International Journal of Pharmacology

ISSN 1811-7775

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OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2018.1145.1152

Research Article
Cardioprotective Efficiency of Tangeretin Against Heart Failure Induced by Isoproterenol in Rats

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Abstract

Background and Objective: Heart failure or myocardial infarction (MI) is one of the deadliest condition which claims many lives globally. This pre-clinical animal study was intended to investigate the beneficial role of Tangeretin (TAN) against isoproterenol (ISO) induced heart failure in a rat model. **Materials and Methods:** Forty healthy male rats were randomly separated into four group with 10 rats in each. Saline treat rats are considered as a control group, whereas ISO (85 mg kg^{-1}, i.p) induced rats (2 consecutive days) are considered as ISO group. The TAN (100 mg kg^{-1} via o.p) pretreated rats for 28 days followed by induction of ISO will be considered as SAB-B+ISO group, only TAN (100 mg kg^{-1}, o.p) received rats for 28 days are considered as TAN group. **Results:** A pronounced increase in the levels of various hemodynamic parameters and the activities of antioxidants were observed in TAN pre-treated rats as compared to ISO-induced rats. However, the levels of infarct size, lipid peroxidation product like MDA, inflammatory markers, apoptotic markers are significantly decreased upon treatment with TAN for 28 days. Furthermore, TAN administration markedly reverted the histomorphological changes (cardiac tissue) caused by ISO induction. **Conclusion:** Taking together, that 28 days of pre-treatment with TAN significantly preserved cardiac function by suppressing oxidative stress, inflammation, apoptosis in ISO-induced heart failure (myocardial infarction) model and hence it can be recommended to treat myocardial infarction/heart failure with conventional (standard) cardio protective agents.

Key words: Tangeretin, myocardial infarction, antioxidant, apoptosis, inflammation, heart failure

Received: August 02, 2018 Accepted: September 03, 2018 Published: October 15, 2018

Citation: Huizhi Lu, Yun Tan, Luyu Yang, Hui Dong, Youxia Liao, Song Cao and Shouzhi Fu, 2018. Cardioprotective efficiency of tangeretin against heart failure induced by isoproterenol in rats. Int. J. Pharmacol., 14: 1145-1152.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Heart failure or myocardial infarction (MI) is one of the deadliest condition which has been estimated to affect around 24 million people by the year 2030[1]. In China morbidity and mortality related to MI has been steeply increased in the past few decades due to increased urbanization, western lifestyle pattern, obesity[2]. The MI is caused due to an insufficient amount of blood supply to heart tissue (myocardium) owing to the blockage of blood vessels (coronary artery) which results in ischemia, oxidative stress (lack of ATP) and subsequently leads to necrosis of cardiomyocytes/myocardial death. Several factors like oxidative stress (excessive free radical generation which end up in decreased endogenous antioxidant activity), hypoxia (lack of oxygen supply), necrosis and apoptosis might contribute to ischemic injury and finally leads to MI[3-5].

Isoproterenol (ISO) is a synthetic non-selective β-adrenergic receptor agonist (catecholamine). When ISO exposed to supramaximal dose it would pose a significant stress in myocardium by eliciting excessive free radical's due to auto-oxidation of catecholamine and subsequently results in inflammatory response and results in necrosis/apoptosis (similar to ischemic injury) and finally end up in myocardial infarction[4,6]. ISO-induced MI or cardiotoxicity is one of the well-accepted standard animal models to assess the cardio-protective function of any synthetic or natural drug[7]. Since, the pathology of ISO-induced cardiotoxicity and MI are almost similar as oxidative stress, inflammation, hypoxia, mitochondrial dysfunction, lack of bioenergetics (lack of ATP), apoptosis and necrosis[3,8].

Tangeretin (TAN, 5,6,7,8,4'-Pentamethoxy flavone) is a flavonoid and mostly found in citrus fruit peel especially mandarin oranges and sweet oranges[9]. Tangeretin shows an array of biological properties including antioxidant, anti-inflammatory, anti-microbial, anti-diabetic and anti-allergic activities as well as neuro and cardioprotective properties[10-12]. It also exerts anti-cancer activity by inhibiting the proliferation and favors cell arrest in various human cancer cell lines[13,14]. Previously, tangeretin has been reported to exhibit hypolipidemic, antioxidant activities and thus effectively protects cardiac function (Cardioprotective action) in an animal model[15,16]. An animal study carried out by Prabha *et al.*[7] have hinted that *Gardenia gummifera* rich in tangeretin showed potent cardio-protective activity against isoproterenol-induced myocardial infarction. As of now, no studies have been carried out with Tangeretin against ISO-induced cardiotoxicity. Therefore, this study was designed to investigate to evaluate the cardioprotective property of tangeretin (TAN) against isoproterenol (ISO) induced heart failure in a rat model.

