肝素处理山羊精子体外获能的研究

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摘 要 系统研究了作用浓度、时间和温度以及输卵管上皮细胞和卵丘细胞对肝素处理山羊精子体外获能后的精子活力、质膜完整性、顶体完整率、获能比例及受精和卵裂的影响，为改善山羊精子体外获能效果和研究获能机理提供了必要的数据。主要实验结果如下：1. 在获能液中添加 5、10、25、50 和 100μg/mL 肝素处理 45min 时，添加 50 和 100μg/mL 肝素精子获能比率最高(分别为 55%和 56%)，但添加 100μg/mL 肝素处理后顶体完整率明显($P < 0.05$)低于对照组。说明山羊精子获能的最佳肝素浓度为 50μg/mL。2. 随肝素作用时间(0，10，20，30，45，60 和 120 min)的延长，获能精子比例逐渐提高。其中，肝素处理 45 ~ 120 min 各组的获能精子比例差异不显著($P > 0.05$)，处理 120 min 组的精子活力和质膜完整率显著低于其它各组。说明 50μg/mL 肝素处理精子获能的最佳时间是 45 ~ 60 min。3. 在 42℃和 38.5℃下处理时，获能精子比例显著高于 15℃和 37℃，但 42℃处理后精子活力和顶体完整率显著低于其它温度。因此，38.5℃为山羊精子获能的最佳温度。4. 与输卵管上皮细胞共培养获能精子比例显著高于对照组和卵丘细胞组，但精子活力、质膜完整率和顶体完整率差异不显著。输卵管上皮组的受精率(91.3%)和卵裂率(72.2%)显著高于对照组(81.2%，65.0%)。说明与输卵管上皮细胞共培养能显著提高肝素处理山羊精子体外获能的效果。

关键词 精子，获能，肝素，体外受精，山羊

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通过卵母细胞体外成熟、体外受精和体外培养(IVM-IVF-IVC)可提供大量优质廉价胚胎。然而，山羊卵母细胞的 IVM-IVF-IVC 技术，系统还不稳定，可重复性差。

目前，虽然已经发明了多种体外获能方法，但关于精子获能的机理及影响因素尚有许多问题值得探讨。肝素是一种高度硫酸化的氨基多糖(GAG)。已证明，体外受精时添加肝素可提高牛[1,2]、绵羊[3]、山羊[4]和马[5]精子的受精能力。尽管有人报道肝素能诱导山羊精子体外获能，但不同实验所用的肝素浓度差异很大[6,7,8]。有人发现，单层输卵管上皮能促进牛[9]和鹿[10]精子获能，但在山羊尚未见同类研究。Gwatkin 等[11]报道，卵丘细胞在体外可以使仓鼠精子获能，但对其在获能和受精中的作用尚存争议。由于细微的温差会使精子膜脂的物理状态发生很大变化[12,13]，故有必要研究温度对精子获能的影响。此外，尚未见有关山羊精子获能处理后的活力、质膜完整性、顶体完整率和获能率变化的报道。

本文系统研究了作用浓度、时间和温度以及输卵管上皮细胞和卵丘细胞对肝素处理山羊精子获能后的精子活力、质膜完整性、顶体完整率、获能比例及受精和卵裂的影响。

1 材料与方法

1.1 体外获能程序

使用假阴道从 3 只成年健康鲁白山羊采集鲜精。先在 6 个 5mL 离心管中装 2mL 改进 DM 液(mDM)[14]，每管在底部加入 70μL 精液进行上游处理[15]。1 h 后，自每个离心管吸取上部 0.7mL 高活力精液，混合后再 200 × g 离心 10min。去除上清后检测精子密度。用获能液(含肝素的 mDM)将精子密度稀释到 80×10^6 个/mL 后，在 38.5℃下孵育获能。

1.2 精子质量参数检测

1.2.1 活力的检测：各实验组精液混匀后取 10μL 放入 1mL 预热的 mTyrode's 液中 38.5℃孵育 20min，

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混匀后，取 10μL 在血球计数板上在 100 倍相差显微镜下观察 5 个视野，分别记录视野中直线运动精子数和精子总数，计算平均直线运动精子百分比。

1.2.2　精子低渗抗性检测（HOST）：按照 Revell 等[16]的方法，从各实验组的精液中取出 25μL，用低渗液稀释（7.35g 柠檬酸钠·$2H_2O$，13.51g 果糖，溶于 1000mL 三蒸水）到 200μL。38.5℃孵育 45min 后，加入 2%的戊二醛 300μL。取 10μL 混合液涂片，在 400 倍相差显微镜下观察 5 个视野，计算弯尾精子数占精子总数的百分率。每次检测时至少数 200 个精子。

