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Metformin induces apoptosis via uterus mitochondrial permeability transition pore opening and protects against estradiol benzoate-induced uterine defect and associated pathophysiological disorder in female Wistar rats

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Abstract

Background: Some antitumor or anticancer agents have been shown to execute cell death by induction of mitochondrial permeability transition (mPT) pore opening in order to elicit their chemotherapeutic effect. Therefore, this study investigated the effect of metformin on cell death via rat uterus mPT pore and estradiol benzoate-induced uterine defect and associated pathophysiological disorder in female rat. Mitochondria were isolated using differential centrifugation. The mPT pore opening, cytochrome c release and mitochondrial ATPase activity were determined spectrophotometrically. Caspases 9 and 3 activities, MDA and estradiol levels and SOD, GSH activities, were determined using ELISA technique. Histological and histochemical assessments of the uterine section were carried out using standard methods.

Results: Metformin at concentrations 10–90 µg/mL, showed no significant effect on mPT pore opening, mATPase activity and release of cytochrome c. However, oral administration of metformin caused mPT pore opening, enhancement of mATPase activity and activation of caspases 9 and 3 significantly at 300 and 400 mg/kg. Metformin protected against estradiol benzoate (EB)-induced uterine defect and other associated pathophysiological disorder. It also improved the antioxidant defense system. The histological evaluation revealed the protective effect of metformin on the cellular architecture of the uterus while the histochemical examination showed severe hyperplasia in the uterine section of EB-treated rats, remarkably reversed by metformin co-treatment.

Conclusion: This study suggests that metformin at high doses induces apoptosis via rat uterus mPT pore opening and protects against EB-induced uterine defect (hyperplasia) and associated pathophysiological disorder.

Keywords: Apoptosis, Cancer, Metformin, Mitochondria, Mitochondrial permeability transition pore

Background

Apoptosis is a process which eliminates unwanted cells and phagocytized by other cells (Green and Llambi 2015; Yan et al. 2020). The mPT plays a critical role in mitochondrial pathway of apoptosis (McIlwain et al. 2013;

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Youle and Strasser 2008). Experimental evidences have shown that some phytochemical compounds elicit their chemopreventive and antiproliferative effects by triggering mPT pore opening. These include betulinic acid (Yong et al. 2013), *Drymaria cordata* (Olowofolahan et al. 2015), *Calliandria portoricensis* (Oyebode et al. 2017), *Mangifera indica* (Olowofolahan et al. 2018), and etc. Metformin is an anti-diabetic drug used for the treatment of type 2 diabetes. However, its effectiveness in suppressing endothelial proliferation at high concentrations has been documented (Kim et al. 2015; Tseng 2017; Habib et al. 2013; Memmott et al. 2010). Nevertheless, its influence on rat uterine mPT pore and estradiol benzoate (EB)-induced uterine pathophysiological disorder are yet to be unraveled. Consequently, this research investigated the influence of metformin on apoptosis via induction of rat uterus mPT pore and its possible protective potential against EB-induced uterine defect and associated pathophysiological disorder in female Wistar rats.

Methods

Chemicals and reagents

These were procured from Sigma-Aldrich Chemical Co. Different doses of metformin (MTF) were orally administered while EB was intraperitoneally administered. The chosen doses were based on pilot study and literature search (Jing et al. 2019; Diniz Vilela et al. 2016; Olowofolahan et al. 2020).

Experimental animals

Two sets of female Wistar albino rats (180–200 g) were kept in clean cages to be acclimatized for fourteen days with free access to pelletized rat chow and water. Their estrous cycles were monitored (Solomon et al. 2010) and the experiment was carried out following the ethical standards (1964 Declaration of Helsinki).

First set

Experimental design Thirty female Wistar rats were randomly assigned into 5 equal groupings and orally administered for 30 days as follows;

- Group 1 (control): distilled water (1 ml/kg).
- Groups 2: MTF(100 mg/kg).
- Group 3: MTF(200 mg/kg).
- Group 4: MTF(300 mg/kg).
- Group 5: MTF(400 mg/kg).

Twenty-four hours post final treatment; the animals were sacrificed by cervical dislocation. Assays were carried out and histological assessment of the uterus sections was performed following standard procedure.

Second set

Experimental design Twenty-eight rats were equally grouped into: A (Control: distilled water), B (metformin (MTF): 300 mg/kg), C (estradiol benzoate (EB): (2 mg/kg) and (EB + MTF). A day post final exposure, the rats were sacrificed by cervical dislocation. Assays were carried out and histochemical study of the uterus sections was performed following standard procedure.

