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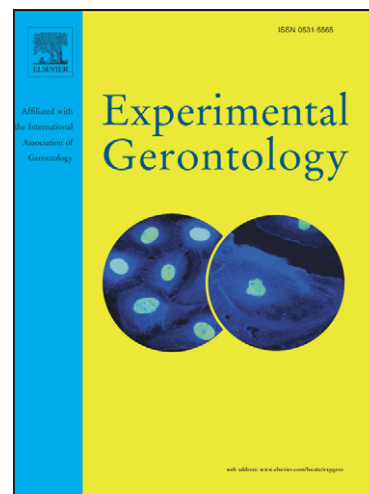
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Comparison of urodynamic effects of phytoestrogens equol, puerarin and genistein with these of estradiol 17 β in ovariectomized rats

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Anke Thielemann, Wolfgang Wuttke, Marcus Wuttke, Dana Seidlova-Wuttke

Department of Endocrinology, University Medical Center Göttingen, Georg-August-University Göttingen, Germany

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Corresponding author:

Prof. Dr. Wolfgang Wuttke
Department of Endocrinology,
University Medical Center Goettingen
Georg-August-University
Robert-Koch-Strasse 40
D-37099 Goettingen/Germany
Tel.: +49-551-396714
Fax: +49-551-396518
E-mail: ufkendo@med.uni-goettingen.de

Abstract

Whether urinary incontinence in the postmenopause can be prevented or delayed by estrogens is currently controversially debated. Ovariectomized (ovx) rats have been successfully used as models in the past years but plant derived substances with estrogenic effects in the lower urinary tract have not been studied so far. Therefore we compared the effects of a 3 months lasting oral administration of estradiol 17 β (E2) with those of the phytoestrogens equol, genistein and puerarin. They were then anaesthetized and catheterized with a biluminal catheter having one outlet in the bladder and another in the urethra at the level of the urethral sphincter. Urethral and bladder pressure were recorded during a 240 sec period of retrograde bladder filling (2 x 0.5 ml within 30 s with 1 min filling intermission).

Bladder and urethra pressures were highest in the E2>puerarin>equol>genistein treated animals. Phytoestrogen and E2 treatment resulted in consistently higher urethral than the bladder pressures during the filling period and in the filled status whereas bladder often exceeded urethral pressures in ovx controls. In summary, we demonstrate significant improvement of urethral closure mechanism under E2 and phytoestrogen administration that can be assumed to be beneficial for prevention or therapy of postmenopausal urge incontinence.

Introduction

Urinary incontinence is a typical but often hidden complaint of postmenopausal women (Irwin et al., 2006). While urge incontinence is based on nervous disregulation (so-called overactive or unstable bladder), stress incontinence is based on an insufficient closure mechanism of the urethral sphincter during increased intraabdominal pressure (Abrams et al., 2003). According to (Swanson et al., 2005) menopause is mostly associated with mixed incontinence in around 50%, stress incontinence in 34% and urge incontinence in 15% of cases.

Since the Women's Health Initiative (WHI) trial was stopped prematurely, hormone replacement therapy (HRT) for treatment of climacteric complaints is widely criticized (Ness et al., 2005). Treatment of women suffering from urinary incontinence with estrogens is also controversially discussed (Hendrix et al., 2005; Kim and Chancellor, 2006; Tseng et al., 2009).

The growing insecurity of women whether or not to practice HRT has increased the request for plant substances as hormone alternatives (Nelson et al., 2006). However, effects of plant substances such as the soy derived isoflavones which have structural similarity with 17 β -estradiol (E2) are not understood in detail. Genistein (Gen) is the main soy isoflavone and has the highest estrogenic potency of all soy constituents as tested in cell biological experiments (Chen et al., 2007). Effects of E2 in the urethra and urinary bladder are exerted via both estrogen receptor (ER) subtypes as the presence of both the ER α and ER β has been demonstrated in the urinary tract (Tikkanen and Adlercreutz, 2000). Equol (Eq) is a biologically active metabolite of daidzein (Daid), which is another main isoflavone of soy extracts. Eq is exclusively produced by intestinal bacteria and only about one third of people possess the proper intestinal flora (Setchell et al., 2002). Puer is an isoflavone derived from the root of *Pueraria lobata*, widely known as Kudzu in traditional Chinese medicine. (Boue et al., 2003) demonstrated the highest estrogenic activity from all Kudzu constituents for Puer. Since Puer is the glycosylated form of Daid it must be deglycosylated by intestinal bacteria, therefore, a depot effect of Puer may be assumed (Park et al., 2006).