1.2.3　Hoechst33258/CTC 染色方法：从各实验组的精液中，取 10μL 用 1mL mTyrode's 液稀释后 38.5℃孵育 20min。（1）取 396μL 精子悬液，加入 4μL Hoechst33258 染色液，混匀，避光室温孵育 3min。（2）1.5mL 离心管中放入 1mL 3%的 PVP 溶液，将精子悬液轻轻铺在 PVP 溶液上，500 × g 离心 6min，去上清。（3）沉淀精子块用 50μL、mDPBS 液悬浮吹打均匀。（4）取 45μL 精子悬液加入 45μL 的 CTC 染液，混匀后加入 8μL12.5%多聚甲醛溶液混匀。（5）把 10μL 精子悬液和等量增光剂滴在载片上，混匀后盖上盖片，在 Leica DMLB 显微镜下放大 400 倍检查。头部不被 Hoechst33258 着色的精子判定为质膜完整。放大 1000 倍油镜下观察质膜完整精子的 CTC 着色情况，判定顶体形态。F 型精子头部均匀着色，为未获能顶体完整精子；B 型精子在顶体后方出现无荧光带，为获能并顶体完整精子；AR 精子头部不发光或在赤道段有一薄层荧光带，为顶体反应的精子。每次检测时数 200 个精子，计算精子质膜完整率、获能率和顶体完整率。

1.3　体外受精

受精液为含有 1μg/mL 牛磺酸的 TALP 受精液。体外成熟卵丘卵母细胞复合体（COC）用受精液洗 1 次后放入 100μL 的受精滴中，每滴 18 ~ 22 枚。然后，每个受精滴加入 5μL 获能精子，最终精子浓度为 3.5×10^6 ~ 4×10^6 个/mL。授精后，在 38.5℃，5% CO_2 的培养箱中孵育。17h 后，一部分卵母细胞压片，检查受精率；另一部分卵母细胞转入 TCM-199 液继续培养，48h 检查卵裂。

1.4　数据处理

每组实验至少重复 3 次。统计时先使用统计软件 SPSS8.0 的 Kolmogrov-Smirnow 和 Levene 方差齐次性检验确定其分布性，不具正态性的数据经过反正弦平方根转换后都符合正态分布型。然后应用统计分析软件 SPSS 8.0 的 ANOVA 模块，进行单因素方差分析。$P < 0.05$ 被认为是差异显著。

2　结果

2.1　肝素浓度对山羊精子获能的影响

在获能液中添加 5、10、25、50 和 100μg/mL 肝素 38.5℃处理 45min，都显著（$P < 0.05$）提高了精子的获能比例。其中，添加 50 和 100μg/mL 肝素的获能比率最高，但添加 100μg/mL 肝素处理后顶体完整率明显低于对照组（表 1）。

表 1　肝素浓度对山羊精子体外获能的影响

Table 1　Effect of heparin concentration on *in vitro* capacitaion of goat spermatozoa

Heparin concentration/(μg/mL)	Sperm motility	Hoechst negative sperm/%	HOST positive sperm/%	Acrosome intact sperm/%	Capacited sperm/%
0	86.1 ± 5.0^a	92.6 ± 3.1^a	93.5 ± 2.2^a	96.1 ± 1.2^a	5.6 ± 5.8^a
5	85.9 ± 6.3^a	92.3 ± 4.5^a	93.0 ± 1.9^a	92.8 ± 4.8^{ab}	14.1 ± 3.3^b
10	84.5 ± 4.5^a	92.9 ± 1.9^a	93.0 ± 2.5^a	92.5 ± 6.2^{ab}	32.5 ± 2.8^c
25	84.8 ± 1.7^a	92.3 ± 5.5^a	92.7 ± 4.6^a	89.1 ± 2.5^{ab}	45.2 ± 4.5^c
50	86.6 ± 6.3^a	92.7 ± 4.1^a	91.5 ± 5.8^a	87.6 ± 3.1^{ab}	54.9 ± 5.2^d
100	86.9 ± 3.9^a	92.0 ± 2.5^a	91.0 ± 2.0^a	73.1 ± 2.2^b	56.0 ± 5.0^d

a,b,c,d：Values with common letters in superscripts did not differ significantly ($P > 0.05$).

2.2　肝素作用时间对山羊精子体外获能的影响

用添加 50μg/mL 肝素的获能液 38.5℃处理 0、10、20、30、45、60 和 120min 时，随肝素作用时间的延长，获能精子比例逐渐提高。其中，肝素处理 45 ~ 120min 各组的获能精子比例差异不显著（$P > 0.05$）。处理 120min 组的精子活力和质膜完整率显著（$P < 0.05$）低于其它各组（表 2）。

2.3　温度对山羊精子体外获能的影响

用添加 50μg/mL 肝素的获能液在 15、37、38.5 和 42℃下分别处理 45min 时，在 42℃和 38.5℃下处理的获能精子比例显著高于其它温度，但 42℃获能处理后精子活力和顶体完整率下降，显著（$P <$

