D-penicillamine prevents ram sperm agglutination by reducing the disulphide bonds of a copper-binding sperm protein

T Leahy¹, JP Rickard¹, RJ Aitken² and SP de Graaf¹

¹Faculty of Veterinary Science, The University of Sydney, Camperdown, New South Wales, Australia and

²Discipline of Biological Sciences, Faculty of Science and IT and Faculty of Health and Medicine, University of Newcastle, Callaghan, New South Wales, Australia

Correspondence should be addressed to T Leahy; Email: tamara.leahy@sydney.edu.au

Abstract

Head-to-head agglutination of ram spermatozoa is induced by dilution in the Tyrode's capacitation medium with albumin, lactate and pyruvate (TALP) and ameliorated by the addition of the thiol D-penicillamine (PEN). To better understand the association and disassociation of ram spermatozoa, we investigated the mechanism of action of PEN in perturbing sperm agglutination. PEN acts as a chelator of heavy metals, an antioxidant and a reducing agent. Chelation is not the main mechanism of action, as the broad-spectrum chelator ethylenediaminetetraacetic acid and the copper-specific chelator bathocuproinedisulfonic acid were inferior anti-agglutination agents compared with PEN. Oxidative stress is also an unlikely mechanism of sperm association, as PEN was significantly more effective in ameliorating agglutination than the antioxidants superoxide dismutase, ascorbic acid, α -tocopherol and catalase. Only the reducing agents cysteine and DL-dithiothreitol displayed similar levels of non-agglutinated spermatozoa at 0 h compared with PEN but were less effective after 3 h of incubation (37 °C). The addition of 10 μ M Cu²⁺ to 250 μ M PEN + TALP caused a rapid reversion of the motile sperm population from a non-agglutinated state to an agglutinated state. Other heavy metals (cobalt, iron, manganese and zinc) did not provoke such a strong response. Together, these results indicate that PEN prevents sperm association by the reduction of disulphide bonds on a sperm membrane protein that binds copper. ADAM proteins are possible candidates, as targeted inhibition of the metalloproteinase domain significantly increased the percentage of motile, non-agglutinated spermatozoa (52.0% $\pm$ 7.8) compared with TALP alone (10.6% $\pm$ 6.1).

Reproduction (2016) **151** 491–500

Introduction

We have previously shown that the extension of ram spermatozoa in the commonly used Tyrode's capacitation medium with albumin, lactate and pyruvate (TALP) causes rapid head-to-head sperm agglutination in a highly specific and persistent manner (Leahy *et al.* 2016). Ram sperm aggregation in TALP does not coincide with a loss of sperm function, as often seen after spermatozoa are exposed to physiochemical stressors such as high dilution or washing, cold shock, and extreme pH or osmotic conditions (Mann 1964). Agglutinated sperm retain their motility, and the phenomenon appears similar to the agglutination that occurs when sera (e.g. follicular fluid and plasma) are added to *in vitro* fertilisation (IVF) media to stimulate cholesterol efflux of ram spermatozoa (Ehrenwald *et al.* 1990, Funahashi & Day 1993). It is uncertain whether this phenomenon is physiologically relevant or an artefact of *in vitro* sperm manipulation;

however, agglutination of rabbit spermatozoa has been noted after placement in the doe uterine lumen (Brown & Senger 1982).

Ram spermatozoa require serum for IVF (Huneau *et al.* 1994, Li *et al.* 2006), as TALP medium alone does not stimulate protein tyrosine phosphorylation and cholesterol efflux in this species (Colas *et al.* 2008, Leahy & Gadella 2015, Leahy *et al.* 2016). Therefore, it is important to understand what factors are driving ram sperm association and disassociation, as it commonly occurs during ram sperm IVF. We have previously shown that the thiol D-penicillamine (PEN) is highly effective in preventing or reversing sperm association in TALP (Leahy *et al.* 2016). The effect of PEN was immediate, long-lasting and had no effect on total motility or the ability of spermatozoa to undergo capacitation. These characteristics make this compound well suited for *in vitro* use in sperm-handling protocols to counteract agglutination issues.

