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Arginase activity of ovarian structures in cows of Brown Swiss and its cross-breeds

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ABSTRACT

Arginase is the last enzyme of the urea cycle. It catalyses the hydrolysis of L-arginine to L-ornitine and urea. The aim of this study was to investigate the presence of arginase activity in ovarian structures such as: Graaf follicles, GF (Medium and Large size, M- and L-size), Corpus Haemorrhagicum (CH), and various types of Corpus Luteum (CL) such as: cyclic CL (CCL), 2-4 month pregnancy CL (2-4 MCL) and 4-7 month pregnancy CL (4-7 MCL). Ovarian tissues of 62 cows (7-10 years old and Brown Swiss or its cross-breeds), collected from a local slaughterhouse, were used as material. The materials were divided into 6 experimental groups, as follows: MGF group (n = 7), LGF group (n = 21), CH group (n = 7), CCL group (n = 6), 2-4 MCL group (n = 9) and 4-7 MCL group (n = 12). Arginase activities were measured as 0.056 ± 0.017, 0.100 ± 0.016, 2.517 ± 0.521, 0.827 ± 0.190, 0.674 ± 0.106 and 0.833 ± 0.093 U/mg protein in all groups, respectively. Arginase activity in the CH group was significantly higher than that in the CCL, 2-4 MCL and 4-7 MCL groups ($P<0.001$). The lowest enzyme activity was in the MGF and LGF groups. Hence, it was concluded that the arginase enzyme might play a crucial role in cell division, proliferation and differentiation in the ovarian tissues (especially the CH) of mature cows.

Key words: arginase activity, Graaf follicle, corpus heamorrhagicum, corpus luteum, cattle

Introduction

Arginase, as determined first by KOSSEL and DAKIN (1904), is the last enzyme of the urea cycle. It catalyses the hydrolysis of L-arginine to L-ornitine and urea (CEDERBAUM et al., 2004; KANDEMIR and OZDEMIR, 2008). With regard to the enzyme's presence, the

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richest organ is the liver. Also, it is present at low levels in the kidney, brain, intestines, thyroid gland, erythrocyte, leukocyte, thrombocyte, placenta, testicular tissue and mammary gland (KANDEMİR and OZDEMIR, 2009). The presence of enzyme in extra hepatic tissues might indicate that these tissues use arginase for their purposes other than the urea synthesis (RAZMI et al., 2005).

The enzyme may play a role in the production of ornithine for the synthesis of polyamines, such as, putrescine, spermidine and spermine, that are required for normal cellular proliferation (PEGG and McCANN, 1982; TABOR and TABOR, 1984; THOMAS and THOMAS, 2001; WELCH et al., 2008) and differentiation (PEGG, 1986; VUOHELAINEN et al., 2010). BACHETTI et al. (2004) indicated that arginase was an important pathway for human endothelial cells to make use of L-arginine, and necessary for normal proliferation. Its activity has been determined in different tissues, including the ovaries with the corpus luteum, the muscular and mucosal layers of the uterine horn, the uterine body, the cervix, vagina and vestibule in cattle. This enzyme is also present at different levels in all parts of the cow's reproductive system, and it may be related to different rates of cell proliferation, differentiation or other potential physiological and biochemical activities, yet the enzyme activity is unknown in reproduction (RAZMI et al., 2005; YUKSEL et al., 2012).

The level of arginase activity may vary depending on the hormonal effect (KUMAR and KALYANKAR, 1984). The oestrus cycle in cattle, as in many other species, is regulated by endocrine and neuroendocrine mechanisms. GnRH has an important role in regulation of the oestrus cycle. It induces the secretion of the gonadotropic complex hormones (FSH, LH) from the hypothalamus. FSH and LH regulate the oestrus cycle by influencing the ovaries. Thus, dynamic ovarian activity occurs in normal (cyclic) cows, unless they have an ongoing pregnancy. In each period of the oestrus cycle, various structures are formed in the ovaries. In cattle, a tertiary follicle (TF) is present in the pro-oestrus period. Under the FSH effect, the TF grows and a Graaf follicle (GF) is then formed during the oestrus period. At the ovarian level, the oestrous period is characterised by high oestrogen secretion, mainly produced by the pre-ovulatory GF. At the end of oestrus, ovulation occurs during the metoestrus phase. The ovulation fossa is filled with blood cells and the corpus heamorrhagicum (CH), consisting of cells of blood, residual follicle, granulosa and theca, is formed during the metoestrus period. The CH turns into CL by the LH effect, that stimulates the granulosa and theca cells to become luteal cells, so that the CL is formed during the dioestrus period in the ovaries (HAFEZ and HAFEZ, 1987; NISWENDER et al., 2000).

