·基础研究·

兔椎间盘器官与脊柱运动节段离体培养条件下髓核组织的变化

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【摘要】 目的:比较离体培养的兔椎间盘器官及脊柱运动节段两种模型椎间盘髓核组织的变化。**方法**:将21只新西兰白兔随机分为器官组8只,节段组13只,处死后在无菌条件下分别取出椎间盘器官和脊柱运动节段各50个,在高渗培养基中进行整体培养(410 mOsm/kg),于培养前及培养后第3、7、14、21天,两组各取10个椎间盘分别进行HE染色、II型胶原免疫组化、蛋白多糖含量和髓核细胞活力测定。**结果**:培养21 d器官组与培养14 d节段组HE染色示椎间盘组织结构基本保持完整,21 d节段组椎间盘组织形态学破坏;21 d器官组与14 d节段组Ⅱ型胶原免疫组化染色强度差异无统计学意义(P>0.05),21 d节段组染色变浅,与之前各时间点及器官组相比差异有统计学意义(P<0.05);蛋白多糖PAS/AB染色7 d内两组强度无降低,14 d两组强度均有所减弱,21 d节段组染色强度进一步减弱,改变比器官组更为明显;髓核细胞荧光检测两组7 d时强度较培养前变化不明显(P>0.05),21 d器官组与14 d节段组强度略有降低,但与之前时间点比较差异无统计学意义(P>0.05),21 d节段组髓核细胞荧光强度减弱,细胞活性降低,与之前各时间点及器官组比较差异明显(P<0.05)。**结论**:14d内脊柱运动节段可作为研究生物力学对椎间盘影响的离体实验模型。

【关键词】 椎间盘; 脊柱; 器官培养技术; 兔
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Changes of nucleus pulposus after in vitro culture of rabbit whole intervertebral disc and spinal motion segment

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ABSTRACT　Objective: To compare the changes of nucleus pulposus after in vitro culture of rabbit whole intervertebral disc and spinal motion segment. **Methods**: Twenty-one New Zealand white rabbits which were randomly divided into organ group with 8 rabbits and segment group with 13 rabbits. Fifty intervertebral discs and 50 spinal motion segments were harvested respectively under aseptic conditions from two groups. These specimens were maintained in organ culture with hyperosmotic media (410 mOsm/kg), then 10 discs of the two groups were observed respectively by HE staining, immunohistochemistry of collagen type Ⅱ, proteoglycan content and cells viability of nucleus pulposus before culture and at 3, 7, 14, 21 days after culture. **Results**: HE staining showed the intervertebral disc tissue structure remained intact after culture of 21 days organ group and 14 days segment group, but there was severely degenerated of 21 days segment group. The intensity value of type Ⅱ collagen immunohistochemical staining in the nucleus pulposus were not changed significantly between 21 days organ group and 14 days segment group (P>0.05), but the staining of segment group at 21 days became shallower, there was significant difference compared with before each time points and organ group at 21 days (P<0.05). PAS/AB staining of proteoglycan of nucleus pulposus showed that there were not decrease of tinting strength of two groups within 7 days, but the strength weakened slightly of two groups at 14 days, and the tinting strength became weaker at 21 days segment group, the change is more obvious than the organ group. The intensity value of fluorescence staining of nucleus pulposus cells was not changed significantly within 7 days of two groups (P>0.05), the intensity value decreased slightly at 21 days organ group and 14 days segment group, but there were no significant difference compared with before time points (P>0.05), however at 21 days segment group the intensity decreased as cells viability of nucleus pulposus decreased, and there was a significant difference compared with before each time points and organ group at 21 days (P<0.05). **Conclusion**: It is not obviously degenerated of the discs of organ group cultured within 21 days and segment group cultured within 14 days, but there was significant degeneration of the intervertebral

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disc of segment group after cultured 21 days, so the rabbit spinal motion segment can be used on research about the biomechanics of intervertebral disc as a vitro experimental model within 14 days.

KEYWORDS　Intervertebral disc；　Spine；　Organ culture techniques；　Rabbits

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椎间盘退变与下腰痛有很大的联系，尽管对椎间盘退变有大量的研究，但对生物和机械途径综合导致椎间盘退变的机制仍不清晰[1],因此构建理想的实验模型对椎间盘退变的发病机制和临床研究有非常重要的意义。将完整的椎间盘在体外进行整体培养，能够在可控的条件下研究椎间盘代谢和退变的相关机制，为观察椎间盘对外界刺激的反应和正常与退变椎间盘的组织学变化创造了良好的实验平台[2-9]。在此基础上,相关研究[10-11]离体培养了脊柱运动节段模型,包括整个椎间盘器官连同椎体,使模型一方面更接近体内环境，另一方面为加载装置提供了固定的施力处，为进一步控制和研究生物力学对椎间盘的影响提供条件。本实验拟比较离体培养的兔椎间盘器官及脊柱运动节段两种模型的椎间盘活性及功能，旨在探讨离体培养连同椎体的椎间盘器官能否达到同不带椎体的椎间盘器官相似的效果，从而为进一步研究椎间盘的生物力学与干预机制提供依据。

