REPRODUCTIVE BIOLOGY

ORIGINAL RESEARCH

The quality of porcine oocytes is affected by sexual maturity of the donor gilt

Piotr Pawlak, Natalia Renska, Emilia Pers-Kamczyc, Ewelina Warzych, Dorota Lechniak[1]

Department of Genetics and Animal Breeding, Poznan University of Life Sciences, Poznan, Poland

Received: 9 October 2010; accepted: 5 January 2011

SUMMARY

Although differences in the quality of oocytes derived from young gilts and adult sows are well documented, evidence concerning gametes of pre-pubertal and cycling gilts is scarce and inconsistent. The aim of this work was to establish whether sexual maturity of gilts affects the quality of their oocytes with the use of the brilliant cresyl blue (BCB) test, oocyte diameter and apoptosis. Ovarian morphology was evaluated, and gonads with corpus luteum or albicans were recognized as originating form cycling gilts (C) and those with follicles as originating form pre-pubertal females (P). Altogether 952 cumulus-oocyte complexes (COCs; group P: 554; group C: 398) were examined, whereas 149 COCs, not subjected to BCB test, served as a control for TUNEL. COCs of proper morphology were evaluated by the BCB test which differentiated two categories of gametes: more

[1]Corresponding author: Department of Genetics and Animal Breeding, Poznan University of Life Sciences, Wolynska 33, 60–637 Poznan, Poland; e-mail: lechniak@jay.au.poznan.pl

competent, BCB+, and less competent BCB- oocytes. The control group comprised oocytes of proper morphology aspirated from ovaries of P and C gilts not subjected to BCB test. Finally five groups of COCs were matured *in vitro:* 1/P-BCB+, 2/P-BCB-, 3/C-BCB+, 4/ C-BCB- and 5/ control. Significantly more large oocytes (≥120 mm), more BCB+ oocytes and more high quality (both BCB+ and ≥120 mm) oocytes originated from ovaries of cycling gilts than pre-pubertal gilts ($p<0.001$). The rate of mature oocytes at the MII stage differed significantly between C-BCB+ (68.5%) and P-BCB+ (32.9%) oocytes. The incidence of apoptosis among BCB-treated oocytes after *in vitro* maturation was 21.4% and was similar to that observed in control oocytes (17.4%). BCB+ oocytes from cycling gilts showed significantly higher (28.7%) incidence of apoptosis than that of the group P (16.2%). Interestingly, high quality oocytes displayed a similar level of apoptosis regardless of the donor puberty. We demonstrated that C gilts provided more BCB+ oocytes as well as more large oocytes than P gilts, although C-BCB+ oocytes showed higher apoptotic rate. In conclusion, high incidence of apoptosis and a big variation in the diameter of more competent BCB+ oocytes make the BCB test a less effective selection tool than previously reported. *Reproductive Biology 2011* ***11*** *1: 1–18.*
Key words: pre-pubertal, pig, TUNEL, diameter, BCB test, IVM

INTRODUCTION

Oocyte quality determines the outcome of the protocol for *in vitro* production of pig embryos generated from cumulus-oocyte complexes (COCs) which are collected from ovaries of slaughtered females. Gilt oocytes differ in many aspects from those of sows [4, 5, 19]. Although meiotic competence and fertilization rate were shown to be similar for gilt and sow oocytes, gametes derived from gilts were more often polyspermic and rarely formed blastocysts [5, 19]. In the study by Bagg et al [6] only oocytes collected from the biggest follicles of gilt ovaries (5–8 mm) developed to the blastocyst stage at the rate similar to that of sow oocytes from all size categories. The population of commercially slaughtered females has been however dominated

by gilts at the age of 6–7 months. Although they are at a similar age, ovulation takes place only in some of them. According to our unpublished data collected at a local abattoir, pre-pubertal gilts may account for up to 80% of slaughtered females. Data comparing the quality of oocytes from pre-pubertal (P) and cycling (C) gilts is rather scarce. According to Brussow et al [8] cycling gilts are better donors of oocytes for the *in vitro* fertilization (IVF) procedure than pre-pubertal and primiparous females. The authors performed a repeated laparotomy on the same female at the ages of 6 months (pre-pubertal) and 9.5 months (cycling). Ovaries of cycling gilts contained more follicles, and their oocytes showed higher meiotic competence when compared to pre-pubertal females.

Two of the main obstacles in the porcine *in vitro* production (IVP) system are high incidence of polyspermy and reduced quality of the resulting blastocysts [9]. Therefore, special attention has been recently placed on developing a protocol for the non-invasive selection of competent oocytes. The method of choice may be the incubation of COCs in brilliant cresyl blue (the BCB test), an approach based on glucose-6-phosphate dehydrogenase activity (G6PD) characteristic for growing oocytes [12]. It is generally accepted that selection of more competent porcine oocytes by the BCB test is a reliable method with no negative effects on their further development after fertilization *in vitro* [17, 24, 28].

Oocyte quality can be assessed by several methods including the detection of apoptosis and size determination. To our knowledge there is no published data on the incidence of apoptosis in porcine oocytes matured *in vitro*. The incidence of apoptotic oocytes prior to *in vitro* maturation (IVM) was low in cattle but increased significantly after IVM [20, 27]. Moreover, the high incidence of apoptotic oocytes (28.4%) has been found in immature oocytes of pre-pubertal goats [2]. With regard to oocyte size, some of the porcine oocytes acquire meiotic competence with a diameter of 100–115 μm, whereas most of oocytes with a diameter ≥120 μm are fully competent [15]. Oocytes of pre-pubertal gilts were smaller (113.1 μm) than those of cycling females (124.7 μm; [4]). Among porcine oocytes evaluated by the brilliant cresyl blue (BCB) test, more competent BCB+ gametes were larger (113.08 μm) than less competent BCB- gametes (100.29 μm; [24]).