0.05)低于其它温度。38.5℃的精子获能率低于42℃,但38.5℃获能时的精子活力、质膜完整率和顶体完整率都显著高于42℃(表3)。

2.4 输卵管上皮细胞和卵丘细胞对精子获能的影响

采用50μg/mL肝素在38.5℃下处理45min发现,在与输卵管上皮细胞共培养30min之内,大部分精子就与输卵管上皮细胞发生贴附,尾部运动加速。但与单层卵丘细胞共培养时,精子不发生贴附。与输卵管上皮细胞共培养获能精子比例显著($P < 0.05$)高于对照组和卵丘细胞组,但精子活力、质膜完整率和顶体完整率差异不显著(表4)。输卵管上皮组的受精率(91.3%)和卵裂率(72.2%)显著($P < 0.05$)高于对照组(81.2%,65.0%)。

表2 肝素作用时间对山羊精子体外获能的影响

Table 2 Effect of treatment duration on *in vitro* capacitation of goat spermatozoa

Heparin treatment time/min	Sperm motility	Hoechst negative sperm/%	HOST positive sperm/%	Acrosome intact sperm/%	Capacited sperm/%
0	91.0±3.5[a]	93.1±6.3[a]	94.5±2.9[a]	95.7±2.5[a]	5.5±6.4[a]
10	89.5±4.2[a]	93.1±4.5[a]	94.0±5.2[a]	93.6±4.1[a]	16.5±6.2[b]
20	88.2±0.5[a]	93.0±1.6[a]	93.8±4.4[a]	91.0±2.5[ab]	29.5±5.0[c]
30	87.6±1.1[a]	92.8±0.4[a]	92.5±3.6[a]	89.5±6.2[ab]	43.9±3.2[d]
45	85.8±2.2[a]	92.5±3.5[a]	92.0±1.5[a]	87.0±5.8[ab]	54.1±2.1[e]
60	83.5±3.0[ab]	90.2±6.1[a]	90.5±4.8[a]	83.8±1.5[b]	56.5±5.9[e]
120	75.2±4.8[b]	80.3±2.5[b]	79.5±2.2[b]	82.5±4.9[b]	60.0±2.8[e]

a,b,c,d,e: Values with common letters in superscripts did not differ significantly ($P > 0.05$).

表3 温度对山羊精子体外获能的影响

Table 3 Effect of temperature on *in vitro* capacitation of goat spermatozoa

Capacitation temperature/℃	Sperm motility	Hoechst negative sperm/%	HOST positive sperm/%	Acrosome intact sperm/%	Capacited sperm/%
15.0	84.9±0.3[a]	92.4±1.5[a]	92.0±1.4[a]	95.0±1.0[a]	9.5±3.0[a]
37.0	84.6±3.5[a]	91.7±0.2[a]	90.3±4.5[a]	92.3±5.8[a]	20.7±2.4[b]
38.5	86.3±5.1[a]	90.7±4.1[a]	89.5±5.8[a]	87.2±2.0[a]	55.2±3.5[c]
42.0	63.5±2.5[b]	80.5±0.8[b]	79.4±3.4[b]	73.4±0.9[b]	67.5±5.9[d]

a,b,c,d: Values with common letters in superscripts did not differ significantly ($P > 0.05$).

表4 输卵管上皮和卵丘细胞对山羊精子体外获能的影响

Table 4 Effect of oviductal epithelial cells (OEC) and cumulus cells (CC) on *in vitro* capacitation of goat spermatozoa

Cell type	Sperm motility	Hoechst negative sperm/%	HOST positive sperm/%	Acrosome intact sperm/%	Capacited sperm/%
Control	86.6±2.5[a]	90.4±6.2[a]	90.0±3.5[a]	88.0±6.4[a]	55.8±2.2[a]
OEC	85.3±5.2[a]	92.9±4.5[a]	93.4±6.5[a]	87.7±3.1[a]	71.2±5.0[b]
CC	85.0±1.5[a]	92.4±0.5[a]	93.0±3.8[a]	87.5±2.8[a]	54.5±3.6[a]

a,b,c,d: Values with common letters in superscripts did not differ significantly ($P > 0.05$).

3 讨论

山羊体外获能时肝素使用的剂量很不一致。旭日干等[17]用10μg/mL肝素处理山羊鲜精和冻精;Palomo等[18]和Mogas等[19]添加的肝素浓度是100μg/mL;Izquierdo等[20]使用50μg/mL的肝素获能。我们通过检测精子活力、质膜完整性、顶体完整性和获能率等参数系统研究了肝素浓度、作用时间和温度对山羊精子获能效果的影响。结果表明,肝素可显著提高精子获能率,浓度为50和100μg/mL时获能率最高。但是,当肝素浓度为100μg/mL时,顶体完整率显著下降。因此,山羊精子获能的最佳肝素浓度为50μg/mL。

