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Oocyte maturation in the presence of randomly pooled follicular fluid increases bovine blastocyst yield *in vitro*

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ABSTRACT

The objective of the study was to examine preimplantation development of the zygotes derived from bovine oocytes matured in the presence of follicular fluid (bFF) randomly pooled from large (>10 mm in diameter) highly vascularised ovarian follicles originating from slaughter-house ovaries. The maturation medium was supplemented with 20% bFF. The controls comprised 20% bFF+10% FCS, and 10% FCS.

The use of three separate batches of bFF have shown that the rates of cleavage (78-87%) and blastocyst formation (about 40%) obtained after bFF-supplemented maturation are similar or higher than those in the FCS control. In another experiment morphological selection of Grade I oocytes was performed after maturation or insemination. Selection of Grade I matured oocytes increased cleavage rate (to 85-91%) and blastocyst yield (to 43%). When Grade I inseminated oocytes were selected, then 92-93% of zygotes divided, and 47% reached blastocyst stage. Blastocyst rates were higher after bFF-supplemented maturation than after FCS-supplemented one, both in Grade I matured (43.0 as opposed to 40.7%) and Grade I inseminated (47.3 against 45.8%) oocytes.

The majority of all zygotes underwent the first cleavage division between 27 and 30 hpi. Zygotes originating from oocytes matured in the presence of bFF or bFF+FCS were found dividing until 48 hpi whereas those obtained from control oocytes no longer cleaved after 33 hpi.In conclusion, oocyte maturation in the presence of bovine bFF randomly pooled from large follicles of slaughter-house ovaries supports as high developmental capacity of presumed zygotes as does the FCS-control.

KEY WORDS: bovine follicular fluid, *in vitro* maturation, *in vitro* insemination, cleavage, blastocyst rate

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INTRODUCTION

Trials undertaken to study the effects of follicular fluid on maturation and subsequent developmental potential in cattle comprised the inhibitory influence as well as stimulation. Inhibitory effects on development appear to be related to small size of follicles from which bFF was obtained (e.g., Wang et al., 1999) and/or to too high a concentration of bFF in the medium. Randomly pooled bFF from small follicles improves blastocyst yield when used at 10-30% concentration, but inhibits development at 60% concentration (Kim et al., 1996). Undiluted bFF used as the maturation medium strongly inhibits development (Avery et al., 2003).

Carolan et al. (1996) have shown that the action of follicular fluid on cytoplasmic maturation of cow oocytes varies with follicle quality. Sirard et al. (1995) noticed that the factors involved in improving development of oocytes seemed to be expressed in selected large follicles. Several studies have shown that dominant follicles have a greater vascular bed and an increased gonadotrophin uptake compared with other antral follicles. Acosta et al. (2003) demonstrated that there is an increase in both vascular area and blood flow in the pre-ovulatory follicle during spontaneous ovulation.

When foetal calf serum in the maturation medium is replaced with bovine follicular fluid, the rate of blastocyst formation and hatching is the same or slightly better than in the presence of serum (Khatir et al., 1997). However, thorough selection of donor follicles - sometimes followed by analysis of batches of collected follicular fluid - is usually involved in the preparation of bFF for culture (Carolan et al., 1996; Romero-Arredondo and Seidel, 1996; Khatir et al., 1997). Indeed, Romero-Arredondo and Seidel (1996) found that cumulus expansion, meiotic maturation and further development were enhanced when bovine oocytes were cultured with bFF obtained from follicles 8 or 20 h after LH surge. In the present work, in an attempt to possibly simplify the procedure, we examine if bFF randomly collected from large follicles effectively supports oocyte maturation and preimplantation development. This is only recently that randomly collected follicular fluid was found to support further development of matured pig oocytes (Algriany et al., 2004).

MATERIAL AND METHODS

Chemicals

All chemicals used in this study were purchased from Sigma (Sigma Chemical Co., Louis, MO), unless stated otherwise.

Oocyte recovery and selection

Bovine ovaries were transported from the slaughterhouse to the laboratory at 30-33°C in phosphate buffered saline. Cumulus oocyte complexes (COCs) were aspirated from follicles sized 2-8 mm by using a vacuum pump connected with 18-gauge needle attached to a 10-ml tube containing 1ml TCM199 washing medium supplemented with 20 IU/ml heparin to prevent clotting. COCs with at least three compact layers of cumulus cells were selected and used for experiments.

