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Running title: PKA and PKC and disordered ZP-induced AR

Capsule

Defective protein kinase A and C pathways are commonly associated with disordered zona
pellucida-induced acrosome reaction in normozoospermic infertile men with normal sperm-
ZP binding.

**Defective protein kinase A and C pathways are common causes of disordered zona
pellucida-induced (ZP) acrosome reaction in normozoospermic infertile men with
normal sperm-ZP binding**

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has nothing to disclose.

Abstract

Objective: To determine association between defective protein kinases C (PKC) and A (PKA) and disordered zona pellucida (ZP)-induced acrosome reaction (DZPIAR) in normozoospermic infertile men with normal sperm-ZP binding.

Design: Sperm from DZPIAR infertile men were treated without (control) or with (test) phorbol myristate acetate (PMA, PKC activator) or dibutyryl cyclic AMP (dbcAMP, PKA activator) under in vitro standard culture condition. The ZP-induced AR was assessed and compared between control and test.

Setting: Public and private hospitals based clinical ART centres.

Patients: A total of 51 DZPIAR infertile men were involved in this study.

Intervention (s): none

Main outcomes measure(s): Sperm-ZP binding and the ZP-induced IAR.

Results: Both PMA and dbcAMP enhanced ZP-induced AR up to a normal level ($\geq 25\%$) in some subjects with DZPIAR: 29 (57%) with PMA and 27 (53%) with dbcAMP. Overall 35 (69%) had the ZP-induced AR enhanced to normal by PMA or dbcAMP but 16 (31%) had little or no response to either agent. Fourteen men responded to the two activators differently: 8 effective only with PMA and 6 effective only with dbcAMP.

Conclusion: Defective upstream of PKC and PKA pathways are highly associated with disordered ZPIAR in normozoospermic infertile men with normal sperm-ZP binding.

Keywords: male infertility, protein kinase pathways, zona pellucida induced acrosome reaction,

Introduction

During the process of human fertilization in vivo and in conventional in vitro fertilization (IVF), sperm must be capable of binding to the zona pellucida (ZP), penetrating the ZP and fusing with oolemma before normal fertilization takes place (1). In humans, it is believed that only acrosome intact sperm bind to the ZP and the acrosome reaction (AR) occurs on the ZP (2,3). In contrast, some other mammalian sperm, such as guinea pig, both acrosome intact and reacted sperm are able to bind and penetrate the ZP (1, 4). Furthermore, recent reports showed that most fertilizing mouse sperm begin the AR before contact the ZP (5) and acrosome reacted sperm recovered from the perivitelline space can bind, penetrate the ZP and fertilize the mouse oocytes in vitro (6).

In conventional IVF, defective sperm-ZP binding and penetration are the most common causes of failure of fertilization in couples with either normal or abnormal semen analysis (7, 8). This is one of the main reasons for the success of intracytoplasmic sperm injection (ICSI) in the treatment of male infertility as ICSI overcomes the inability of sperm to penetrate the ZP. Defective sperm-ZP binding and penetration are commonly associated with abnormal semen, particularly severe teratozoospermia. However, some of patients with defective sperm-ZP binding and penetration can have normal semen analysis (9,10). Furthermore, in normozoospermic infertile men with normal sperm-ZP binding, failure of sperm-ZP penetration is mostly due to disordered ZP-induced AR (DZPIAR) which sperm are able to bind to the ZP but unable undergo the AR on the ZP (10,11). Diagnosis of these men with DZPIAR has important implications in clinical ART as they have low or zero fertilization rates with conventional IVF. Testing couples with idiopathic infertility for DZPIAR can direct successful treatment with ICSI (12). However the cause of DZPIAR is

poorly understood.