Fukui等[21]在牛的体外受精实验中发现,在一定肝素浓度下,处理时间超过60min,精子的受精能力显著下降。Gliedt等[22]发现,当牛精子的肝素获能时间增至4h时,体外受精卵裂率显著降低。我们的研究证实,在肝素浓度为50μg/mL时,处理时间超过60min,山羊精子活力、质膜完整性和顶体完整率均显著降低。这可能是获能时间过长造成精子受精能力下降的原因。

研究证明，在 37 ~ 38℃下，很难使绵羊和猪的精子发生获能。但把温度提高到 39℃就可以使这两种动物精子获能[23,24]。本研究发现，37℃时山羊精子获能比率较低，但当温度提高到 38.5℃和 42℃时，精子获能率显著提高。然而，42℃处理时精子活力下降。这种细微温差对获能的影响，可能与精子膜脂的物理状态发生变化[12,13]有关。

Guyader 等[25]发现，单层输卵管上皮细胞可诱导牛精子获能。本实验证明，与单层输卵管上皮细胞共培养能提高山羊精子获能、体外受精和卵裂率，但卵丘细胞的这种作用不明显。本实验发现，在孵育 30min 之内大部分精子就与输卵管上皮细胞发生贴附，尾部运动加速，但与单层卵丘细胞共孵育时则不发生贴附。Li 等[26]发现，兔精子与输卵管上皮共同孵育的最初几分钟内，一些精子就贴附在上皮上。Gutierrez 等[27]发现，绵羊精子在与绵羊输卵管或仓鼠输卵管单层上皮细胞接触时，不超过 1h 就发生贴附，尾部表现出强烈的运动，但与单层猪肾细胞共培养时没有精子贴附现象。Ellington 等[28]提出，牛输卵管细胞与精子在体外接触时会改变精子质膜状态。这些结果说明，输卵管上皮细胞具有独到的促精子获能作用，且此作用需要精子与上皮细胞的接触。虽然有研究证明，单层输卵管上皮细胞可以维持精子活力，但我们的研究并未能证实这一点。

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Studies on *in vitro* Capacitation of Goat Spermatozoa by Heparin Treatment

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Abstract Systematical studies are lacking on the influencing factors and mechanisms of the heparin enhanced sperm capacitation, although many studies have shown that heparin enhanced sperm capacitation. The effect of heparin concentration and exposure time, incubation temperature and co-culture with oviductal epithelial cells or cumulus cells on goat sperm capacitation were investigated in this study. The motility, membrane and acrosome integrity and capacitated percentage of goat spermatozoa were assessed after different heparin treatments, and rates of fertilization and embryo cleavage were compared after *in vitro* insemination of oocytes with spermatozoa capacitated by different heparin treatments. The major results are summarized as follows: 1) When spermatozoa were capacitated with heparin at 5, 10, 25, 50 and 100μg/mL for 45 min, 50 and 100μg/mL heparin treatments produced the highest capacitated percentages of 55% and 56%, respectively, but the percentage of spermatozoa with intact acrosomes in the 100μg/mL heparin treatment decreased significantly ($P < 0.05$) in comparison with that in the control group, indicating that the optimal heparin concentration for goat sperm capacitation would be 50μg/mL. 2) Capacitated percentage of spermatozoa increased with extension of treatment time when goat sperm were treated with 50μg/mL heparin for 0, 10, 20, 30, 45, 60 or 120 min. Although heparin treatments for 45 to 120 min did not differ significantly ($P > 0.05$) in capacitated sperm percentages, sperm motility and membrane integrity decreased significantly when treated with heparin for 120 min. This suggested that the optimal exposure time of heparin at 50μg/mL for goat sperm capacitation would be 45 to 60 min. 3) Significantly higher capacitated percentages of spermatozoa were obtained when goat sperm were treated at 42 and 38.5 ℃ than at 15 and 37 ℃, but sperm motility and acrosome integrity were significantly lower when spermatozoa were treated at 42 ℃ than they were treated at other temperatures. Temperature of 38.5 ℃ would, therefore, be the optimal temperature for goat sperm capacitation. 4) The capacitated percentage of spermatozoa was significantly higher when goat sperm were co-cultured with oviductal epithelial cells than when treated with heparin alone or co-cultured with cumulus cells, but sperm motility and membrane and acrosome integrity did not differ significantly among the three treatments. Rates of fertilization (91.3%) and cleavage (72.2%) were significantly higher in the oviductal epithelial cell co-culture group than those in the heparin alone group. This indicated that co-culture with oviductal epithelial cells significantly enhanced goat sperm capacitation by heparin treatment.

