Fig. 1a, $p < 0.05$). Based on these findings, we selected 2, 8, and 32 µg/mL ECF concentrations as optimal for the pre-treatment procedure.

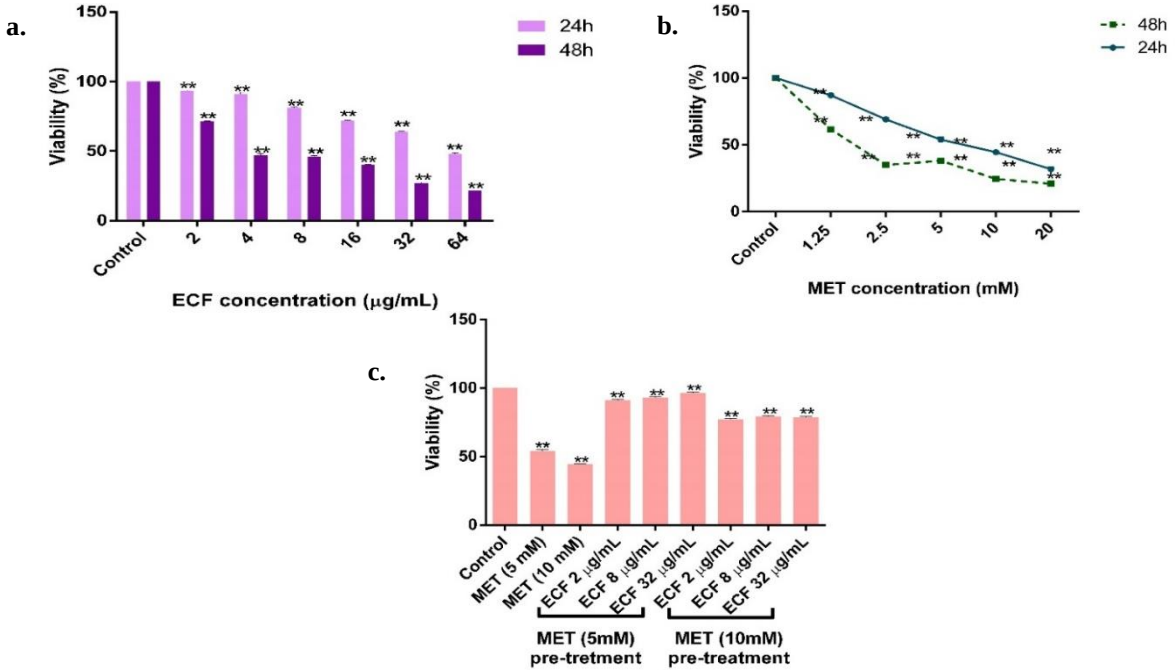

Fig. 1. Evaluation of HUVEC viability using the WST-1 assay. **a.** Effect of varying concentrations of ECF (2-64 µg/mL) on HUVEC viability, **b.** effect of MET at concentrations ranging from 1.25-20 mM on HUVEC viability, **c.** protective effects of ECF pre-treatment (2, 8, and 32 µg/mL for 24 h) on HUVECs exposed to MET (5 mM) for an additional 24 h. Significant differences were observed at $^{**}p < 0.0001$.

Table 1. Statistical analysis results of HUVEC viability assay. Two-way ANOVA analysis with Dunnett's and Sidak's multiple comparisons test was used for multiple comparisons.