During human sperm-oocyte interaction, acrosome intact sperm bind to the ZP and then the AR is induced by the ZP. However, the mechanism of the human sperm-ZP interaction is not fully understood. The human ZP likely contains four glycoproteins, hZP1, hZP2, hZP3 and hZP4 (13, 14). Although it has been suggested that both the ZP3 and ZP4 may have a role as the primary ZP receptor for binding acrosome intact sperm and inducing the AR (13, 15), transgenic mouse experiments showed that neither human ZP3 nor ZP4 is sufficient to support human sperm binding to the ZP and other human ZP proteins may be necessary for human sperm-oocyte interaction (16). In mouse, gamete recognition depends on the cleavage status of the ZP2 and binding of sperm to the surface of the ZP may not sufficient to induce sperm AR (17). On the other hand, so far no human sperm receptors for the ZP proteins have been well defined despite many candidates being reported to be able to interact with either solubilised or intact ZP (18-21). As well as multiple receptors other processes involved in the human sperm-ZP induced AR include protein kinase A (PKA) and C (PKC) signal transduction pathways, phosphorylation of protein SRC, actin polymerization and depolymerisation, ion flux particularly calcium, and membrane fusion (1, 22-31). Activation of PKC pathways by the PKC activator, phorbol 12-myristate 13-acetate (PMA), can significantly enhance ZP-induced AR in mouse sperm (22). Tollner et al (23) found that PMA can enhance sperm-ZP binding and the ZP-induced AR in macaque sperm *in vitro*.

We have developed tests for human sperm oocyte interaction using oocytes which failed to fertilise (32). In fertile men on average 48% of ZP-bound sperm undergoes the AR *in vitro* (33). In contrast, with DZPIAR very low proportions of sperm undergo ZP induced AR (mean 6%, range 1-16%) (11). Extending the time of pre-incubation of sperm does not

1 increase the ZP-induced AR with DZPIAR, suggesting it is not simply due to delayed
2 capacitation. Furthermore, DZPIAR sperm usually respond normally in calcium ionophore
3 A23187 induced AR tests suggesting it is unlikely to be caused by lack of responsiveness to
4 calcium influx (11).

5 In human sperm we found the PKC activator, phorbol 12-myristate 13-acetate (PMA)
6 induces acrosomal ruffling and increases the ZP-induced AR of ZP bound sperm but it is
7 unable to stimulate the AR of sperm in medium (25,26). In contrast, activation of PKA
8 pathway using dbc-AMP has very small effect on ZP-induced AR of sperm from
9 normozoospermic men with normal ZP-induced AR (25). In this study we have recalled 51
10 DZPIAR infertile men diagnosed previously to determine if defective PKA and PKC
11 pathways is associated with DZPIAR.

12 13 **Materials and methods**

14 **Chemicals and culture medium**

15 Human tubal fluid (HTF, Irvine Scientific, Irvine, CA, USA) supplemented with 2%
16 human serum albumin (Irvine Scientific) was used as culture medium for all the experiments.
17 Dibutyl cyclic adenosine monophosphate (dbcAMP), phorbol 12-myristate 13-acetate
18 (PMA), 4 α -phorbol 12,13-didecanoate (4 α PDD), dimethyl sulfoxide (DMSO), and *Pisum*
19 *Sativum* agglutinin conjugated with fluorescein isothiocyanate (PSA-FITC) were purchased
20 from Sigma Chemical Company (St Louise, MO, USA). Stock solution of 200 mM dbcAMP
21 was made using HTF medium and aliquots 20 μ l/tube were stored at -20°C. Stock solution 5
22 mM PMA or 4 α PDD was made using DMSO and aliquots 20 μ l/tube were stored at -70°C.

Human oocytes

Oocytes used for the sperm-ZP interaction tests were obtained from the clinical IVF program as described previously. We used both unfertilized oocytes with no evidence of pronuclei or cleavage at 60 h after insemination and immature oocytes with either germinal vesicle or metaphase I. Degenerate, activated or morphologically abnormal oocytes, as well as oocytes with >10 sperm penetrated in the ZP were not used for the test. All the oocytes were obtained on day 3 after collection and the oocytes were pooled from several patients and used for the test on the same day or kept in the 5% CO₂ incubator and used within the next 3 days. Use of group of 4 oocytes for each test, the results of ZP-induced AR was highly reproducible (10,34).

