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A simple method for assessing hyaluronic acid production by cumulus-oocyte complexes

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ABSTRACT: The cumulus expansion of cumulus-oocyte complex (COC) is an essential regulating process of oocyte maturation and as such it is a possible biomarker of the *in vitro* maturing oocytes quality. Cumulus expansion is usually assessed by non-invasive methods based on visual evaluation with many inaccuracies. On the other hand, analytical measurement of the quantity of hyaluronic acid (HA), the most abundant compound of expanded cumuli, is one of possible methods to evaluate cumulus expansion precisely. Therefore, this study aimed to verify the applicability of HA analysis for evaluating the cumulus expansion and testing oocyte maturation. The COCs were cultured in modified M199 medium for 8–48 h. The samples for the HA analysis were prepared on an 8-hour time scale, and HA retained in COCs was measured using a spectrophotometric method adapted for this purpose. We observed an increasing quantity of HA during the *in vitro* cultivation. A comparison with expanded COCs' classification or expansion area proved the proposed method of HA analysis suitable for the evaluation of cumulus expansion *in vitro*. Our findings consider the quantity of HA-expressed cumulus expansion to be a valuable marker of COC quality enabling an adequate oocyte meiotic stage estimation.

Keywords: oocyte; meiotic maturation; cumulus expansion; glycosaminoglycans; spectrophotometry

INTRODUCTION

A sufficient number of successfully *in vitro* matured oocytes, enclosed in cumulus cells and creating a cumulus-oocyte complex (COC), is key for advances in assisted reproduction. While oocyte maturation occurs, mucification of surrounding cumulus cells, called cumulus expansion, takes place simultaneously. Therefore, cumulus expansion is a significant regulating process of oocyte maturation (summarized in Nevoral et al. 2014).

Gonadotropins and growth factors stimulate cumulus expansion, as well as cumulus expansion enabling factors (CEEFs) incoming from the

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oocyte (Dekel et al. 1979; Prochazka et al. 1998, 2011; Nemcova et al. 2007; Huang and Wells 2010). Cumulus expansion consists of the enlargement of the extracellular matrix based on glycosaminoglycan synthesis, in particular hyaluronic acid (HA) (Nakayama et al. 1996). Cumulus expansion results in the disruption of cumulus cells–oocyte cross-talk and in the suppression of the effect of meiosis inhibiting factors (Yokoo et al. 2007, 2010).

Cumulus expansion intensity and the expression of cumulus expansion markers positively correlate with successful oocyte *in vitro* maturation and subsequent embryonic development (Qian et al. 2003; Flechon et al. 2003; Assou et al. 2010; Davachi et al. 2012). It has been shown that the expression of genes associated with cumulus expansion (*HAS2*, *PTGS2*, *TNFAIP6*, *PTX3*) is a marker of adequate cumulus expansion and thus oocyte quality and its developmental competence (McKenzie et al. 2004; Zhang et al. 2005; Assidi et al. 2010; Yuan et al. 2011). However, simple and less difficult approaches to oocyte quality estimation are more appropriate. As such, general cumulus expansion intensity can be used as the biomarker for the aforementioned changes.

Visual evaluation of expanded COCs (Fagbohun and Downs 1990; Vanderhyden et al. 1990) or their area measurement (Daen et al. 1994) are still used for the evaluation of cumulus expansion (Appeltant et al. 2015; Kubo et al. 2015; Machado et al. 2015). Nevertheless, these methods do not affect the three-dimensional structure of expanded cumuli and cannot uncover differences in HA synthesis after COC treatment. Therefore, the use of radioisotope-labelled HA precursors (Eppig 1980) or analytical assessment of HA by hyaluronidase-induced β-elimination (Volpi 2000) enable to utilize the mentioned advantages. Spectrophotometric analysis of β-eliminated HA measurable at 232 nm (Yosizawa et al. 1983), in particular, offers a simple approach for HA-predicted evaluation of expanded cumuli. Surprisingly, analytical methods of HA analysis are not being used, although more precise results of cumulus expansion can be determined in this way. Based on best knowledge, we hypothesized that introducing the HA analysis of *in vitro* cultured COCs can serve as a predictable marker of cumulus expansion and the corresponding stage of oocyte maturation.

Our results indicate that the spectrophotometric HA analysis is a possible method for the evaluation of cumulus expansion expressed by HA in COCs. The use of a simple analytical method of HA measurement comes out as a suitable tool for oocyte quality prediction by cumulus expansion.

MATERIAL AND METHODS

Chemicals. Unless otherwise stated, all chemicals used for the analytical analysis of HA were purchased from Sigma-Aldrich Co. (St. Louis, USA): phosphate buffered saline, pH 7.4 (PBS; P5368), polyvinyl alcohol (PVA; 341584), hyaluronic acid sodium salt (49775), hyaluronidase from *Streptomyces hyalurolyticus* (SHH, H1136), and bovine testicular hyaluronidase (BTH, H3506).