The follicular fluids play a major role in the physiological, biochemical and metabolic views of the nuclear and cytoplasmic maturation stages of the oocyte (HAFEZ and HAFEZ, 1987). Therefore, the potential presence of arginase enzyme and its activity in different

ovarian structures (such as follicles and luteal ones) in cattle were investigated in this study.

Materials and methods

Collection of ovary samples. Ovaries were obtained from a local slaughterhouse. In this way, healthy cows (7-10 years old) of Brown Swiss or its cross-breeds were monitored and the cycle periods of appropriate cows was identified by inspecting the post-mortem uterine and ovarian findings. The ovaries of 62 cows were selected as the material. After the slaughtering process, the ovaries were collected, kept on ice, and transferred to the laboratory immediately. Materials were classified according to the varying status of the reproductive tract as: Group 1: medium Graaf follicle (n = 7) (MGF), Group 2: large Graaf follicle (n = 21) (LGF), Group 3: corpus heamorrhagicum (n = 7) (CH), Group 4: cyclic corpus luteum (n = 6) (CCL), Group 5: 2-4 month pregnancy corpus luteum (n = 9) (2-4 MCL) and Group 6: 4-7 month pregnancy corpus luteum (n = 12) (4-7 MCL). Follicles were classified as MGF (both small and large diameters of <10 mm) and LGF (both small and large diameters of >10 mm). The CH, CCL, 2-4 MCL and 4-7 MCL groups were determined according to the status of the reproductive tracts.

Ultrasonographic measurement of ovarian structures. To measure the diameters of ovarian structures, the water bath technique was used. The ovaries were placed in the water bath after they were brought into the laboratory. The vertical and horizontal diameters of ovarian structures (Figs 1 and 2) were measured by ultrasonography (260 Corvus, Pie Medical®) at 5 MHz. Diameters were classified as large and small according to their lengths. Bearing in mind the fact that only the follicles that have enlarged to over 10 mm can ovulate in cows (HATZIRODOS et al., 2014), the follicles were classified as large- (11-15 mm) and medium-size Graaf follicles (6-10 mm) with regard to their diameters, as described by MACHATKOVA (2004).

After the measurement of fluids of large- and medium-size Graaf follicles, the follicles were placed into eppendorf tubes by means of sterile injectors and stored at -20 °C. Also, all the CL and CH tissues were separated; the inessential material of the ovary was removed by surgical blade and kept at -20 °C until arginase activity was determined.

Determination of arginase activity. For determination of arginase activity in solid tissues that included CH, CCL, 2-4 MCL and 4-7 MCL, the samples were homogenised in a Potter Elvehjem (glass-glass) homogenisator by dilution with $MnCl_2$ (1/6 w/v) after drying between the two filter papers. The homogenate was centrifuged at +4 °C, 14,000 rpm for 13 min, and the supernatants of the samples were used as the enzyme source. Follicular fluids were used as the source for the MGF and LGF groups.

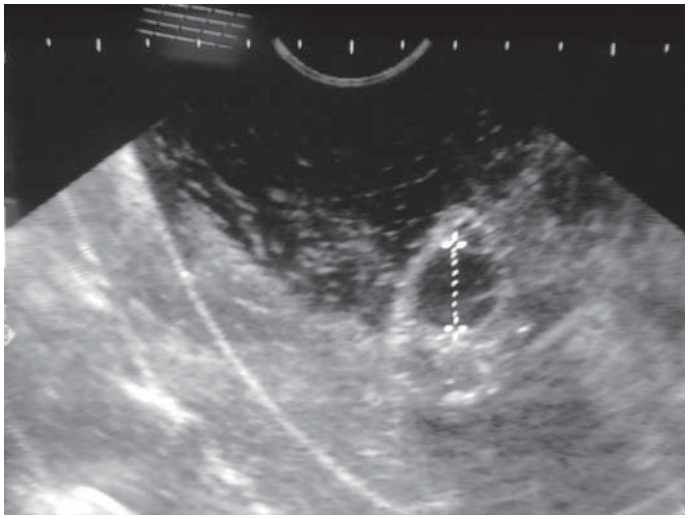

Fig. 1. Vertical measurement of the diameter of an ovarian structure (Graaf follicle)

