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精原干细胞体外分离培养的研究进展

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摘 要 为探索提高精原干细胞分离和体外培养效率的技术方法,以建立长期稳定的动物精原干细胞培养体系,对动物精原干细胞的分离纯化和体外培养的研究进展进行综述。以"Spermatogonial stem cell,Isolation,Culture,Enrichment,*in vitro*"和"精原干细胞、分离、培养和体外扩增"为检索词,查阅精原干细胞相关文献,并进行归纳整理。精原干细胞分离主要有机械分离法和酶消化法,纯化精原干细胞的方法有差速贴壁法、密度梯度离心法、免疫磁珠法、流式细胞法等,目前大动物精原干细胞的富集常使用酶消化法、差速贴壁法和 Percoll 梯度离心法相结合。精原干细胞体外培养需模拟体内生境,通常在基础培养基中添加血清、饲养层细胞和各类生长因子等,培养条件会影响精原干细胞培养的效果。小鼠精原干细胞已建立较完善的培养体系,而人和多数大动物还缺乏完善的精原干细胞体外培养体系。

关键词 精原干细胞;A 型精原细胞;分离;纯化;体外培养

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Progress on the research of spermatogonial stem cells cultured *in vitro*

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Abstract In order to explore the technology and methods to improve the efficiency of isolation and culturing of spermatogonial stem cells, and to establish long-term stable animal spermatogonial stem cell culture system, the isolation, purification and culture in vitro of animal spermatogonial stem cells. Literatures were searched using keywords of "spermatogonial stem cell, isolation, culture, enrichment, *in vitro*" both in Chinese and in English, and the relevant literatures were collated. The results show that: Spermatogonial stem cells can be isolated by mechanical separation and enzymatic digestion; Purification of spermatogonial stem cells are differential plating, density gradient centrifugation, magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS); At present, large animals spermatogonial stem cells are commonly enriched by enzyme digestion, differential adherence method and Percoll gradient centrifugation; Culture *in vitro* of Spermatogonial stem cells should be simulated *in vivo*, which usually added serum, feeder cells and various growth factors in the basal medium; Conditions affect the results of spermatogonial stem cell culture. It was found that a perfect system has been established for mouse spermatogonial stem cells culture *in vitro*, while there is still a lack of fine spermatogonial stem cells culture system for human and the majority of the large animals.

Keywords spermatogonial stem cells; type A spermatogonium; isolation; purification; *in vitro* culture

分离精原干细胞(Spermatogonial stem cells, SSCs)在体外进行富集和培养,可以在人和动物的幼龄阶段保存精原干细胞,在发生不育时进行自体移植恢复生精能力[1-2],或是体外定向分化为精子来

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治疗不育[3]。精原干细胞体外培养冻存后解冻还可用于转基因动物的制备[4-5]和濒危物种的种质资源保存[6]。改善精原干细胞分离纯化技术和鉴定方法，建立高效的精原干细胞体外培养体系，有助于精原干细胞的生物学特性和增殖分化机制的进一步研究以及精原干细胞在转基因和治疗男性不育方面的应用。

精原干细胞作为一种生殖干细胞，可增殖并分化产生大量精子[7]；且精原干细胞是一种成体干细胞，可以在动物的体外经过诱导，重编程后变成多潜能干细胞[8]。精原干细胞可在无外源基因操作下诱导产生胚胎样多潜能干细胞，并进一步转分化为其他类型细胞[9]，因此SSCs被认为是合乎道德伦理、安全可靠的多潜能干细胞来源。在小鼠上，精原干细胞自我更新[10]和分化机制[11]已经研究得比较清楚。近年来，精原干细胞体外分离培养技术不断改进，能富集到大量细胞，促进了SSCs诱导多能干细胞和体外诱导精子的研究，Kanatsu-Shinohara[12]首次成功将小鼠精原干细胞诱导为多潜能细胞。陈庭锋等[13]成功将猪精原干细胞诱导为神经元样细胞、成骨细胞和脂肪细胞。

Kanatsu-Shinohara实验室在2003年成功建立了小鼠精原干细胞体外培养体系[14]，经过10余年的发展，小鼠SSCs分离培养技术日臻成熟。在此基础上，精原干细胞自我更新以及增殖分化的机制研究也越来越深入。人精原干细胞也已建立体外的长期培养体系，但培养时选取的起始细胞常为掺杂睾丸体细胞的混合细胞[15]，亟需建立更完善和稳定的培养体系。大动物的SSCs体外分离培养技术也逐渐兴起，但尚未完全成熟[16-17]。

