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Research Article

Effect of Quercetin on Cortisol and Oxytocin Levels, Oxytocin Receptor Gene Expression and Morphometry of Uterus in Rats Exposed to Bisphenol A

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Abstract

This study was carried out to evaluate effects of quercetin and bisphenol A (BPA), alone and in combination, on plasma cortisol and oxytocin levels, oxytocin receptor gene expression and morphometry of uterus in rat. After one week of acclimatization, twenty female rats were divided into four treatments as: control, received ethanol saline as injection intraperitoneally and gavaged hydroxypropyl-methylcellulose (HPMC). The second group injected with 50 µg BPA per kg body weight dissolved in ethanol saline two times per week for 4 weeks. The third group gavaged 30 mg quercetin per kg body weight suspended in aqueous solution of HPMC two times per week for 4 weeks, while the fourth group was treated with quercetin along with BPA. Two days after final injection and gavage, rats were anesthetized and uterine and blood samples were collected. BPA alone increased plasma MDA level, decreased total antioxidant capacity (TCA) and had no effect ($P>0.05$) on oxytocin and cortisol levels in the plasma compared with control group. Quercetin along with BPA decreased MDA level, but had no effect ($P>0.05$) on TCA, oxytocin and cortisol levels compared to control. Quercetin and BPA, alone or in combination, resulted in increase ($P<0.05$) oxytocin receptor gene expression. Treatment with quercetin along with BPA increased ($P<0.05$) endometrium, but had no effect ($P>0.05$) on myometrium and epimetrium. It was indicated that quercetin may be a potential compound for reducing oxidative stress damages in uterus layer and increasing blood oxytocin level and its receptor in uterine, but cannot completely ameliorate the negative effects of BPA.

Keywords: *Bisphenol, Oxidative stress, Oxytocin, Quercetin, Rat*

Bisfenol A Verilen Ratlarda Kuersetinin Kortizol ve Oksitosin Seviyeleri İle Oksitosin Reseptör Gen Ekspresyonu ve Uterus Morfometrisi Üzerine Etkileri

Özet

Bu çalışma birlikte ve ayrı ayrı kuersetin ve bisfenol A (BPA) uygulamalarının ratlarda plazma kortizol ve oksitosin seviyelerine, oksitosin reseptör gen ekspresyonuna ve uterus morfometrisine etkilerini araştırmak amacıyla yapılmıştır. Bir haftalık alıştırma dönemi sonrasında 20 dişi rat dört gruba ayrıldı. Kontrol grubuna intraperitoneal etanol-salin enjeksiyonu ile birlikte hidroksipropil-metilselüloz (HPMC) gavaj yoluyla uygulandı. İkinci gruba 4 hafta boyunca haftada iki kere olmak üzere etanol-salinde çözdürülmüş BPA 50 µg/vücut ağırlığı dozunda enjekte edildi. Üçüncü gruba 4 hafta boyunca haftada iki kere olmak üzere HPMC içinde çözdürülmüş edilmiş kuersetin 30 mg/vücut ağırlığı dozunda gavaj yoluyla verilirken dördüncü gruba kuersetin ile birlikte BPA verildi. Son enjeksiyon ve gavaj uygulamasından 2 gün sonra ratlar anestezi uygulanarak uterus dokuları ve kan örnekleri toplandı. Tek başına BPA uygulaması plazma MDA seviyesini artırırken total antioksidan kapasiteyi düşürdü (TCA). BPA uygulaması plazma oksitosin ve kortizol seviyelerinde ise konrol grubu ile karşılaştırıldığında bir değişime neden olmadı ($P>0.05$). Kuersetin ve BPA'nın birlikte uygulanması kontrol grubuyla karşılaştırıldığında MDA seviyesini düşürürken TCA, oksitosin ve kortizol seviyelerinde ise bir değişime neden olmadı ($P>0.05$). Kuersetin ve BPA'nın tek başlarına veya birlikte uygulanması oksitosin reseptör gen ekspresyonunun artmasına neden oldu ($P<0.05$). Kuersetin ve BPA'nın birlikte uygulanması endometrium kalınlığını artırırken ($P<0.05$) myometrium ve epimetriuma etki etmedi. Kuersetinin uterusda oksidatif stres hasarını azaltmada ve kan oksitosin seviyesi ile oksitosin reseptör gen ekspresyonunu artırmada potansiyel bir madde olabileceği ancak BPA tarafından oluşturulan negatif etkileri tümüyle ortadan kaldıramadığı belirlenmiştir.

