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**THE HUMAN OOCYTE-DERIVED SPERM CHEMOATTRACTANT IS A HYDROPHOBIC MOLECULE
ASSOCIATED WITH A CARRIER PROTEIN**

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Capsule

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3 Treating oocyte-conditioned media with proteases and hexane provided evidence for the
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5 oocyte-derived chemoattractant being a hydrophobic non-peptide molecule which, in an oocyte-
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7 conditioned medium, is associated with a carrier protein.
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Abstract

Objective: To characterize the nature of the human oocyte-derived chemoattractant.

Design: Laboratory *in-vitro* study.

Setting: Academic research institute.

Patients: Ten healthy sperm donors. Oocyte-conditioned media from women undergoing IVF treatment due to male factor infertility.

Interventions: Sperm samples were processed by the migration–sedimentation technique. Oocyte-conditioned media were collected 2-3 h after oocyte stripping.

Main Outcome Measure(s): Sperm chemotaxis was assayed in a μ -slide chamber according to the direction of swimming relative to that of the chemical gradient.

Results: Oocyte-conditioned media treated with proteases did not lose their chemotactic activity; on the contrary, they became more active, with the activity shifted to lower concentrations. When oocyte-conditioned media were subjected to hexane extraction, chemotactic activity was found in both the hydrophobic and aqueous phases. Known mammalian sperm chemoattractants were ruled out as oocyte-derived chemoattractants.

Conclusions: Our results suggest that the oocyte-derived chemoattractant is a hydrophobic non-peptide molecule which, in an oocyte-conditioned medium, is associated with a carrier protein that enables its presence in a hydrophilic environment.

Key words

Sperm chemotaxis; Chemotaxis; Oocyte-derived chemoattractant

Introduction

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4 In recent years it became clear that mammalian spermatozoa must be guided for reaching
5 the oocyte [for a review, see (1)]. Thus far, three potential sperm guidance mechanisms have been
6 recognized in humans: chemotaxis (2), thermotaxis (3), and rheotaxis (4). All these mechanisms only
7 function in capacitated spermatozoa, i.e., ripe spermatozoa that acquired the ability to fertilize the
8 egg (3-5). Thermotaxis and rheotaxis are assumed to be long-range mechanisms (1,4) guiding
9 spermatozoa to the fertilization site, and chemotaxis is thought to be a short-range, more accurate
10 mechanism that acts in the immediate surroundings of the oocyte-cumulus complex, guiding
11 spermatozoa to the oocyte (1). This short-range mechanism appears to be a two-step process, first
12 chemotaxis to the cumulus cells that surround the oocyte and then chemotaxis within the cumulus
13 mass towards the oocyte itself. This is based on the finding that, in humans, there are two origins of
14 sperm chemoattractants: the mature oocyte and the surrounding cumulus cells (6). The cumulus cells
15 secrete a single chemoattractant, progesterone (7,8). This chemoattractant is highly active, with
16 chemoattraction being observed at a concentration range as low as 10^{-11} – 10^{-10} M (7,8). The identity
17 of the chemoattractant(s) secreted from the oocyte is not known, in spite of intensive efforts to
18 identify it. It is known, however, that it is more potent than the cumulus-derived chemoattractant
19 progesterone (6), meaning that it is probably active at concentrations lower than 10^{-11} M. Realizing
20 that the identification of a substance present at such a low concentration is extremely difficult, our
21 aim in this study was to characterize the nature of the oocyte-derived chemoattractant.
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Materials and Methods

Chemicals

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48 Modified human tubal fluid (HTF) medium and human serum albumin (HSA) solution were
49 obtained from Irvine Scientific (Santa Ana, CA, USA); polyvinylpyrrolidone (PVP, 25K) was from Fluka
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1 (Buchs, Switzerland); progesterone, Pronase E and undecanal were purchased from Sigma (Rehovot,
2 Israel); Proteinase K and *n*-hexane were from Merck (Darmstadt, Germany) and prostaglandin E1 was
3 purchased from Avanti Polar Lipids (AL, USA).
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7 **Spermatozoa**

