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Kefir as a Prevention of Arsenic-mediated Toxicity in Uterine Female Rats

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Abstract
Background: kefir is a fermented milk product that demonstrates numerous health benefits including antioxidant and immunomodulatory. Aim: to study the protective effect kefir on the expression of estrogen receptor alpha (ERα) in endometrial stromal cells and endometrial thickness on female rats that were exposed to arsenic. Methods: twenty-five female Wistar rats (*Rattus norvegicus*) were divided into five groups (CRL, As, T1, T2, T3). Control group (given a normal diet), As group (given the normal diet and exposed to arsenic trioxide 2 mg/kgBW/day). The T1; T2; T3 were exposed to arsenic trioxide 2 mg/kgBW/day and treated with different doses of kefir (1.25; 2.5; and 5 mL/kgBW/day, respectively) for 35 days. The rats of group As treated with arsenic trioxide only and group CRL served as control with normal feed in water. Cytological samples were taken after 35 days of treatment and examined every day to see the rat oestrus phase, and the proestrus phase of the oestrous cycle was chosen for termination. Uterine tissue fixed in 10% neutral buffered formalin for tissue preparation. ERα expression in endometrial stromal cells was analized using immunohistochemistry method, endometrial thickness was observed using histopathological methods. Results: significant reduction of ERα expression in endometrial stromal cells and endometrial thickness in female rats exposed to arsenic were observed in groups on treated rats ($p \leq 0.000$; 0.009, respectively). Conclusion: the administration of kefir in female Wistar rats exposed to arsenic had shown significantly differences on ERα expressions and endometrial thickness. The smallest dose of kefir (1.25 mL/kgBW/day) could increase ERα expression and endometrial thickness in female Wistar rats with arsenic exposure. Therefore kefir has protective effect related to female reproductive system.

Keywords: Arsenic, ROS, Kefir, Antioxidant, Endometrium, Estrogen Receptor Alpha

1. Introduction

Inorganic arsenic with high toxicity increasingly spread in the surrounding environment, such as air, water, industrial waste, cigarette smoke, and even food in the last decade (WHO, 2018). The widespread distribution of arsenic makes it difficult for humans to avoid its exposure (Sun et al., 2016). The World Health Organization

(2018) states that arsenic contamination is the biggest threat to global public health. The Agency for Toxic Substances and Disease Registry (ATSDR) mentions that exposure to high doses of arsenic can cause death, while exposure in a small dosage and for a long time can cause toxic, carcinogenic, and mutagenic effects (ATSDR, 2007; Tchounwou, Centeno, & Patlolla, 2004).

Exposure to arsenic, an Endocrine Disrupting Chemical (EDC), can disrupt the function and structure of the human reproductive system via the hypothalamus-pituitary-ovarian axis (HPO) pathway (Deyhoul, Mohamaddoost, & Hosseini, 2017; Rattan et al., 2017). Several studies reported the toxic effects of heavy metal arsenic (As) associated with decreased fertility and female reproductive health problems. In previous cross-sectional research by Lei et al. (2015) the concentration of arsenic in the blood of infertile women was significantly higher than that of pregnant women.

Decreased fertility and impaired female reproductive health associated with arsenic toxicity are primarily the results of oestrogen receptor damage and oestrogen signalling pathways disruption followed by morphological change and proliferation disruption of the endometrium (Chatterjee & Chatterji, 2010; Ronchetti, Bianchi, Duvilanski, & Cabilla, 2016; Sun et al., 2016). Oestrogen receptor alpha (ERα), the dominant mediator of oestrogenic action on endometrial tissue, is downregulated at the mRNA and protein levels by arsenic exposure. In addition, histologically, there is a degeneration of luminal epithelial cells and endometrial glands together with a reduction in the thickness of the uterine longitudinal muscle leading to a reduction in uterine thickness (Akram et al., 2010; Chatterjee & Chatterji, 2010). There was a significant decrease in endometrial thickness found in rats after the exposure of arsenic for 28 days in the study by Akram et al. (2010). Reproductive organs, especially the uterus, are one fertility determinant in which endometrium is receptive to embryo implantation (Strowitzki, Germeyer, Popovici, & von Wolff, 2006).

