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Immunolocalization and the Effect of Cryopreservation on M1 Antigen of Ram Spermatozoa

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M1 antigen is a protein located at the equatorial segment (ES) of hamster sperm, involved in hamster sperm-egg fusion during fertilization. Recently the antigen were detected on ram sperm specifically located at the anterior acrosome. In this research work, further immunolocalization and the effect of cryopreservation procedure on the M1 antigen were investigated. BWW medium and Tyrode's medium were used respectively to induce capacitation and acrosome reaction of a ram sperm. Indirect immunofluorescent (IIF) and Giemsa staining were performed in parallel to analyze the status of M1 antigen as well as the acrosomal cap of the sperm. The results confirmed our previous finding that the M1 antigen was present on fresh ram sperm. Interestingly two types of pattern were displayed, type I ; on the whole anterior acrosome, type II; on marginal part of anterior acrosome. The pattern is related to the status of acrosomal cap of the sperm. Different medium exhibited different percentages of type I and type II staining pattern of M1 antigen. In contrast M1 antigen was failed to detect on frozen-thawed sperm. Observation on giemsa staining sperm, revealed the status of M1 antigen was dependent on the status of the acrosome cap. The localization of M1 antigen on ram sperm head suggested that the antigen involved during sperm-zona interaction at fertilization.

Key words: Ram sperm, M1 antigen, cryopreservation, Immunolocalization

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Introduction

Fertilization is achieved following the successful completion of a complex sequence of events by a spermatozoan and an egg. Although these gamete interactions have been studied extensively, the molecular details of these events, particularly those concerning the sperm cell, have remained exclusive. This has been due to the lack of probes specific for individual sperm components. But with the hybridoma technology, monoclonal antibody can be generated to identified the specific sperm protein. Monoclonal antibodies (mab) are proven to be useful in delineating the sperm antigens involved in the regulation of fertility. For example several sperm fusion proteins such as fertilin on guinea pig sperm was identified by PH30 mab (Primakoff *et al.*, 1987) equatorin on mice sperm identified by MN9 mab (Toshimori *et al.*, 1998) DE protein on rat sperm (Rochwerger *et al.*, 1992) and M1 protein was also identified by mab (Mat Noor and Moore, 1995).

Such antibodies which have been used intensively to characterize the sperm protein of specific animal species were also used as a probe to identify the protein on other animal species in case of cross-reactivity. Saling *et al.*, 1985 for example had developed several mab (against mice) which demonstrated cross-reactivity with sperm from the other species tested (human, hamster, rabbit). Like M29 mab in mice (Saling *et al.*, 1985), M1 mab was used to screen several samples of sperm from different animal species (rat, mice, rabbit, human guinea-pig and ram). None of them except guinea-pig and ram sperm revealed the presence of M1 antigen on sperm head.

Cryopreservation of sperm, at which sperm might be preserved more or less indefinitely has opened a new era in the breeding of livestock, the treatment of human infertility and preservation of endangered species (that is, through sperm banks). For human sperm this technique is a complimentary to artificial reproductive technology (ART). Improvements in cryopreservation protocol have resulted from an empirical approach of slowly modifying an existing method (Weidel and Prins, 1987) However, despite many years of research, the fertility rates using frozen-thawed sperm remain consistently lower than those obtained using fresh sperm (Bordson *et al.*, 1986). This may due to the acrosomal swelling whereby frequently the acrosomal membrane and some of the contents were lost. There is also a evidence that the corresponding freezing and thawing rates during cryopreservation protocol are linked and have reciprocal effect on motility, membrane integrity and mitochondrial function (Mazur, 1984).

As reported earlier cryopreservation affects sperm membrane integrity (Bwanga, 1991 and Watson, 1995). Eventhough the sperm motility assesment is one of the best indicators of fertility and can be quantified reliably (Holt *et al.*, 1989) the status of certain fertilization molecules (especially the one associated/integrated with sperm membrane) upon cryopreservation procedure never been reported. Therefore, this work was an attempt to determine the effect of cryopreservation on M1 ram sperm protein (which associated in acrosomal membrane) through immunolocalization studies.

