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Research Article
The Effect of Different Physiological Reproduction Conditions on Estradiol (E2) Residues in Local Raw Milk

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Abstract

Objective: The aim of this study was to evaluate the estradiol (E2) residues found in milk from local dairy farms of cows in different physiological reproduction states to determine whether the E2 residues were still within normal limits for consumption. **Materials and Methods:** This research used 22 adult Friesian Holstein Crossbreed (PFH) cows that were divided into 3 groups; A: Productive cows (non-pregnant and non-estrus conditions), B: Pregnant cows in the second trimester of gestation and C: Estrus cows. All of the cows were in the productive age range of 2-4 years old or in the second to fourth parity. Blood and milk samples were collected twice a day. Blood samples were kept at room temperature for 24 h to collect the serum and then stored in a freezer. The milk was centrifuged at 2000 rpm for 10 min and the supernatant was collected and then kept frozen. The estradiol assay was conducted with an ELISA kit from Calbiotech. The body weight was estimated with Rondo measuring tape and the daily milk production was evaluated. The data were analyzed in a completely randomized design with IBM SPSS 23. **Results:** Reproduction conditions affected the E2 residues in milk ($p<0.05$). The estradiol residues found in milk were greater than in serum due to an unknown mechanism. The highest E2 residues were observed in the milk of pregnant cows (187.425 ± 27.315 pg mL^{-1}). **Conclusion:** It can be concluded that theE2 residues found in milk in every group was very low compared with the normal estradiol levels consumed by humans.

Key words: Estradiol (E2), milk, reproduction, dairy cattle, pregnant cows

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Estrogen, especially estradiol (E2), is a common hormone that is important for reproductive activity in dairy cows. Gestational age affects the production of the estradiol (E2) hormone and a greater gestational age causes higher E2 hormone production. Estrus also affects E2 production to more when compared to that produced under non-estrus conditions. The frequency of parity affects the amount of milk production and the number of components present in milk. In the first parity, the amount of milk production and milk fat is low and milk production and the milk components will slowly increase in the third to fifth parity[1]. The amount of daily milk production will increase during the third parity and further increase during the fifth parity. Parity affects the urea residues in milk in the post-partum stage, which contributes to reproductive physiological changes because it was found to be greater during estrus than during pregnancy[2]. These conditions might affect hormone residues, especially those of E2, found in milk.

Currently, the arguments that claim milk is a cancer agent in the human body are debated. Some research has found that E2 hormone residues need to be monitored because they can turn into metabolites that cause cancer in the human body or are dangerous for human consumption[3-5]. Some argue that the carcinogenic hormone in milk are impossible to find and that there is a blocking mechanism of these hormones in the liver[6]. The amount of E2 produced in the human body is greater than that detected in cow milk and the amount of E2 produced in prepubescent girls (8 years old) was approximately 400 ng per day[7].

Some studies have found that there was a relationship between increasing cases of prostate cancer and increasing milk consumption[8,9]. Additionally, the consumption of milk containing high levels of estrogen increased the chances of breast cancer in menopausal women[10]. The consumption of milk as a dairy product was positively associated with the risk of estrogen receptor (ER)-negative breast cancer[11]. These controversial results are the basis for investigating E2 residues found in the milk from cows experiencing different reproductive physiological conditions.

MATERIALS AND METHODS

Animals: This research used serum samples and milk samples from 22 Friesian Holstein Crossbreed (PFH) cows from local breeders in the Ngablak District, Magelang Regency, Central Java, Indonesia, which were collected twice a day at 07.00 a.m. and 02.00 p.m. The cows were divided into 3 groups:

A: Productive cows (non-pregnant and non-estrus conditions)
B: Pregnant cows in the second trimester of gestation
C: Estrus cows

All of the cows were in the productive age range of 2-4 years old or in the second to fourth parity. The A group contained 9 cows, the B group, 9 cows and the C group, 4 cows. Cows were maintained traditionally, so they were fed using forages of *Pennisetum purpureum* and vegetable waste twice a day, as well as concentrate feed from a local commercial concentrate for dairy cows consisting of fermented coffee skins, soybean meal, corn and bran. Drinking water was administered *ad libitum*. Body weight was estimated with RONDO measuring tape made in Germany.

Blood and milk collection: Blood and milk samples were collected twice a day. Blood was collected with a G 21 venoject needle and then kept at room temperature for 24 h to collect the serum, which was taken with a Socorex Acura 815 100-1000 µL micropipette and kept frozen in PCR tubes until analysis via Enzyme-linked immunosorbent assay (ELISA) hormone detection. Milk samples of approximately 100 mL were collected twice a day and centrifuged at 2000 rpm for 10 min with a PLC Series K Centrifuge. The supernatant (1.5 mL)was collected with a micropipette and kept frozen in PCR tubes until analysis via ELISA hormone detection.

