Praca oryginalna *Original paper*

Association between the number and quality of bitch COC's and selected donor factors*)

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Summary

The aim of the present study was to investigate whether selected factors attributed to oocyte donors can affect the number and quality of canine cumulus-oocyte-complexes (COC). The following parameters were considered: female age and body mass, ovarian weight, presence of functional ovarian structures (eg. corpus luteum, visible follicles), and ovarian and uterine pathology. Altogether 10 077 COCs were collected, on average 125.6 per bitch.

The number and quality of collected COCs was significantly affected by female age and the presence of functional ovarian structures. We found an increased total number of COCs in up to 8-month-old bitches as compared to up-to-3-year-old and up-to-7-year-old females ($P < 0.001$). Moreover, senile females produced more high quality COCs than the younger ones. However, the differences between those groups were not statistically significant ($P = 0.469$, $P = 0.346$). A higher number of COCs was collected from ovaries with a smooth surface (202.8) or with visible follicles (121.1), in comparison to ovaries with corpus luteum (97.6), bitches with pyometra and pathologies of the ovaries or the uterus (82.8). No influence of the bitch's body mass and ovarian weight on the number of COCs was observed, although a higher number of oocytes was usually collected from the right ovary.

The quantity and morphological quality of bitch COCs varied significantly among individual females in an age-dependent manner. The greater number of COCs, isolated from younger bitches, may be associated with a higher reproductive potential and hormonal activity of these females. The study suggests that the age affects the total number of collected COCs but has no influence on the quality of bitch oocytes.

Keywords: oocytes, individual factors, bitch, donor age

Since the first artificial insemination in the dog was applied, assisted reproductive technologies (ART) have undergone several modifications (3, 11, 12). The implementation of *in vitro* techniques led to the development of *in vitro* maturation (IVM) and *in vitro* embryo production (IVP) procedures (14, 17, 25). Since that time, dogs have been used for physiological and genetic studies of human diseases. One of the most important concerns in studies involving IVM and IVF of bitch oocytes is the quality of gametes (1, 4, 18). The classification of dog oocytes is based on the analysis of the cytoplasmic appearance and the presence or absence of cumulus cells.

The number and quality of canine cumulus-oocyte complexes (COC) that are suitable for *in vitro* maturation and consequent embryo culture depend on several factors. Moreover, the method of COC collection is considered to be one of the most important factors. An average number of COCs collected from a bitch may range from 10 up to several dozen or even over 100 (3, 11, 22). On the other hand, regardless of the oocyte collection method, the number and morphology of collected COCs are influenced by some bitch-related factors such as: season, breed, age, size and weight of ovaries, presence or lack of functional structures on the ovarian surface, stage of oestrus cycle, and physiological or pathological condition of the uterus (2, 6, 16, 22). Previously published data on the importance of the above-mentioned factors for the oocyte number and quality are quite often contradictory.

Pyometra is a pathological condition of the uterus, occurring predominantly in bitches under the influence of progesterone at metoestrus. However, the plasma progesterone concentration in bitches with pyometra

*) This study was supported by grant No 2923/B/P01/2009/37 from the Polish Ministry of Scientific Research and High Education.

is not different from those of pregnant or normally cycling females (15, 21, 23). The most frequently preferred treatment of this disease is ovariohysterectomy (24). Therefore, the collection and morphological assessment of COCs and IVF are applied to salvage the genetic potential of these females.

The development of ART in dogs may be important in the future to salvage gametes from genetically and evolutionarily precious bitches of other canid species. The aim of the present study was to investigate whether selected factors related to oocyte donors influence the number and quality of collected COCs.