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10 Human semen samples were obtained from ten healthy donors after 3 days of sexual
11 abstinence. (The study was approved by the Bioethics and Embryonic Stem Cell Research Oversight
12 Committee of the Weizmann Institute of Science. Informed consent was obtained in writing from
13 each sperm donor.) Semen samples were allowed to liquefy for 30–60 min at room temperature and
14 verified for normal sperm density, motility and morphology [according to WHO guidelines (9)].
15 Human spermatozoa were separated from the seminal plasma by the migration–sedimentation
16 technique (10) using the commercially available HTF medium supplemented with 0.3% (w/v) HSA.
17 Following this procedure, the sperm concentration was adjusted to $4\text{--}5 \times 10^5$ cells/ml in HTF medium
18 containing 0.3% HSA and 3.5% (w/v) PVP. The sperm suspensions were incubated under an
19 atmosphere of 5% (v/v) CO₂ at 37°C for an additional 1 h (2 h in total with the separation procedure)
20 to obtain capacitated spermatozoa (5).
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37 **Oocyte-conditioned media**

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40 Oocyte-conditioned media and the respective control medium (G-1, Vitrolife, Sweden) were
41 obtained from the *In-vitro* Fertilization (IVF) Unit at Assaf Harofeh Medical Center (with the approvals
42 of the hospital's Helsinki committee and the Weizmann Institute Bioethics and Embryonic Stem Cell
43 Research Oversight Committee). Cumulus-oocyte complexes were retrieved from women undergoing
44 transvaginal oocyte aspiration for *in vitro* fertilization. The women were treated with GnRH analog
45 and gonadotrophin for ovarian stimulation. Oocyte retrieval was performed 35–38 h after human
46 choriogonadotrophin injection. The cumulus cells were stripped from the cumulus-oocyte complexes
47 by employing both mechanical pipetting and 10–30 s treatment with 40 units/mg hyaluronidase
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1 (type IV-S, Sigma, USA) followed by washing, thus avoiding leftovers of cumulus cells. Groups of
2 stripped oocytes (6–8 per group) were cultured in 25 µl drops of G-1 medium supplemented with
3
4 10% (v/v) HSA for 2-3 h before undergoing intra-cytoplasmic sperm injection. The conditioned media
5
6 were collected and frozen just prior to the injection.
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10 **Progesterone determination**

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12 Oocyte-conditioned media were analyzed for the presence of progesterone using a
13 progesterone ELISA kit (Enzo Life Sciences, Farmingdale, NY, USA) according to manufacturer
14 instructions. The concentration of progesterone was calculated according to an experimental
15 standard curve. The lowest concentration of the standard was 15.62 pg/ml, which set up the lowest
16 limit of detection. Media from six different women pooled in pairs (total of three measured samples)
17 were subjected to analysis. Samples were diluted 1:14 and run in duplicates. The negative control
18 was G-1 medium supplemented with 10% (v/v) HSA.
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30 **Hexane extraction**

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32 An equal volume of n-hexane was added to the samples of oocyte-conditioned medium and
33 control medium. The tubes were vortexed for 2 min and then spun (3000 × g) until a clear
34 differentiation between two phases was observed. The upper phase, containing the hexane fraction,
35 was transferred to another tube. Both fractions were then subjected to chemotaxis assays.
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44 **Chemotaxis assays**

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46 Chemotaxis assays were performed as described (11), using a disposable µ-slide chemotaxis
47 chamber (Ibidi GmbH, Munich, Germany). Each assay included a negative control experiment in the
48 absence of the test substance. In experiments with oocyte-conditioned media this negative control
49 consisted of a gradient of the oocyte-incubating medium [G-1 medium supplemented with 10% (v/v)
50 HSA] treated the same way as the oocyte-conditioned medium (e.g., protease treatment). In
51 experiments with prostaglandin E1 the negative control contained a gradient of the diluent.
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Chemotaxis assessment and statistics

Chemotaxis was assessed, as described (12), according to the distribution of the instantaneous directionality angles (γ_{inst} , the angle between the vector of the cell frame-to-frame displacement and the gradient direction; the video frequency was 25 frames/s). For each treatment the total number of angles in the gradient direction (N+) and in the opposite direction (N-) were summed from a number of experiments. A combined odds parameter was calculated as the ratio between these sums (combined Odds = $\Sigma N+ / \Sigma N-$). The odds parameter yields values close to 1 when the swimming is random; it is > 1 when the swimming is biased in the gradient direction. The intensity of the chemotactic response was reflected in the combined odds ratio (O.R.) parameter (combined O.R. = combined Odds_{treatment} / combined Odds_{control}).

