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Gissa M Jose
MVSc Scholar, Department of Veterinary Pharmacology and Toxicology, NTR College of Veterinary Sciences, Sri Venkateswara Veterinary University, Gannavaram, Andhra Pradesh, India

K Bharavi
Professor, Department of Veterinary Pharmacology and Toxicology, NTR College of Veterinary Sciences, Sri Venkateswara Veterinary University, Gannavaram, Andhra Pradesh, India

Afroz Jahan
Assistant Professor, (Contractual), Department of Veterinary Pharmacology and Toxicology, NTR College of Veterinary Sciences, Sri Venkateswara Veterinary University, Gannavaram, Andhra Pradesh, India

G Srinivasa Rao
Professor and University Head, Department of Veterinary Pharmacology and Toxicology, College of Veterinary Sciences, Sri Venkateswara Veterinary University, Tirupati, Andhra Pradesh, India

Corresponding Author
K Bharavi
Professor, Department of Veterinary Pharmacology and Toxicology, NTR College of Veterinary Sciences, Sri Venkateswara Veterinary University, Gannavaram, Andhra Pradesh, India

Quercetin ameliorates the Cadmium induced structural and functional changes in myometrium of estrus mice

Gissa M Jose, K Bharavi, Afroz Jahan and G Srinivasa Rao

Abstract
Cadmium, a heavy metal and an environmental toxicant, is regarded as a potent endocrine disruptor resulting in uterine abnormalities. Quercetin, a powerful antioxidant flavonoid, has heavy metal chelating property, smooth muscle relaxant property and estrogenic effect. Present study designed to evaluate the ameliorative effect of quercetin on cadmium induced structural and functional changes in mice myometrium. Swiss albino mice were divided into four groups with 6 mice in each viz control, cadmium treated (administration of cadmium only), cadmium and quercetin treated and quercetin alone treated for one month. The *ex vivo* study on uterine horn strip revealed a decrease in the mean EC50 value for oxytocin in cadmium group, which might be the implication of estrogen mimicking effect of cadmium. The co-administration of quercetin and cadmium increased mean EC50 value, suggesting the reversal effect of quercetin in cadmium induced changes. But, quercetin treatment alone showed a reduced EC50 value, which was possibly due to its estrogenic property. Thus, quercetin exhibited a contradictory behavior in alone administration and co-administration with cadmium. Histopathology of cadmium group in estrus animals revealed degenerative changes in the glandular epithelium, congestion and desquamation of lining epithelium. The co-administration of quercetin and cadmium showed normal structure with lining epithelium and glands. Our study revealed that quercetin might have some ameliorative effect on cadmium induced structural damages in uterus, even though it exhibited a contradictory effect on functional changes.

Keywords: Cadmium, uterine functional and structural changes, myometrium, quercetin

Introduction
Industrialization intensifies the exposure to various environmental toxicants like pesticides, herbicides, heavy metals etc. Cadmium (Cd) is one among the xenobiotic, obligatory toxic to animals and humans that enters the body by inhalation, ingestion and dermal contact [1]. It is having deleterious effect on various organs like liver, kidney, bones, brain and gonads [2,3,4].
Cadmium exposure is strongly associated with reproductive toxicity in females of both animal and human populations, culminating infertility and cancers of the reproductive tissues[5]. Cadmium can accumulate in human endometrial tissue and its levels are increased in female with history of smoking [6] and low iron stores in the body [7].
Cadmium is a metalloestrogen, possessing estrogen mimicking and potent endocrine disruptor, which can bind and stimulate estrogen α receptor, thereby stimulating it and also it up regulates the progesterone receptors [8]. The metalloestrogenic property of Cd is responsible for estrogen dependent diseases like endometriosis, spontaneous abortions, endometrial and breast cancers etc [6]. Cadmium exposure is associated with hypertrophy and hyperplasia of endometrium and increased levels of blood cadmium is reported in women with endometriosis [5].
There are many studies regarding the effect of Cd on smooth muscle. Chronic Cd exposure (3 months) impairs neurogenic and myogenic contractility of rat detrusor muscle as well as orally ingested Cd also accumulates in myometrium and alters its responsiveness to oxytocin, phenylephrine, histamine and KCl[9]. Oral administration of herbal adaptogens prevents the bioaccumulation of Cd and reversed Cd-induced oxidative tissue damage [10].
Quercetin is a flavanoid derived from plants such as apples, tea, onions, nuts, berries, cauliflower, cabbage etc. It exerts its antioxidant activity by scavenging free radicals [11] and also having metal chelator activity [12]. Quercetin is classified as phytoestrogen as quercetin can bind to type 1 estrogen receptors [13].
Quercetin produces a relaxant effect in the smooth muscles of various arteries [14] and smooth muscles of bronchus contracted by acetyl choline and histamine [15].