Anahtar sözcükler: *Bisfenol, Oksidatif stres, Oksitosin, Kuersetin, Rat*

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INTRODUCTION

Bisphenol A (BPA), a monomer of plastics, causes cytotoxicity and adverse effects on brain, reproductive system, and liver, because of oxidative stress [1-3]. Patisaul et al.[4] reported that adolescent rats exposed to BPA exhibited significantly higher levels of anxiety, because of oxytocin reduction by BPA exposure. They also found that adolescent rats on the soy-rich diet did not exhibit anxiety. Finally, Patisaul et al.[4] suggested that the phytoestrogens may mitigate the effects of BPA.

Quercetin, a potent phytoestrogen, acts as an antioxidant and has been identified to occur naturally in many vegetables and fruits at relatively high concentrations [5]. Both BPA and quercetin have a week estrogen-like feature and disrupt or mimic the normal action of estradiol [6,7]. According to the previously reported studies [8,9], estradiol could increase the frequency and duration of uterine contractility. It was reported that BPA could stimulate uterine contraction via oxytocin-related pathway in immature rats [6]. Wolstenholme et al.[10] showed that BPA exposure results in change of oxytocin receptor gene expression in brain.

In the literature, information concerning effect of BPA and quercetin, alone or together, on blood oxytocin level and gene expression of oxytocin receptor in uterine is scarce. It was hypothesized that estrogenic activity of these compounds involved the oxytocin secretion and its receptor gene expression in the uterine. Therefore, the present study was carried out to evaluate the effects of BPA and quercetin, alone or together, on the concentration of cortisol and oxytocin levels, oxytocin receptor gene expression and morphometry of uterus in rat.

MATERIAL and METHODS

The study was approved by the Ethics and Research Committee of Islamic Azad University, Science and Research Branch (Approval date: 15.04.2014; no: 1424).

Chemicals and Reagents

Quercetin (CAS Registry No. 117-39-5) used in this assays was provided by Merck KGaA (Darmstadt, Germany). Quercetin was suspended in 10 mL of a 0.25% aqueous solution of hydroxypropyl methylcellulose (HPMC) to a final concentration of 30 mg quercetin/mL. Bisphenol A was purchased from Sigma Chemical Company (CAS Registry No. 80-05-7). BPA was dissolved in 5% ethanol solution. The solvents of quercetin and bisphenol were used as the negative control. All other materials were of analytical grade, obtained from standard sources.

Animals and Experimental Design

Twenty female Wistar rats (age, 28 days; average body weight, 80 g) were supplied from the Razi Institute (Karaj, Iran). The animals were kept in plastic cages, fed a standard diet and access to free and fresh water. Rats were maintained at 20 ± 2°C, and exposed to a 12 h light/dark cycle. The animals were quarantined for 7 days before beginning the experiments. Rats were handled in accordance with the standard guide for the care and use of laboratory animals. After one week of acclimatization to the laboratory conditions, rats were randomly divided into four treatments with five rats in each. Treatments were as: rats of the first group served as a control group and was injected ethanol saline intraperitoneally and gavaged HPMC solution, both two times per week (Sunday morning and Wednesday evening) for 4 weeks. The second group (BPA) received bisphenol A at a dose of 50 µg/kg body weight two times per week (Sunday morning and Wednesday evening) for 4 weeks intraperitoneally and received HPMC solution via gavage. Rats in the third group (Q) received quercetin at a dose of 30 mg/kg body weight two times per week for 4 weeks (Sunday morning and Wednesday evening) via gavage and injected ethanol saline intraperitoneally. The fourth group (Q + BPA) received in combination, bisphenol and quercetin. Treatment of quercetin was started five days (two gavages at days 1 and 4 of experiment) before bisphenol injection and continued throughout the experiment (eight gavages during 28 days). Other rats received aqueous solution of HPMC via gavage two times during mentioned period. Bisphenol A was injected eight times and quercetin was gavaged 10 times during experiment period. The doses of bisphenol and quercetin were calculated according to the animal's body weight before each administration.

