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Some Vasovasostomized Men Are Characterized by Low Levels of P34H, An Epididymal Sperm Protein

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ABSTRACT: During epididymal transit, sperm surface proteins involved in the fertilization process can be added or modified. P34H, a human epididymal-sperm protein, is proposed to be involved in the interactions between spermatozoa and the zona pellucida. We have previously demonstrated that P34H is present in men of proven fertility and is absent in 50% of men presenting with idiopathic infertility. Spermatozoa with a low amount of P34H exhibit a dramatic reduction in their ability to interact with zona pellucida. Even if the surgical success of vasectomy reversal is high, fertility is not always reestablished, possibly due to epididymal damage caused by vasectomy. In this study, western blot analyses were performed to determine the level of P34H present on spermatozoa of men who underwent vasectomy reversal. Spermatozoa obtained from different semen samples from a given individual had similar P34H levels; however, samples from different men were highly variable. When quantified by densitometric scanning, P34H levels from vasovasostomized men varied between 1.5% and 149% compared with that from a fertile donor who represented 100%. Eighteen of 25 vasovasostomized men had a P34H level lower than 30% of the normal value, while the remaining 7 males were in the normal range. Furthermore, the population of vasovasostomized men with P34H levels lower than 30% was significantly different from the control group of 19 fertile men. The high variation of P34H levels observed in vasovasostomized men did not correlate with the spermiogram values ($P > 0.05$). An important factor in determining sperm P34H level appears to be the period of time elapsed between the vasectomy and vasovasostomy. In summary, our results show that the P34H level varied from one man to another and that low levels of the epididymal sperm protein is associated with vasectomy reversal.

Key words: Spermatozoa, vasectomy.

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Though a condom is the most commonly used male contraceptive method, vasectomy is also widely used, especially in North America (Harrison and Rosenfield, 1996). The reestablishment of fertility within a relatively short period of time is an essential characteristic of contraception. In the case of vasectomy, this property has been questioned. Even though vasovasostomy, or surgical vasectomy reversal, is becoming more frequent, vasectomy is often referred to as a male sterilization procedure. The surgical success of vasectomy reversal is higher than the recovery of fertility. During the last 15 years, successful vasovasostomy rates have been reported to range widely. Controversy still exists concerning the results that can be expected following this procedure, and differences may exist in technical skill and microsurgical expertise of the surgeon. It has been reported that the best pregnancy rate expected following surgical vasectomy reversal is approximatively 66% (Sharlip, 1993). One third of the vasovasostomized men will, therefore, fail to father. This failure of fertility recovery may be attributed to partner infertility, sperm antibodies, epididymal dysfunctions, and other unknown factors (Silber, 1989; Belker et al, 1991; Sharlip, 1993; Nieschlag, 1997).

Interaction between spermatozoa and the zona pellucida is a critical step in the early events of fertilization (Wassarman and Litscher, 1995; McLeskey et al, 1998). During epididymal transit, sperm surface proteins involved in this fertilization process can be added or modified. We have previously described a 34-kDa human epididymal sperm protein (P34H) that is proposed to be involved in the interaction of spermatozoa with the zona pellucida. P34H first appears in the the corpus epididymidis and is restricted to the sperm acrosomal cap (Boué et al, 1994, 1996). In a previous study, we showed that P34H is present in men of proven fertility and is absent in 50% of men presenting with idiopathic infertility. Spermatozoa with a low amount of P34H exhibit dramatically decreased ability to interact with the zona pellucida (Boué and Sullivan, 1996).

The aim of the present study was to determine the level of P34H on spermatozoa of men who had undergone surgical vasectomy reversal. Our results demonstrate that many vasovasostomized men are characterized by low P34H levels similar to those observed in men with idio-

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pathic infertility. These results are discussed with regards to the epididymal damage that potentially occurs following vasectomy.