To assess the statistical significance of elevated O.R. we used a block-bootstrapping algorithm as described earlier (13). Briefly, we resampled a large control data set to build a distribution of control O.R. for different sample sizes. The distribution provided the probability that a treatment O.R. is above a control O.R. randomly drawn from this distribution. The assay variability was assessed by the width of the control confidence intervals, assuming similar variability for the treatments and the controls. The dependence of the confidence intervals of the control O.R. values on the sample size is shown in Table 1.

Results

Wishing to employ for the characterization the most physiologically relevant source of oocyte-derived chemoattractant, we used human oocyte-conditioned media, i.e., media in which mature oocytes, stripped from the surrounding cumulus cells, were incubated for 2–3 h. We assayed chemotaxis by determining the swimming bias in the direction of the chemoattractant gradient, and calculating the ratio between the biases in the presence and absence of a gradient (termed combined odds ratio — see Materials and Methods). A value of 1 means no chemotaxis; a value >1

1 means chemotaxis towards the chemoattractant (12,13). Fig. 1A shows a representative chemotactic
2 response of oocyte-conditioned media at various dilutions, demonstrating that an oocyte-
3 conditioned medium is usually chemotactically active at 10^5 – 10^6 dilution.
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8 To verify that these conditioned media had no remains of progesterone from the cumulus
9 cells, we performed a highly sensitive, progesterone-specific ELISA assay. We assayed three different
10 samples of oocyte-conditioned media, each being a mixture of oocyte-conditioned media obtained
11 from two women (6–8 oocytes each). In two of the samples, no progesterone was detected by the
12 assay. In the third sample, progesterone at a concentration of 2×10^{-9} M was detected. Since oocyte-
13 conditioned media are chemotactically active at 10^5 – 10^6 dilutions (Fig. 1A), the effective
14 concentration of progesterone in the diluted sample was in the range of $2 \times (10^{-15}$ – $10^{-14})$ M, which is
15 four orders of magnitude below the threshold progesterone concentration that can be sensed by
16 human spermatozoa and stimulate a chemotactic response [10^{-11} – 10^{-10} M (7,8)].
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30 To characterize the nature of the oocyte-derived chemoattractant, we first determined
31 whether or not it is a peptide. We incubated oocyte-conditioned media with a broad-spectrum
32 protease, Proteinase K. In each experiment we verified the activity of the protease by running the
33 oocyte-conditioned media on SDS-PAGE prior and subsequent to the proteolysis. The
34 chemoattractant activity of the oocyte-conditioned media was not lost by the proteolysis (Fig. 1B;
35 similar results were obtained with trypsin — data not shown). On the contrary, the activity shifted to
36 a lower concentration (i.e., higher dilution). Since Proteinase K cleaves peptide bonds adjacent to the
37 carboxyl group of both aliphatic and aromatic amino acids, these results suggest that the
38 chemoattractant in oocyte-conditioned media is not a peptide (excluding a very short peptide).
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52 To verify the unexpected observation that the proteolysis increased the chemotactic activity
53 of the medium from $1:10^5$ dilution to $1:10^6$, we carried out another set of experiments, using this
54 time a mixture of two proteases, Proteinase K and Pronase E in higher concentrations than those
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1 used for Proteinase K alone, thus eliminating any possibility of peptide leftovers (excluding very small
2 peptides). In this set of experiments the sample of oocyte-conditioned media (prior to treatment)
3 happened to be chemotactically inactive. (This happened occasionally due to the fact that we used a
4 pool of media from different women.) Nevertheless, these inactive media became active after
5 proteolysis (Fig. 1C). Clearly, proteolysis elevated the chemoattractant concentration. This could be
6 the outcome of two scenarios. One is that the chemoattractant is an amino acid or a very short
7 peptide (such as a di- or tri-peptide) and proteolysis produces more of it. The other is that the
8 chemoattractant is a hydrophobic molecule which, in an oocyte-conditioned medium, is associated
9 with a carrier protein that enables its presence in a hydrophilic environment. According to this
10 scenario, proteolysis destroys the carrier protein and frees the hydrophobic chemoattractant to the
11 medium (the high concentration of albumin in the medium likely enables presence of the
12 chemoattractant in the aqueous solution).