Materials and Methods

Semen Collection and Handling: All laboratory works were conducted at Universiti Kebangsaan Malaysia during, June, 1999 - June, 2000. Fresh ram semen were collected from MARDI Research Station at Serdang, Selangor, Malaysia while the cryopreserved semen were obtained from MARDI and Institute Bioteknologi Haiwan Kebangsaan, Jerantut, Pahang, Malaysia. Fresh semen were collected using an artificial vaginal and the samples were induced in Tyrode's and BWW medium at 37°C and 5% CO_2 for three hours respectively. This step is important to induce the capacitation and acrosome reaction of the sperm. Before inducing capacitation and acrosome reaction, frozen sperm were aliquot into two samples: Sample 1 was washed with Percoll to eliminate any cryopreservative agent and sample 2 without Percoll washing step. All samples were subjected to the IIF procedure and Giemsa staining technique:

Indirect Immunofluorescent Procedure (IIF): After inducing the sperm in two different media Tyrode's and BWW the IIF staining procedure were undertaken following the methods as described by Ellis *et al.* (1985). Briefly 20µl of induced sperm were smeared on the glass slides and fixed with 100% methanol. The slides were then incubated with primary antibody (M1 mab). The incubation were carried out in the incubator at 37°C and 5% CO_2 for 1hr. The slides were washed three times in phosphate buffer saline (PBS) followed by second incubation with secondary antibody (antimouse-FITC). After one hour incubation, the slides were washed twice in PBS and were mounted with 100% glycerol. Viewing were carried out using fluorescent microscope to detect the localization of M1 antigen on ram sperm.

Giemsa Staining: Giemsa staining on methanol fixed sperm were undertaken following the standard method of Casarett (1953). Briefly, sperm slides were smeared with 500µl giemsa and incubated for 90 min at room temperatures. Then washed with distilled water before mounted with DPX and viewed under light microscope.

Statistical Analysis: Statistical analyzes were performed by categorical data analyses (the SAS system) to compare the effect of BWW and Tyrode's medium to staining pattern and Chi-Square (SPSS system) were used to test the statistical significant.

Results

Immunolocalization of M1 protein: The investigation focused on the immunolocalization of M1 protein on fresh and frozen-thawed ram sperm. According to IIF staining result, M1 antigen was detected only on the fresh ram sperm but not on frozen-thawed sperm. This result, confirmed that M1 antigen also present on the ram sperm but located at the acrosome cap (anterior acrosome) while in hamster sperm, it is located on the equatorial segment (posterior acrosome). Interestingly, M1 antigen on fresh ram sperm showed two types of staining pattern depending on which medium were used for incubation. The two types of staining pattern showed as fluorescent staining on whole anterior region (Type I) and fluorescent