Therefore, we assumed, that an oocyte displaying both, BCB+ phenotype and a diameter ≥120 μm can be considered as high quality gamete.

The aim of the study was to establish whether puberty of gilts affects the quality of oocytes. We analyzed the quality of COCs aspirated from ovaries of pre-pubertal and cycling gilts and selected by the BCB test. We also compared oocyte diameter and the incidence of apoptosis between these two groups of gilts.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA). A pair of ovaries with corresponding reproductive tracts were collected after slaughter in a local abattoir from commercial cross-bred pigs of unknown origin. The animals were 6–7 months old and weighed 100–110 kg. Each uterus was carefully examined for size and the corresponding ovaries were examined for the presence of corpus luteum or albicans. Such approach allowed us to exclude females which had been previously pregnant. 70% of collected gonads did not show the presence of corpus luteum or albicans and therefore were classified as originating from pre-pubertal gilts.

Aspiration and morphological evaluation of oocyte-cumulus complexes

Dissected ovaries were transported to the laboratory in an isolated container within two hours of animal slaughter. One medium size, randomly selected uterus was also included to keep the temperature of the material in the range between 25–29°C. Then, the ovaries were divided into two categories [4] originating from: 1/ pre-pubertal (P) gilts (with small follicles only), and 2/ cycling (C) gilts (with corpus luteum or albicans). COCs were aspirated from non-atretic follicles 2–4 mm in diameter, washed twice in Hepes-Talp and examined under a stereomicroscope (magnification 20×). Only COCs displaying at least 3–4 layers of compact cumulus cells and a homogenous ooplasm (fig. 1A) were subjected to the BCB test.

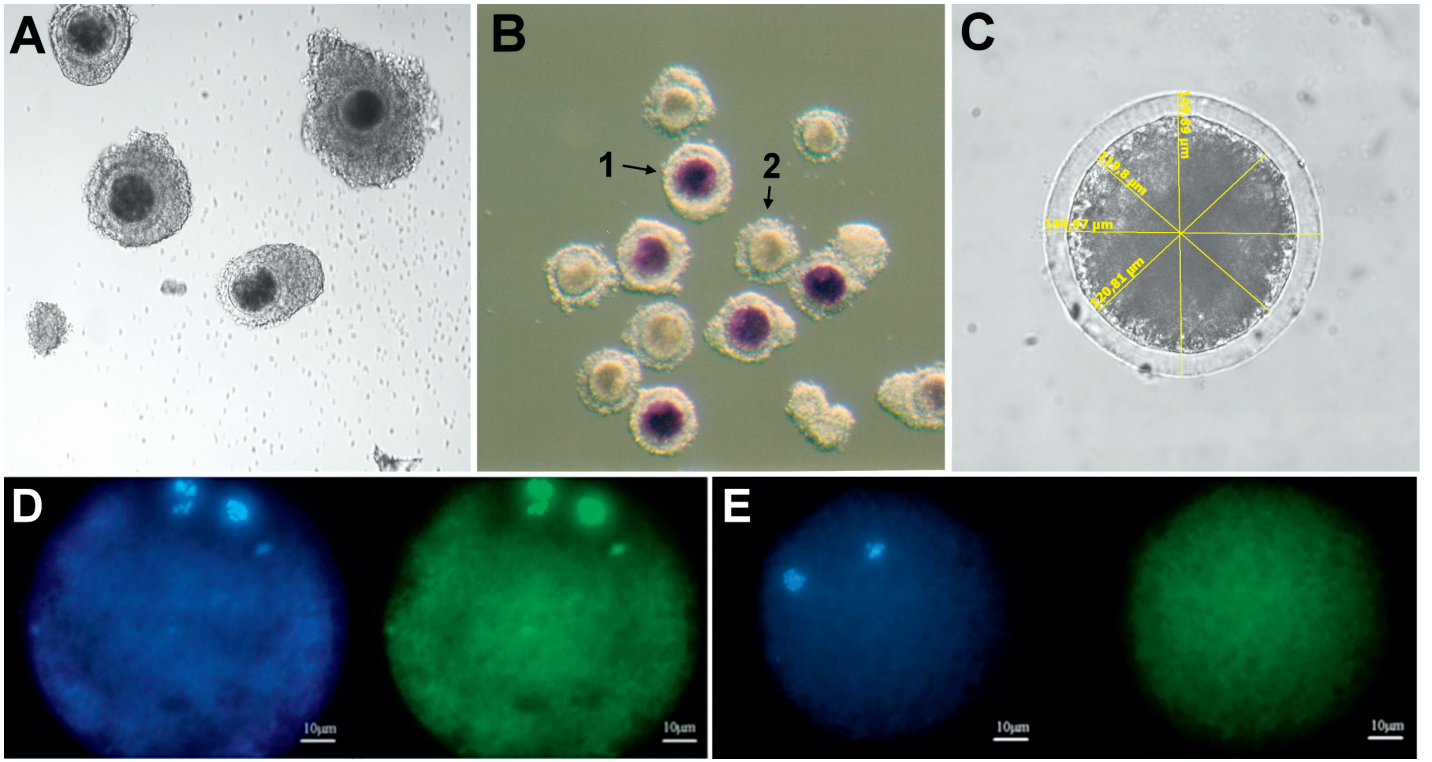

Figure. 1. Quality assessment of gilt oocytes performed by the following methods: A/ morphology of the cumulus-oocyte complex; B/ brilliant cresyl blue (BCB) test (1: BCB+ stained oocyte, 2: BCB- colorless oocyte); C/ size determination; D/ TUNEL positive (apoptotic) oocyte with a distinct FITC signal; and E / TUNEL negative (non-apoptotic) oocyte with no FITC signal; DAPI – blue, FITC – green.