ECF (µg/mL)	Mean Difference	95% CI of Difference	Significant?	Summary	Adjusted *p* Value
24h					
Control vs. 2	6.747	4.843 - 8.652	Yes	****	< 0.0001
Control vs. 4	8.968	7.063 - 10.87	Yes	****	< 0.0001
Control vs. 8	18.80	16.90 - 20.71	Yes	****	< 0.0001
Control vs. 16	28.05	26.15 - 29.96	Yes	****	< 0.0001
Control vs. 32	36.01	34.11 - 37.92	Yes	****	< 0.0001
Control vs. 64	51.83	49.92 - 53.73	Yes	****	< 0.0001
48h					
Control vs. 2	28.72	26.81 - 30.62	Yes	****	< 0.0001
Control vs. 4	52.99	51.09 - 54.90	Yes	****	< 0.0001
Control vs. 8	53.82	51.91 - 55.72	Yes	****	< 0.0001
Control vs. 16	59.97	58.06 - 61.87	Yes	****	< 0.0001
Control vs. 32	72.96	71.05 - 74.86	Yes	****	< 0.0001
Control vs. 64	78.49	76.59 - 80.40	Yes	****	< 0.0001
MET (mM)					
24h					
Control vs. 1.25	12.93	10.99 - 14.88	Yes	****	< 0.0001
Control vs. 2.5	30.92	28.98 - 32.87	Yes	****	< 0.0001
Control vs. 5	46.02	44.08 - 47.97	Yes	****	< 0.0001
Control vs. 10	55.42	53.47 - 57.36	Yes	****	< 0.0001
Control vs. 20	68.14	66.20 - 70.09	Yes	****	< 0.0001
48h					
Control vs. 1.25	38.26	36.32 - 40.21	Yes	****	< 0.0001
Control vs. 2.5	65.00	63.06 - 66.95	Yes	****	< 0.0001
Control vs. 5	61.83	59.88 - 63.77	Yes	****	< 0.0001
Control vs. 10	75.48	73.53 - 77.42	Yes	****	< 0.0001
Control vs. 20	79.10	77.15 - 81.04	Yes	****	< 0.0001
ECF (µg/mL) pre-treatment + MET (mM)					
Control vs. MET 5	23.01	21.85 - 24.18	Yes	****	< 0.0001
Control vs. MET 10	27.71	26.55 - 28.87	Yes	****	< 0.0001
Control vs. ECF 2+MET 5	4.385	3.221 - 5.550	Yes	****	< 0.0001
Control vs. ECF 8+MET 5	3.441	2.276 - 4.605	Yes	****	< 0.0001
Control vs. ECF 32+MET 5	1.753	0.5883 - 2.917	Yes	**	0.0019
Control vs. ECF 2+MET 10	11.39	10.23 - 12.55	Yes	****	< 0.0001
Control vs. ECF 8+MET 10	10.37	9.203 - 11.53	Yes	****	< 0.0001
Control vs. ECF 32+MET 10	10.63	9.466 - 11.79	Yes	****	< 0.0001

* CI: confidence intervals.

Similarly, MET treatment (5 and 10 mM) exhibited a dose-dependent reduction in HUVEC viability (Fig. 1b, $p < 0.0001$). For instance, treatment with 5 mM MET for 24 h resulted in a cell viability of approximately 45-53% (Fig. 1b, $p < 0.0001$). This concentration was deemed sufficient to induce significant toxicity for further analysis. To evaluate the protective effects of ECF against MET-induced cytotoxicity in HUVECs, cells were pre-treated with 2, 8, or 32 µg/mL ECF for 24 h before exposure to 5 mM MET for an additional 24 h. As expected, MET-alone treatment significantly decreased cell viability compared to the control group. However, ECF pre-treatment notably improved cell viability compared to the MET-alone group (Fig. 1c, $p < 0.0001$). In summary, these findings demonstrate that ECF pre-treatment mitigates MET-induced cytotoxicity in HUVECs, thereby preserving cell viability.

The Effect of ECF Pre-treatment after MET-induced Cell toxicity on ANXA5, ROS, and Gene Expression in HUVECs