Data regarding the therapy of urinary incontinence in menopause with phytoestrogens is rare. (Manonai et al., 2006) demonstrated a significant increase of complaints regarding urge incontinence and vaginal atrophy after a 12 week soy-free diet of 36 climacteric Thai women. A soy-rich diet however, in contrast could not relieve the pertained complaints. (Tomaszewski et al., 2003) showed that fibroblasts out of the pubocervical fascia of climacteric women have a higher proliferation activity under E2 and Daid compared to skin fibroblasts

1 It is known that a dedicate equilibration between detrusor muscle pressure of the urinary
2 bladder and the sphincter pressure of the urethra are of importance for continence
3 (DeLancey, 1990; Delancey and Ashton-Miller, 2004; Keane and O'Sullivan, 2000). A good
4 animal model to collect urodynamic data was shown previously to be the ovariectomized
5 (ovx) rat in which irregular contractions of the detrusor of the bladder occurred such that the
6 intravesicular pressure often exceeded the urethral pressure which resulted in urine leakage
7 (Seidlova-Wuttke et al., 2004). Hence, this overactivity of the bladder of ovx rats is
8 reminiscent of the instable bladder in women suffering from urge incontinence (Keane and
9 O'Sullivan, 2000). In our earlier study E2 treatment of ovx animals both increased
10 intravesicular and intraurethral pressures but in the urethra to a higher degree than in the
11 bladder. Therefore, unwilling voiding during the filling periods of the urinary bladder occurred
12 much less frequently and at lower volumes in the estrogen treated animals (Seidlova-Wuttke
13 et al., 2004). In the present study we investigated the effects of Gen, Eq and Puer on urethral
14 and urinary bladder pressures during and after 2 artificial filling periods and compared them
15 with those in ovx but otherwise untreated and with those in E2 treated animals.

17 **Material and Methods**

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20 The study was performed as part of a larger trial designed to investigate a number of
21 endpoints in chronically substituted ovariectomized rats. Permission to perform experiments
22 was obtained from the Regional Administrative Authority in Braunschweig (AZ 33.42502-
23 032/06). Sixty 3 months old female Sprague Dawley rats with an average weight of 227 g
24 were Purchased from Winkelmann (Borchen, Germany). Rats were kept under standard
25 conditions in the animal experimental facilities of the University of Goettingen (6 rats per
26 Makrolon® IV cage, 20° C, 50% relative humidity, light on from 6 am to 6 pm, water and food
27 ad libitum). After a two week acclimatization period on a soy- and phytoestrogen-free diet, all
28 experimental rats were ovariectomized under isoflurane anaesthesia. From this point in time,
29 rats in groups of 12 animals were kept under special diet supplied by Ssniff (Ssniff
30 Spezialdiaeten GmbH, Soest, Germany). In control group Co-SF, feeding with soy- and
31 phytoestrogen-free pellets continued. All other groups received pelleted food supplemented
32 with the phytoestrogens to be tested. On the basis of the twice weekly measured food intake
33 per cage the approximate daily intake of each individual rat and consequently the amount of
34 test substances could be calculated (Table I).

37 Urodynamic procedures

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39 After 12 weeks of food supplementation, rats were anaesthetised with isoflurane and the
40 urodynamic experiment was performed. In a supine position, the bladder was catheterized
41 transurethrally using a biluminal catheter (CS-14402: Pediatric Two-Lumen Central Venous
42 Catheterization Set with Blue FlexTip® Catheter: 4 Fr. x 5-1/8" (13 cm), Arrow). As soon as
43 the catheter reached the bladder during catheterization, residual urine volume was released.
44 Both openings of the biluminal catheter were connected to electromechanical pressure
45 monitoring devices and in that way to a computer for recording measured values. The
46 proximal catheter opening, positioned in the bladder, was connected to an infusion pump
47 with saline solution. Correct position of proximal catheter opening was checked by automatic
48 urine release and decreasing pressure values down to zero after catheterization. The distal
49 catheter opening was positioned in the urethra in juxtaposition to the internal sphincter.
50 Correct positioning was ensured by reaching highest possible pressure values during
51 catheter insertion. Having ensured correct catheter positioning, the urodynamic experiment
52 was started by infusion of 0.5 ml saline solution over 30s. Infusion was then stopped for 60s.
53 A second infusion period of 0.5 ml saline solution over 30s succeeded. The experiment
54 ended with a 120s observation period. Pressure values were monitored over the entire time
55 span of 240s. Animals were sacrificed after another life span of 2 weeks, uteri were removed
56 and wet weight was determined. Blood was collected for later determination of E2
57 concentrations by a commercially available assay (Elecsys Roche, Mannheim, Germany).