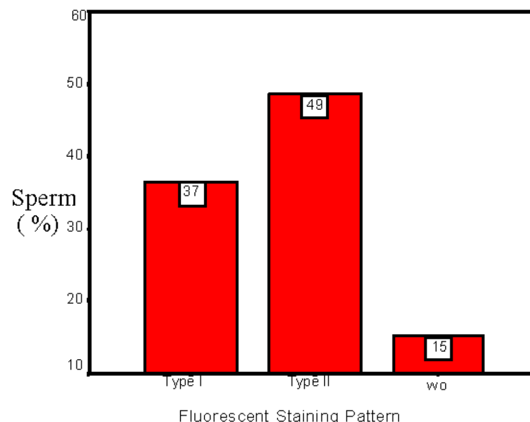

Fig. 1: Percentage (%) of sperm with different staining patters

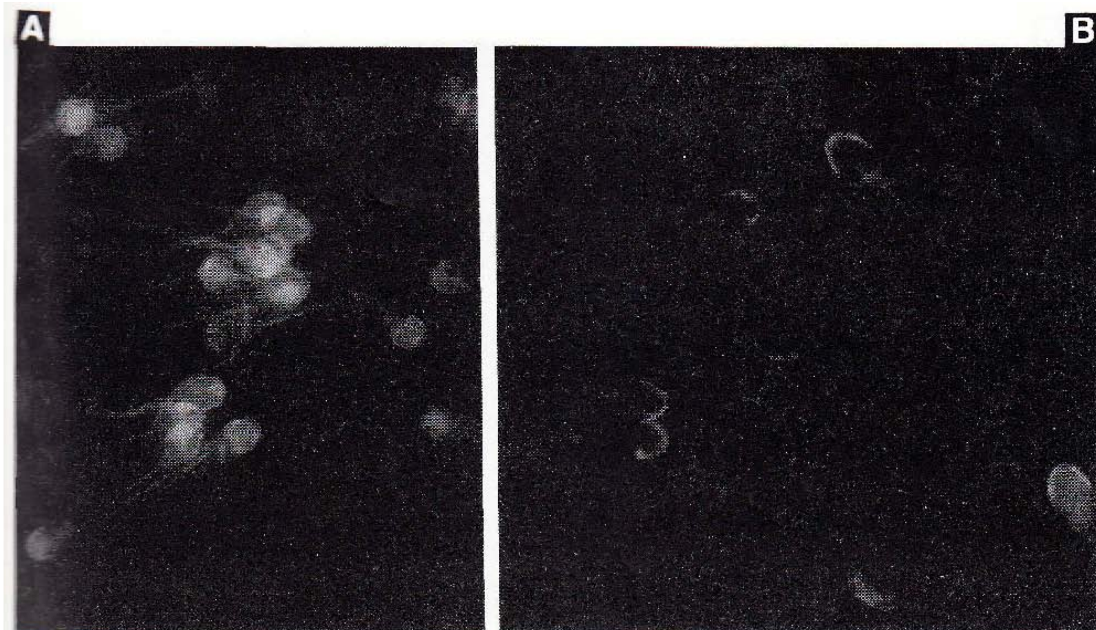

Fig. 2: Fresh ram sperm under epifluorescent microscope. Incubation in (a) BWW medium, fluorescent staining exhibited M1 protein located at anterior acrosome (x400) (b) Tyrode's medium, M1 protein restricted on anterior margin of anterior acrosome (x1000).