MATERIALS AND METHODS

Chemicals: Tangeretin (TAN), isoproterenol hydrochloride (ISO-HCL), triphenyl tetrazolium chloride (TTC), formalin, hematoxylin and eosin (H and E), xylene were bought from Sigma-Aldrich All the other chemicals used in this study are of analytical grade.

Experimental animals: Forty male Sprague-Dawley (SD) rats were procured from a local experimental animal agent (Lab animals corp., Wuhan, China) and maintained under standard laboratory condition (22-24°C with 55-60% humidity) in a polycarbonate cage with free access to water and rat standard pellet (food). All the animal experimental protocols/procedures were carried out as per the regulation of NIH guidelines (revised 1978) and approved by institutional ethical board members of Tongren Hospital of Wuhan University (06/BAC1-2017).

Animal grouping: After 2 weeks of assimilation period, rats were randomly divided into four group with 10 rats in each group. Control rats received only saline (0.9%), whereas ISO-induced rats were intraperitoneally (i.p) injected with ISO (85 mg kg^{-1}, i.p) on 29 and 30th day. Treatment rats were pre-treated with TAN (100 mg kg^{-1} orally, o.p by dissolving with saline) for 28 days and followed by induction of ISO (85 mg kg^{-1}, i.p) on 29 and 30th day. Drug control rats received only TAN (100 mg kg^{-1}, o.p) for 28 days.

Sample collection: After 30 days of experiments, all the overnight fasted rats (on 31st day) were weighed as well as blood pressure are noted. Then the rats were anesthetized with pentobarbital sodium (45 mg kg^{-1}, i.p) and a blood sample was collected after cardiac puncture into the non-anticoagulant tube and sacrificed by cervical decapitation. The cardiac tissue (myocardium) was excised immediately and rinsed in ice-cold saline and dried and weighed. A portion of the cardiac tissue was homogenized (potter-Elvehjem type homogenizer) with phosphate buffered solution (PBS, 7.4 pH). The homogenate was centrifuged at 12000×g for 10 min at 4°C, the supernatant portion was used for biochemical analysis. Remaining cardiac tissue was fixed in 10% formalin to assess any morphological changes. A collected blood sample was allowed to clot and serum samples were separated by centrifuging at 3500×g for 15 min at 4°C. All the samples were stored at -80°C until they used it.

Measurement of hemodynamic parameters: After 24 h of last ISO induction on 31st day (before sacrifice), the hemodynamic parameters like mean arterial pressure

(MAP), Systolic arterial pressure (SAP), Diastolic arterial pressure (DAP) were measured using commercial NIBP200Atail-cuff non-invasive blood pressure monitor bought from BIOPAC Systems, INC., (CA, USA).

Evaluation of myocardial infarct size: The myocardial infarct size in experimental rats (Ventricular slice) was measured after staining with TTC reagent by the method of Wang *et al.*[17] with slight modification. In brief, the left ventricle of heart was transversely cut (base apex axis) into 2-3 mm thick slices and incubated in 1% TTC solution (made from PBS) for 15 min at 37°C and fixed with 10% formalin. The viable ischemic tissue slice was stained red (non-infracted), whereas non-ischemic tissue slice appeared white or pale grey (infracted). The image of each slice was captured by NIKON digital camera (Eclipse 50i) and the (%) of infarct size were analyzed with Image-Pro PLUS Software (Ver 6.0) from Media Cybernetics (MD, USA).

Assay of cardiac antioxidants and lipid peroxidation product: The activities of cardiac antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) as well as lipid peroxidation product like malondialdehyde (MDA) were assessed using a commercial kit from Sangon Biotechnology (Shanghai, China) based on supplier protocol.