1　材料与方法

1.1　实验动物

4~6 月龄健康新西兰兔 21 只,雌雄不限,体质量 2.5~3 kg（中国中医科学院实验动物中心提供),根据随机数表法分为器官组 8 只,节段组 13 只。

1.2　实验方法及步骤

实验新西兰兔麻醉后耳缘静脉给予肝素钠，5 min 后空气栓塞处死,带入超净工作台,立即在无菌条件下自背部纵切口，自尾部完整取出腰段及下位胸段脊柱,入高渗肝素 PBS 液冲洗。在 2.5 倍放大镜下应用咬骨钳、手术刀片切取，其中器官组切取 $T_{12}L_1$-L_6S_1(7 节段)椎间盘器官,最大限度剔除椎骨,自软骨终板与骨性终板结合处分离,得到 50 个完整椎间盘器官,包括髓核(NP)、纤维环(AF)及上下软骨终板(EP)；节段组切取 $T_{12}L_1$、$L_{2,3}$、$L_{4,5}$、L_6S_1(4 节段)椎间盘运动节段,自相邻椎间盘的软骨终板与骨性终板结合处锐性分离,得到 50 个完整椎间盘运动节段,包括髓核(NP)、纤维环(AF)及上下软骨终板(EP)及相邻椎体(VB)。用带有 18 号针头的无菌注射器吸取含肝素的高渗 PBS 液冲洗标本表面的碎屑及终板上的血凝块,于含肝素的 HBSS 液(含 1 000 U/ml 青霉素,1 mg/ml 链霉素）中漂洗 2 min 后放入 6 孔板中。

两组 6 孔板中予细胞培养液 DMEM(含有 20% 胎牛血清、25 μg/ml 抗坏血酸、50 mg/ml 庆大霉素，并用 NaCl 将细胞培养液的渗透压调整到 410 mOsm/kg[9])。两组均置于 5% CO_2、37 ℃恒温培养箱进行整体培养,每 2 d 更换培养液。分别在培养前(0 d)和培养后第 3、7、14、21 天,两组各取 10 个椎间盘分别进行椎间盘组织学观察、Ⅱ型胶原免疫组化、蛋白多糖含量和髓核细胞活力测定。

1.3　观察项目与方法

1.3.1　组织学观测　每个时间点两组取椎间盘 5 个,10%甲醛固定,EDTA 脱钙,石蜡包埋,切取一半行连续切片,厚 4~6 μm,另一半行免疫组化检测,切片应用标准 HE 染色方法观察两组椎间盘的组织学变化。

1.3.2　免疫组织化学染色　每个时间点用 5 个组织学观测中的另一半椎间盘，使用Ⅱ型胶原抗体对髓核组织切片行免疫组织化学染色观察,应用 NIS-Elements D2.30 图像分析系统对Ⅱ型胶原免疫组织化学染色切片行Ⅱ型胶原半定量分析。

1.3.3　髓核蛋白多糖检测　每个时间点应用组织学观测中的 5 个椎间盘，切片后常规脱蜡，脱水入水,3% 醋酸水溶液(pH2.5)略漂洗,阿利新蓝染液(pH2.5)染 5 min,流水冲洗后入蒸馏水,1%高碘酸水溶液 5 min,蒸馏水充分漂洗,雪夫试剂 15 min,流水冲洗 10 min,苏木精淡染后水洗使胞核显淡蓝色,无水酒精冲洗及脱水,二甲苯透明,中性树胶封片,倒置显微镜下观察拍片。

1.3.4　细胞活性检测　每个时间点各组取椎间盘 5 个,尖刀切开椎间盘纤维环,用无菌小刮匙将髓核刮出至含有 5 μmol/L Mitotracker Green 工作液的无血清培养基中,在 37 ℃培养箱中染色 1 h,离心并倾倒上清液,用不含荧光染料的无血清培养液吹洗 2 次,在新鲜无血清培养基中培育 1 h,倾倒上清液并用 PBS 吹洗 2 遍后，将组织浸入载玻片上的 PBS 液中,盖上盖玻片。荧光显微镜进行观察,髓核组织中有活性的细胞线粒体被 Mitotracker Green 染色而发出绿色荧光。采用 NIS-Elements D2.30 图像分析系统进行灰度值比较。