Histopathology

The uteruses were harvested and processed for histopathology using standard laboratory procedures (Luna 1968; Masson 1929). The harvested uteruses were dehydrated in an ascending grade of (ethanol), cleared in xylene and embedded in paraffin wax. Serial sections of 5–6 microns thick were obtained using a rotator microtome and stained with hematoxylin and eosin for histological assessment and Masson's trichrome for histochemical study. The histological pictures were taken using an Olympus microscope, Japan.

Isolation of rat uterine mitochondria

This was performed following the method of Costa et al. (2006). The uteruses were excised, cleaned of blood and fat, minced and homogenized on ice in 8 mL of isolation buffer consisting of 70 mM of sucrose, 1 mM of EDTA, and 5 mM of HEPES (pH 7.2). The homogenate was centrifuged in an MSE refrigerated centrifuge (Progen Scientific, UK) for 7 min at 1000 g and temperature of 4 °C. The supernatant obtained was separated and centrifuged for 7 min at 12,000g and temperature of 4 °C. The pellet was suspended in isolation buffer (containing no EDTA) and kept on ice (4 °C). All experiments with isolated mitochondria were performed within 4 h of the preparation.

Mitochondrial protein

Procedure of Lowry et al. (Lowry et al. 1951) was employed to determine the mitochondrial protein using bovine serum albumin as standard.

Mitochondrial F_0F_1 ATPase activity

The mitochondrial F_0F_1 ATPase activity was determined following the method of Olorunsogo and Malomo (Olorunsogo and Malomo 1985). Each reaction mixture contained 65 mM Tris-HCl buffer pH 7.4, 0.5 mM KCl, 1 mM ATP and 25 mM sucrose. The reaction mixture was made up to a total volume of 2 mL with distilled water. Mitochondrial suspension was added to the reaction medium in a shaker water bath and allowed to proceed for 30 min at 27 °C. Aliquot amount (1 mL) of 10 percent sodium dodecyl sulphate (SDS) solution was

added to stop the reaction at 30 s intervals. 2, 4 Dinitrophenol (2, 4 DNP) was used as a standard uncoupling agent. Aliquot of each solution (300 µL) was dispensed into fresh test tubes, followed by the addition of 300 µL of distilled water. To each of the test tube, 1 mL of 5% ammonium molybdate and 1 mL of 9% freshly prepared solution of ascorbic acid were added. The tube was well mixed and allowed to stand for 20 min. The absorbance was read at 680 nm. Water blank was used to set the spectrophotometer at zero.

Cytochrome c release

This was determined following the method of Appaix et al. (Appaix et al. 2000). Mitochondria were preincubated in the presence of 0.8 µM rotenone in a medium containing 210 mM mannitol, 70 mM sucrose and 5 mM HEPES–KOH (pH 7.4) for 30 min at 27 °C in the presence of different concentrations of metformin, using 24 mM calcium as the standard (Triggering Agent). After the incubation, the mixture was centrifuged at 15,000 rpm for 10 min. The optical density of the supernatant was measured at 414 nm which is the solet (γ) peak for cytochrome c.

Determination of caspases 9 & 3 levels

The rat uterus was excised, weighed, rinsed with phosphate buffered saline thoroughly and processed according to the manufacturer’s protocol for ELISA (Elabscience biotechnology Ltd. China).

Oxidative indices

Malondialdehyde (MDA) level, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities were detected using ELISA technique.

Determination of estradiol and progesterone levels

The levels of estradiol (E2) and progesterone (PGR) were determined using ELISA kits obtained from abcam, Shanghai, China.

Statistical analysis

Data were expressed as mean ± SD and analyzed using ANOVA (at α 0.05) followed by Tukey’s post test.

Results

Figure 1a shows non significant induction of mPT by intact mitochondria (NTA), significant induction upon calcium (TA) addition and evident reversal by spermine