Analysis of HA by spectrophotometry and High-Performance Liquid Chromatography (HPLC). To determine the HA content, the HA polymer was enzymatically digested by specific hyaluronidase from *Streptomyces hyalurolyticus* (hyaluronan lyase, SHH) (Vanderhyden 1993) with the ability to create β-elimination products. In this reaction, the HA polymer was enzymatically digested to the dimers (HA2) and HA oligomers, creating double bonds. Concurrently, standards of HA tetramers (HA4), hexamers (HA6), octamers (HA8), and decamers (HA10), overall named HA oligomers, were analyzed. These HA oligomers were detected by spectrophotometric measurement in an ultraviolet (UV) absorption spectrum. The solutions were spectrophotometrically measured in Einmalküvetten cuvettes (UV-Küvette mikro, 7592 00, Plastibrand) using Spectronic Helios Gamma UV-Vis spectrophotometer (Thermo Fisher Scientific, Waltham, USA) at 190–280 nm against blank consisting of HA-free adequate enzyme-medium solution.

HPLC was performed simultaneously. The HPLC analysis was performed using an UltiMate 3000 system (Thermo Fisher Scientific) equipped with an autosampler (10°C), column oven (35°C), and PDA detector, monitoring at wavelengths 210 and 235 nm for detection of unsaturated and saturated bonds, respectively, where they show the highest absorption (Dr. M. Hermannová, pers. comm.). The injection volume was 25 µl, mobile phase flow 1.5 ml/min, total run time 40 min. Analyte separation was achieved on a Shodex anion exchange column IEC QA-825 (Shodex, Munich, Germany) using gradient elution. The mobile phase consisted of (a) 0.02M sodium chloride in water, and (b) 0.25M sodium chloride in wa-

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ter. The gradient was as follows: 0 min – 100% A, 25 min – 24% A, 26–32 min – 100% B, re-equilibration 33–40 min – 100% A. A polynomic eight-point calibration curve (from 7 to 1000 µl/ml) for both approaches was used.

In vitro cultivation of COCs and evaluation of oocyte maturation. Porcine ovaries were obtained from non-cycling gilts at the local slaughterhouse (Jatky Plzeň a.s., Czech Republic) and kept at 39°C until arrival at the laboratory. Cumulus-oocyte complexes (COCs) were collected from ovarian follicles with a diameter of 2–5 mm by a 20-gauge aspirating needle. Only fully grown oocytes with intact cytoplasm surrounded by compact cumuli were used in further experiments. The COCs were matured in a modified M199 medium (Sigma-Aldrich) supplemented with 32.5mM sodium bicarbonate, 2.75mM calcium L-lactate, 0.025 mg/ml gentamicin, 6.3mM HEPES, 13.5 IU eCG : 6.6 IU hCG/ml (P.G. 600; Intervet International B.V., Boxmeer, the Netherlands) and 5 % (v/v) foetal bovine serum (Sigma-Aldrich). The COCs were matured for 0–48 h in 4-well Petri dishes (Nunc, Thermo Fisher Scientific) containing 1.0 ml of culture medium, at 39°C in a mixture of 5.0 % CO_2 in air.

At the end of culture, oocytes were denuded by repeated pipetting as described below and mounted on microscope slides with vaseline, covered with a cover glass, and fixed in ethanol acetic acid (3 : 1, v/v) for at least 48 h. The oocytes were stained with 1.0 % orcein in 50 % aqueous-acetic acid and their meiotic stage was examined under a phase contrast microscope. Five groups of meiotic maturation stages were determined in accordance with the criteria published by Motlik and Fulka (1976): GV – germinal vesicle, LD – late diakinesis, MI – metaphase I, AITI – anaphase I to telophase I transition, MII – metaphase II.

Classification of expanded cumuli. Cumulus expansion was visually assessed using a subjective scoring system (Vanderhyden et al. 1990). Scores 0 to +4 were attributed to increasing degrees of expansion. Score 0 indicated no observable expansion, +1 minimal observable expansion, +2 extended expansion to several layers, +3 complete expansion excluding *corona radiata*, and +4 indicated complete expansion including *corona radiata*. The data were expressed as percentage portion of COC population.

Area measurement of COCs. For the area measurement, the approach by Daen et al. (1994) was used with slight modifications. In brief, images of the same 25× COCs (25 COCs in group) were prepared using a monochromatic CCD camera ProgRes CT1 (Jenoptik, Jena, Germany) with NIS Elements software (Laboratory Imaging s.r.o., Prague, Czech Republic) on an 8 h time scale. The image analysis based on thresholding and area measurement of 25× COCs including oocyte areas was performed. The data were expressed relative to COCs after 48 h of cultivation.

Expanded cumuli isolation and HA extraction. Groups of 25× COCs were cultured in 1 ml M199 culture medium under the above described conditions, at intervals from 0 to 48 h. At the end of the culture period, the COCs were washed four times in 500 µl PBS-PVA with their gentle transferral, and oocytes were mechanically removed by repeated pipetting during the last wash. To determine the HA retained in the expanded cumuli, the expanded cumuli were transferred into an Eppendorf tube in 500 µl of PBS-PVA and enzymatically digested by SHH (2 IU/ml; Sigma-Aldrich) at 39°C overnight. Samples of HA isolated from the COCs were stored at –20°C until measurement. After spectrophotometric analysis, the synthesis of HA was expressed as HA concentration (µg/ml) in the solution obtained.