Material and methods

Animals. Ovaries were collected from 84 sexually mature pure bred bitches and mongrels aged from 18 months to 13 years. The animals originated from a local dog shelter or were directed to our veterinary clinic (Department of Agricultural Veterinary, University of Agriculture, Poznan, Poland) due to various reasons. Ovaries, oviducts and uteri were surgically removed under general anesthesia induced with ketamine hydrochloride (Ketalar™, Sankyo Co., Ltd., Tokyo, Japan) administered i.m., following pre-anesthetic medication with atropine sulphate (Fuso Pharmaceutical Industries, Ltd., Osaka, Japan), and maintained by inhalation of halothane, nitrous oxide and oxygen. The surgery was performed at the Department of Agricultural Veterinary, University of Agriculture, Poland. Bitches were diagnosed with pyometra on the basis of clinical signs and a clinical examination, i.e. hemogram, cytology of vaginal smears, radiography, and ultrasonography. All bitches with pyometra showed polydipsia, complete lack of appetite or its reduction (anorexia), apathy, peripheral leukocytosis, enlarged abdominal integuments, and increased pulse and respiration rate; additionally, 75% of them developed purulent discharge from the reproductive organs. In all investigated bitches, abdominal ultrasound examination with a 5/7.5 MHz microprobe (Honda electronics, Japan 1500) revealed enlarged uteri of diameters ranging from 3.5 to 6 cm with hypoechogenic content. After being removed by ovariohysterectomy, ovaries were weighed and subjected to morphological evaluation. Uterine fluid ranged from yellow to green or brown. Additionally, data concerning age, body mass, and sterilization reasons were collected.

Experimental groups. Animals were divided into four groups according to morphological structures on the ovarian surface: (1) 18 healthy bitches with no morphological structures detected macroscopically (GL); (2) 33 healthy bitches with visible follicles (F); (3) 12 bitches with wrinkles, folds and corpus lutei (CL); (4) 21 bitches with various pathological changes on ovaries (cysts, neoplastic tumours) and with pyometra (EPC).

With respect to age, bitches were divided into 3 groups: (1) 46 bitches aged 8 months to 2 years and 11 months; (2) 24 bitches aged 3 to 6 years and 11 months; (3) 14 bitches aged 7 to 13 years. According to body weight, females were divided into 3 groups: (1) 21 bitches with a body weight of 25-60 kg; (2) 27 with a body mass of 16-25.5 kg; (3) 36 with a body weight of 7-15.9 kg.

COC collection. After ovariohysterectomy, the ovaries, oviducts and uteri were transported to the laboratory in 0.9% NaCl solution at room temperature immediately after removal. To preserve high quality of oocytes, the ovaries were then surgically removed from surrounding tissues and placed into HEPES-buffered Tyrode's medium (TALP--HEPES) supplemented with 3 mg/ml BSA (Fraction V, Sigma, St. Louis, MO, USA), 0.2 mM sodium pyruvate (Sigma, St. Louis, MO, USA) and 50 µg/ml gentamicin sulfate (Sigma, St. Louis, MO, USA) in a 60 mm plastic dish (Falcon 1007, Becton Dickinson Laboware, Lincoln Park, NJ, USA). The COCs were recovered by multiple multiangle scarification with a special device comprising 4-6 razor blades fixed vertically. After collection, the COCs were washed twice in TALP-HEPES. The diameter of COCs was measured using a micrometer on a video monitor connected with an inverted microscope (Olympus BO61) at 16 × magnification. The morphological assessment of COCs was performed using the Quick Photo 5.0 program with a video micrometer. All of the recovered oocytes were collected during the spring.

Quality assessment. COCs were evaluated under a stereomicroscope at 400 × magnification and divided into 3 groups according to the criteria described by Hewitt et al. (7). The following criteria were included: (1) Class I (DI) – COCs of high and very high quality, suitable for *in vitro* maturation, with compact, multi-layered cumulus cells displaying no signs of expansion and an evenly granulated ooplasm. Insignificant changes in ooplasm granularity or color were acceptable; (2) Class II (DII) – COCs of sufficient quality, with less compact, fairly dispersed cumulus cells. Their transparent theca was complete, dark, of high granularity, with the ooplasm showing an unclear structure and uneven color; (3) Class III (DIII) – oocytes of decreased quality, showing various extent of ooplasm degeneration, signs of expansion or absence of cumulus cells. Zona pellucida was dark, uneven, or damaged, having a contracted ooplasm with some fragmentation.