Estradiol (E2) detection method: ELISA estradiol analysis was conducted in the Animal Physiology Laboratory, Department of Animal Physiology, Animal Veterinary Faculty, Universitas Gadjah Mada in September 2019. The serum and milk E2 concentrations were determined using a Calbiotech Estradiol ELISA kit and 96-well plates. Samples and 25 µL of a standard were added to the wells with 50 µL of a working solution of estradiol biotin conjugate. The mixture was mixed on a plate shaker and incubated at room temperature for 45 min. Then, 100 µL of estradiol enzyme was added and incubated at room temperature for 45 min on the plate shaker. The wells were washed with 300 µL of a washing solution 3 times, inverted and vigorously tapped dry on absorbent paper to ensure that all residual moisture was removed. TMB substrate was added to the dry wells and then incubated under room temperature and low light conditions for 30 min on the plate shaker.

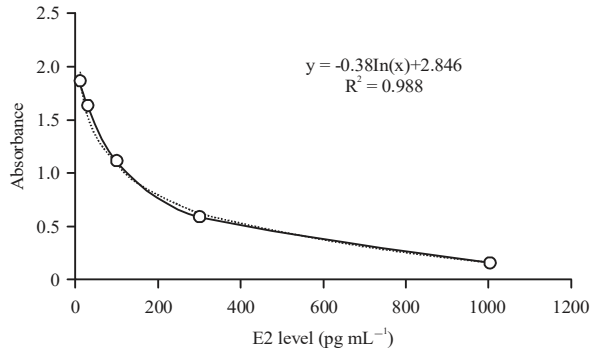

Fig. 1: Standard estradiol reference absorbances

The reaction was stopped with 50 µL of a stopping solution and mixed gently until the colorfully changed to yellow. All of the prepared wells were read with a Zenix microplate reader 320 at 450 nm for 15 min. The results of the standard reference absorbance were calculated and utilized to generate a calibration curve and then, the R^2 value and the regression equation were determined, as shown in Fig. 1. The data were analyzed using a completely randomized design using SPSS IBM 23 followed by the Duncan multiple range test (DMRT).

RESULTS

The results in Table 1 shows that E2 was found in both the milk and serum samples of the cows. Group B (pregnant cows) showed the highest E2 levels in milk and the lowest were observed in the A group (productive cows). E2 found in the serum samples was higher in the C group (cows in estrus) than in the A and B groups. For the pregnant cows, the E2 residue in milk was higher than that of the other samples (187.425±27.315 pg mL^{-1}) and it was also higher than the E2 level found in the serum of pregnant cows. This phenomenon was also observed for the cows in estrus, where the E2 level observed in milk (148.832±47.311 pg mL^{-1}) was greater than that found in the serum (100.249±12.798 pg mL^{-1}) (Fig. 2).

Table 2 shows that the body weights of all groups ranged from 431.700±53.244 kg to 456.250±45.959 kg and the milk production was approximately12.778±2.645 L per day to 14.778±5.911 L per day. There was no significant difference between groups (A, B and C) regarding body weight and milk production. No significant correlations were found between body weight and milk production, body weight and E2 levels in serum and milk production and E2 levels in milk.

DISCUSSION

Physiological reproduction conditions affected the E2 residues found in milk and serum. Gestation and estrus are

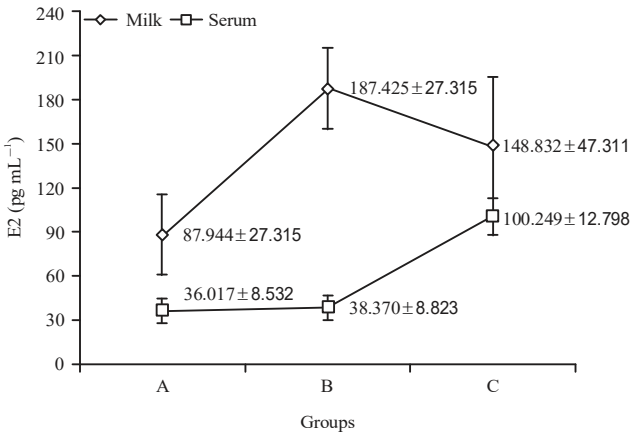

Fig. 2: Comparison of E2 observed in milk and serum samples (pg mL^{-1}), (a) Productive cow, (b) Pregnant cow and (c) Estrus cows

Different superscripts within the same row indicate significant differences (p<0,05)

Table 1: Estradiol concentrations in milk and serum samples (pg mL^{-1})

	A	B	C
Milk samples	87.944±27.315[a]	187.425±27.315[a]	148.832±47.311[a]
Serum samples	36.016±8.532[a]	38.369±8.823[a]	100.249±12.798[b]

[a-c]Different superscripts within the same row indicate significant differences (p<0.05)

Table 2: Body weight (kg) and milk production profiles (L per day)

	A	B	C
Body weight	440.111±68.725	431.700±53.244	456.250±45.959
Milk production	14.778±5.911	13.000±4.323	12.778±2.645

There was no significant difference between (1) the physiological reproduction condition and body weight or (2) the physiological reproduction condition and milk production and there was no significant correlation between body weight and milk production (3) (1 = 0.204, 2 = 0.272, 3 = 0.255)

reproductive conditions that are managed by hormones, especially estrogen, which includes estradiol (E2)[12]. A large amount of E2 normally occurs in cows in estrus because E2 supports ovulation. Daily consumption ofE2 during the luteal phase does not support ovulation[13] because it is supported during follicle development.