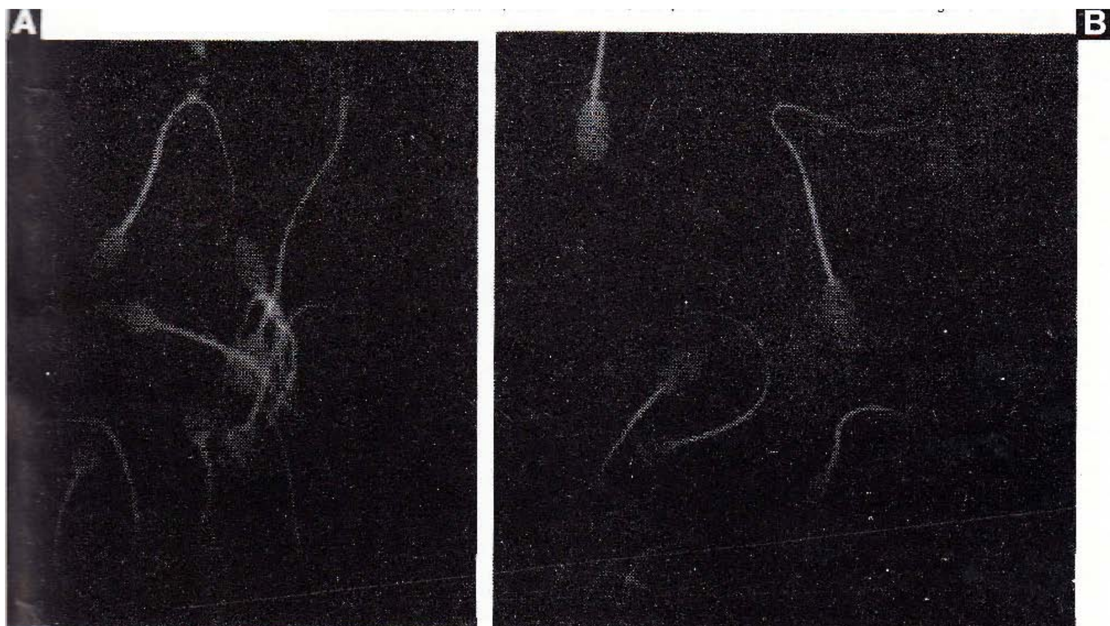

Fig. 3: Frozen-thawed ram sperm (percoll treatment) under epifluorescent microscope. Incubation in (a) BWW medium (b) Tyrode's medium. None of the sperm displayed the presentation of M1 protein (x1000).

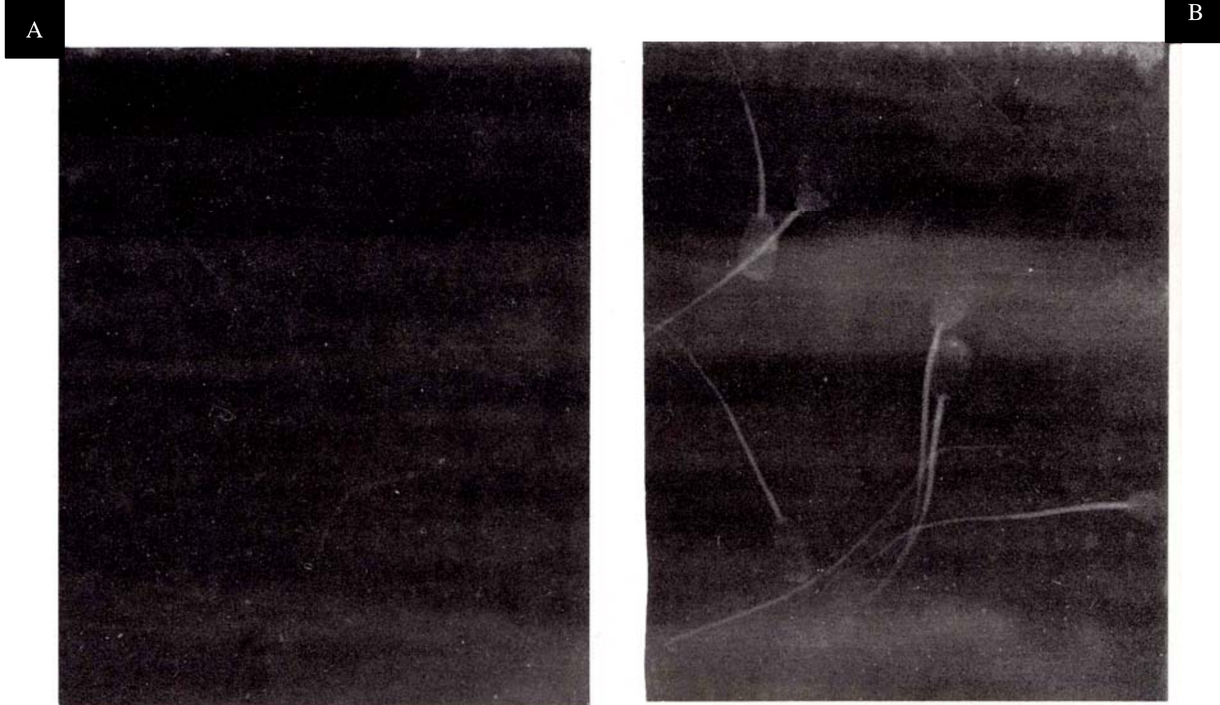

Fig. 4: Frozen-thawed ram sperm (without percoll treatment) under epifluorescent microscope. Incubation in (a) BWW medium (b) Tyrode's medium. None of the sperm displayed the presentation of M1 protein (x1000).

