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·基础研究·

Ventral Prostate Glands Modification of Sperm Membrane Proteins in Golden Hamster

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Abstract: 【Objective】 To investigate the effect of the secretory proteins of the ventral prostate glands on the sperm membrane proteins in golden hamsters. 【Methods】 The sperm was collected from female hamsters uteri after mated with the males with or without ventral prostate gland. Male golden hamsters were divided into four experimental groups: (i) all accessory sex glands (ASG) removed; (ii) ventral prostate gland removed; (iii) all ASG removed except ventral prostate gland and (iv) sham-operated controls. Each group contained 6 hamsters. The sperm membrane proteins were extracted from uterine sperm and analyzed by SDS-PAGE and two dimension electrophoresis. 【Results】 The SDS-PAGE results of sperm membrane showed that the ventral prostate glands added up some proteins or increased the quantity of some proteins, which contained molecular mass (MM) 15 k, 29 k, 38 k, 55 k, and 91 k proteins, to the sperm membrane. 2D-electrophoresis of sperm membrane showed that some extra protein spots were appeared in with ventral prostate gland groups, their MM and isoelectric point (IP) corresponding to 16 k/8.60, 16.6 k/9.20, 28 k/5.88, 28 k/6.10, 29 k/5.98, 32 k/6.35, 32 k/6.50, 32 k/7.20, 61 k/5.90, and 83 k/6.40, respectively. The quantity of some protein spots increased in sperm membrane of ventral prostate glands group, their MM/IP coresponding to 17 k/5.95, 17.5 k/6.50, 24 k/7.20, 26 k/5.40, 26 k/5.60, 27 k/7.20, 27 k/7.50, 28 k/5.70, 29 k/8.50, 42 k/6.50, and 42 k/7.00, respectively. 【Conclusion】 The ventral prostate gland secretion plays a role in regulating the sperm membrane proteins and the latter may in turn affect the male fertility or embryo development.

Key words: golden hamster; ventral prostate; uterin sperm; protein
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金黄地鼠腹侧前列腺在体修饰精子膜蛋白作用研究

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摘 要: 【目的】在整体水平探讨前列腺分泌蛋白对金黄地鼠精子膜蛋白的影响。【方法】雄性鼠手术分为 4 组,分别为附属性腺全去组,腹前列腺摘除组,去除其他附属性腺仅保留腹前列腺组和假手术组,每组动物 6 只。收集与保留前列腺及去除组的雄性鼠交配后的子宫腔精子, 抽提精子膜蛋白, 膜蛋白经聚丙烯酰胺凝胶 (SDS-PAGE)和二维电泳凝胶分析,比较前列腺存在与否精子膜蛋白组分的异同。【结果】与不同组雄鼠交配后收集的子宫腔精子膜蛋白 SDS-PAGE 电泳谱主要条带类似,含腹前列腺组的精子膜组分中相对分子质量 15 k、29 k、38 k、55 k 和 91 k 多肽的量较去除腹前列腺组的子宫腔精子膜相应组分增加。二维电泳结果示与含腹前列腺组雄鼠交配后收集的子宫腔精子膜蛋白较无腹前列腺组的精子膜蛋白多出 10 个蛋白斑点 (MM/IP 分别为 16k/8.60、16.5k/9.2、28k/5.88/6.10、29k/5.98、32k/6.35/6.50/7.20、61k/5.90、83k/6.40),另外蛋白斑点量增加有 11 个。【结论】腹前列腺可修饰和调节精子膜蛋白,进而这些蛋白可能对雄性生殖力及胚胎的发育起作用。