Determination of cardiac inflammatory markers: The supernatant of the cardiac tissue homogenate was used to extract the nuclear and cytosolic fractions using nuclear and cytoplasmic extraction kit (Guge Biotechnology, Wuhan, China). The concentration of nuclear factor kappa B p65 subunit (NF-κB p65) in the nuclear fraction of cardiac tissue homogenate was measured by NF κB p65 ELISA kit (abcam., Cambridge, UK). The levels of pro-inflammatory cytokines like Interleukins 1 (IL-1β), Tumour necrosis factor (TNF-α) and Interleukins 6 (IL-6) in cardiac tissue homogenate was determined using commercial ELISA kit from Cayman Chemicals (MI, USA) based on manufacturer instruction.

Evaluation of cardiac apoptotic markers: The cardiac tissue apoptosis (cardiac tissue homogenate) were evaluated by checking the activity of major caspases like caspase-3 and 9 using ELISA kit purchased from Beyotime Int., Biotech (Jiangsu, China) by following the manufacturer's instruction.

Detection of morphological changes: As mentioned in the sample section the formalin fixed cardiac tissues were dehydrated, fixed and embedded in liquid paraffin (wax) and made as a tissue block. Those cardiac tissue blocks were sliced into 3-4 μm diameter using ultra-microtome from Jinhua Huiyou Equipment and Instrument co., Ltd. (Zhejiang, China) and bound to slide. Finally, the cardiac tissue slides were stained with H and E stain and detected for any histo-morphological changes using a light microscope (Nikon, Eclipse 50i, Tokyo, Japan) at 100× magnification.

Data analysis: Data are expressed as the Mean±Standard Error of Mean (SEM). The statistical difference between the experimental groups were compared to ISO-induced group vs. Control group and TAN+ISO group vs. ISO-induced group by One-Way Analysis of Variance (ANOVA) followed by Dunnett multi-comparison test using GraphPad-Prism (Ver. 6) a statistical program software from GraphPad Software, Inc., (CA, USA). Probability (P) value<0.05 (95% confidence) was recognized as significant.

RESULTS

Efficacy of TAN on hemodynamic parameters in experimental rats is epitomized in Table 1. The mean levels of various hemodynamic parameters including mean arterial pressure (MAP), systolic arterial pressure (SAP) and diastolic arterial pressure (DAP) were significantly declined after 2 days of ISO induction. Whereas, pre-treatment with TAN for 28 days before the induction of ISO could considerably enhance the levels of various hemodynamic parameters on comparison with ISO-induced rats.

Efficacy of TAN on myocardial infarct size in experimental rats were portrait in Fig. 1. As compared with control rat's infarct size, the MI-induced (ISO) rat's infarct size was substantially increased (shows large non-viable/ischemic non-stain white regions). However, the infarct size was

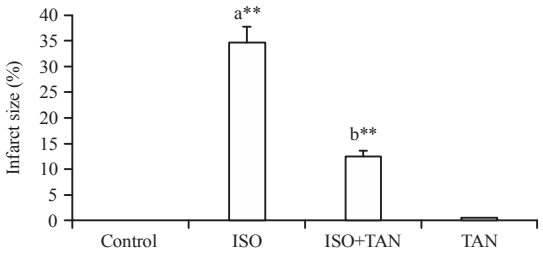

Fig. 1: Efficacy of TAN on infarct size in experimental animals

Data are expressed as the Mean±Standard error of Mean (SEM). p-value (**p<0.01) a: ISO-induced rats vs. Control rats, b: TAN+ISO rats vs. ISO-induced rats. ISO: isoproterenol, TAN: Tangeretin

Table 1: Efficacy of TAN on hemodynamic parameters in experimental animal

Parameters (mmHg)	Control	ISO	ISO+TAN	TAN
MAP	110.05±6.93	67.20±5.10^{a**}	98.55±7.33^{b*}	108.45±6.50
SAP	129.50±7.29	88.60±10.08^{a**}	113.60±9.70^{b**}	130.81±9.90
DAP	84.60±7.59	53.70±5.10^{a**}	74.30±6.80^{b*}	86.20±7.00

Data are expressed as the Mean±Standard Error of Mean (SEM). p-value (*p<0.05, **p<0.01) a: ISO-induced rats vs. Control rats, b: TAN+ISO rats vs. ISO induced rats. ISO: Isoproterenol, TAN: Tangeretin, MAP: Mean arterial pressure, SAP: Systolic arterial pressure, DAP: Diastolic arterial pressure