Arsenic bioaccumulates in essential organs, Sun et al. (2016) and Ronchetti et al. (2016) explained that arsenic accumulated in the anterior pituitary gland and gonads might block the development and function of reproductive organs. Several studies have stated that the dominant mediator of arsenic cytotoxicity occurred by the formation of oxidative stress responses and Reactive Oxygen Species (ROS) caused by the bioaccumulation of arsenic (Flora, 2011; Rao et al., 2017; Ronchetti et al., 2016; Sun et al., 2016). It causes an imbalance of endogenous antioxidants in the body. Ronchetti et al. (2016) and Rao et al. (2017) reported that the administration of external antioxidants could improve and prevent the adverse effects of arsenic toxicity.

Kefir is a probiotic product derived from the milk fermentation of goats and cows, using kefir grains with a complex microbiological composition. Kefiran, a type of potential exopolysaccharide in kefir grains, has active antioxidant activities. A potent antioxidant, kefir also functions as an antimutagenic, and antitumor, an anti-inflammatory, a radical scavenging system, and an oxidative stress-reducing agent (Chen et al., 2015; Farnworth & Mainville, 2008; Prado et al., 2015). Kefir is a probiotic, used in the appropriate dose, is expected to prevent damage to the endometrium due to arsenic toxicity through free-radical scavenging pathways. In addition, there is still a lack of research on the effect of kefir in preventing the toxicity of heavy metals, especially arsenic, to the female reproductive organs. Based on the explanation above, in this study, we aimed to investigate the effects of kefir on the female reproductive system, especially on the endometrium and ovaries of female Wistar rats (*Rattus norvegicus*).

2. Method

2.1 Chemical

Arsenic Trioxide (As2O3) powder is the product of Loba Chemie Pvt. Ltd., Mumbai, India. The powder dosage was 2 mg/kgBW/day (Mershiba et al., 2013). As much as 210 mg of Arsenic Trioxide (As2O3) powder was then dissolved in 700 mL of 0.9% NaCl using a magnetic stirrer at a temperature of 50º C for 3-4 hours until the arsenic powder dissolved completely.

2.2 Kefir

Kefir used in this study is made of fermented milk of goat, which was purchased from the Natural Probiotic Laboratory, Faculty of Animal Husbandry, Brawijaya University, Malang. Upon the receiving kefir was aliquot and frozen in -80º C until use.

2.3 Animals and experimental protocols

A female Wistar rat (*Rattus norvegicus*) aged 8-10 weeks weighing 100—150 g was purchased from Rattus Breeding Centre in Malang. Rats were kept and adapted in the laboratory for seven days at constant room temperature (20-25ºC) with regular light and dark cycles. The cycles include the 12-hour light cycle and the 12-hour dark cycle. For the housing of the animals, four rats were kept in a plastic box, covered with wire and given a husk mat. Every three days, the researchers change the husk mat to prevent infection. The dietary requirement for adult rats was 45 g/day/head. The regular diet consists of 67% Comfeed PAR-S (PT JAPFA *COMFEED* INDONESIA, Jakarta, Indonesia), 33% flour, and water as well as *ad libitum*. The experimental protocol has been reviewed and approved by the Ethical Committee of the Faculty of Medicine, Brawijaya University (Certificate No. 95/EC/KEPK-S2/04/2020).

Twenty-five female rats (*Rattus norvegicus*). (n=25) were divided into five groups (5 rats per group) : Control group, given a standard diet and 1 mL Normal Saline per day/rat ; As group, exposed to As_2O_3 2 mg/kgBW/day, and, T1, T2, and T3 groups, exposed to As_2O_3 and kefir at doses of 1.25, 2.5, and 5 mL/kgBW/day, respectively, for 35 days (Fahmy & Ismail, 2015). Arsenic and kefir were administered orally for 35 days.