精原干细胞体外分离培养技术是研究其生物学特性、诱导分化及在转基因和种质资源保存上应用的基础。目前对高效分离非啮齿动物精原干细胞的技术及成熟的精原干细胞培养体系的研究报道较少。本研究对迄今为止研究中常见的动物精原干细胞分离手段及体外培养富集的培养条件进行了总结，旨在为人和动物精原干细胞的体外分离培养及扩增提供参考，为建立较完善的大动物SSCs体外长期稳定的培养体系提供理论依据。

1 精原干细胞的分离纯化

精原干细胞具有自我更新和分化的能力，理论上可以在动物各年龄段分离，但其比例随动物年龄增加而明显降低[18]，例如SSCs在新生小鼠睾丸中可达到约1.41%，而在成年小鼠睾丸中降低至0.02%～0.03%[19]，这增加了精原干细胞的获取及富集难度。因此分离精原干细胞多选用青春期前的动物，如5～8 d小鼠[20-22]、9～10 d大鼠[23-24]、1～3月龄猪[25-26]、1～4月龄山羊[27-30]、3～7月龄牛[31-33]、3～14月龄水牛[34-37]。精原干细胞分化后形成的A型精原细胞具有很相似的生物学特征，以至于目前还没有合适方法来分离不同的A型精原细胞，因而很多研究者将A型精原细胞都视为干细胞[24,38-39]。

1.1 精原干细胞初步分离

目前精原干细胞的分离方法主要有酶消化法和机械分离法[40]。酶消化法得到的细胞活力高，杂细胞少，但不同品牌或批次的酶消化时间不易把握，常用的酶包括胰酶、透明质酸酶、DNaseI、Ⅳ型胶原酶等；用胶原酶和胰蛋白酶两步酶消化，可收集到90%以上的精原干细胞[41]，两步酶消化法中第一步酶消化的时间非常重要，既不能过长（消化过度使得精原干细胞数量减少，活性降低）也不能过短（管周肌样细胞和间质细胞消化不充分，杂质过多）。

机械分离法一般适应于牛、羊和猪等大动物，根据睾丸的组织特点进行分离，操作简单，成本低，但对细胞损伤大，杂细胞多，张守全等[42]通过机械分离法获得了猪精原干细胞，细胞克隆速度较快，细胞生长状态良好。经典的两步酶消化法通常先用机械法将睾丸剪碎，再将剪碎的组织用Ⅳ型胶原酶、透明质酸酶和DNase等3种酶消化成单细胞悬液[43]。Yang等[44]对分离猪精原细胞的不同方法进行比较，发现采用酶消化法与机械法结合的方式能获得更高的活细胞率，约为仅使用机械法的10倍。

1.2 精原干细胞纯化

为获得更多数量、更高纯度的精原干细胞，在进行初步分离后还应对其进行富集和纯化。纯化精原干细胞的方法有差速贴壁法（Differential plating）、密度梯度离心法（Density gradient centrifugation）、免疫磁珠分选法（MACS）、流式细胞分选法（FACS）、干细胞克隆挑选法等[45]。各种分离方法都有各自的优点和不足，将不同分离纯化方法结合使用可弥补这些不足从而获得纯度更高的精原干细胞，因此目前精原干细胞的分离纯化常使用2种或2种以上方法相结合。Percoll密度梯度离心和差异贴壁法因简便易操作、且成本低、效率高，在大动物

精原干细胞纯化中广泛应用[29]；免疫磁珠法和流式细胞法需要特定的分子标记，目前还缺乏在各物种中通用的精原干细胞特异性分子标记[46]故而应用受到限制。

1.2.1 差速贴壁法

精原干细胞在层粘连蛋白上比支持细胞贴壁速度快，而在明胶上比支持细胞贴壁慢[47]，差速贴壁法（也称差异贴壁法）根据这一特点将二者分开。此法分离精原干细胞通常纯度不高，可能掺杂较多体细胞[31]；但因不需特殊仪器、操作简便、成本较低，差速贴壁法在小鼠、人和大家畜精原干细胞的纯化中应用非常广泛。Izadyar 等[39]采用差速贴壁法分离牛精原细胞，纯度＞50％；此后研究者用该方法分离新生牛[48]、猪[49]的精原干细胞，纯度分别达到72％、90％。用单位重力速度沉降法与差速贴壁法结合纯化猪的 A 型精原细胞，可得到纯化率高达95％～98％的细胞[25]。陈庭锋等[13]成功使用差速贴壁法分离出猪精原干细胞，并且分离的细胞具有多能性。