Competence evaluation by the brilliant cresyl blue test

COCs collected from C and P ovaries were initially washed twice in BCB medium (PBS supplemented with 4 mg/ml BSA and 13 mM of BCB) and incubated for 90 minutes at 39°C in a humidified 5% CO_2 atmosphere [12]. Following exposure to BCB, the COCs were transferred to PBS supplemented with 0.4% BSA and washed twice. After washing, the COCs were examined under a stereomicroscope and divided into two groups: COCs with an ooplasm stained blue were classified as more competent (BCB test positive – BCB+; fig. 1B1), whereas COCs with a colorless ooplasm were classified as less competent (BCB test negative – BCB-; fig. 1B2). After selection, the COCs were matured *in vitro* in five groups: 1/ P-BCB+, 2/ P-BCB-, 3/ C-BCB+, 4/ C-BCB-, and 5/ control oocytes (non-BCB-treated from both P and C gilts).

IN VITRO MATURATION

COC incubation was carried out according to the protocol described by Gupta et al [14]. Follicular fluid (FF) was aspirated from non-atretic follicles (2–6 mm in diameter), centrifuged (30 min, 1 400×g), filtered (Milipore, Millex GP 0.22 μm), frozen (-70°C) and stored for a maximum of two weeks. The maturation medium consisted of basic TCM199 supplemented with 10% FF, 25 mM $NaHCO_3$, 0.57 mM cystein, 0.22 mg/ml sodium pyruvate, 0.5 mg/ml FSH, 10 mg/ml estradiol-17β, 10 ng/ml EGF, 25 mg/ml gentamicin sulfate. *In vitro* maturation was performed in 500 ml of IVM medium (100 COCs per droplet) for 44 h (39°C; humidified 5% CO_2 atmosphere).

After culture, COCs were shortly incubated (3–5 min) in 0.025% hyaluronidase and subjected to vigorous pipetting in order to remove all attached follicular cells. The denuded oocytes from C and P ovaries were randomly separated into two populations: 1/ subjected to size determination, and then washed in PBS+0.2% PVP, and 2/ subjected to TUNEL analysis, and then fixed in a 4% paraformaldehyde solution in PBS (30 min) and washed twice in PBS+0.2% PVP.

Oocyte size measurement

Denuded oocytes were individually placed into separate wells of a 96-well dish in a 150 µl of PBS+0.2% PVP. Oocyte diameter was measured using a computerized microscopic system (AxioVision, Zeiss, Germany) connected to a camera (AxioCam MRm, Zeiss, Germany). A mean of two measurements of oocyte diameter without zona pellucida made perpendicular to each other was used for calculations (fig. 1C). Two size categories were distinguished: 1/ small oocytes with diameter <120 µm and 2/ large oocytes with diameter ≥120 µm [18].

Apoptosis (TUNEL)

Apoptosis was investigated in all oocytes after maturation *in vitro*. A group of high quality oocytes (BCB+; ≥120 µm) was added in order to address a question whether puberty affects the incidence of apoptosis in such gametes. Apoptotic cells were identified using the DeadEnd™ Fluorometric TUNEL System (Terminal dUTP Nick-End Labeling with FITC fluorochrome; Promega, Madison, WI, USA) following the manufacturer's instructions with some modifications [27]. Fixed oocytes were permeabilized by incubation in 0.2% Triton® X-100 in PBS for 5 min, washed twice in PBS+0.2% PVP and covered with an equilibration buffer for 15 min. The oocytes were then placed in the TUNEL reaction mixture, covered with mineral oil and incubated at 37°C for 60 min in a humidified dark chamber. The reaction was terminated by washing the oocytes in 2×SSC for 15 min. Afterwards oocytes were washed three times in PBS+0.2% PVP and placed on slides in groups of 20–30. Oocytes that served as a positive control were incubated in DNase I solutions (5U/50 µl). An oocyte was scored as TUNEL positive when a distinct, green fluorescence signal was observed (fig. 1D). In order to stain chromatin, oocytes were mounted in 20 µl of VECTASHIELD with 4,6-diamido-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA), covered with a glass coverslip and stored at 4°C until further analysis. The second metaphase (MII) stage was recognized by the presence of two groups of chromosomes: clumped together belonging to the first polar body (I pb) and spread-out haploid set of oocyte chromosomes (fig. 1D). When only

blue signal (DAPI) was identified with no corresponding green fluorescence (FITC), an oocyte was classified as TUNEL negative (fig. 1E).

Statistical analysis

The effect of the ovarian status (group P vs. C) on the quality of oocytes determined by the BCB test, diameter measurement and apoptosis detection was analyzed by the Fisher exact test (SigmaStat 2.0 statistical program; Jandel scientific software, San Rafael, CA, USA). The p value less than 0.05 was considered as significant.