Materials and Methods

Semen Processing

Twenty-five men who underwent postvasovasostomy spermiogram analysis were included in this study. Patients who underwent vasoepididymostomy were excluded from this study, as well as those exhibiting anti-sperm antibodies in seminal plasma or on sperm, as determined by indirect and direct immunobead assay, respectively (WHO, 1992). These healthy men were between 28 and 53 years of age. Between 2 and 7 days of sexual abstinence were required before semen collection. A comparable group of 19 fertile volunteers aged between 24 and 49 years were included for comparison. These men had fathered children within the last 3 years and had shown normal spermiogram values as defined by the World Health Organization (1992). The usual spermiogram values were determined after a liquefaction period of 30–60 minutes at room temperature and included light-microscopic evaluation by the same technician who evaluated sperm concentration, percentage of motility, and morphology. Motility was classified according to the WHO (1992) "a" to "d" scale; sperm in the "a" and "b" categories were considered as forward motile. Aliquots of semen were centrifuged, and sperm-free seminal plasma was stored at −20°C until colorimetric determination of neutral alpha-glucosidase concentration, as described by Cooper et al (1990). This enzyme is used as a marker of epididymal patency.

P34H Determination

Spermatozoa were washed three times by centrifugation in Dulbecco's phosphate-buffered saline (D-PBS; Gibco, Grand Island, New York) and resuspended in sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) sample buffer (50 mM Tris-HCl, pH 6.8; 2% SDS; and 5% ß-mercaptoethanol). Samples were heat denatured at 95°C for 5 minutes and processed by centrifuge, after which supernatants were recovered and stored at −80°C until used.

Proteins extracted from 10^7 spermatozoa were submitted to SDS-PAGE according to Laemmli (1970). Electrophoretic patterns were electrotransferred onto a nitrocellulose membrane as described by Towbin et al (1979). These western blots were saturated for 2 hours, or overnight, in PBS containing 5% defatted milk. After three washes with 0.1% Tween 20 in PBS (PBS-Tween 20), membranes were incubated for 2 hours with P26h antiserum diluted 1:1000 in PBS supplemented with 2.5% goat serum. This antiserum has been produced against a hamster sperm protein (Bérubé and Sullivan, 1994) and showed cross-reactivity with the human sperm protein, P34H (Boué et al, 1994). The amino acid sequences of these two sperm proteins shows a high level of homology. As described by Bérubé et al (1994), the anti-P26h polyclonal antiserum was previously adsorbed on human keratin. After three washes with PBS-Tween 20, membranes were incubated for 45 minutes with a peroxidase-conjugated anti-rabbit IgG diluted 1:3000 in PBS containing 2.5% goat serum followed by three washes with PBS-Tween 20. The immune complexes were revealed with a Biomax film (Eastman Kodak, Rochester, New York) after incubation with a chemiluminescent substrate of peroxidase as described in the supplier's instruction (ECL kit; Amersham, Buckinghamshire, United Kingdom).

In order to quantify P34H, two western blots were performed on sperm from each individual in both groups, i.e., fertile controls and vasovasostomized men. The blots were scanned with an UltroScan XL laser densitometer (LKB, Rockville, Maryland). The area under the curve corresponds to the amount of P34H in samples containing 10^7 spermatozoa. Within each electrophoretic gel from each patient, a reference lane from the same fertile donor was included. Each P34H level was compared with this fertile donor, for whom the level of P34H was defined as 100%. This fertile donor was chosen based on his P34H concentration, which was in the middle range of the control fertile group. This procedure of P34H quantification in semen samples from fertile and idiopathic infertile men has been previously described (Boué and Sullivan, 1996). Based on the fertile population, P34H values under 30% are considered abnormal (Fig. 1). Such low P34H levels have been previously shown to be associated with an inability of spermatozoa to bind to homologous zonae pellucidae *in vitro* (Boué and Sullivan, 1996).

Statistical Analysis

The distribution-free Mann-Whitney *U*-test was performed to evaluate the difference in P34H levels between the populations of vasovasostomized and fertile men. Correlations between P34H level and different parameters, such as time of P34H determination postsurgery, total number of spermatozoa, percentage of motility, and alpha-glucosidase activity, were estimated by linear regression analyses.

