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# 兔组织工程同种异体气管支架三种制备方法的比较

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**摘要 目的:**探讨深低温冷冻-酶洗法制备的气管支架在去除抗原性、维持生物力学及保护细胞外基质方面的效果。**方法:**健康新西兰兔24只随机分为气管未处理作为对照A组,深低温冷冻法处理B组,玻璃化法处理C组,深低温冷冻-酶洗法D组,各组样本数均为6。处理后将各组标本行HE染色后光镜观察,戊二醛固定后电镜扫描,并测量气管最大拉伸力、破裂力和变异率等生物力学性能。**结果:**组织学观察显示对照A组有大量完整的粘膜上皮细胞;B组和C组可见部分气管粘膜上皮细胞;D组标本未见气管粘膜上皮细胞,且细胞核碎裂。电镜显示A、B、C、D组气管支架可见丰富的细胞基质,未暴露胶原纤维。组间两两比较,气管支架的最大拉伸力、最大破裂力和变异率均无统计学差异。**结论:**综合组织学、扫面电镜和生物力学分析,应用深低温冷冻-酶洗法制备气管支架D组可以有效地去除抗原,维持生物力学性能,并具有较完整的细胞外基质。

**关键词:**深低温冷冻法;玻璃化法;深低温冷冻-酶洗法;组织工程气管支架;细胞外基质

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# Three Preparation Methods of the Rabbit tissue Engineering Allogeneic Tracheal Stent

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**ABSTRACT Objective:** Although it has been proved that detergent-enzymatic is a proper method to obtain non-immunogenic tracheal matrices, it could not maintain allograft tissue engineering prefectly and protect biomechanics extra cellular matrix completely. This study mainly focused on finding out the optimal method to obtaine rabbit histological engineering tracheal matrix. **Methods:** 24 adult rabbits were divided into 4 groups (each n=6), group A were not treated as control group,groupB was treated with the method of cryopreservation, group C was treated with vitrificational method, group D was treated with the method of cryopreservation-detergent-enzymatic. HE dyes and scanning electron microscopy were used to observe the morphology and ultrastructure of the treated tracheal matrices. The biomechanical properties including maximum tensile force, rupture force and aberration rate of the treated tracheal matrices were measured. **Results:** There were a lot of epithelial cells in the tracheas of group A and some epithelial cells in the tracheas of group B and group C. But the tracheas of group D could not be seen complete epithelial cells. Under scanning electron microscopy, there were abundant extracellular matrix and collagen fiber in group A, B, C and D. Finally, no statistical differences were found in the maximum tensile force, rupture force and aberration rate of all groups. **Conclusion:** The method of cryopreservation-detergent-enzymatic, by which the antigen can be removed, and extracellular matrix and biomechanical properties can be maintained effectively, is a better way to prepare tissue engineering tracheal matrix.

**Key words:** Allograft tissue engineering; Detergent-enzymatic method; Vitrificational method; Cryopreservation-detergent-enzymatic; Biomechanics extra cellular matrix (SEM)

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## 前言

随着气管外科技术的发展,同种异体组织工程气管由于其免疫排斥和材料来源等方面的优势,已成为气管重建外科的研究热点。气管支架是组织工程重建外科的关键技术之一,制备同种异体组织工程化气管支架较常用方法有有深低温冷冻法[1,2]、玻璃化法[3]和酶消化法[4,5],但都有自身的缺陷。本实验探讨深低温冷冻-酶洗法处理后的气管支架在生物力学、去除抗原性及气管细胞基质保护方面,与深低温冷冻法和玻璃化法有无差别。

## 1 材料和方法

### 1.1 实验材料

健康新西兰白兔购自中国人民解放军第四军医大学实验动物中心,脱氧胆酸钠购自北京奥博星生物技术有限责任公司,脱氧核糖核酸酶-Ⅰ(DNase-Ⅰ)购自德国Roche公司,两性

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霉素 B 购自美国 Amresco 公司,M199 培养基购自 Gibco 公司、二甲基亚砜购自 Gibco 公司、乙二醇购自 Gibco 公司、蔗糖购自Gibco 公司、乙酰胺购自 Gibco 公司、小牛血清白蛋白购自 Gibco 公司、青霉素购自哈药集团制药总厂。蔡司正置显微镜 Axioskop 40 购自德国 ZEISS 公司，钨灯丝扫描电镜 VEGA3 LMH 购自捷克 Tescan 公司，电子实验机购自日本 Shimadzu 公司。

### 1.2 方法

**1.2.1 实验动物分组** 健康新西兰兔 24 只，雌雄不限，体重 2.5～3.0kg,购自第四军医大学实验动物中心。将其随机等分为 4 组,每组 6 只。A 组是对照组,行新鲜气管检测；B、C、D 是实验组,分别进行玻璃化液冷冻法、酶洗法、深低温冷冻 - 酶洗法处理。