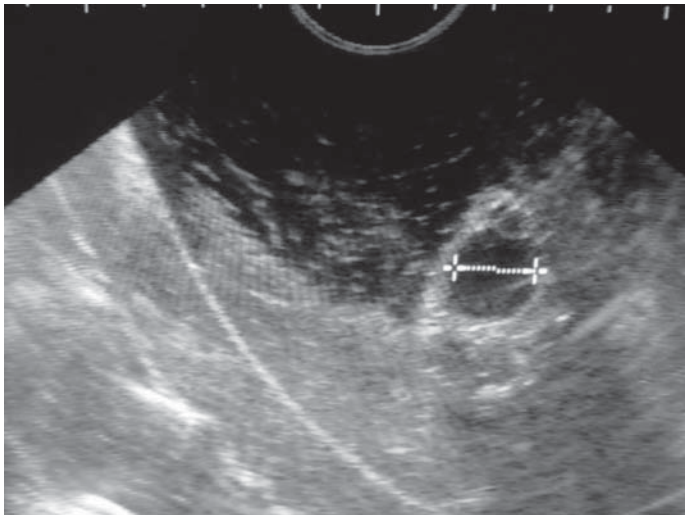

Fig. 2. Horizontal measurement of the diameter of an ovarian structure (Graaf follicle)

The arginase activity of the ovarian structures was determined spectrophotometrically using a modification of the Thiosemicarbazide-Diacetylmonoxime urea (TDMU) method, as described by GEYER and DABICH (1971). Measurements were performed in duplicate. All chemicals (L-arginine, Mangan chloride, Sodium carbonate, Sodium bicarbonate, Thiosemicarbazide and Diacetyl monoxime had analytical purity) used in the study were obtained from Sigma Chemical Co. Briefly, 0.1 mL follicular fluid/supernatant (the enzyme source) was diluted with 1 mM $MnCl_2$ at the rate of 1:40 (v/v), along with pre-incubation at 55 °C for 12 min. Tubes containing 0.3 mL of the mixture, 0.3 mL L-arginine (120 mM, pH 9.5) and 0.4 mL carbonate buffer (200 mM, pH 9.5) were incubated at 37 °C for 10 min. By adding 3 mL acid reagent to the tubes at the end of the incubation period, the reaction was halted. Thereafter, a 2 mL colour reagent was added to the tubes that were kept in a boiling water bath for 10 min. The tubes were then removed from the bath, cooled and the absorbance was recorded at 520 nm. The protein concentration was determined by the method of LOWRY et al. (1951), using bovine serum albumin as standard. Briefly, tubes containing 1 mL alkaline copper reagent and 0.1 mL supernatant samples were mixed, and incubated at room temperature for 10 min. Afterwards, 4 mL folin and Ciocalteu's phenol reagent were added into the tubes, mixed and incubated at 55 °C for 5 min. The absorbance of the samples was recorded at 650 nm, using a Shidmadzu UV 240 spectrophotometer.

The principle of this determination of arginase activity relies on the spectrophotometric measurement of urea, produced by the hydrolysis of L-arginine, by arginase. One unit of arginase activity was stated as the quantity of enzyme catalysing the formation of 1 μmol of urea/h, at 37 °C. The results were then expressed as 'units/mg' of protein (specific activity).

Statistical analyses. All values are presented as the mean ± standard error of means (S.E.M.). The differences were considered to be significant when $P<0.05$. Statistical analyses were performed using analysis of variance (One-way ANOVA) and the post hoc Tukey test, using the SPSS/PC (Version 20) software programme.

Results

Arginase activity in all groups is presented in Tables 1 and 2. In terms of arginase activity, no statistical difference was found between the MGF and LGF groups ($P>0.05$). However, the activity in the CH group was significantly higher than in the CCL, 2-4 MCL and 4-7 MCL groups ($P<0.001$).

Small and large diameter values in all the groups are also presented in Tables 1 and 2. Regarding both diameter values, there was a significant difference between the MGF and LGF groups ($P<0.001$). The small diameter in the CH group was significantly lower than in the CCL, 2-4 MCL and 4-7 MCL groups ($P<0.001$). Further, the large diameter

in the CH group was lower than in the CCL, 2-4 MCL and 4-7 MCL groups (P<0.001). However, the large diameters of the CCL and 2-4 MCL groups were similar to each other (P>0.05) and the large diameter value in the 4-7 MCL group was significantly higher than in the CCL and 2-4 MCL groups (P<0.05).

Table 1. Arginase activity in small and large diameters of follicles

	n	Arginase (U/mg protein)	Small diameter (mm)	Large diameter (mm)
Medium Graaf follicle	7	0.056 ± 0.017	8.2 ± 0.3^{b}**	10.4 ± 0.8^{b}**
Large Graaf follicle	21	0.100 ± 0.016	12.2 ± 0.4^{a}	13.8 ± 0.4^{a}

$^{a-b}$: Different superscript letters within the same row show significant differences between the groups. *P<0.05, **P<0.001.