Results

Western blots were performed on a fixed number of spermatozoa obtained from 25 men who had previously undergone vasovasostomy. These P34H levels were determined by comparison with a positive internal control, a fertile donor, designated as 100%. When quantified by densitometric scanning, levels of P34H from samples obtained from vasovasostomized men were between 1.5 and 149%. These are arithmetic means of determinations performed on two different western blots. There was less than 10% variation between the P34H quantities determined from two different western blots of the same semen sample (data not shown). Eighteen of the vasovasostomized men studied revealed a P34H level lower than 30% of the normal value, while the other seven men were in the normal range (Fig. 1). The same quantification performed on semen samples from fertile donors showed P34H values between 55 and 125%. Two populations of vasovasostomized patients were therefore defined according to the amount of P34H detected. Those with a P34H

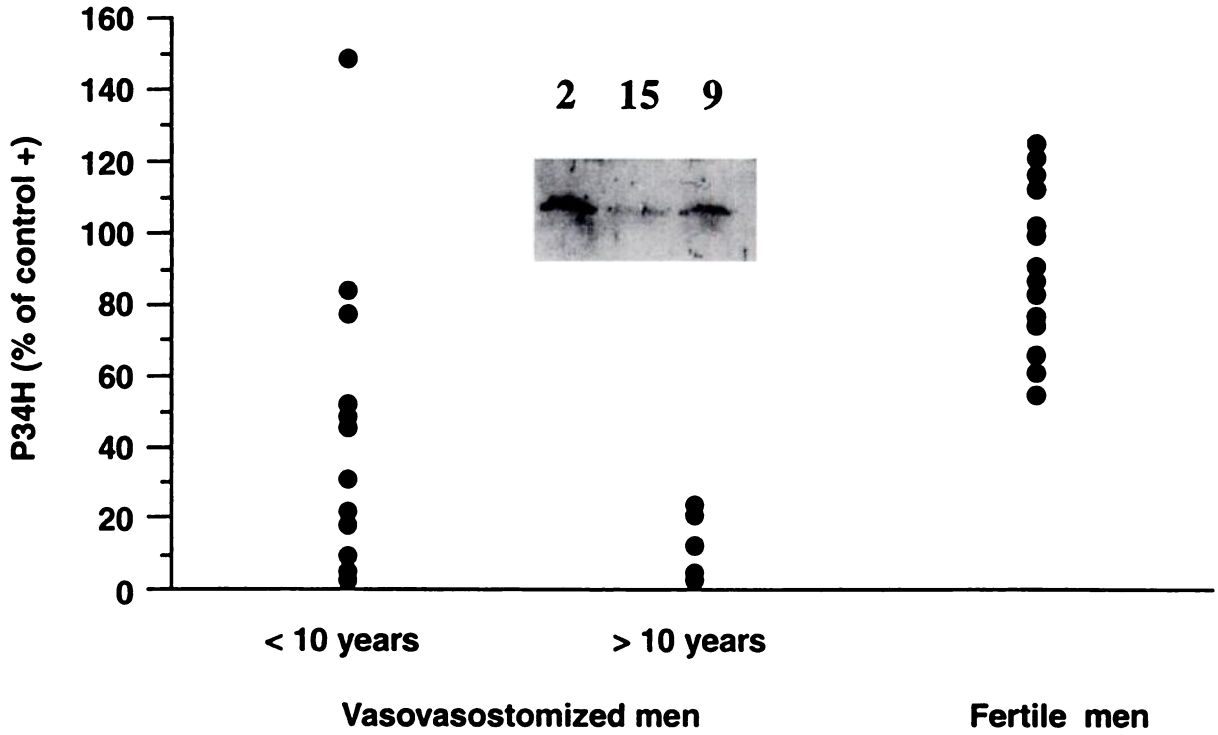

FIG. 1. Amount of P34H as determined by densitometric scanning of immunoblots of proteins extracted from 10^7 spermatozoa from vasovasostomized men who have been vasectomized for less (<) or more (>) than 10 years, as well as from fertile men. The results are expressed as a percentage of an internal positive control (100%). The group of vasovasostomized men was significantly different from the group of fertile men ($P < 0.001$). Inset shows examples of western blot determination of P34H in semen samples from men vasovasostomized 2, 15, and 9 years after vasectomy.

value lower than 30% were significantly different from the control fertile group ($P < 0.0001$).