Penicillamine was named so because it is a degradation product of penicillin that contains an amine group (Fig. 1) (Abrahams *et al.* 1943). Penicillamine (PEN) also contains two other functional groups, a carboxyl and a sulphhydryl (Fig. 1). Although it possesses no antibacterial properties, these functional groups impart a range of biochemical behaviours that have been therapeutically exploited for more than 50 years to treat copper toxicities (Walshe 1956). The sulphhydryl group is of greatest biological significance, as it can combine with a multitude of compounds and act as a chelator or antioxidant as well as a participant in redox reactions as a reducing agent (Netter *et al.* 1987). In this study, we investigate how PEN perturbs sperm agglutination to shed some light on the factors involved in autologous sperm binding.

Materials and methods

Chemicals

Unless otherwise stated, products were sourced from Sigma-Aldrich and were of the highest reagent grade available. The base medium used was modified Tyrode's medium supplemented with albumin, lactate and pyruvate (TALP) (Parrish *et al.* 1988), which consisted of 2 mM CaCl_2 , 3.1 mM KCl, 0.4 mM MgCl_2 , 95 mM NaCl, 0.3 mM NaH_2PO_4 , 10 mM HEPES, 21.6 mM Na lactate, 5 mM glucose, 1 mM Na pyruvate, 25 mM NaHCO_3 , 0.03 mM phenol red and 3 mg/ml BSA (fatty acid free). The final pH and osmolarity of the various diluents was 7.4 and 300–310 mOsm/L respectively.

Sperm preparation

Ram semen was collected from two Merino rams and one Merino cross ram ($n=3$) using an artificial vagina with project approval from the University of Sydney's Animal Ethics Committee (Protocol No: 2013/5854). Two ejaculates were collected from each ram, three times a week. Only ejaculates that had a wave motion score of 4 (out of 5) or higher were used. Spermatozoa were diluted to 50×10^6 spermatozoa/mL in 1 mL aliquots and held for 3 h in an Eppendorf tube in a humidified incubator set at 38.5 °C in an atmosphere of 5% CO_2 .

Figure 1 Structure of D-penicillamine.

Experiment design

Each experiment was replicated six times using two ejaculates from three Merino rams. Motility and agglutination were assessed immediately following sperm dilution (0 h) and after 3 h of incubation. Except in Experiment 7, all compounds of interest were added to TALP at the desired concentration and then the sperm samples were diluted to 50×10^6 spermatozoa/mL.

Experiment 1. As PEN is an effective copper chelator, the influence of copper ions (Cu^{2+}) on sperm agglutination in TALP was assessed. A dose course of copper sulphate (0–500 μM) was added to TALP supplemented with 250 μM PEN.

Experiment 2. Experiment 2 assessed the specificity of the effect of copper on inducing sperm agglutination by comparing it to a range of other heavy metals. A standard dose of 10 μM of the following metals was added to TALP supplemented with 250 μM PEN: iron sulphate heptahydrate, zinc sulphate heptahydrate, manganese sulphate monohydrate, cobalt sulphate heptahydrate and copper sulphate.

Experiment 3. A Cu^{2+} -dependent lectin associated with agglutination of goat spermatozoa has been described (Roy *et al.* 2014). The lectin was sialic acid-specific and agglutinated cells could be dissociated with neutralised sialic acid (Roy *et al.* 2014). The aim of Experiment 3 was to assess the response of 0, 1, 10, 20 or 30 mM *N*-acetylneuraminic acid (sialic acid; Cayman Chemicals) on TALP-induced ram sperm agglutination.

Penicillamine (PEN) can act as a chelator, an antioxidant and a reducing agent. Experiments 4–6 were designed to compare the ability of PEN to reduce agglutination of ram spermatozoa in TALP with that of other chelators, antioxidants and reducing agents.

Experiment 4. Experiment 4 compared the effects of the broad-scale chelator, ethylenediaminetetraacetic acid disodium salt (EDTA) and the copper-specific chelator bathocuproinedisulfonic acid disodium salt (BCS) to PEN. To broaden the basis for comparisons between the effects of each substance studied, standardised low (0.1 mM) and high (1 mM) concentrations were tested.

Experiment 5. Experiment 5 compared the effects of four antioxidants to PEN (1 mM). L-ascorbic acid (VIT C; 1 mg/mL) was supplemented at the physiological concentration in ram semen (Mann 1964). Superoxide dismutase (SOD; 800 IU mL^{-1}), catalase (150 IU mL^{-1}) and α -tocopherol (VIT E; 1 mM; solubilised in ethanol with a final concentration of 0.5% (v/v)) were supplemented at levels that were previously shown to counteract sperm oxidative stress in vitro (Aitken & Clarkson 1988, Maxwell & Stojanov 1996, Leahy *et al.* 2010).