RESULTS

Altogether 1101 COCs of proper morphology were collected from ovaries of pre-pubertal (P) and cycling gilts (C). A group of 149 COCs served as a control for TUNEL staining and these oocytes were not subjected to the BCB test. The remaining 952 COCs (398 COCs from C gilts and 554 COCs from P gilts) were subjected to the BCB test. The experiment aimed to compare the quality of the oocytes collected from ovaries of C gilts with those of P gilts.

As a result of BCB staining we demonstrated that cycling gilts produced significantly more BCB+ oocytes than pre-pubertal gilts ($p<0.001$). Overall, BCB+ gametes predominated among the oocytes collected from both groups of gilts (C ovaries: 89.7% and P ovaries: 77.8%). The rate of less competent BCB- oocytes was two times higher in P ovaries (22.2%) when compared to that of group C (10.3%; $p<0.001$; fig. 2; tab. 1).

Next, we were interested in the ability of oocytes originating from C and P gilts to mature *in vitro* and to reach the MII stage. The data on the stage of meiotic division (the rate of MII oocytes) in a representative group of 227 BCB-treated oocytes (BCB+: 168; BCB-: 59) was collected during the examination of slides subjected to the TUNEL procedure. The overall rate of meiotic maturation was higher for C oocytes (62.1%, 64/103) than for their P counterparts (25%, 31/124). A significantly higher number of C-BCB+ oocytes (68.5%, 61/89) reached the MII stage compared to P-BCB+

Table 1. Distribution of porcine oocytes according to their size (diameter in µm), BCB category and sexual maturity of the donor gilt

BCB category	Number of small oocytes (<120 µm)			Number of large oocytes (≥120 µm)			Total
	Group P	Group C	Groups P+C	Group P	Group C	Groups P+C	
BCB+	145	48	193	286	309	595	788
BCB-	76	15	91	47	26	73	164
Total	221	63	284	333	335	668	952

P: pre-pubertal gilts; C: cycling gilts; BCB+ : more competent oocytes selected by the BCB test; BCB- : less competent oocytes selected by the BCB test.

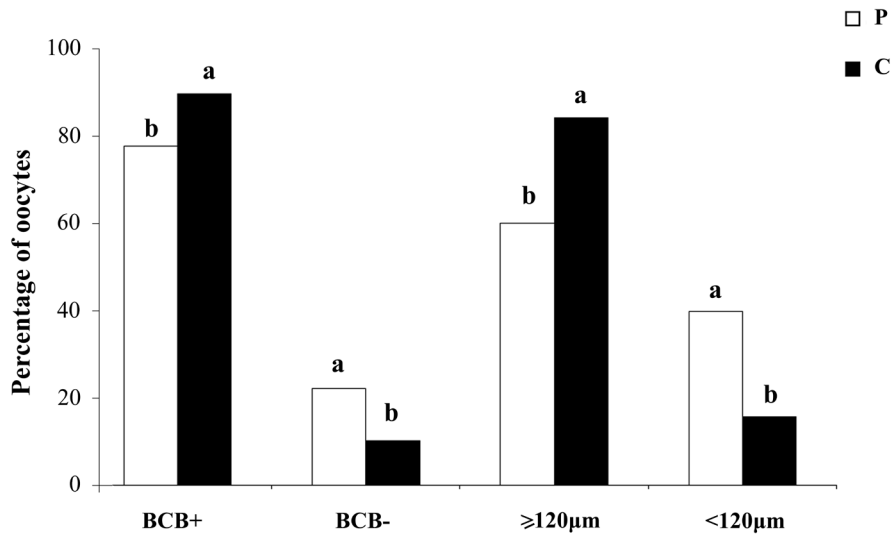

Figure 2. Distribution of oocytes collected from cycling (C) and pre-pubertal (P) gilts according to the BCB test (BCB+, BCB- oocytes) and oocyte size (small oocytes with a diameter <120 µm, large oocytes with a diameter ≥120 µm). Different superscripts show statistically significant differences between oocytes from C and P gilts within each oocyte category; $p<0.001$.

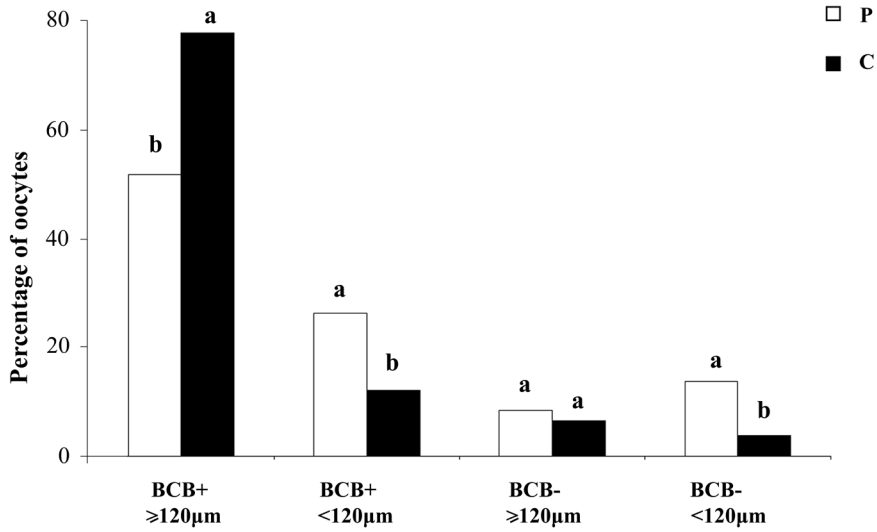

Figure 3. Distribution of oocytes collected from cycling (C) and pre-pubertal (P) gilts: 1/ BCB+ oocytes with a diameter ≥120 µm, 2/ BCB+ oocytes with a diameter <120 µm, 3/ BCB- oocytes with a diameter ≥120 µm, and 4/ BCB- oocytes with a diameter <120 µm. Different superscripts show statistically significant differences between oocytes from C and P gilts within each oocyte category; $p<0.001$.

