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Conference Proceedign

CARDIOTOXIC POTENCIALOF PIOGLITAZONE/AZOLE ANTIFUNGALS DRUGS INTERACTION IN RATES

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ABSTRACT

Objective: Drug-drug interactions could augment or develop cardiotoxicities. Drug induced cardiotoxicityconsidered one of the major concerns that lead to serious and life threatening clinical situations The current study was carried out to investigate and compare the innate cardiotoxic potential of pioglitazone, an antidiabetic drug, and selected azole antifungals e.g. fluconazole / itraconazole in non-diabetic healthy rats either administrated alone or concomitantly.

Method: Adult male *Sprague dawely* ratsrandomly and equally assigned into six groups 10 rats each. The first was kept as control and received the vehicle (0.25% tween 80/distilled water), three groups were received single drug treatment;pioglitazone at (10 mg/kg), fluconazole at (20 mg/kg) and Itraconazole at (18 mg/kg). While animals of fifth group-received co-treatments of pioglitazone (10 mg/kg) and fluconazole (20 mg/kg) and animals of sixth group received combination of pioglitazone (10 mg/kg) and itraconazole (18 mg/kg).all drugs were administrated orally for 28 consecutive days.

Results: single drug treatments induced morphological electrocardiographical (ECG) changes,elevated serumCreatine kinase-MB (CK-MB),elevated serum troponin-T (cTnT) except fluconazole, elevated tissue GSH, diminished catalase activity (CAT) and only single fluconazole and pioglitazone showed elevation in cardiacmalondialdehyde (MDA).Single azoles showed significant increase in apoptotic marker caspase-3, while combined treatments showed morphological ECG changes, prolonged QTc interval, elevation of CK-MB and cTnT, elevated GSH and diminished CAT activity.

Conclusion: Pioglitazone and azole antifungals have the potential to develop cardiomyopathical and electrophysiological toxicity in healthy rats either single or combined.

Keywords: Cardiotoxcity, Drug Interaction, Pioglitazone, Fluconazole, Itraconazole, ECG.

INTRODUCTION:

Pioglitazone (PIO) is one of the most widely used and successful antidiabetic drug belonging to thiazolidinediones class of medications. A peroxisome proliferator activated receptor gamma (PPAR-γ) agonist serving as insulin sensitizer improving insulin resistance(Jiang et al., 2012; Murphy & Holder, 2000). PIO possess a lot of beneficial actions i.e. amelioration of glucose homeostasis, increase HDL cholesterol concentration, slowing progression of atherosclerosis(Zinn et al., 2008).However, pioglitazone cardiovascular risks remain controversial and its safety became a matter of concern especially after withdrawal of other class members e.g. troglitazone and rosiglitazone, on regard of their hepatotoxicity and cardiotoxicity respectively (Consoli and fFormoso, 2013).

Moreover, pioglitazone was found to developcongestive heart failure (Juurlink et al., 2009; Zinn et al., 2008). PIO provoked cardiac damage in isoproterenol induced heart failure in rat model and induced mice ventricular hypertrophy in acute toxicity experiments(Biswas et al., 2012; Chinnam et al., 2012) and aggravated doxorubicin induced Cardiomyopathy. (Saraogi et al., 2011) .

Cardiotoxcity could result as an unintended consequence of drug therapy and considered as one of medication-related adverse events and a major toxic effect that in some occasions leads to drug withdrawal from the market. Drug-drug interaction (DDI) could augment or aggravate adverse drug actions. Consequently, researches about pharmacodynamics and pharmacokinetics have been widely performed in an attempt to create a pre- knowledge on possible DDIsthat could be in some circumstances life threatening i.e. torsade de pointe (TdP) ventricular arrhythmia, myocardial infarction.

A drug induced or drug aggravated cardiac arrhythmia could be a dreadful side effect related to administration of some drugs. The most common form of proarrhythmia is drug induced long QT syndrome(Haverkamp et al., 2000; Yap a Camm, 2003). Acquired QT prolongation syndrome associated with drug administration appears to be concentration dependent, so variables that interfere with the pharmacokinetics of a drug may further increase the risk of TdP which is a potentially fatal polymorphic ventricular tachyarrhythmia.(Sung et al., 2012).

The azoles are a widely used antifungal agents used for treatment of fungal infections effectively. They inhibit fungal cytochrome P450 (CYP)–dependent enzyme lanosterol 14-demethylase, which blocks the formation of ergosterol an essential component of fungal lipid membranes (Dodds-Ashley, 2010).

Itraconazole (ITZ) and fluconazole (FLU) are broad spectrum antifungal triazole, ITZ is used to treat invasive fungal infections like onychomycosis, aspergillosis, blastmycosis and histoplasmosis (Cleary et al., 2013; Okuyan and Altin, 2013), FLU is used in the treatment and prevention of superficial and systemic fungal infections (Yu et al., 2005)

Azole antifungals are known with their life threatening QT prolongation side effect which could be direct or indirect through potentiating other QT prolonging drug effect by interfering with CYP450 metabolic enzyme system(Goldstein et al., 2006)

Patients suffering metabolic disorder like diabetes, usually complain fungal infections and other opportunistic microbial infections due to their compromised immune system (Casqueiro et al., 2012).

Risk of possible DDI between the hypoglycemic drug in use and the prescribed antimicrobial are considerably high. Consequently, the objective of the current study was to investigate and compare the innate cardiotoxicity potential and hazards of single administration of pioglitazone, fluconazole and itraconazole and their combined administration in healthy rats.

MATERIALS AND METHODS

Animals

Adult Male Sprague dawely rats (200–220 g, n = 60) obtained from National Organization for Drug Control and Research where housed on Standard rats' diet, water was supplied *ad libitum*, temperature (25 ± 2ºC), humidity (60 ± 10%), and alternating 12 h light-dark cycle. All procedures of the current experiment have been approved by research ethics committee for experimental and clinical studies at Faculty of pharmacy, Cairo University, Egypt (permit number: PT 1804).