关键词: 金黄地鼠; 前列腺; 子宫腔精子; 膜蛋白

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The cell surface is an important functional part of the sperm. Ultrastructural studies have shown that the appearance and topographic configuration of the sperm plasma membrane are altered during the epididymal transit [1]. Some of the epididymal proteins may be absorbed onto the sperm surface or may modify pre-existing molecules through the action of proteases, glucosidases, glucosaminidases, or glycosyltransferases. During ejaculation, the sperm comes into contacted with seminal plasma that is the bulk of male accessory sex glands (ASG) secretions. The secretions can influence the surface properties of sperm[2]. A role for the ASG secretions in male fertility has long been suspected, but no single factor has been implicated. ASG have been shown to be essen- tial for the maintenance of fertility in the mouse, rat and golden hamster [3]. More evidence indicated the proteins of seminal plasma are related with the fertility, two proteins (26 k/IP 6.2 and 55 k/IP 4.5) predominated in higher-fertility bulls and a protein prevalent in the seminal plasma of lower-fertility males [4]. Factors put forward to explain fertility are largely related to the effects of ASG on such prefertilizational events of spermatozoa as activation, motility and transport in the female reproductive tract in vivo [5]. In vitro experiments suggested that ASG secretions temporarily decapacitated spermato- zoa, activated spermatozoa and provided the meta-bolic fuel for the sperm's motility and survival, as well as suppressed immunogenicity of spermatozoa in the female genital tract [6]. Of the five sets of ASG in the golden hamster, only ampullary gland (AG) and ventral prostate gland (VPG) are important in fertility because of their effect on embryo develop-ment, even though fertilization rate is not affected [7]. To find out which aspects of the sperm membrane are affected by the VPG, we characterized the property of sperm membrane ejaculated from males whose VPG left or VPG removed. Although spermatozoa are incapable of protein synthesis, their plasma membrane is postulated to be modified during developmental process at contacted with ASG. So the present study is to investigate how sperm membrane proteins are changed after ablation of the VPG in the golden hamster in vivo. Since it is extremely difficult to obtain ejaculated sperm in rodents, uterine sperm recovered after mating were used.

1 Materials and methods

1.1 Chemicals and Animals

All chemicals used were of analytical grade and were obtained from Sigma (USA) unless otherwise indicated. All random bred golden hamsters (Mesoc-ricetus auratus) were obtained from the Laboratory Animal Unit of the University of Hong Kong. Animal housing, care, and application of experimental pro-cedures were in accordance with the institutional guidelines and approved by the University Commi-ttee on the Use of Live Animals in Teaching and Research for the University of Hong Kong. The hamsters were kept under a 14-h light∶10-h dark cycle (lights on 11 00 AM～01 00 AM) at 25℃ and were fed a standard laboratory diet. All adult female hamsters (7～8 weeks old) were checked daily for vaginal secretion at least two consecutive normal cycles before mating. The female hamsters were chosen to carry out mating on the second day of the cycle.

1.2 Operation of hamster

Male hamsters were operated according to a method previousely established in this laboratory [8]. According to the glands removed, four treatment groups were designed. Group SH: Sham-operated animals; Group TX: Total removal of the AG, coagulating glands, ventral and dorsal prostate glands and seminal vesicles; Group VP: Total removal of all ASG except VP; Group VPX: Removal of ventral prostate gland.

1.3 Epididymal sperm and Uterine sperm

Normal 14～20 weeks male hamster was sacrificed with an intraperitoneal injection of over dosage sodium pentobarbital (Rhone Merieus Com-pany, Australia). The cauda epididymis was isolated. A cut was made and sperm were flushed out by retrograde perfusion through the vas deferens with 5 mmol/L HEPES [N-[2-Hydroxyethl] piperazine-N'-[4-butanesulfonic acid]] / 0.264 mmol/L sucrose buffer pH 7.4 containing 2 mmol/L phenylmethyl-sulfonyl fluoride (PMSF) and 10 mmol/L iodoaceta-mide. The sperm were washed three times (600 ×g, 5 min, 4℃) in PBS (containing 2 mmol/L PMSF, 10 mmol/L iodoacetamide). The sperm pellet was resuspended in PBS and the number of sperm was counted by a haemocytometer. The sperm con- centration was adjusted to about 2×10^6 sperm/mL. For collecting of uterine sperm, the males (SH, TX, VP and VPX) were hand-mated with female hamsters for 15 min and then the females were kept separately in cages. Each male group contained 6 hamsters. The female hamster was sacrificed and the uterus was dissected at 45 min p.c. (post coitus). The sperm were flushed out of the uterus with 2 mL PBS (containing PMSF and iodoacetamide) and washed for 3 times with PBS. The sperm pellet was suspended and adjusted to 2×10^6 sperm/mL.