Table 2: Efficacy of TAN on cardiac antioxidants and lipid peroxidation products (MDA) in experimental animals

Parameters	Control	ISO	ISO+TAN	TAN
SOD (U mg^{-1} pro)	7.02±0.92	4.05±0.45^{a**}	6.12±0.80^{b**}	7.10±0.85
CAT (U mg^{-1} pro)	15.32±1.10	8.92±0.80^{a**}	13.87±1.50^{b**}	15.46±1.78
GPx (μg mg^{-1} pro)	9.35±1.00	7.40±0.88^{a**}	8.42±1.10^{b*}	9.67±1.20
MDA (nmols mg^{-1} pro)	0.47±0.05	0.92±0.10^{a**}	0.58±0.08^{b**}	0.51±0.05

Data are expressed as the Mean±Standard Error of Mean (SEM). p-value (*p<0.05, **p<0.01) a: ISO-induced rats vs. Control rats, b: TAN+ISO rats vs. ISO induced rats. ISO: Isoproterenol, TAN: Tangeretin, Pro: Protein, SOD: Superoxide dismutase, CAT: Catalase, GPx: Glutathione peroxidase, MDA: Malondialdehyde

Table 3: Efficacy of TAN on serum cardiac markers in experimental animals

Parameters	Control	ISO	ISO+TAN	TAN
cTn T (ng mL^{-1})	0.54±0.06	1.68±0.19^{a**}	0.75±0.09^{b**}	0.51±0.05
CK-MB (IU L^{-1})	65.09±5.20	157.94±17.00^{a**}	95.24±10.05^{b**}	70.45±6.80
LDH (IU L^{-1})	91.93±9.10	162.00±15.52^{a**}	109.35±11.70^{b**}	89.50±9.40

Data are expressed as the Mean±Standard Error of Mean (SEM). p-value (**p<0.01) a: ISO-induced rats vs. Control rats, b: TAN+ISO rats vs. ISO induced rats. ISO: Isoproterenol, TAN: Tangeretin, cTn T: cardiac troponin T, CK-MB: Creatine phosphokinase, LDH: Lactate dehydrogenase

Table 4: Efficacy of TAN on cardiac inflammatory markers in experimental animals

Parameters	Control	ISO	ISO+TAN	TAN
TNF-α (ng mg^{-1} pro)	108.70±12.50	212.30±17.95^{a**}	141.10±12.82^{b**}	112.30±13.00
IL-1β (ng mg^{-1} pro)	62.40±07.00	160.71±17.05^{a**}	85.66±10.55^{b**}	64.00±7.090
IL-6 (pg mg^{-1} pro)	72.60±08.14	178.93±20.40^{a**}	112.47±16.99^{b**}	70.22±9.900
NF-κb p65 (pg mg^{-1} pro)	80.90±11.00	192.62±21.83^{a**}	115.83±13.00^{b**}	84.70±9.220

Data are expressed as the Mean±Standard Error of Mean (SEM). p-value (**p<0.01) a: ISO-induced rats vs. Control rats, b: TAN+ISO rats vs. ISO induced rats. ISO: isoproterenol, TAN: Tangeretin, Pro: Protein. TNF-α: Tumor necrosis factor alpha, IL-1β: Interleukin one beta, IL-6: Interleukin six, NF-κb p65: Nuclear Factor kappa b p65 subunit

remarkably decreased (shows large viable/non-ischemic red stain regions) in rats administered with TAN at a dose of 100 mg kg^{-1} for 4 weeks as equivalent with ISO-induced MI model rats.

The data in Table 2 showed the efficacy of TAN on cardiac antioxidants and lipid peroxidation products (MDA) in experimental animals. ISO-induced rats showed increased levels of lipid peroxidation product like MDA with decreased activity of various cardiac endogenous antioxidants like SOD, GPx and CAT than non-ISO induced control rats. Between the TAN pre-treated group and induced group, the levels of MDA were markedly decreased with a significant increase in the activities of other cardiac endogenous antioxidants like SOD, GPx and CAT.

The efficacy of TAN on serum cardiac markers in experimental animals (Table 3). As shown in Table 3 the average values of serum cardiac markers like LDH, CK-MB, cTnT were notably escalated in ISO injected rats than control rats. The average levels of these serum cardiac markers were markedly reverted to near normal (normalcy) after 28 days of pre-treatment with TAN on comparison ISO rats.