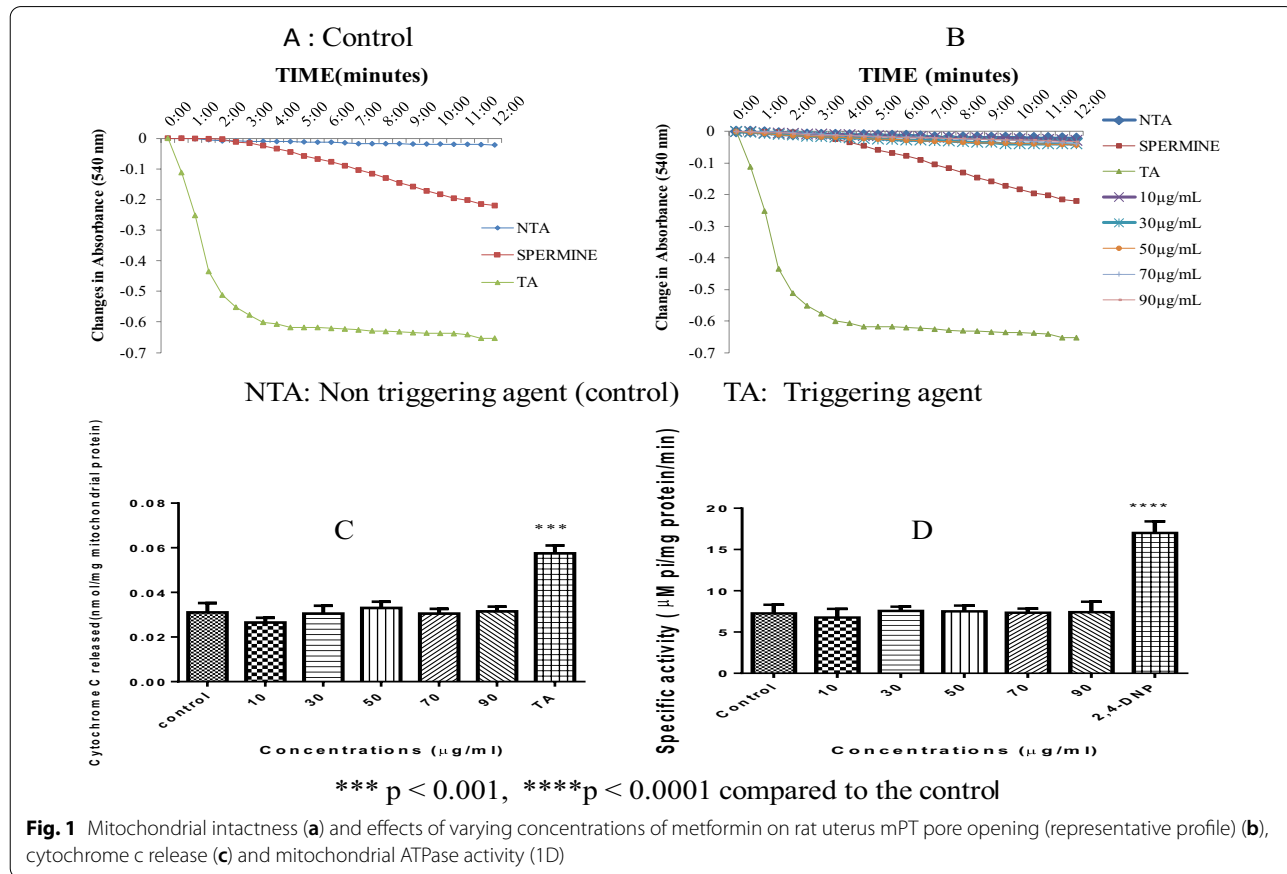


Fig. 1 Mitochondrial intactness (a) and effects of varying concentrations of metformin on rat uterus mPT pore opening (representative profile) (b), cytochrome c release (c) and mitochondrial ATPase activity (1D)

(standard inhibitor), over the experimental period. Metformin (0-90 µg/mL) had no remarkable effect on mPT, cytochrome c release and mitochondrial ATPase activity when compared with the control as shown in Fig. 1b–d. Also, oral administration of metformin at lower doses (100 and 200 mg/kg) had no notable influence ($P < 0.05$). However, at 300 and 400 mg/kg, induction of mPT pore opening and mATPase activity were significant as revealed in Fig. 2a and b. The 300 and 400 mg/kg induced pore opening by 8.8 and 10.4 folds, respectively, while mATPase activity was elevated by 60 and 80%, respectively ($P < 0.05$). Caspases 9 and 3 activities as depicted in Fig. 2c and d were significantly increased at 300 and 400 mg/kg while there was no significant effect at lower doses ($P < 0.05$). Influence of metformin on the antioxidant status in normal and EB-treated rats is shown in Fig. 3a–c. There was elevated serum MDA level in the EB-treated rats ($P < 0.05$). However, co-treatment with metformin caused significant reduction in the MDA level when compared with the EB-treated group ($P < 0.01$) as illustrated in Fig. 3a. The SOD and GSH-Px activities were significantly lowered in the EB-treated group ($P < 0.05$). Nevertheless, metformin co-treatment significantly increased their

activities as opposed to the EB-treated group. This is depicted in Fig. 3b and c. As shown in Fig. 4a and b, the EB-treated group showed significant increase in estradiol and progesterone levels in comparison to control. Nonetheless, metformin co-treatment caused a remarkable reversal in their levels in contrast to the EB-treated group.