Quercetin inhibits intracellular calcium induced contractions by PGF2α, oxytocin, carbachol, KCl and Bay K 8644 by blocking the Ca^{2+} through receptor operated channels and voltage operated channels [16].

Quercetin effectively prevents Cd induced haematological impairment, testicular hormone and sperm abnormalities [17], neuronal damage [18] and hepato-renal damage [19]. The protective effect of quercetin on Cd induced functional derangement of various organs have been studied, but very less reports are available concerning the female reproductive system. Cd accumulation induced oxidative stress in the uterus and ovaries of rats by increased caspase-3 activity which can be attenuated by quercetin through antioxidant and antiapoptotic action [20]. Considering this scenario, our study was focused on the effect of Cd on structural and functional changes in mice myometrium and also the ameliorative effect of quercetin on cadmium induced myometrial changes.

Materials and Methods

Chemicals

Quercetin, cadmium chloride monohydrate, oestradiol benzoate, oxytocin were procured from M/s Sigma Aldrich, India. Quercetin suspension (1%) for oral administration was prepared by triturating quercetin in tween 80 and distilled water in 1:9 ratio. Cadmium (30 ppm) solution was prepared by mixing 5.04 mg cadmium chloride in 100 ml distilled water. Oestradiol benzoate solution to induce estrus was prepared with 3:7 ratio of absolute alcohol and normal saline. Dilutions of oxytocin were made using modified Krebs-Henseleit solution.

Experimental animals

The experimentation was done on 24 adult female Swiss albino mice (weighing 25-30g) which were procured from M/s Sanzyme (P) Ltd, Telangana State (1837/PO/RcBT/s/15/CPCSEA). An acclimatization period of one week was given to mice before starting the experiment. Animals were divided randomly into four groups of 6 each and group 1 mice were treated with normal drinking water, group 2 mice were treated with 30 ppm of Cd as $CdCl_2$ in drinking water for one month [9], group 3 mice were treated with both Cd (30 ppm) and quercetin (50 mg/kg BW)[19] for a period of one month and group 4 mice were treated with only quercetin @ dose 50 mg/kg BW for one month. After 30 days of treatment, estrus was induced in mice by administering estradiol benzoate @ dose 1mg/kg BW/day subcutaneously for three consecutive days [21].

The experimentation was started after approval from Institutional Animal Ethics Committee of NTR CVSc vide reference number 3/ IAEC /NTR CVSc / 2018 dated 01-09-2018 as per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Collection of tissue

Experiment was designed in such a way that mice of each group would be slaughtered on alternate day. At the end of experiment, the mice in estrus stage of estrus cycle were sacrificed humanely as per CPCSEA standard procedure. Uterus was dissected out and transferred to a dish containing Kreb's solution consisting of sodium chloride (NaCl) 118 mM, sodium bicarbonate ($NaHCO_3$) 25 mM, anhydrous dextrose 11.1 mM, potassium dihydrogen orthophosphate (KH_2PO_4) 1.2 mM, potassium chloride (KCl) 4.8 mM, magnesium sulphate heptahydrate ($Mg_2SO_4.7H_2O$) 1.2 mM and calcium chloride ($CaCl_2$) 1.2 Mm and having pH 7.4. The uterus was cleaned by removing the fascia and connective tissue.

Mounting of tissue for isometric tension studies

A small longitudinal strip of uterine horn was mounted in stainless steel hook attached to the S-shaped notch in oxygen delivery tube and was suspended in tissue chamber containing 20 ml modified Krebs-Henseleit solution[21]. The other end of the uterine strip was tied to an isometric force transducer. The bath temperature was maintained at 37°C and aerated continuously with carbogen. The uterine strip was mounted under a resting tension of 1g and allowed to equilibrate for about 45 minutes. During the equilibration period, it was washed thrice with modified Krebs- Henseleit solution for every 15 minutes before proceeding for actual work. The myometrial contractile response to oxytocin at 0.110×10^{-12} to 3.53×10^{-12} M from all four groups of mice were recorded using a polygraph digital data acquisition system linked to isometric transducer connected to a recorder (physiopac PC-2004, Medicaid systems) [9].