Statistical analysis. All the experiments were repeated at least three times. Data were analyzed using the General Linear Models (GLM) procedure of SAS software (Statistical Analysis System, Version 9.3, 2012). Significant differences between groups with equal and diverse numbers of repetition were determined using the *t*-test and Sheffé's test, respectively. The level of significance was set at $P < 0.05$.

Experimental design

Testing of enzymes with hyaluronidase activity and spectrophotometry optimization. The aim of this experiment was to evaluate the most suitable combination of HA-digesting enzyme and incubation medium. An assessment of spectrophotometric measurement wavelength for maximal absorbance yield was performed. Two enzymes creating heterodimers and oligomers from HA polymer in different ways (with or without involvement of water molecules) in various media were used: (a) non-hydrolytic hyaluronan lyase SHH with β-elimination activity as an enzyme specifically degrading HA (Vanderhyden 1993), with unsaturated bound creation absorbing light

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in a UV absorption spectrum at 232 nm (Yosizawa et al. 1983); (b) concurrently, bovine testicular hyaluronidase (BTH), hydrolytic enzyme creating saturated heterodimer bounds non-absorbing ultraviolet (UV) light (negative control). The presence of HA-degrading products was proved by HPLC. The optimization of spectrophotometry, focused on wavelength finding with maximal yield absorbance, was simultaneously performed.

Verification of measurement of β-elimination products. The aim of this experiment was to prove the absorbance of standards of HA oligomers (HA4–HA10) without enzyme digestion. Concurrently, HA polymer with and without SHH (SHH^+ and SHH^-, respectively) digestion was measured for spectrophotometry specificity to β-elimination products. The absorbance was spectrophotometrically measured at 216 nm.

Verification of HA content according to COC abundance. The aim of this experiment was to verify HA content in dependence on COC number in the 15×, 25× or 50× COCs per sample, prepared after 48 h of *in vitro* cultivation, and to substantiate the specificity of HA analysis to COC abundance-derived production of HA. The HA was spectrophotometrically analyzed at the above optimized wavelength. Simultaneously, an evaluation of oocyte maturation was performed.

Comparison of the cumulus expansion evaluation methods. The aim of the last experiment was to compare the results of visual and analytical methods of cumulus expansion evaluation. Three methods for cumulus expansion assessment were tested on the same population of 25× COCs: visual evaluation of expanded COCs (Vanderhyden 1993), area measurement of COCs (Daen et al. 1994) and, finally, spectrophotometrical HA analysis. For this experiment, the COCs were tested on an 8-h time scale for 48 h. Results from oocyte maturation were simultaneously collected.

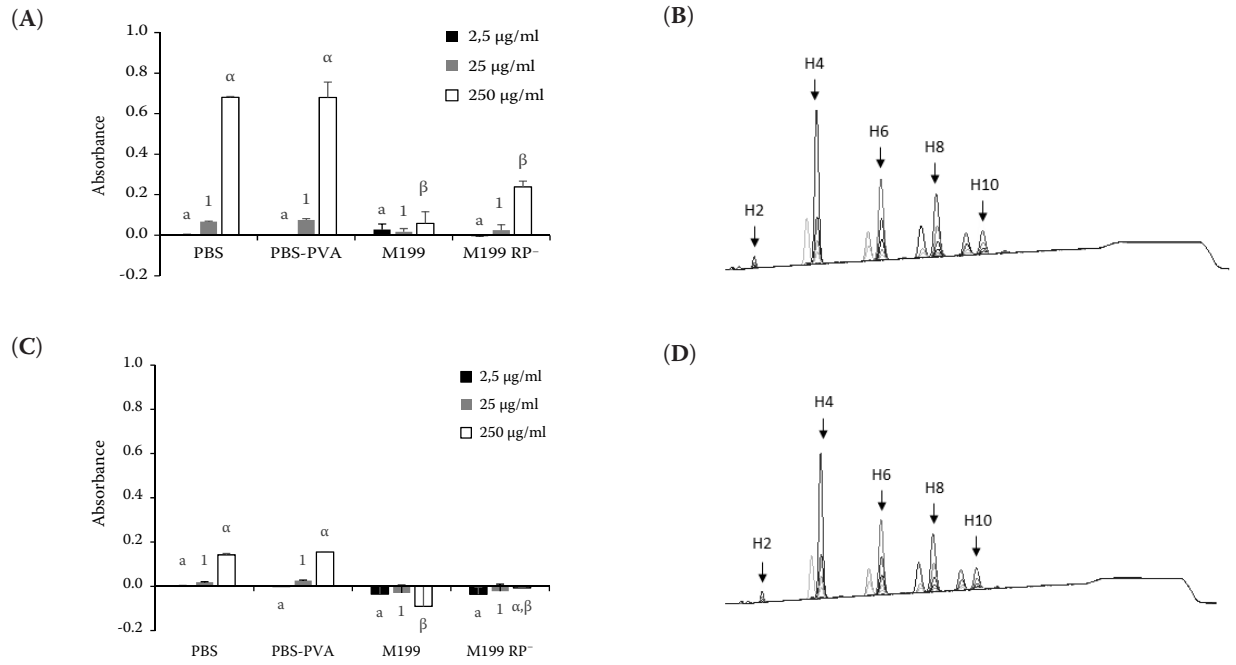

Figure 1. Presence of digested hyaluronic acid (HA) products by hyaluronan lyase from *Streptomyces hyalurolyticus* (SHH) (**A**, **B**) and bovine testicular hyaluronidase (BTH) (**C**, **D**). Comparison of different enzyme–medium combinations for HA digestion and spectrophotometric measurement (wavelength 232 nm). SHH with β-elimination activity (**A**) and hydrolytically digested BTH (**C**) were used. Data were verified using HPLC (210 and 235 nm wavelength for detecting unsaturated and saturated bounds, respectively), all solutions were diluted in PBS and representative chromatograms are shown (**B**, **D**)

bars show the means of three independent experiments ± SEM

GLM procedure followed by *t*-test was performed and different letters indicate significant differences ($P < 0.05$)