The effects of ECF pre-treatment on MET-induced cell toxicity in HUVECs were assessed by ANXA5 ELISA, ROS production assays, and RT-PCR analysis (Fig. 2 and Table 2). The level of cell death following ECF pre-treatment was determined using ANXA5 ELISA (Fig. 2a). ANXA5 is a protein that binds phosphatidylserine (PS), a hallmark of apoptosis, making it a reliable marker for detecting apoptotic cells (Crowley et al. 2016). The ANXA5 ELISA results revealed that ANXA5 levels significantly decreased following MET (5 mM) treatment compared to the control group (Fig. 2a, $p < 0.0001$). However, pre-treatment with ECF (2, 8, and 32 µg/mL) followed by MET exposure increased ANXA5 levels, with the most pronounced effect observed at the 2 µg/mL ECF concentration (Fig. 2a, $p < 0.0001$). In terms of oxidative stress, MET treatment alone resulted in a significant 1.8-fold increase in ROS levels compared to the control group (Fig. 2b, $p < 0.0001$). ECF pre-treatment at all concentrations (2, 8, and 32 µg/mL) significantly reduced ROS levels compared to the MET-alone treatment group (Fig. 2b, $p < 0.0001$). RT-PCR analysis further demonstrated that ECF pre-treatment modulated the expression of apoptotic and anti-apoptotic genes. Specifically, Bax mRNA levels were significantly

decreased, while BCL-2 mRNA levels were significantly increased, particularly in the 2 µg/mL ECF pre-treatment group, compared to the MET-alone group (Fig. 2c, $p < 0.0001$). These findings suggest that ECF exerts a protective effect against MET-induced cell death and oxidative stress in HUVECs by modulating apoptosis to related pathways and reducing ROS levels.

The Effect of ECF Pre-treatment After MET-induced Cell toxicity on Cell and Nuclear Morphology in HUVECs

The effects of ECF pre-treatment on MET-induced cell and nuclear morphology changes in HUVECs, AO and DAPI stainings were performed. The staining results revealed a slight increase in apoptotic cell morphology characterized by cell shrinkage, nuclear condensation, and membrane blebbing following treatment with MET alone compared to the control group (Fig. 3). In contrast, pre-treatment with 2 and 8 µg/mL ECF followed by MET exposure reduced apoptotic morphology and improved overall cell morphology, resembling that of the control group. However, pre-treatment with 32 µg/mL ECF followed by MET exposure did not restore apoptotic cell morphology to the level observed in control cells (Fig. 3). These findings suggest that the lowest ECF concentration (2 µg/mL) is the most effective in protecting HUVECs from MET-induced cell toxicity and preserving normal cell morphology.

Table 2. Statistical analysis results of ANXA5, ROS and gene expression analysis. Two-way ANOVA analysis with Dunnett's and Sidak's multiple comparisons test was used for multiple comparisons.

ANXA5 level	Mean Difference	95% CI of Difference	Significant?	Summary	Adjusted *p* Value
ECF (µg/mL) pre-treatment + MET (mM)					
Control vs. MET 5	5.097	5.069 - 5.124	Yes	****	< 0.0001
Control vs. ECF 2+MET 5	-5.245	(-5.272) - (-5.217)	Yes	****	< 0.0001
Control vs. ECF 8+MET 5	-0.4276	(-0.4551) - (-0.4001)	Yes	****	< 0.0001
Control vs. ECF 32+MET 5	6.997	6.969 - 7.024	Yes	****	< 0.0001
ROS level					
ECF (µg/mL) pre-treatment + MET (mM)					
Control vs. MET 5	-0.3925	(-0.4540) - (-0.3310)	Yes	****	< 0.0001
Control vs. ECF 2+MET 5	-0.3125	(-0.3740) - (-0.2510)	Yes	****	< 0.0001
Control vs. ECF 8+MET 5	-0.1825	(-0.2440) - (-0.1210)	Yes	****	< 0.0001
Control vs. ECF 32+MET 5	-0.0650	(-0.1265) - (-0.003474)	Yes	*	0.0384
Gene (mRNA) expression level					
ECF (µg/mL) pre-treatment + MET (mM)					
Bax					
Control vs. MET 5	0.6350	0.5582 - 0.7118	Yes	****	< 0.0001
Control vs. ECF 2+MET 5	0.6650	0.5882 - 0.7418	Yes	****	< 0.0001
Control vs. ECF 8+MET 5	0.6500	0.5732 - 0.7268	Yes	****	< 0.0001
Control vs. ECF 32+MET 5	0.5350	0.4582 - 0.6118	Yes	****	< 0.0001
BCL-2					
Control vs. MET 5	0.7850	0.7082 - 0.8618	Yes	****	< 0.0001
Control vs. ECF 2+MET 5	-2.115	(-2.192) - (-2.038)	Yes	****	< 0.0001
Control vs. ECF 8+MET 5	-0.0600	-0.1368 - 0.01677	No	ns	0.1311
Control vs. ECF 32+MET 5	0.3900	0.3132 - 0.4668	Yes	****	< 0.0001

* CI: confidence intervals.