Statistical evaluations included a unidirectional analysis of variance (ANOVA), as well as Tukey's multiple comparisons (NIRt) contained in the Analysis Toolpak package. Two levels of significance ($P < 0.05$, $P < 0.001$) were considered.

Results and discussion

Altogether, 10 077 oocytes were analyzed in the present study; class I, II, and III oocytes comprised 2353 (23.4%), 2640 (26.2%), and 5084 (50.4%) COCs, respectively. The average number of COCs collected from one bitch was 125.6, ranging from 11 to 588, whereas the average number of DI from one bitch was 29.4 COCs. The weight of the left and right ovaries was on average 1.5 g and 1.7 g, respectively, and was not related to the number and quality of collected COCs. The average number of COCs collected from the right ovary was 150, and from the left 133. The rate of DI COCs originating from the right and left ovaries was 24.7% and 22.2%, respectively.

The presence of morphological structures significantly influenced the number of collected COCs (tab. 1). The mean number of COCs was 202.8 in bitches with no structural growths (group GL) and was significantly higher ($P < 0.001$) than the number of COCs collected from bitches with uterine pathology, cysts or neoplastic tumours (82.8, group EPC). In females with visible follicles (group F) or corpus luteum (group CL), the mean number of collected COCs was 121.1 and 97.6, respectively (tab. 1).

The female's age had a significant influence on the number of collected COCs. The number of COCs in the youngest bitches (group I) reached 163.4 and was higher than in the middle (group II) and old age (group III) groups (84.4 and 83.9, respectively), ($P < 0.001$), (tab. 2).

Female body weight had no significant influence on either the number or the quality of the collected COCs (tab. 3).

The present study describes the reproductive potential of several bitches in relation to the number and the rate of high quality follicular oocytes. The average number of collected COCs was 125.6 per female, which we consider as high in comparison to other reports of 37.2, 45.7, 71 and 18.5 (6, 9, 16, 22), respectively. The observed variation may be due to different collection methods, e.g. ovum pick up (22) or with scalpel (6), which are known to significantly affect the number of collected cells. In this study, a significant relation between the condition of the ovary (presence of functional structures) and the number of collected COCs was noted. The highest number of COCs (202.8) was collected from bitches with ovaries displaying a smooth surface, while the lowest (82.8) in the case of ovarian and/or uterine pathologies. However, no relationship was observed with regard to COC quality and these conditions. A similar opinion was presented by Songasen et al. (22), who did not find any influence of pathological changes within the uterus and the presence of various functional ovarian structures on the number of collected COCs. In this study we demonstrated a higher number of COCs from ovaries without morphological structures in comparison to earlier studies. It can be explained by a specific group of investigated animals as well as individual characteristics of the bitches. In addition, Lopes et al (13) showed a higher number of grade I COCs collected from the youngest bitches. In our study we presented a higher total number of recovered COCs, although we did not find significant differences in grade I COCs between the age-dependent groups of bitches. One of the explanations of these two cases may be the progesterone specific concentration, which, as it was proved by Hossein et al. (10), has an important influence on the number and quality of collected COCs. Probably, several other factors, which have not been focused on till the present day, have a more important influence on the quality and number of recovered COCs from bitches.