oocytes (32.9%, 26/79; $p<0.01$). All BCB- oocytes showed a significantly lower maturation rate (C-BCB-: 21.1%, 3/14; P-BCB-: 11.1%, 5/45) with the majority of oocytes arrested at the germinal vesicle breakdown (GVDB) or the metaphase (MI) stages of the first meiotic division.

We have also evaluated the size of the oocytes and compared the BCB+ and BCB- oocytes from C and P gilts. Over 60% of analyzed BCB+ oocytes were large gametes with the diameter ≥120 µm (62.5%, 595/952; fig. 3). The overall frequency of large oocytes was 84.2% in C gilts and 60.1% in P gilts. The distribution of small gametes (diameter <120 µm) was over 2.5 times higher in P gilts (39.9%) than in C gilts (15.8%). The predominant number of large oocytes fell within the group of BCB+ oocytes (75.5%, 595/788), while BCB- oocytes were almost equally distributed between the two examined size categories (≥120 µm: 44.5%, 73/164; <120 µm: 55.5%, 91/164).

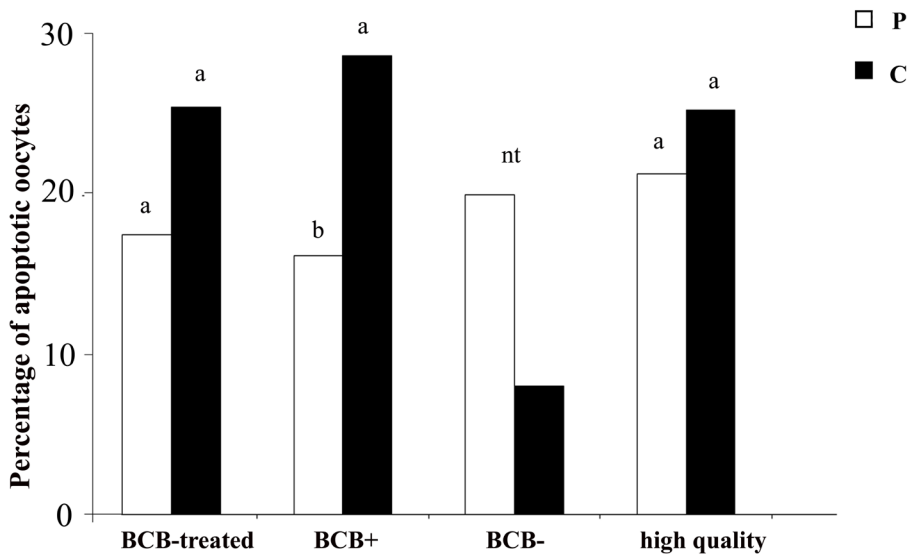

Figure 4. The incidence of apoptosis in oocytes collected from cycling (C) and pre-pubertal (P) gilts after *in vitro* maturation: 1/ in all BCB-treated oocytes, 2/ in BCB+ oocytes, 3/ in BCB- oocytes, 4/ in high quality oocytes (BCB+ and diameter ≥120 μm). Different superscripts show statistically significant differences between oocytes from C and P gilts within each oocyte category; $p<0.05$; nt: data not analyzed statistically due to a very low number (2/25) of oocytes in the BCB- category.

When the puberty of the donor was considered, significantly more large oocytes were found in the C-BCB+ (77.6%) than in the P-BCB+ group (51.6%). We also noted that the incidence of small oocytes was over two times higher (26.2%; 145/554) in the P-BCB+ group compared to the C-BCB+ group (12.1%; 48/398). With respect to BCB- oocytes, the frequency of large gametes was equal in both groups of gilts (group P: 8.5%, group C: 6.5%), whereas small oocytes were 3.5 times more abundant in C-BCB- (13.7%) than P-BCB- group (3.8%).

Altogether 476 oocytes were subjected to TUNEL analysis (BCB-treated: 327; control non-BCB-treated: 149; fig. 4). We observed a similar incidence of apoptosis in all BCB-treated oocytes derived from P (17.4%) and C gilts (25.5%) with an average of 21.4%. Moreover, no significant differences were

Table 2. Incidence of apoptosis* in porcine oocytes matured *in vitro* collected from cycling (group C) and pre-pubertal (group P) gilts according to BCB category

	BCB+ oocytes			BCB- oocytes			High quality oocytes (BCB+ and ≥120 μm)			Control group
	Group P	Group C	Groups P+C	Group P	Group C	Groups P+C	Group P	Group C	Groups P+C	
Apoptotic oocytes	18	39	57	11	2	13	37	55	92	26
Analyzed oocytes	111	136	247	55	25	80	174	217	391	149