The proestrus phase of the oestrous cycle was chosen for termination. Thus, after day 35 cytological samples were taken and examined every day to see the rat oestrus phase. The process included staining the samples using blue methylene, observing the morphology of epithelial cells under a microscope and choosing the proestrus phase of the oestrous cycle for termination (Khatun, Maity, Perveen, Dash, & Chattopadhyay, 2018). On the proestrus phase, rats were sacrificed through cervical dislocation and uterine tissue was collected.

2.4 Tissue Preparation

Uterine tissues are fixed in 10% neutral buffered formalin for at least 24 hours to prevent autolysis and tissue decomposition. After fixation, the tissue was sliced into 2—3 mm using a scalpel and inserted the tissue into the cassette and labeled it. Then, the tissue was processed into microscopic parts through the dehydration, clearing and embedding stages. Dehydration processed the tissue by immersing it in several concentrations of alcohol to remove water and formaldehyde. Then, the clearing process proceeded with the cleansing of the remaining alcohol. The final step was embedded, which was inserted the tissue into a paraffin block to produce a thin piece of tissue. A microtome (4-5 micrometres) formed a paraffin block to construct a thin layer of tissue and put it in a warm water bath (Llewellyn, 2013).

2.4.1 The Histopathological Preparations

The histopathological preparations use the paraffin block method. The method involved rinsing the fused uterine tissue for at least 1.5 hours. The tissue was added with 70% alcohol for 1 hour, 80% alcohol for 1 hour, 99% alcohol for 1 hour and absolute alcohol for 2x1 hours. Then the tissue was immersed in a mixture of xylol; absolute alcohol = 1: 1 for 0.5 hours, and xylol PA for 2x30 minutes. The tissue was cut as thin as possible and put in melted paraffin; xylene = 1: 1 for 1 hour, paraffin (54-58) for 2x1 hour. The liquefied paraffin was placed into a cube-shaped mould, placing the paraffin in the desired position. After watering it again with sufficient paraffin, the chilled paraffin block was outed from the mould. Then, the back of the paraffin block was on the microtome. The indicator position should show the thickness of the cut in 4-5 micrometres. The cut paraffin, in ribbons form, was then transferred into a warm water bath (45ºC), stretching the paraffin parts. A preparate or glass object was on the underside of the selected tissue and then removed from the warm water bath, after which it was allowed to dry for 24 hours, preferably in a thermostatic laboratory oven at 37° C (Slaoui & Fiette, 2011)

2.5 Immunohistochemistry (IHC)

During uterine tissue preparations, retrieval antigen was given using citrate buffer, rinsed using PBS for 3x5 minutes. After that, the endogenous enzyme block used 3% of H2O2, producing primary antibody (*oestrogen receptor alpha antibody* (C-311): sc-787 Santa Cruz), then dissolved in PBS 5% with a ratio of 1: 100 and incubated in a staining chamber at 40C overnight. PBS was essential for washing. Then the preparate uterine transversal section was diluted with biotinylated anti-rabbit immunoglobulin G and diluted in PBS with a ratio of 1: 200, containing 1% bovine serum albumin, for 2 hours. Horseradish peroxidase streptavidin (SA-HRP) was applied in a dilution of 1:200 in PBS-BSA for 2 hours. Immune precipitates were visible with diaminobenzidine (DAB). After staining the immune precipitate for 5 minutes, the preparate was rinsed in distilled water and applied a counterstain with Meyer's haematoxylen and mounted in Entellan.

The following process was observing ERα expressions quantitatively. The immunohistochemical stain was observed and photographed using an Olympus BX51 microscope with a magnification of 400 times and proceeded using the software image J using the Cell Counter plugin. ERα expressions were expressed in the number of endometrial stromal cells, brown in the nucleus of the cells, in ten visual fields.