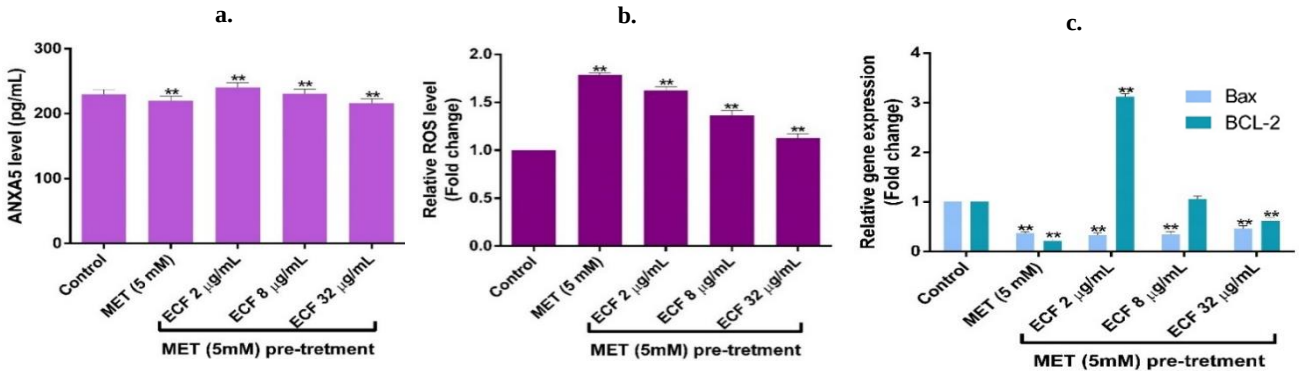

Fig. 2. Effects of ECF pre-treatment on MET-induced cell toxicity in HUVECs. **a.** ANXA5 levels, **b.** relative ROS levels and **c.** relative gene expression levels were evaluated, **c.** HUVECs were pre-treated with 2, 8, and 32 µg/mL ECF for 24 hours, followed by exposure to MET (5 mM) for an additional 24 hours. Significant differences were observed at $^{**}p < 0.0001$.