There was no linear correlation between P34H levels and the period of time that had elapsed between vasectomy and vasovasostomy. However, P34H levels in all men who had been vasectomized for a time period of 10 years or more ($n = 5$) was less than 30% of the internal positive control. On the other hand, the time between surgical vasectomy reversal and semen collection showed no correlation with P34H levels (Fig. 2). The age of the vasovasostomized as well as of the fertile control men had no effect on the level of P34H associated to a constant number of spermatozoa (data not shown).

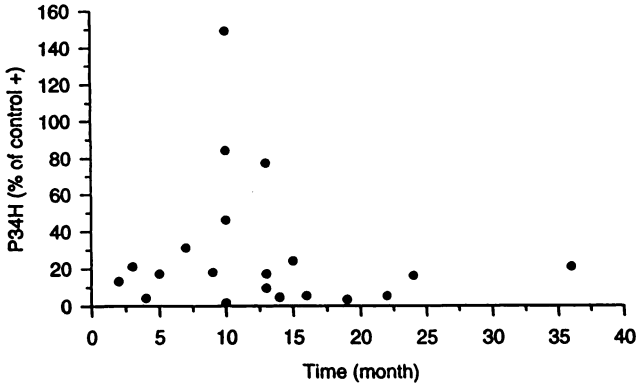

FIG. 2. Amount of P34H as determined by densitometric scanning of immunoblots of proteins extracted from 10^7 spermatozoa from vasovasostomized men in relation to the time between vasovasostomy and P34H determination.

Spermiogram values were determined in order to evaluate the surgical success of vasovasostomy and to determine if these parameters influenced P34H quantity. Each parameter was examined by a simple regression analysis and compared to the theoretical slope by Student's *t*-test to evaluate the significance of P34H level on these parameters. In all situations, simple regression probability showed no relationship between P34H level and sperm parameters ($P < 0.001$). Total sperm count in vasovasostomized men varied from 20×10^6 to more than 200×10^6 and did not correlate with the level of P34H determined on a constant number of spermatozoa (Fig. 3). In a similar manner, the percentage of motile sperm, as well as the percentage of forward motile spermatozoa, did not correlate with P34H quantity (Fig. 4). Semen neutral alpha-glucosidase is a marker of epididymal patency, and to assess the excurrent duct patency, alpha-glucosidase was determined in the same semen used to evaluate sperm P34H. Only one of the 25 samples analyzed showed alpha-glucosidase activity lower than the normal value of 20 mU or more per ejaculate (WHO, 1992). Therefore, P34H was not affected by alpha-glucosidase values (Fig. 5).

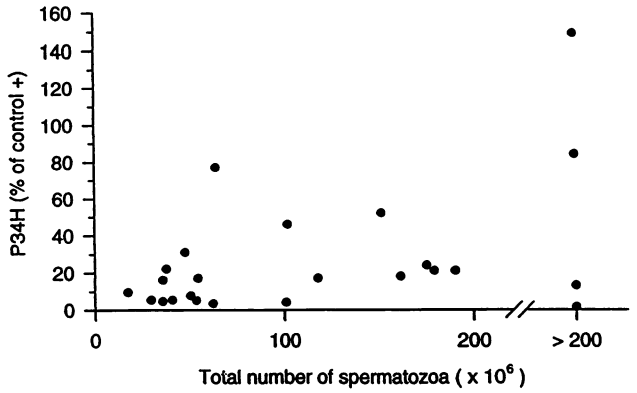

FIG. 3. Amount of P34H as determined by densitometric scanning of immunoblots of proteins extracted from 10^7 spermatozoa from vasovasostomized men in relation with the total number of spermatozoa ($\times 10^6$) in semen samples.