1.4　统计学方法

应用 SPSS 16.0 统计学软件，计量资料以均数±标准差 $\bar{x}\pm s$ 表示,组间比较采用单因素方差分析,两组数据之间比较采用 t 检验。以 $P<0.05$ 为差异有统计学意义。

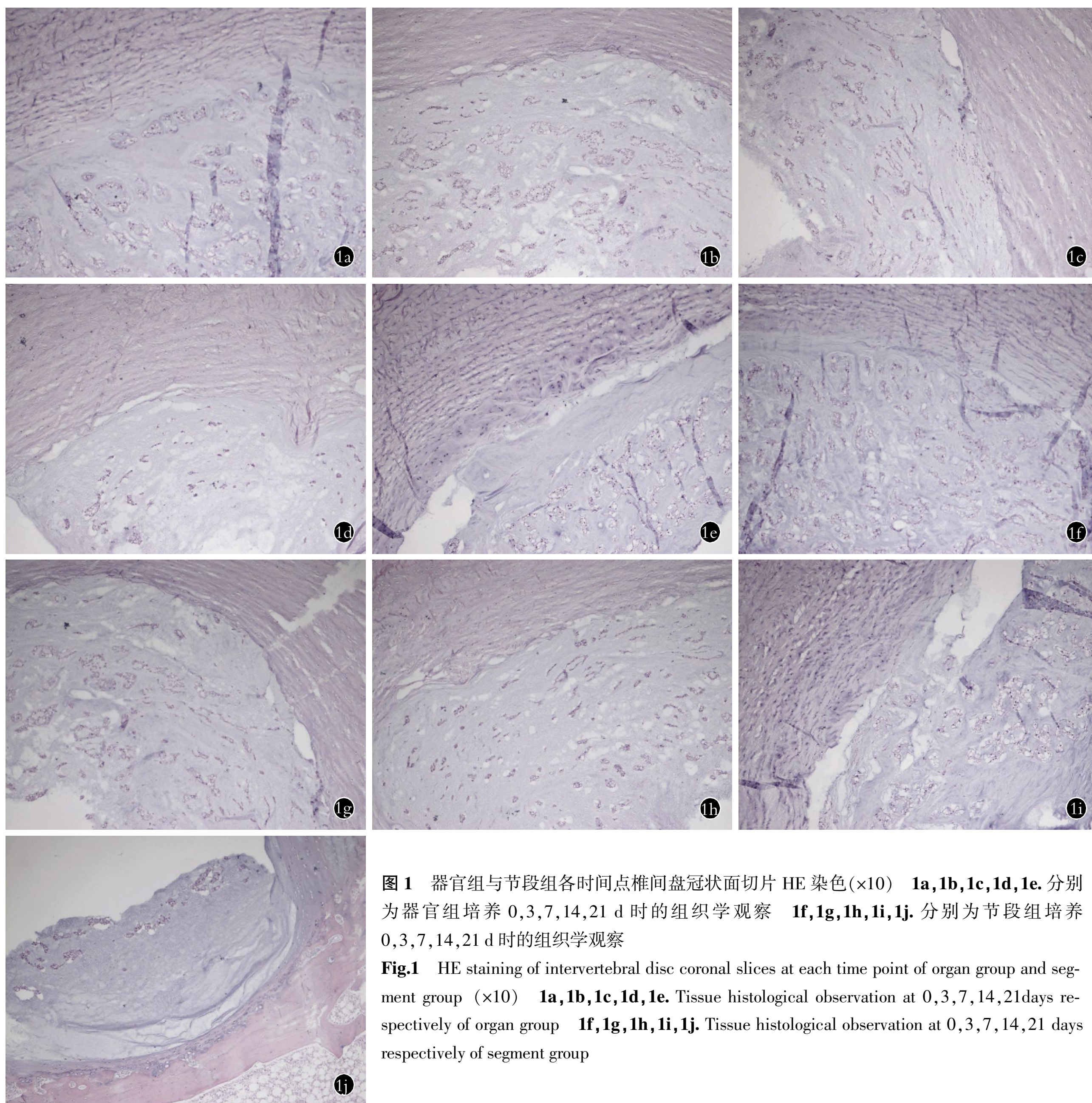

图 1 器官组与节段组各时间点椎间盘冠状面切片 HE 染色(×10) **1a,1b,1c,1d,1e.** 分别为器官组培养 0,3,7,14,21 d 时的组织学观察 **1f,1g,1h,1i,1j.** 分别为节段组培养 0,3,7,14,21 d 时的组织学观察