Sperm samples and preparation

Sperm samples were obtained by masturbation after 2 to 5 days abstinence from 51 unexplained infertile men with previous diagnosed with DZPIAR as defined as the AR of ZP-bound sperm <16% after incubation of 2×10^6 /ml motile sperm with a group of 4 oocytes for 2 h (11). Under this experimental condition, sperm from fertile men will have an average 48% ZPIAR (33). Semen analysis was performed after liquefaction within one hour of collection of semen according to the World Health Organization manual (35). Percent normal sperm morphology was assessed according to strict criteria under oil immersion with magnification x1000 and bright field illumination (35).

Motile sperm were selected by colloidal silica gradient centrifugation (PureSperm, Nidacon International AB, Molndal, Sweden) using two layers of 1ml of 40% and 1ml of 80% PureSperm. The pellet of motile sperm obtained from PureSperm was washed once with 1 ml

of HTF supplemented with 2% human albumin. The washed sperm pellet was resuspended with the HTF medium to a sperm concentration of 2×10^6 /ml for sperm-ZP interaction test.

All patients signed consent forms permitting use of their gametes (unfertilised or immature oocytes and sperm) for this research project. The Royal Women Hospital Research and Ethics Committees approved the project.

Effect of dbc-PMA and PMA on the ZP-induced AR of sperm in DZPIAR men

Our previous studies showed that concentration of 2 mM dbcAMP and 15 μ M PMA had maximum effect on sperm-ZP interaction without impact on sperm motility (25,36). Therefore, we used 2mM dbcAMP and 15 μ M PMA in this study. dbcAMP solution was made in medium and the same medium was used for control. For PMA, we used the biologically inactive phobol ester (4 α PDD) for control.

Motile sperm (2×10^6 in 1 ml medium) selected by PureSperm were incubated with a group of 4 oocytes with or without (control) the agents in 4-well culture plates (Nunc, Roskilde, Denmark) for 2 h at 37 °C in 5% CO₂ in air. After 2 h incubation, each group of 4 oocytes was transferred to PBS containing 2 mg/ml BSA. The oocytes were then flushed several times in three separate wells containing 0.5 ml PBS with 0.2% BSA to dislodge loosely adherent sperm. The number of sperm bound to each of the 4 oocytes was counted using an inverted contrast microscope and average number of sperm bound per ZP was used as endpoint. **All the** DZPIAR men had normal sperm-ZP binding (average ≥ 50 sperm bound /ZP) under these experimental conditions according to definition of our previous studies (9, 33).

After counting and recording the number of sperm bound on the ZP, all sperm bound

to the 4 ZP were then removed by repeated vigorous aspiration of the oocytes using a narrow gauge micropipette with an inner diameter that was slightly smaller than that of the oocyte (approximately 120 μ m). This was performed on a glass slide with about 3 μ l PBS containing 0.2% BSA and the removed ZP-bound sperm were smeared in a limited area (~4 x 4 mm). The smear was then air-dried and marked with a glass pen on the back of the slide to help find the sperm under the microscope.

Sperm in the medium were washed with 10 ml normal saline, the sperm pellet resuspended in about 20 μ l saline and then 5 μ l smeared on a glass slide for assessment of acrosome status as described the below.

Assessment of the AR

The AR of ZP-bound sperm and sperm in medium were assessed using PSA-FITC as described previously (11). Briefly, sperm smears were fixed in 95% ethanol for 30 minutes after air-drying and then stained with 25 μ g/ml PSA-FITC in PBS for 2 h at 4°C. The slides were washed and mounted with distilled water and 200 sperm per sample were examined under a fluorescence microscope (excitation wavelengths of 450-490 nm) with 400x magnifications. Acrosome intact sperm have a uniform bright fluoresce covering half or more sperm head and the AR sperm had only a fluorescing band at the equatorial segment.

