Rocz Panstw Zakl Hig 2017;68(3):303-307

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ORIGINAL ARTICLE

ESTROGENIC ACTIVITY OF COMMERCIAL MILK AS REVEALED IN IMMATURE HAMSTER UTEROTROPHIC ASSAY– PILOT STUDY

Lidia Radko[*]*, Maria Minta, Sylwia Stypuła-Trębas*

National Veterinary Research Institute,
Department of Pharmacology and Toxicology, Pulawy, Poland

ABSTRACT

Background. The risk for public health posed by endocrine disruptors present in food is relatively new issue. Our current understanding of human exposure is mainly based on the residue analysis of selected compounds. With such approach potential, effects of mixtures, including so-far unidentified compounds are not taken into consideration. Therefore, the knowledge of overall hormonal activity in food samples is of big importance.

Objective. Milk and dairy products are a rich source of estrogens but very rarely undergo testing for estrogenic activity. For this reason the rodent uterotrophic bioassay is one of the most useful tool. This preliminary study was conducted in immature hamsters to assess commercially available milk. The endpoint measured was uterine weight increase.

Material and methods. Fifteen-day old females received *ad libitum* throughout 7 days commercially available milk i.e. raw goat's, raw cow's, processed 3.2% UHT, and for comparison soy milk. The animals of negative control group received water but positive control group got 17β - estradiol (E_2) at the concentration of 100 ng/ml.

Results. All samples of milk showed estrogenic activity as follow: goat's >cow's >soy >processed milk. Significant increase of uteri weights were recorded in goat's ($p<0.001$) and cow's milk ($p<0.01$). However, the activity was approximately 5-fold lower than induced by 17β-estradiol. The ratio uterine weight/body weight (%) in negative control was 0.096%, in milk experimental groups ranged from 0.112% to 0.153% and in positive control this value was 0.493%.

Conclusion. The results suggest that commercially available milk has a weak uterotrophic activity. Further *in vivo* and *in vitro* studies are warranted to gain more insight into the estrogenic risk from milk and other dairy products.

Key words: *milk, estrogenic activity, immature hamster, uterotrophic assay*

STRESZCZENIE

Wprowadzenie. Zagrożenie ze strony związków hormonalnie aktywnych pobieranych z żywnością jest nowym wyzwaniem w ochronie zdrowia publicznego. Jak dotąd ocena narażenia ludzi opiera się na podstawie analizy pozostałości wybranych związków. Takie podejście nie pozwala na przewidywanie efektów oddziaływania związków niezidentyfikowanych oraz ich mieszanin. Dlatego też znajomość ogólnej aktywności hormonalnej w próbkach żywności ma duże znaczenie.

Cel pracy. Mleko i jego produkty są obfitym zewnętrznym źródłem estrogenów lecz rzadko podlegają badaniu w kierunku aktywności estrogennej. Jednym z bardziej przydatnych biotestów umożliwiających taką ocenę jest test wzrostu macicy wykonywany na gryzoniach.

Celem pracy była wstępna ocena aktywności estrogennej mleka dostępnego na rynku krajowym z wykorzystaniem niedojrzałych płciowo samic chomika złocistego.

Materiał i metody. Samicom w wieku 15 dni podawano *ad libitum* przez 7 dni mleko surowe kozie, krowie, przetworzone 3,2% UHT i dla porównania mleko sojowe. Zwierzęta kontroli negatywnej i pozytywnej otrzymywały odpowiednio wodę lub 17β- estradiol (E_2) w stężeniu 100 ng/ml.

Wyniki. Wszystkie rodzaje badanego mleka wykazywały aktywność estrogenną (mleko kozie>krowie>sojowe>3,2% UHT). Statystycznie istotny wzrost masy macicy zanotowano dla mleka koziego ($p<0,001$) i krowiego ($p<0,01$). Jednakże wartości te był około 5-krotmie mniejsze niż w kontroli pozytywnej. Stosunek masy macicy/masy ciała (%) w grupie kontroli negatywnej wynosił 0,096%, grupach otrzymujących mleko zawierał się w zakresie 0,112% -0,153%, a kontroli pozytywnej – 0,493%.