*TUNEL positive cell = apoptotic cell ; BCB+: more competent oocytes selected by the BCB test; BCB-: less competent oocytes selected by the BCB test; the high quality oocytes: gametes displaying two features: BCB+ phenotype and large size (≥120 μm).; the control group: non BCB-treated oocytes of proper morphology derived from C and P gilts.

noticed in the rate of apoptotic cells between BCB+ (23.1%; 57/247) and BCB- oocytes (16.3%; 13/80). After including donor puberty factor into the analysis, apoptosis appeared more frequently in C-BCB+ (28.7%) than in P-BCB+ oocytes (16.2%). An opposite relation was observed for BCB- oocytes, where apoptotic cells were more abundant in P-BCB- (20.0%, 11/55) in comparison to C-BCB- oocytes (8%; 2/25). The incidence of apoptosis in the population of high quality oocytes (BCB+ and ≥120 mm) was equal in both groups of gilts with an average of 23.5%. Therefore the rate of apoptotic oocytes was close to that for all BCB-treated oocytes (21.4%; fig. 4; tab. 2). The incidence of apoptosis in the control (17.4%) and BCB-treated gametes was similar.

DISCUSSION

The quality of oocytes is a complex trait and as such is not easily evaluated by non-invasive means. COC morphology evidently does not fully predict oocyte competence, so we used a two-step selection. Aspirated COCs were first classified morphologically and then selected by the BCB test. We demonstrated that gilt puberty affected the quality of oocytes. We have also shown that ovaries of cycling (C) gilts yielded more BCB+ and large (diameter ≥120 μm) oocytes, and those oocytes were more often apoptotic when compared to BCB+ oocytes of pre-pubertal (P) gilts.

The available data on the quality of oocytes of pre-pubertal and cycling gilts is scarce [4, 23]. Bagg et al [4] did not observe any differences in the rates of meiotic maturation, cleavage and blastocyst formation in oocytes of P and C gilts. On contrary, our results showed different meiotic competence displayed by morphologically normal oocytes from cycling (62.1% of oocytes at MII stage) and pre-pubertal gilts (25%). Our findings support those of Paczkowski et al [22] who revealed significant differences in the pattern of 14 proteins related to glucose metabolism, cellular stress and ovulation in oocytes of these two gilt categories. Unfortunately, these authors did not provide data on meiotic competence of oocytes.

Since distinct differences in the rate of MII oocytes from P and C gilts had been observed we addressed a question whether more competent BCB+ oocytes

would differ in this respect. Although bovine and caprine oocytes positive for the BCB test display higher developmental potential than BCB negative cells, the evidence concerning pigs is not consistent [1, 25]. In the recent study by Egerszegi et al [11] only BCB+ oocytes were penetrated after fertilization *in vitro*. Wongsrikeao et al [28] observed satisfactory developmental competence for BCB+ oocytes (blastocyst rate of 12%), whereas none of the fertilized BCB- oocytes reached the blastocyst stage. In other studies, BCB- oocytes were characterized by lower penetration as well as drastically reduced maturation (2.3%) and fertilization (1%) rates [24, 28]. Similarly, meiotic competence of BCB- oocytes analyzed by Ishizaki et al [17] was reduced (58.1%) when compared with that of BCB+ oocytes (77.2%) however, the differences were not as distinct as in our experiment. We have noticed a lower MII rate among BCB- oocytes (13.5%) when compared with BCB+ group (51.8%). In addition, we observed a significant influence of the donor puberty on meiotic competence of oocytes selected by BCB test. More BCB positive oocytes from cycling gilts reached the MII stage (68.5% vs. 32.9% pre-pubertal). A similar tendency was observed for the BCB negative gametes (C 21.4%, P 11.1%).

Some of the described discrepancy in the parameters attributed to the quality of oocytes selected by the BCB test may be related to their heterogeneity as was previously observed among murine BCB+ oocytes [30]. It was reported that bigger BCB+ oocytes (>70 μm) had better developmental potential than small oocytes (<60 μm). A majority (95%) of small oocytes were BCB+ but failed to mature *in vitro*. Our data also shows a variation in the quality of BCB+ oocytes with respect to their size and origin. Generally, 24.5% of the BCB+ cells displayed a diameter <120 μm, the trait attributed to oocytes of reduced competence [15]. This contrasts with data presented by Roca et al [24] who found 60% of BCB+ oocytes with a diameter smaller than 115 μm. In our study, significantly more large BCB+ oocytes were aspirated from ovaries collected from C gilts (77.6%) than P gilts (51.6%). This confirms the previous findings of Bagg et al [4] who demonstrated that cycling ovaries yielded bigger oocytes (124.7 μm) than their pre-pubertal counterparts (113.1 μm). In our experiment the rate of small BCB+ oocytes ranged from 12.1% in C gilts to 26.2% in P gilts with an average of 20.3%. The observed differences between mice and pigs may be species-related. For the above reasons we se-

lected a sub-population of BCB+ oocytes standardized with respect to their size and derived from both categories of gilts in order to examine the quality of a homogenous group of gametes. This group named "high quality oocytes" was assessed for apoptosis by TUNEL.