The enhanced ZP-induced AR $\geq$ 25% was defined as normal since sperm from fertile men had ZP-induced AR ranged 20-95% (with average of 48%) and those with ZP-induced AR $\geq$ 25% had no significant impact on sperm-ZP penetration (33,34). Our previous studies in 176 men with two different ejaculates showed the ZP-induced AR results between ejaculates in the same men were very consistent and highly reproducible (10).

Statistical analysis

Statistical power calculation estimated that minimum 14 samples were required to achieve the P value 0.05 based on 80% power level, two-tailed t-test of paired samples with anticipation of ZP-induced enhanced by the activators from 10% in control to 25% in test groups. In this study we had 51 samples with actual enhancement of ZP-induced AR by the activators from 8% in control to 28% (for dbcAMP) or 34% (for PMA) in test groups. Non-parametric (Wilcoxon rank) test was used for comparison of the number of sperm bound/ZP, the ZP-induced AR (AR of ZP bound sperm) or spontaneous AR (sperm in medium) between test (dbcAMP and PMA treatments) and control. Mean and standard deviation (SD) was presented between test and control groups. Proportion of DZPIAR men with various levels of responses to PKA or PKC activators was expressed in the percentage.

Results

Sperm test results for 51 infertile men with DZPIAR were summarised in Table 1. All men had normal semen analysis and normal sperm-ZP binding but DZPIAR (ZP-induced AR $\leq 16\%$). Both dbcAMP and PMA significantly enhanced the ZP-induced AR but not spontaneous AR of sperm in the medium or the number of sperm bound/ZP (Table 2).

Of the 51 DZPIAR men, proportion of men with enhanced the ZP-induced AR up to normal range (AR $\geq 25\%$) was 57% (29 of 51) by PMA or 53% (27 of 51) by dbcAMP (Fig 1). When combined results of both activators treatment samples together a total of 69% (35 of 51) of the DZPIAR men had the ZP-induced AR enhanced to normal level by either PMA

or dbcAMP (Fig 1). Twenty-one of 51 (41%) men was highly response to both dbcAMP and PMA alone (Fig 2A). Fourteen DZPIAR men responded to PMA and dbcAMP differently: eight had ZP-induced AR enhanced to normal level by PMA and not by dbcAMP, and on the other hand six had ZP-induced AR enhanced to normal level by dbcAMP and not by PMA (Fig 2B). Only 31% (16 of 51) of them had no or very little responses to either of these activators (Fig 2C).

Discussion

This study showed that defective PKA and PKC signal transduction pathways are the major cause of DZPIAR in normozoospermic infertile men with normal sperm-ZP binding. In 51 DZPIAR infertile men studied, 69% of them responded effectively to either PMA or dbcAMP enhancement of the ZP-induced AR up to normal level and only 31% had very low or no response to the both activators. DZPIAR men with less or no response to PMA or dbcAMP may be associated with other defects unrelated to these pathways. This data provides further evidence that both PKA and PKC pathways play critical roles in human ZP-induced AR after sperm bind to the ZP.

In the present study, 21 of 51 (41%) DZPIAR men highly responded to both PKA and PKC activators with the ZP-induced AR enhanced up to normal range. Doherty et al (37) reported that there is a convergent mechanism of crosstalk between the PKA and PKC pathways leading to the human AR. Defective one pathways may also have negative impact on another pathway for the human AR. In addition, Visconti et al. (38) reported that PMA may stimulate PKA pathway in sperm. However, 14 of 51 DZPIAR men responded to the two activators differently; 8 effective only with PMA and 6 effective only with dbcAMP. It is

likely that those DZPIAR may be associated with only single PKC or PKA pathway defect or the crosstalk between PKA and PKC were lacking and further study is required.

In comparison with our previous study in normozoospermic infertile men with defective sperm-ZP binding, only about 13% or 33% of these men found to be associated with defective pathways of either PKA or PKC pathways (36). While sperm-ZP binding and the ZP-induced AR are obviously different stage of sperm-ZP interaction, it is clear that defective PKA and PKC pathways are more frequently associated with DZPIAR than defective sperm-ZP binding in normozoospermic infertile men. Thus use of PKA and PKC activators to identify which of men without defective PKA or PKC pathways will be useful to identify patients for future study of sperm receptors or other causes of defective sperm-ZP interaction.