Blood Sampling and Measurements

Two days after final injection and gavage, rats were anesthetized with diethyl ether and blood sample was collected into heparinized tubes from heart. The blood was then centrifuged and the plasma was collected and kept at -20°C for the determination of malondialdehyde (MDA), total antioxidant, oxytocin, and cortisol levels. Oxytocin level was measured using ELISA kits that provided from Enzo Life Sciences (Farmingdale, NY, USA). Cortisol level was measured using ELISA kit that provided from Mono Bind Company (Lake Forest, CA, USA). Plasma MDA level was determined using commercial kit (Pars Azmoon, Tehran, Iran) based on the method described by Dropper et al.[11]. Total antioxidant capacity of plasma was measured according to the method of Benzie and Strain [12].

Tissue Processing

Left uterine corn was fixed in buffered 10% formalin solution. After 72 h fixation, uterine corn was cut to 5 mm sections. Sections were dehydrated by passing them through increasing concentrations of ethanol (70%, 80%, 95% and 100%) for 1 min each, and then placed in xylene for 1 min to clear. Then sections were stabilized in paraffin and sliced by a microtome (Leica M20, Leica Microsystems,

Germany) with 6 µm thickness. At least 6 slices per section were fixed on a glass slides, processed by hematoxylin-eosin and then mounted by entellan rapid mounting medium. Slides were evaluated under computer-connected microscope using image analyzer software.

Quantification of Oxytocin Receptor Gene Expression

At the end of experiment, uterus horns of anesthetized rats were removed and immediately stored in liquid nitrogen for messenger RNA (mRNA) extraction using extraction kit (Vivantis Company, Malaysia). cDNA synthesis was done by reverse transcriptase according to the kit (Vivantis Company, Malaysia). Real time PCR for oxytocin receptor gene was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA).

Statistical Analysis

Data were subjected to analysis of variance procedures appropriate for a completely randomized design using the General Linear Model procedures of SAS software. Mean comparison was done using Tukey test at $P<0.05$.

RESULTS

The effect of different treatments on plasma MDA levels is shown in *Fig. 1*. There were significant differences (P=0.0003) among treatments for plasma MDA level. There was no significant difference for MDA level between rats received quercetin and the control group. Injection of BPA increased significantly MDA as compared with the control group. Rats received quercetin along with BPA had lower MDA as compared to those received BPA alone.

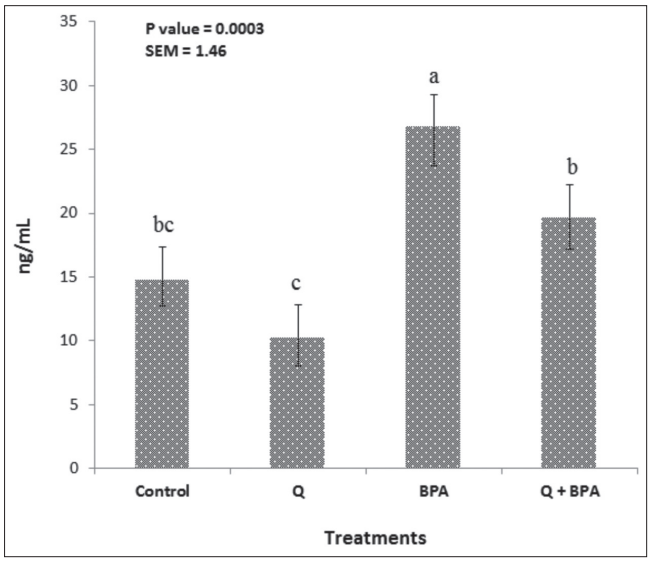

Fig 1. The effect of treatments (Q, quercetin; BPA, bisphenol) on plasma malondialdehyde level of rats

Şekil 1. Ratlarda uygulamaların (Q, kuersetin; BPA, bisfenol) plazma malondialdehit seviyelerine etkileri

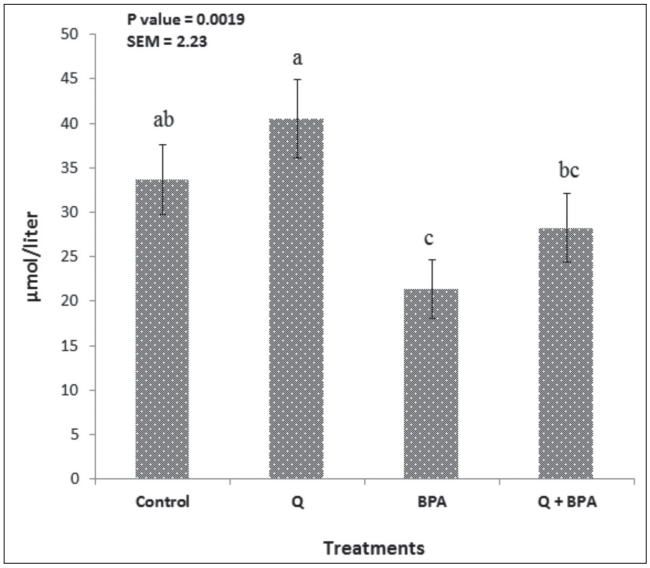

Fig 2. The effect of treatments (Q, quercetin; BPA, bisphenol) on plasma total antioxidant activity level of rats