Drugs

Pioglitazone (99 % purity) was obtained from Unipharma Pharmaceutical Industries Company (Egypt) at a dose of 10 mg/kg according to previous studies of Janadri et al., 2009 and Saraogi et al., 2011. fluconazole (98%) was administered at 20 mg/kg (Fisher et al., 1989; Ramzan and Chan, 1993) was obtained from AL Rowad Pharmaceutical Industries Company (Egypt).itraconazole (98%)was obtained as kind gift from ADWIA Pharmaceutical Industries Company and given at a dose of 18 mg/kg (Janadri et al., 2009).All drugs suspended in 0.25% tween 80

Experimental design

After one week of acclimatization, rats were divided into 6 groups (10 rats each): The 1st group was administered 0.25% tween 80 served as control. The 2nd,3rd,4th groups were given pioglitazone at a dose of 10mg/kg, fluconazole at 20 mg/kg and itraconazole at 18 mg/kg body weight, respectively.5th and 6th group received combination of pioglitazone and fluconazole or itraconazole respectively. All treatments suspended in tween 80 and administered orally. The animals subjected to their respective treatments for 28 consecutive days.

Sample collection

At the end of experiment, rats were anesthetized using thiopental 50 mg/kg IP, for ECG examinations. Blood samples were drawn from the retro-orbital vein(Cocchetto & Bjornsson, 1983), centrifuged at 3000 rpm for 15 min, and the obtained sera was stored at -20 ˚C till used in detection of cardiac biomarkers. Following sacrificing by decapitation, hearts were rapidly dissected, washed in ice-cold saline, blotted dry, weighed and then divided into two longitudinal halves, the first half (left) of hearts were immersed in 10% formalin and kept at room temperature for histopathological examination. The remaining heart tissue (right side) was stored at -80 ˚C for subsequent homogenization.

The frozen heart samples homogenized in cold 10% (w/v) 0.1M Tris-HCl at PH 7.4 using Teflon coated homogenizer submerged in ice. Heart homogenates were centrifuged at 3000 rpm for 15 minutes at 4 ℃ using cooling centrifuge (Sigma 3K30 Germany) and the clear supernatant was kept and used later to determine the alteration in antioxidant enzymes.

Electrocardiographical examinations.

After anesthesia, the rats were fixed on a pad on their back, the skin of the abdomen was shaved and was wiped with alcohol, and a conducting gel was added on the electrodes and adhered on the skin. The

electrodes inserted in a lead II position. ECG recordings made usingMultimedia biofeedback biograph infinity SA7900PD version 1.1.2 apparatus (Thought Technology Ltd., Canada). Changes in heart rate (HR), R-R interval, R-amplitude and QT interval were considered.QT interval was corrected using Fridericia formula***(Fridericia, 1921)***:

$$QTc = \frac{\mathrm{QT}}{\sqrt[3]{RR}}$$

Biochemical analysis

Serum cardiac biomarkers; Creatine kinase (CK-MB) activities and cardiac troponin (cTnT) concentration, were determined using commercially available diagnostic kits (BIOLABO SA Maizy, France) and (Cloud-Clone Corp. ,USA) respectively.

Oxidative stress biomarkers; reduced glutathione (GSH)was determined by method of Ellman, 1959, using 5,5'-dithiobis-(2-nitrobenzoic acid) as substrate, the lipid peroxidation marker malondialdehyde (MDA) was determined by method of Uchiyama and Mihara, 1978 using thiobarbituric acid reactive substances as substrates ,catalyse activity was determined using the method of Aebi, 1974 using H_2O_2 as substrate.

Histopathological and immune-histopathological examinations

In 10% neutral-buffered formalin, fixed specimens from heart left side were cleaned in xylene, embedded in paraffin then sectioned (4mm). Selected sections (5 per animal) were stained with hematoxylin and eosin and examined by light microscope to determine histopathological changes. For immune-histopathology, 5 microns thick paraffin embedded tissue sections were prepared. Immunohistochemical staining (caspase-3) was conducted according to the manufacturer's protocols.

Statistical analyses

The data were expressed as mean ± SEM. Statistical analysis included the calculation of the mean values, standard deviation and standard error using IBM SPSS version 20.0 statistical package (SPSS Inc, Chicago, IL) and graphs were plotted using Graphpad prism 8 software. Data were analyzed by one-way analysis of variance (ANOVA) followed by tukey's test as a *Post hoc* for multiple comparisons. The differences were considered significant at $p \leq 0.05$.

Non-parametric data were analyzed by kruskal Wallis test followed by a *post hoc* Dunn test for caspase-3 Immunohistochemical examination.

RESULTS

Relative heart weight

Statistical analysis revealed that both single and combined treatment regimens significantly decreased the relative heart weight without significant changes in the absolute heart weight indicting increase in body weight gain (Fig 1)

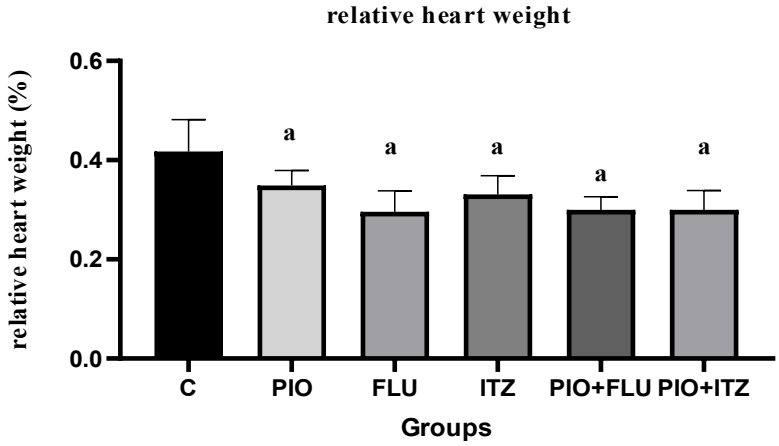

Figure 1: Relative heart weight of control (C) and drug treated groups, (mean ± SEM). Values carrying different letters considered significant (P < 0.05).

Cardiac antioxidant status

The statistical analysis showed that single fluconazole and pioglitazone increased cardiac MDA content significantly by 69% and 32% (P≤0.001) and (P≤0.05) respectively, compared to control groups (Fig.2). However, single itraconazole and combined treatments could restore cardiac MDA level to normal without significant influence (Fig.2).