PBS = phosphate buffered solution, PBS-PVA = 0.01 % polyvinyl alcohol in phosphate buffered solution, M199 = M199 culture medium, M199 RP^- = M199 without red phenol

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RESULTS

Testing of enzymes with hyaluronidase activity in different media and spectrophotometry optimization. The aim of this experiment was to assess the optimal medium for hyaluronan lyase (SHH) digestion and optimal wavelength for spectrophotometric analysis with maximum absorbance. Based on our results, SHH in PBS has been defined as an optimal combination for real sample preparation. Moreover, PVA addition, necessary for COC manipulation, offers comparable results to SHH-PBS usage. The M199 and M199 without red phenol (M199 RP$^-$) culture media manifested unsuitable conditions for SHH digestion and spectrophotometry analysis, where absorbance values were significantly lower (Figure 1A). At the same time, BTH usage, with presumed no detection of β-elimination products in UV light, was verified and no detectable absorbance was measured (Figure 1C). The detection of HA dimers and HA oligomers, regardless of β-elimination enzyme ability, was revealed by retention time by HPLC (Figure 1B, D). A wavelength of 232 nm in the ultraviolet spectrum was used. Thereafter, the SHH-PBS was selected for standard preparation and a 190–280 nm wavelength was used for optimal wavelength evaluation. The highest absorbance was measured at 216 nm in all the HA standards used (Figure 2), and this wavelength was used in subsequent experiments. The detection limit of spectrophotometry was checked concurrently, and absorbance equalling ~0.13 (i.e. 60 µg/ml HA polymer) was established as the minimal detectable concentration of β-elimination products for spectrophotometry (Figure 2).

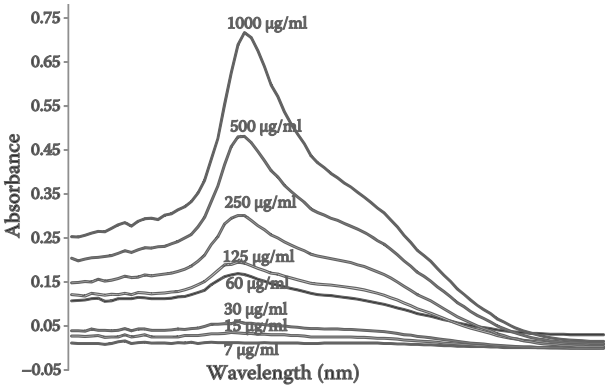

Figure 2. Wavelength optimization of spectrophotometric measurement. The 7–1000 µg/ml HA standard solutions were digested by SHH in PBS and β-elimination products were measured in wavelength range 190–270 nm

HA = hyaluronic acid, SHH = hyaluronan lyase from *Streptomyces hyalurolyticus*, PBS = phosphate buffered solution

Measurement of standard solutions of HA polymer and its oligomers. The standards of HA oligomers (4HA–10HA containing 2–5 glucuronic acid-glycosaminoglycan heterodimers, respectively) were used for verification of the spectrophotometry analysis of digested HA polymer. The present data support previous SHH-digested HA polymer measurements as the result of the presence of HA-digesting products of β-elimination with a comparable absorbance response (Figure 3). Absorbance of non-digested HA polymer was

Table 1. Meiotic maturation of oocytes during *in vitro* cultivation of cumulus-oocyte complexes

	0 h	8 h	16 h	24 h	32 h	40 h	48 h
GV	94.0 ± 8.5^a	96.8 ± 3.9^a	58.7 ± 11.6^b	3.0 ± 2.2^c	1.3 ± 1.3^c	–^{c}	–^{c}
LD	–^{b}	3.2 ± 0.9^b	22.0 ± 6.1^a	1.0 ± 0.5^b	–^{b}	–^{b}	–^{b}
MI	–^{b}	–^{b}	15.7 ± 5^b	91.0 ± 3.8^a	42.7 ± 20.5^{a,b}	29.5 ± 15.3^b	4.0 ± 1.4^b
AI/TI	–^{c}	–^{c}	–^{c}	5.0 ± 5.0^b	19.3 ± 5.0^a	14.9 ± 7.5^a	4.5 ± 2.3^b
MII	–^{c}	–^{c}	–^{c}	–^{c}	36.7 ± 20.8^b	49.2 ± 19.9^b	90.5 ± 4.2^a
Degenerated	6.0 ± 2.6^a	–^{a}	3.6 ± 3.3^a	–^{a}	–^{a}	6.4 ± 5.3^a	1.0 ± 0.4^a
n	75	200	200	250	150	250	250