Experiment 6. Experiment 6 compared the reducing agents, reduced L-glutathione (GSH), L-cysteine (CYS) and DL-dithiothreitol (DTT) to PEN. To broaden the basis for comparisons between the effects of each substance studied, standardised low (0.1 mM) and high (1 mM) concentrations were tested.

Experiment 7. ADAM (A disintegrin and metalloproteinase) proteins are involved in cell adhesion, are abundant on the extracellular sperm membrane (Loechel & Wewer 2001, Byrne *et al.* 2012) and may be activated by copper (Loechel & Wewer 2001). The aim of Experiment 7 was to assess the effect of targeting either the “integrin” or the “metalloproteinase”

functions of ADAM proteins through the addition of a RGDS sequence blocking peptide (0.1 and 1 mM; In vitro technologies, Lane Cove West, NSW, Australia) or the ADAM metalloproteinase inhibitor tumour necrosis factor- α protease inhibitor-2 (TAPI-2, 25 μ M; Sapphire Bioscience, Waterloo, NSW, Australia) respectively. The dose of TAPI-2 (Tousseyn *et al.* 2009) and RGDS peptide (Mould *et al.* 1991) utilised were based on the studies on somatic cells that showed physiological responses in these concentration ranges. Only in Experiment 7, samples were pre-incubated with various treatments at 500×10^6 spermatozoa/mL for 15 min at 37 °C in PBS and then diluted to 50×10^6 spermatozoa/mL in TALP supplemented with the same treatments.

Sperm auto-agglutination assay

Spermatozoa were observed under light microscopy using phase-contrast optics (400 $\times$ magnification) with an Olympus BX51 microscope equipped with an Olympus DP73 digital camera. Four fields of view were recorded over 1 min. Samples were randomised and coded, so the assessor was blind to sample treatment. The videos were watched frame by frame using the Olympus cellSens imaging software. Spermatozoa (200 cells counted per sample) were assigned to one of four categories: motile and non-agglutinated, motile and agglutinated, non-motile and non-agglutinated, or non-motile and agglutinated.

Statistical analysis

Statistical analysis was performed using linear mixed model regression in GENSTAT (version 16; VSN International, Hemel Hempstead, UK). Interactions between treatment effects and time were assessed with ejaculate and ram incorporated into the blocking structure. Data are presented as the model-derived mean $\pm$ standard error of the mean (S.E.M.) of six replicates. Results are expressed as significant ($*P < 0.05$) and highly significant ($**P < 0.001$) compared with control samples.

Results

Low concentrations of cupric ions cause mass agglutination of motile spermatozoa

TALP was supplemented with 250 μ M PEN to produce a non-agglutinated, control population (Fig. 2A & Supplementary Video 1; control, representative sample at 0 h). Supplementation of 10 μ M (Fig. 2B & Supplementary Video 2; copper, representative sample at 0 h) and 50 μ M Cu^{2+} ions had no effect on total motility but caused an immediate switch from a non-agglutinated motile state to an agglutinated motile state (Fig. 3). Copper-induced sperm agglutination occurred in a head-to-head manner in the area overlying the acrosome (Fig. 2B & Supplementary Video 2, copper) and the association persisted throughout the 3-h test period. Copper was toxic from 100 μ M onwards and resulted in an increase in the percentage of non-agglutinated, non-motile spermatozoa

Figure 2 A representative image of ram spermatozoa in TALP + 250 μ M PEN supplemented with (B) or without (A) 10 μ M Cu^{2+} . Video files of these two sperm populations can be viewed in supplementary materials. Viewed under 400 $\times$ magnification at 0 h of incubation.

at 100 μ M and an increase in both agglutinated and non-agglutinated, non-motile spermatozoa at 250 μ M (Fig. 3).

Compared with other heavy metals, copper is a significantly stronger agglutination agent

Copper caused significantly higher levels of sperm agglutination than the other heavy metals tested (Fig. 4). Copper-induced sperm agglutination was immediate, specific and broad scale. In the presence of copper, less than 1% of the sperm population were motile and non-agglutinated at both 0 and 3 h (Fig. 4). This effect was not evident in the other heavy metals tested. Zinc and manganese had no effect on sperm agglutination, and cobalt and iron only showed a moderate decrease in the percentage of non-agglutinated, motile spermatozoa at 3 h. None of the heavy metals tested had an effect on total motility at the dose tested (10 μ M).