Wniosek. Uzyskane wyniki wskazują, że mleko dostępne w sprzedaży wykazuje aktywność biologiczną w teście wzrostu macicy. Aby uzyskać więcej informacji o potencjalnym ryzyku niezbędne są dalsze badania nad oceną aktywności estrogennej mleka z uwzględnieniem także przetworów mlecznych

Słowa kluczowe: *mleko, aktywność estrogenna, chomik, test wzrostu macicy*

[*] **Corresponding author:** Lidia Radko, National Veterinary Research Institute, Department of Pharmacology and Toxicology, Partyzantow 57, 24-100 Pulawy, Poland, phone: +48 81 889 3246; fax: +48 81 886 2595, e-mail: lidia.radko@piwet.pulawy.pl

INTRODUCTION

In last decades increasing concern has been raised over the adverse effects from exposure to endocrine disruptors (EDs) [14, 19]. So far, compounds with estrogenic-like action have attracted the most attention because they may play a role in the etiology of cancers and reproductive disorders [7]. Estrogens, both synthetic and natural, are ubiquitous in the environment and the main way of human exposure is food [2, 13] and water [30]. Within the last decade, it has become accepted that Food Contact Materials (FCMs) including food packaging are important contributors to human EDs exposure [6]. In recent years special attention was paid on the risk from milk and dairy products [17]. Since modern practices involve milking of cows during pregnancy significantly elevated endogenous estrogens and their metabolites may be detected in milk [8]. Their concentrations depend on cow`s physiological stage, age and type of diet [5, 9, 10, 17]. Additionally, xenoestrogens and phytoestrogens could be present in milk [16] but estrogenic steroids produced endogenously by the food producing animals possess a much more profound estrogenic activity [25, 27].

Our current understanding of human exposure to estrogens is mainly based on the analytical detection of few specific chemicals [26]. With such approach potential mixture effects and the effects caused by so-far unidentified compounds are not taken into consideration. Nowadays, assessment of the total estrogen load in the sample is increasingly used [1, 3]. Both, *in vitro* and *in vivo* short bioassays are introduced. The rodent uterotrophic bioassay, long considered the "gold standard" for determining estrogenicity, is identified as a preferred *in vivo* screen [22]. The assay is based on the principle that the growth phase of the uterus in the natural estrous cycle is under the control of estrogens. When the natural source of estrogens is not available, either because the animal is immature or because it has been ovariectomized, then the growth of the uterus becomes sensitive to external sources of estrogens. Due to animal welfare concerns regarding survival surgery the OECD recommends the use of immature animals. Moreover, past experience suggests that immature uterotrophic assay is more sensitive to estrogenic compounds than the ovariectomized model [15, 23, 28].

The aim of this preliminary study was to assess biological activity of commercially available milk in immature hamster uterotrophic assay which was previously found to be very sensitive to reference estrogen agonists [24].

MATERIALS AND METHODS

Animals, housing, diet

Golden hamsters (*Mesocricetus auratus*) of own colony were housed in clear polypropylene cages (48 x 26.5x 21 cm) and under controlled conditions: temperature (22±2°C), lighting (light: dark cycle 14h:10h) and humidity (40-60%). Distilled water and certified hamster phytoestrogen-free diet Altromin 7010 (ALTROMIN Spezialfutter GmbH&Co., Germany) were provided *ad libitum* until the pups were weaned. On the 15 postnatal day (PND) female pups were weaned, weight and randomly assigned to experimental groups (12 per cage).

Uterotrophic assays

The experimental protocol was approved by the Local Ethics Committee at the University of Life Sciences in Lublin (no. 72/2013).

The females were kept *ad libitum* for seven days (15-22 PND) on one of four different milk: processed milk (3.2%); raw milk samples from individual farm cow's or goat's; soy milk, and water (negative control) or 17β - estradiol (E_2) (Sigma-Aldrich, Poland) at the concentration of 100 ng/ml water (positive control).

Approximately 24 h after the last treatment the animals were killed by an overdose of pentobarbital. The uteri were carefully dissected and weighed immediately (wet weight) and after drying at 60°C for at least 24h (dry weight).

Statistical analysis

The results are presented as a mean and standard deviations (n=12). The statistical significance of the difference between the mean values was evaluated with analysis of variance (ANOVA). All statistical analyses were performed using the GraphPad Software (San Diego, CA, USA). Values of $P<0.05$ were considered significant.

RESULTS

As presented in Table 1 initial animal body weights were comparable among the 6 groups. Comparison of terminal weights showed that out of four experimental groups in two, receiving raw goat's and cow's milk animal body gains were significantly higher than in negative control group. In the same groups higher absolute wet and dry uteri weights were recorded. The induction over negative control wet uteri weights were 1.79-fold in group received goat milk and 1.61-fold in group received cow milk. The mean weight of absolute wet uterus weight in the positive control group was 7.43-fold over negative control.

Another endpoint taken into account in our study was the ratio uterine weight/body weight (UW/BW %). In comparison with negative control value (0.096 %) in all experimental groups the values were higher, and ranged from 0.112 % (raw cow's milk and soy milk) to 0.114 % (raw goat's milk). The highest value 0.153% was calculated for processed UHT milk.

The solution of E_2 (100 ng/ml) induced a significant effect ($P<0.001$) on all endpoints measured including the ratio UW/BW (0.493%).