61 Analysis of urodynamic data

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1 Analysis of Urodynamic data was performed with GraphPad Prism TM Version 4.00 for
2 Windows (GraphPad Software, San Diego, California, USA). Figure 2 to 6 show that the
3 experiment consisted of different periods. The 2 infusion periods lasted 30 seconds and in
4 between these 2 infusion periods 2 times 30 seconds relaxation was allowed. Following the
5 2nd infusion period, recording of intravesicular (vP) and intraurethral pressure (uP)
6 commenced for four 30 second periods. During these periods, the mean values of 2 second
7 time segments per group were calculated and shown in Fig. 2 to 6. In addition, the
8 differences between urethral and intravesicular pressures ($\Delta uP - vP$) were calculated to
9 demonstrate putative beneficial effects of the different treatments. Hence, a large difference
10 between these two pressures indicated highest continence whereas low differences between
11 these two pressures or even negative differences were indicative of an incontinent situation.
12 Pressure values were analysed in 2-sec intervals and the means over 30-sec periods were
13 calculated followed by a one-way ANOVA using Dunnett's post test for significance. A p-
14 value of less than 0.05 was considered significant.
15

16 Results

17
18 Average body weight (BW) of animals at the beginning of the experiment was $227,29 \pm 2,79$
19 g SD. Mean daily food intake per animal was significantly decreased under Eq, Puer and E2
20 supplementation (Table I) in comparison to animals fed with unsubstituted chow and this
21 resulted in significantly decreased body weights (Table II) . This effect was not seen in the
22 Gen treated animals.
23

24 Supplementation with E2 resulted in serum E2 concentrations of 40.2 ± 4.6 pg/ml vs
25 undetectable levels in the control animals. The E2 treated animals had highest uterine
26 weights compared to the control group (Fig. 1) and increased uterine weights were also
27 recorded under Gen and Puer but not under Eq supplementation.
28

29 Fig. 2 details mean bladder and urethra pressures during and after the 2 filling periods in the
30 control animals (Fig. 2a) and in the E2 treated rats (Fig. 2b). In comparison to the control
31 group urethral pressure curves under E2 supplementation were consistently higher than the
32 intravesicular pressures except during the 2nd filling period when the mean urinary bladder
33 pressure exceeded the urethral pressure. In the control animals urethral and vesicular
34 pressures were significantly lower than in E2 treated animals during the first filling period and
35 the values overlapped at many time points as indicated by the frequent overlapping of the
36 SD. During and after the 2nd filling period the means of uP and vP were almost identical in
37 control animals whereas bladder pressure in the E2 treated rats dropped dramatically to
38 values well below those recorded in the urethra at all times after the 2nd filing period.
39

40 In Fig. 3, bladder and urethral pressure changes during the experiment's course are shown.
41 In comparison to the control group Co-SF (see Fig. 2a), urethral pressures in the
42 phytoestrogen treated animals fluctuate less during the infusion times and were similar to
43 those observed in the E2 treated animals. Therefore, as in the E2 treated animals pressures
44 in the urethra were usually higher than in the bladder. Fig. 4 depicts mean urethral pressures
45 per 30s interval. At each time point they were significantly higher under supplementation with
46 E2, Puer, Gen and Eq in all study periods compared to the control group.
47

48 In Fig. 5 mean bladder pressures per 30s intervals are shown. Under E2 and phytoestrogen
49 supplementation, bladder pressures were significantly higher compared to control group in
50 the first study phases, whereas values dropped significantly below control group's values in
51 the relaxation phases at the experiment's end.
52

53 Fig. 6 details mean differences of pressures in urethra minus those in the bladder in 30s
54 intervals. In cases of similar or even negative values the bladder pressures were higher than
55 in the urethral which facilitated unwilling voiding. In all study phases values for these
56 differences were lowest in the control animals and significantly higher in the E2 and
57 isoflavones treated animals.
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Discussion

1 Purpose of our experiments was to determine the influence of the phytoestrogens Eq, Puer
2 and Gen on the urodynamic profile of ovariectomized rats. Thus, consequences for therapy
3 of urinary incontinence after menopause may be determined. Special interest was focussed
4 on previously not studied urethral pressures (uP) and the ovariectomized rats were used as
5 they constitute an established model for menopause research (Wu et al., 2005).