1.2.2 密度梯度法

不同类型的生精细胞存在大小和重力上的差异，可在分离介质（如 Percoll 液和 BSA）中沉降于不同密度梯度层面，可在富含精原干细胞的层面收集较高纯度目的细胞。由于 Percoll 不连续梯度离心分层较不清晰，所以易丢失大量精原干细胞，而这一缺陷常由该方法与其他方法相结合使用的方式进行消除，如 Lee 等[50]用 Percoll 密度梯度离心法，在30％梯度处分离得到小鼠精原干细胞，并结合免疫磁珠法进一步筛选，得到较纯的精原干细胞。王永彬等[51]采用 Percoll 梯度离心分离精原细胞，再用差异贴壁法进一步纯化得到了较纯的小鼠精原干细胞。Shi 等[52]使用差异贴壁法和 Percoll 密度梯度法结合分离长白山小猪（出生后 1～5 d）的精原干细胞，在 19％～27％密度梯度收集的细胞数量最多且含大量精原干细胞，碱性磷酸酶染色阳性率在87.47％ 左右。Heidari 等[38]用 Percoll 不连续梯度离心法分离山羊精原干细胞，在 32％梯度处得到较多山羊 A 型精原细胞，经检测细胞阳性率约为 94.60％。

BSA（牛血清白蛋白）连续密度梯度离心法（也叫 STA-PUT 重力沉降法）不同于 Percoll 不连续梯度法，形成的是连续的密度梯度，可分选出更高纯度的精原干细胞[53]。Liu 等[40]首次用 STA-PUT 重力沉降法分选人精原干细胞，经验证分离的细胞纯度高达 90％，但可能混有次级精母细胞（人的部分次级精母细胞与精原干细胞的大小和重力非常接近）。虽然很多研究者用密度梯度离心法分离出高纯度精原干细胞，但不可否认的是离心对细胞的机械损伤比较大，分离出的细胞活力不高，可能会对纯化精原干细胞后的长期培养造成不利影响，所以该法是否还具有应用前景值得斟酌。

1.2.3 免疫磁珠法

免疫磁珠分选法（MACS）是通过包被抗体的磁珠识别精原干细胞表面的特异性抗原将目标细胞分离[46]。其主要特点是：分选效率随抗原抗体亲和力大小和可利用表面抗原的数量而变化；磁珠对细胞无损伤，分选后不影响细胞活性，得到的精原干细胞浓度较高[54]；纯化时需要专门的设备、特异性较高的表面抗原、抗体标记的磁珠及一次性的分离柱，费用较高。Kubota 等[55]用 MACS 法纯化小鼠精原干细胞，使用 Thy-l 抗体的磁珠富集到纯度比较高的细胞。Conrad 等[56]用整合素 α6 磁珠分选富集到了 90％的人精原干细胞。利用免疫磁珠法进行人精原干细胞分选，纯度可达到 95％以上[57]。

1.2.4 流式细胞术

流式细胞分选技术（FACS）是将带有免疫荧光的抗体与精原干细胞相结合，依据荧光信号来收集目的细胞，具较快测量速度和较高成功率，可进行多参数的测量，精度高、准确性好；但需要流式细胞仪，且对操作技术有较高要求，重复性不高[46]。流式细胞术分选法可高倍富集小鼠精原干细胞[47]，Kubota 等[58]用流式细胞仪筛选 MHC-I 阴性细胞，将小鼠 SSC 富集了 6 倍；而结合筛选 MHC-I 阴性、c-kit 阴性和 Thy-1 阳性的细胞，将小鼠 SSCs 富集了 6 倍。Hamra 等[24]用 FACS 分选获得约 90％的大鼠 A 型精原细胞。Shi 等[52]用流式细胞法富集得到了具有多能性的猪睾丸干细胞。Malecki 等[59]采用流式细胞仪分选 $POU5F1^{+}$、$SSEA\text{-}4^{+}$、$Sox2^{+}$、$Nanog^{+}$ 和 $TRA\text{-}1\text{-}60^{+}$ 的细胞富集到了睾丸多潜能干细胞。

1.2.5 挑克隆法

挑克隆法是根据精原干细胞在体外培养会形成克隆，已分化的生精细胞则不能形成，来分离未分化的精原细胞[60]。Sadri-Ardekani 等[61]用差异贴壁法与干细胞克隆挑选法结合分离人精原干细胞，并将其异种移植到裸鼠睾丸内从而证实了获得的细胞为人精原干细胞。