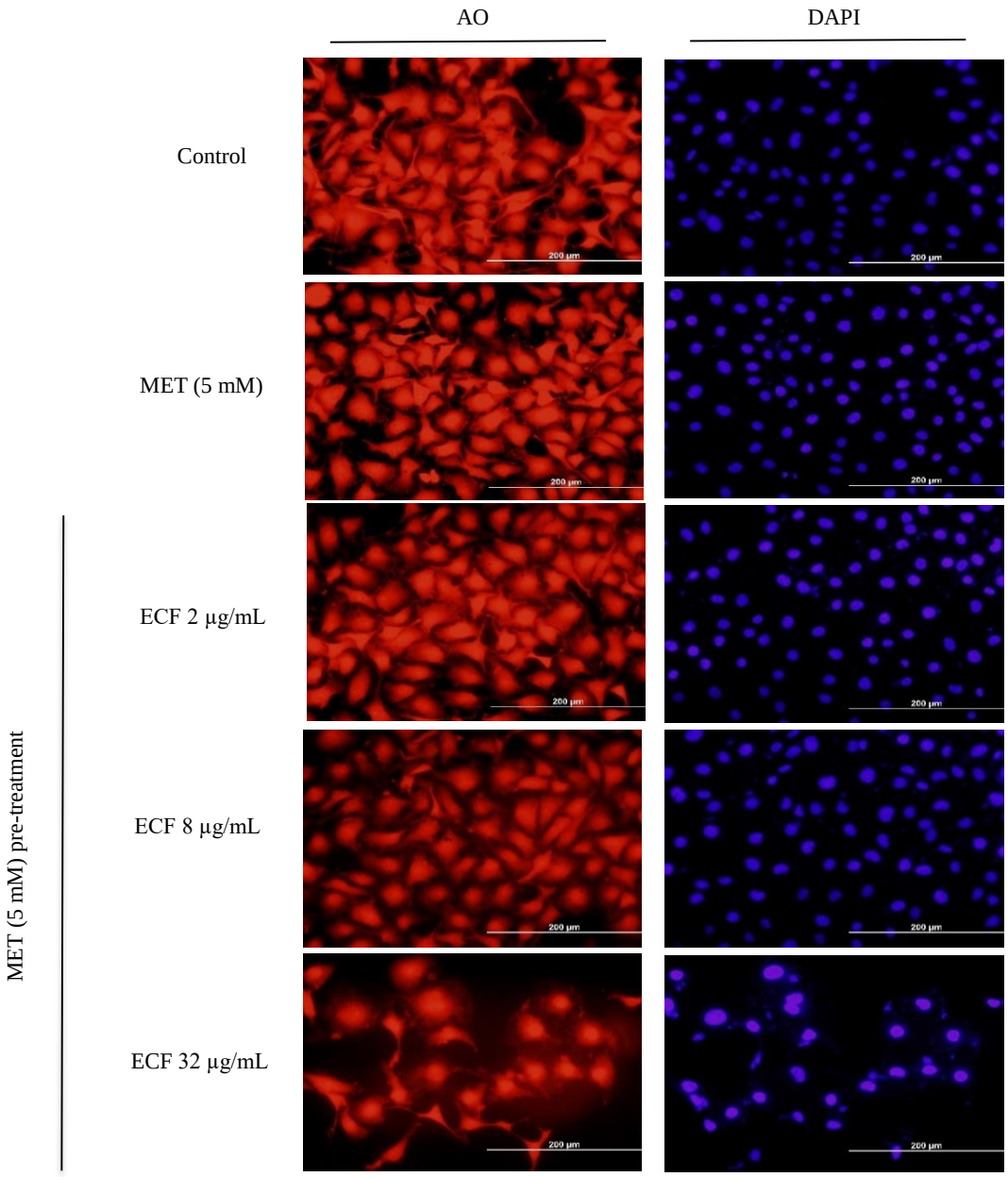

Fig. 3. Effects of ECF pre-treatment on cell and nuclear morphology in HUVECs following MET-induced cell toxicity, as assessed by AO and DAPI stainings. HUVECs were pre-treated with 2, 8, and 32 µg/mL ECF for 24 h, followed by exposure to MET (5 mM) for an additional 24 h. The staining highlights changes in apoptotic morphology and nuclear integrity.

Discussion

In this study, the *in vitro* protective effect of *E. foetida* coelomic fluid against MET-induced toxicity was determined in HUVECs. According to our findings, low ECF pre-treatment concentrations protect cell viability, preserve cell and nuclear morphology, reduce ROS production, and decrease apoptotic cell death against MET-induced toxicity.

The biguanide drug MET is commonly used to treat high blood sugar in people with type 2 diabetes mellitus, despite its well-known toxicity and side effects (Hasanvand *et al.* 2016). In addition to its beneficial effects on heart failure, MET therapy has positive effects on the organisms oxidative stress activities and lowers endotoxemia and improves insulin signaling pathways in animals (Ghosh 2017). Furthermore, it exhibits a number of characteristics that make MET appealing for repurposing as an anti-cancer treatment, including melanoma and pancreatic cancer cells (Romero *et al.* 2017). Interestingly, our findings showed that ROS generation levels were particularly high in the MET alone treated group but were much lower in the cells pre-treated with the ECF group. Additionally, ANXA5 levels, as an apoptotic marker of the cells, did not exhibit higher differences among the ECF pre-treatment and MET alone treated groups. In one study, the preventive and curative effects of garlic and MET combination were evaluated on Gentamicin (GM) induced tubular toxicity in Wistar rats

and the results demonstrated that MET and garlic combination have curative and protective activity against GM nephrotoxicity (Rafieian-Kopaei *et al.* 2013). These results and studies referred so far demonstrate that MET maintains its own protective and beneficial features, although a cytotoxic dose damage model was created for MET in the current study. However, in our findings, it is very clear that ECF has the potential to exert a protective effect when MET has a therapeutic dose-related adverse event. In this case, the presence of MET suggests that the protective effect of ECF as a pre-treatment increases, although not very much.