GV = germinal vesicle oocytes, LD = late diakinesis oocytes, MI = metaphase I oocytes, AI/TI = anaphase I or telophase I oocytes, MII = metaphase II oocytes

data are expressed as a percentage proportion of meiotic stages and show the means of at least three independent experiments ± SEM

GLM procedure followed by Sheffé's test was performed and different letters indicate significant differences in the same stage of meiotic maturation, i.e. in rows ($P < 0.05$)

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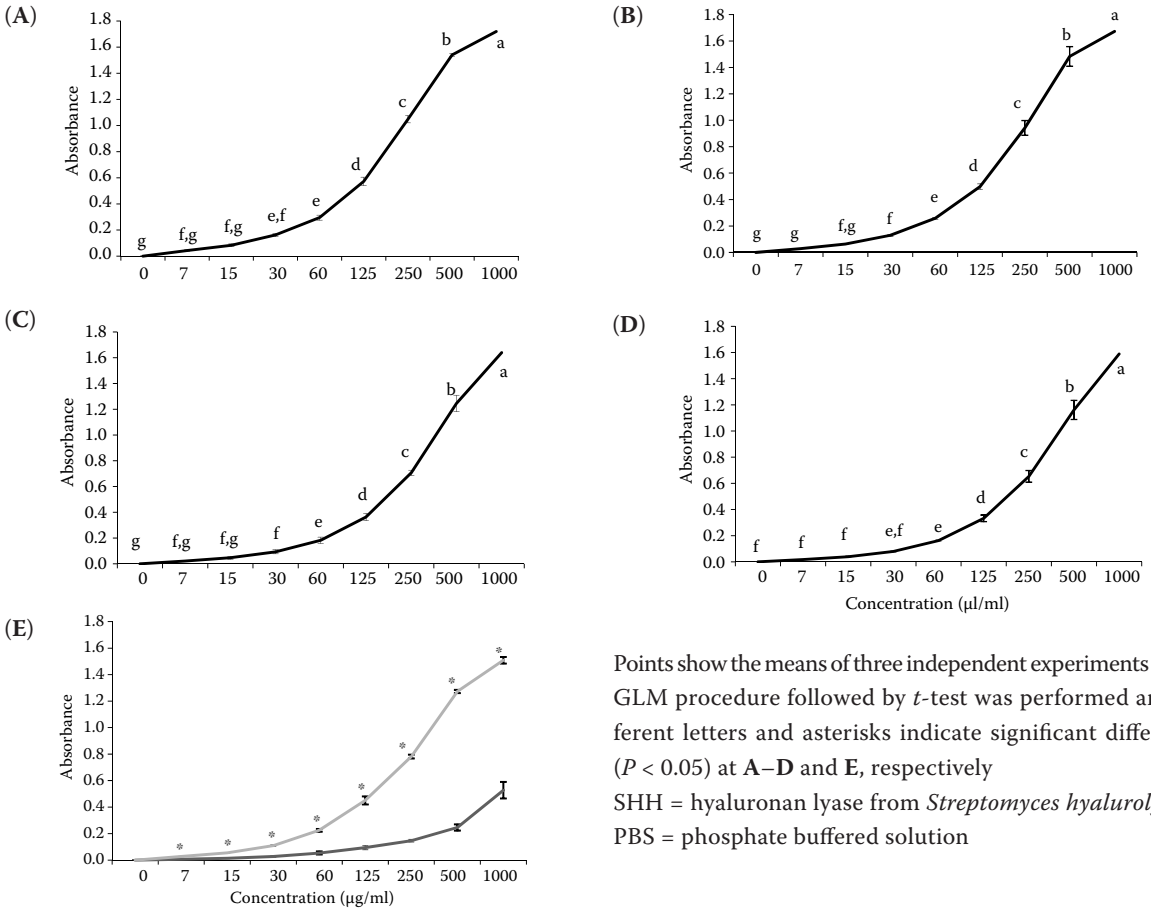

Figure 3. Measurement of standards of undigested hyaluronic acid (HA) oligomers – 4HA (**A**), 6HA (**B**), 8HA (**C**), and 10HA (**D**). The basal undigested HA polymer absorbance was measured (SHH^-) and compared with SHH^+ digestion (**E**). Standard solutions were diluted in PBS and spectrophotometrically measured at 216 nm wavelength

also revealed and weak absorbance was detected. Therefore, approximately 500 µg/ml undigested HA revealed an absorbance value equal to 60 µg/ml of SHH-digested HA polymer (Figure 3E). The standards of 4HA–10HA showed approximately twice as high a detection limit compared to β-elimination products of HA polymer with equal absorbance values (Figure 3A–D).

COC abundance-derived HA content in a sample. The aim of this experiment was to show that HA content is an applicable marker for evaluating cumulus expansion. Therefore, populations of 15×, 25× or 50× COCs were washed and digested for measuring HA retained in COCs after a 48-h *in vitro* cultivation. No significant difference was revealed in HA between the 15× and 25× COCs samples and a significant increase was found in the 50× COCs sample, where HA content showed an almost four-times higher HA sample concentration (213.58 ± 82.15 vs 854.83 ± 113.47 µg/ml for 25× and 50× COCs sample, respectively). The data are summarized in Figure 4.