Apoptosis is considered a marker of quality of both oocytes and embryos. Its incidence also reflects sub-optimal culture conditions since apoptotic oocytes were more abundant (17.1% – 52.3%) among human oocytes matured *in vitro* [29] than in their ovulated counterparts (2%; [26]). Moreover, a significantly higher proportion of apoptosis was described in immature (1.4%, 7%) than *in vitro* matured bovine oocytes (11.2%, 23%; [20, 27]). One of the factors affecting apoptosis was the puberty of the oocyte donor. A very high proportion (28.4%) of immature oocytes collected from 2-month old pre-pubertal goats showed signs of apoptosis [2]. To our knowledge there is no published evidence on the incidence of apoptosis in porcine oocytes matured *in vitro*. In this experiment, the average percentage of apoptosis in BCB-treated oocytes of gilts after IVM was 21.4%. The general incidence of apoptosis was affected by neither the donor puberty nor the size of the oocyte itself. Furthermore, the high quality oocytes displayed a similar level of apoptosis in the C (25.3%) and P (21.3%) groups which was close to the average level (21.4%). Interestingly, BCB+ oocytes derived from C gilts displayed a higher incidence of apoptosis (28.7%) than those collected from P gilts (16.2%). This finding is controversial since cycling females produce more competent oocytes [3]. On the other hand, some previous studies have shown that the incidence of apoptosis was greater in cumulus cells accompanying oocytes collected from adult sheep (39.2%) compared with those of juvenile females (21.9%; [7]). However the extent of apoptosis in cumulus cells does not fully predict the quality of the oocyte [31]. In antral follicles, a common source of oocytes for IVF, the oocyte was shown to be the last follicular compartment affected by apoptosis [10]. This phenomenon was confirmed by Feng et al [13] who demonstrated a significant elevation in developmental potential of oocytes derived from antral follicles (4–8 mm) that showed early signs of atresia in comparison with non-atretic and late atretic bovine follicles. Moreover, Hussein et al [16] demonstrated the ability of bovine oocytes to prevent apoptosis in cumulus cells by secreting some

oocyte-specific factors (e.g. BMP15). Therefore, we suggest that apoptosis in oocytes is rather a questionable marker of the quality of BCB-tested oocytes in gilts. Our suggestion is supported by data concerning the expression of genes regulating apoptosis (Bax and Bcl 2) in bovine oocytes [21]. In that study, no significant differences in transcript and protein levels between BCB+ and BCB– oocytes were observed. Therefore, the question arises as to what causes the reduction in development competence of BCB- oocytes observed after fertilization and during embryonic development. Assuming a similar level of apoptosis in BCB+ and BCB- porcine oocytes, some other factors than apoptosis must be responsible for the reduction of oocyte quality.

In conclusion, a high incidence of apoptosis and a big variation in the diameter of more competent BCB+ oocytes make the BCB test a less effective selection tool as previously reported. The BCB+ oocytes from P and C gilts still differ in many aspects. We suggest that the reliable evaluation of the quality of pig oocytes should consider several factors. The selection of gilt oocytes by the means of the BCB test yields a highly heterogeneous group of oocytes in terms of their intrinsic features like size, meiotic competency or susceptibility to apoptosis. Therefore, the rates of fertilized oocytes and developing embryos still remain the most reliable markers of oocyte quality.

ACKNOWLEDGEMENTS

Research conducted within the statutory activity of the Department of Genetics and Animal Breeding (no. 34–1), Faculty of Animal Breeding and Biology, Poznan University of Life Sciences, Poland.

REFERENCES

1. Alm H, Torner H, Lohrke B, Viergutz T, Ghoneim IM, Kanitz W **2005** Bovine blastocyst development rate in vitro is influenced by selection of oocytes by brilliant cresyl blue staining before IVM as indicator for glucose-6-phosphate dehydrogenase activity. *Theriogenology* **63** 2194–2205.

2. Anguita B, Paramio MT, Morató R, Romaguera R, Jiménez-Macedo AR, Mogas T, Izquierdo D **2009** Effect of the apoptosis rate observed in oocytes and cumulus cells on embryo development in prepubertal goats. *Animal Reproduction Science* **116** 95–106.
3. Armstrong DT **2001** Effects of maternal age on oocyte developmental competence. *Theriogenology* **55** 1303–1322.
4. Bagg MA, Vassena R, Papasso-Brambilla E, Grupen CG, Armstrong DT, Gandolfi F **2004** Changes in ovarian, follicular, and oocyte morphology immediately after the onset of puberty are not accompanied by an increase in oocyte developmental competence in the pig. *Theriogenology* **62** 1003–1011.
5. Bagg MA, Nottle MB, Grupen CG, Armstrong DT **2006** Effect of dibutyryl cAMP on the cAMP content, meiotic progression, and developmental potential of in vitro matured pre-pubertal and adult pig oocytes. *Molecular Reproduction and Development* **73** 1326–1332.
6. Bagg MA, Nottle MB, Armstrong DT, Grupen CG **2007** Relationship between follicle size and oocyte developmental competence in prepubertal and adult pigs. *Reproduction Fertility Development* **19** 797–803.
7. Boni R, Cocchia N, Silvestre F, Tortora G, Lorizio R, Tosti E **2008** Juvenile and adult immature and in vitro matured ovine oocytes evaluated in relation to membrane electrical properties, calcium stores, IP3 sensitivity and apoptosis occurrence in cumulus cells. *Molecular Reproduction and Development* **75** 1752–1760.
8. Brüssow KP, Rátky J, Torner H, Egerszegi I, Schneider F, Solti L, Tuchscherer A **2002** Follicular and oocyte development in gilts of different age. *Acta Veterinaria Hungarica* **50** 101–110.
9. Coy P, Romar R **2002** In vitro production of pig embryos: a point of view. *Reproduction Fertility Development* **14** 275–286.
10. Driancourt MA, Thatcher WW, Terqui M, Andrieu D **1991** Dynamics of ovarian follicular development in cattle during the estrus cycle, early pregnancy and response to PMSG. *Domestic Animal Endocrinology* **8** 209–221.
11. Egerszegi I, Alm H, Ratky J, Heleil B, Brussow KP, Torner H **2010** Meiotic progression, mitochondrial features and fertilization characteristics of porcine oocytes with different G6PDH activities. *Reproduction Fertility Development* **22** 830–838.
12. El Shourbagy SH, Spikings EC, Freitas M **2006** Mitochondria directly influence fertilisation outcome in the pig. *Reproduction* **131** 233–245.
13. Feng WG, Sui HS, Han ZB, Chang ZB, Zhou P, Liu DJ, Bao S, Tan JH **2007** Effects of follicular atresia and size on the developmental competence of bovine oocytes: A study using the well-in-drop culture system. *Theriogenology* **67** 1339–1350.
14. Gupta MK, Uhm SJ, Lee HT **2008** Sexual maturity and reproductive phase of oocyte donor influence the developmental ability and apoptosis of cloned and parthenogenetic porcine embryos. *Animal Reproduction Science* **108** 107–121.
15. Hunter MG **2000** Oocyte maturation and ovum quality in pigs. *Reviews of Reproduction* **5** 122–130.
16. Hussein TS, Froiland DA, Amato F, Thompson JG, Gilrichst RB **2005** Oocyte prevent cumulus cell apoptosis by maintaining a morphogenic paracrine gradient of bone morphogenetic proteins. *Journal of Cell Science* **118** 5257–5268.