Data showed significant tremendous elevations in tissue glutathione levels by 8.5, 8 and 5 folds (all P≤0.001) for pioglitazone, fluconazole and itraconazole, respectively than control group (Fig.3). While combined treatments induced pronounced increases in cardiac GSH level by 5.6 and 7.2 folds (both p≤0.001) compared to control (Fig.3).

All the utilized drugs regimens induced significant exhaustion of cardiac catalase activity (Fig.4). Single treatments decreased CAT by 52% (P≤0.001), 28% (P≤0.05) and 30% (P≤0.01) for pioglitazone, fluconazole and itraconazole, respectively, compared to control group. Combined pioglitazone and fluconazole decreased cardiac CAT activity by 27% (p≤0.05) while pioglitazone and itraconazole by 42% (p≤0.001) than control group.

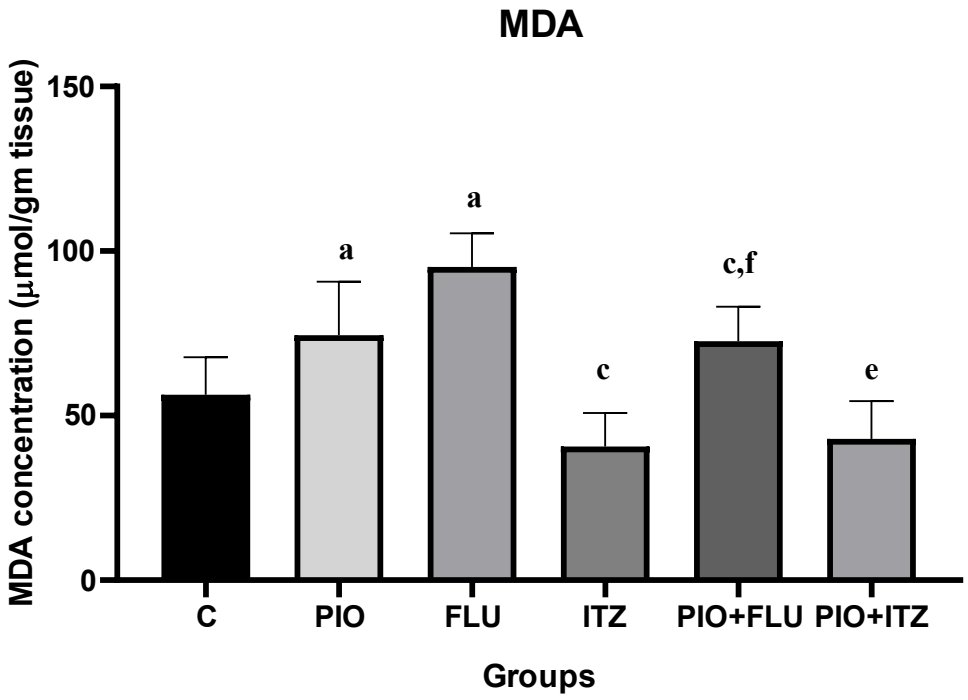

Figure 2: Cardiac malondialdehyde (MDA) content in control (C) and drug treated groups, (mean ± SEM).Values carrying different letters considered significant (P < 0.05).

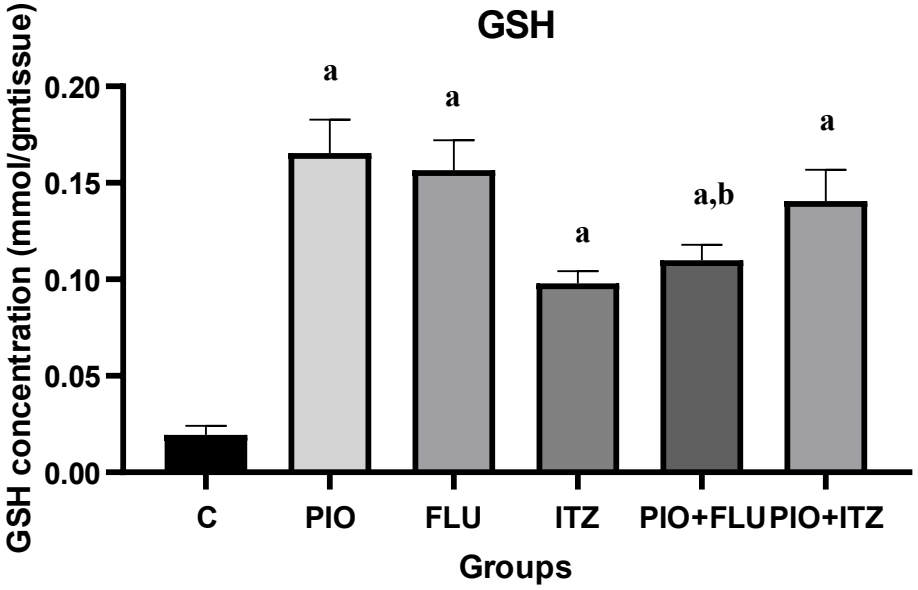

Figure 3: Cardiac reduced glutathione (GSH) content in control (C) and drug treated groups, (mean ± SEM). Values carrying different letters considered significant (P < 0.05).