In this study, a relatively large number of high quality COCs was collected from bitches with pyometra. A large number of oocytes from bitches with pyometra were also collected by Hishinuma et al. (8) and Ptaszyńska (16). However, these authors indicated the ambivalent relationship between the pathology and

Tab. 1. Number and quality of collected COCs depends on detectable morphological growths on the ovarian surface

Objectives	Structure of the surface of ovaries			
	Smooth (without visible follicles)	Follicular structure	Corpus lutei	Uterine and ovarian pathology
	Groups			
	I	II	III	IV
No. of bitches (n)	18	33	8	21
Mean number of COCs	202.8^{a}	121.1^{b}	97.6^{C}	82.8^{D}
DI oocytes (%)	22.9	21.1	24.2	25.7
DII oocytes (%)	25.5	27.8	24.1	26.6
DIII oocytes (%)	51.6	51.1	51.7	47.7

Explanations: means with different superscript letters differ significantly at $p < 0.05$ (a, b, c, d), $p < 0.01$ (A, B, C, D)

Tab. 2. Mean number and quality of oocytes collected from different age groups of bitches

Objectives	Age		
	8 mths to 2 yrs and 11 mths	3 yrs to 6 yrs and 11 mths	7 to 13 yrs
	Groups		
	I	II	III
No. of bitches	46	24	14
Mean No. of COCs	163.4^{a}	84.4^{B}	83.9^{C}
DI oocytes (%)	23.0	23.6	24.2
DII oocytes (%)	27.1	24.5	22.5
DIII oocytes (%)	49.9	51.9	53.3

Explanations: as in tab. 1.

Tab. 3. Number and quality of bitch oocytes collected from females of different body mass

Objectives	Body mass		
	25-60 kg	16-25.5 kg	7-15.9 kg
	Groups		
	I	II	III
No. of bitches	21	27	36
Mean No. of COCs	131.7	149.8	108.6
DI oocytes (%)	33.2	34.3	33.7
DII oocytes (%)	27.4	26.8	25.3
DIII oocytes (%)	39.4	38.9	41.0

Explanations: as in tab. 1.

a decreased number of high quality oocytes. In their work, they observed that this tendency may be due to a great variation among females (age, condition etc), which slightly increased the number of DI and DII COCs isolated from bitches with pyometra, although the total number of COCs aspirated from bitches with this pathology was significantly smaller compared to bitches with various ovarian structures. Our observations suggest that pyometra is a pathologic disease that significantly influences the number of total COCs recovered but is not statistically associated with the collection of DI and DII oocytes.

Analyses of the relationship between the age and the number and quality of bitch COCs have only been presented in a few studies (19). However, there is a tendency to hypothesize that the female's age is among the factors significantly influencing the number of collected COCs. In this study, a higher number of oocytes was collected from the youngest adult bitches (up to 3 years old), and the lowest from the oldest (more than 7 years old). The greater number of COCs isolated from younger adult bitches and a significantly lower number of COCs isolated from adult and senile bitches may be associated with a decreased reproductive potential of these females, which is age--dependent. A similar tendency was described by Hewitt and England (6), who collected 54.2 oocytes from 1- to 6-year-old bitches and 26.4 from older ones (7- to 13-year-olds). The role of the female's age has also been described by Rocha et al. (19), who compared the quality of COCs between young (45-60 days), adult (2-6.5 years) and senile (9.5-13) females. They did not find oocytes in the ovaries of pre-pubertal donors. However, they collected COCs from adult and senile bitches. Contrary to our results, they observed an increased number of total oocytes collected from senile females as compared to adults. Similarly, they found a statistically increased number of degenerative oocytes (DIII) in senile bitches. These observations were also confirmed by Hay et al. (5), who demonstrated that oocytes from senile bitches have a lower quality.

In this study, a varying reproductive potential of adult and senile bitches was described. Moreover, we compared two different age groups of adult females and observed statistically significant differences in the quality of COCs collected from these bitches. These results suggest that the reproductive potential of up--to-3-year-old bitches is closer to senile females than to younger adult females.

In conclusion, this study confirms the hypothesis of the important influence of age and uterine/ovarian pathology on the quantity and quality of recovered oocytes. A clear demonstration of the reproductive potential of females in different age groups, and especially those with conditions such as pyometra, may be crucial to the salvage of oocytes from genetically significant canids and to the preservation of their genetic potential.

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