The role of Zn- α 2 glycoprotein in sperm motility is mediated by changes in cyclic AMP

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Abstract

Sperm motility is essential for male reproduction or natural fertilization. The cyclic AMP (cAMP)/cAMP-dependent protein kinase A (PKA) signaling pathway is generally recognized as one of the significant signaling pathways in the regulation of mammalian spermatozoan motility. Since Zn- α 2-glycoprotein (ZAG) activity in mammalian adipose tissue is mediated via the β_3 -adrenoreceptor, with upregulation of the cAMP pathway, we hypothesize that ZAG may play the same role in sperm motility regulation, a new factor of regulation of sperm motility. Therefore, the gene encoding human ZAG was cloned and polyclonal antibodies were generated, and then laser scanning confocal microscopy and flow cytometry were employed to identify this protein in human spermatozoa. The results showed that ZAG protein was mostly localized on the pre-equatorial region covering the acrosome, neck, and middle piece of the flagellum of spermatozoa. Furthermore, using computer-assisted sperm analysis, we found that anti-human ZAG antibodies could significantly reduce the motility of human swim-up spermatozoa after 90- or 120-min incubation ($P < 0.05$ and $P < 0.01$ respectively), together with the decreasing of intracellular cAMP and PKA levels. In conclusion, these data suggest that ZAG is present in human spermatozoa and may be involved in the regulation of sperm motility via the cAMP/PKA signaling pathway.

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Introduction

It is generally accepted that vigorous sperm motility is a central component for successful natural fertilization. Both the long-distance journey through the female reproductive tract and the subsequent penetration of the extracellular matrices surrounding the egg rely on the motility of the spermatozoa (Luconi *et al.* 2006). Thus, sperm motility provides an ideal target mechanism to exercise reproductive control, either by enhancing the fertilization success rate or reducing it through the development of novel, targeted contraceptives (Brito *et al.* 2005, Love 2005, Turner 2006).

Sperm motility is generated by the extremely long flagellum that comprises >90% of the length of a mammalian sperm (Vernon & Woolley 2004, Turner 2006). Such motility is regulated by the surrounding environment; obvious changes in motility include activation, hyperactivation, and chemoattraction in various species (Ahmad *et al.* 1995, Ho & Suarez 2001, Wang *et al.* 2001, Wade *et al.* 2003). The cyclic AMP (cAMP)/cAMP-dependent protein kinase A (PKA) signaling pathway and the calcium signaling pathway are generally recognized as the two signaling pathways most central to the regulation of mammalian sperm

motility (Brokaw 1987, Wishart & Ashizawa 1987, San Agustin & Witman 1994, Wade *et al.* 2003). Even though the role of these signaling pathways in sperm motility has been demonstrated in many experiments, the specific underlying molecular mechanisms have never been fully explored.

Zn- α 2-glycoprotein (ZAG) is a 43 kDa soluble glycoprotein, originally isolated for human plasma in 1961, which was named for its tendency to precipitate with zinc salts and its electrophoretic mobility in the α 2-globulins (Burgi & Schmid 1961, Sanchez *et al.* 1999). It has subsequently been detected in various physiological and pathological fluids (Jirka *et al.* 1978, Ohkubo *et al.* 1990, Bundred *et al.* 1991, Sanchez *et al.* 1997). Acting as a lipid-mobilizing factor, ZAG is expressed and secreted in mammalian adipose tissue and is markedly upregulated with cancer cachexia (Hale *et al.* 2001, Sanders & Tisdale 2004, Tisdale 2004). The expression level of ZAG is regulated through tumor necrosis factor- α and the peroxisome proliferator-activated receptor- γ nuclear receptor (Bao *et al.* 2005).

Recently, it has been reported that ZAG activity in rodents is mediated via the β_3 -adrenoreceptor, with upregulation of the cAMP pathway (Russell *et al.* 2004,

Tisdale 2004, McDermott *et al.* 2006). In a previous study, we reported that ZAG was primarily related to sperm forward motility in human semen (Ding *et al.* 2007). Our data supported the fact that ZAG in human seminal plasma might be integrated with α -1-antitrypsin (AAT) and was capable of initiating forward motility in immature immotile spermatozoa. However, it is still unclear whether ZAG is present in human spermatozoa, and what the relationship is between ZAG and sperm motility.