Table 2. Arginase activity in small and large diameters of CH, CCL, 2-4 MCL and 4-7 MCL

	n	Arginase (U/mg protein)	Small diameter (mm)	Large diameter (mm)
Corpus haemorrhagicum	7	2.517 ± 0.521^{a}**	11.8 ± 0.8^{b}**	13.7 ± 0.5^{c}**
Corpus luteum	6	0.827 ± 0.190^{b}	16.8 ± 0.9^{a}	19.2 ± 0.6^{b}*
2-4 months pregn. CL	9	0.674 ± 0.106^{b}	17.3 ± 1^{a}	19 ± 0.7^{b}*
4-7 months pregn. CL	12	0.833 ± 0.093^{b}	19.4 ± 0.7^{a}	22.4 ± 0.5^{a}

$^{a-c}$: Different superscript letters within the same row show significant differences between the groups.* P<0.05, ** P<0.001.

Discussion

The sexual cycles in cattle are regulated by endocrine and neuroendocrine mechanisms. During the cycle, the ovaries of the cattle include different structures that are the MGF, LGF, CH and CCL. Also, the CCL refers to the pregnancy corpus luteum if conception occurs. The GF, CH and CL have different structures and properties from each other. The GF contains the theca interna, granulosa cells, and follicular fluid. Follicular fluid in cattle includes various components such as elongase and Δ9-desaturase enzymes and some fatty acids (WARZYCH et al., 2014), oestradiol, insulin-like growth factor, progesterone, androstenedione, total inhibin, and inhibin-A (BEG et al., 2002), glycoproteins (heparin, glucosamine, hyaluronic acid, plasminogen etc.), gonadotropins (FSH, LH) and elements (Na, K, Zn, Ca, Cu etc.) (HAFEZ and HAFEZ, 1987). Some parameters of follicular fluid are related to the diameter of the follicle, such as inhibin-B and activin-A. The CH appears following the ovulation of GF, and it includes lymph, blood and residual follicular cells. The CL is formed by the luteinisation of granulosa cells, containing small and large luteal

cells, under the influence of the LH (NISWENDER et al., 2000). Also, these tissues are present in various cycle periods, regulated by different hormones.

L-Arginine serves as a precursor for many molecules (such as proline, glutamate, creatine, nitric oxide and polyamines) that have an important role in cellular physiology. L-Arginine is converted into ornithine through the action of arginase, when converted into nitric oxide (NO) via NO synthase (WU and MORRIS, 1998). Both NO and polyamines have vital roles in cellular proliferation. There are some studies about the effect of L-arginine on the female reproductive system. WU et al. (1996) reported that an extraordinary abundance of L-arginine in porcine allantoic fluid, and they suggested that L-arginine plays a role in fetoplacental nutrition. Dietary L-arginine supplementation has been revealed to enhance the reproductive performance of rats (ZENG et al., 2008), gilts (MATEO et al., 2007) and mice (GREENE et al., 2012). Also L-arginine supplementation improves the luteal function by decreasing CL blood flow impedance. As is known, CL blood flow is a critical factor for the continuity of luteal function (TAKASAKI et al., 2009).

Arginase may play a crucial role in the production of ornithine, as required for normal cellular proliferation (PEGG and McCANN, 1982; TABOR and TABOR, 1984) and differentiation (PEGG, 1986). The enzyme is found profoundly in tissues with a high proliferation and differentiation rate (PEGG, 1986). Arginase may be an important indicator for male fertility. There are some studies about arginase activity and semen parameters. TURK et al. (2011) suggested that there is a positive correlation between seminal plasma arginase activity and sperm mass activity, sperm motility and sperm concentration, and there is also a negative correlation between seminal arginase activity and abnormal sperm rates in Saanen goats. Similarly, GUR and KANDEMIR (2012) suggested that there is a positive correlation between seminal plasma arginase activity and semen volume, semen mass activity, sperm motility and sperm concentrations, and a negative correlation between arginase activity and abnormal sperm rates in Awassi rams. ELGUN et al. (2000) suggested that there was a positive correlation between seminal plasma arginase activity and sperm motility and a negative correlation had been detected between between seminal arginase activity and sperm count in infertile men. However, there has been no scientific study to investigate the arginase activity of ovarian structures in different oestrus periods.