This fact is unique because it is related to an unknown mechanism that regulates the release of E2 into milk. The integrity of tight junctions and the presence of a transport protein, such as albumin, might also in uence the E2 concentration in milk. It is possible that mastitis could in uence milk E2 concentrations if the integrity of tight junctions within the mammary gland was compromised, allowing for increased concentrations of albumin (a non specific carrier of estradiol) into the alveolar lumen, which might increase milk E2 concentrations in infected glands relative to those of non-infected glands but it is a complex process and is not well de

ned[14]. Some factors that affect the composition of milk in addition to reproduction condition are season and parity. Seasons affect the environmental conditions that lead to the initiation of pathogens, especially in the mammary glands and affect the composition of raw milk[1]. Additionally, during the first parity, milk production and fat and protein components are lower than those of milk produced during the third parity[1]. Usually, during the third parity, the amount of daily milk production will also increase the amount of urea in milk, which is affected by the physiological conditions of reproduction in post-partum dairy cows during preparation for the next estrus period[2].

The presence of E2 in milk might pose a risk to human health because of its ability to travel through the body with transport proteins or affect protein receptors. Cancer development has been one of the stated risks of consuming hormone residues, which might increase the amount of protein receptors that initiate the physiological mechanism of cancer development in the human body. The physiological response in the human body that occurs when estrogen is consumed is initiated by the enzyme cytochrome P450 monooxygenase, which oxidizes estradiol and estrone to 2- or 4-catechol estrogens. Both of these metabolites can also be methylated into methoxy-estrogens by catechol-o methyltransferase. Eventually, methoxy-estrogens will be converted into semiquinones and quinones, which can act as cancer agents when depurination of DNA occurs[4]. Estradiol levels on dairy products that are safe for consumption by humans are 0-50 ng kg^{-1} body weight (50,000 pg kg^{-1})[15]. Pape-Zambito *et al.*[14] found that estrogen produced during puberty in girls reached 54,000 ng mL^{-1}. Milk consumption does indeed increase the metabolites of some hormone residues, such as estrogen metabolites, which can become cancer agents and can increase estrogen hormone levels in consumers but it cannot be determined that milk consumption causes cancer due to various factors that need to be further proven[16]. Based on the research data of E2 residues found in the milk from cows during every reproduction stage (groups A, B and C), these levels were very low compared with those of normal estradiol consumption by humans.

The physiological reproduction of cows from local farms in the Ngablak District was difficult to determine and some of the adult cows experienced silent estrus because of a lack of nutrition in the feed. As shown in Table 2, the weight of the adult cows from each group was lower than normal, especially that of the pregnant cows. The pregnant cow body weight at 8 weeks before parturition ranges between 547 and 607 kg and usually increases following the parities[17]. The feed for almost all lactation cows from local farms in Ngablak was supplemented with remnant vegetables that were collected from farms because the citizens work as farmers. In addition, some of the cows were not supplied with quality concentrate feed, which might be the main reason why the body weight was lower than normal. In addition, the milk production shown in Table 2 was still at the normal level. The average milk production was 12-14 L per day. A dairy farm in Enrekang, South Sulawesi (Eastern side of Indonesia) found that the milk production of the dairy cows was 4-6 L per day[18] and dairy production levels reached up to 19.63 L per day on commercial farms in west Java (west side of Indonesia)[19]. Normal milk production for FH crossbreeds in Indonesia is 15-20 L per day[20]. However, a normal quantity of milk production does not indicate a normal milk composition, the milk might have a low solid content and a high water content. This may be due to a lack of nutrition during the lactation period.

CONCLUSION

Different physiological reproduction conditions, such as pregnancy and estrus, affected the levels of E2 residues found in milk. There is an unknown mechanism that affects the E2 residues found in milk, including mastitis, which affected the tight junction within the mammary glands. The E2 residues found in milk was very low when compared with normal estradiol levels consumed by humans.

SIGNIFICANCE STATEMENT

This research discovered the E2 residue level in milk that could be correlated with the maximum E2 value allowed for human consumption, which may be beneficial for the government to determine policies regarding the use of local milk. This study will help researchers develop the critical areas of E2 metabolism effects that many researchers have not been able to explore. Thus, a new theory on the interactions of these metabolites can be determined.

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