Histopathological examination of uterus

A piece of uterus was collected at the time of sacrifice and fixed in 10% formalin in neutral buffer for histopathological studies. The fixed tissues were processed and paraffin embedded and sectioned at a thickness of 5 µm. The sections were stained by Harris Haematoxylin and Eosin (H&E) stain for histopathological studies.

Data analysis

The contractile effect of oxytocin on mice myometrium was calculated as percent contractile response. The percentage response was calculated by taking the maximum contraction as 100% and the rest of the contractions were converted into respected percentages [22]. The results were graphically represented as Mean ± SEM, which were calculated using Microsoft Excel programme. The concentration producing 50% of the maximal effect (EC50) was calculated for oxytocin using 'ED50 plus software' that calculates ED50 by linear regression method (Mario H. Vargas, version 1.0). The EC50 values were presented as geometric means accompanied by their respective 95% confidence limits. The differences between the groups were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests (GraphPad prism software, version 7.05). A value of $P<0.05$ was considered significant.

Results

Estrus mice uterus cumulative contractile dose response to oxytocin

Estrus mice uterus cumulative contractile dose responses to oxytocin in various groups were represented in figure 1. Oxytocin induced cumulative contractile dose response of uterus was expressed as EC50 by using the frequency and amplitude of contraction. In the estrus mice, by comparing both frequency and amplitude of contraction (Fig.1), the mean EC50 value of oxytocin contraction in cadmium group is significantly lower than the control. Basing on amplitude of contraction there was significant increase in the EC50 value of group 3 mice compared to group 2 mice (Table 1). Hence, there is a rightward shift in the dose-response curve (Fig.2). Even though the EC50 value was increased with frequency in

group 3 mice compared to group 2 mice with a rightward shift in dose-response curve, it is not significant. The EC50 value of quercetin treated group 4 mice is less compared to group 1 mice (Table 1).

Histopathology of uterus

Group 2 (cadmium) mice uterus in estrous stage showed congestion and desquamation of lining epithelium and degenerative changes in glandular epithelium (Fig 3a, b). However group 3 mice, the estrus uterus showed normal structure with lining epithelium and glands along with infiltration of neutrophils (Fig. 3c).

Discussion

The present study evaluated the protective effect of quercetin on Cd induced myometrial damage in mice especially on functional changes. The experiment was conducted on four groups of mice with treatment like control, Cd treated, Cd and quercetin co-administrated and quercetin alone respectively. Supplemented 30 ppm of Cd orally for 30 days to all the mice in group 2 to induce Cd toxicity. The dose used in the current study was sufficient enough to produce Cd mediated structural and functional changes in the uterus as per the previous studies in rat [9]. The dose for quercetin was chosen on the basis of previous studies with regard to the protective role of quercetin on Cd induced toxicity [19]. There was an increased frequency and amplitude of oxytocin induced contraction in Cd treated estrus mice than the control which could be possibly due to the estrogen mimicking effect of Cd on the uterus [8, 23]. This is in accordance with a previous study stating the metalloestrogenic effect of Cd which leads to the stimulation of estrogen receptors in the uterus, resulting in increased frequency and amplitude of oxytocin induced contraction [24]. There are studies mentioning the relation between estrogen administration and oxytocin action. The *in vitro* studies showed an increased oxytocin mRNA levels in the rat myometrium after 48 hours of estrogen administration [25]. This might be the reason for increased response of Cd treated myometrium to oxytocin, as Cd is having metallo-estrogenic behavior. There is increased frequency and amplitude of contraction of quercetin group than the control group. The quercetin is reported to have estrogenic property as it is a phytoestrogen and the above observation in our study is in accordance with the previous study stating an increased frequency and amplitude of contraction by the quercetin in onion peel extract on mice uterine strip [21]. The simultaneous administration of quercetin along with Cd decreases the amplitude of contraction than the Cd group which can be correlated with the metal chelation and relaxant effect of quercetin on smooth muscles. Ravichandran *et al*. [12] reported the metal chelation ability of quercetin, as they found high stability constant for the cadmium-quercetin complex [12]. Moreover, there are studies demonstrating the inhibitory action of quercetin on intracellular calcium induced contractions by $PGF_{2\alpha}$, oxytocin, carbachol, KCl as well as blockage of the influx of calcium through receptor operated calcium channels (ROCs) and voltage operated calcium channels (VOCs) [16]. Thus, both the metal chelation ability and relaxation effect of quercetin on smooth muscles on simultaneous administration with Cd could be the main factor for the less contraction amplitude in cadmium & quercetin group in our study. Thus, quercetin shows a contradictory behavior in single administration and co-administration with Cd with regard to the myometrial contraction induced by oxytocin. The mechanism of this conflicting behavior of quercetin is not understood and hence, it becomes a matter of subject for future researches.