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29 If, indeed, the oocyte-derived chemoattractant is hydrophobic and it is chemotactically
30 active both when it is in complex with its carrier protein and when unbound, it should exhibit
31 chemotactic activity both in hydrophilic and hydrophobic environments. To determine whether or
32 not this is the case, we extracted by hexane the hydrophobic substances from the oocyte-
33 conditioned media, and examined the chemotactic activity of both the hexane fraction and the
34 remaining aqueous fraction. Consistently with the carrier-protein scenario, both fractions were
35 chemotactically active (Fig. 2).

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47 Two sperm receptors apparently can act as chemotaxis receptors. One of them is the CatSper
48 channel (or a protein closely associated with it), shown to be the sperm receptor for the
49 chemoattractant progesterone (14,15). The other is OR1D2, suggested to mediate the chemotactic
50 response to the odorant bourgeonal (16) [though a later study raised the possibility that the
51 response to bourgeonal is mediated by CatSper (17)]. Since the ligands of these receptors are quite
52 hydrophobic, we examined whether any of them is the chemoattractant in oocyte-conditioned
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1 medium. CatSper is also activated by prostaglandin E1 (though via a binding site different from that
2 of progesterone) (14,15) and is known to be secreted from the cumulus-oocyte complex (18).
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4 Prostaglandin E1 exhibited no chemotactic activity at any tested concentration (Fig. 3A), even though
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6 it bound to its receptor (reflected in stimulating intracellular Ca^{2+} rise — not shown). Likewise,
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8 preincubation of oocyte-conditioned media with undecanal — an antagonist of the OR1D2 receptor
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10 (16), did not affect the chemotactic activity of the medium (Fig. 3B). These results indicate that
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12 neither prostaglandin E1 nor an OR1D2 ligand is the oocyte-derived chemoattractant.
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18 Discussion

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21 The results of this study show that the human oocyte-derived sperm chemoattractant does
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23 not lose its activity by proteases (Fig. 1), it can be present in both hydrophobic and hydrophilic
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25 environments (Fig. 2), and it is not one of the known ligands of the recognized chemotaxis receptors
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27 (Fig. 3). The first two observations could be explained by a number of scenarios: a hydrophobic
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29 chemoattractant in complex with a carrier protein, two (or more) chemoattractants — one
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31 hydrophobic and one hydrophilic, a chemoattractant of an amphipathic nature, and a
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33 chemoattractant which is an amino acid or a very short peptide. The finding that the activity of the
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35 oocyte-conditioned medium increased by proteolysis, taken together with the conservation of
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37 activity in both hydrophobic and hydrophilic environments, is consistent with the possibility of a
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39 hydrophobic chemoattractant associated with a carrier protein in an aqueous environment.
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41 According to this possibility, both the hydrophobic molecule and its complex with the carrier protein
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43 are chemotactically active. The free hydrophobic molecule is expected to be more active because,
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45 otherwise, proteolysis would not increase the activity. The other mentioned scenarios do not seem
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47 probable. Thus, the possibility of an amphipathic chemoattractant or two chemoattractants, one
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49 hydrophobic and one hydrophilic, is inconsistent with the proteolysis-dependent increase in
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51 chemotactic activity. Likewise, the possibility of an amino acid chemoattractant or a very short
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1 chemoattractant peptide is inconsistent with the proteolysis results by trypsin, which generates
2 relatively long peptides. The anticipation in such a case would be no change in activity. The results
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4 further indicate that the oocyte-derived chemoattractant is not one of the peptides reported to act
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6 *in vitro* as chemoattractants for human spermatozoa [e.g., CCL5 (19), Allurin (20), CCL20 (21) and
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8 atrial natriuretic peptide (22)] as all are expected to undergo proteolysis by the proteases used. Thus,
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10 it seems probable that the chemoattractant is a hydrophobic molecule associated with a carrier
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12 protein.
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17 It is reasonable to speculate that the receptor for the oocyte-derived chemoattractant is
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19 different from the known receptors for sperm chemoattractants. Otherwise, the receptor for the
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21 oocyte-derived chemoattractant might be saturated with cumulus-derived progesterone when
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23 passing through the mass of cumulus cells, thus interfering with the binding of the oocyte-derived
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25 chemoattractant to this receptor.
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30 To conclude, the chemoattractant is likely a hydrophobic non-peptide molecule which, when
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32 secreted from the oocyte, is in complex with a carrier protein. Apparently, the extreme potency of
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34 the chemoattractant compensates for its low concentration in the medium, and the carrier protein
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36 enables the presence of the chemoattractant in aqueous solutions and prevents its adsorption to
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38 non-specific locations.
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43 **Acknowledgments**