**1.2.2 获取气管** 耳缘静脉空气栓塞致死，无菌条件下取出气管,迅速剥离气管上的疏松结缔组织。经生理盐水冲洗,并在 200000 U/L 青霉素、200 mg/L 庆大霉素溶液中浸泡 3 min。

**1.2.3 标本制备** 玻璃化液冷冻法制备 B 组气管支架：以 M199 培养基溶液为基础配置冻存液,加入 3.2 mol / L 二甲亚砜、3.2mol / L 乙二醇、0.5 mol / L 蔗糖和 2.5 mol / L 乙酰胺,于超净台中滤过除菌后,加入 20%小牛血清白蛋白、100 U / ml 青霉素、100 mg / L 链霉素和 2.5 mg / L 二性霉素 B,4℃ 预冷。将获取气管在依次在 50%、75% 、85% 、95% 的梯度玻璃化冻存液 (4℃ )中冷平衡 8～10 min 后转入 100%玻璃化冻存液中冷平衡 30 min,迅速投入液氮中保存。4 周后投入 37℃ 恒温水浴箱中 2 min 使其复温，先移入 4℃ 含 1.0 mol / L 蔗糖的解冻液中反复冲洗约 5 min，后移至含 20%小牛血清的 M199 溶液反复冲洗后浸泡于 PBS 缓冲液中准备检测。酶洗法制备 C 组气管支架：将获取的气管置入 4℃ 纯净水中浸泡 48 小时后,在含有 4%去氧胆酸钠生理盐水中浸泡 3 小时，然后在含有 DNase-I2000KU/L 的生理盐水中浸泡 3 小时,如此循环 3 个周期,然后浸泡在 4 ℃ 的 PBS 溶液中准备检测。深低温冷冻 - 酶洗法制备 D 组气管支架：气管支架前期处理同 B 组气管支架,至 -80℃ 后投入 -196℃ 液氮冷冻 6 周。冷冻后将气管在含有 4% 去氧胆酸钠生理盐水中浸泡 3 小时，再在含有 DNase-I2000KU/L 的生理盐水中浸泡 3 小时,然后浸泡在 4 ℃ 的 PBS 溶液中准备检测。

**1.2.4 生物力学检测** 用直尺测量 A、B、C、D 组气管的长度；参照 Macchiarini 等生物力学检测标准,将 A 组气管与 B、C、D 组制备好的气管支架固定在电子实验机上，测出最大拉伸力、破裂力、破裂点,并算出变异率。

**1.2.5 组织学显微镜观察粘膜上皮细胞** 将处理后的气管在福尔马林中浸泡 24 h,行脱水、浸纳、蜡块包埋、切片处理后,HE 染色,光镜观察。

**1.2.6 扫面电镜观察细胞外基质完整性** 戊二醛固定 A 组气管和处理后的 B、C、D 组气管支架,梯度脱水,然后用 8。

**1.2.7 统计学分** 采用 SPSS 13.0 软件进行统计分析,定量数据以均数± 标准差 ($\bar{x}$± s) 表示，组间比较采用单因素方差分析 (ANOVA),以 $P<0.05$ 为差异有统计学意义。

## 2 结果

### 2.1 生物力学检测

各组气管长度均数不相等,差异无统计学意义($P>0.05$)。组间两两比较显示,最大拉伸力、破裂力、变异率无统计学意义($P>0.05$)(表 1)。

**表 1 气管支架生物力学比较**

Table 1 Biomechanical Comparison of tracheal stent

| 组 别 Groups | 气管长度 Pipe length | 最大拉伸力(N) The maximum tensile force | 破裂力(N) Breakout Force | 破裂点(cm) Breaking point | 变异率(%) Mutation rate |
|---|---|---|---|---|---|
| 对照组 A The control group A | 5.00± 0.61 | 4.485± 0.55 | 0.665± 0.070 | 9.74± 0.44 | 200± 10 |
| 实验组 B Experimental group B | 4.88± 0.58 | 4.445± 0.45 | 0.635± 0.072 | 9.68± 0.52 | 196± 08 |
| 实验组 C Experimental group C | 4.98± 0.58 | 4.475± 0.50 | 0.625± 0.066 | 9.78± 0.72 | 198± 12 |
| 实验组 D Experimental group D | 5.02± 0.60 | 4.455± 0.40 | 0.650± 0.072 | 9.88± 0.66 | 197± 10 |

### 2.2 组织学显微镜观察粘膜上皮细胞

气管粘膜上皮组织有大量完整的粘膜上皮细胞,成软骨细胞密集均匀分布于气管软骨(图 1a)；气管粘膜上皮组织仍有完整粘膜上皮细胞(图 1b)；气管粘膜上皮组织仍有完整粘膜上皮细胞(图 1c)；气管粘膜上皮组织未见完整的粘膜上皮细胞,可见一些残存的细胞基质(图 1d)。