The histological assessment of uterine sections using hematoxylin and eosin (H&E) staining revealed normal histological structure in the control and all the metformin treatment groups. No visible lesion was observed in any of the groups as depicted in Fig. 5. Furthermore, histochemical analysis carried out on the uterine sections using Masson’s trichome stain revealed a high deposition of collagen fiber accompanied with severe uterine hyperplasia in the EB-treated category. This was evidently reversed by metformin co-treatment as shown in Fig. 6a. The histochemical analysis was quantitatively evaluated using histomorphometry as illustrated in Fig. 6b. There was remarkable increase in the fibroblast cell count/µm² in the EB-treated category which was mitigated upon co-treatment with metformin.

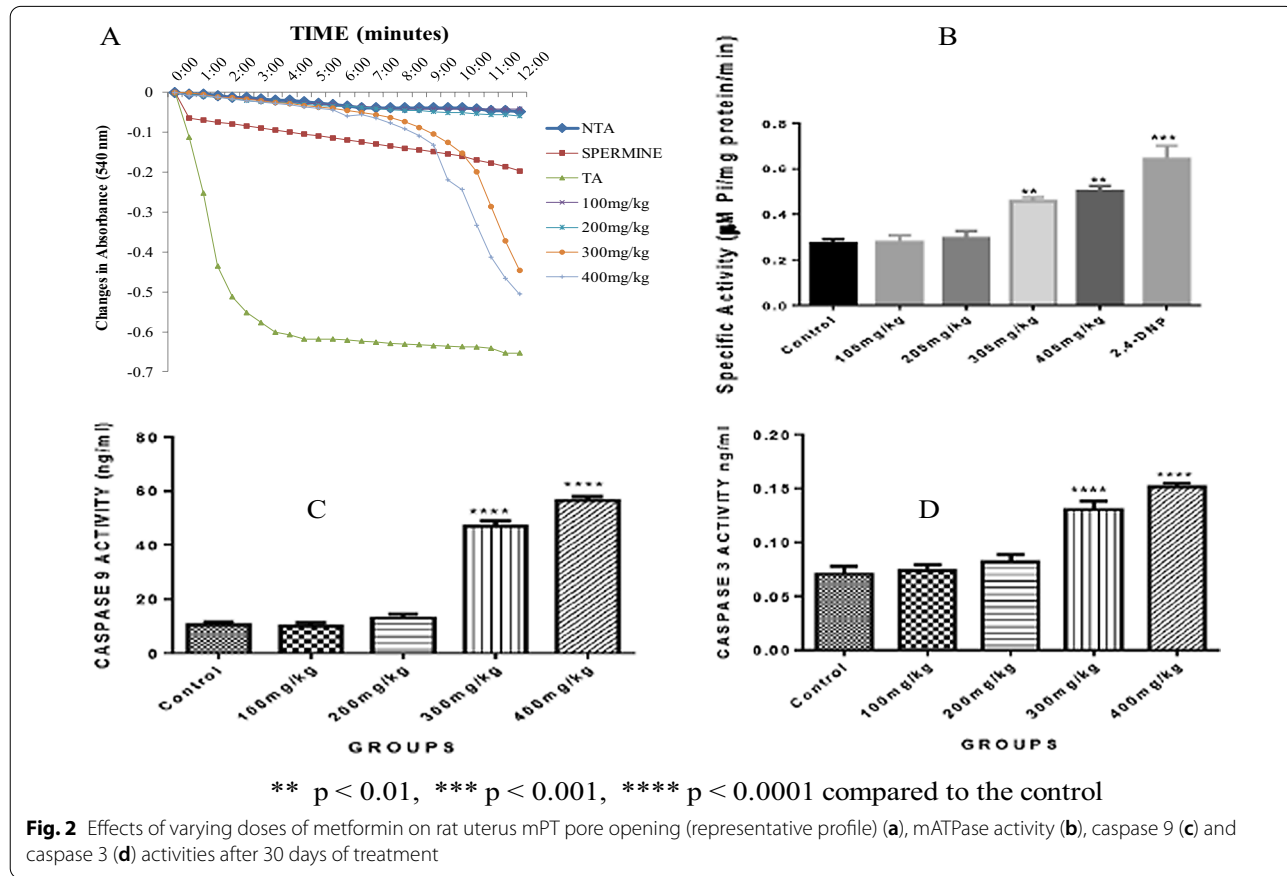
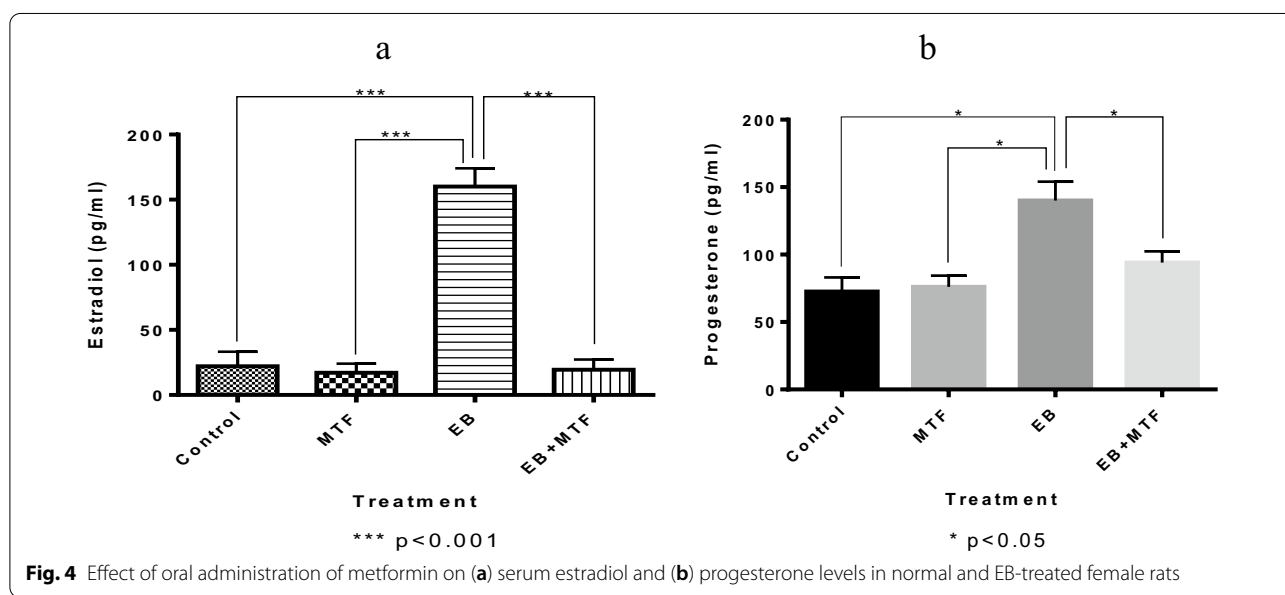
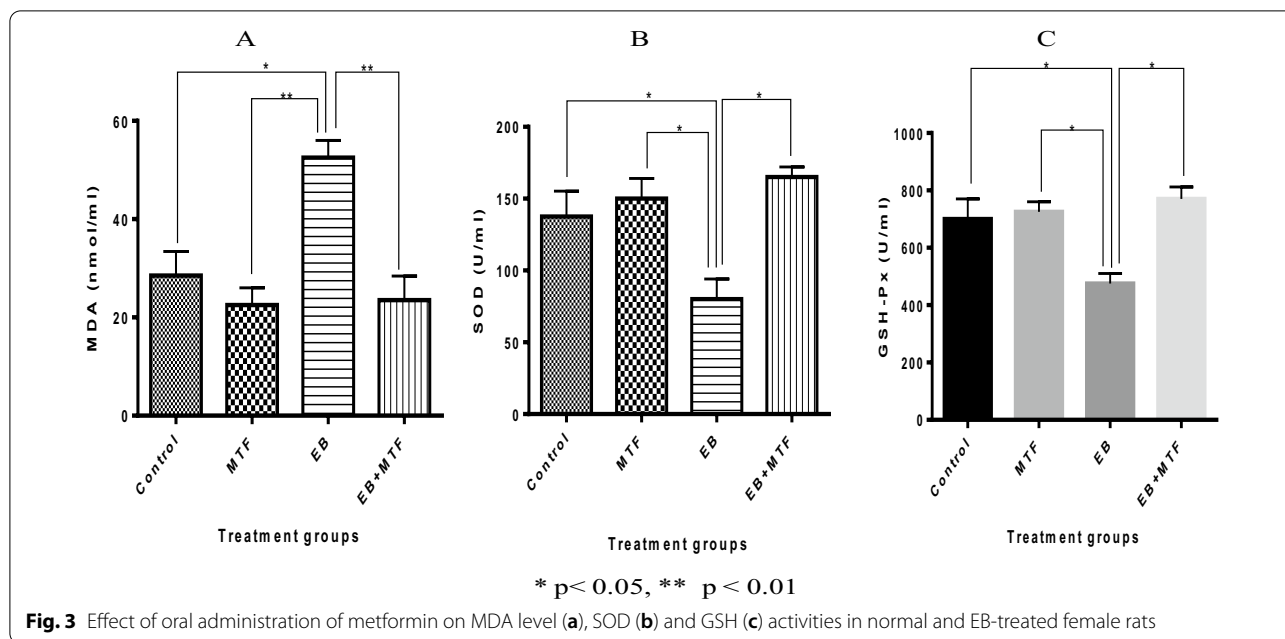