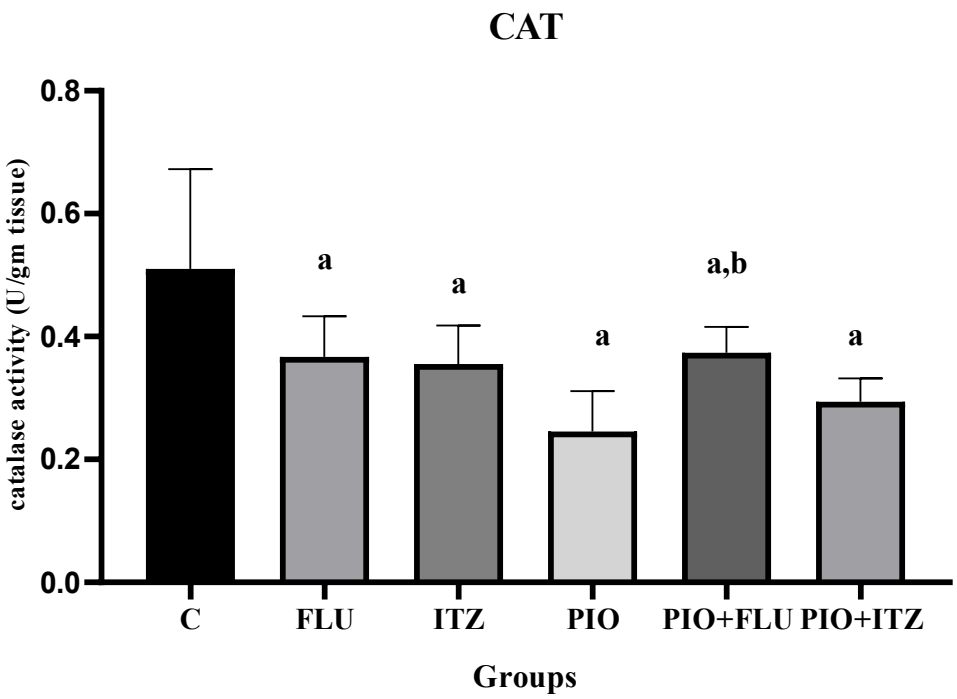

Figure 4: Cardiac catalase (CAT) activity in control (C) and drug treated groups, (mean ± SEM). Values carrying different letters are considered significant ($P < 0.05$).

Cardiac biomarkers

The statistical analysis showed that pioglitazone and azole antifungals treatment either alone or concomitantly administered caused significant increases in the cardiac biomarker CK-MB level in sera of all treated groups (Fig.5). Additionally all the utilized drug regimens resulted in significant increases in serum cTnT level, except single treated fluconazole rats, which had no influence on this cardiac biomarker (Fig.6)
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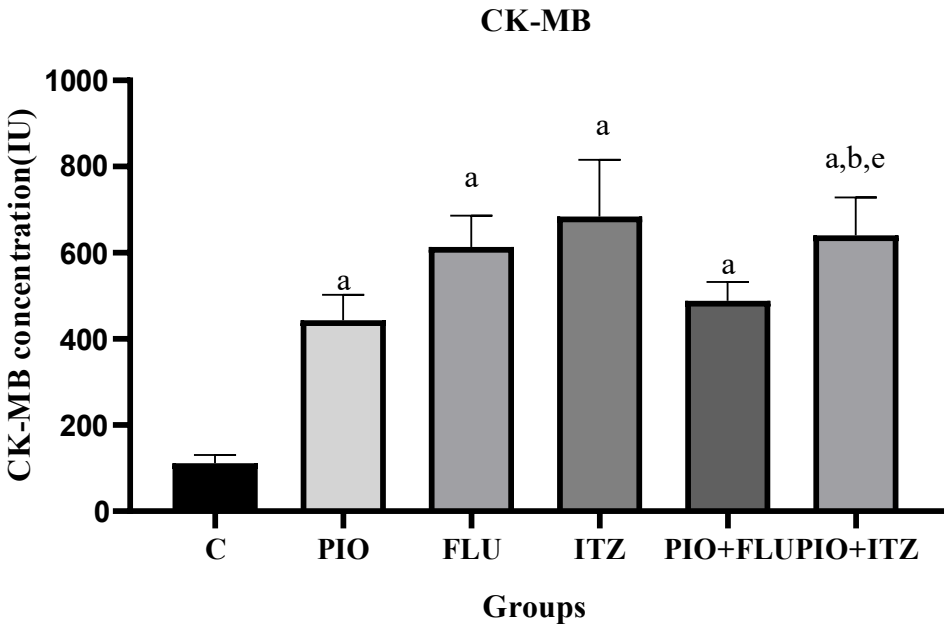


Figure 5: Cardiac Creatine kinase-MB (CK-MB) concentration in control (C) and drug treated groups, (mean ± SEM). Values carrying different letters considered significant (P < 0.05).

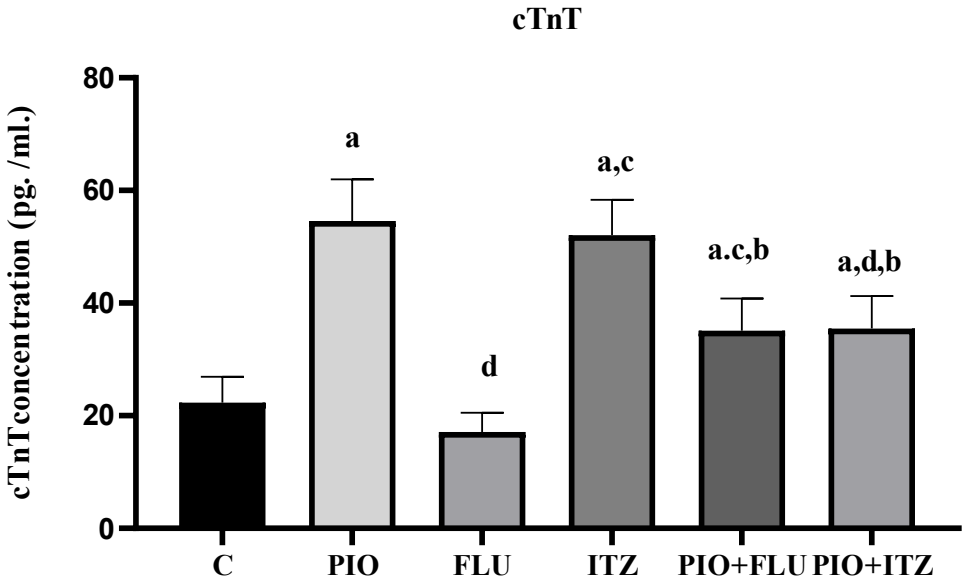


Figure 6: Cardiac troponin-T (cTnT) concentration in control (C) and drug treated groups, (mean ± SEM). Values carrying different letters considered significant (P < 0.05).

Electrocardiographic (ECG) examination

Statistical analysis for ECG calculated parameters showed thatneither HR nor R-R interval had a significant difference among all treated groups and control group (Fig. 7a, c). However, statistical analysis revealed a significant increase in the R-amplitude among pioglitazone and fluconazole co-treated rats as compared to

control rats (Fig. 7b). Additionally, calculated QTc interval, in accordance to Fridericia's formula, showed significant prolongation among combined treated rats as compared to control rats (Fig.7d). Where combined pioglitazone and fluconazole treatment induced QTc prolongation by 22 % (P≤0.05) while combined pioglitazone and itraconazole by 32% as compared to control group (Fig.7d).