Key words spermatozoa, capacitation, heparin, *in vitro* fertilization, goat

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Purification and Characterization of Recombinant Human anti-HAV Monoclonal Antibody

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Abstract In order to obviate the drawbacks of plasma immunoglobulins, the whole molecular recombinant human anti-HAV (hepatitis A virus) monoclonal antibody (anti-HAV IgG) produced and secreted by rCHO cells was purified and its physicochemical properties were extensively characterized. The rCHO cells were cultured in serum-free medium and the supernatants were collected. The recombinant human IgG molecules were sequentially purified by ultrafiltration, rProtein A Sepharose Fast Flow affinity chromatography, ion exchange chromatography and diafiltration. In affinity chromatography, prior to the target protein elution, an intermediate high salt wash step was inserted, different pH and salt concentrations were evaluated for the capacity of removing host cell DNA. The yield of the downstream purification process was approximately 40%. The purity of anti-HAV IgG thus generated was assayed with SEC-HPLC method, integration result showed that the monomeric IgG content was more than 99%. Western-blot was carried out with AP-antiHuman IgG (Fab specific) and AP-antiHuman IgG (Fc specific) respectively, the blot result demonstrated that the anti-HAV IgG is human antibody with Fab and Fc structure. The specific anti-HAV activity determined by ELISA was 100 IU/mg, with anti-HAV immunoglobulin as the working standard reference. Ligand leakage in the eluate of the affinity column was approximately 32 ng/mg IgG, while after further purification steps, it was decreased to less than 2 ng/mg IgG. Residual host cell DNA was monitored with solid dot blot assay, DNA can be removed effectively with intermediate high salt wash step in the affinity chromatography. Free sulfhydryl content of anti-HAV IgG was assayed with fluorescent spectrophotometer, the low molecular weight bands appeared in non-reducing SDS-PAGE may be caused by the presence of free sulfhydryl. The endotoxin content was less than 1EU/mg examined by standard LAL test procedures. Anti-HAV IgG prepared with this process is able to fulfill the regulatory requirements of State Food and Drug Administration for recombinant products.

Key words recombinant human anti-HAV monoclonal antibody, protein purification, affinity chromatography, residual rProtein A content

Recombinant human or humanized monoclonal antibodies (mAbs) are becoming increasingly important as therapeutic pharmaceuticals in biotechnology corporations, especially in the prevention and therapy of cancer and virus-induced diseases. Hepatitis A virus (HAV) is a wide spread pathogenic agent. In developing countries and in rural areas of developed countries, hepatitis A is still a kind of severe infectious disease, it can cause nearly 200 ~ 300 thousand people infected each year, and sometimes it can even become prevalent in certain area. To date, there is no specific therapeutic pharmaceutical for HAV infection. The biological products usually used clinically are mainly of plasma source, such as immunoglobulin which has been used in clinic for many years[1]. These plasma products may contain some potential undetectable pathogenic agents and the concentration of specific antibody protein is relatively low. To overcome these drawbacks, we have developed a recombinant human anti-HAV monoclonal antibody preparation for pre-exposure prophylaxis of normal and risky people in HAV prevalent area, short term prophylactic immunization of domestic and foreign tourists, and for the treatment of HAV infectious patients.

HAV vaccines are becoming popular recently, including inactivated[2] and attenuated or recombinant[3] products, these vaccines are effective in pre-exposure prophylaxis, but can do little under post-exposure conditions. At this time the passive immunization is needed, so anti-HAV IgG can makeup. We report in this study the establishment of a practical downstream purification process of anti-HAV IgG, and the characterization of the physicochemical properties of the product.

1 Materials and Methods

1.1 Cell culture with serum free medium

The recombinant cell line secreting anti-HAV mAb was provided by professor Liang Mifang[4], Chinese

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Center for Disease Control and Prevention. The anti-HAV mAb is a human derived mAb of IgG1 subtype. The medium used for cell propagation is DMEM (GIBCO) containing 10% FBS and 5×10^{-7} mol/L Methotrexate (MTX), the serum free medium used for production is CCM-5 (Hyclone).

Cells from the cell bank were propagated in a series of T-flasks and finally inoculated into roller bottles, roller bottles and roller bottle apparatus were obtained from BELLCO Corporation. After inoculation, cells were cultured in DMEM for 5 to 7 days, and then the medium was changed to serum free and supernatants were collected and replaced every other day.

1.2 Chromatography equipment

Waters™ 650E Advanced Protein Purification System was purchased from Waters Chromatography Division of Millipore Corporation, GradiFrac FPLC System was obtained from Amersham Biosciences (Uppsala, Sweden).

1.3 Chromatography medium

rProtein A Sepharose Fast Flow, Sephadex G-25 Medium and SP Sepharose Fast Flow were all products of Amersham Biosciences (Uppsala, Sweden).