Table 1. Uterotrophic effects of different kind of milk in immature hamsters

Animal body weight (g)			Uteri				
Group	Initial	Terminal	Wet uterine weight (mg)	Fold induction over control	Wet uterine weight/ body weight (%)	Dry uterine weight (mg)	Dry uterine weight mg/100g
Negative control – water	11.9 ± 1.6	18.0 ± 2.8	17.1 ± 3.7	1	0.096±0.022	3.4 ± 0.4	19.2±3.8
Raw goat'milk	14.0 ± 2.3	26.8 ± 4.2*	30.6 ± 8.6***	1.79	0.114 ± 0.024	5.5 ± 1.3***	20.6 ± 2.9
Raw cow's milk	12.7 ± 1.4	25.2 ± 2.8*	27.5 ± 5.3**	1.61	0.112 ± 0.021	4.9 ± 0.8**	19.6 ± 3.5
Processed 3.2% UHT milk	10.4 ± 2.5	14.4 ± 4.7	21.3 ± 5.2	1.25	0.153 ± 0.030*	4.6 ± 1.1	32.9 ± 6.8***
Soy milk	12.2 ± 1.7	21.7 ± 3.2	24.1 ± 5.4	1.41	0.112 ± 0.022	4.8 ± 1.0**	22.4 ± 4.1
Positive control- E_2 100 ng/ml	12.6 ± 2.8	26.0 ± 5.8*	127 ± 24.6***	7.43	0.493±0.065***	19.6±4.1***	76.3 ± 11.7***

Data is presented as mean ± SD (n=12); $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ *vs* negative control.

DISCUSSION

There is growing concern of whether or not exposure to the estrogens from milk can cause adverse effects in the consumer [11]. However, a number of publication have reported that hormones present in cow`s milk may be responsible for the adverse effect on human health. Available literature data on estrogenic activity of cow's milk and dairy products, either *in vitro* and *in vivo*, are inconsistent.

One of the most frequently cited limitations of *in vitro* tests for assessing health effects are qualitative and quantitative deficiencies in the biotransformation of test chemicals, in comparison with the *in vivo* situation [4]. In this place it is interesting to mention the results of our previous work *in vitro* in which the estrogenic activities of cow's milk samples without hydrolysis were below the detection limit, whereas in 50% of the deconjugated samples they were detectable and varied between 0.29 and 0.49 ng EEQ/ml [27]. To avoid 'false negative' results, *in vivo* bioassays are very desirable.

In the present study we evaluated activity of different kind of milk available on the Polish market. The model system was immature hamster uterotrophic assay. As it is recommended [22, 29] before conducting the assay the sensitivity of this model was confirmed in our laboratory in the previous experiments with reference compounds [24]. The animals were fed with phytoestrogen-free diet, Altromin which was shown not to affect the sensitivity of the uterotrophic assay [20]. In this study the variance in body weights at the start of the experiments was insignificant (Table 1) and these values were in line with our historical data for immature hamsters [24]. Significant differences (increase of terminal body weight and uterine weight) were recorded in groups receiving raw milk (goat's and cow's). In the same groups significant uterotrophic activity was observed. Comparing with the effects in positive control (E_2, 100 ng/ml) observed activity was low, however similar to those induced by weak estrogen BPA (*p.o.* 160 mg/kg/d ≈ 3days) what was revealed in our previous study [24].

We observed that significant differences were noticed in the groups in which wet uterine weight increase was at least 1.4-fold over control. The induction of uteri weights in raw milk groups was 1.79 and 1.61-fold over negative control (Table 1), and 1.76-fold for BPA [24], respectively.

Until now just few authors utilized uterotrophic assay to assess total estrogenic activity of milk. *Ganmaa* et al. showed that commercially available low-fat milk has uterotrophic effects in both young ovariectomized rats and sexually immature rats [12]. More recently commercial milk was also shown to have weak uterotrophic and also estrogenic effects on immature ovariectomized rats [31]. In opposite, lack of biologically active estrogens in commercial milk was confirmed after 2 week exposure of ovariectomized rats [11]. Similar results were obtained on gonadally intact immature mouse, however activation of the estrogen receptor in *in vitro* assay took place [21].

The current study on immature hamsters was designed in a similar manner to those previously published on immature mice [21]. Seven day oral *ad libitum* exposure of milk and reference estrogen agonist (E2) were applied. The increase of the mean uterus weight of immature hamsters in positive E2 control was 7.43-fold over control and was higher than those (approximately 5-fold) observed in immature mice by *Nielsen* et al. [21]. You can therefore believe that hamster model is more sensitive than mice.

It seems to be that the existed differences are due to different models used and different endpoints assessed.

CONCLUSIONS

1. The final conclusion of the study is that milk has a weak estrogenic activity.
2. Further studies are warranted to evaluate the significance of milk and dairy products as a source of estrogens for humans.
3. There is an urgent need to harmonize research in this field and the most important key learnings were defined from the Endocrine Disruptor Screening Program (EDSP).

Conflict of interest

The authors declare no conflict of interest.

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Received: 11.04.2017
Accepted: 14.07.2017