1.4 Extraction of sperm membrane proteins

Sperm membrane protein extracts were prepared as described previously [8,9]. Two mililiter of the epididymal sperm or uteri sperm suspension was centrifuged at 600 × g, 5 min, 4℃. The sperm pellet was resuspended in 2 mL of the 0.5% NP-40, 5 mmol/L HEPES/0.264 mmol/L pH 7.4, sucrose buffer, containing 2 mmol/L PMSF, 10 mmol/L iodoa- cetamide and kept on ice for 20 min. The sperm suspension was centrifuged at 10 000 ×g for 15 min at 0℃. The supernatant containing the solubilized membrane protein was concentrated with Centricon-3 (Amicon, USA) and its protein concentration was determined by the Bradford method.

1.5 SDS-PAGE

Sperm membrane proteins (5 μg) were separated by SDS-PAGE (10% gels) under denaturing conditions with Hoefer SE250 (Hoefer, USA). After electro-phoresis, the gels were stained with Coomassie Brilliant Blue R250. The molecular mass was deter- mined by using the Middle and Low Molecular Weight Calibration Kit (Pharmacia, Sweden). The band absorbent intensity was determined by an image densitometer (Model GS-690, BIO-RAD USA). The area occupied by each peak in the absorbent intensity profile was measured with a manual image analyzer (Molecular Analysis Software). The quantity of protein in each band was represented by an absorbent intensity index (area ×absorbent optical density).

1.6 Two-dimensional gel electrophoresis

Electrophoresis was performed on a Multiphor® II Electrophoresis System. The first dimension sepa-ration was done with an immobilized pH gradient. The electrophoresis gel was stained with silver staining.

1.7 Gel scanning and computer analysis

Spots that consistently appeared in three gels were considered valid. The spots were scanned with an imaging densitometer (Bio-RAD, Model GS-690, USA) and semi-quantitative analyzed by eye. Spot matching in different gels were performed manually. Several reference spots (2-D-markers) were manually matched in a pair of gels. The IP values and relative molecular mass of all spots were then identified with reference to the markers.

1.8 Statistical analysis

Data was analyzed by Student's test or analysis of variance (ANOVA), followed by the Newman-Keuls multiple comparisons test. Significance was set at $P < 0.01$ and $P < 0.05$.

2 Results

2.1 SDS-PAGE of sperm membrane proteins

The SDS-PAGE analysis of proteins extracted from epididymal (EPI) or uterine sperm membrane revealed polypeptides with MM 14 k to 116 k (Fig.1). The protein profile from epididymal sperm is different from that of the uterine sperm. Quantitative analyses of the polypeptide bands from uterine sperm were summarized in Table 1.

2.2 Interaction of sperm plasma membrane with ventral prostate gland in vivo

A parallel in vivo study was carried out by comparing uterine sperm from females mated with TX and VP males. The major protein profiles of uterine sperm plasma membrane from mating with different male groups were slightly different. 12 major polypeptides (MM as 15 k, 17 k, 27 k, 29 k, 32 k, 34 k, 38 k, 43 k, 55 k, 67 k, 91 k and 116 k) were present in TX and VP sperm membrane. Quantitatively, the 15 k, 29 k, 55 k, 67 k, and 91 k polypeptides of the VP group were significantly increased, but those of the 34 k and 43 k were reduced although no statistics significance (Fig.1, Table 1). The results indicated that the ventral prostate gland secretion modified the sperm membrane. The major effect of the VPG secretion was to add up some polypeptides to sperm membrane.