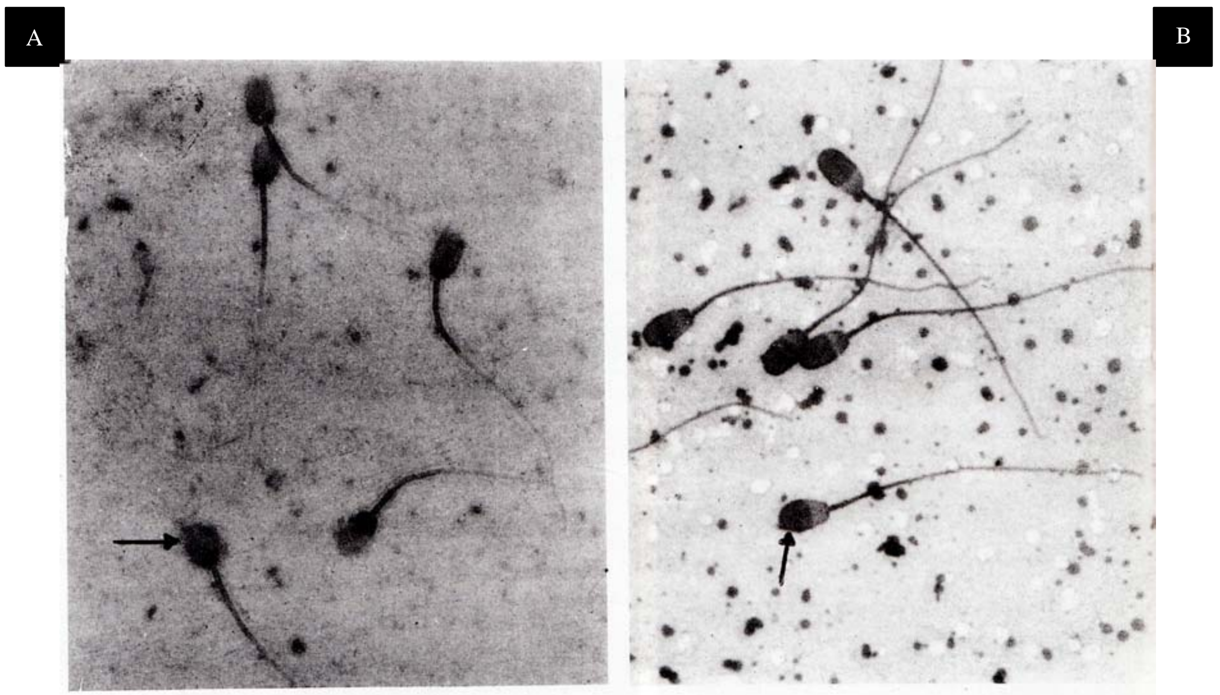

Fig. 5: Fresh ram sperm under light microscope. Acrosome intact sperm in (a) BWW medium, and acrosome reacted sperm in (b) Tyrode's medium (x400).

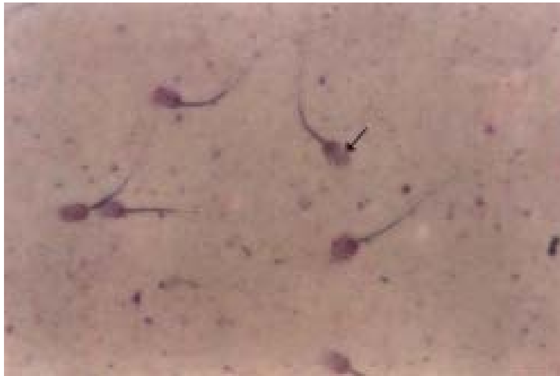

Fig. 6: Typical of frozen-thawed ram sperm (under light microscope) with free acrosome cap after 3 hours in incubation medium (x 400).