Figure 3 Dose response (0–250 μ M) of Cu^{2+} ions on ram sperm agglutination in TALP supplemented with 250 μ M PEN. Copper caused mass agglutination of motile spermatozoa (open bars) at the lowest dose tested (10 μ M), which was accompanied by a reduction in the percentage of non-agglutinated, motile spermatozoa (black bars). Toxic effects are evident from 100 μ M onwards with an increase in the percentage of non-agglutinated, non-motile spermatozoa (hatched bars) and an increase in non-motile, agglutinated spermatozoa (grey bars) at the highest dose tested. Data correspond to model derived mean $\pm$ S.E.M. for six replicate samples assessed at 0 and 3 h and pooled over time. $*P < 0.05$ and $**P < 0.001$ differences compared with control (0 μ M).

Figure 4 The influence of heavy metals on ram sperm agglutination. The effect of 10 μ M of the following metal ions was tested: cobalt (Co^{2+}), copper (Cu^{2+}), iron (Fe^{2+}), manganese (Mn^{2+}) and zinc (Zn^{2+}). Agglutination was assessed at 0 and 3 h and the data for both time points are shown (double columns within treatment). Data correspond to model derived mean $\pm$ s.e.m. for six independent samples. * $P < 0.05$ and ** $P < 0.001$ different compared with corresponding hour of control sample (TALP + 250 μ M PEN).

Sialic acid has no effect on sperm agglutination

The supplementation of neutralised sialic acid to TALP had no effect on sperm motility or agglutination. The percentage of motile, non-agglutinated spermatozoa supplemented with 0 ($3.2 \pm 1.1\%$), 1 ($3.0 \pm 1.3\%$), 10 ($3.5 \pm 0.9\%$), 20 ($3.8 \pm 0.9\%$) or 30 ($3.8 \pm 1.0\%$) mM sialic acid was low and statistically similar. This suggests that the mechanism of sperm binding described herein does not involve a sialic acid-specific lectin as shown in goat spermatozoa (Roy *et al.* 2014).

Chelation of extracellular free cupric or cuprous ions is unlikely to be the sole mechanism by which PEN prevents sperm association

In comparison to the control (TALP only), both 1 mM EDTA and 1 mM BCS significantly increased the percentage of non-agglutinated, motile spermatozoa; however, this was only by a moderate amount compared with both 0.1 and 1 mM PEN (Fig. 5A). This indicates that chelation is unlikely to be the sole mechanism by which PEN prevents sperm association.

Protection from oxidative stress is unlikely to be the sole mechanism by which PEN prevents sperm association

Catalase (CAT) and α -tocopherol (VIT E) had no effect (Fig. 5B) on sperm agglutination compared with the control (TALP). L-ascorbic acid (VIT C; 3 h only) and superoxide dismutase (SOD; 0 and 3 h) significantly increased the percentage of non-agglutinated, motile spermatozoa compared with the control, but only by a moderate amount in comparison to 1 mM PEN (Fig. 5B). This indicates that protection from oxidative damage is unlikely to be the sole mechanism by which PEN prevents sperm association.

Figure 5 The influence of chelators, antioxidants and reductants on sperm agglutination in TALP. PEN has previously been shown to prevent or reverse agglutination of ram spermatozoa in TALP, but its mechanism of action is unknown. As D-penicillamine (PEN) can act as a chelator, an antioxidant or a reducing agent, the ability of PEN to prevent ram sperm agglutination in TALP was compared with these compounds. Agglutination was assessed at 0 and 3 h and data for both time points are shown (double columns within treatment). The control sample contains TALP medium alone. (A) shows the effects of the copper-specific chelator bathocuproinedisulfonic acid disodium salt (BCS; 0.1, 1 mM), the broad-scale chelator, ethylenediaminetetraacetic acid disodium salt (EDTA; 0.1 and 1 mM) and PEN (0.1, 1 mM). (B) shows the effects of five antioxidants: L-ascorbic acid (VIT C; 1 mg/mL), catalase (CAT; 150 IU/mL), superoxide dismutase (SOD; 800 IU/mL), DL- α -tocopherol (VIT E; 1 mM) and 1 mM PEN. (C) shows the effects of the reducing agents L-cysteine (0.1, 1 mM), DL-dithiothreitol (DTT; 0.1, 1 mM), reduced L-glutathione (GSH; 0.1, 1 mM) and PEN (0.1, 1 mM). Data correspond to model derived means $\pm$ s.e.m. for six replicate samples. * $P < 0.05$ and ** $P < 0.001$ different compared with corresponding hour of control sample (TALP).