According to the above introduction, ZAG is supposed to be a special factor related to human sperm motility. There is a possibility that the signal transduction pathway of ZAG in adipose tissue will be similar to that in the spermatozoa. Thus, in the present study, we prepared polyclonal antibodies against recombinant human ZAG. Laser scanning confocal microscope (LSCM) and flow cytometry (FCM) were then employed to identify ZAG protein in human spermatozoa. Furthermore, these antibodies were applied to the experiments of sperm motility monitored by computer-assisted sperm analysis (CASA), together with the investigation of intracellular cAMP and PKA levels.

Results

Prokaryotic expression and purification of ZAG

To overexpress ZAG protein and raise antibodies, a protein fragment of good antigenicity was selected by DNASTar and DNAssist (Fig. 1A). A 345 bp fragment was amplified from human cDNA by PCR and separated on a 1% agarose

Figure 1 Cloning the target fragment of ZAG. (A) Antigenicity of the ZAG fragment was analyzed by DNASTar software. (B) RT-PCR products of ZAG fragment on 1% agarose gel. Lane M, marker; lane 1, ZAG fragment; lane 2, negative control. (C) Schematic of the recombinant plasmid pET-28a(+)/ZAG.

gel (Fig. 1B). The target fragment of ZAG was then cloned into the prokaryotic expression vector, which was named pET-28a(+)/ZAG (Fig. 1C). This construct is predicted to encode a 143 amino acid-containing recombinant protein with molecular weight of ~ 18 kDa. It was successfully overexpressed by inducing with IPTG; this 18 kDa protein was absent in non-induced cells, shown on 15% SDS-PAGE gel (Fig. 2A). The recombinant protein was purified and then confirmed by SDS-PAGE analysis (Fig. 2A) and Western blotting using anti-His monoclonal antibody (MAB) (Fig. 3A).

Production of polyclonal anti-ZAG antibodies in rabbits

Polyclonal antibodies against the recombinant ZAG protein were generated by immunization of white New Zealand rabbits. After immunization, rabbit anti-ZAG serum was drawn every week. By day 35, s.c.

Figure 2 Prokaryotic expression and purification of ZAG, and production of polyclonal anti-ZAG antibodies in rabbits. (A) Expression and purification of recombinant ZAG fragment in *E. coli* BL21 (DE3) host cells. Different fractions of *E. coli* were separated on a Coomassie-blue-stained 15% SDS-PAGE gel. Lane M, protein molecular weight markers; lane 1, total cell lysates carrying pET-28a(+)/ZAG fragment before IPTG induction; lane 2, total cellular protein after IPTG induction; lane 3, pellet of cell lysate after ultrasonic treatment; lane 4, supernatant of cell lysate after ultrasonic treatment; lane 5, protein purified based on its His₆-tag by affinity chromatography employing a Ni²⁺-NTA His-binding resin; lane 6, protein eluted in PBS by cutting certain protein bands from the gel after preparative electrophoresis. (B) Values of ELISA absorbance at 405 nm using anti-ZAG antibodies in both rabbit sera were higher than that of pre-immunized serum (control). (C) Anti-sera from immunized rabbits (diluted 1:1000) were determined weekly using ELISA after immunization.

Figure 3 The purified recombinant ZAG protein was analyzed by Western blot. (A) The purified recombinant ZAG protein (~18 kDa) was confirmed by immunoblotting, using anti-His monoclonal antibody (1:1000 dilution) and rabbit anti-ZAG IgG purified from sera (1:1000). (B) ZAG (~41 kDa) was distinctly detected in human sperm proteins using rabbit anti-ZAG IgG (1:1000) but not with IgG from pre-immunized sera. His, anti-His monoclonal antibody; Ab, rabbit anti-ZAG IgG purified from sera; Pre, IgG from preimmunized sera.

immunization of rabbits with ZAG induced a very strong IgG response against the antigen, as determined by ELISA at 405 nm. Results from the indirect ELISA indicated that the titers of rabbit anti-ZAG sera were fairly high, reaching 1:819 200 (Fig. 2B and C). Anti-ZAG antibodies (without sodium azide) were then purified from crude rabbit sera, confirmed by Western blotting (Fig. 3A) and stored at 4 °C for functional analysis *in vitro*.

Identification and localization of ZAG in spermatozoa

Using indirect immunofluorescence, spermatozoa staining with/without fluorescence were discerned and counted. The fluorescence intensity and the ratio of fluorescent sperm to non-fluorescent sperm were significantly higher in spermatozoa stained with ZAG antibody than in the control group (stained with normal rabbit IgG; $P < 0.001$; Fig. 4A and B). The results were confirmed using LSCM (Fig. 4C), LS-50B luminescence spectrometer (Fig. 4D), and further confirmed by the fact that ZAG was distinctly detected from human sperm proteins by Western blot (Fig. 3B). Furthermore, intense immunoreactivity was localized in the head and tail region of spermatozoa, while the most intense immunoreactivity was observed in the pre-equatorial region covering the acrosome, neck, and middle piece of flagellum (Fig. 5).