Fig.1 HE staining of intervertebral disc coronal slices at each time point of organ group and segment group (×10) **1a,1b,1c,1d,1e.** Tissue histological observation at 0,3,7,14,21days respectively of organ group **1f,1g,1h,1i,1j.** Tissue histological observation at 0,3,7,14,21 days respectively of segment group

2 结果

2.1 组织学观察

器官组培养至 14 d 椎间盘髓核和纤维环结构完整致密、分界清楚、髓核组织染色均匀、胞核明显、均匀分布,培养 21 d 髓核与纤维环轻度分离、完整性仍好; 节段组培养至 7 d 基本有效维持了椎间盘髓核和纤维环出现部分裂伤,培养 21 d 纤维环排列紊乱、与髓核组织明显分离、髓核细胞数目减少、轻度分散、组织结构分解,与器官组相比有明显改变(图 1)。

2.2 免疫组织化学观察

培养 21 d 器官组及培养 7 d 节段组髓核细胞及基质组织内Ⅱ型胶原免疫组织化学染色均呈均匀的棕褐色阳性反应(图 2),经图像分析软件测得平均灰度值,差异无统计学意义(P>0.05);培养 14 d 节段组棕褐色染色不均匀,但染色强度与培养前及培养 14 d 器官组相比差异无统计学意义(P>0.05);培养 21 d 节段组染色变浅,与之前各时间点及同时间点器官组比较差异均有统计学意义(P<0.05),见表 1。

2.3 蛋白多糖检测

PAS/AB 染色法中酸性黏多糖呈深蓝色,中性黏多糖呈紫红色,基质呈淡蓝色。该法可以将组织中的蛋白多糖全部呈现出来。培养前与培养第 3、7 天,两组相同时间点蛋白多糖着色均匀一致,强度无降