17. Ishizaki C, Watanabe H, Bhuiyan MM, Fukui Y **2009** Developmental competence of porcine oocytes selected by brilliant cresyl blue and matured individually in a chemically defined culture medium. *Theriogenology* **72** 72–80.
18. Lechniak D, Szczepankiewicz D, Kauss D, Szulc J, Szydłowski M **2005** IVM media, oocyte diameter and donor genotype at RYR1 locus in relation to the incidence of porcine diploid oocytes after maturation in vitro. *Theriogenology* **64** 202–212.
19. Marchal R, Feugang JM, Perreau C, Venturi E, Terqui M, Mermillod P **2001** Meiotic and developmental competence of prepubertal and adult swine oocytes. *Theriogenology* **56** 17–29.
20. Matwee C, Betts DH, King WA **2000** Apoptosis in the early bovine embryo. *Zygote* **8** 57–68.
21. Opiela J, Katska-Ksiazkiewicz L, Lipiński D, Słomski R, Bzowska M, Ryńska B **2008** Interactions among activity of glucose-6-phosphate dehydrogenase in immature oocytes, expression of apoptosis-related genes BC-2 and Bax, and developmental competence following IVP in cattle. *Theriogenology* **69** 546–555.
22. Paczkowski M, Krisher R **2010** Aberrant protein expression is associated with decreased developmental potential in porcine cumulus-oocyte complexes. *Molecular Reproduction and Development* **77** 51–58.
23. Paczkowski M, Bidwell C, Craig B, Lipka A, Waddell J, Krisher R **2007** Effect of puberty and follicle size on gene expression in porcine oocytes. *Biology of Reproduction* **77** 229a.
24. Roca J, Martinez E, Vazquez JM, Lucas X **1998** Selection of immature pig oocytes for homologous in vitro penetration assays with the brilliant cresyl blue test. *Reproduction Fertility Development* **10** 479–485.
25. Rodriguez-Gonzalez E, Lopez-Bejar M, Izquierdo D, Paramio MT **2003** Developmental competence of prepubertal goat oocytes selected with brilliant cresyl blue and matured with cysteamine supplementation. *Reproduction Nutrition Development* **43** 179–187.
26. Van Blerkom J, Davis PW **1998** DNA strand breaks and phosphatidylserine redistribution in newly ovulated and cultured mouse and human oocytes: occurrence and relationship to apoptosis. *Human Reproduction* **13** 1317–1324.
27. Warzych E, Peippo J, Szydlowski M, Lechniak D **2007** Supplements to in vitro maturation media affect the production of bovine blastocysts and their apoptotic index but not the proportions of matured and apoptotic oocytes. *Animal Reproduction Science* **97** 334–343.
28. Wongsrikeao P, Otoi T, Yamasaki H, Agung B, Taniguchi M, Naoi H, Shimizu R, Nagai T **2006** Effects of single and double exposure to brilliant cresyl blue on the selection of porcine oocytes for in vitro production of embryos. *Theriogenology* **66** 366–372.
29. Wu J, Zhang L, Wang X **2000** Maturation and apoptosis of human oocytes in vitro are age-related. *Fertility Sterility* **74** 1137–1141.
30. Wu YG, Liu Y, Zhou P, Lan GC, Han D, Miao DQ, Tan JH **2007** Selection of oocytes for in vitro maturation by brilliant cresyl blue staining: a study using the mouse model. *Cell Research* **17** 722–731.
31. Zeuner A, Muller K, Reguszynski K, Jewgonew K **2003** Apoptosis within bovine follicular cells and its effect on oocyte development during in vitro maturation. *Theriogenology* **59** 1421–1433.