Measurement of sperm kinematics

For the motility inhibition experiment, human swim-up spermatozoa (adjusted to 10×10^6 sperm/ml) were incubated with or without the prepared rabbit anti-ZAG antibodies or normal rabbit IgG at different concentrations (Fig. 6A). Sperm and forward motility indices were evaluated after 0, 60, 90, and 120 min of incubation in the different experimental conditions described above using CASA. The following parameters were measured: ratio of motile sperm and straight line

Figure 4 Identification of ZAG on human spermatozoa by indirect immunofluorescent staining. The ratio of fluorescent sperm to non-fluorescent sperm was significantly higher in samples stained with ZAG antibody than with samples stained with normal rabbit IgG as primary antibodies (Con.; $**P < 0.001$) using FCM (A) and LSCM (C). The spermatozoa fluorescence intensity was significantly higher in spermatozoa stained with ZAG antibody than the control group ($**P < 0.001$) as monitored under FCM in (B) and LS-50B luminescence spectrometer in (D).

velocity (VSL; time-average velocity of a sperm head along the straight line between its first and last positions). At 90 or 120 min of incubation, the motility and VSL of spermatozoa treated with the rabbit anti-ZAG IgG (50.0 µg) were significantly lower than those from sperm treated with normal rabbit IgG or those treated with Tyrode solution with 3% BSA alone (Fig. 6B and C). These results indicated that anti-ZAG antibodies were able to reduce swim-up spermatozoa motility *in vitro*.

Measurement of intracellular cAMP and PKA levels

When we measured levels of intracellular cAMP, we found that rabbit anti-ZAG antibodies decreased cAMP levels in spermatozoa to 5.87 pmol/ 10^8 cells, compared with a normal level of about 10.36 pmol/ 10^8 cells. However, the addition of cAMP, co-incubated with the antibodies *in vitro*, mostly minimized this reduction (Fig. 7A). For the measurement of intracellular PKA activity, the fluorescence intensity measured in the assay is inversely correlated with kinase activity. Thus, the PKA activity of swim-up spermatozoa incubated with anti-ZAG IgG was significantly lower than that in sperm treated with normal rabbit IgG (Fig. 7B). Therefore, both intracellular cAMP levels and PKA activity in human sperm were reduced by the anti-ZAG antibodies.

Figure 5 Localization of ZAG on human spermatozoa by LSCM. (A) ZAG was observed on the head and tail region of spermatozoa. (B) Differential interference contrast (DIC) images corresponding to (A). (C) Two spermatozoa in (A) were magnified to show the precise localization of immunofluorescent staining on pre-equatorial region covering the acrosome, neck and middle piece of flagellum. (D) DIC images corresponding to (C). (E) Normal rabbit IgG as primary antibodies. (F) DIC images corresponding to (E). (G) Secondary antibodies alone. (H) DIC images corresponding to (G).

Discussion

A high percentage of poorly motile or immotile spermatozoa in semen is obviously detrimental to male fertility. A recent investigation of 1085 sperm samples from infertile patients revealed that 81% had defects in motility and 19% had asthenozoospermia without any other defects in sperm number or morphology (Curi *et al.* 2003). Thus, the analysis of sperm motility has become a central part of male fertility evaluation (Turner 2006). In spite of its importance for fertility, poor sperm motility is only a descriptive symptom for which the underlying cause is poorly understood.

In this study, we try to explore the possible relationship between ZAG and sperm motility. Since previous studies have demonstrated the presence of ZAG in seminal and prostate fluid (Ohkubo *et al.* 1990, Ahlgren *et al.* 1995), there is a possibility that ZAG was adsorbed to the sperm. Furthermore, ZAG was reported to be capable of initiating forward motility in immature immotile spermatozoa, integrated with AAT (Ding *et al.* 2007). Thus, it was reasonable to investigate whether ZAG affects the motility of sperm individually. We cloned the gene encoding human ZAG and generated the relevant polyclonal antibodies. We not only identified these antibodies in the tail region of spermatozoa by immunofluorescence but also found that they reduced sperm motility. In addition, the anti-ZAG antibodies decreased intracellular cAMP levels and PKA activity.