Table 1 The absorbent optical intensity times area of each bands of uterine sperm membrane from different groups ($\bar{x} \pm s$, n=6)

	Relative molecular mass/k									
	15	29	32	34	38	43	55	67	91	116
VP	2.98 ±0.18[1)]	1.10 ±0.12[1)]	0.65 ±0.06	0.39 ±0.04	0.72 ±0.06[1)]	0.67 ±0.07	0.91 ±0.08[1)]	2.11 ±0.19	0.44 ±0.05[1)]	0.19 ±0.03
TX	2.33 ±0.16	0.63 ±0.04	0.69 ±0.06	0.46 ±0.04	0.45 ±0.04	0.79 ±0.08	0.53 ±0.06	1.92 ±0.13	0.12 ±0.02	0.12 ±0.02
SH	2.27 ±0.19[2)]	0.91 ±0.08[2)]	0.38 ±0.04	0.19 ±0.03	0.66 ±0.06[2)]	0.74 ±0.06	0.83 ±0.07[2)]	2.41 ±0.17	0.33 ±0.04[2)]	0.21 ±0.02
VPX	2.05 ±0.21	0.82 ±0.09	0.57 ±0.06	0.24 ±0.03	0.49 ±0.03	0.62 ±0.04	0.71 ±0.06	2.30 ±0.18	0.25 ±0.04	0.15 ±0.01

1) VP vs TX, $P < 0.05$; 2) SH vs VPX, $P < 0.05$

2.3 2-D electrophoresis of uterine sperm mem-brane proteins

2-D electrophoresis of the detergent-extracted sperm proteins from the different groups were shown in Fig.2a, 2b,

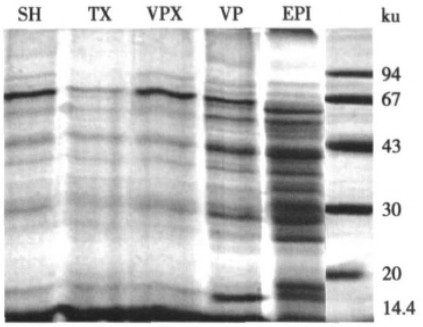

Fig.1 10% SDS-PAGE of sperm membrane proteins

The sperm membrane proteins were extracted from epididymal sperm （EPI） and uterine sperm collected from the females mated with males of the various operated groups. Each lane was loaded with 15 μg proteins.

2c and 2d. 2-D markers reference spots were manually matched in each gel. The IP values and MM of all spots were then identified with reference to the standard.

2.4 The effect of VPG on the sperm membrane in vivo

Sperm membrane of VP or SH compared with that of TX or VPX The protein patterns of the sperm membrane from the VP （14 spots） were compared to TX group （06 spots）, and SH （19 spots） to VPX （11 spots） （Fig.2a, 2b, 2c, and 2d）. The MM range covered from 15 k to 94 k. The resolved proteins spanned a IP range from 4.20 to 9.20. All spots and the trains （more spots with same MM but different IP） were semi-quantitative and summarised on the Table 2. The results showed that 3 spots （MM/IP respectively as, 27 k/4.54, 47 k/8.90, 94k/5.70） were present in TX or VPX, while absent in VP or SH group （in Fig.2aB or 2bB, the blue circles indicated）. 10 spots （6 k/8.60, 16.5 k/9.2, 28 k/5.88/6.10, 29 k/5.98, 32 k/6.35/6.50/7.20, 61 k/5.90, and 83 k/6.40） were present in VP or SH and not in TX or VPX