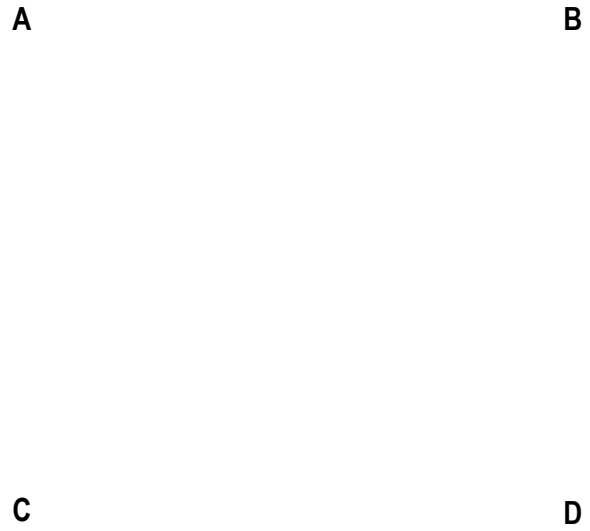


Figure 7: Calculated electrocardiographic patterns :(A) Heart rate (HR), (B) R-amplitude (R-amp.), (C) R-R interval, corrected QT interval (QTc), (mean ± SEM). Values carrying different letters considered significant (P < 0.05).

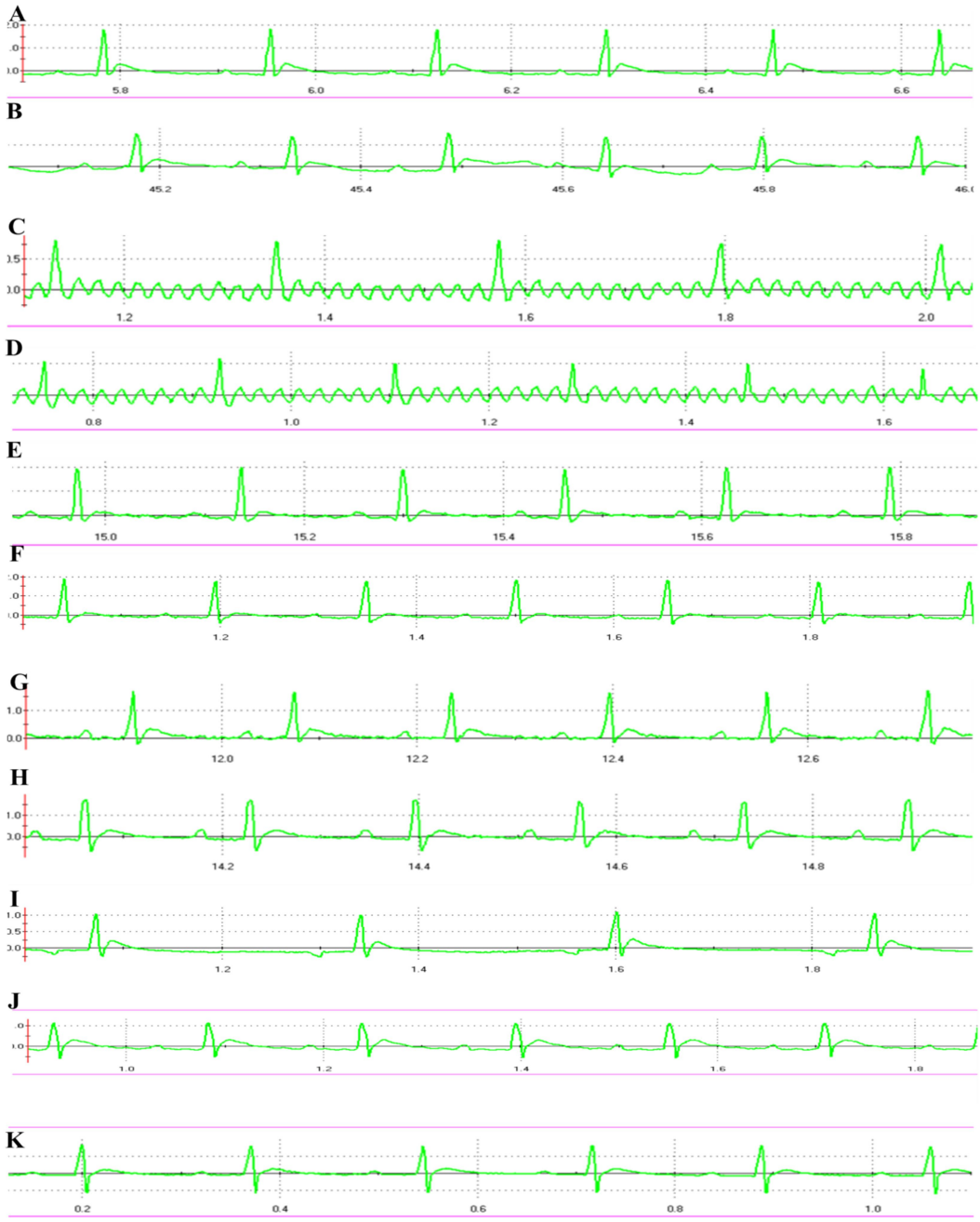


Figure 8: Electrocardiographs of A: Control group showing normal morphological structure of PQRST with normal amplitudes, B: Pioglitazone treated rats showed elevated ST segments, C: Fluconazole treated rats suffered atrial flutter, D: Itraconazole treated rats showed atrial flutter (a) E: Itraconazole treated rats showed flattened T-wave (b). F: Pioglitazone and fluconazole treated rats showed flattened t-wave (a), G: elevated ST segment (b) and H: prolonged QTc, I: Pioglitazone and itraconazole showed Nodal rhythm (a), J: elevated ST segment (b) and K: prolonged QTc.

Histopathological Examination

Cardiac tissue stained by H&E showed well-organized normal histological structure of cardiomyocytes with intact cellular details and striation (Fig 8a). Cardiac tissue of pioglitazone treated rats showed alternated areas with degenerated or necrotic cardiomyocytes and apparent intact cells, Congested cardiac blood vessels, intramuscular hemorrhages and perivascular edema with focal hyalinization and vacuolation of blood vessels wall with occasional perivascular inflammatory cells infiltrates (Fig. 8b). Heart tissue of fluconazole treated rats showed degenerative changes with pyknotic nuclei and fragmentation of myofibrils accompanied with intramuscular hemorrhages as well as congested blood vessels (Fig.8c). Cardiac tissue examination of itraconazole treated rats revealed almost the same records as fluconazole in addition to higher records of dispersed necrotic muscle fibers (Fig.8d).