In the regulation of mammalian spermatozoa motility, the cAMP/PKA signaling pathway is usually recognized as one of the most significant signaling pathways. This pathway is also known to play a major role in intracellular signaling during mammalian sperm capacitation (Brokaw 1987, Si & Okuno 1995, Fraser & Osiguwa 2004). The cAMP-dependent phosphorylation of flagellar proteins is at least partially responsible for the initiation and maintenance of activated sperm motility in mammals (San Agustin & Witman 1994). In mice with a targeted deletion of the sperm-specific isoform of the

catalytic (C) subunit of PKA, male infertility is highly correlated with poor sperm motility (Skalhegg *et al.* 2002). Thus, it is very likely that one mechanism of cAMP action is to affect sperm motility via activation of PKA. On the other hand, cAMP has also been associated with gated ion channels, involved in the calcium signaling pathway. Therefore, increased intracellular cAMP through both the cAMP/PKA and calcium signaling pathways could lead to hyperactivated motility via upregulation of the flagellar wave (Ahmad *et al.* 1995, Ho & Suarez 2001).

However, how does ZAG affect the cAMP/PKA signaling pathway in human sperm? The cAMP signaling pathway is reported to be involved in the process of lipolysis in adipocytes in many mammals, for which the β -adrenergic receptor is directly induced by ZAG (Russell *et al.* 2002, 2004, Sanders & Tisdale 2004, Tisdale 2004, McDermott *et al.* 2006). The crystalline structure of ZAG resembles a class I major histocompatibility complex (MHC) heavy chain, but, unlike other MHC-related proteins, ZAG does not bind the class I light-chain β_2 -microglobulin (Sanchez *et al.* 1997, 1999). ZAG structure includes a large groove analogous to the class I MHC peptide-binding grooves. However, instead of a peptide, ZAG's groove contains a non-peptidic compound that might be implicated in lipid catabolism under normal or pathological conditions (Delker *et al.* 2004, McDermott *et al.* 2006). The mechanism involved in lipid catabolism is that ZAG can stimulate lipolysis in adipocytes through the β_3 -adrenoreceptor, which directly results in the stimulation of adenylate cyclase and an increase in intracellular cAMP (Russell *et al.* 2004, Tisdale 2004, Russell & Tisdale 2005). Interestingly, β -adrenergic receptors have already been detected in human sperm (Adeoya-Osiguwa & Fraser 2005, 2007, Adeoya-Osiguwa *et al.* 2006), and β_3 -adrenoreceptor also has a similar localization to ZAG. Thus, we presume that ZAG is able to stimulate cAMP signaling via the β -adrenoreceptor in human

Figure 6 Sperm kinematics measurement in human swim-up spermatozoa using CASA. (A) Human ($n=35$) swim-up spermatozoa were incubated with purified rabbit anti-ZAG IgG (without sodium azide) at different concentrations for up to 2 h at 37 °C. The antibodies inhibited sperm motility at the concentration of 50.0 μg. At the concentration of 50.0 μg, sperm motility and forward motility index were evaluated during incubation. (B) After 90 or 120 min of incubation, the motility of spermatozoa treated with anti-ZAG IgG was significantly lower than in sperm treated with normal rabbit IgG or Tyrode solution with 3% BSA alone. (C) A similar tendency was found for straight line velocity (VSL) at 120-min incubation.

sperm, a potential pathway of motility regulation. This could explain why anti-ZAG antibodies can inhibit sperm motility, together with the decreasing of intracellular cAMP and PKA levels.

In conclusion, our current investigations demonstrate that ZAG is present in human spermatozoa, and observed mostly in the pre-equatorial region covering the acrosome, neck, and middle piece of the flagellum.

Figure 7 Measurement of intracellular cAMP and PKA levels. (A) The cAMP ELISA was performed on human ($n=18$) spermatozoa lysates treated with rabbit anti-ZAG antibodies, normal rabbit IgG, and rabbit anti-ZAG antibodies plus cAMP. Relative cAMP levels in swim-up sperm incubated with anti-ZAG antibodies decreased compared with sperm treated with normal rabbit IgG (Con.) or Tyrode solution with 3% BSA alone (N). (B) The fluorescence intensity measured in the assay is inversely correlated with kinase activity, according to the manufacturer's instructions of ProFluor™ PKA Assay. The PKA activity of swim-up spermatozoa incubated with anti-ZAG IgG was significantly lower than those from sperm treated with normal rabbit IgG. However, the addition of cAMP *in vitro* minimized this reduction. * $P<0.05$.