6 Uterine weights as measurement for estrogenic effects in ovariectomized rats are proposed
7 in the OECD guidelines regarding as the uterotrophy assay (Owens and Koeter, 2003) In our
8 experiments, uterine wet weights were not only significantly increased in the Estradiol group,
9 but also in animals treated with Puer, Gen and Eq. However, increase in those groups was
10 lower than under E2 and increase under Eq was not statistically significant. (Zheng et al.,
11 2002) supplemented different doses of Puer orally in mice and ovx rats for 5 to 9 days to
12 determine estrogen-like effects of Puer. Estrogenic effects were quantified by vaginal
13 cytology, uterine and ovarian weights and a dose-dependent stimulation of uterine weight
14 and vaginal cornification was demonstrated. Vaginal weight of mice previously treated with
15 E2 decreased under Puer administration; on this basis (Zheng et al., 2002) concluded that
16 Puer has estrogen-agonistic effects in estrogen depleted and estrogen-antagonistic effects in
17 E2 substituted animals. The estrogen-agonistic characteristics of Puer in this study are
18 comparable to the results of our trial.

19 OvX is known to result in obesity (Wade and Gray, 1979) and this can be prevented by
20 estrogens. This is confirmed in the present study and also the phytoestrogens had an obesity
21 preventive effect which was strongest for Puer>Eq>Gen whereby the effects of Gen and Eq
22 were not statistically significant. The observation that Gen has a relatively strong uterotrophic
23 effect but a minor obesity preventive effect is of interest and points to some tissue specificity
24 of this isoflavone. Puer with a molecular weight (MW) of 416 is the glycosylated form of Daid
25 (MW 254) and is deglycosylated by the intestinal flora (Rong et al., 1998). Hence, the active
26 principle is Daid and the mean daily uptake of 44 mg Puer would amount to 27 mg Daid after
27 deglycosylation. These amounts had similar uterotrophic effects as Gen but were much more
28 effective to prevent obesity. If this difference occurs also in postmenopausal women a
29 Puer/Daid containing preparation would be preferable to Gen containing products.

30 Catheterizing of the urinary bladder with the biluminal catheter of which one lumen was
31 located within the bladder the other within the sphincter of the urethra, allowed recording of
32 intravesicular and intraurethral pressure during two filling periods. This was elaborated in
33 previous experiments (Seidlova-Wuttke et al., 2004). As demonstrated there and confirmed
34 in the present experiments E2 had significant tonus stabilizing effects on both urethral and
35 vesicular pressures, which were both low in the ovariectomized animals. The present study
36 shows that from the 3 phytoestrogens tested, Puer had the strongest estrogenic activity in
37 urinary bladder/urethral system.

38 By investigating isoflavones contained in *Pueraria lobata* (Kudzu) with high-performance
39 liquid chromatography coupled to photodiode array and to mass spectroscopy, (Rong et al.,
40 1998) showed that Puer is the glycosylated form of Daid and they concluded that estrogenic
41 activity occurs only after deglycosylation by gut bacteria which takes time. It may therefore
42 be assumed that Puer has a depot effect. Such a depot effect of Puer would be a possible
43 explanation for the stronger estrogenic effects in the urinary tract in comparison to Gen or
44 Eq. As such depot effect cannot be assumed for Eq and Gen, the difference in pressure
45 levels may be explained in this way.

46 Voiding is the result of bladder pressure exceeding urethral pressure and this may occur
47 unwillingly which results in incontinence. Hence, the differences between urethral minus
48 bladder pressure are indicators of continence, if negative or close to zero, i.e. overlapping,
49 unwilling voiding may be the result. As illustrated paradigmatically in Fig. 2 vP and uP in ovx
50 control animals are considerably lower than in E2 treated animals which confirms our
51 previous observation. The increased bladder and urethral pressure are most likely due to
52 increased muscular activity as a higher muscle mass of the detrusor and sphincter and
53 higher blood supply was reported in ovx-E2 in comparison to untreated animals (Aikawa et
54 al., 2003; Lin et al., 2006a; Lin et al., 2006b; Lin et al., 2006c). It appears that the
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1 phytoestrogens have similar, though weaker effects in the detrusor than in the sphincter
2 apparatus.