staining restricted on anterior margins of acrosome (Type II) (Fig. 2). Fresh sperm which induced in BWW medium showed higher percentage in Type I staining compared to Type II with 61 and 26% respectively. Whereas, the fresh sperm induced with Tyrode's medium showed higher percentage in Type II pattern compared to Type I with 71 and 12% respectively (Table 1). In contrast frozen-thawed samples (treated with or without Percoll) (Figs. 3 and 4) which undergone the same procedure showed negative results, M1 antigen unsuccessfully detected on any region of frozen-thawed ram sperm. Statistical analyses also proved that there is a significant association between medium and florescent staining on fresh ram sperm ($P< 0.001$). Regardless of medium, there is a strong evidence that the highest percentage of sperm (49%) showed type II staining pattern (Fig. 1).

Table 1: Percentage of fluorescent staining, type I and type II on fresh ram sperm

Medium	Fluorescent Staining Acrosome (Type-I) %	Anterior margin acrosome (Type-II) %	Without fluorescent Staining(WO)%
BWW	61	26	13
Tyrode's	12	71	17

Detection of Acrosome Reaction: Giemsa staining showed that fresh sperm incubated in BWW medium for 3hr. were remained intact while sperm in Tyrode's medium were acrosome reacted (Fig. 5). Surprisingly the frozen-thawed samples were acrosome free (> 95%) after incubation for 3hr. in both medium respectively. The result suggested that cryopreservation procedure seriously affect the sperm acrosome membrane (Fig. 6).

Discussion

Like many cells ultimately involved in recognition, binding and signaling events, the mammalian sperm is a highly differentiated cell, exhibiting a high degree of molecular mosaicism on its surface. The heterogenous nature of sperm surface has been shown by the studies of surface charge, lectin binding to specific sugar moieties, freeze-fracture patterns and antibody labeling. Based on IIF staining, MI antigen was detected on the ram sperm but localized on different region of sperm head, therefore, the M1 mab was not only cross react with sperm of other animal species, but also distributed at different part of sperm acrosome. On ram sperm, M1 protein localized at acrosomal cap (anterior acrosome) while in hamster sperm it was on the equatorial segment (posterior acrosome). This result represents the mosaicism of sperm surface membrane.

It is not uncommon when one mab can recognized more than one sperm antigen from other animal species. Mouse M29 mab for example showed cross-reactivity with sperm antigen of human, rabbit and hamster. This is not surprise, eventhough mabs are often specific to particular molecules, they may also recognize epitopes shared by many molecules (Brown *et al.*, 1983). Furthermore, even mabs that label only one domain on the sperm surface it may immunoprecipitate proteins more than one molecular weight and this was happened to M1 mab on hamster sperm (Mat Noor and Moore, 1999). In some cases the proteins are subunits of a molecular complex that co-precipitate under such conditions, but in other cases they might be functionally unrelated molecules that share a common epitope recognized by the antibody. This results, based on M1 localization on sperm head region, we believed the ram M1 antigen has different function from M1 antigen in hamster sperm, perhaps it involves in sperm-zona interaction rather than sperm-egg fusion. Exactly the function of M1 protein in ram sperm remains to be determined.

We also found the presentation of M1 antigen on ram sperm are not acrosome reaction dependent as compared to the M1 antigen on hamster sperm. M1 antigen on hamster sperm can only be detected after thesperm had undergone the acrosome reaction (Mat Noor and Moore, 1999). On ram sperm, it was believed the M1 antigen was already exist on acrosome cap but when and how it's become activated remained unknown. Since M1 antigen appear and can be detected before the acrosome reaction occur, we believed this provide a tool to evaluate the acrosomal status of ram sperm as the 4D4 antibody to proacrosin of human sperm (Gallo *et al.*, 1991). The monoclonal antibodies to acrosomal internal antigens have been suggested as a good probe for the evaluation of the acrosomal status. This can be useful on the diagnosis of infertility since some cases of males infertility are due to impaired acrosome reaction (Topfer-Peterson *et al.*, 1987). Another application is the screening of sperm samples to be used *in vitro* fertilization (Kallajoki *et al.*, 1986). The validity of the quantitative evaluation of the acrosomal status with such antibodies has been demonstrated (Byrd *et al.*, 1989)