2.6 Endometrial Thickness Measurement

The examination endometrial thickness was done by staining Haematoxylin Eosin (H&E) uterine samples were fixed in 10% formalin buffer. A thin tissue, cut by a microtome of 2-3 mm thickness, was inserted into a cassette. The result is a thin tape, dipped in Haematoxylin, seen under the microscope Dot slide Olympus XC 10 and then measured with Dot Slide Software.

2.7 Statistics

The researchers analysed the data using SPSS 23 software for windows. Oestrogen receptor alpha (ERα) expression and endometrial thickness were parametric numbers and presented by measuring mean and standard deviation (SD). The researchers used ANOVA to compare the mean of variables in the control group with the mean of the treated group. The result showed a significant difference with a p-value <0.05. The data was then analysed using post hoc analysis with the least significant difference (LSD).

3. Results

3.1 ERα Expression in Endometrial Stromal Cells

The study described the effects of kefir on ERα expression by calculating the cells mean in each treatment group. There were statistically significant differences in ERα expression (p<0.000) (Table 1).

Table 1: Effect of kefir on ERα expression in endometrial stromal cells of female Wistar rats (*Rattus norvegicus*) exposed to arsenic

Variable	Group	Mean ± SD	*p- value*
Expression of ERα	CRL	81.29 ± 3.15[a]	
	As	70.12 ± 5.50[b]	
	T1	83.86 ± 4.85[a]	0,000[*]
	T2	83.33 ± 2.19[a]	
	T3	82.72 ± 1.42[a]	

Note. Expression of ERα (%); CRL=control group; As=Arsenic group (As_2O_3 2 mg/kgBW/day); T1=arsenic+kefir 1.25mL/kgBW/day; T2=arsenic+kefir 2.5 mL/kgBW/day; T3=arsenic+kefir 5 mL/kgBW/day. * = significantly different

IHC results demonstrated a significantly lowered expression of ERα in As group compared to the control group (Figure 1A and B). Rats in T1-T3 group showed an increase in number of stromal cells expressing ERα, which was significantly different from the As group (Figure 2). However, there was no significant difference among the three doses of kefir. Therefore, the interpretation of the data shows that the three doses of kefir have the same ability to increase ERα expression.