Collection of follicular fluid

Translucent and highly vascularized large follicles (diameter >10 mm) from slaughterhouse ovaries were evacuated by aspiration. Mixed follicular fluid was retrieved from them, irrespective of the oestrous cycle stage. The pooled bFF was then centrifuged at 200 g for 15 min to remove cellular debris and the supernatant was filtered through 0.22 µm filter, aliquoted and stored at -20°C until use (after 7 to 70 days). The method of bFF clearing differs from that by Avery et al. (2003), in which higher speed centrifugation (4000 rpm) was not followed by filtration. The pools of follicular fluid were collected from three separate supplies of ovaries. Each pool (batch of bFF) was used for maturation in 2 or 3 replicates.

Oocyte maturation

COCs were matured in TCM199 supplemented with 0.2 mM pyruvic acid, 0.4 mM glutamine, 20 IU/ml PG 600 (PMSG 400 IU+ HCG 200 IU) (Intervet, Holland) and 50 µl/ml gentamycine. Also, 20% bFF and/or 10% FCS (Gibco) was added to the medium. COCs were cultured in 4-well culture plates (50 COCs in 500 µl medium in each well). The medium was preincubated for a minimum of 3 h (humidified atmosphere of 5% CO_2 in the air at 38.5°C) and then the COCs were added and cultured for 24 h.

Morphological assessment after maturation

COCs were vortexed 30 sec at 2000 rpm to remove some cumulus cells. Matured oocytes were classified according to cumulus investments and morphology of cytoplasm. Cumulus-enclosed oocytes irrespective of cytoplasm morphology, and corona-enclosed oocytes with even, dense, granular cytoplasm were classified as Grade I. Denuded oocytes or corona-enclosed oocytes with uneven granulated cytoplasm morphology were assessed as Grade II. This classification partly overlaps with that by Fukui (1990), which includes cumulus investments morphology.

In vitro fertilization

COCs were vortexed in Hepes-TALP medium for 30 sec to remove some cumulus cells. IVF was done in droplets composed of IVF-TALP medium supplemented with 6.0 mg/ml BSA, 0.2 mM Na-pyruvate, 50 μg/ml gentamycine, 30 μg/ml heparin, 20 μM penicillamine, 10 μM hypotaurine and 1μM epinephrine. Frozen semen from a single bull of proven fertility was used for IVF. Spermatozoa were thawed in a 35°C water bath for 1 min and then washed in a discontinous Percoll gradient prepared in a 15-ml tube by adding 2 ml of 90% Percoll under 2 ml of 45% Percoll. The semen samples were added onto the Percoll gradient and centrifuged at 200 g for 25 min. The supernatant was removed and the pelleted spermatozoa were washed twice with sperm TALP followed by centrifugation at 200 g for 10 min. After removal of the supernatant, spermatozoa were resuspended in sperm TALP and counted in a hemocytometer chamber (final concentration = 2×10^6/ml). Of the final sperm suspension 10 μl was added to each droplet (90 μl) of IVF medium containing 15 oocytes. Matured oocytes were coincubated with the sperm suspension for 18 h in humidified atmosfere of 5% CO_2 in the air at 38.5°C.

Morphological assessment of presumptive zygotes

For morphological selection, presumptive zygotes after insemination were vortexed 45 sec at 2000 rpm to remove spermatozoa and cumulus cells. Presumptive zygotes with dense even cytoplasm and/or moderate perivitelline space were classified as Grade I and the others (uneven cytoplasm and small or large perivitelline space) were classified as Grade II. Selection of presumptive zygotes on the basis of quality of cytoplasm was earlier described (Hawk and Wall, 1994).