1.4 Purification process

The cell culture supernatants collected were microfiltered to remove cell debris and then ultrafiltered using membrane with MWCO 50 kD to 10-folds of initial concentration. The concentrated fluid thus produced was loaded on rProtein A SFF column pre-equilibrated with 20 mmol/L PB, pH7.0. After sample loading, the column was first washed with equilibration buffer until the ultraviolet absorbance at 280nm decreased to baseline, then washed with a high salt solution of 20mmol/L PB + 1mol/L NaCl pH8.0 for 4 column volumes. Finally, the anti-HAV mAb was eluted with 100 mmol/L Glycine pH3.0. The eluate was immediately neutralized with neutralization buffer of 2 mol/L Tris·HCl pH7.5.

After buffer exchange using Sephadex G-25 Medium, the sample was loaded on an ion exchange column SP SFF pre-equilibrated with 20 mmol/L NaAc-HAc pH5.2. The target protein was eluted with discontinuous salt gradient steps. The eluate collected was finally diafiltered to the formulation buffer.

1.5 Anti-HAV IgG immunological activity assay

Regular ELISA assay was carried out, with inactivated HAV as the antigen coating solution, and anti-HAV immunoglobulin as working standard reference (76 IU/mL, National Institute for the Control of Pharmaceutical and Biological Products).

1.6 Protein concentration assay

The Bradford method was used according to the method described in reference [5].

1.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The reducing and non-reducing SDS-PAGE were run according to the method outlined in reference [6].

1.8 Size exclusion chromatography (SEC-HPLC)

The purity of anti-HAV IgG was measured by gel filtration on an analytical column of Superdex 200 HR 10/30 (V_t = 23.6 mL, Amersham Biosciences, Uppsala, Sweden) run on Waters HPLC system (Waters Associates, Milford, USA). The column was equilibrated with 100 mmol/L PB, pH7.0, the flow rate was 0.5 mL/min, the volume of sample loaded was 50μL, the monitored wavelength was 280nm.

1.9 Western blot

After non-reducing SDS-PAGE, the protein was electrotransfered from the polyacrylamide gel to nitrocellulose membrane. The membrane was incubated with AP-antiHuman IgG (Fab specific) and with AP-antiHuman IgG (Fc specific) respectively, then substrate was added for color development.

1.10 Isoelectric focusing

PHARMACIA PHAST System and precast IEF 3-9 gel (all were obtained from Amersham Biosciences, Uppsala, Sweden) were used, the gel was silver stained after focusing. The focusing of the gel and subsequent staining were performed according to the recommended procedure of the manufacturer.

1.11 Residual DNA content assay

Total DNA extraction from host cells was carried out following the steps outlined in reference [7].

Solid dot blot assay was used to monitor the residual DNA using DIG High Prime Labeling and Detection Starter Kit (Boehringer Mannheim). The assay was conducted following the kit instruction manual.

1.12 Residual rProtein A content assay

Protein A ELISA Kit (IMMUNSYSTEM AB, Uppsala, Sweden) was used. The assay was executed in accordance with the kit instruction manual.

In the presence of IgG, the Protein A content assay may be interfered to some extent. To avoid this disturbance, the standard references of Protein A were made with known amounts of IgG. The IgG solution added to the standard references was Human Immunoglobulin for Intravenous Use purchased from Liuzhou Biological Product Division of Guangxi Beisheng Pharmaceutical Corporation. The IgG concentrations in standard references and in samples were all adjusted to 0.25 mg/mL.

1.13 Free sulfhydryl assay

The assay was done according to the method described in reference [8]. Free sulfhydryl was fluorescent derived and then assayed with HITACHI F-2500 fluorescent spectrophotometer.

1.14 Endotoxin test

The Tachypleus Amebocyte Lysate was from Zhanjiang A&C Biological Ltd, the sensitivity was 0.25 EU/mL. Standard LAL test procedures were followed in accordance with Chinese Pharmacopoeia.

2 Results

2.1 Purification process

2.1.1 rProtein A SFF affinity chromatography: In the

intermediate high salt wash step before the target protein elution, we had tested with different pH and salt concentrations, i.e. 20 mmol/L PB + 0.5 mol/L NaCl pH7.0, 20 mmol/L PB + 1 mol/L NaCl pH7.0, 20 mmol/L PB + 0.5 mol/L NaCl pH8.0 and 20 mmol/L PB + 1 mol/L NaCl pH8.0. The flow through solution under each wash condition was collected and residual DNA content was assayed. In respect to the salt concentration, 1.0 mol/L NaCl gave much better results than 0.5 mol/L NaCl in removing host cell DNA, while raising the salt concentration to 1.5 mol/L gave no further improvement; in respect to pH, pH8.0 gave better results than pH7.0.

2.1.2 SP SFF ion exchange chromatography：The eluate of rProtein A SFF was processed on Sephadex G-25 for buffer exchange, then loaded on SP SFF column pre-equilibrated with 20mmol/L NaAc-HAc pH5.2. A continuous salt gradient from 0 to 1 mol/L NaCl was applied and anti-HAV IgG was eluted in the place of about 0.3 mol/L NaCl. To get higher purity, we used 0.15 mol/L salt concentration to wash out traces of the impurities, then applied 0.3 mol/L salt to elute anti-HAV IgG.