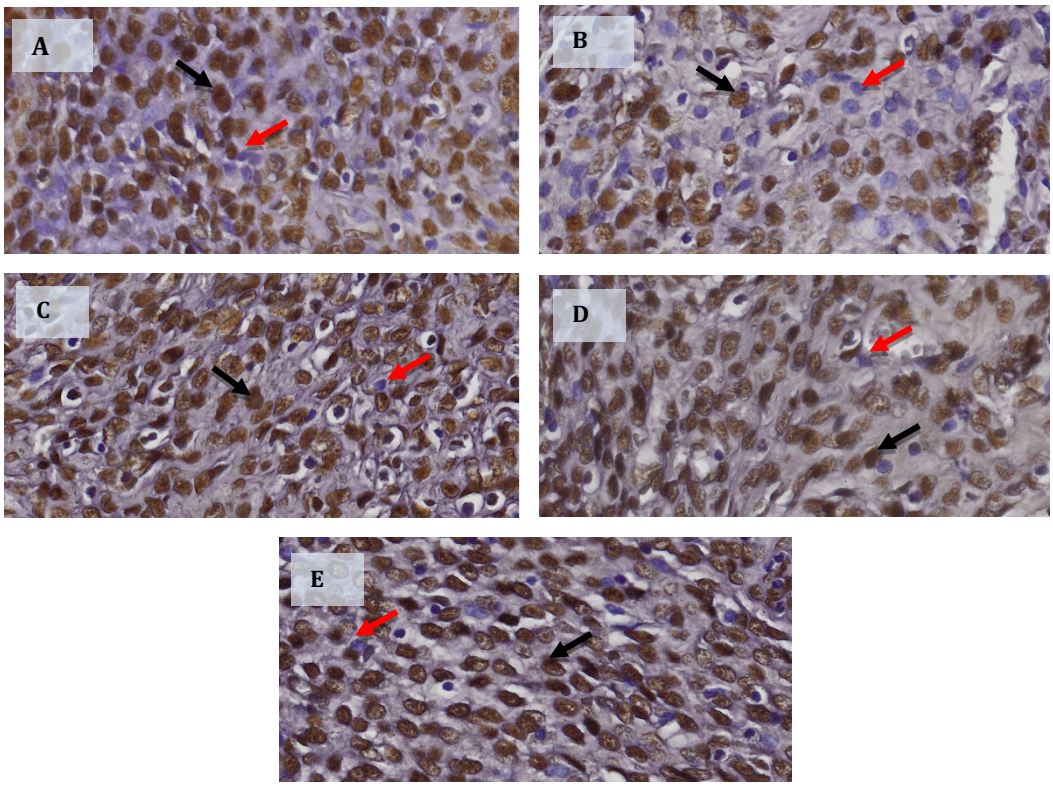

Figure 1: Immunohistochemical localization of ERα expression in the rat endometrial stromal cells.

Note. Black arrow showed positive ERα cell staining with brown color and red arrow showed negative ERα cell staining with blue color; A. CRL: control group, B. As: arsenic 2 mg/kgBW, C. T1: arsenic 2 mg/kgBW + different doses of kefir kefir; 1.25 mL, D. T2: 2.5 mL, D. T3: 5 mL (magnification 400x).

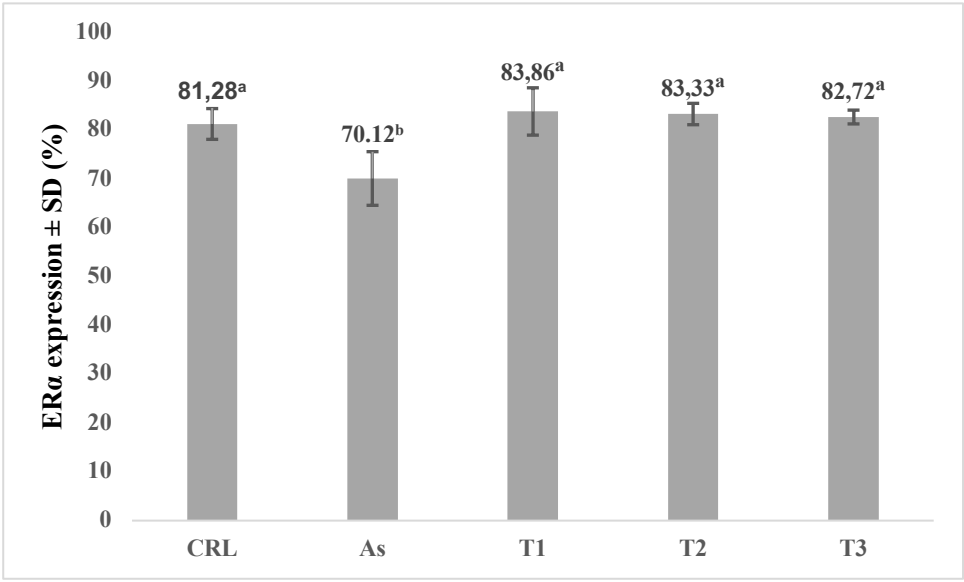

Figure 2: Histogram of ERα expression in the rat endometrial stromal cells.

Note. LSD Test. If it contains different letters, it means that there is a significant difference ($p \leq 0.05$)

3.2 Endometrial Thickness

Histological examinations showed significant differences in each treatment group ($p<0.009$) (Table 2). The controls exhibited normal endometrial tissue. In contrast, the arsenic-intoxicated rats demonstrated significant thinning of the uterine endometrial. The T1-T3 group exhibited no degenerative changes in the uterine endometrial layers (Figure 3). Compared to the As group, the endometrial thickness in T-T3 group was significantly higher and had a similar value compared to the control group (Figure 4).