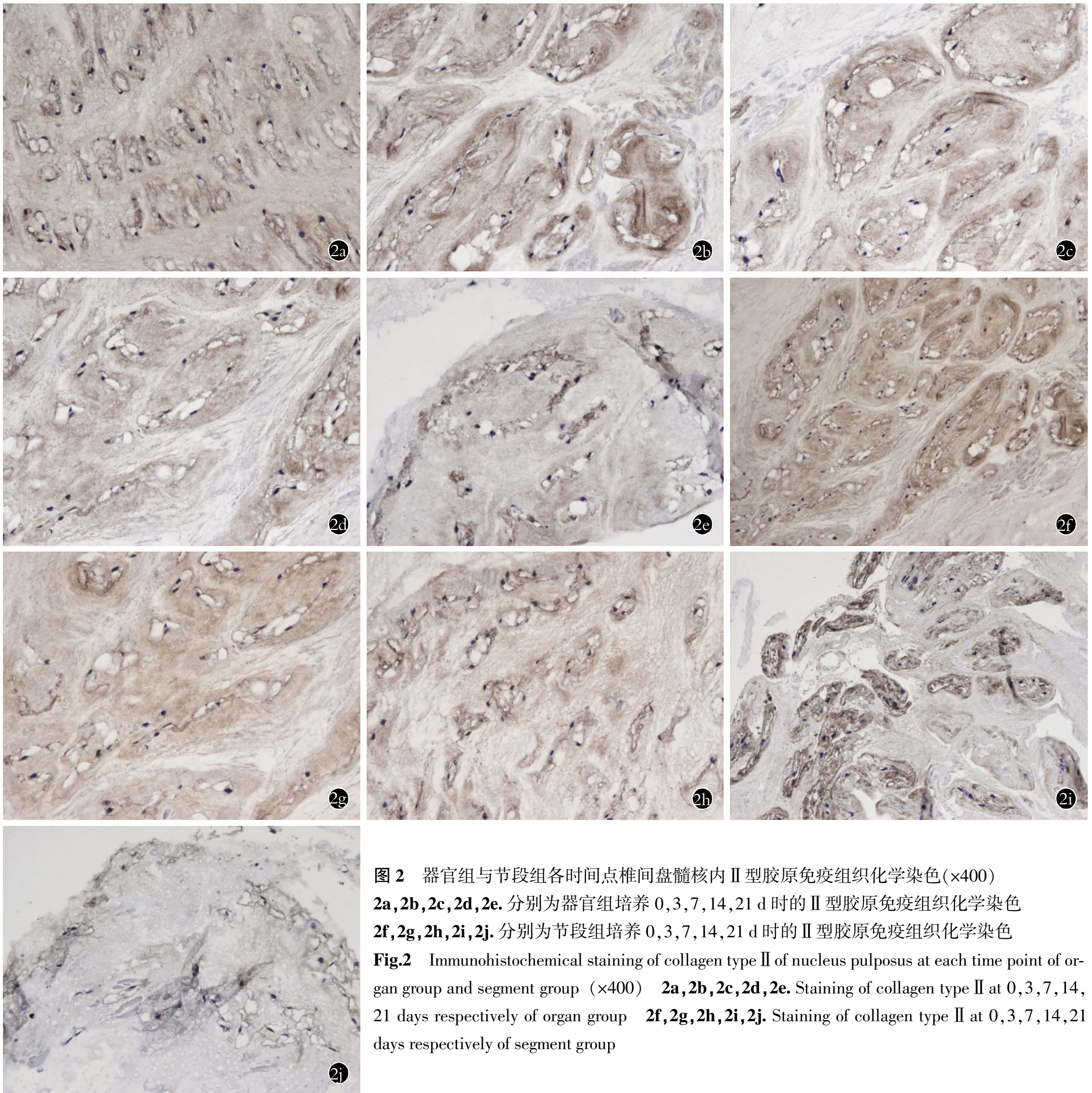

图 2　器官组与节段组各时间点椎间盘髓核内Ⅱ型胶原免疫组织化学染色(×400)
2a,2b,2c,2d,2e. 分别为器官组培养 0,3,7,14,21 d 时的Ⅱ型胶原免疫组织化学染色
2f,2g,2h,2i,2j. 分别为节段组培养 0,3,7,14,21 d 时的Ⅱ型胶原免疫组织化学染色
Fig.2　Immunohistochemical staining of collagen type Ⅱ of nucleus pulposus at each time point of organ group and segment group (×400)　**2a,2b,2c,2d,2e.** Staining of collagen type Ⅱ at 0,3,7,14,21 days respectively of organ group　**2f,2g,2h,2i,2j.** Staining of collagen type Ⅱ at 0,3,7,14,21 days respectively of segment group

表 1　不同时间点两组髓核内Ⅱ型胶原免疫组织化学染色强度值($\bar{x}\pm s$)

Tab.1　Intensity value of type Ⅱ collagen immunohistochemical staining in the nucleus pulposus at different time points between two groups($\bar{x}\pm s$)

时间	器官组 (n=10)	节段组 (n=10)	t 值	P 值
培养前	157.62±2.08	157.48±1.41	0.303	0.770
培养 3 d	156.04±2.59	155.86±2.27	0.674	0.519
培养 7 d	155.96±3.44	155.27±3.87	0.777	0.460
培养 14 d	155.48±2.97	153.84±1.87	1.923	0.091
培养 21 d	154.54±2.81	142.54±1.82	7.997	0.000
F 值	0.902	20.785	–	–
P 值	0.481	0.000	–	–

低,含量无明显区别;培养 14 d 两组着色强度均有所减弱,细胞数目减少;培养 21 d 时节段组改变明显(见图 3)。

2.4　细胞活性检测

Mitotracker Green 荧光检测,培养前兔椎间盘髓核细胞活力好、数量多,荧光强度较高(图 4)。培养 3、7 d 时检测,两组髓核细胞的荧光强度较培养前变化不明显(P>0.05)。培养 14 d 时器官组荧光强度仍无明显变化,节段组髓核细胞荧光强度略有降低,但与培养 3、7 d 比较两组差异无统计学意义(P>0.05),两组之间相同时间点比较差异也无统计学意义(P>0.05)。培养 21 d 时器官组荧光强度略有降低,与其