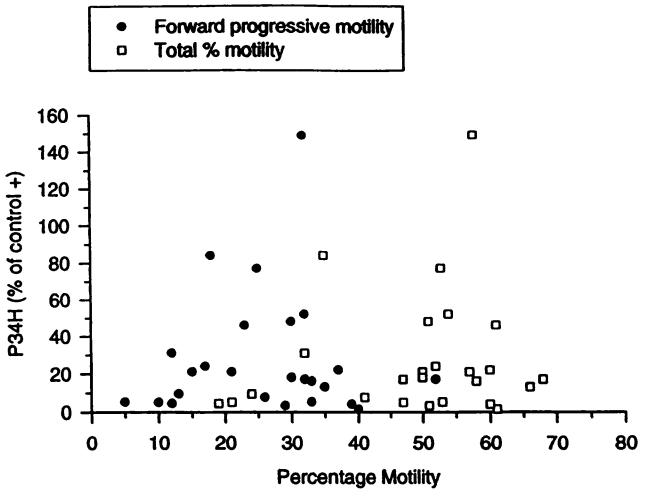

FIG. 4. Amount of P34H as determined by densitometric scanning of immunoblots of proteins extracted from 10^7 spermatozoa from vasovasostomized men in relation to the percentage of total motile (closed circle) and forward motile (open square) spermatozoa in semen samples.

Discussion

In man, as in other mammalian species, spermatozoa emerge highly differentiated from the testis but continue to undergo major biochemical and biophysical changes during their passage through the epididymis. These are essential for acquiring mature motility patterns and fertilizing ability (Hinrichsen-Kohane et al, 1984; Cooper, 1986). The sperm–zona interaction is a species-specific event involving complementary protein receptors on both the surface of sperm and the egg's zona pellucida (Hinrichsen-Kohane et al, 1984; Yanagimachi, 1994). Sperm surface proteins involved in interaction of spermatozoa with the zona pellucida can be added or modified during their journey through the epididymis. P34H, a human epididymal sperm protein, has been proposed to be implicated in this process (Boué et al, 1994, 1996). We have previously demonstrated that this sperm protein is present in all men of proven fertility and is absent in 50% of idiopathic infertile men (Boué and Sullivan, 1996). Spermatozoa with low amounts of P34H exhibit a marked reduction in their ability to interact with zona pellucida. These observations are in agreement with the role of the P34H in the processes of sperm–egg interaction and in the involvement of the epididymis in the acquisition by sperm of fertilizing ability. Some cases of male infertility are thus associated with a suboptimal processing of sperm maturation occuring during the epididymal journey.

Vasectomy is a widely used form of male contraception. The demand for surgical vasectomy reversal can be extrapolated from the number of vasectomies and the divorce rate. It is obvious that the number of surgical vasectomy reversals increases with time (Nieschlag et al, 1997). Surgical success of this intervention is convincing; however, fertility is not always reestablished (Silber, 1989). Certainly, the vas deferens patency rate obtained by microsurgical vasovasostomy depends on the surgeon's skill (Hendry, 1994; Fox, 1997). It is generally recognized that a patency rate of 80% is expected. The recovery of fertility, however, is thought to be 20% lower (Belker et al, 1991). This could be associated with epididymal damage occuring over time in vasectomized men. The epididymis undergoes anatomical sequelae following vasectomy in men (McDonald, 1996). Physiological injuries that can compromize sperm maturation may also occur during this period.

In order to understand the discrepancy between patency success and fertility recovery rate following vasovasostomy, we have searched for P34H. This marker of sperm maturation is predominantly synthesized in the corpus segment of the epididymis and is added to the sperm surface covering the acrosome (Boué et al, 1994, 1996). Spermatozoa obtained from different semen samples from a given vasovasostomized individual had similar P34H

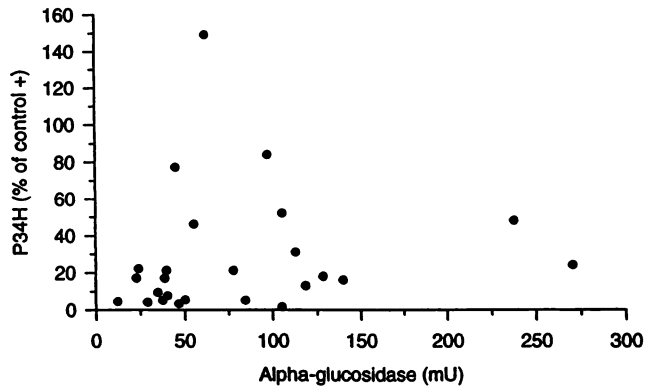

FIG. 5. Amount of P34H as determined by densitometric scanning of immunoblots of proteins extracted from 10^7 spermatozoa from vasovasostomized men in relation to the total neutral alpha-glucosidase activity (mU) in semen samples.