Table 2: Effect of kefir on endometrial thickness in female Wistar rats (*Rattus norvegicus*) exposed to arsenic

Variable	Group	Mean ± SD	*p- value*
Endometrial thickness	CRL	504.13 ± 54.40[a]	
	As	332.57 ± 100.38[b]	
	T1	512.81 ± 69.79[a]	0,009*
	T2	521.84 ± 74.82[a]	
	T3	526.42 ± 57.45[a]	

Note. Endometrial thickness (µm); CRL=control group; As=Arsenic group (As_2O_3 2 mg/kgBW/day); T1=arsenic+kefir 1.25mL/kgBW/day; T2=arsenic+kefir 2.5 mL/kgBW/day; T3=arsenic+kefir 5 mL/kgBW/day. * = significantly different

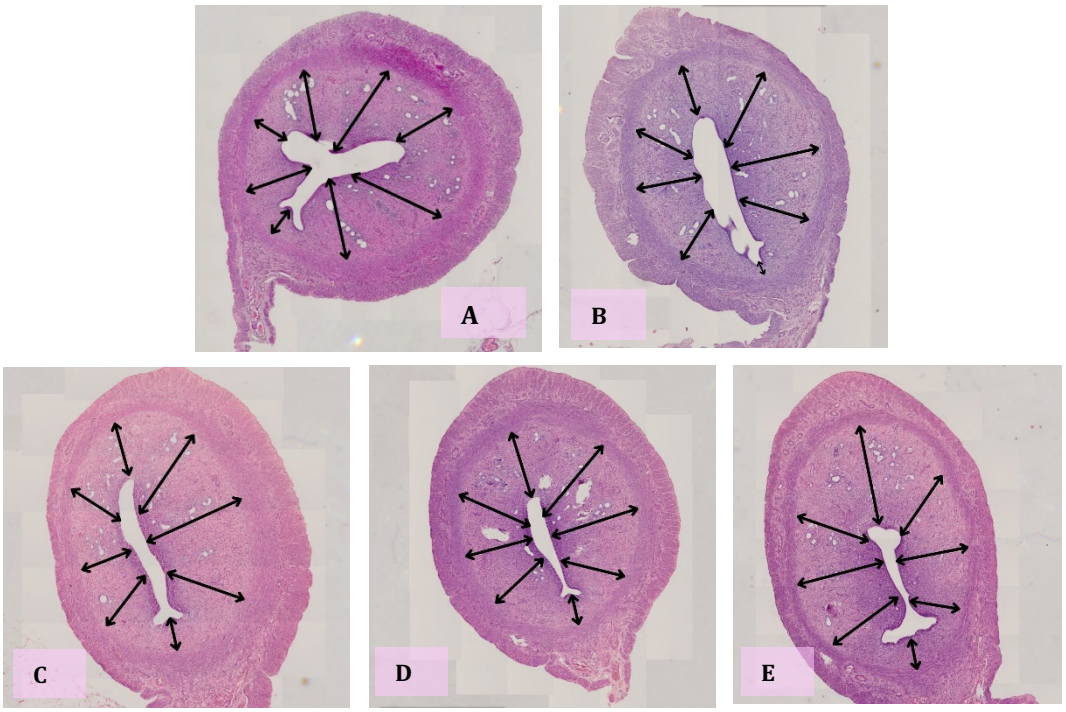

Figure 3: Endometrial thickness measurement of female rats by H&E staining

Note. A. CRL: control group, B. As: arsenic 2 mg/kgBW, C. T1: arsenic 2 mg/kgBW + different doses of kefir kefir; 1.25 mL, D. T2: 2.5 mL, D. T3: 5 mL (magnification 200x).