Comparison of expanded cumulus evaluation during COC in vitro cultivation. Three different methods of expanded cumulus evaluation were tested: (1) classification of expanded cumuli, (2) area measurement of COCs, and (3) HA analysis. All the methods used showed an increasing trend during *in vitro* cultivation of COCs (Figures 5–7). Cumulus classification and area measurement showed 16 h as the first time point with a significant increase of visible expansion and area enlargement, respectively (Figures 5 and 6). On the other hand, no differences were detected in HA content and no significant increase was measured between 16 and 24 h (Figure 7). A further significant increase in HA production was observed at 40–48 h, while cumulus classification and area measurement differences were not seen. At the same time, evaluation of oocyte maturation

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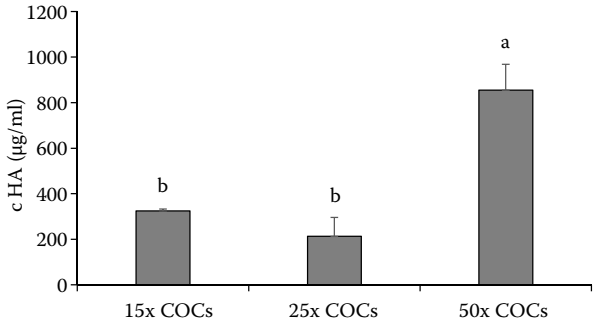

Figure 4. Verification of hyaluronic acid (HA) content depending on cumulus-oocyte complexes (COCs) abundance after a 48-h *in vitro* cultivation. Samples from COCs were prepared using SHH digestion in PBS-PVA overnight. Absorbance measured at 216 nm wavelength and HA concentration (µg/ml) in samples was calculated using polynomic curves of HA standards digested under equal conditions

bars show the means of three independent experiments ± SEM
GLM procedure followed by *t*-test was performed and different letters indicate significant differences ($P < 0.05$)
SHH = hyaluronan lyase from *Streptomyces hyalurolyticus*, PBS-PVA = 0.01 % polyvinyl alcohol in phosphate buffered solution

was verified and the standard course of meiotic maturation was observed (Table 1).

DISCUSSION

An adequate cumulus expansion is essential for successful oocyte maturation. As such, the intensity of cumuli expansion is a biomarker for oocyte quality prediction, key for subsequent *in vitro* fertilization efficiency. Some subjective non-invasive approaches have been used for this purpose (Vanderhyden et al. 1990; Daen et al. 1994). However, some disadvantages (e.g. subjectivity in evaluation, three-dimensional structure non-recognition, inability to indicate cumulus quality) make these methods less attractive. Therefore, a more precise method for the exact study of cumulus expansion and for oocyte quality estimation would be suitable. Analytical measurement of HA, the most abundant compound of expanded cumulus, offers a tool for the objective quantification of cumulus expansion.

In accordance with previous studies (Salustri et al. 1989, 1995; Nakayama et al. 1996), our results indicate that HA, as an abundant compound of

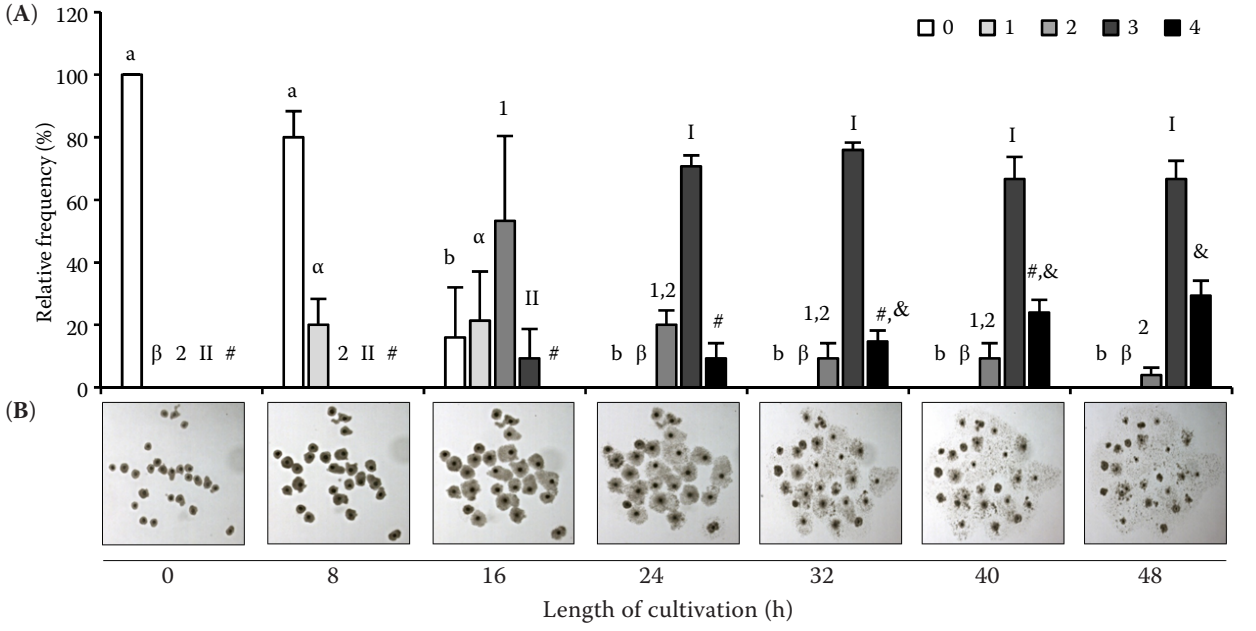

Figure 5. Visual evaluation of expanded cumulus-oocyte complexes (COCs) on an 8-h time scale for 48 h (score 0 = no observable expansion, +1 = minimal observable expansion, +2 = extended expansion to several layers, +3 = complete expansion excluding *corona radiata*, and +4 = complete expansion including *corona radiate*) (**A**); representative images of COCs at each time point (**B**)