Fig. 2 Effects of varying doses of metformin on rat uterus mPT pore opening (representative profile) (a), mATPase activity (b), caspase 9 (c) and caspase 3 (d) activities after 30 days of treatment



Discussion

Pharmacological relevance of mPT pore to the development of cytotoxic drug for diseased conditions involving tumors and cancers has been established (Giorgio et al. 2018; Bernardi et al. 2015). The results on the isolated mitochondria showed that they were intact and not compromised. Varying concentrations of metformin used in this study had no significant effect on mPT pore, cytochrome c release and mATPase activity. This probably suggests that metformin at the concentration range

used in the in vitro study may not be able to interrelate with the mitochondrial components to effect pore opening which could lead to subsequent cytochrome c release and bioenergetic ATP hydrolysis (Porporato et al. 2018).

However, when it was orally administered, the opening of the pore at higher doses suggests its increase in bio-availability at the target site and interaction with some pore components to elicit its opening. Recently, the mitochondrial adenosine triphosphate (ATP) synthase was suggested to be the mPT pore (Bernardi and Lisa 2015;

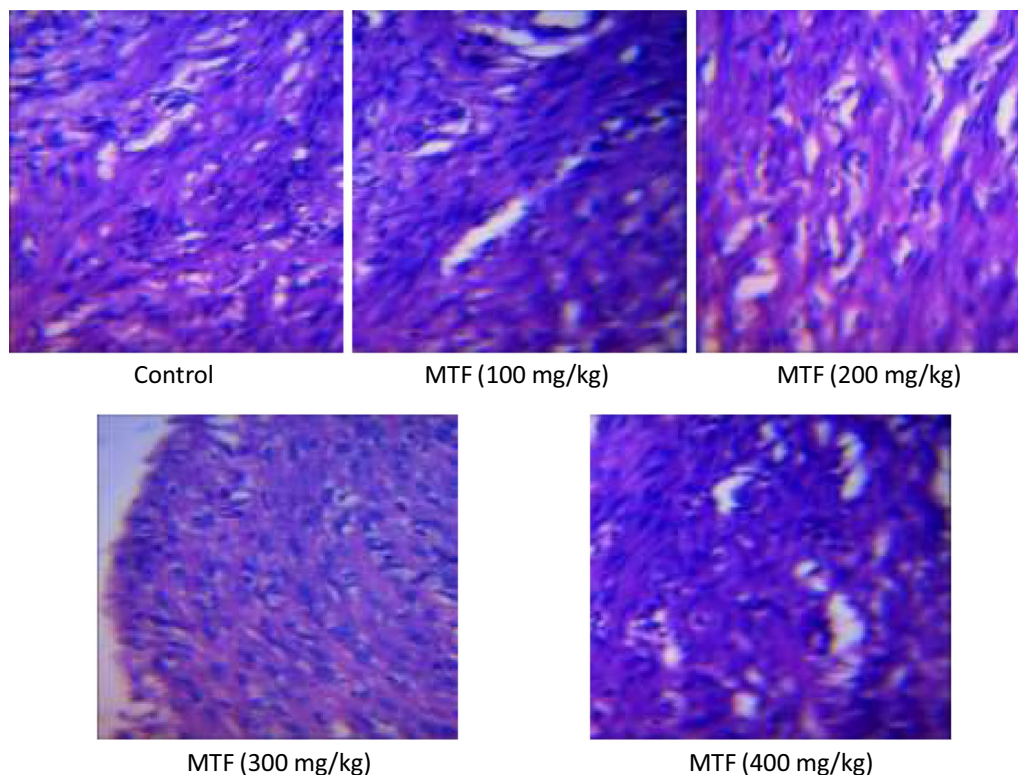


Fig. 5 Histological study on the effect of oral administration of metformin on the rat uterine sections using H&E staining (Mag. X 400). Control: The cellular architecture of myometrium was normal. MTF (100 mg/kg): There was no significant lesion. MTF (200 mg/kg): There was no significant lesion. MTF (300 mg/kg): There was no visible lesion. MTF (400 mg/kg): There was no visible lesion