ZAG may be a novel factor involved in the regulation of sperm motility, mediated by activation of the cAMP/PKA signaling pathway.

Materials and Methods

Specimens and reagents

Human semen specimens were obtained from Shanghai Jiaji Genetics and IVF Institute-China USA Center. The Ethics Committees of this unit approved the use of these samples for this project, and all donors gave written informed consent for the use of their leftover semen samples. Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Corporation.

Prokaryotic expression and purification of ZAG

Human cDNA was kindly gifted by Dr Min Wang from the Shanghai Key Laboratory for Reproductive Medicine (Shanghai, China). The segment of ZAG was amplified with the forward primer 5'-GCGGATCCGGAAGGTTGGTTGTGA-GAT-3' (containing BamHI site, underlined) and the reverse primer 5'-GCAAGCTTCCCTGGGTAGAAAGTCGTAG-3' (containing HindIII site, underlined). The PCR product was

purified from the agarose gel and cloned into the pMD18-T vector (TaKaRa Biotech, Dalian, China) to transform *Escherichia coli* (*E. coli*, strain DH16B). The recombinant plasmid was digested with BamHI and HindIII, and ligated into the pET-28a(+) expression vector (Novagen, Darmstadt, Germany) at the same restriction site. The target fragment of ZAG was confirmed by restriction enzyme digestion, PCR, and sequencing. The new recombinant prokaryotic expression vector was named pET-28a(+)/ZAG, and propagated in *E. coli* BL21(DE3) host cells cultured with 0.1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) for 5 h at 37 °C with gentle shaking. Bacterial cells were harvested by centrifugation (5000 g) for 15 min at 4 °C, and disrupted by gentle ultrasonic treatment (50 W, 20 s $\times$ 10) with a 50% duty cycle on ice. Recombinant protein was purified based on its His₆-tag by affinity chromatography using a Ni²⁺-NTA His-binding resin (Pierce, Rockford, IL, USA; Joshi & Puri 2005). For further purification, the recombinant protein was separated on preparative electrophoresis, and the corresponding band was cut from the gel and eluted in PBS. The eluted protein was freeze-dried in a Heto Freeze Dryer (Thermo Electron Corporation, Waltham, MA, USA), and its identity was confirmed by 15% SDS-PAGE analysis and Western blotting using anti-His MAB.

Production of polyclonal rabbit anti-ZAG antibodies

Two healthy white New Zealand rabbit males (~6 months old, body weight ~2.5 kg; purchased from Shanghai SLAC Laboratory Animal Co., Shanghai, China) were accommodated in the animal facility for at least for 1 week prior to immunization. The pre-immunized rabbits' blood was obtained, and the serum was separated and kept at -80 °C for later use (negative control). The rabbits were immunized a total of three times with the recombinant ZAG protein and the adjuvants to raise antibodies (Hu *et al.* 2002). The titer of the antiserum was measured using an indirect ELISA at 405 nm using an Anthos Zenyth 1100 multimode detector (Anthos Labtec Instruments GmbH, Wals, Austria) with a 5-s pre-read shake. Finally, the anti-ZAG IgG was purified through immunoaffinity chromatography from crude rabbit sera, using the ImmunoPure (G) IgG Purification kit (Pierce). In the meantime, normal rabbit IgG was purified from pre-immunized rabbit sera.

Preparation of spermatozoa and sperm proteins

Semen samples were collected via masturbation after 3–5 days of sexual abstinence from young healthy donors (20–30 years of age). Individual semen samples were allowed to liquefy at 37 °C, and the mature spermatozoa were separated from seminal plasma, immature germ cells, and non-sperm cells by Percoll density ingradient centrifugation (Fraser & Osiguwa 2004). In sperm motility inhibition experiments, motile, intact spermatozoa were obtained using the swim-up method, as previously described (Bronson & Fusi 1990).

Purified spermatozoa were solubilized in a lysis buffer containing: 8 M urea, 1.5% Triton X-100, and 1 mM phenyl

methyl sulfonyl fluoride. The sperm suspension was then vortexed for 10 min at 4 °C and kept in a rotating ice bath for at least 2 h in order to allow proteins to dissolve completely. After protein dissolution, samples were ultrasonicated (30 W, 10 s $\times$ 3, VC600, Sonics and Materials, Newtown, CT, USA) and centrifuged at 12 000 g for 20 min to remove the sediment. Protein concentration was determined by the BCA Protein Assay Kit (Pierce), using BSA to generate the standard curve.