Several histo-pathological changes have been observed in the uterus of Cd treated mice such as degenerative changes in the glandular epithelium along with infiltration of neutrophils and extensive desquamation of lining epithelium of uterus. The simultaneous treatment with quercetin along with cadmium almost restored the normal intact structure of uterus with normal epithelium and endometrial glands. Nna *et al*. [20] also reported the reversal of histopathological changes of cadmium by quercetin in rats treated with $CdCl_2$ (5 mg/kg BW/ day) for 14 days. Further studies on this are warranted to elucidate the mechanism behind the reversal of Cd induced structural damages by quercetin.

Table 1: Mean EC50 values of oxytocin induced dose frequency and amplitude of contractile response on estrus mice myometrium of various groups of mice.

Group	Treatment	Frequency	Amplitude
1	Control	$2.013x10^{-12\ a}$ (0 to $2.329x10^{-12}$)	$2.039x10^{-12\ a}$ (0 to $2.371x10^{-12}$)
2	Cadmium	$8.864x10^{-13\ b}$ ($8.064x10^{-13}$ to $9.717x10^{-13}$)	$9.344x10^{-13\ b}$ ($8.473x10^{-13}$ to $1.029x10^{-12}$)
3	Cadmium with quercetin	$1.048x10^{-12\ b}$ ($9.166x10^{-13}$ to $1.194x10^{-12}$)	$1.306x10^{-12\ c}$ ($1.142x10^{-12}$ to $1.489x10^{-12}$)
4	Quercetin	$8.402x10^{-13\ b}$ ($7.496x10^{-13}$ to $9.415x10^{-13}$)	$9.788x10^{-13\ b}$ ($8.633x10^{-13}$ to $1.11x10^{-12}$)

Values are expressed as Mean with range, n=6. Two way ANNOVA (GraphPad Prism 7.05). Means with different superscripts differ significantly, $p<0.05$

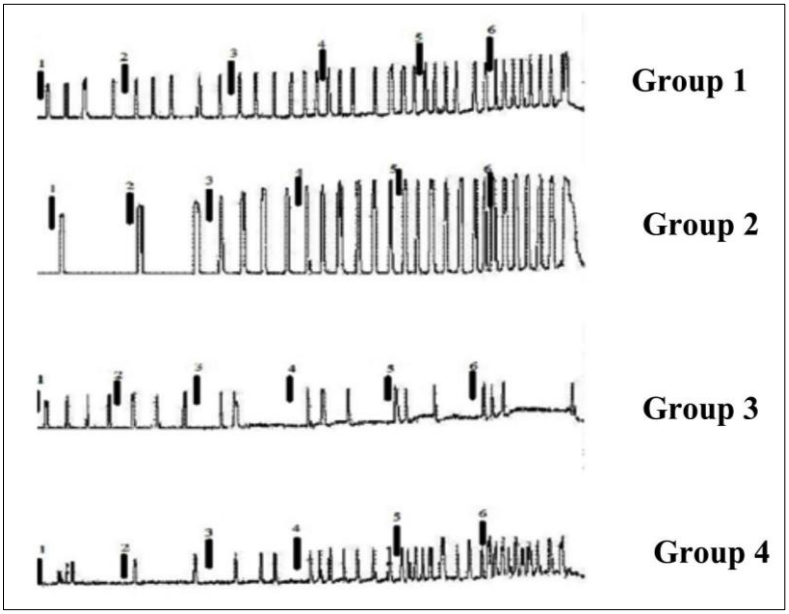

Fig 1: Cumulative contractile dose response to oxytocin in group 1 (control), group 2 (cadmium), group 3 (cadmium & quercetin), group 4 (quercetin). 1, 2, 3, 4, 5, 6 in the graph represents the concentrations of oxytocin from $0.11x10^{-12}$, $0.221x10^{-12}$, $0.441x10^{-12}$, $0.883x10^{-12}$, $1.76x10^{-12}$, $3.53x10^{-12}$.