Şekil 2. Ratlarda uygulamaların (Q, kuersetin; BPA, bisfenol) plazma total antioksidan aktivite seviyelerine etkileri

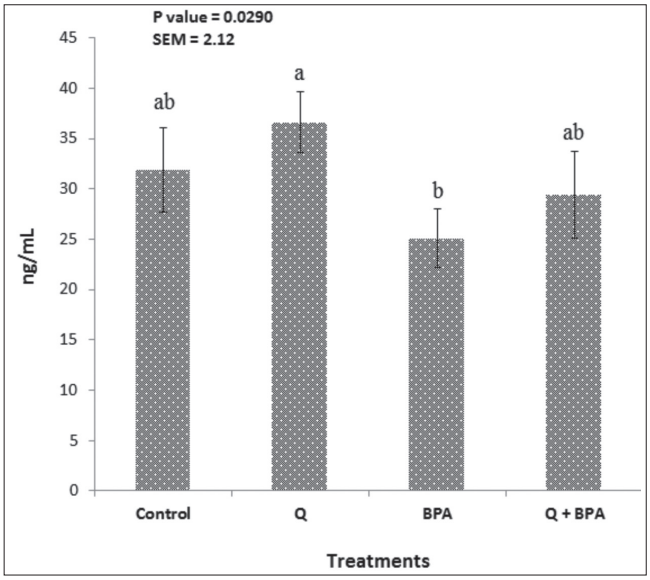

Fig 3. The effect of treatments (Q, quercetin; BPA, bisphenol) on plasma oxytocin level of rats

Şekil 3. Ratlarda uygulamaların (Q, kuersetin; BPA, bisfenol) plazma oksitosin seviyelerine etkileri

Total antioxidant capacity in plasma was differ (P=0.0019) among treatments *(Fig. 2)*. A significant difference found between rats received quercetin and BPA alone. Quercetin alone had no effect (P>0.05) on TAC, but injection of BPA decreased it as compared to the control group. Treatment of rats exposed to BPA with quercetin could not increase (P>0.05) the plasma antioxidant capacity as compared with those received BPA alone.

Plasma oxytocin level of rats is presented in *Fig. 3*. Injection of BPA alone reduced the oxytocin level

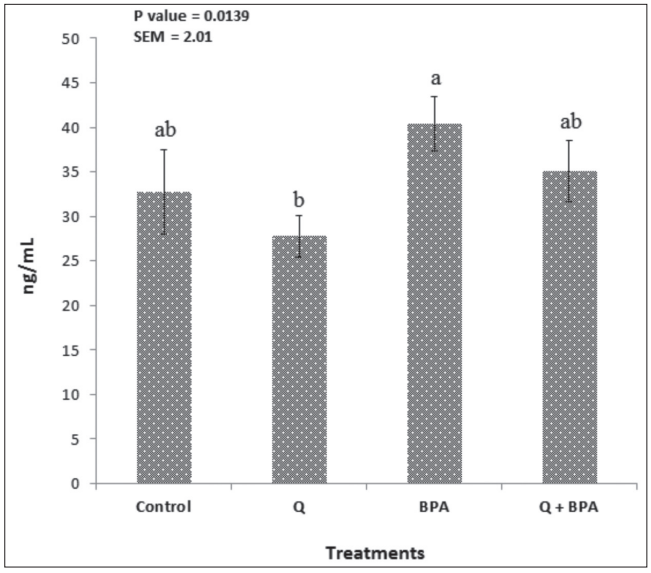

Fig 4. The effect of treatments (Q, quercetin; BPA, bisphenol) on plasma cortisol level of rats

Şekil 4. Ratlarda uygulamaların (Q, kuersetin; BPA, bisfenol) plazma kortizol seviyelerine etkileri