3 Studies of estrogen effects in the urethra and the urinary bladder of animal origin are scarce.
4 Morphological changes in response to ovariectomy and E2 replacement have been reported.
5 For functional studies most authors used either isolated urinary bladder tissue strips or intact
6 bladders of ovariectomized (ovx) rats and rabbits, both kept under in vitro conditions, and
7 these few experiments indicated that E2 affects the function of the voiding mechanisms. It
8 was shown that detrusor contractility in rodents and its innervation are sensitive to estrogen
9 deficiency. Estrogen withdrawal causes an over reactivity of urinary bladder tissue, which
10 can be "smoothened" by estrogens, and it appears that β 2- or α 3-adrenergic receptor
11 subtypes may be involved in this process. This effect may involve sympathetic and
12 parasympathetic preganglionic neurons innervating the bladder, which express the estrogen
13 receptor of the α - and β -subtype. Owing to the presence of ER β in the urothelium and ER α
14 in the fibroblasts and detrusor muscle cells, a direct action of estrogens on these cell types is
15 also likely and, indeed, histological changes in ovx estrogen-replaced animals have been
16 demonstrated when compared to ovx controls.

17 In isolated rabbit bladder preparations weak estrogens such as genistein caused also
18 contractile response induced by muscarinergic/purinergic receptor activation (Ratz et al.,
19 1999) and in a recent publication it was shown that detrusor overactivity in ovx rats was also
20 reduced by an application of a mixture of commercially available isoflavones given orally at
21 approximately the same amounts as in the present study (Okada et al., 2009).

22 The temporal aspects of bladder and urethral pressures during and after the 2 filling periods
23 under the different treatments deserve also some discussion. The vP was similarly high
24 throughout the initial 30 sec filling phase and the following 60 sec in the treated groups. Even
25 though the absolute values for uP and vP were significantly higher in the E2 treated animals
26 during the 2nd 30 sec filling period which filled the bladder maximally with 1 ml, the Δ uP-vP
27 was lowest in all groups, hence in many animals the vP was higher than the uP which
28 indicated incontinence. Interestingly the Δ uP-vP in all following phases (in which the bladder
29 remained filled) remained low or even negative indicative for incontinence. This was neither
30 seen in the E2 nor in the phytoestrogens treated animals in which the vPs decreased
31 progressively while the uPs stayed stable. Hence, the Δ uP-vP stayed high indicating
32 continence. It was also interesting to note that the magnitude of Δ uP-vP after the 2nd filling
33 period did not differ significantly.

34 Using estrogen receptor binding assays, it was repeatedly shown that isoflavones have a
35 slightly higher affinity to ER β than to ER α (Kuiper et al., 1998; Mueller et al., 2004) though
36 their transactivational efficacy was similarly strong via both ER subtypes (Mueller et al.,
37 2004). Dominant occurrence of ER β in urethral epithelium of adult rats was shown by
38 (Makela et al., 2000) using immunohistochemistry and Northern Blot (Salmi et al., 2001)
39 found that ER β is predominant in urethral muscle of rats. Therefore, increased urethral
40 pressures under E2 and isoflavone supplementation might be an effect of their affinity to
41 ER β . As a result of high affinity, muscle tone of the urethral sphincter might rise and lead to
42 increased uPs. (Strauss et al., 1998) investigated estrogen-like effects of Gen on the
43 reproductive tract of male mice. Newborn and adult mice received Gen injections over 9
44 days. Control group received s.c. E2. Histology and quantitative RNA amount in urethral
45 epithelium showed an increase in c-fos, which is interpreted as typical estrogenic effect. E2-
46 like effects of Gen on urethral epithelium were thus shown. These results of (Strauss et al.,
47 1998) fit to our findings in which Gen significantly increased uPs in an estrogen-like way.

48 In the urinary tract our study showed strong E2-like effects under all isoflavones with some
49 minor quantitative differences. Differences in the urethral pressure minus bladder pressure
50 indication for the functionality of the urethral closure mechanism. In literature, the
51 incompetence of the urethral closure mechanism is seen as a major factor for incontinence
52 (Keane and O'Sullivan, 2000).

53 For all tested compounds, E2 and the phytoestrogens, a significant increase in the difference
54 of pressures (Δ uP-vP) could be shown. Since a higher uP level can improve urethral closure,
55 an improved urethral closure mechanism under E2 and the phytoestrogens can be
56 postulated. In control animals, vesicular pressure often exceeded urethral pressure such that
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1 a negative difference between the two pressures resulted which indicated an incontinence
2 situation. This was obvious during both filling periods as well as in each of the periods after
3 the second filling period.

4 Our results do not indicate the location of action of E2 and of the phytoestrogens. Presence
5 of estrogen receptors was not only shown for genitourinary tract but also for parts of the
6 central and peripheral nervous system involved in micturition (VanderHorst et al., 2001) and
7 (Woolley, 1999). Therefore, pressure changes due to investigated substance in our study
8 may reflect the total activity of the estrogens in all of the estrogen sensitive structures
9 involved in regulation of micturition. Taken together our data indicate urethral affects of E2
10 and of the phytoestrogens on continence behaviour of ovx rats. Future clinical studies need
11 to be performed to test whether such effects are also mediated in incontinent
12 postmenopausal women.