High percentage (61%) of fresh ram sperm induced in BWW medium showed type I pattern (M1 antigen on the anterior acrosome) compared to type II pattern (anterior margin acrosome), 26% (Fig. 2a) and giemsa staining showed majority of the type I pattern were acrosome intact sperm. This result suggested that the expression of M1 protein on ram sperm head are not dependent on acrosome reaction. Unlike in BWW medium, fresh sperm in Tyrode's medium demonstrated higher percentage (71%) of type II M1 protein staining pattern (Fig. 2b). Giemsa staining revealed that > 95% of the sperm in Tyrode's medium were acrosome reacted. As reported earlier, Tyrode's medium has the similar composition with fluid in t he ewe reproductive tract compared to the BWW, therefore it is suitable to induce acrosome reaction of ram sperm. We believed the presence of MI antigen at the anterior margin acrosome was due to incomplete formation of vesicle during acrosome reaction process.

In contrast, frozen samples of ram sperm showed a negative result when M1 antigen unsuccessfully detected neither in BWW nor in Tyrode's medium. There are several factors involved to explain the results, such as freezing and thawing effect during cryopreservation procedure. According to Watson (1990) freezing can deactivated a few sperm

protein. Mellrose (1962) reported that deeply freezing can inhibit protein activity and suggesting that M1 antigen had been changed configuration or protein folding during cryopreservation. Additionally, the used of antibacterial agent in cryopreservation medium may cause a toxic effect (Watson, 1990). Previously we believed that Percoll which had been used to wash the sperm from cryopreservative agent can be one of the factor preventing the M1 antibody to conjugate with M1 antigen (Mat Noor *et al.*, 1999). Unfortunately we found exclude Percoll in washing method did not improve our present results.

As observed by Giemsa staining technique, we discovered the unexpected result that perhaps can explain the absence of M1 antigen on frozen-thawed sperm. It was found that all frozen-thawed sperm were free from acrosomal cap. We believed this was due to cryoinjury. According to the "salt injury" theory of the Lovelock (1953) the intracellular solute concentration would be expected to rise during the freezing of the diluent then as the cells are suddenly expose to the melting diluent during thawing, most frozen-thawed spermatozoa believed to be suffer from structural damaged by osmotic swelling. It is possible that perhaps the rate of ice crystal growth might be causing the intracellular damage (Woolley and Richardson, 1978).

Schill and Wolff (1974) reported that the sperm organelle obviously altered by freezing and thawing is the acrosome membrane. According to Holt and North (1994), sperm acrosome had been damaged because of the thawing process and finally the acrosome cap become fully vesiculate. Therefore, we believed disruption of the acrosome membrane is the main factor which cause the M1 antigen can not be detected on the frozen-thawed ram sperm.

It can be concluded that:

M1 antigen was not only present in hamster sperm but also in ram sperm and the localization was on the acrosomal cap (anterior acrosome). The antigen on ram sperm is not acrosome reaction dependent and believed to be exist on acrosome cap during spermatogenesis. Tyrode's medium is the best medium to induce acrosome reaction of ram sperm and the acrosome reacted sperm revealed type II pattern in fluorescence staining. The localization of the antigen on ram sperm suggests that the antigen may be important at primary binding during fertilization. Freezing and thawing procedure in cryopreservation technique cause severe cryoinjury to the acrosomal membrane that cause disruption of the M1 antigen. Monoclonal antibody (mab) designated M1 has proved particularly valuable as a specific marker of the ram acrosome and also can be used for diagnosis of certain forms of infertility.

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