In vitro culture

Eighteen hours after insemination, presumptive zygotes were denuded of surrounding cumulus cells by vortexing for 45 sec and washed twice in Hepes-TALP and once in CR1aa medium before being transferred to droplets (50 μl) under mineral oil and cultured at 38.5°C in 5% CO_2, 5% O_2 and 90% N_2. In all experiments, presumptive zygotes were cultured in CR1aa medium for four days and for the remaining period (4 days) in SOFaa. CR1aa and SOFaa medium were made of chemicals in the laboratory. Culture media were supplemented with BSA (3 mg/ml) during the first two days followed by FCS supplementation (5%) during the remaining period of culture. Cleavage was assessed at 24, 27, 30, 33, 42 and

48 hpi. Development to the blastocyst stage and cell numbers (after staining with Hoechst 33342) was recorded at 144 h of culture, and blastocysts were classified as early blastocysts, mid-blastocysts, expanded blastocysts and hatching blastocysts (Hasler et al., 1995). Hatched blastocysts were assessed at 192 h of culture.

Statistical analysis

Differences in embryo development in culture were analysed using general linear model (GLM) procedure of SAS.

RESULTS

Comparison of cleavage dynamics and developmental competence of oocytes matured in the presence of bFF derived from three separate batches

In this experiment oocytes matured in the presence of bFF were not morphologically graded thereafter or later, as presumptive zygotes. The first cleavage of such zygotes occurred in about 80% (Total divided; Table 1) both after maturation in the presence of bFF as well as after FCS-supplemented maturation. Despite variation between the three batches in the percentage of cleavage at any given time, overall efficiency of the first cleavage falls within the range 78-87%. Variations in the percentages of cleavage between the batches

TABLE 1

First cleavage of oocytes matured in the presence of three different batches of follicular fluid

Duration of culture, hpi	Divided zygotes, %											
	bFF				bFF+FCS				FCS			
	1	2	3	mean	1	2	3	mean	1	2	3	mean
24 h	2.7	2.6	2.4	2.6	0.4[b]	0.9	2.5	1.3	3.5[a]	2.5	2.4	2.8
27 h	45.6	27.9	26.5	33.4	47.0	43.9	34.1	41.7	48.6	42.7	40.5	43.9
30 h	26.7	35.4	26.5	29.5	20.1	34.1	21.8	25.3	28.5	33.1	27.5	29.7
33 h	6.7	6.7	15.7	9.7	8.9	3.8	14.6	9.1	1.4	2.9	6.6	3.5
42 h	3.9	5.9	7.2	5.7[a]	4.4	3.5	3.9	4.0	0.4	0.0	0.0	0.13[b]
48 h	1.4	0.0	3.6	1.7	2.3	0.0	2.6	1.6	0.4	0.0	0.0	0.13
Total divided	87.0	78.5	81.9	82.6	83.1	86.2	79.5	83.0	82.8	81.9	77.0	80.2
No of zygotes	216	113	83		222	98	78		208	116	90	

bFF - bovine follicular fluid; FCS - foetal calf serum

hpi - hours after insemination

values with different superscripts (a,b) within a row are different ($P<0.05$)

of bFF might rather indicate differences between the batches of oocytes. For example, the highest percentage of division at 27 hpi was observed in batch 1 of bFF (45.6%), but also in the corresponding FCS control group 1 as well (48.6%); similarly, at 30 hpi batch 2 is the best in bFF group (35.4%) and also in number 2 control FCS group (33.1%).

In the majority of fertilized oocytes, the first cleavage occurred between 27 and 30 hpi. However, the groups differed in the kinetics of cleavage. In FCS group, the division was completed until 33 hpi, whereas in the bFF and bFF+FCS groups it continued till 42 or 48 hpi.

No significant differences between bFF samples were found in cleavage rates (Table 2). Blastocyst rate and quality tended to be comparable in either bFF sample, whereas variations were found within FCS groups. Cleavage and blastocyst rates were higher in oocytes matured in the presence of bFF+FCS than in FCS group ($P<0.05$). Blastocyst cell number was lower in FCS group than in bFF groups ($P<0.05$).