Moreover, Pioglitazone and fluconazole co-treated group revealed sever congestion of cardiac blood vessels and hemorrhagic patches, moderate degenerative changes of some cardiomyocytes with occasional hyalinization of few muscle fibers (Fig.8e). Meanwhile, pioglitazone and itraconazole co-treated group showed many congested intramuscular blood vessels, scattered intramuscular hemorrhages, focal hyalinization and vacuolation of blood vessels wall, moderate records of degenerative changes in surrounding cardiomyocytes and occasional focal marked swelling of cardiomyocytes (Fig.8f).

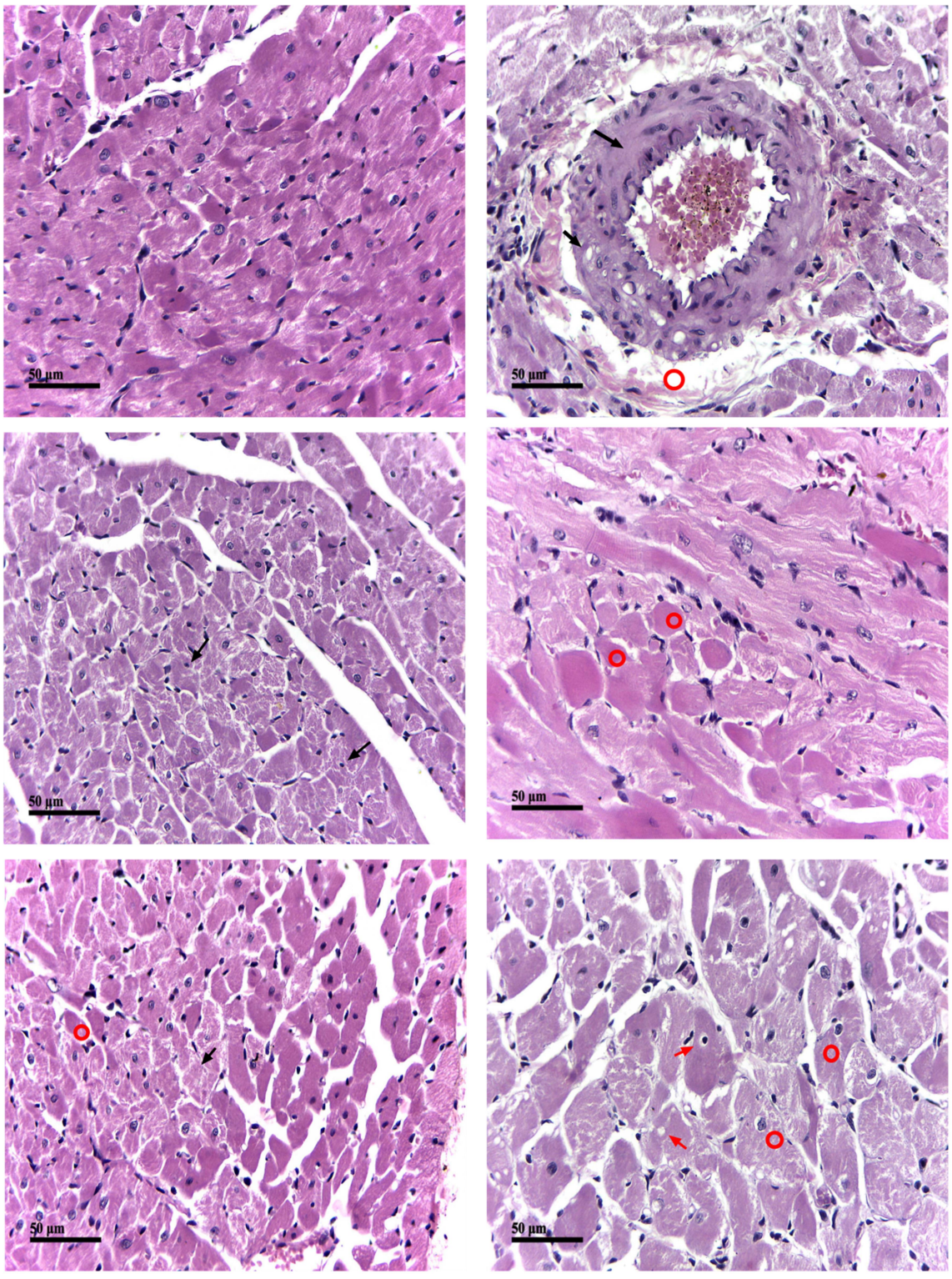
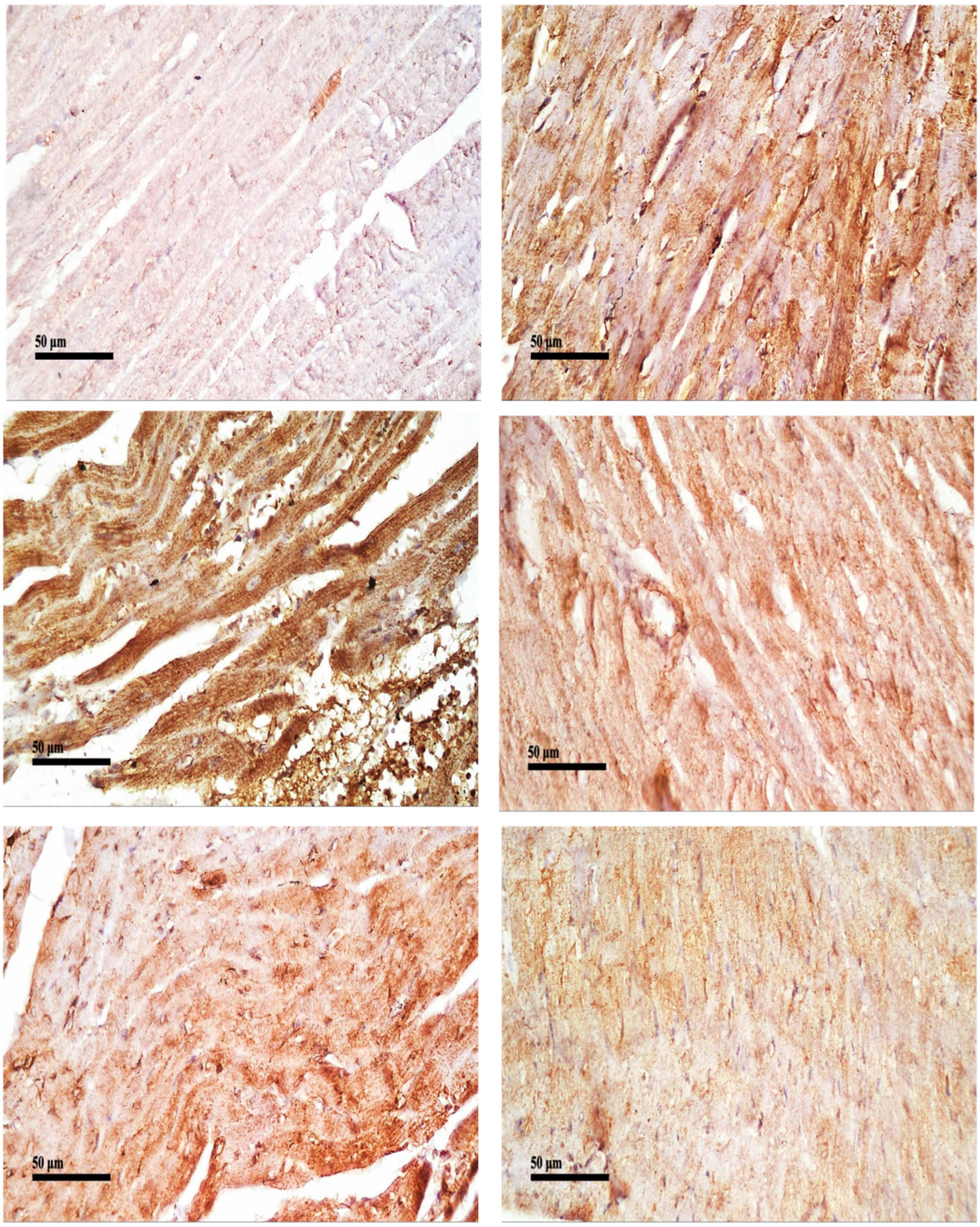