The reducing action of PEN is the most likely mechanism by which PEN prevents sperm association

The reductants showed the strongest anti-agglutination effect of all the compounds tested. Supplementation of

Figure 6 Effect of the ADAM metalloproteinase inhibitor tumour necrosis factor- α protease inhibitor-2 (TAPI-2, 25 μ M) and the RGD sequence integrin blocking peptide (0.1 and 1 mM) on sperm agglutination in TALP. Data correspond to model derived mean $\pm$ S.E.M. for six independent samples. Samples were assessed at 0 and 3 h and pooled across time period. * $P < 0.05$ and ** $P < 0.001$ differences compared with control.

1 mM cysteine (CYS) and DL-dithiothreitol (DTT) at 0 h caused a highly significant increase in the percentage of motile, non-agglutinated spermatozoa, which was equivalent to 1 mM PEN (Fig. 5C). However, at the lower dose of 0.1 mM, the most effective reducing agent was still PEN.

Some of the reducing agents showed a striking drop-off in effectiveness from 0 to 3 h. For example, free-swimming spermatozoa decreased by 72% from 0 to 3 h in 0.1 mM PEN, whereas 1 mM PEN did not change over time. It is important to note here that the large decrease in free-swimming spermatozoa seen in 1 mM DTT at 3 h was not associated with an increase in agglutinated, motile spermatozoa, but a result of sperm toxicity evidenced by an increase in non-motile, non-agglutinated spermatozoa. With the exception of this DTT dose at this time point, no other compound decreased total sperm motility, and all other increases in non-agglutinated, motile spermatozoa were accompanied by decreases in agglutinated, motile spermatozoa.

Inhibition of the metalloproteinase domain of ADAM proteins significantly increases the percentage of non-agglutinated, motile spermatozoa

Targeted inhibition of the metalloproteinase domain with tumour necrosis factor- α protease inhibitor-2 (TAPI-2, 25 μ M) significantly increased (Fig. 6) the percentage of motile, non-agglutinated spermatozoa compared with TALP alone (control). Pre-incubation with either 0.1 or 1 mM RGD peptide to block RGD-binding sequences of the disintegrin domain of ADAM proteins had no effect on sperm agglutination (Fig. 6).

Discussion

In this study, we show that low concentrations of Cu^{2+} ions cause mass agglutination of ram spermatozoa

(Figs 2, 3, 4 and Supplementary Videos 1 and 2). In the presence of 10 μ M Cu^{2+} , more than 90% of the motile sperm population was agglutinated (Figs 3 and 4). Copper-mediated sperm agglutination was similar to TALP or sera-induced sperm agglutination (Senger & Saacke 1976, Aalseth *et al.* 1978, Leahy *et al.* 2016) in that the association was immediate, persistent and occurred in an area overlying the acrosome and did not influence total motility. Plasma and follicular fluid contain relatively high levels of copper (14.16–18.25 μ M) (Ng *et al.* 1987, Twomey *et al.* 2005, Picco *et al.* 2012). This may explain why the inclusion of sera in ovine *in vitro* fertilisation protocols causes similar agglutination to that induced by cupric ions and why PEN can also prevent sera-induced agglutination of ram sperm in IVF media (Leahy *et al.* 2016). It can be assumed that the agglutination of ram spermatozoa requires only trace amounts of copper as mass agglutination occurred when 10 μ M Cu^{2+} was supplemented to TALP containing a 25-fold higher dose of the copper chelator, PEN.

Ram spermatozoa have been previously noted to be highly reactive to copper. The motility of ram spermatozoa was significantly decreased by 20 μ M Cu^{2+} , whereas a 100-fold higher dose (2 mM) was required to achieve a similar effect in both bull and human spermatozoa (White 1955). In this study, co-supplementation with 250 μ M PEN provided some protection from excess free copper ions as copper toxicity was not detected until the level of supplementation reached 100 μ M Cu^{2+} (Fig. 3). Interestingly, the aforementioned study (White 1955) also reported that copper caused head-to-head agglutination of ram spermatozoa, and this effect was not noted in any of the other species tested.

The influence of copper on sperm agglutination was shown to be highly specific as supplementation with a range of other heavy metals did not cause mass agglutination (Fig. 3). It is intriguing that ram spermatozoa are so susceptible to exogenous copper because they have higher sperm-bound copper levels (estimated to be 18.9 μ M compared with 0.94–3.78 μ M for humans) and higher seminal plasma copper levels than a range of species including the bull, boar, stallion and human (Mann 1964, Massanyi *et al.* 2003).