13 In summary, in the present experiments we demonstrated that oral treatment of
14 ovariectomized rats over a period of 3 months with E2 resulted in massively increased
15 uterine weights and reduced obesity. Similarly, though less potently, Gen and Puer had
16 significant stimulatory effects on the uterine weights.
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Figure Legends**Fig. 1**

Effects of 14-week feeding with soy-free food (Co-SF), or food containing Eq, Gen, Puer or E2 on uterine wet weight of ovx rats. Means and SD are shown. * $p < 0.05$ vs. Co-SF

Fig. 2

Mean bladder (vP) and urethral pressures (uP) in ovx animals kept under a) soy-free (Co-SF) or b) E2 enriched diet. The vertical lines show the segments (intervals) during which means of all values were calculated. Means \pm SD are shown.

Fig. 3

Mean vesicular pressure (vP) and urethra pressure (uP) in ovx animals kept under Eq, Gen or Puer enriched diet for 3 mo. Time flow after retrograde infusion of 0.5 ml saline solution at 0s and 90 s over 30 s (details see legend Fig. 1). For the sake of clarity only means are shown.

Fig. 4

Mean urethra pressures (uP) in experiment at phases of 30s; Means and SD are shown; in some cases SD were so small that they cannot be seen. * $p < 0.05$ compared to control group Co-SF

Fig. 5

Mean vesicular pressures (vP) in experiment at phases of 30s; Means and SD are shown; in some cases SD were so small that they cannot be seen. * $p < 0.05$ compared to control group Co-SF

Fig. 6

Mean differences of pressures in urethra minus bladder ($\Delta uP-vP$) in experiment at phases of 30s; a large difference indicates larger urethral than bladder pressure which suggests a higher continence. Consequently, a low or negative value indicates a higher predisposition for incontinence. In some cases SD were so small that they cannot be seen. Means and SD are shown; * $p < 0.05$ compared to control group Co-SF

Table 1

Mean daily food and substance intake, mean body weight (BW) at the end of the experiment.

Table 1

Group	Mean food intake per animal per day	Mean substance intake per animal per day	Mean BW at the end of the experiment (starting BW: 227,3 ± 0,9)
Co-SF	16.7 ± 0,6 g		325,4 ± 5,7 g
Equol	15,0 ± 0,5 g *	16,0 ± 0,18 mg	307,3 ± 5,0 g
Genistein	16.5 ± 0,5 g	16.5 ± 0,50 mg	322,3 ± 7,7 g
Puerarin	14.7 ± 0,4 g *	44.2 ± 1,20 mg	271,8 ± 4,0 g*
E2	14,0 ± 0,5 g *	0.06 ± 0,002 mg	278,3 ± 5,3 g *

Mean daily food and substance intake, mean body weight (BW) at the end of the experiment. * p<0.05 vs. Co-SF