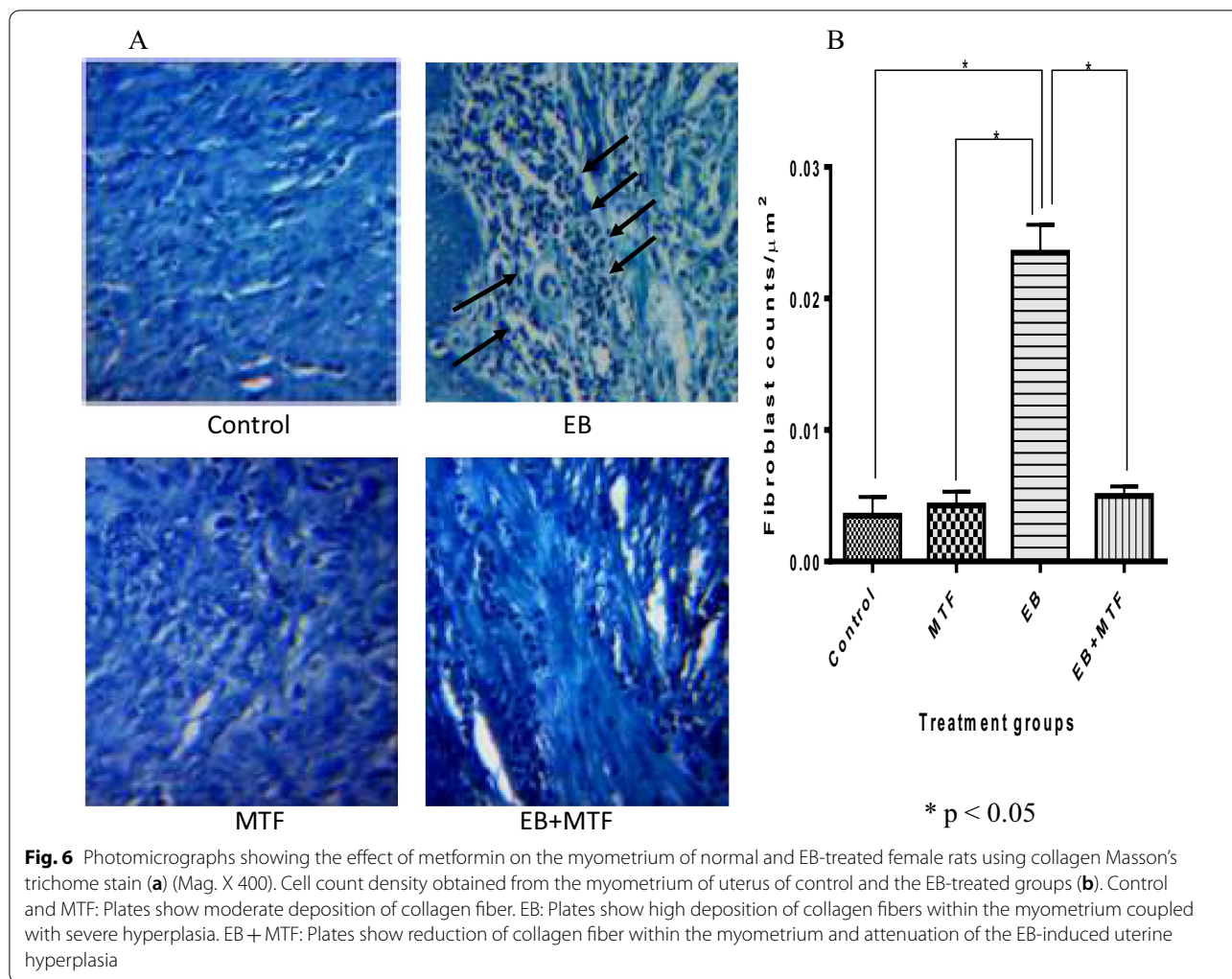
Seidlmayer et al. 2012). The enhanced mitochondrial ATPase activity at higher doses could be linked to pore opening at higher doses of administration. Meanwhile, studies have shown that metformin is effective at high concentrations as an antitumor agent (Gao et al. 2016).