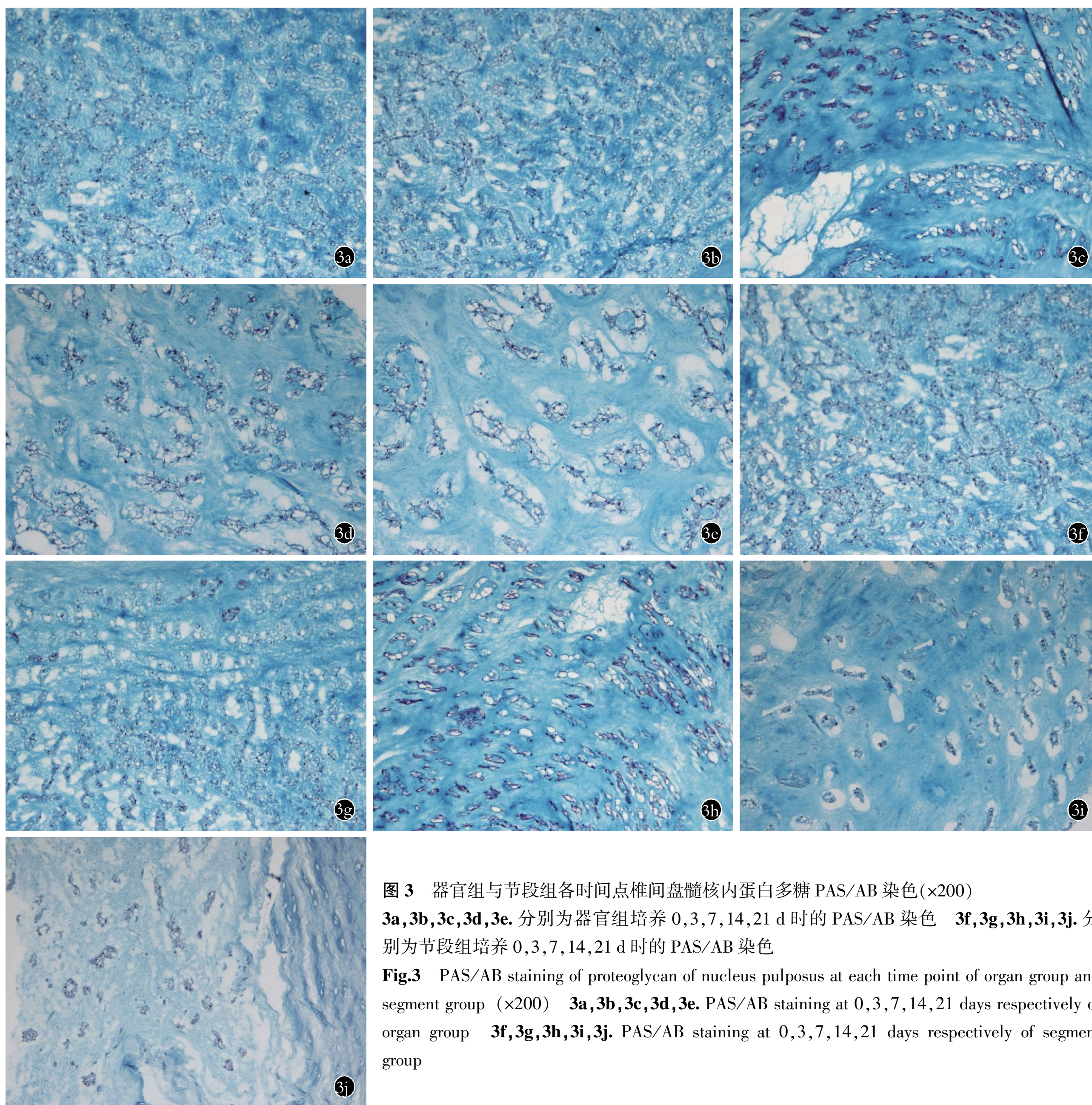

图 3　器官组与节段组各时间点椎间盘髓核内蛋白多糖 PAS/AB 染色(×200)
3a,3b,3c,3d,3e. 分别为器官组培养 0,3,7,14,21 d 时的 PAS/AB 染色　**3f,3g,3h,3i,3j.** 分别为节段组培养 0,3,7,14,21 d 时的 PAS/AB 染色
Fig.3　PAS/AB staining of proteoglycan of nucleus pulposus at each time point of organ group and segment group (×200)　**3a,3b,3c,3d,3e.** PAS/AB staining at 0,3,7,14,21 days respectively of organ group　**3f,3g,3h,3i,3j.** PAS/AB staining at 0,3,7,14,21 days respectively of segment group

余时间点比较差异仍无统计学意义($P>0.05$)；节段组荧光强度降低明显，与其余各时间点及同时间点器官组比较差异有统计学意义($P<0.05$)，见表 2。

3　讨论

椎间盘器官的体外培养模型作为体内试验和细胞培养的联系能够在原生细胞外基质中维持细胞活性[12]，从而保留了重要的细胞与细胞和细胞与基质的相互作用，由于没有炎性反应的干扰，便于研究椎间盘代谢和退变的相关机制，以及不同力学负荷和渗透负荷等环境因素与椎间盘退变的相关性[13-16]。为研究正常与退变椎间盘对外界刺激的反应及其组织学变化创造了良好的实验平台[16-20]。

表 2　不同时间点两组髓核细胞荧光染色强度值($\bar{x}\pm s$)

Tab.2　Intensity value of fluorescence staining of nucleus pulposus cells at different time points between two groups ($\bar{x}\pm s$)

时间	器官组(n=10)	节段组(n=10)	t 值	P 值
培养前	136.08±1.81	135.78±2.43	0.957	0.367
培养 3 d	135.12±3.49	135.28±1.19	0.426	0.681
培养 7 d	135.00±2.94	134.90±2.44	0.644	0.538
培养 14 d	134.46±3.09	132.28±6.08	0.714	0.495
培养 21 d	133.14±2.30	127.08±2.87	6.121	0.000
F 值	0.353	7.706	–	–
P 值	0.839	0.001	–	–