Data in Table 4 showed that the efficacy of TAN on cardiac inflammatory markers in experimental animals. The values of various inflammatory markers like TNF-α, IL-1β, IL-6 and NF-κb p65 in the nuclear and cytosolic fraction of cardiac tissue homogenate of ISO administered rats were significantly peaked. While, TAN supplemented rats showed a decrease in the values of various inflammatory markers like TNF-α, IL-1β, IL-6 and NF-κb p65 as compared with those of ISO-induced MI model rats.

The Fig. 2 illustrated the efficacy of TAN on apoptotic markers like Caspase 3 and 9 in experimental animal's A pronounced increase in the concentration of various apoptotic markers like caspase-3 and 9 were observed in ISO injected rats. Upon pre-treatment with TAN for 28 days the concentration of apoptotic markers like caspase-3 and 9 were concomitantly decreased than ISO-induced rats.

As Fig. 3 represented the efficacy of TAN on histo-morphological changes after ISO induction in cardiac tissue stained with H and E in experimental animals. The transection of control cardiac tissue represents normal myofibrillar structure without any other pathological

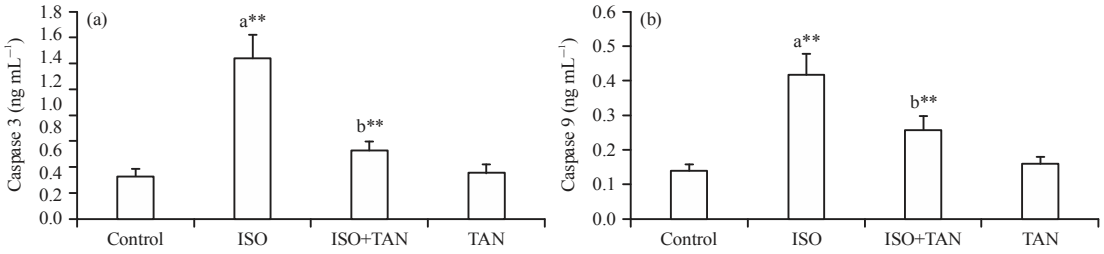

Fig. 2(a-b): Efficacy of TAN on apoptotic markers like (a) Caspase 3 and (b) 9 in experimental animals

Data are expressed as the Mean±Standard Error of Mean (SEM). p-value (**p<0.01) a: ISO-induced rats vs. Control rats, b: TAN+ISO rats vs. ISO-induced rats, ISO: Isoproterenol, TAN: Tangeretin