levels. The amount of P34H on a constant number of spermatozoa was, on the other hand, highly variable from one man to another (Fig. 1). Over 72% of men (18 of 25 in this study) who underwent vasovasectomy reversal were characterized by low levels of P34H. This high proportion of vasovasostomized men with low levels of this sperm protein may be overestimated because of the fact that P34H determination was performed on semen samples produced for postsurgical follow-up. This group probably represents more men who did not father compared to the population of all men that underwent vasovasostomy during the same period. A large proportion of vasovasostomized men may not present themselves for the postsurgery follow-up after successfully fathering. Nevertheless, these low levels of P34H are never found in fertile men (Fig. 1 and Boué and Sullivan, 1996). This suggests that at least in some men, the time period postvasectomy affects the epididymis such that spermatozoa are not processed in a proper way to acquire P34H, a necessary antigen for successful sperm–zona pellucida interaction.

Following vasectomy, epididymal damage may increase with time and compromize the recovery of fertility following surgical vasectomy reversal. In fact, the best pregnancy rate is obtained if vasovasostomy is performed within 2 years of vasectomy. Pregnancy rates decrease by another 20% after 5 years. Chances of fertility recovery are drastically lower after a 10-year period since vasectomy (Belker et al, 1991). No linear correlation was observed between P34H and the time between vasectomy and vasovasostomy. Nevertheless, the men that have been vasectomized for more than 10 years showed P34H values lower than 30% of the normal values. When the duration of vasectomy was less than 10 years, interindividual variation in P34H levels was much greater (Fig. 1). On the other hand, there was no correlation between P34H and the time between vasectomy reversal and postoperative semen sample analysis, at least for a time period of 36 months (Fig. 2). Follow-up evaluations over a longer period of time will be necessary to determine if vasovasostomized men with low level of P34H are able eventually to recover this epididymal marker on their spermatozoa.

Total seminal plasma–neutral alpha-glucosidase activity is a marker of epididymal function in men, as shown by a drastic decrease of this enzyme activity in semen following vasectomy (Chapdelaine et al, 1978). Low semen glucosidase activity is also associated with epididymal obstruction or dysfunction (Kret et al, 1995). Furthermore, the presence of alpha-glucosidase can indicate the surgical success of vasovasostomy. In the semen of the vasovasostomized men investigated, there was no correlation between total seminal glucosidase activity and the level of P34H associated to spermatozoa present in the sample (Fig. 5). This suggests that vasectomy affects epididymal physiology in such a way that sperm maturation is suboptimal but that other functions of the excurrent duct are not necessarily affected. This is also supported by the spermiogram values determined following surgical vasectomy reversal.

Men presenting spermiogram values with a percentage of total motile sperm under 50% or a percentage of progressive motility under 25% are traditionally classified as asthenospermic and considered to be subfertile (Liu and Gordon Baker, 1992). A previous study on vasovasostomized men demonstrated that pregnancy rates declined with motility <20%. On the other hand, the postoperative semen parameters have been reported to be similar to prevasectomy values (Silber, 1989). In the present study, the percentage of sperm motility in almost all men who underwent vasovasostomy was >20%, and the percentage of spermatozoa with progressive motility was highly variable from one individual to another; from 5 to 52%. The majority of the vasovasostomized men (20/25) demonstrated a percentage of sperm with forward progressive motility higher than 50% (Fig. 4). P34H determination did not correlate with spermiogram values in our group of vasovasostomized men. Together with alpha-glucosidase determination, these semen parameters suggest that P34H is independent of patency success of the surgical vasectomy reversal and that the epididymis is still able to support sperm modifications involved in the induction of motility but not able to support all the biochemical surface modifications necessary for the acquisition of complete fertilizing ability in sperm.

P34H is added onto the acrosomal cap of the spermatozoa during epididymal maturation, and this antigen is essential in the interaction with the zona pellucida (Boué et al, 1996). This study demonstrated that a high proportion of vasovasostomized men produce spermatozoa deficient in this essential sperm surface proteins similarly to that observed in idiopathic infertile men. This supports the hypothesis that epididymal dysfunction can, in part, be responsible for the discrepancy between the surgical success and the pregnancy rates observed following vasovasostomy.

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