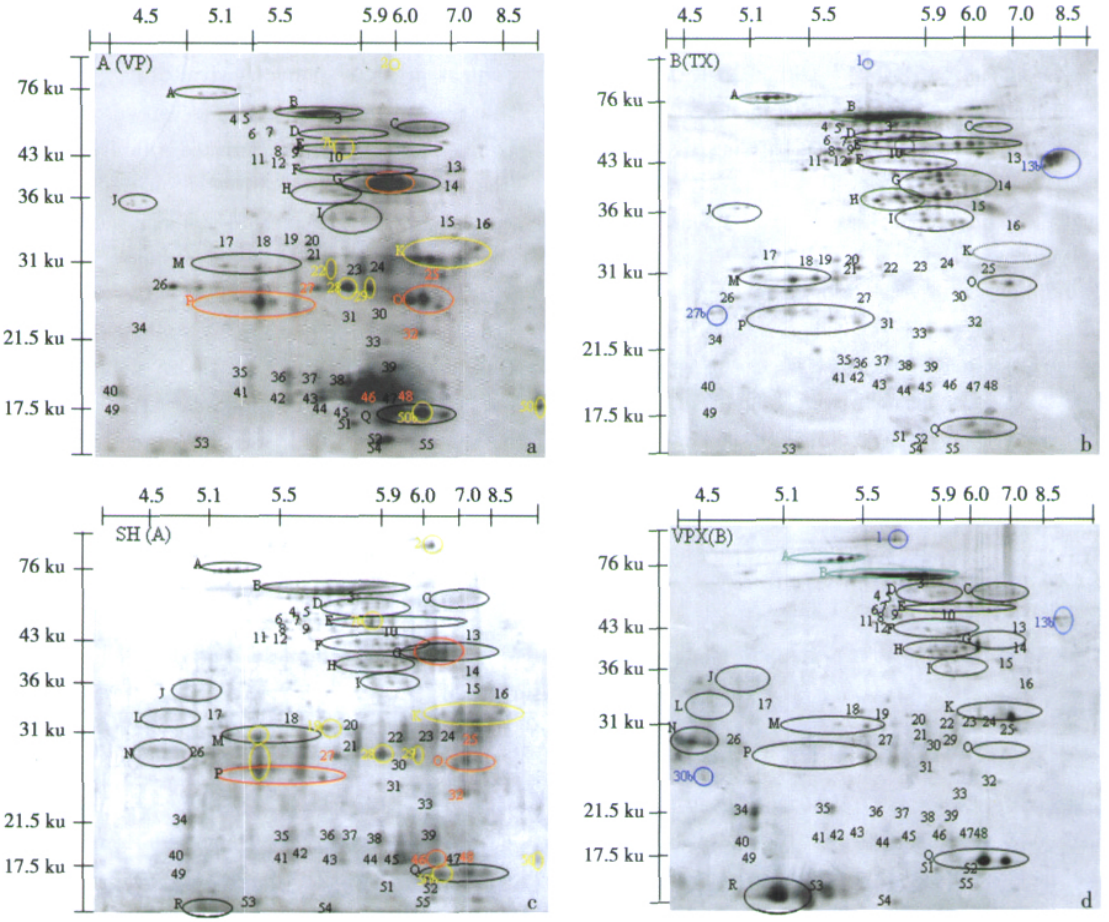

Fig. 2 2D-electrophoresis of VP, TX, SH and VPX group sperm membrane proteins

10 spots （yellow circle indicated） were only present in VP or SH group （a, c）. The red circle or number in VP or SH indicated that the semi-quantitative of the spots increased. The green circle in TX or VPX indicated that the semi-quantitative of spots increased （b, d）. The blue number indicated that the spots were only present in TX or VPX group.

Table 2　The list and characteristics of the different train and spots of sperm membrane proteins separated by 2D-electrophoresis with or without ventral prostate gland

	Spot number/Train lablled																			
	1	2	TB	7bE	13b	TH	TK	17bcd	22	25	27	28	29	30b	TO/P	32	46	48	50	50bO
VP	-	+	++	++	-	+	++	++	+++	++	++	+++	++	-	++	++	+++	+++	+	++
TX	+	-	+++	-	++	++	-	-	-	+	+	-	-	+	+	+	+	+	-	-
SH	-	++	+++	+	-	+	++	++	++	+++	++	++	++	-	++	++	+++	++	+	++
VPX	+	-	++++	-	++	++	+	-	+	+	+	-	±	+	+	+	+	+	-	-

-：undetectable；±：very weak；+：weak (detectable)；++：moderate；+++：strong；T：train；Spot number or train lablled corresponding to MM/IP as 1 (94 k/5.70), 2 (83/6.10), TB (75 k/5.60-5.90), 7bE (61 k/5.90), 13b (47 k/8.90), TH (89 k/5.70-5.92), TK (82 k/6.35-8.50), 17bcd (82 k/6.35/6.50/7.20), 22 (29 k/5.98), 25 (29 k/8.50), 27 (28 k/5.70), 28 (28/5.88), 29 (28 k/6.10), 30b (27 k/4.54), TO/P (27 k/7.00-8.50; 26 k/5.15-5.70), 32 (24 k/7.20), 46 (7.5 k/5.95), 48 (7.5 k/6.50), 50 (6.5 k/9.20), and 50bO (6 k/8.60)

group (Fig.2a or 2c, yellow circles indicated major spots). Semi-quantitatively, the spots (7.5 k/5.95/6.50, 24 k/7.20, 26 k/5.40/5.60, 27 k/7.20/7.50, 28 k/5.70, 29 k/8.50, 42 k/6.50/7.00) of the VP or SH group were increased, but those of the spots (86 k/5.86, 75 k/5.60/5.65/5.70/5.80) were reduced compared with that of TX or VPX group.