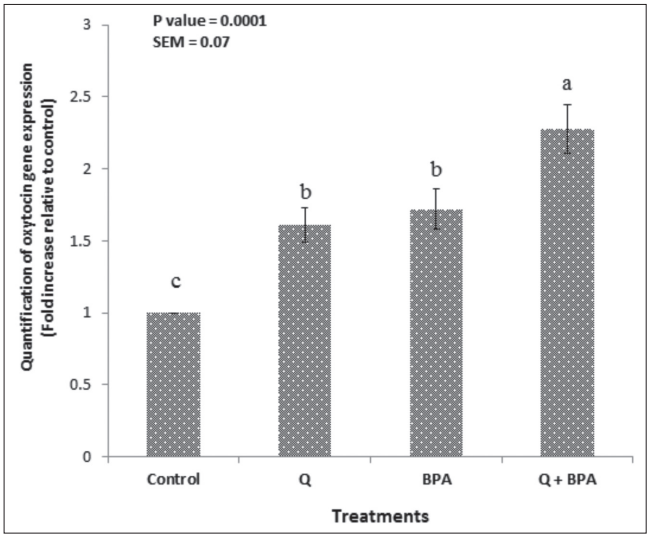

Fig 5. The effect of treatments (Q, quercetin; BPA, bisphenol) on gene expression of oxytocin receptor in uterine

Şekil 5. Ratlarda uygulamaların (Q, kuersetin; BPA, bisfenol) oksitosin reseptörü gen ekspresyonu *üzerine* etkileri

as compared to quercetin alone (P=0.029). Treatment with quercetin along with BPA had no significant effect (P>0.05) on oxytocin level as compared with the control group and also those received these compounds alone.

There was a significant difference (P=0.0139) for plasma cortisol level among treatments *(Fig. 4)*. BPA increased the plasma cortisol level compared to quercetin. Administration of quercetin to rats exposed to BPA could not decreased (P>0.05) cortisol level as compared to those received BPA alone.

Table 1. *The effect of different treatments (Q, quercetin; BPA, bisphenol) on diameter of various layers of uterine*

Table 1. *Uygulamaların (Q, kuersetin; BPA, bisfenol) uterus katmanlarının kalınlıklarına olan etkileri*

Treatments	Endometrium µm	Myometrium µm	Perimetrium µm
Control	255±15.7^{b}	275±10.7^{ab}	18.3±2.07^{b}
Q	355±17.5^{a}	307±13.1^{a}	23.6±2.08^{a}
BPA	193±16.2^{c}	260±12.0^{b}	11.5±1.52^{c}
Q + BPA	238±15.1^{b}	262±16.1^{b}	13.6±1.32^{bc}
SEM*	9.29	7.61	1.032
P value	0.0001	0.0089	0.0001

*^{a,b,c} Means without a common superscript letter differ within each part of a column (P<0.05); * SEM: standard error of means*

The gene expression of oxytocin receptor in uterine is shown in *Fig. 5*. The gene expression was differ (P=0.0001) among treatments. Administration of quercetin and BPA, alone or in combination, resulted in increase the expression of oxytocin receptor gene. Quercetin and BPA in combination had higher impact on gene expression.

The effect of different treatments on various layers of uterine is shown in *Table 1*. Treatment with quercetin resulted in significant increase and exposure of rats to BPA resulted in significant decrease in various layers of uterine. Treatment with quercetin along with BPA significantly increased endometrium, but had no effect on myometrium and epimetrium.

DISCUSSION

The main objective of this study was to evaluate the effect of bisphenol and quercetin administration, alone and together, on plasma cortisol and oxytocin levels, oxytocin receptor gene expression and morphometry of uterus in rat. In the literature, there was limited information about the effects of administration of bisphenol and quercetin, alone or in combination, on these parameters in animals and we conducted this research with hypothesis that quercetin could ameliorate the adverse effects of bisphenol.

The level of plasma MDA in rats exposed to BPA increased and in those received quercetin decreased. In agreement to our results, Sangai et al.[13] reported a significant increase in the serum MDA level in rats exposed to BPA compared to the control group. Exposure of animals to BPA resulted in increase of reactive oxygen species and decrease in antioxidant content and activity [14-16]. A study [17] revealed that BPA increase the free radical formation and decrease body ability to detoxify reactive oxygen species. It was reported [18] that BPA can induce cytotoxicity by impairing mitochondrial function and a consequent decrease in the cellular levels of ATP. Thus, changes in energy metabolism and glutathione redox balance could

be considered as potential mechanisms for inducing adverse effects by bisphenol A. This condition leads to lipooxidation and cell membrane damages and finally increases in plasma MDA.