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Figure legends

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4 **Fig. 1. Chemotactic response of human spermatozoa to oocyte-conditioned medium (OCM) in the**
5 **absence and presence of proteases. A:** Dependence of the chemotactic response on the OCM
6 dilution. **B:** Chemotactic activity of OCM following treatment with Proteinase K (20 µg/ml, 3 h, 37⁰C).
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8 **C:** Chemotactic activity of OCM following treatment with a mixture of Proteinase K and Pronase E (50
9 µg/ml each, 3 h, 37⁰C). The data are presented as combined odds ratios (O.R.) of 6900-9500 tracks
10 [(91–121)×10⁴ angles; A] and 5000-5800 tracks [(70–80)×10⁴ angles; B,C]. The negative control is
11 defined as O.R. = 1. Note that error bars are missing because the combined O.R. value is calculated
12 from a pool of the whole data and is not an average. Also note that, due to the large variations
13 between sperm samples [even of the very same sperm donor — a well known phenomenon in sperm
14 biology (23-25)], a quantitative comparison is allowed within each panel but not between panels. **P*
15 < 0.05 versus the appropriate control.
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30 **Fig. 2. Chemotactic response of human spermatozoa to oocyte-conditioned medium (OCM) before**
31 **and after extraction with hexane.** The data are presented as combined O.R. of 1400-2900 tracks
32 [(18–35)×10⁴ angles]. Note that error bars are missing because the combined O.R. value is calculated
33 from a pool of the whole data and is not an average. **P* ≤ 0.06; ***P* < 0.01 versus the appropriate
34 control.
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43 **Fig. 3. Examination of sperm receptors suspected to be involved in human sperm chemotaxis. A:**
44 Chemotactic response to Prostaglandin E1 (PGE1). The data are presented as combined O.R. of 4200-
45 5300 tracks [(58–73)×10⁴ angles]. **B:** Chemotactic response to oocyte-conditioned medium (OCM) in
46 the presence of undecanal. The data are presented as combined O.R. of 2500-3600 tracks [(9–
47 15)×10⁴ angles]. Note that error bars are missing because the combined O.R. value is calculated from
48 a pool of the whole data and is not an average. **P* ≤ 0.02 versus the appropriate control.
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Table 1

Confidence intervals (95%) of O.R. values according to sample size

Sample size (number of angles)	Confidence interval ^a
5×10^4	0.86-1.16
10×10^4	0.90-1.11
100×10^4	0.97-1.03