2 精原干细胞的体外培养

精原干细胞所处的环境由睾丸、曲细精管、血睾屏障憩室和睾丸间质三者组成。体外培养精原干细胞的早期，主要采取模拟体内生境的方式。精原干细胞分离富集后的适当培养，可使精原干细胞完成自身损伤修复和增殖，并为细胞的遗传修饰及受体动物准备争取时间，提高移植和转基因效率[62]。培养效果取决于最佳培养条件(例如基本培养基、血清、饲养层细胞和生长因子)的探索和选择。

目前在小鼠[14]和大鼠[63]等啮齿动物精原干细胞相关研究中，较为完善精原干细胞的长期体外培养体系均已成功建立。科学家一直为建立明确成分的体外培养体系而努力，以避免不确定因素对精原干细胞的影响。Kanatsu-Shinohara 等[47]最早使用无血清无饲养层的培养体系培养小鼠精原干细胞；Kubota 等[64]建立的精原干细胞体外培养体系将胎牛血清(FBS)的体积分数降低到 1%；2005 年，Kanatsu-Shinohara[65]简化了无血清无饲养层培养体系；2008 年，Kanatsu-Shinohara 等[66]建立了第 1 个成份明确的小鼠精原干细胞培养体系，该体系使用层粘连蛋白涂层的培养皿，不含饲养层细胞和血清，并添加生长因子，为精原干细胞的诱导分化等研究奠定了坚实的基础。相比之下，猪[67-70]、牛[32-33,71-73]、山羊[27,30,74]、水牛[75-77]的精原干细胞只能在体外培养较短时间(不超过 2 个月)。

2.1 培养基

根据不同的动物类别、年龄及 SSCs 的培养方式，应选择不同的基础培养基来进行精原干细胞的体外培养，主要的培养基有 DMEM、MEM、SFM 等。Kanatsu-Shinohara 等[14]用 StemPro-34SFM 体外培养小鼠精原干细胞长达 6 个月。目前猪[67]、山羊[73]、牛[70]等大动物精原干细胞的培养常用 DMEM/F12 和 DMEM(高糖)培养基；Lzadyar 等[39]分离牛的 A 型精原细胞并用胎牛血清含量为 2.5%的 MEM 培养基培养，比用 KSOM 培养基及其他浓度血清的 MEM 获得了较纯的精原干细胞。人精原干细胞的培养常用 StemPro 培养基[78]，可实现体外的长期培养[79]；Conrad 等[56]使用经过改良的 StemPro 培养基培养人生殖干细胞，体外培养人精原干细胞长达 14 个月。

2.2 血清

血清包含多种调节精原干细胞增殖和分化的因子，可以补充基础培养液中缺少的营养物质，为 DNA 合成提供一些必要的营养成分，减少细胞代谢及凋亡产生的有毒有害物质，对精原干细胞早期建系时发挥作用；而维持其特征则可无血清参与，且血清还有可能使精原干细胞分化[80]。血清的具体成分不明确且受品牌批次影响，还存在某些对细胞有不利影响的因子，可能造成细胞形态变化和基因变异。精原干细胞体外培养时是否添加血清、添加的浓度存在争议，且血清的具体作用机制尚未完全清楚，目前大部分研究证实血清对 SSCs 体外培养利大于弊[81]。Brinster 实验室首次用含血清和小鼠胚胎成纤维细胞(PMEF)饲养层的培养基在体外长时间培养 SSCs[82]；此后 Kanatsu-Shinohara 等[83]建立了小鼠精原干细胞的无血清培养体系，在该培养体系下进行了 6 个月体外培养，移植后产生了可育的后代鼠。

Dovere 等[84]在含不同体积分数血清的培养基培养小鼠精原干细胞，发现在低体积分数(0.3%～2.0%)血清中均在 5～7 d 后出现精原干细胞克隆；而在高体积分数(5%～15%)血清中则没有出现。在山羊[85]和猪[86]上的研究与小鼠相似，高体积分数血清不利于精原干细胞增殖。Guan 等[80]使用胎牛血清添加体积分数为 1%的 OL-MEM 培养基在体外培养小鼠精原干细胞，精原干细胞成功增殖，细胞存活时间长达 113 d。血清对不同物种精原干细胞培养有不同程度的影响，明确血清的具体成分和其对精原干细胞增殖分化的调控机理是下一步研究中亟待解决的问题。