Figure 9: histopathological examination

Immunohistochemical cardiac tissue caspase-3 reactivity

Statistical analysis revealed strong positive staining of caspase-3 immunostaining among single fluconazole and itraconazole groups (fig.10) by 46 (P≤0.001) and 35.6 folds (P≤0.01), respectively as compared to control goup



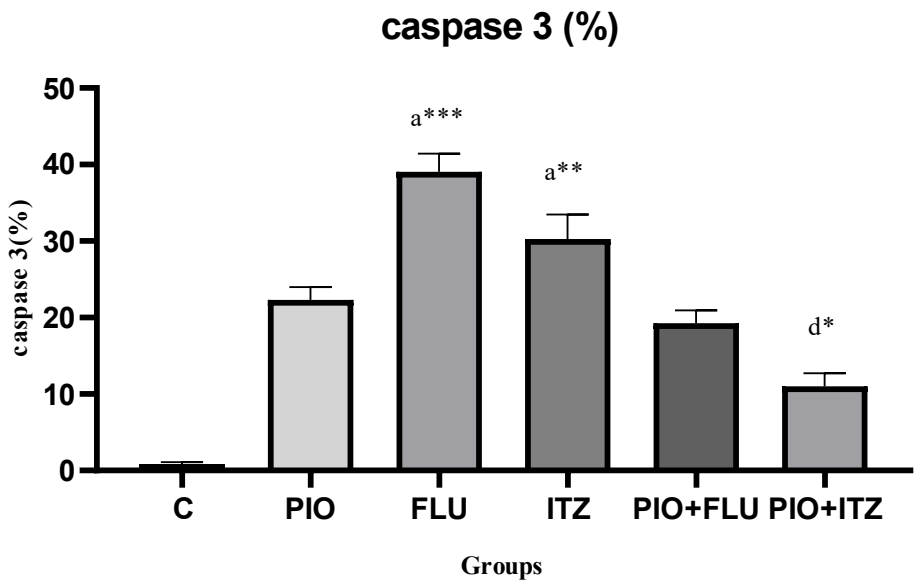


Figure 10: Immunohistopathology

DISCUSSION

Diabetic patient usually suffer from opportunistic fungal infections. Hence, risk of DDI is highly considered between the hypoglycemic drug in use and the antimicrobial used to treat such infection.

In the current study, we aimed to explore the possible cardiotoxicity that could develop from single use of hypoglycemic drug pioglitazone and antifungal drugs; fluconazole or itraconazole and the possible hazards that could be evoked from combined administration.

Single drugs resulted in significant increase in serum cardiac biomarkers e.g. CK-MB and cTnT, especially PIO and ITZ. They induced significant elevation in both biomarkers unlike FLU which induced a significant increase in CK-MB only.

CK-MB could be released in multiple types of myocardial injury such as myocarditis , trauma, cardiac surgery while cTnT was considered indicative of myocardial cell death***(Archan and Fleisher, 2010)***

It could be interpreted that PIO and ITZ induced cardiomyocyte cell death while FLU induced cell injury, which induced apoptotic pathway.

Confirmatively, histopathological examination of PIO and ITZ showed sever necrotic degenerative changes and hyalinization which is a vascular lesion causes a small foci of myocardial infarction ***(Miller & Gal, 2016)***.while FLU showed multiple signs of cell injury; pyknosis of some nuclei, congestion, intramuscular hemorrhage and signs of degenerative changes.

Inductions of cell death through apoptotic pathwaywere examined through caspase-3 immunostaining where FLU showed the highest significant casp-3 immuno-reactivity, indicating induction of cell death possibly through necroptosis or necrosis.