A copper-dependent lectin that causes sperm agglutination has been observed on the outer membrane of mature caprine spermatozoa (Roy *et al.* 2014). This lectin was sialic acid specific, and agglutinated cells could be dissociated with neutralised sialic acid. In this study, supplementation of neutralised sialic acid to TALP had no effect on sperm motility or agglutination, suggesting that the mechanism of sperm binding described herein does not involve a sialic acid-specific lectin as shown in goat spermatozoa.

We have previously shown that the thiol D-penicillamine (PEN) is highly effective in preventing or reversing sperm association in TALP (Leahy *et al.* 2016). PEN can act as a reducing agent, chelator or antioxidant;

however, it is unclear how PEN reduced agglutination. Cell aggregation has previously been linked to oxidative damage. Copper-induced lipid peroxidation has been implicated in platelet cell aggregation (Gorog & Kovacs 1995, Neiva *et al.* 1997) and protein aggregation in Alzheimer's and prion disease (Moir *et al.* 1999, Viles 2012). Copper catalyses the generation of reactive oxygen species (ROS), which attacks polyunsaturated fatty acids (PUFAs) of the sperm membrane and causes lipid peroxidation, sperm membrane destabilisation and cell death (Aitken *et al.* 1989). PEN can chelate transition metals that catalyse lipid peroxidation (Aitken *et al.* 2012a,b) or directly neutralise ROS (Gupte & Mumper 2007), limiting their ability to interfere with PUFAs of the sperm membrane. However, the range of antioxidants tested in this study showed no or minimal ability to prevent the agglutination of ram spermatozoa (Fig. 5B). L-ascorbic acid and superoxide dismutase (SOD) significantly increased the percentage of non-agglutinated, motile spermatozoa compared with the control but only by a moderate amount in comparison to PEN. Since L-ascorbic acid is a known reducing agent and SOD can chelate copper (Nordberg & Arnér 2001, Ottaviano *et al.* 2008), it is possible that these secondary functions, as a reductant or chelator, contributed to this response (Fig. 5B). The variable results obtained with the antioxidants tested indicate that protection from oxidative stress is unlikely to be the sole mechanism by which PEN prevents sperm association.

As excess free copper ions cause agglutination, and the well-known copper chelator PEN prevents agglutination, it would follow that chelation of copper is a likely mechanism by which PEN prevents sperm association. However, other chelators, such as EDTA and BCS, that have a high affinity for copper were not highly effective in preventing sperm agglutination (Fig. 5A). EDTA is a membrane-permeable chelator that binds Cu^{2+} with a high copper stability constant (18.8) due to its six chelate rings (Ding *et al.* 2011). BCS is an impermeable chelator with a copper stability constant of 6.1, which selectively chelates Cu^+ but will also bind Cu^{2+} (Patel *et al.* 1997). PEN has a similar copper stability constant (7.1) to BCS but is membrane permeable and can reduce Cu^{2+} to Cu^+ and bind to either of these copper forms (Smith *et al.* 1994, Ding *et al.* 2011). Interestingly, despite displaying higher or similar affinity for copper as PEN, both BCS and EDTA showed minimal effects on sperm agglutination. Multiple studies have shown that PEN has lower affinity for copper *in vitro* than a range of other common chelators (e.g. EDTA and cysteine) but significantly higher copper affinity *in vivo* (Tran-Ho *et al.* 1997, Ding *et al.* 2011). For this reason, PEN remains the chelator of choice for the treatment of patients with copper accumulation due to Wilson's disease or other heavy metal intoxication (Walshe 2011). The superiority of PEN over EDTA *in vivo* implies that copper chelation

therapy is unlikely to depend on complexation alone (Tran-Ho *et al.* 1997). The reasons why PEN is superior to EDTA for *in vivo* copper chelation therapy have been the focus of immense interest and are proposed to rely on two factors (1) that PEN can liberate Cu^{2+} from metalloproteins by reduction to Cu^+ (May & Williams 1977) and (2) that PEN can form stable complexes with multiple ligands (Cu^{2+} , Cu^+ and other thiols) and produce a stable anionic multivalent copper cluster $(\text{Cu}_6^{2+}\text{Cu}_8^+\text{PEN}_{12}\text{Cl})^{5-}$ (Birker & Freeman 1977, Kato *et al.* 1999). EDTA can prevent copper-dependent biological events when it is added to media containing free copper but cannot despoil copper from previously formed copper—protein complexes (Jimenez & Speisky 2000). Therefore, it is likely that it is not free Cu^{2+} or Cu^+ ions in the TALP medium that caused sperm agglutination, as the chelators EDTA or BCS were added to the diluent before sperm extension. Rather it is copper that is bound to sperm proteins that is being modified by the extension of spermatozoa in TALP, as EDTA would not be able to chelate copper in this form (Jimenez & Speisky 2000).