Although proliferation and differentiation are commonly seen in reproductive organs (HAFEZ and HAFEZ, 1987), only a few scientific articles exist about the presence of arginase activity within the female reproductive system. Arginase activity of CL, ovaries, uterine tube, as well as both the mucosa and muscles of the uterine horn, uterine body, cervix, vagina and vestibule, were determined at different ranges in cattle (RAZMI et al., 2005). In cows, YUKSEL et al. (2012) suggested that the uterus enzyme levels remain unchanged in different stages of the oestrous cycle. Also, the activity has been detected in female rabbits (WILSON, 2003) who suggested that the treatment with a small amount

of arginase inhibitor increased the blood flow in both male and female genital organs in rabbits.

There appears to be a correlation between the diameters given and some components of follicles (BEG et al., 2002). However, enzyme activity had no correlation with the diameters in both the MGF and LGF groups studied herein. Also, the activity of the LGF group was only numerically higher than in the MGF group.

In our study, the arginase activity of CCL was higher than the values reported by RAZMI et al. (2005). This difference might stem simply from the use of different methods of evaluation. The activity in the CH group was markedly higher than that in the CCL, 2-4 MCL and 4-7 MCL groups ($P<0.001$). These differences might arise from the luteinisation of granulosa and theca cells. Arginase may play a critical role in transforming the CH into CL during the differentiation period, and also the blood cells that are involved in the structure of CH could have been affected by the enzyme activity of this tissue. However, there were no marked differences in the activities between the CCL, 2-4 MCL and 4-7 MCL.

Values of arginase activity in the MGF and LGF groups were the lowest in all the groups. This may depend on the material of the follicular fluid that may dilute the concentration of the arginase enzyme when compared to the enzyme activity of solid structures in the ovary, including the CCL, 2-4 MCL and 4-7 MCL.

For small diameters, there was no marked difference of activity between the CCL, 2-4 MCL and 4-7 MCL groups. However, in the large diameter of 4-7 MCL it was markedly higher than that in the CCL and 2-4 MCL groups ($P<0.05$). This may simply depend on the advanced on-going pregnansy period of support to a greater size of CL.

There was no marked correlation between the arginase activity and diameters (small or large) in all the groups studied. The lack of a marked correlation between the arginase activity in the luteal structures and diameters might indicate the transformation of CCL into pregnancy CL following conception. It may also imply the lack of considerable proliferation and differentiation capabilities of the luteal cells.

In conclusion, different arginase activities were observed in all the ovarian structures at different stages of the oestrus cycle. Further, the enzyme may play a crucial role in cell division, proliferation and differentiation in the ovarian structures concerned (especially in the corpus heamorrhagicum). The data presented may shed light on future studies to be conducted on the actual effects of arginase and other enzymes, not only in cattle but also in other ruminant species with great economic and nutritional importance.

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SAŽETAK

Arginaza je posljednji enzim u ciklusu ureje koji katalizira hidrolizu L-arginina u L-ornitin i ureju. Cilj istraživanja bio je utvrditi prisutnost aktivnosti arginaze u ovarijskim strukturama kao što su Graafovi folikuli, GF (srednje veliki - M i veliki - L), corpus haemorrhagicum (CH) i različiti tipovi corpus luteum (CL) kao što su ciklični (CCL), 2-4 mjeseca graviditetni (2-4 MCL) i 4-7 mjeseci graviditetni (4-7 MCL). Tkiva ovarija od 62 krave (švicarske smeđe pasmine i križanaca u dobi od 7 do 10 godina) prikupljena su u lokalnoj klaonici. Materijali su bili podijeljeni u 6 pokusnih skupina kako slijedi: MGF skupina (n = 7), LGF skupina (n = 21), CH skupina (n = 7), CCL skupina (n = 6), 2-4 MCL skupina (n = 9) i 4-7 MCL skupina (n = 12). Slijedom navedenih skupina, aktivnost arginaze bila je 0,056 ± 0,017, 0,100 ± 0,016, 2,517 ± 0,521, 0,827 ± 0,190, 0,674 ± 0,106 i 0,833 ± 0,093 U/mg. Aktivnost arginaze u CH skupini bila je signifikantno viša u odnosu na skupine CCL, 2-4 MCL i 4-7 MCL ($P<0,001$). Najniža aktivnost enzima bila je u skupinama MGF i LGF. Zaključeno je o mogućoj ključnoj ulozi aktivnosti enzima arginaze u diobi stanica, proliferaciji i diferencijaciji ovarijskih tkiva (osobito CH) kod odraslih krava.

Ključne riječi: aktivnost arginaze, Graafov folikul, corpus heamorrhagicum, corpus luteum, govedo