MET side effects include gastrointestinal disorders, vitamin B12 deficiency, and hemolytic anemia. Although metformin offers many advantages such as being effective, affordable, and widely available, its use is often limited by kidney function issues commonly observed in individuals with type 2 diabetes mellitus. Decreased renal function increases the risk of lactic acidosis, a dangerous condition. Although MALA is the lowest, the probability of growth with MALA is high (Bennis *et al.* 2020). Excessive lactate production, inadequate lactate clearance, or both can cause lactic acidosis. The most common cause of excessive lactate production is the switch from aerobic to anaerobic glucose metabolism in hypoxic environments (Adeva-Andany *et al.* 2014). It is still unclear how MET may accelerate lactic acidosis, but one theory is that it may do so by inhibiting mitochondrial respiratory chain complex 1, the first enzyme in the mitochondrial electron transport chain (Fontaine 2018, Vial *et al.* 2019). MALA may also occur as a result of mitochondrial glycerophosphate dehydrogenase (mGPDH). Maintenance of toxicity should include additional therapies such as extracorporeal techniques and metabolic rescue to reduce MET body loss (Wang & Hoyte 2019). Considering the antioxidant and cytoprotective properties of ECF and the data obtained from the current study, ECF is thought to reduce MET-induced toxicity by alleviating the risk of lactic acidosis.

The literature states that many compounds with potential protective effects have been investigated in relation to various cell types (Vargas *et al.* 2014, Shi *et al.* 2021). Vargas *et al.* (2014) assessed how different amounts of vitamin E alpha-tocopherol (α-T) isomer protected dental pulp cells from hydrogen peroxide (H_2O_2) damage and they found that the pulp cells of the immortalized MDPC-23 were protected from the negative effects of H_2O_2. Shi *et al.* (2021) found that pre-treatment with Cryptotanshinone (CTS) may enhance cell survival and promote the expression of the *Bcl-2* anti-apoptotic gene in neonatal rat cardiomyocytes. Furthermore, CTS may stop H_2O_2-induced NO synthesis and stop the production of ROS and MDA (Shi *et al.* 2021). Our findings are in line with previous research; as the concentration of ECF pre-treatment increased, oxidative damage from MET-induced toxicity decreased, free ANXA5 protein levels were reduced, and anti-apoptotic *Bcl-2* mRNA expression was enhanced. Additionally, AO and DAPI staining demonstrated that these findings were supported. In a previous study, we demonstrated how ECF impacted the molecular level of oxidative damage in MCFto7 cells produced by the chemotherapeutic agent NaBu, which is used to treat breast cancer (Ozkan *et al.* 2022). Our findings that ECF may be a potential therapeutic and supportive molecule with fewer side effects in cancer treatment and reduce the side effects of treatment in the future are consistent with this study's finding that ECF pre-treatment against NaBu-induced toxicity significantly reduced the amount of ROS production.

The findings of our study are preliminary and have limitations. More detailed molecular analyses are needed to show that MET toxicity is reduced by ECF. In particular, mitochondrial function and lactate production analysis should be performed by selecting cells that are sensitive to the effects of MET, such as liver or kidney cell lines. After cell culture experiments, the effect of ECF on MET-induced lactic acidosis should be tested in *in vivo* models. In conclusion, both the data we obtained and the methods we suggested can help us understand the potential protective effects of ECF on MET-induced lactic acidosis at the molecular, cellular and systemic levels. In this sense, these preliminary in vitro findings we obtained showed that ECF has great additional treatment potential. Considering the limitations of the study, future studies should focus more on in vivo validation, mechanistic analyses and long-term safety assessments.

Ethics Committee Approval: Since the article does not contain any studies with human or animal subject, its approval to the ethics committee was not required.

Data Sharing Statement: All data are available within the study.

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