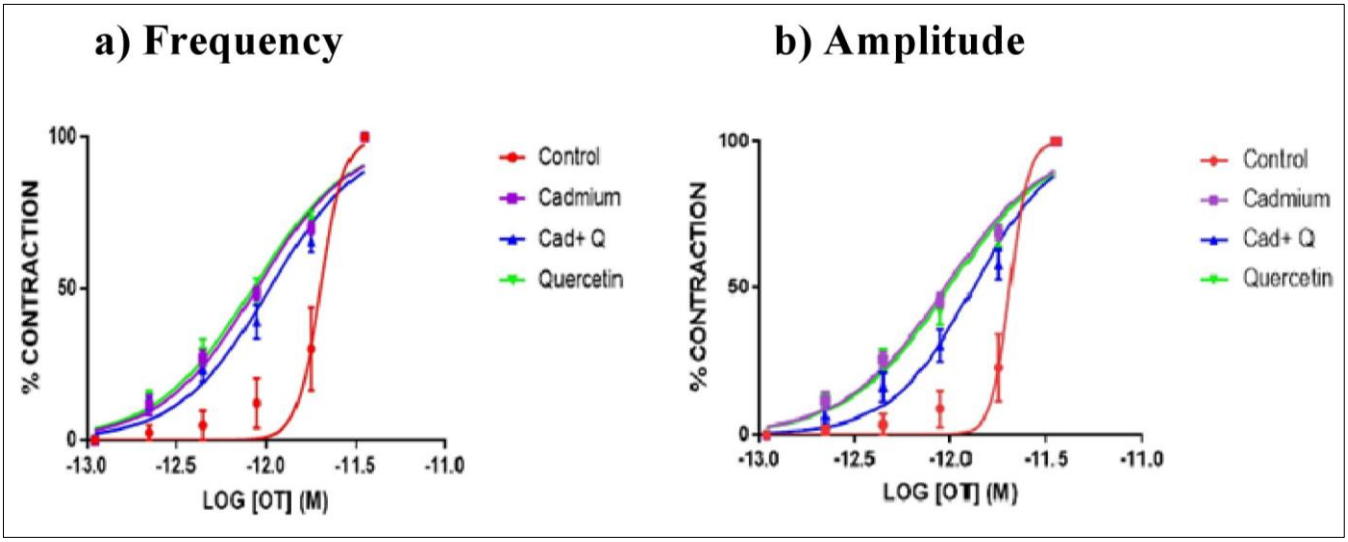

Fig 2: Log dose of oxytocin, frequency and amplitude of contractile response curves of estrus mice myometrium of various groups.

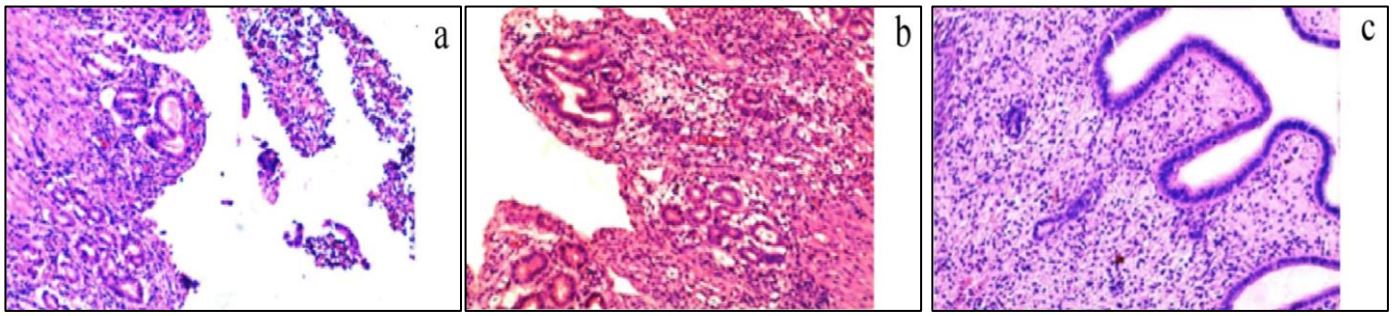

Fig 3: H & E staining section of mice estrus uterus.
a: Group 2 (Cadmium treated) showing degenerative changes and desquamation of lining epithelium.
b: Group 2 (Cadmium treated) congestion and degenerative changes in glandular epithelium
c: Group 3 mice (Cadmium along with quercetin) showing normal lining epithelium, glands infiltration of neutrophils (c)

Conclusion

Quercetin treatment helped to regain the structural damages caused by Cd on the mice uterus. The functional studies revealed a contradictory effect of quercetin on Cd induced changes in uterus, which may be due to the multiple effects of quercetin on uterus while administering alone and in co-administration. In this regard, our study is preliminary, giving some implications about the discrepancy of the action of quercetin in Cd induced changes. Hence, further studies are required for elucidating the contradictory behavior of

quercetin on cadmium induced myometrial functional changes.

Disclosure statement

The authors declare that there is no conflict of interest.

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