Although this study showed that both PMA and dbcAMP can enhance ZP-induced AR in majority of DZPIAR men, it is unlikely that this would have a therapeutic effect in clinical ART, as both dbcAMP and PMA are not recommended for use *in vivo*. This study was mainly to identify what proportion of DZPIAR men is associated with defects of PKA and PKC pathways. However, patients diagnosed with DZPIAR can be successfully treated by ICSI (12). Thus in clinical ART program, it is very critical to diagnose this condition in unexplained infertile couples before they commence the ART treatment as they require ICSI instead of conventional IVF to achieve optimal outcome. Usually, couples at the first cycle with normal semen analysis are treated by conventional IVF. Unfortunately, some of the patients may have DZPIAR which will have low fertilization results in IVF because sperm from those men are less or unable to penetrate the ZP of oocytes inseminated (9, 12).

Frequency of DZPIAR can be diagnosed in 20-25% normozoospermic infertile men with normal sperm-ZP binding (12). At the present, human oocytes are required for sperm-ZP

1 interaction test to diagnose DZPIAR and no artificial human ZP or other substitute is available
2 to replace native human ZP. Recombinant human ZP (rhZP) proteins are easily produced by
3 several groups but rhZP does not bind to human sperm and also very low biological activity in
4 inducing the AR compared with native human ZP (39-45). This is also a main reason for non
5 progress of development of commercial rhZP kit for clinical diagnosis of male infertility with
6 either defective sperm-ZP binding or DZPIAR. Currently, DZPIAR is also unable to be
7 predicted using other chemical or biological inducers such as calcium ionophore A23187 or
8 progesterone since there is no correlation between the ZP and A23187 or progesterone
9 induced AR (34, 46, Liu et al unpublished data).

10 Early study showed that activation of the PKC pathway by PMA can induce the AR of
11 human sperm *in vitro* as assessed by a modified triple stain method, but effects were usually
12 very small (47). When we used electron microscopy and PSA-FITC to examine the AR of
13 sperm after treatment with PMA, PMA did not significantly induce the AR of sperm in
14 medium (25). However, our electron microscope results revealed that PMA significantly
15 induced acrosomal ruffling which may result from activation of actin polymerization (26). The
16 proportion of sperm with PMA-induced acrosomal ruffling is highly correlated with PMA-
17 enhanced ZP-induced AR of human sperm (26). Studies from several independent groups
18 also showed that PMA significantly enhanced the ZP-induced AR of sperm in the mouse,
19 macaque monkey and human (22, 23 25, 26).

20 In conclusion, cause of DZPIAR in most normozoospermic infertile men with normal
21 sperm-ZP binding may be due to defective signal transduction pathways upstream of either
22 PKA or PKC. However, some (31%) of these men, DZPIAR may be due to other defects
23 unrelated PKA and PKC pathways. DZPIAR are experiments of nature indicating the

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1 importance of the ZP-induced AR for human fertilisation.

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FIGURE 1. Proportion of DZPIAR men had ZP-induced AR enhanced to normal ($\geq 25\%$) by the activators: 57% by PMA, 53% by dbcAMP and a total of 69% enhanced by either PMA or dbcAMP. There was 31% of DZPIAR men had no effective response to either PMA or dbcAMP.

FIGURE 2. Variable results of ZP-induced AR responses to PMA and dbcAMP in sperm from 51 DZPIAR men: (A) enhancement of ZP-induced AR to normal ($\geq 25\%$) by both activators in 21 men; (B) enhancement of ZP-induced AR to normal ($\geq 25\%$) by either activators in 14 men: 8 by PMA and 6 by dbcAMP; (C) No or little effect ($< 25\%$ ZP-induced AR) of either activators in 16 men.

1 **Fig 1**

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Fig 2