In this study, BPA treatment caused a statistically significant reduction in total antioxidant capacity compared with the control group. In a study [13], BPA treatment reduced the activities of catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase, also glutathione and total ascorbic acid contents compared with control. Reduction in anti-oxidants activity and content resulted in decrease of plasma total antioxidant activity.

Co-treatment with quercetin and BPA could not alleviate the changes in plasma MDA level, as well total antioxidant activity. It decreased plasma MDA, but its effect was not significant. In contrast to our results, Sangai et al.[13] reported that quercetin could ameliorate the oxidative stress created by BPA through lowering MDA levels and increasing enzymatic and non-enzymatic anti-oxidants in mice. This discrepancy may be related to higher dose of quercetin, as they treated mice with 60 mg/kg body weight. Another study [1] showed that concurrent addition of bisphenol A and quercetin caused concentration-dependent amelioration in bisphenol A-induced cytotoxicity. Also, it was shown [13] that treatment with quercetin along with bisphenol A could significantly ameliorates bisphenol A-caused changes in the activities of ATPase in liver and kidney of mice. The restoration of ATPase activity suggests the ability of quercetin to protect the sulfydryl group from oxidative damage through inhibition of lipid peroxidation [18]. In our study, the diameter of uterus layers for rats exposed to BPA decreased, because of oxidative damage. Quercetin had positive effect on them by antioxidant activity and co-treatment results in ameliorating the negative effects of BPA.

Plasma cortisol level decreased in rats treated with quercetin as compared with the control group. A study demonstrated [19] that during times of prolonged stress, quercetin has been shown to suppress the release of cortisol. Over time the quercetin dose had the strongest suppression on cortisol levels. They then examined the rats' brains and observed that quercetin can inhibit the release of corticotropin-releasing hormone [20]. One of the reasons for decrease in cortisol level with quercetin treatment is elevation of oxytocin. Oxytocin is a hormone that helps relax and reduce blood pressure and cortisol levels.

The plasma level of cortisol increased in group received BPA. Increases in cortisol level in rats received BPA may be related to estrogenic activity. The study of Edwards and Mills [21] showed that estrogen administration lead to elevated plasma cortisol level. An interesting study [22] demonstrated that bisphenol A, similar to estrogen [23], could increase cortisol production by enhancing phosphorylation of CREB (cAMP response element-binding protein) in normal human adrenocortical cells. Another study [24] showed that BPA could induce corticotropin-releasing hormone expression in the placental cells. A study [25] demonstrated that BPA exposure to the dam throughout gestation and lactation led to elevated corticosterone concentrations in female litters. Further, BPA-treated female rats exhibited higher basal corticosterone than control [26]. Also, Kawabata et al.[27] demonstrated that quercetin have suppressive effect on acute stress-induced hypothalamic-pituitary-adrenal axis response in rats. Quercetin co-treatment with BPA could not ameliorate its effect on cortisol level. In the literature, there was no study concerning effect of BPA on blood oxytocin levels and uterus oxytocin receptor gene expression, but many studies were found about its effects on brain oxytocin level, brain oxytocin receptor gene expression and behavior.

Brains from 18.5 days post-coitus male mice exposed to BPA express less oxytocin, oxytocin receptor gene expression, and vasopressin than control males [28]. In their study, expression of oxytocin receptor gene in brain of female mice was higher than control that is consistent with our finding in uterine. The estrogenic effect of BPA may be related to increase in gene expression of oxytocin in uterine.

Quercetin treatment in this study increased plasma oxytocin level and its gene expression in uterine. Information about quercetin effects on oxytocin level and gene expression is limited. A study [29] showed that quercetin could inhibit the PGF2α-induced uterine contraction. They reported that quercetin could reduce oxytocin level that is inconsistence with our finding. Co-treatment of quercetin and BPA resulted in increase of oxytocin gene expression in uterine 2.3 folds higher that control. The additive estrogenic effects of these compounds may be resulted to increase the gene expression higher than their individual effect.

It was indicated that quercetin may be a potential compound for reducing oxidative stress damages in uterus layer and increasing blood oxytocin level and its receptor in uterine, but cannot completely ameliorate the negative effects of BPA.

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