Fig. 1

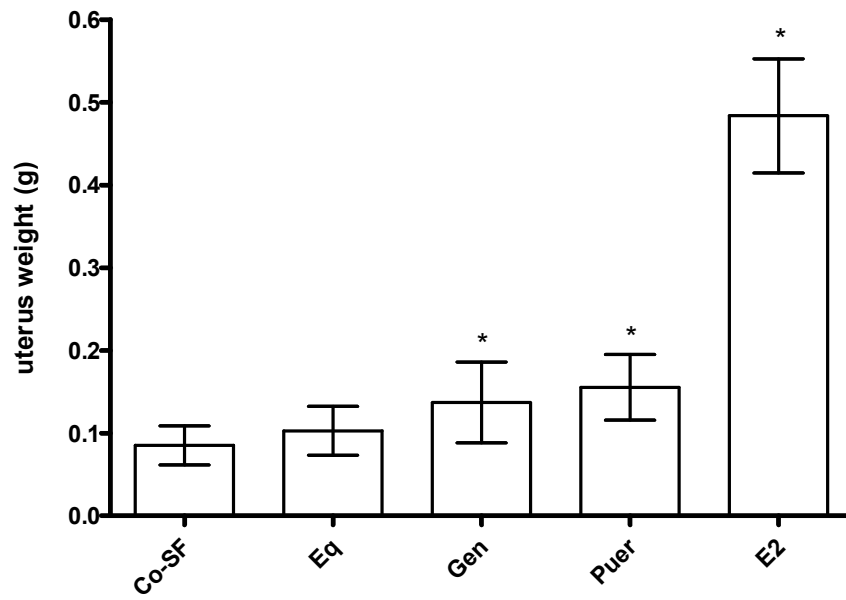


Fig. 2a

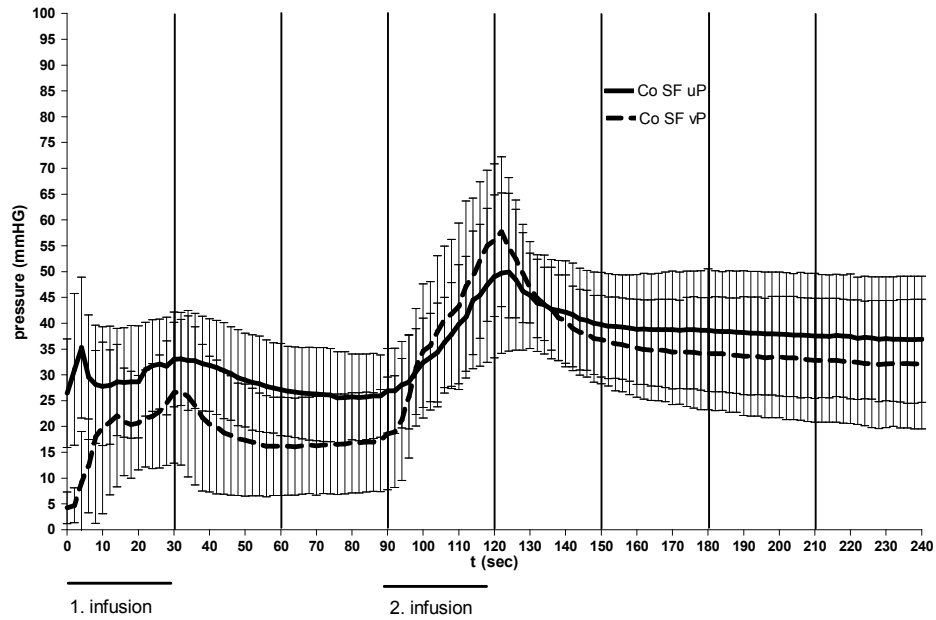


Fig. 2b