TABLE 2

Developmental competence of oocytes matured in the presence of three different batches of follicular fluid (1, 2, 3) and fertilized

	bFF				bFF+FCS				FCS			
	1	2	3	mean	1	2	3	mean	1	2	3	mean
Cl, %	79.1	88.2	82.8	83.4	83.3	91.5	85.7	86.8 [a]	75.1	80.2	78.2	77.8 [b]
Bl, %	40.8	41.2	39.2	40.5	39.16	48.2	49.2	45.5[a]	31.2	38.7	41.6	37.1[b]
HBl, %	24.1	22.4	17.1[b]	21.21	22.7	37.4	35.3 [a]	31.8	16.6	25.0	31.6 [a]	24.4
Bl	95±	105	95±	99±	101±	95±	96±	97±	75±	91±	83±	83±
no of	5[c]	±5.9	6.5	3.4[a]	4.7 [c]	6.2	6.5	3.4 [a]	4.7[d]	6.9	7.4	3.7[b]
cells[1]	(9)	(10)	(17)	(36)	(9)	(11)	(15)	(35)	(7)	(8)	(17)	(32)
No of oocytes	153	85	84		143	83	130		157	88	131	

bFF - bovine follicular fluid; FCS - foetal calf serum

Cl - cleavage; Bl - blastocyst; HBl - hatched blastocyst;

values of means (a,b) and batches (c,d) with different superscripts within a row are different ($P<0.05$)

[1]mean cell number of blastocyst ± standard error; in parentheses the numbers of blastocysts are given

Comparison of cleavage dynamics and developmental competence between Grade I and Grade II oocytes matured in the presence of bFF

In this experiment oocytes matured in the presence of bFF were morphologically graded thereafter (Grade I/II matured oocytes) or later, as presumptive zygotes (Grade I/II inseminated oocytes).

The highest percentage of Grade I matured oocytes was found divided at 27 hpi in bFF groups (38.6 and 46.1%; Table 3). This clearly contributed to overall higher cleavage rate of Grade I as compared to Grade II matured oocytes (85.1

TABLE 3

Timing of the first cleavage of Grade I and Grade II matured oocytes after fertilization

Duration of culture, hpi	Divided zygotes %					
	bFF		bFF+FCS		FCS	
	Grade I	Grade II	Grade I	Grade II	Grade I	Grade II
24 h	2.9	2.3[c]	1.9	0.0[d]	2.9	2.3[c]
27 h	38.6	33.9[d]	46.1	38.4	42.4	48.8[c]
30 h	29.6	27.8	25.3	21.5	33.2	25.7
33 h	7.7	10.2[c]	10.5[a]	6.7	3.1[b]	2.3[d]
42 h	4.8[a]	5.8[c]	4.6[a]	3.2[c]	0.0[b]	0.4[d]
48 h	1.5	1.8	1.3	2.7	0.0	0.4
Total divided	85.1	81.8	89.7	72.5	81.6	79.9
No of oocytes	231	181	228	172	246	168

bFF - bovine follicular fluid; FCS - fetal calf serum
hpi - hours after insemination
values of percentages for Grade I oocytes (a,b) and Grade II oocytes (c,d) with different superscripts within a row are different ($P<0.05$)

as opposed to 81.8% in bFF group and 89.7 against 72.5% in bFF+FCS group, respectively). In control FCS group this difference between cleavage rates was less pronounced (81.6.5 against 79.9%). A conclusion then can be drawn that bFF maturation environment together with selecting Grade I matured oocytes yield high cleavage rates of zygotes.

Overall cleavage rate was higher in Grade I presumed zygotes than in Grade II ones within all experimental groups (Table 4). Also in Grade I inseminated oocytes the percentage of the first cleavage at 27 hpi was higher than in Grade II

TABLE 4

Timing of the first cleavage of Grade I and Grade II presumed zygotes

Duration of culture, Hpi	Divided zygotes, %					
	bFF		bFF+FCS		FCS	
	Grade I	Grade II	Grade I	Grade II	Grade I	Grade II
24 h	4.0[a]	0.8	0.9[b]	1.0	4.4[a]	0.4
27 h	41.6	29.0	51.8	29.4	52.1	34.2
30 h	34.7	20.3	28.5	19.2	32.0	25.9
33 h	7.0	11.0[c]	9.2[a]	8.6	2.7[b]	3.3[d]
42 h	4.8[a]	5.4[c]	2.8	5.7[c]	0.0[b]	0.4[d]
48 h	1.1	2.0	0.4	4.3[c]	0.0	0.4[d]
Total divided	93.2	68.5	93.6	68.2	91.2	64.6
No of oocytes	252	160	232	166	239	175

bFF - bovine follicular fluid; FCS - foetal calf serum
hpi - hours after insemination
values of percentages for Grade I oocytes (a,b) and Grade II oocytes (c,d)) with different superscripts within a row are different ($P<0.05$)

ones (Table 4). Both Grade I matured oocytes and Grade I inseminated oocytes (presumed zygotes) that matured in the presence of bFF or bFF+FCS, continued the first cleavage until 48 hpi (Tables 3 and 4).