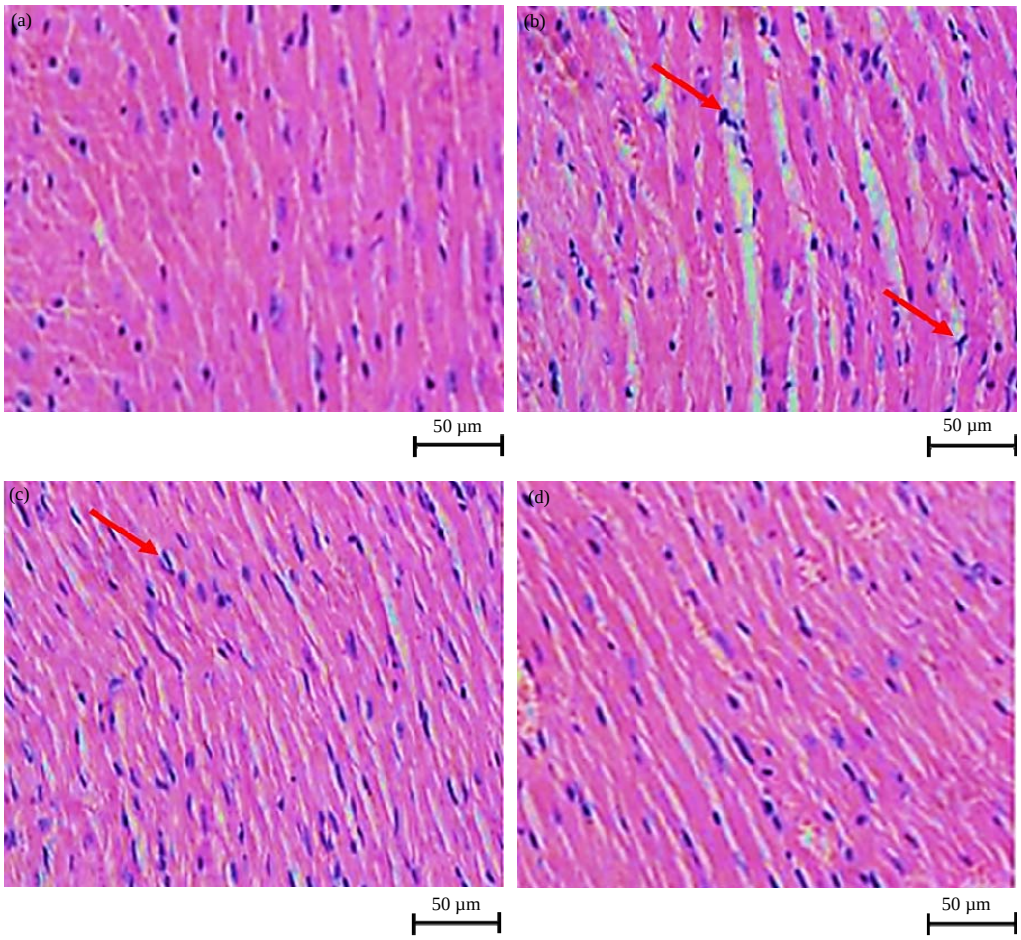

Fig. 3(a-d): Efficacy of TAN on histo-morphological changes after ISO induction in cardiac tissue stained with H and E in experimental animals, (a) The transection of control cardiac tissue represents normal myofibrillar structure without any other pathological condition, (b) Transection of ISO-induced cardiac tissue portrait considerable myofibrillar degeneration/disruption characterized with high neutrophil granulocytes infiltration (inflammation-indicated by a circle), necrosis, perivascular cuffing and myocytolysis (indicated by red arrow mark), (c) Whereas, transection of cardiac tissue treated with TAN for 28 days and followed by ISO induction (29th and 30th days) display lesser myofibrillar degeneration/disruption with very few neutrophil granulocytes infiltration, necrosis and myocytolysis and (d) Transection of the cardiac tissue of TAN alone treated rat revealed the presence of normal cardiac architecture with prominent myofibrillar structure

Scale bar 50 µm

condition (Fig. 3a). Transection of ISO-induced cardiac tissue portrait considerable myofibrillar degeneration/disruption characterized with high neutrophil granulocytes infiltration (inflammation-indicated by a circle), necrosis, perivascular cuffing and myocytolysis (indicated by red arrow mark) (Fig. 3b). Whereas, transection of cardiac tissue treated with TAN for 28 days and followed by ISO induction (29th and 30th days) display lesser myofibrillar degeneration/ disruption with very few neutrophil granulocytes infiltration, necrosis and myocytolysis (Fig. 3c). However, transection of the cardiac tissue of TAN alone treated rat revealed the presence of normal cardiac architecture with prominent myofibrillar structure (Fig. 3d).

DISCUSSION

Several studies have indicated that administration of ISO (supramaximal dose) would trigger excessive free radical generation (auto-oxidation of catecholamine) and eventually results in inflammatory response, necrosis/apoptosis and finally end up in myocardial infarction which is almost similar to human MI[4,6]. Therefore, for the present study ISO-induced MI animal model was used to investigate the cardioprotective role of Tangeretin (TAN) by evaluating hemodynamic parameters, myocardial infarct size, antioxidant status, inflammatory and apoptosis cascade as well as histopathological changes. The outcome of this study showed that pre-treatment for 28 days with TAN after ISO induction could improve the levels of various hemodynamic parameters and the activities of antioxidants were observed with decrease in the levels of infarct size, lipid peroxidation product like MDA, inflammatory markers, apoptotic markers as well as preserve cardiac function and morphology.

Ample amount of studies reported that ISO induction results in ventricle contractile dysfunction (cardiac remodeling) which could alter the pattern of cardiac output and hemodynamic parameters like blood pressure[18,19]. The values of various hemodynamic parameters (MAP, SAP and DAP) were exponentially lowered after exposed to ISO. Interestingly, rats administered with TAN for 28 days before the induction of ISO could markedly improve the levels of various hemodynamic parameters on comparison with ISO-induced rats. Also, MI-induced (ISO) rat's shoed increased infarct size but on treatment with TAN would significantly decrease the infarct size with many viable cardiomyocytes than ISO injected rats. The above results depicted that TAN could preserve cardiac function by alleviating ventricular dysfunction/myocardial degeneration and cardiac output (blood pressure) owing to its antioxidant and anti-inflammatory properties[10,11].