Caspases are known key players in apoptosis (Sadowski-Debbing et al. 2002; Pisani et al. 2020). On assessment of caspases 9 and 3 levels, the significant increase in levels of the enzymes recorded at 300 and 400 mg/kg suggests that metformin actually induces cell death via mitochondria-mediated pathway. In addition, its ability to induce mitochondrial-mediated apoptosis occurs at higher doses of metformin administration. These could be correlated with the findings of He and Wondisford (He and Wondisford 2015), who reported the effectiveness of metformin at higher doses against cancer cells. This is in agreement with our findings which show opening of the pore by metformin at higher doses. Based on literature search (Jing et al. 2019; Diniz Vilela et al. 2016) and the findings in this study (which showed the effectiveness of metformin to induce rat uterus mitochondrial-mediated apoptosis at higher doses), the effect of metformin at 300 mg/kg was investigated on estradiol benzoate

(EB)-induced uterine defect and other associated pathophysiological disorder/defect.

It has been reported that unopposed high estrogen treatment can cause increase in reactive oxygen species (ROS) resulting to DNA, protein and lipid damage (Gupta et al. 2015; Pescatori et al. 2021). The elevated MDA level in the EB-treated rats could be attributed to EB-induced oxidative stress (Olowofolahan et al. 2020; Pejic et al. 2009). This was reversed by metformin co-administration. Also, the EB-induced decrease in SOD and GSH-Px activities which could be as a result of accumulation of ROS (Peji et al. 2003; You et al. 2017) was significantly increased with metformin co-treatment. This probably suggests the antioxidant potential of metformin in EB-induced pathophysiological disorder in rats. This result is similar to a recent finding where methyl palmitate was shown to reverse estradiol benzoate-induced endometrial hyperplasia with concomitant increase in the antioxidant status of the cell (Olowofolahan et al. 2020).

Studies have shown that elevation of ROS as a result of high estrogen exposure could promote genetic instability, tumorigenesis, development of endometrial hyperplasia



and endometrial cancer (Pescatori et al. 2021; Lian et al. 2001; Moloney and Cotter 2018). The prolonged EB inducement could have resulted to elevated estrogen level. Nevertheless, it was remarkably mitigated by metformin. The increase in progesterone level could probably be linked to elevated luteinizing hormone. However, administration of metformin ameliorated the EB-induced rise in estrogen and progesterone levels. This probably suggests the potential of metformin to protect against estrogen-dependent uterine pathophysiological disorder (Deng et al. 2020). This finding is consistent with the findings of La Marca (Marca et al. 1999) and Nestler and Jakubowicz (Nestler and Jakubowicz 1997), where metformin treatment decreased serum estradiol and progesterone levels in polycystic ovary syndrome (PCOS) and also Pimentel et al. (2021), where metformin treatment lowered estradiol levels in Canadian Cancer Trials Group (CCTG) MA.32; a phase III trial of nondiabetic breast cancer subjects.

The histopathology results on the uterine sections of female rats exposed to varying doses of metformin showed no visible lesion in all the treatment groups in comparison to the control. This probably suggests that metformin administration does not cause uterine cellular damage or distortion.

The histopathological abnormalities found in the uterine sections of EB-treated rats (using Masson's trichome staining) suggests that EB administration resulted in an increase in estrogen level which lead to ROS generation thereby causing damage to the uterus; thus, promoting uterine tumorigenesis or hyperplasia. These histopathological changes are compatible with the discoveries of Yang et al. (Yang et al. 2015) and Refaie and El-Hussieny (Refaie and El-Hussieny 2017). However, the group that was co-treated with metformin showed improvement on the pathological changes. This is similar to the discoveries of Elia et al. (Elia et al. 2009) where metformin was shown to restore uterine cellular architecture and

function in hyperandrogenized BALB/c mice and Tseng (Tseng 2019) where metformin administration is associated with a lower risk of uterine leiomyoma. This study suggests the ability of metformin to reverse the EB-induced uterine tumor/hyperplasia.

Conclusion

The data generated in this study suggest that metformin induces apoptosis via uterus mPT pore opening at high doses and also protects against estradiol benzoate-induced uterine defect (hyperplasia) and other associated pathophysiological disorder in female rats.

Abbreviations

NTA: Non triggering agent; TA: Triggering agent; mPT: Mitochondrial permeability transition; mATPase: Mitochondrial ATPase activity; MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase; E2: Estradiol; PCOS: Polycystic ovary syndrome.

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Authors' contributions

Conceptualization: AOO. Material preparation, data collection and analysis: AOO, OMP, HMD. Supervision: OOO. Writing of the manuscript: AOO. All authors read and approved the final manuscript.

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Availability of data and material

On request.

Declarations

Ethics approval and consent to participate

The study was approved by animal use and care research ethics committee, university of Ibadan, with approval number UI-ACUREC/190065 and carried out following ethical standards (1964 Declaration of Helsinki).

Consent for publication

Not applicable.

Competing interests

There was no conflict of interest among the authors.

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