The results obtained in experiments on ungraded oocytes (previous section) were compared with that comprising graded oocytes. Grade I matured oocytes (M) yielded improved cleavage and blastocyst rates as compared to ungraded oocytes (Table 5). Even more improved rates came from Grade I inseminated oocytes (I)

TABLE 5

Developmental competence of ungraded oocytes (UG) as compared with Grade I matured oocytes (M) and Grade I inseminated oocytes (I)

	Developmental competence								
	bFF			bFF+FCS			FCS		
	UG[1]	M	I	UG[1]	M	I	UG[1]	M	I
Cleavage, %	83.4	91.3	91.8	86.8[a]	86.2	95.0	77.8[b]	82.4	91.4
Blastocysts, %	40.5	43.0	47.3	45.5[a]	46.0	51.9	37.1[b]	40.7	45.8
Hatching, %	21.2	17.0	22.0	31.8	27.8	30.5	24.4	20.3	24.3
Blastocyst	99[a]	110[c]	108[e]	97[a]	110[c]	107[e]	83[b]	88[d]	90[f]
No of cells [2]	(36)	(23)	(23)	(35)	(18)	(23)	(32)	(21)	(23)
No of oocytes	322	231	252	356	228	232	376	246	239

[1] values from Table 2

bFF - bovine follicular fluid; FCS - foetal calf serum

values of UG (a,b), M (c,d) and I (e,f) with different superscript within a row are different ($P<0.05$)

[2] in parentheses the numbers of blastocysts are given

matured in the presence of bFF or bFF+FCS. The percentage of blastocysts was close to 50% (47% in bFF group and 51.9% in bFF+FCS group). Hatching of blastocyst was not correlated with high grade oocytes. Interestingly, Grade I matured oocytes (M) seemed as predictive of high blastocysts cell numbers as Grade I inseminated (I) ones. Both types of Grade I oocytes yielded significantly higher blastocyst cell numbers in bFF groups than in FCS group ($P<0.05$).

DISCUSSION

The method of randomly pooling follicular fluid from large follicles proved reasonable, since it supports high developmental potential of oocytes. Combined with the selection of Grade I inseminated oocytes, the random pooling method provided up to 47% of blastocysts in bFF group and 51.9% in bFF+FCS group. Also in the pig randomly collected follicular fluid was found to support

developmental potential of matured oocytes, since cleavage rate reached 45%, and cleaved embryos formed blastocysts in 31% (Algriany et al., 2004).

Evidence has been presented that the composition of bFF changes between 8 and 20 h after the LH surge, and that bFF contains a factor(s) by 20 h after LH surge that stimulates the resumption of meiosis (Kato and Seidel, 1998). Upon supplementation of IVM medium with bFF collected at 0, 8, and 20 h after LH surge, the lowest cleavage and blastocyst rates were found with the 0-h bFF; the combination of 0-h and 20-h bFF was similar to 20-h bFF in cleavage and blastocyst formation rates, suggesting that a stimulator in the 20-h bFF overcame an inhibitor in 0-h bFF (Romero-Arredondo and Seidel, 1996). It can be hypothesied that a dominant stimulatory effect overcomes weaker inhibitory influences in all bFF batches randomly collected from large, highly vascularized follicles used in the present study.

Randomly collected mixed follicular fluid supported development at least as well as did foetal calf serum. This may be partly due to follicular-synthesized components such as hyaluronan or midkine. Granulosa and expanding cumulus cells in the follicle secrete large amounts of hyaluronan, and when hyaluronan is added to the maturation and culture media, it improves the developmental potential of bovine oocytes and embryos (Marquant-Le Guienne et al., 2002) and the blastocysts' cell numbers (Stojkovic et al., 1999). Stojkovic et al. (2002) found that blastocysts cultured in the presence of hyaluronan contained a similar total number of cells as *in vivo* derived embryos. Addition of midkine during oocyte maturation influenced cytoplasmic maturation of oocytes and increased blastocyst yield as compared to untreated controls (Ikeda et al., 2000).