Of all the compounds tested, only the thiols (proteins with a sulphhydryl bond), which act as reducing agents, showed strong anti-agglutination effects (Fig. 5C). This suggests that the mechanism of action of PEN is conferred by its ability to donate an electron to another molecule and act as a reductant in redox reactions (Fig. 7). Cysteine and DTT (1 mM) displayed similar levels of non-agglutinated spermatozoa at 0 h compared with 1 mM PEN but were less effective after 3 h. At the lower dose of 0.1 mM, PEN was also the most effective reducing agent. The analysis of the mechanisms underlying the oxidation of thiols explains why PEN may be more effective in initiating sperm disassociation and maintaining spermatozoa in a disassociated state. PEN has been proposed to undergo a higher rate of oxidation under physiological conditions (e.g. pH 7) compared with other thiols (e.g. cysteine 8.3 and glutathione 8.8) because the pKa of its SH-functional groups (7.9) is lower (Netter *et al.* 1987), making it more effective at lower doses. How PEN maintains the sperm population in a non-agglutinated form by keeping the copper complex in a reduced state is more complex. Smith *et al.* (1994) outlined the following reaction sequence for the oxidation of thiols by copper. Cupric ions react with RSH (where R denotes the R group of the thiol and SH denotes the sulphhydryl bond of the thiol) to form a Cu^{2+} —RSH complex in which Cu^{2+} is reduced to Cu^+ and RSH is oxidised ($\text{RS}\bullet$) (Reaction 1). Two of the radicals ($\text{RS}\bullet$) react to form a disulphide (Reaction 2) and Cu^+ reacts with a second thiol to form a Cu^+ thiol complex (Reaction 3). The rate-limiting step of the process is considered (Smith *et al.* 1994) to rely on the reoxidation of the RSCu^+ complex (Reaction 4), as it results in the release of Cu^{2+} where it can participate in further redox cycling (Reaction 1).

Figure 7 Proposed model of ram sperm agglutination. The data presented in this article suggest a model by which PEN (denoted as PSH to show sulphhydryl bond) prevents or reverts sperm association by reducing the disulphide bonds (S–S) of a sperm protein to free sulphhydryls (SH). Copper (Cu^{2+}) causes the opposite effect by oxidising free sulphhydryl bonds to disulphide bonds.

The rate of reoxidation of the RSCu^+ complex is dependent on the R-group of the thiol (Smith *et al.* 1994). Reoxidation of Cu^+ (Reaction 4) in a PEN complex is considered to occur at a slower rate, compared with other thiols, as the Cu^+ is partially protected from further oxidative attack by steric shielding from the two methyl groups (CH_3 ; Fig. 1) of the PEN side chain (Birker & Freeman 1977).

If sperm disassociation is occurring due to the reduction of copper bound to a sperm protein, or the reduction of a disulphide bond to free sulphhydryl groups in a sperm protein, then the re-association of spermatozoa is due to the re-oxidation of these proteins over time in the highly oxidising extracellular environment. Such a response is clearly evident when PEN is provided at a rate-limiting dose (0.1 mM) but not when excess PEN is present at the higher dose of 1 mM (Fig. 5). Reversible thiol–disulphide redox reactions are a functional switch for various important fertilisation-related signalling events. The oxidation of thiols to form disulphide bridges and the reduction of disulphide bridges to free sulphhydryls on the sperm surface have been shown to regulate capacitation-related sperm proteins (O'Flaherty 2015). PEN and other thiols have also been shown to initiate sperm release through the reduction of sperm surface disulphides to sulphhydryls (Talevi *et al.* 2007, Gualtieri *et al.* 2009).