^a Calculated by block-bootstrapping

Figure 1
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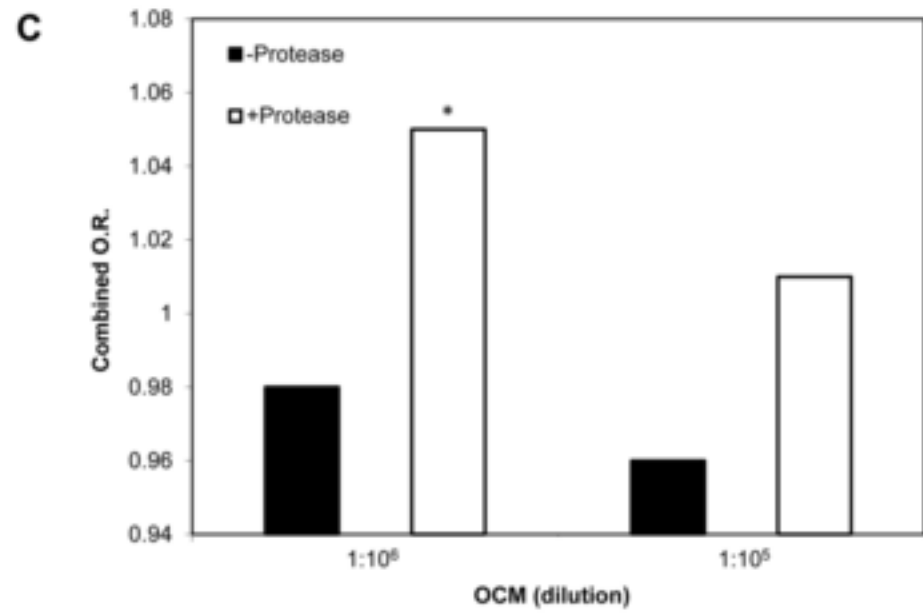
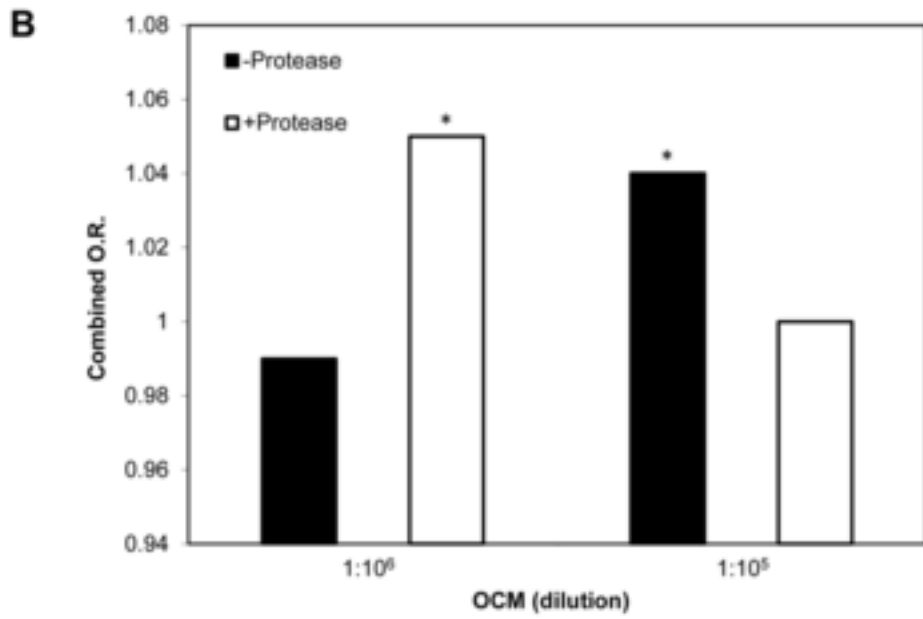
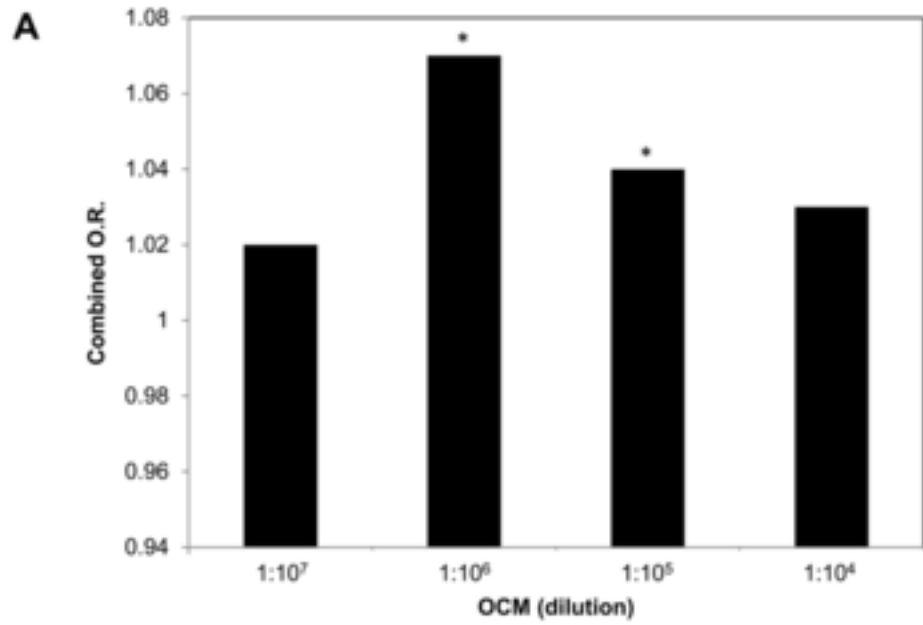