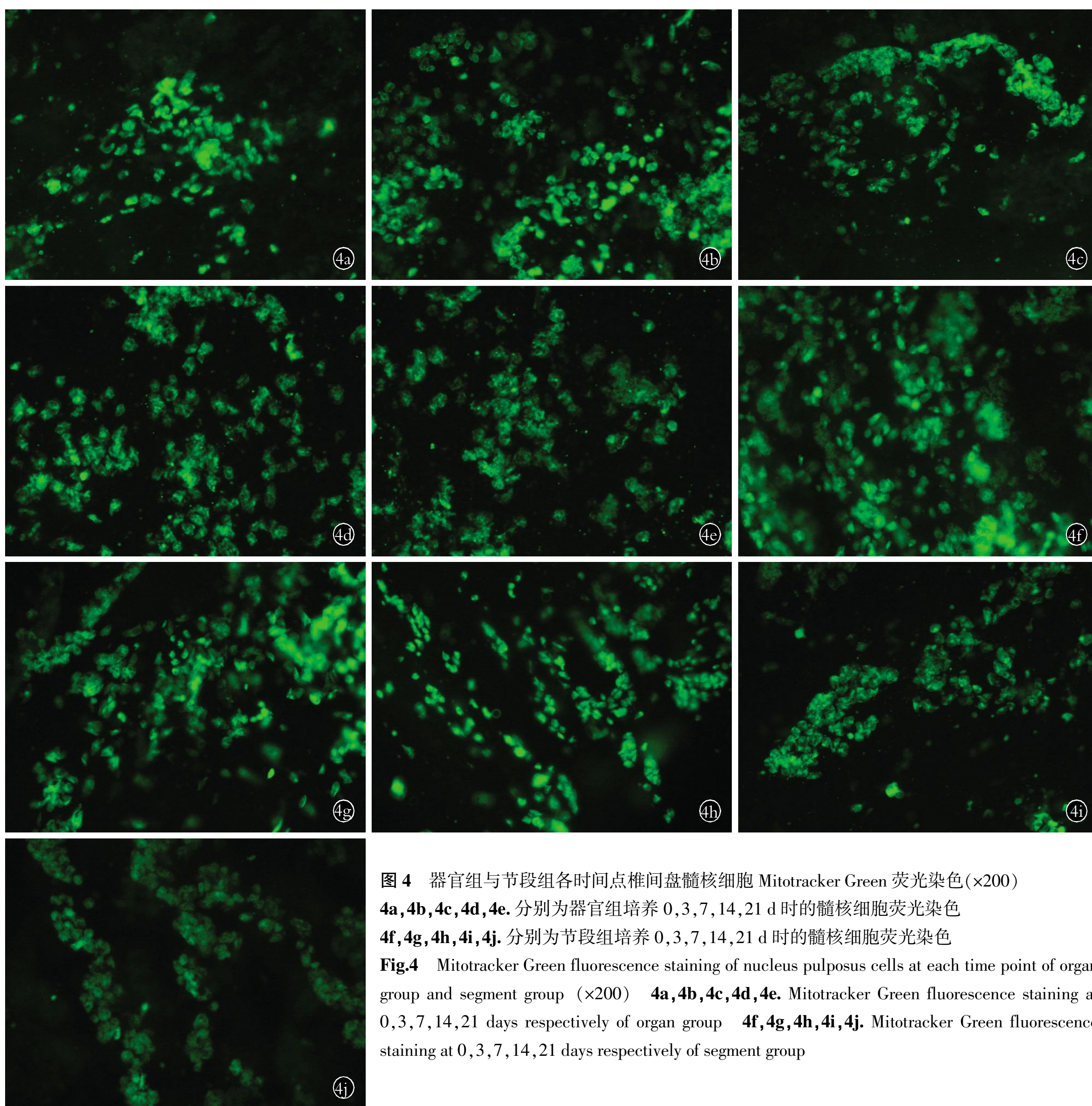

图 4　器官组与节段组各时间点椎间盘髓核细胞 Mitotracker Green 荧光染色(×200)
4a,4b,4c,4d,4e. 分别为器官组培养 0,3,7,14,21 d 时的髓核细胞荧光染色
4f,4g,4h,4i,4j. 分别为节段组培养 0,3,7,14,21 d 时的髓核细胞荧光染色
Fig.4　Mitotracker Green fluorescence staining of nucleus pulposus cells at each time point of organ group and segment group (×200)　**4a,4b,4c,4d,4e.** Mitotracker Green fluorescence staining at 0,3,7,14,21 days respectively of organ group　**4f,4g,4h,4i,4j.** Mitotracker Green fluorescence staining at 0,3,7,14,21 days respectively of segment group