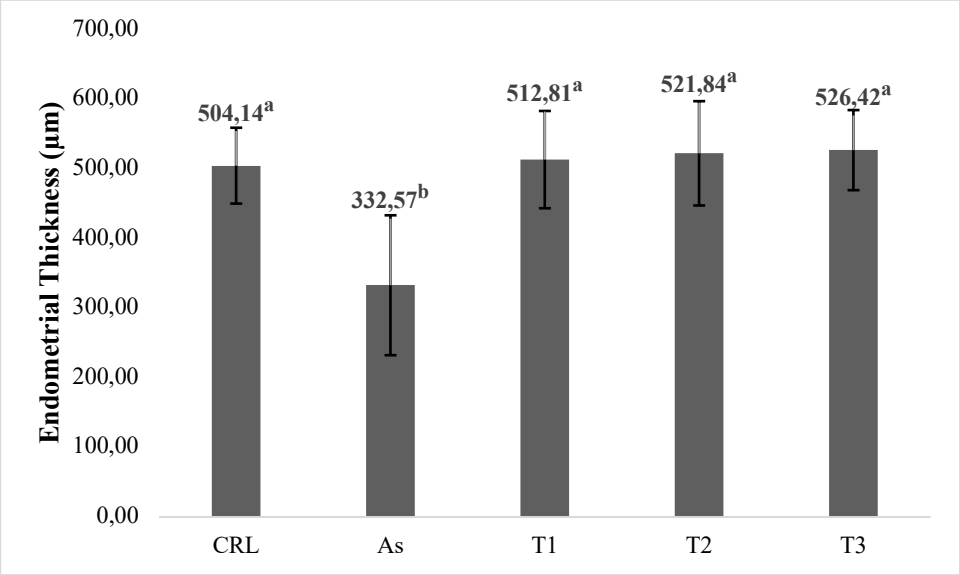

Figure 4: Histogram of Endometrial Thickness.

Note. LSD Test. If it contains different letters, it means that there is a significant difference ($p \leq 0.05$)

4. Discussion

Inorganic arsenic exposure causes arsenic bioaccumulation in intracellular and body tissues, especially in the anterior pituitary gland and gonads. Consequently, these organs can not perform their function as regulators of reproductive physiology accurately due to the production of Reactive Oxygen Species (ROS) and oxidative stress (Flora, 2011; Lushchak, 2014; Ronchetti et al., 2016). Huff et al. (2016) demonstrate that when exposed to arsenic, high ROS was produced in the body and inhibited the expression of oestrogen receptor genes through the MAPK/ERK pathway disruption. Mitogen-activated protein kinases (MAPKs) are protein kinases families that play the leading role in the transduction of extracellular signals from cell surface membranes to the nucleus via phosphorylation cascades (Bae-Jump, Chunxiao Zhou, Boggess, & Gehrig, 2008; Son et al., 2011). The disruption of the MAPK/ERK pathway, according to Bae-Jump et al. (2008), leads to the rapid phosphorylation of MAPK, inducing hyperactivation of the MAPK pathway that results in an ERα expression decrease. Therefore, arsenic exposure inhibits ERα mRNA transcription and decreases ERα protein expression in the endometrium via potentiation of the MAPK pathway. Our result indicated that exposure of rats to arsenic leads to the decrease of ERα expression in endometrial stromal cells.

Little is known whether there is association of the immune system in the regulation of arsenic-mediated toxicity. Considering that production of ROS is the most distinct mechanism of arsenic toxicity, it was assumed that compounds having antioxidant properties might be effective against arsenic toxicity. Several studies have demonstrated that kefir exerts extensive pharmacological actions in various diseases, including immune suppression in mice.

In this study, administration kefir into arsenic exposed rats, showed a significant increase in ERα expression leading to preventive action against the toxic effects of arsenic on the rat endometrium. The three doses of kefir, 1.25; 2.5; and 5 mL/kgBW/day, were significantly able to increase ERα expression in female rats exposed to arsenic. Moreover giving kefir with the smallest dose of 1.25 mL/kgBW/day to the rats had been able to increase both ERα expression and endometrial thickness. The repair effect of kefir is probably due to the antioxidant activity of kefir.