data were expressed as a percentage proportion of COC population, bars show the means of three independent experiments ± SEM
GLM procedure followed by *t*-test was performed and different letters indicate significant differences in the same score grade of cumulus expansion ($P < 0.05$)

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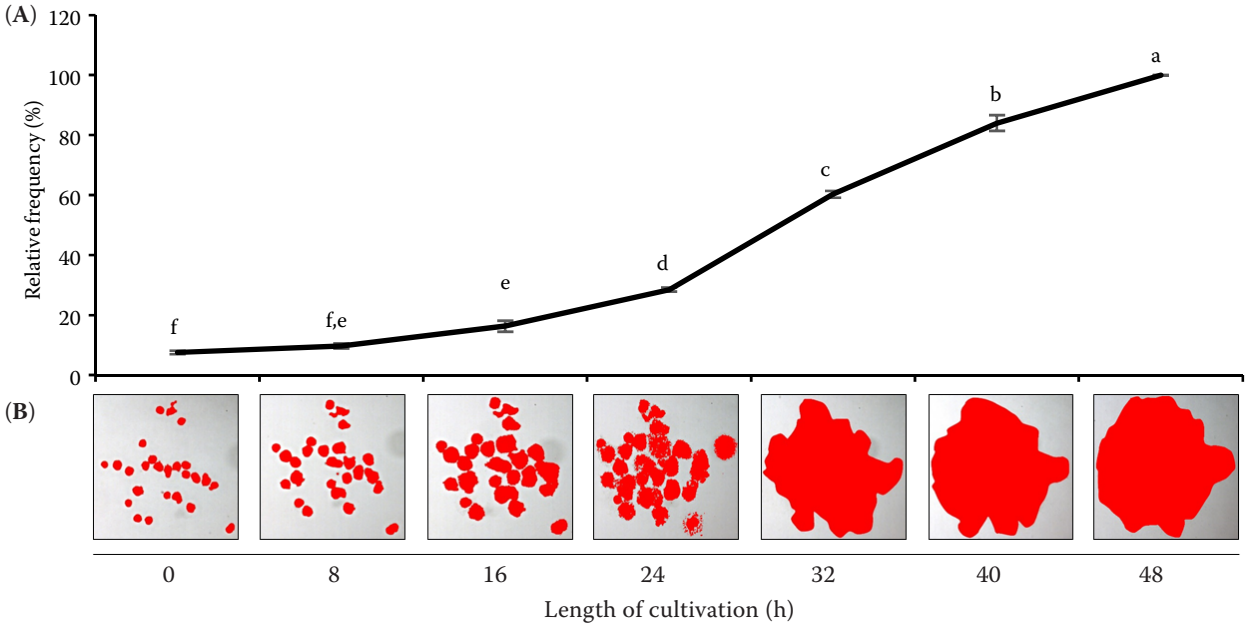

Figure 6. Area measurement of cumulus-oocyte complexes (COCs) on an 8-h time scale for 48 h (**A**); representative images of threshold COCs at each time point (**B**). Data were related to COCs after a 48-h cultivation (i.e. 100%)

points show the means of three independent experiments ± SEM

GLM procedure followed by *t*-test was performed and different letters indicate significant differences ($P < 0.05$)

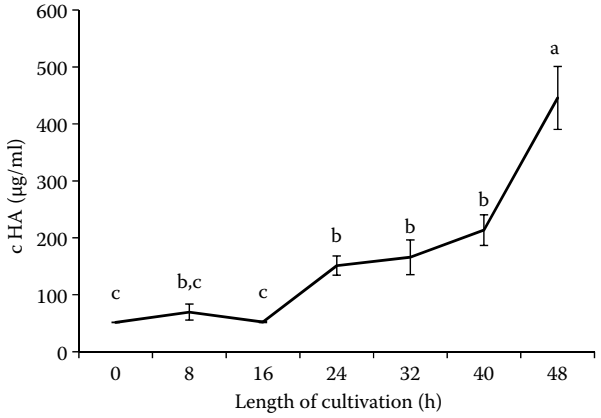

Figure 7. Analysis of hyaluronic acid (HA) retained in cumulus-oocyte complexes (COCs) on an 8-h time scale for 48 h. COCs were prepared using SHH digestion in PBS-PVA overnight. Absorbance was measured at 216 nm wavelength and HA concentration (µg/ml) in samples was calculated using polynomic curves of HA standards digested under equal conditions

points show the means of at least three independent experiments ± SEM

GLM procedure followed by Sheffé's test was performed and different letters indicate significant differences ($P < 0.05$)