What the spots with MM 17.5 k, 29 k, 32 k and 61 k were only present or increased in VP or SH group sperm were agreement with the results of SDS-PAGE that the 15 k, 29 k and 55 k polypeptides increased in VP or SH group. What the spots with MM 39 k, and 47 k were only present or increased in TX or VPX group sperm were agreement with that MM 34 k and 43 k polypeptides increased in TX or VPX group in SDS-PAGE gel (no significant reduced).

The spots (5 k/4.80/5.00/5.10, 27 k/4.30/4.60/4.70, 32 k/4.40/4.80) that were present both in SH and VPX group, but absent in both VP and TX group indicated that the secretion of male ASG except the VPG added proteins onto epididymal sperm.

Taken together, this set of data indicated that VPG secretion could react with proteins on the sperm surface. The modification effect was related to add up the ventral prostate gland secretory proteins by the proteins interaction or protease effect.

3 Discussion

3.1 Sperm membrane protein extracts

Preparation of sperm membrane is important for studying the composition and property of the sperm surface. Membrane fraction recovered from sperma-tozoa have been disrupted by sonicaton, hypotonic shock, homogenization or nitrogen cavitation. Although all of these procedures release plasma membrane, membranes from intracellular organelles (e.g. acrosome, mitochondria) and their contents could also be present in varied amounts. Detergent extraction, if done too aggressively, could also have the tendency to solubilize the cytosolic fraction, the acrosomal and mitochondrial matrices, as well as the remaining cytoplasmic droplet [8,9]. In the present study, a mild extraction procedure used in which sperm membrane protein is solubilized with 0.5% NP-40 on ice. The sperm pellet and their dislodged membrane proteins have been examined by transmission electron microscopy which shows that only the outer layer of the sperm membrane is disrupted [8]. So it is a good way to obtain plasma membrane proteins to study their properties and to compare the effects that the VPG might have on the sperm surface.

3.2 Analyisis of sperm membrane extracts by electrophoresis

The profile the uterine sperm proteins (TX, VP, VPX and SH) after SDS-PAGE separation contains 14 major bands and appears to be slightly different. The protein bands profile consists with the previous observation [8]. In addition to two proteins (91 k and 116 k) appear in the sperm in this study. The difference is probably due to the fact that previous electrophoresis was carried out on the minigel in which only 1.5 μg protein sample was loaded. A 27 k protein, which may correspond to the 25 k protein [8], is found in the sperm membrane from all four groups. It may be the same as the putative hamster zona binding protein (26 k) [10]. Its persistent presence in the various experimental groups supports our repeated observations that fertilizing ability of spermatozoa is not compromised regardless of the presence of the VPG and AG [11].

Quantification of the polypeptides indicates that VPG increases the amount of proteins of sperm membrane with MM 15 k，29 k，55 k，67 k and 91 k. The previous study revealed that the sperm membrane proteins are modified by VPG with increases in the 17 k，28 k and 56 k proteins [8]. The results are consistent with the previous report， based on relative molecular mass except the 67 k and 91 k proteins. The profile of the epididymal sperm is different from that of the uterine sperm. This observation indicates that uterine secretion also modifies the surface of ejaculated sperm by addition or and by removing some proteins from sperm. The biological role of the uterine secretion modification remains to be investigated.