A disintegrin and metalloproteinase (ADAM) proteins are good candidates for involvement in autologous sperm agglutination because they bind metals, are involved in cell adhesion (Seals & Courtneidge 2003, White 2003, Weber & Saftig 2012), contain a

cysteine-rich domain and are abundant on the extracellular surface of the sperm membrane (Byrne *et al.* 2012). In this study, targeted inhibition of the ADAM metalloprotease domain significantly increased (Fig. 6) the percentage of motile, non-agglutinated spermatozoa ($52.0\% \pm 7.8$) compared with TALP ($10.6\% \pm 6.1$), suggesting that this domain may be involved in autologous sperm binding.

ADAMs are zinc-binding proteins; however, there are known examples that are activated by copper as metal binding sites of proteins are likely sites for oxygen-based reactions (Wilcox *et al.* 2001). For example, copper oxidises the zinc binding Cys residue of the HIV retrovirus nucleocapsid p7 protein (Yu *et al.* 1995). Oxidation of the Cys thiolate ligand does not affect binding of the zinc to the metalloprotease domain but results in three specific disulphide bonds among the six cysteines (Yu *et al.* 1995). Copper oxidation of one of the amino acid residues in the zinc binding site has also been shown to change the conformation of ADAM12-s to its mature, active form (Loechel & Wewer 2001). The ADAM metalloprotease domain participates in ectodomain shedding of multiple substrates including amyloid precursor protein, prion protein, heparin binding epidermal growth factor, tumour necrosis factor, L-selectin and various interleukins (Seals & Courtneidge 2003, White 2003, Weber & Saftig 2012). ADAMs cleave and remodel extracellular matrix proteins through the proteolytic release of these signalling factors (White 2003). Interestingly, many of these substrates have been shown to interact with copper (Hesse *et al.* 1994, Moir *et al.* 1999, Viles 2012, McDonald *et al.* 2014), and physiological concentrations of copper have been shown to stimulate zinc metalloproteinase ectodomain shedding of cell surface proteins. For example, copper-induced zinc metalloproteinase shedding of two key signalling and adhesion proteins, Jagged 1 and E-cadherin, was shown to be post-translationally modulated and enhanced the invasion of a PC3 cancer cell line (Parr-Sturgess *et al.* 2012).

However, targeting of the integrin domain by pre-incubation with either 0.1 or 1 mM of the RGD blocking peptide had no effect on sperm agglutination (Fig. 6). Although this suggests that sperm agglutination is not dependent on RGD, it does not rule out ADAM-mediated cell adhesion. Only a subset of ADAM proteins, such as ADAM15, display the integrin binding motif Arg-Gly-Asp (RGD) in their disintegrin domain with the majority displaying an XCD (where X is an amino acid) sequence (Lu *et al.* 2010). Cell adhesion is also not restricted to the disintegrin domain of ADAM proteins. The cysteine-rich domain of ADAM proteins can bind directly to cell surface syndecans (ADAM12) (Iba *et al.* 2000) and the Von Willebrand factor (ADAM13) (de Groot *et al.* 2015). The mechanism by which ADAM proteins may mediate autologous sperm binding remains far from

certain. Ongoing investigation on the influence of ADAM proteins in other fertilisation-related events, such as sperm-zona pellucida binding, will shed further light on the role of these multifunctional proteins in cell association and signalling within the reproductive tract (Dun *et al.* 2012, Okabe 2015).

Conclusion

In this study, we show that trace levels of copper ions cause mass agglutination of ram spermatozoa in a manner similar to that of TALP-induced sperm agglutination. The effect is highly specific as a similar response was not provoked by other heavy metals. The nucleophilic thiol, penicillamine, can act as an antioxidant, a reducing agent or a chelator and has previously been shown to prevent or reverse ram sperm agglutination. Comparison of PEN to compounds in these classes showed that only the reducing agents (particularly cysteine and DTT) displayed similar levels of non-agglutinated spermatozoa as PEN. This suggests that the mechanism of action of PEN is conferred by its ability to donate an electron to another molecule and act as a reductant in redox reactions. Together, these results indicate that PEN prevents sperm association by the reduction of disulphide bonds of a sperm membrane protein that binds copper. ADAM proteins are possible candidates, as targeted inhibition of the metalloproteinase domain of these proteins reduces agglutination; however, further research is required to elucidate how ADAM proteins mediate ram sperm agglutination.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by funding from the NSW Stud Merino Breeders Association Trust and Australian Wool Innovation Limited.

Acknowledgements

The authors are grateful for the technical assistance of K. Heasman.

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Received 24 December 2015

First decision 11 January 2015

Revised manuscript received 21 January 2016

Accepted 8 February 2016