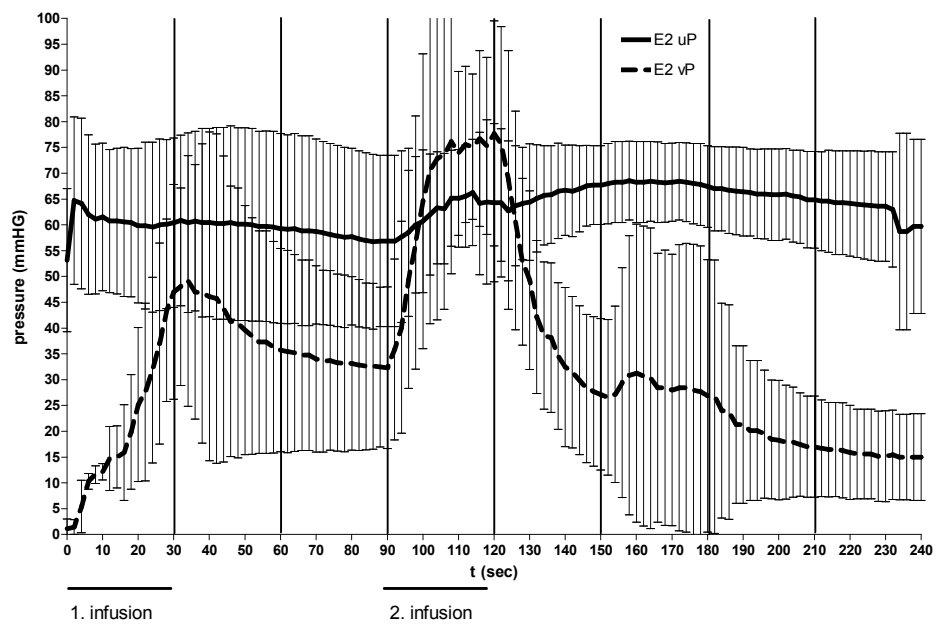


Fig.3

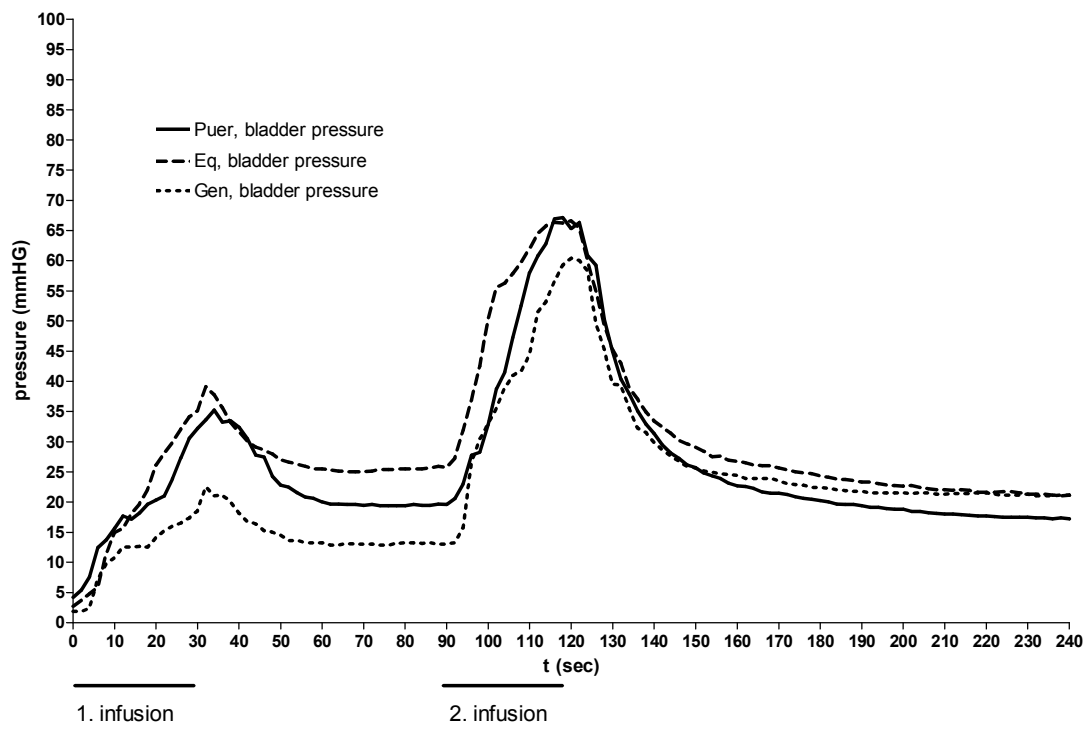


Fig. 4

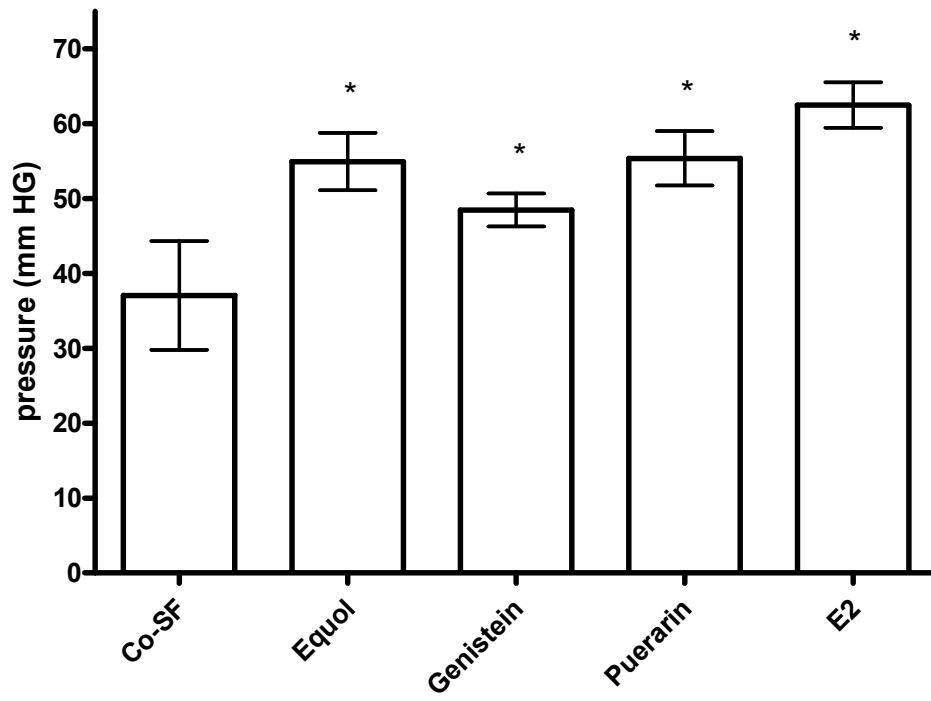


Fig. 5