Both PIO and ITZ inhibit glycosylation and phosphorylation of vascular endothelial growth factor receptor-2 (VEGFR-2) which play an important role in angiogenesis and it's inhibition induces apoptosis ***(Nacev et al., 2011; Zhong et al., 2018)***

PIO also induces cell death through depletion of ATP pool inside the myocyte , which eventually, lead to subsequent myocardial dysfunction, PIO interact with electron transport chain, resulting in uncoupling of electron transport from ATP production ***(Sarraf et al., 2012)***

It's worth note to know that apoptosis is process that require ATP while necrosis is not ***(Tsujimoto, 1997)***

Generation of reactive oxygen species (ROS) and depletion of antioxidant capacity is also another pathway from inducing cardiomyocyte damage and possibly death. Similarly, doxorubicin (DOX)an anticancer drug, induces Cardiotoxcity through ROS generation and antioxidant depletion ***(Kang et al., 1996; Octavia et al., 2012)***

In present study, both single and combined drug administration induced both CAT depletion and MDA elevation, indicating increased production of ROS.

Unexpectedly, reduced glutathione (GSH) witnessed a tremendous elevation, this elevation possibly a defensive and compensatory mechanism in an attempt to overcome continuous ROS generation***(Colak et al., 2012; Deng et al., 2015; Salvemini et al., 1999)***

Similarly, ***Ammar et al., 2011*** reported a significant increase in GSH content in myocardium of DOX-treated rats.This elevation in GSH content explained as over-expression stimulus done because of continuous ROS generation to overcome DOX-induced oxidative stress

On the other hand, GSH plays an important role in development of cardiometabolic and cardiovascular diseases, through shifting Redox homeostasis towards reductive stress, prolonged antioxidant state or reductive stress can similarly lead to such cardiovascular complications ***(Bajic et al., 2019)***,this reductive stress leads to protein aggregation cardiomyopathy and cardiac hypertrophy***(Rajasekaran et al., 2007).***

Combined PIO/Azole administration also showed a significant increase in both cardiac biomarkers. Interestingly, combined treatment regimen pointed to less pronounced cTnT serum concentration, indicating attenuated cardiomyocyte damage. Confirmatively, immune-histochemical examination showed insignificant casp-3 immuno-reactivity.

These results could be understood in terms of inhibition of PIO metabolism leading to accumulation of parent drug rather than its metabolites. PIO metabolites was found to exert more pronounced toxicities and side effects than the parent drug as described by **Baughman *et al*., 2005 and *Camposet al*., 2018.**

PIO was considered as a substrate and inhibitor to CYP2C8 and to lesser extent to CYP3A4 ***(Eckland & Danhof, 2000)***,while FLU was considered a strong inhibitor of CYP2C8 and itraconazole a strong inhibitor of CYP 3A4. Considerably, a risk of DDI evokes***(Gupta et al., 1999; Janadri et al., 2009 and Patel et al., 2016).***

On the other hand, combined treatment regimens pointed to more electrophysiological cardiotoxicity. Co-treated PIO and FLU showed a significant elevation in R-amplitude, while both combined treated groups showed a prolonged QTc interval.

The R-amplitude elevation varies under a Varity of conditions such as abnormal left ventricular function ***(David et al., 1981)***,recent evidences suggests that the sub-chronic use of PIO leads to cardiac muscle hypertrophy and ventricular hypertrophy ***(Elshama et al., 2016).*** a potential volume expansion, leading to volume overload cardiac hypertrophy ***(Arakawa et al., 2004; Pickavance et al., 1999; Sena et al., 2007)***. PIO have been shown to block ATP sensitive potassium channel (KATP),thereby leading to increased incidence of ventricular fibrillation during ischemia in pigs ***(Sarraf et al., 2012).***PIO also aggravated DOX induced cardiomyopathy in rats ***(Saraogi et al., 2011)***

Co-treatment with PIO and azole antifungals pronounced the QTc prolongation side effect known to occur during azole administration ***(Kikuchi et al., 2005; Tamargo, 2000)***.Hence, it could be interpreted that PIO increased the bioavailability of azoles where ITZ and FLU are substrates for CYP3A4 which has been inhibited by PIO.

On the other hand, ITZ absorption is potentially affected by any drug modulating gastric acidity e.g. increasing gastric acidity enhances ITZ absorption and vice versa,PIO induces gastric acid secretion accordingly increasing ITZ absorption***(Rotte et al., 2009)***.

Although concentrations of ITZ in the myocardium have not been described, the tissue concentrations in most organs are much higher than that in plasma because of the high affinity of ITZ for plasma protein and its highly lipophilic features ***(Felton et al., 2014)***.

The observed QTc prolongation among rats that were co-treated with PIO and ITZ/FLU could be attributed to ITZ and FLU inhibitory effect on rapidly activating delayed rectifier potassium current (I_{Kr}) encoded human-ether-a-go-go-related gene ($HERG^+$) ***(Wang et al., 2016; Yap and Camm, 2003).***

Beside the K^+ channels, Na^+ and ca^{2+} channels play an important role in cardiac action potential, slowing the inactivation of voltage-gated Na^+ or Ca^{2+} currents may also contribute to prolonged action potential and generation of prolonged QT or torsade de pointes***(Farkas et al., 2009; Yoon et al., 2004)***. However, FLU and ITZ had relatively little effect on ion currents when compared to other azole antifungals ***(Sung et al., 2012)***

The current study has concluded that single treatment by PIO, FLU or ITZ is capable to cause cardiotoxicity in healthy rats through affecting cardiomyocyte function and to a lesser extent cardio-electrophysiological functions. This may be due to triggering apoptotic and/or necrotic cell death pathways through induction of oxidative stress.

On the other hand, co-treated groups with either PIO and FLU or PIO and ITZ exhibited a more pronounced electrophysiological cardiotoxicity than cardiomyocyte toxicity and impairment of channel trafficking resulting in QTc prolongation which could develop to torsade de pointe and fatal ventricular arrhythmia.

Finally, a toxic interaction in *Sprague dawely* rats had been witnessed; drug toxicity and drug interaction drug toxicity can be a major factor limiting the usefulness of widely used agents. A pre-knowledge with the possible drug interaction is necessary for preventing life threatening or fatal adverse effects.

CONCLUSION

Pioglitazone and azole antifungals have the potential to develop cardiomyopathical and electrophysiological toxicity in healthy rats either single or combined.

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