2.2 Purity assay

2.2.1 SEC-HPLC：Anti-HAV IgG produced from the purification steps was assayed with SEC-HPLC, the monomeric IgG content was more than 99% (the small peak in the profile represented solvent in sample) (Fig. 1).

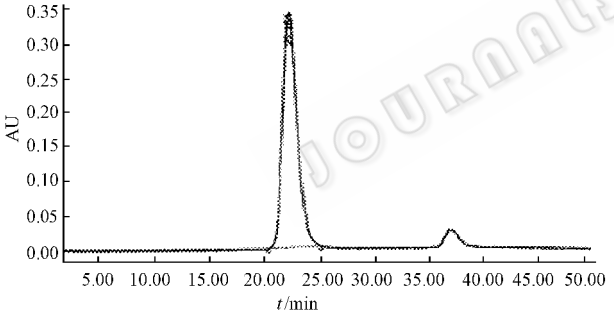

Fig. 1 SEC-HPLC analysis of purified anti-HAV IgG
The small peak at retention time of 37min represented solvent, was induced by the buffer used in anti-HAV IgG sample

2.2.2 SDS-PAGE：The reducing SDS-PAGE assay of anti-HAV IgG revealed two distinct bands corresponding to heavy and light chain respectively (Fig. 2). While in non-reducing SDS-PAGE, except the main band corresponding to 150 kD, there were still several regular faint bands below (Fig. 2).

2.2.3 Isoelectric focusing：The isoelectric point of IgG is relatively high since there are abundant basic amino acids in IgG molecule. The pI of anti-HAV IgG was not homologous, but distributed in a range of 8.4 to 9.3, the distribution range of samples prepared from different batches was identical (Fig. 3).

2.2.4 Western blot：Anti-HAV IgG can bind specifically with AP-antiHuman IgG (Fab specific) and AP-antiHuman IgG (Fc specific) respectively, demonstrating that anti-HAV IgG contains human IgG Fab and Fc structure, is human whole molecule antibody.

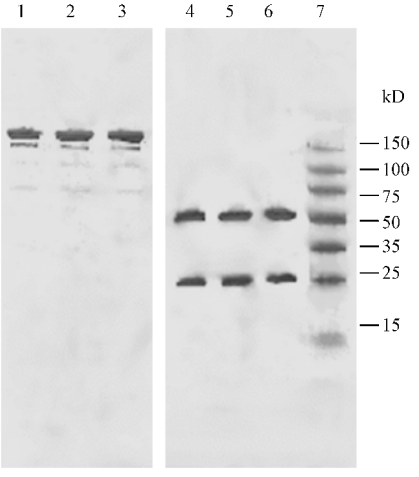

Fig. 2 Reducing and non-reducing SDS-PAGE analysis of purified anti-HAV IgG (10% ~ 15% gradient gel)
1,2,3：purified anti-HAV IgG, non-reduced;
4,5,6：purified anti-HAV IgG, reduced;
7：molecular weight standard (Sigma, Product No. M 0671)

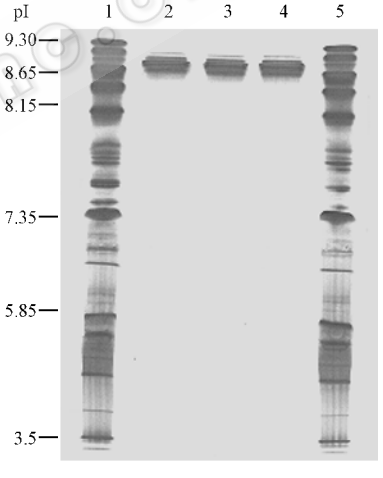

Fig. 3 IEF analysis of anti-HAV IgG (precast IEF3-9 gel)
1,5：pI calibration marker (Amersham biosciences);
2, 3, 4：purified anti-HAV IgG

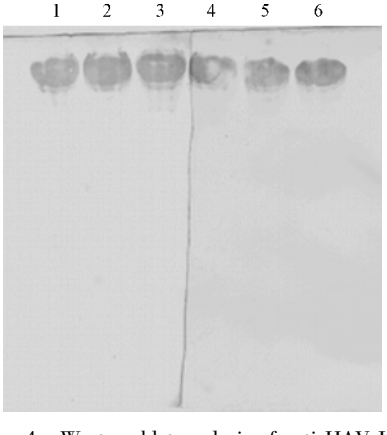

Fig. 4 Western blot analysis of anti-HAV IgG
1,2,3：incubated with AP-antiHuman IgG (Fc specific);
4,5,6：incubated with AP-antiHuman IgG (Fab specific)

2.2.5 Free sulfhydryl content: Free sulfhydryl content of anti-HAV IgG determined in nondenatured condition (in phosphate buffered saline) was 0.2 mol/mol, in denatured condition (6mol/L guanidine hydrochloride) was 0.7 mol/mol.