An interesting outcome of the presented results is the common action of bFF+FCS: cleavage and blastocysts rates in this group are systematically higher than in bFF and FCS groups. No additive effect was earlier found in terms of blastocyst yield when bovine oocytes matured in the presence of 10% bFF+10% FCS were fertilized and cultured in synthetic oviduct fluid (Carolan et al., 1996). The difference between these two groups of results may come from the use of different culture media, serum batches, but also from using 20% bFF in our study. There is a significant correlation between the composition of serum and follicular fluid in cows (Leroy et al., 2004). However, the interplay between the two (FCS and bFF) in the maturation medium seems not to be of the type of synergy, since in that case blastocyst rates in our experiment could be doubled in bFF+FCS group as compared to single-factor groups.

The kinetics of the first cleavage reveals common characteristics, which is independent from the supplementation of maturation medium, namely that the majority of zygotes divided between 27 and 30 hpi. Lately dividing oocytes appeared in bFF and bFF+FCS groups. Since they were present among high

graded oocytes, it can be suggested that they are physiologically normal. Why they are absent from FCS group, remains to be elucidated.

Grade I matured oocytes (M) yielded improved blastocyst rate and quality as compared to ungraded oocytes (Table 5) in each supplementation group. Grade I matured oocytes are cumulus-enclosed or corona-enclosed oocytes. Cumulus cells benefit bovine oocyte development either by secreting soluble factors which induce developmental competence or by removing an embryo development-suppressive component from the medium (Hashimoto et al., 1998). Moor et al. (1998) suggested that the key to the maturation and embryo viability resides in the follicle cell compartment rather than the oocyte itself.

Selecting Grade I inseminated oocytes (I) is considerably more effective in yielding high cleavage and blastocyst rates than selecting Grade I matured oocytes (M) or not selecting at all.

CONCLUSIONS

Maturation supplemented with pooled follicular fluid (bFF) collected from highly vascularized large follicles and aided by morphological grading of matured or inseminated oocytes selects for increased developmental competence.

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STRESZCZENIE

Dojrzewanie oocytów bydła w obecności losowo zbieranego płynu pęcherzykowego zwiększa produkcję blastocyst *in vitro*

Przedmiotem pracy było zbadanie rozwoju przedimplantacyjnego zygot uzyskanych z oocytów bydła, które dojrzewały w obecności płynu pęcherzykowego (PP) zebranego losowo z dużych (>10 mm średnicy) dobrze unaczynionych pęcherzyków jajnikowych pochodzących z jajników pobranych w rzeźni. Do pożywki do dojrzewania dodano 20% PP. Kontrole obejmowały 20% PP+10% surowicy (FCS) oraz 10% surowicy.

Używając trzech oddzielnych porcji PP wykazano, że współczynniki bruzdkowania (78-87%) i tworzenia blastocyst (około 40%) uzyskane po dojrzewaniu wspomaganym przez PP, są podobne lub wyższe niż uzyskane w kontroli FCS. W drugim doświadczeniu na podstawie morfologii selekcjonowano oocyty dojrzałe klasy I i oocyty inseminowane klasy I. Selekcja oocytów dojrzałych klasy I zwiększyła współczynnik bruzdkowania (do 85-91%) i tworzenia blastocyst (do 43%). W wyniku selekcji oocytów inseminowanych klasy I uzyskano 92-93% podzielonych zygot, a 47% osiągnęło stadium blastocysty. Współczynniki tworzenia blastocyst były wyższe po dojrzewaniu wspomaganym przez PP niż przez FCS, zarówno wśród oocytów dojrzałych klasy I (43,0 i 40,7%), jak oocytów inseminowanych klasy I (47,3 i 45,8%).

Większość zygot przeszła pierwszy podział bruzdkowania między 27 i 30 godz. po inseminacji. Zygoty pochodzące z oocytów dojrzałych w pożywce z PP i PP+FCS dzieliły się do 48 godz. po inseminacji, podczas gdy kontrolne nie później niż do 33.

Podsumowując, dojrzewanie oocytów w obecności PP losowo zebranego z dużych pęcherzyków z jajników pochodzących z rzeźni zapewnia wyższy potencjał rozwojowy potencjalnych zygot niż w obecności surowicy.