Figure 2
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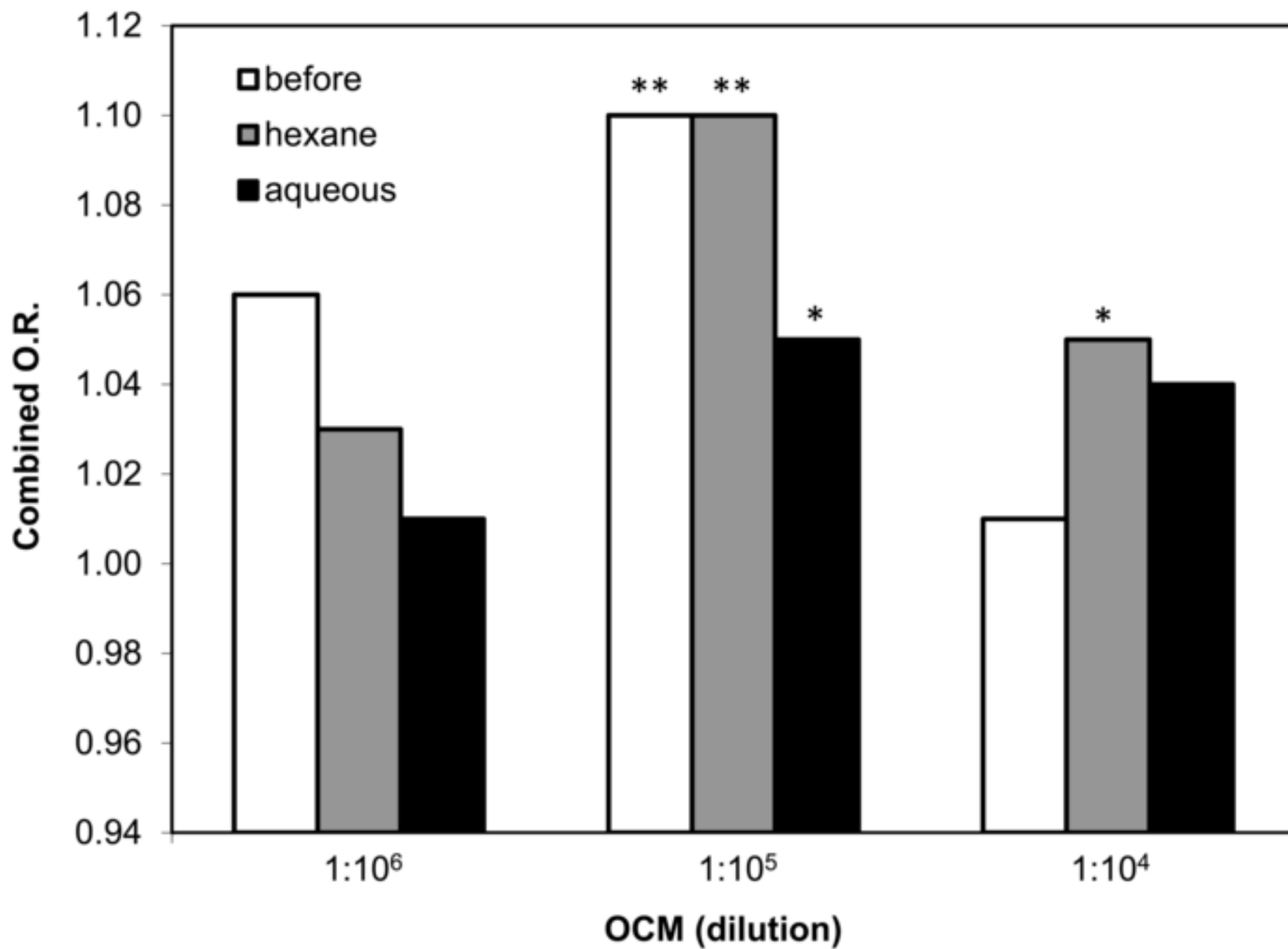


Figure 3
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