The content of kefiran, exopolysaccharides (EPS) formed by kefir, shows high antioxidant activity in protecting protein from oxidative and neutralizing superoxide radicals, as a Fe^{2+} chelating agent (Chen et al., 2015; Radhouani, Gonçalves, Maia, Oliveira, & Reis, 2018a, 2018b). The bioactive components of goat kefir are closely related to the properties of goat milk. Recent studies have also focused on the element and antioxidant activities of milk and dairy products, such as amino acids (mainly tyrosine and cysteine), vitamins (A, C and E), carotenoids,

and endogenous enzymatic systems, mainly by superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). Fermented milk products have antioxidant capacity and can act as radical scavengers for free radicals or ROS (Deeseenthum, Luang-In, John, Chottanom, & Chunchom, 2018; Farnworth & Mainville, 2008; Liu, Lin, Chen, Chen, & Lin, 2005; Yilmaz-Ersan, Ozcan, Akpinar-Bayizit, & Sahin, 2016). Some *Lactococcus* and *Staphylococcus thermophilus* bacteria in kefir, expressed the antioxidant activities of SOD. *Lactobacillus rhamnoses* bacteria are also known to inhibit the oxidation of linoleic acid then inhibit in vitro lipid peroxidation due to the chelation effect of the iron ions and the scavenging ability in superoxide anions (Kesenkas, Dinkcİ, Seckin, Kinika, & Gonc, 2011; Liu, Chen, & Lin, 2005).

The prevention of ROS accumulation by antioxidants is also proven to inhibit MAPK activation. It indicates the involvement of ROS in MAPK pathway activation (Bae-Jump et al., 2008). In line with the previous research conducted by Choi et al. (2015), ROS inhibition with antioxidants can downregulate the MAPK/ERK pathway. In turn, it can stabilize the function and expression of ERα. Based on the above explanation, kefir with potent antioxidant activity is proven to increase the expression of ERα in the endometrium of female rats exposed to arsenic.

In addition, the inhibition of lipid peroxidation is essential to prevent human diseases involving free radicals. According to Liu et al. (2005), when compared to milk, kefir showed significant inhibitory effects on linoleic acid peroxidation. The level of kefir inhibition of goat milk on linoleic acid peroxidation was 76.0%, indicating that by inhibiting lipid peroxidation, kefir has high antioxidant activities (Liu, Chen, et al., 2005; Liu, Lin, et al., 2005). Therefore, giving kefir can increase the thickness of the endometrium. Our result indicated demonstrated that administration of kefir independent from the dose could increase the endometrium thickness.

Morphologically, the endometrium is the most dynamic tissue in women and is very responsive to changes in reproductive hormones. Endometrial integrity is a reflection of the endometrial proliferation level in a healthy uterus. Thus, it displays the most compelled prognostic indicator for endometrial receptivity examination to implantation outcomes (Bromer, Aldad, & Taylor, 2009; Cunningham et al., 2013; Kovacs, Matyas, Boda, & Kaali, 2003). The thinning endometrium tends to fail to respond to cyclical hormonal changes resulting in implantation failure. In different words, after implantation, a miscarriage could happen due to the lack of blood supply that transports nutrients (Akram et al., 2010; Baradwan, Shafi, Baradwan, Bashir, & Al-Jaroudi, 2018).

Kefir, given in the appropriate dose, has desirable effects on the body. Meanwhile, too much dosage of kefir can increase the risk of a reaction change of antioxidants to pro-oxidants that potentially may cause harmful impacts (Laily, 2020; Raras, Hidayati, & Wardhani, 2021). The balance between oxidant production and antioxidant protection is indispensably essential in maintaining the health of biological systems. Physiological doses of exogenous antioxidants are required to maintain or re-establish redox homeostasis (Bouayed & Bohn, 2010; Raras et al., 2021).

5. Conclusion

Sub-chronic exposure to arsenic was able to decrease ERα expression and endometrial thickness. Giving kefir, containing antioxidant activities, was able to protect the reproductive organ system of female rats by increasing ERα expression and endometrial thickness. Therefore, the appropriate dose of kefir would benefit the body.

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