SHH = hyaluronan lyase from *Streptomyces hyalurolyticus*, PBS-PVA = 0.01 % polyvinyl alcohol in phosphate buffered solution

expanded cumuli, is thus a possible candidate biomarker of expansion quantity. In our experiments, we used high specific hyaluronan lyase produced by *Streptomyces hyalurolyticus* (Eppig 1980; Vanderhyden 1993), and a mixture of 2HA and longer HA oligomers, in general called β-elimination products, were produced and subsequently measured. Only double bounds of SHH-derived 2HA/HA oligomers are capable of absorbing UV light (Alkrad et al. 2003). However, our spectrophotometric analysis showed lower absorbance values of SHH-digested HA polymer compared with undigested 4-6HA. This observation, supported by HPLC analysis, suggests that SHH digestion of HA polymer loses absorbance in the occurrence of 8–10HA oligomers, and the detection limit of digested HA polymer is slightly decreased. In addition to HPLC improvement, optimization of our measurement wavelength was performed, and 216 nm was established as the optimal wavelength and subsequently used. A different optimal wavelength compared with the initially tested 232 nm has been confirmed by previous studies (Takagaki et al. 1994; Alkrad et al. 2003), which describe various wavelengths for spectrophotometric measurement of HA-digestion product, pointing out divergent laboratory conditions. Overall, the results suggest that the original

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product measured by spectrophotometry after HA digestion equals β-elimination products, and that the real sample from COCs reflects their HA content.

The presented evaluation of cumulus expansion based on HA analysis offers an expression of HA production retained in COCs. We have proven the dependence on COC abundance when HA retaining by COCs significantly increased in samples containing 50× COCs. Although 15× and 25× COCs did not show any differences in the HA content, 25× COCs samples gave a more precise analysis with respect to the variability of COCs. Besides tritium-labelled glycosaminoglycans (Solursh 1976; Eppig 1979), this work supports the measurement of HA production as a usable approach for evaluating cumulus expansion. In spite of radioisotope usage, HA analytical measurement is based on real HA production, without the addition of radioisotope-labelled precursors at the beginning of COC *in vitro* cultivation (Eppig 1979). Moreover, spectrophotometric HA analysis becomes more available due to the absence of radioisotope needs. On the other hand, our suggested method does not respect total HA production and the HA released by COCs into the surrounding medium is not affected, so the method should be further developed.

In our experiments, we compared HA analysis with commonly used cumulus expansion evaluation methods: the subjective classification of COCs and image-derived area measurement, and HA analysis. Based on the results of cumulus expansion classification and area measurement, a different time schedule of cumulus expansion as measured by these two methods should be pointed out. Evidently, area measurement seems to be more consistent through repeated experimentation. On the other hand, this quite consistent and non-invasive simple method of area measurement does not enable the observation of the three-dimensional structure of the expanded cumuli. This phenomenon can be affected by various compounds tested during the *in vitro* COC cultivation. Changes in HA level can suggest modifications of HA binding proteins (HABPs) which are responsible for expanded cumulus structure (Yokoo et al. 2002; Nemcova et al. 2007), resulting in a change in HA quantity and not a change in the visible quality of COCs.

Based on our knowledge, the quality of cumulus expansion can determine oocyte meiotic maturation and its developmental potency (Qian et al. 2003; Feuerstein et al. 2012; Bergandi et al. 2014). Conversely, cumulus expansion physiologically reflects the meiotic stage of oocyte secreting CEEFs (Prochazka et al. 1998; Dragovic et al. 2005). Our findings are in accordance with those when we detected meiosis re-initiation taking place after approximately 16 h of *in vitro* culture, when cumulus expansion was beginning. This observation is reported in previous studies describing the significance of cumulus expansion for re-initiation of oocyte meiosis (Dekel et al. 1981; Chen et al. 1990), as well as the effect of CEEFs on cumulus expansion intensity (Nakayama et al. 1996). In addition to the above-mentioned, further meiotic progress can be regulated by other methods, such as the HA-activated CD44 receptor, one of HABPs (Kimura et al. 2002; Yokoo et al. 2007). As such, following HA production can be required in further meiotic maturation, although cumulus expansion for 24 hours and longer seems to be a passive process as a result of the momentum of primary gonadotropin- or growth factor-initiated cumulus expansion, as previous studies have described (Abeydeera et al. 1998).

This study monitored the course of cumulus expansion together with oocyte maturation in *in vitro* conditions. Cumulus expansion was evaluated by three different methods out of which the quantification of HA seems to be the best method for precise cumulus expansion study and oocyte quality estimation. HA-evaluated cumulus expansion was identified as a suitable biomarker which responds to the stage of meiotic progression of oocyte maturation.

CONCLUSION

Cumulus expansion reflects meiotic maturation and its features refer to the quality and developmental potential of the oocyte. HA-assessed cumulus expansion and its measurement in COCs offer a suitable tool for estimating COC and oocyte quality. This work presents a simply applicable method for the isolation and measurement of HA retained in COCs.

Potentially, HPLC analysis seems to be a helpful alternative when longer digested HA products of β-elimination can be detected, regardless of the efficiency of enzyme digestion. Moreover, a higher sensitivity of HPLC can provide an analysis of HA released by COCs on to the culture medium. Further experiments are needed for verification of these modifications, which could lead to an improvement in COC-produced HA measurement.

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