Western blot analysis

For Western blot analysis, samples containing 20 μ g protein were separated on 15% SDS-PAGE gel (denatured). Proteins were then transferred to PVDF membranes (GE Healthcare, Waukesha, WI, USA) using a semi-dry transfer apparatus (Bio-Rad), and membranes were blocked with 5% BSA. Immunoblotting was performed with primary antibody at a suitable dilution in Tris-buffered saline containing 0.1% Tween 20 and 5% BSA overnight at 4 °C. Blots were then incubated with a goat-anti-rabbit secondary antibody conjugated with horseradish peroxidase (HRP) at a 1:1000 dilution for 1 h at room temperature. Signals were detected by ECL (ECL Plus, GE Healthcare), following the manufacturer's protocol.

Indirect immunofluorescence of human spermatozoa

For the immunofluorescence studies, fresh human spermatozoa were harvested over a discontinuous 47/90% Percoll density gradient and subsequently washed thrice with PBS. The sperm solution (2×10^5 sperm/ml) was then added onto slides, fixed with acetone for 30 min. All subsequent incubations were performed in a humid chamber. The slides were blocked in 5% BSA in PBS for 30 min at room temperature, and later incubated either with the purified rabbit anti-human ZAG antibody (1:500) overnight at 4 °C or with normal rabbit IgG (1:500 dilution) as negative control. After washing, samples were incubated with a secondary antibody linked with fluorescein isothiocyanate (FITC; Biosource, Burlingame, CA, USA) diluted 1:300 for 3 h at 37 °C. Finally, the fluorescent-stained sperm samples were viewed and counted under a LSCM (Carl Zeiss LSM-510, Jena, Germany).

Flow cytometry (FCM)

Purified spermatozoa were washed twice with HEPES buffer (pH 7.3) containing 1% BSA and 0.5 mM CaCl₂, and then adjusted to the concentration of 1×10^6 cells/ml. Cells were incubated with rabbit anti-human ZAG antibody diluted 1:1000 overnight at 4 °C or with normal rabbit IgG (1:500 dilution) as negative control. After samples were washed twice with HEPES buffer, FITC-labeled goat anti-rabbit antibody diluted 1:1000 was used as a secondary antibody and incubated for 1 h at room temperature. Finally, fluorescence was measured by FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA), and the percentage of fluorescent cells was analyzed using CellQuest software (Becton Dickinson). The same treated samples were also analyzed on a LS-50B luminescence spectrometer (Perkin-Elmer, Norwalk, CT, USA) to measure fluorescent intensity at 510 nm (490 nm excitation).

Measurement of sperm kinematics

Donors' sperm isolated by the swim-up method were collected, washed, and resuspended in Tyrode solution with 3% BSA. Before the experiment of kinematics, the sperm concentration was adjusted to $10 \times 10^6/\text{ml}$. In total, 35 individual sperm samples from different healthy donors were used in this motility experiment. Swim-up spermatozoa were incubated in the presence or absence of prepared and purified rabbit anti-ZAG antibodies (without sodium azide) or normal rabbit IgG at different concentrations (1, 10.0, 50.0, and 100.0 μg) for up to 2 h at 37 °C. Sperm and forward motility indices were evaluated after 0, 60, 90, and 120 min of incubation with the antibodies using CASA (Hamilton-Thorn Research, Beverly, MA, USA).

Measurement of intracellular cAMP and PKA levels

For the measurement of intracellular cAMP and PKA levels in human spermatozoa, 18 individual swim-up spermatozoa populations (every six individual samples for each experiment and three experiments were repeated totally) were incubated with or without rabbit anti-ZAG antibodies (or added cAMP at the concentration of 15 pmol/ 10^8 cells) for 2 h at 37 °C. Subsequently, the samples were washed with PBS and centrifuged for 20 min at 4 °C. Sediments were then collected and assayed for intracellular cAMP using cAMP Immunoassay kit (R&D Systems, Minneapolis, MN, USA), as well as for PKA activity using ProFluor PKA Assay (Promega), according to the manufacturer's instructions.

Statistical analysis

For each sample, kinematical parameters were expressed as the median of analyzed sperm tracks per man. Data were recorded and analyzed using the SAS 8.2 statistical software. Sperm kinematics was analyzed using the Student–Newman–Keuls one-way ANOVA test. $P < 0.05$ was considered statistically significant. Values reported were mean $\pm$ S.E.M.

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