2.2.6 Host cell DNA content: Residual DNA content of anti-HAV IgG was less than 50 pg/mg. In rProtein A SFF affnity chromatography, high concentration of host cell DNA could be detected in flow through fluids during the intermediate high salt wash step.

2.2.7 Residual rProtein A content assay: During the purification process, the rProtein A content of anti-HAV IgG differed significantly. The average level of rProtein A content in eluate of rProtein A SFF was 32 ng/mg IgG, while after ion exchange chromatography, the content decreased to below 2 ng/mg IgG.

2.2.8 Endotoxin content assay: The endotoxin content of anti-HAV IgG preparation was less than 1EU/mg.

2.3 Immunological activity assay

The specific immunological activity of purified anti-HAV IgG was 100 IU/mg. The yield of anti-HAV activity in purification process was in average 40%.

The specific activity and yield of every process step was listed in table 1.

Table 1 The specific activity and yield of every process step

Process step	Specific activity/(IU/mg)	Purification factor	Yield/%
Cell culture supernatant	12	1	100
Ultrafiltration concentration	13	1.1	92
Affinity chromatography	95	7.9	65
Sephadex G-25 desalting	95	7.9	62
Ion-exchange chromatography	100	8.3	50
Diafiltration	100	8.3	40

3 Discussion

Protein A affinity chromatography is the principal method for purification of antibodies mainly due to the high selectivity and recovery. But the affinity ligand Protein A may sometimes leak from the column[9] and contaminate the preparation. Immunoglobulins react with Protein A *in vivo* and may cause anaphylactic reactions. Contamination of Protein A may also cause false results in immunological assays. Thus Protein A ligand leakage into the elution pool is a major issue in the pharmaceutical industry.

From the result of our study, the Protein A ligand leakage did exist in a relatively high level (about 32ng/mg IgG), but most of the leaked affinity ligand could be cleared from the antibody preparation by the followed purification steps (to the level of less than 2 ng/mg IgG). To date, there is no defined limitation requirement from SDA as to the Protein A content in recombinant antibody product that is destined for use in clinical applications, the secure content range should be acquired depending on the accumulation of animal experiment results.

As to the formation of low molecular weight bands in non-reducing SDS-PAGE, there are several kinds of explanations[10, 11]. We think it is more reasonable to explain this phenomenon as arising from the presence of free sulfhydryl in antibody molecules[8]. It 's well known that IgG mAbs are composed of two heavy and two light chains covalently linked by interchain disulfide bonds, and each domain of the heavy or light chain contains one additional disulfide bond. Native IgG mAbs with completely formed disulfide bonds should not bear any free sulfhydryl. But during the culture process and/or protein folding process in the endoplasmic reticulum, the formation of some mAbs containing free sulfhydryl may occur, and the majority of these sulfhydryls reside within a solvent-inaccessible region. So under nondenaturing conditions such as in SEC-HPLC, the IgG molecules containing free sulfhydryl can maintain intact four-chain structure through the strong non-covalent interchain interactions and seem to be homologous, while under denaturing conditions such as in SDS-PAGE, the non-covalent interaction was disrupted and the chains without disulfide bond linkage become dissociated, so faint bands with low molecular weights were revealed. This can be verified from free sulfhydryl determination. Under nondenaturing condition (in PBS solution), the free sulfhydryl content was 0.2 mol/mol IgG, while under denaturing condition (6mol/L guanidine hydrochloride), the content of detected sulfhydryl was 0.7 mol/mol IgG. The value was much higher than that of previously reported by Zhang Wei *etc*[8], the reason for this difference calls for further investigation.

The isoelectric points of antibody molecules are usually high, which is characterized by the amino acids composition. Within the stabilizing pH range, the IgG molecules usually charged with abundant cations, and can bind strongly with negatively charged endotoxin and nucleic acid. In large scale purification process of recombinant mAbs, the usually used reagent for DNA removing is endonuclease (Benzonase). But Benzonase is expensive and the residual Benzonase may become a new contaminant of mAbs, since there has been evidence that Benzonase can induce chromosomal aberrations in CHO cells[12,13]. In order to clear endotoxin and nucleic acid cost-effectively, we introduced the high salt wash step to disrupt the static interaction between the IgG and endotoxin or nucleic acid. This wash step was very effective, the level of residual host cell DNA left in anti-HAV IgG was much lower than required by SDA, and the endotoxin level could meet the requirement of SDA for injection pharmaceuticals.

The purity index of anti-HAV IgG produced with above process could fulfill the criteria of SDA. From ELISA results that the specific immunological activity of anti-HAV IgG was about 100 IU/mg, we can predict that 2 mg of anti-HAV IgG will be enough to provide full protection for adult. All these data demonstrated that we could get anti-HAV IgG with high purity and high activity.