3.1　模型选择

为了研究椎间盘生物力学 Lim 等[10]培养了脊柱运动节段包括两端完整椎体及椎间盘器官，该模型培养 14 d 后组织形态保持良好,故认为该模型可被广泛应用于椎间盘生物力学的研究。Seol 等[11]还对体外培养的 SD 大鼠和新西兰白兔腰椎运动节段进行了比较,结果表明兔的椎间盘更加稳定,对于生物力学的研究兔脊柱运动节段更加适合。因此本实验选择新西兰兔脊柱运动节段作为培养对象。

3.2　培养条件

研究表明[21]离体后椎间盘器官容易出现肿胀现象。在体外培养中缺少负荷将使间盘不受约束的膨胀,引发蛋白多糖减少导致失衡。一些研究采取了控制膨胀的方法:如用聚乙二醇调整介质的渗透浓度[22],利用藻酸盐凝胶降低组织膨胀[23],通过压缩负荷避免膨胀[24],利用高渗环境持细胞的形态[12,25]等。本研究在培养液中加入 NaCl 调整培养液渗透压至高渗态，结果表明短时期内在高渗状态下能够维持器官组与节段组的椎间盘结构与功能。

3.3　培养的评价指标

椎间盘组织和细胞活力、功能及代谢的检测是评价椎间盘体外培养系统优劣的必备条件。

3.3.1　组织学检查　HE 染色法能显示椎间盘组织的结构形态[26],本实验培养 2 周内两组均基本有效

维持了椎间盘髓核和纤维环组织结构的完整性,培养 21 d 节段组纤维环出现裂伤、排列紊乱,与髓核组织分界模糊,髓核细胞数目减少、轻度分散,组织结构分解,完整性消失,与器官组相比出现明显退变。

3.3.2 基质的变化 椎间盘基质的变化能够反映椎间盘合成代谢功能, 相关研究表明Ⅱ型胶原含量下降可导致椎间盘结构变形、破坏,与椎间盘退变程度密切相关[27]。本实验应用免疫组化检测Ⅱ型胶原含量,图像分析结果显示培养 14 d 内节段组及培养 21 d 内器官组髓核细胞及基质组织内Ⅱ型胶原含量较高,培养 21 d 节段组Ⅱ型胶原含量显著减少。

3.3.3 细胞代谢合成功能的检查 蛋白多糖具有重要的功能,同时能够反映椎间盘细胞的合成能力,多种方法可以检测 GAG 含量[11,14,17,28]。本实验应用 PAS/AB 染色法观察 GAG 的变化,该法中中酸性黏多糖呈深蓝色, 中性黏多糖呈紫红色, 基质呈淡蓝色,故可以将组织中的蛋白多糖全部呈现出来[29],培养 14 d 内两组 GAG 含量无明显改变, 培养 21 d 两组着色强度均有所减弱,且节段组下降更为明显。

3.3.4 细胞活性检查 荧光标记法[12]与复染法[10,30]为常用的检测手段。本实验用 Mitotracker Green 荧光测定髓核细胞活性,Mitotracker Green 用于活细胞线粒体特异性荧光染色, 活细胞能够被染色并在荧光显微镜下发出绿色荧光[31]。牛朋彦等[32]用该法测定,显示降低椎间盘软骨终板的通透性,短期内髓核细胞的活性下降。本实验培养前兔椎间盘髓核细胞活力好、数量多,有较高的荧光强度,培养 14 d 内器官组荧光强度无明显变化, 培养 21 d 略有降低,节段组培养 14 d 后荧光强度逐渐降低,至培养 21 d 时降低明显。

综上所述, 本研究比较了离体培养的兔椎间盘器官及脊柱运动节段两种模型的椎间盘活性及功能,结果表明培养 14 d 两组椎间盘活性与功能与正常椎间盘相似,培养 21 d 时器官组活性与功能仍维持良好,但节段组显示出明显的退变,这可能是由于营养通路受阻, 即使我们在取材过程中加入抗凝药以保持营养供应的通畅, 可椎体在一定程度上还是阻碍了培养液的渗透。因此培养 21 d 椎间盘器官与培养 14 d 脊柱运动节段均可作为研究椎间盘对外界刺激反应与退变的离体培养模型, 其中脊柱运动节段模型为加载装置提供了固定的施力处, 更接近体内环境,所以在短期内(14 d)可作为研究生物力学对椎间盘影响的理想模型, 为进一步控制和观察机械刺激对椎间盘生化与退变的研究提供了条件。

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