The 2-D electrophoresis of the sperm membrane showed that the hamster sperm membrane proteins are focused in a range IP 4.2 ～9.0，MM 15 k ～94 k and there are about 110 spots in the membrane extract. The different species of sperm membrane 2-D electrophoresis protein profiles have been reported，such as rat epididymal sperm membrane proteins containing 60 spots （IP 4.4 ～6.0/MM 14 k ～120 k），human sperm membrane proteins extracted with CHAPS containing 50 spots （IP 3.0 ～9.0 / MM 10 k ～130 k） [2]. Xu et al reported that 600 spots can be found in human sperm homogenate proteins but a much simpler pattern，with only 64 major protein spots，is seen in highly enriched sperm membrane vesicle extract [3]. Comparison of the 2-D electro-phoresis profile of different species is difficult， since different sperm membrane preparation and different 2-D electrophoresis methods result in different pattern. Although analysis of sperm membrane by 2-D electrophoresis in some species， there is still a lack of understanding in the function and biochemical character of the sperm membrane proteins. We obtained better reproducibility of hamster sperm membrane protein profiles using detergent to extract the sperm membrane and immobilized pH gradient used as the first dimension.The current 2D-electrophoresis study shows that 10 spots are only present in VP or SH group. These spots appear to be closely related to VPG because they are intensified in VP or SH group， but reduced in the TX or VPX group. The results are in agree-ment with the SDS-PAGE results and our previous findings that VPG supplies more proteins to the sperm membrane under physiological conditions [8]. The 2D-electro-phoresis analysis gives more detail changes of proteins，especially those proteins having same molecular mass with different isoelectric point [4-16]. The results supply the evidence and confirm our previous findings that the VPG secretory proteins bind to the sperm surface.

3.3 The effect of male accessory sex glands on sperm membrane，embryo development，and fertillity

ASG of rodents consist of seminal vesicle， coagulating gland， dorsal， and VP and amullary gland. Spermatozoa are coated with a wide variety of macromolecules upon exposure to seminal plasma. Specifically，proteins from seminal vesicles and coagulating glands interact with the surface of human and mammalian epididymal spermatozoa [7]. Each individual gland with distinct structures might have a distinct function. Ventral prostate is known to have an ultrastructure remarkably well suited for the production and secretion of proteins. The concentra-tion of the proteins in VPG is higher than that any other individual gland of male AGS [1]. VPG secretory proteins were found to the midpiece of sperm both in vitro and in vivo study with proteinprotein interaction mode [4]. The proteins identified from seminal plasma bind to the head or anterior acrosomes of the sperm，such as clusterin，P12 （A kazal-type trypsin inhibitor，caltrin-like protease inhibitor），steroid-binding protein （prostatein） and IGF-I （insulin-like growth factor I）. Our previous study indicates that VPG secretory proteins do not bind to head of hamster sperm. Only Manjunath et al. reported that the major proteins of bovine seminal vesicles （BSP） bind to midpiece of spermatozoa，but the proteins bind specifically to choline phospholipids，and the binding modifications occur during capacitation [8]. So the binding proteins from VPG are different from that identified proteins binding to sperm from the seminal plasma.

Although it is clear that pregnancy can proceed in the absence of exposure to semen， studies in various species suggest a role for both sperm and seminal plasma in optimizing pregnancy success. The importance of ASG is unequivoally established when alation of some or all ASG reduced fertility and fecundity in animal models [1,5,11]. However，only the VPG is relevant to maintaining fertility [1]. Surgical ablation of sire VPG or all ASG is associated with delayed entry to the zygote into the first cell cycle， reduces preimplantation embryonic cell numbers，early embryo transits into the uterus from oviduct，and decreases implantation rate. Bulls with detectable seminal fluid heparin-binding proteins （HBP） on sperm membranes were more fertile than bulls with undetectable HPB in sperm membranes [9]. The molecular mechanisum of ventral prostate glands affecting the fertility is unknown.Our hypothesis is that VPG may affect fertility or embryonic development by modification of sperm membrane protein. Analysis of the uterine sperm membrane extracts from

mated with or without VPG supplies a support evidence that VPG modifies the sperm membrane.

It can be concluded from the present results that, in the hamster, VPG secretory proteins modify sperm membrane protein by adding up polypeptides to sperm membrane. Further investigation will be necessary to identify the function of these proteins, will ascertain whether or not the modified proteins participates in activation of oocyte or early embryo development.

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