As aforementioned oxidative stress is the major contributor for ISO-induced cardiotoxicity and hence the levels of lipid peroxidation products and cardiac antioxidant levels were assessed. A pronounced increase in the levels of lipid peroxidation product like MDA with decreased activity of various cardiac endogenous antioxidants like SOD, GPx and CAT were observed in ISO-induced MI model rats because of oxidative stress. However, TAN pre-treated rats showed decreased levels of MDA (anti-peroxidative activity) with a significant increase in the activities of cardiac antioxidants like SOD, GPx and CAT were noted due to potent antioxidant activity. TAN possess strong anti-peroxidative and free radical scavenging activity (antioxidant) owing to methoxy moiety and thus lower lipid peroxidation and oxidative stress[11]. In addition, tangeretin could positively regulate Nrf2 signaling pathway and thereby enhance the production of various cyto-protective agent and antioxidants in HepG2 cell line[20]. Meanwhile, the levels of serum cardiac markers in were notably increased in ISO injected rats than control rats owing to cardiotoxicity. Nevertheless, pre-treatment with TAN for 28 days after ISO induction could considerably revert those cardiac markers to normalcy due to cardio-protective and anti-peroxidative properties[11]. Previously, Sundaram *et al.*[9], demonstrated that treatment with tangeretin in STZ induced rat showed a significant decrease in the activities of serum cardiac marker enzymes (CPK, LDH) due to cardioprotective and anti-lipid peroxidation activity of Tangeretin.

The values of both inflammatory and apoptotic markers are significantly elevated in ISO administered rats. While TAN supplemented rats the values of various inflammatory and apoptotic markers are dramatically decreased due to anti-inflammatory and anti-apoptotic activities. Few animal and cell line studies also hinted that administration of tangeretin could considerably lower the different inflammatory markers in cardiac tissue[9,12]. Likewise, Tangeretin is reported to diminish apoptosis by improving the oxidative status and inflammatory response which are evidenced by decreased levels of caspases (3/7) in cisplatin-induced hepatic injury rat model[21]. Thus, from the above data it's clear that TAN can protect the cardiomyocytes from free radical-induced damage by exerting antioxidant, anti-peroxidative, anti-inflammatory and anti-apoptotic activities.

The cardiac tissue morphological changes in experimental rats exposed to ISO and TAN treatment were detected by staining with H and E stain to confirm the biochemical changes. Transection of ISO-induced cardiac tissue portrait considerable morphological changes like myofibrillar degeneration/disruption characterized with

elevated neutrophil granulocytes infiltration, necrosis, perivascular cuffing and myocytolysis as compared to control rats which showed normal myofibrillar structure without any pathological changes. Whereas, transection of cardiac tissue treated with TAN for 28 days and followed by ISO induction (29th and 30th days) display lesser myofibrillar degeneration/disruption with very few neutrophil granulocytes infiltration, necrosis and myocytolysis due to antioxidant, anti-necrosis/apoptosis properties which results in preserve cardiac function. A major limitation of this animal study is the avoidance of mRNA and protein expression of various apoptotic and inflammatory markers.

CONCLUSION

To conclude that pre-treatment of TAN for 28 days after ISO induction could significantly improve the levels of various hemodynamic parameters and the activities of antioxidants as well as considerably reduced the levels of infarct size, lipid peroxidation product like MDA, inflammatory markers, apoptotic markers as well as preserve cardiac function and morphology. Nevertheless, the mechanism underlining the cardioprotective property are needed to be explored in future.

SIGNIFICANCE STATEMENT

The outcome of the study clearly shows that tangeretin exhibit potent cardioprotective activity by improving the antioxidant status and suppress infarct size, inflammatory and apoptosis cascade and thereby preserve cardiac function. The above results would aid researchers to produce a novel natural cardioprotective agent and can be helpful for combating various cardio related dysfunctions.

ACKNOWLEDGMENT

Authors would like to thank the Project of Health and Family Planning Commission of Wuhan Municipality (WX18D18) for funding this study.

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