### 2.3 扫面电镜观察细胞外基质完整性

气管粘膜上皮组织见丰富细胞外基质，均未见胶原纤维(图 2a、2b、2c、2d)。

## 3 讨论

气管支架是构建组织工程气管的关键环节[4-9]。理想的气管支架应满足以下条件：管腔密封不漏气；易弯曲成形,且不致塌陷；具有良好的组织相容性,能与宿主组织紧密结合；允许呼吸道上皮细胞沿管腔生长；内壁光滑,防止成纤维细胞和细菌的侵入；引起的炎性反应最小,无致癌性[10-16]。同种异体气管支架被公认为组织工程气管理想的气管来源,但是其免疫排斥影响组织工程再细胞化和再血管化,必须通过适当的处理去除免疫

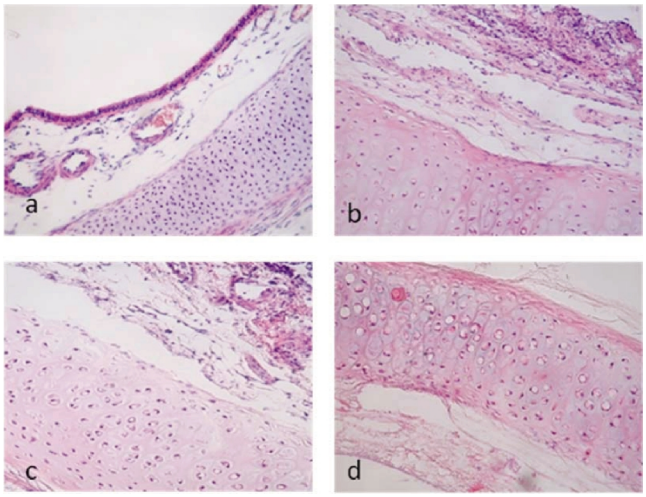


图 1a、图 2b、图3c、图4d

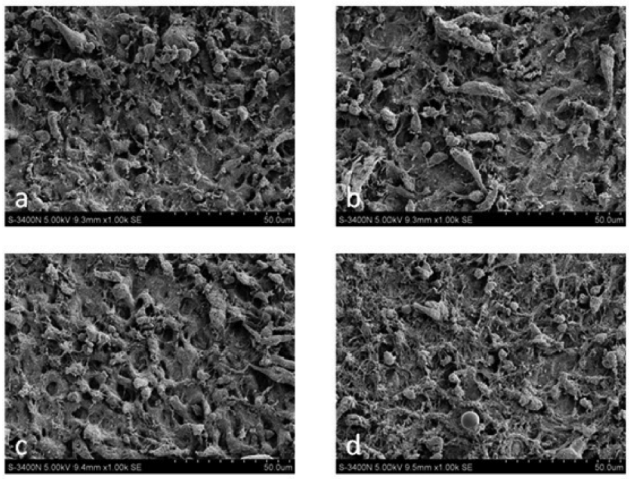


图 2a、2b、2c、2d

原性，保持力学性能，保护细胞外基质[17]。深低温冷冻 - 酶洗法是制备同种异体气管支架的一种有效方法，Macchiarini 等运用酶洗法制备气管支架完成了世界首例同种异体组织工程气管移植，为气管重建指明了方向[18]。然而，患者移植后 8 个月发生气管塌陷，说明酶洗法制备气管支架仍需改进[19]。我国学者李建忠[20,21]等已证实深低温冷冻 - 酶洗法较单纯酶洗法制备的兔气管支架更适合构建组织工程气管。玻璃化法是制备组织工程气管支架的另一种常用方法，但是存在不能完全去除抗原，影响组织工程气管的再细胞化和再血管化[22]。

本研究是用深低温冷冻法、玻璃化法和深低温冷冻 - 酶洗法处理兔气管。行组织学检查，结果示经过深低温冷冻 - 酶洗法获得的气管支架未见完整的上皮粘膜细胞，并且细胞核破碎。由此可以推断，深低温冷冻 - 酶洗法与深低温冷冻法、玻璃化法相比较，深低温冷冻 - 酶洗法制备的气管支架在生物力学和保留细胞外基质方面未见明显差别，但前者较后者能够更彻底的去除气管支架免疫原性。因此，经过深低温冷冻 - 酶洗法较经过深低温冷冻法、玻璃化法获得的气管支架更适合于组织工程同种异体气管的制备，可能更有助于气管粘膜上皮细胞和成软骨细胞的再细胞化，以及促进供体气管的再血管化。

综上所述，深低温冷冻 - 酶洗法获取的气管支架更适合于制备组织工程同种异体气管，其实际效果有待于动物实验的进一步研究。

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