2.3 饲养层

饲养层(滋养层)细胞分泌许多生长因子，能促进有丝分裂从而促进精原干细胞增殖[87]或抑制细胞分化[88]，并且饲养层有助于干细胞建系[89]。在精原干细胞体外培养的早期培养体系中不可缺少饲养层，若直接在培养皿中培养，细胞数量 1 周后仅剩 10%～20%[81]。常用的饲养层有睾丸支持细胞、小鼠原代胚胎成纤维细胞(PMEF)、BEF 细胞、小鼠胚胎成纤维细胞耐乌苯苷亚系(STO 细胞)、MSC-1 细胞和 SNL 细胞等。小鼠精原干细胞体外培养常用 STO 细胞作为饲养层，STO 细胞可以分泌 LIF(白血病抑制因子)，维持精原干细胞的自我更新[90]；Nagano 等[81]用 STO 细胞作为饲养层培养小鼠精原干细胞 3 个月。大鼠精原干细胞在间充质干细胞(Mesenchymal stem cells，MSC)饲养层上比在小鼠

胚胎成纤维细胞(Mouse embryonic fibroblast，MEF)饲养层上培养时的细胞活性高[19,91]。牛精原干细胞的体外培养也可用 STO 细胞作为滋养层[92]。支持细胞可以经直接分泌或旁分泌产生某些因子，对精原干细胞的自我更新和分化有促进作用[93]，例如可经旁分泌产生胶质细胞源性神经营养因子(Glial cell line derived neurotrophic factor，GDNF)并作用于精原干细胞[94]。冉竞超等[95]用胶原酶和透明质酸酶混合酶液除去间质细胞，从而获得较纯的支持细胞作饲养层，并添加 GDNF、LIF 和 bFGF 等因子后，精原干细胞克隆明显增加。Liu 等[96]用 hAECs (人羊膜上皮细胞)作饲养层，长期体外培养小鼠精原干细胞获得成功。

近年来，越来越多的研究采用无饲养层培养体系，去除了饲养层细胞中的一些不确定因素对精原干细胞的影响，但精原干细胞的增殖能力和稳定性不好。无饲养层的培养体系中，需添加生长因子等成分才能保持干细胞特性及维持干细胞增殖，且无饲养层体系不利于干细胞的贴壁，易使细胞漂浮[97]。采取明胶、层粘连蛋白和人工基膜等包被的方法，更有利于细胞贴壁，如 Kanatsu-Shinohara 等[65]无饲养层的鼠雄性生殖干细胞培养体系中，采用层粘连蛋白包被培养皿。

2.4　生长因子

第一个被发现调控精原干细胞自我更新和分化的细胞因子是 GDNF，其过量表达会导致未分化精原细胞的大量积累[93]，促进精原干细胞自我更新[98]，且作用效果与添加浓度有关[99-100]。支持细胞分泌的其他生长因子如碱性成纤维生长因子(bFGF 或 FGF2) 和表皮生长因子(EGF)也可促进精原干细胞的体外增殖[101]，在仅含 GDNF 和 bFGF 2 种因子的基础培养基中培养小鼠精原干细胞就能形成克隆[102]。Kanatsu-Shinohara 等[14]建立的小鼠精原干细胞培养体系中，添加了 EGF、bFGF、GDNF 等几种生长因子。LIF(白血病抑制因子)能促进体外培养鼠睾丸原始生殖细胞的自我更新[103]，干细胞因子(Stem cell factor，SCF)能促进 A 型精原细胞的形成[104]。培养基中添加 EGF、GDNF、LIF、bFGF 和 SCF 中的单个或组合生长因子均能促进精原干细胞的体外增殖，其中 GDNF 和 bFGF 抑制精原干细胞分化的效果最好[25]。此外还有一些非必需的生长因子，如 GFRA1 可使精原干细胞增殖速度加快[105]。

2.5　添加剂

体外培养精原干细胞时添加剂需求量往往很小，但具有不可或缺的作用。丙酮酸与乳酸可作为精原干细胞生长的能量底物，维生素 C 和维生素 E 有助于体外培养的精原干细胞存活，维生素 A 对精原干细胞具有抑制分化和促进增殖的作用；非必需氨基酸、L-谷氨酰胺、β-巯基乙醇、转铁蛋白和亚硒酸盐等也常作为精原干细胞体外培养的添加剂[106]。Wang 等[107]研究表明 40 μg/mL 的维生素 C 可以使细胞内活性氧保持在一定的生理水平，促使抗凋亡基因 *Bcl-2* 表达，抑制促凋亡基因 *Bax* 的表达和 p53 通路，从而促进山羊精原干细胞的体外增殖。

2.6　培养温度

多数研究者在 37 ℃下培养动物精原干细胞，培养温度高于动物睾丸内温度，而哺乳动物阴囊温度比体温低 4～5 ℃，低于体温的温度有利于精子发生，因此也有研究采取 30～34 ℃的温度培养精原干细胞。多数哺乳动物精原干细胞在睾丸位于体腔内时就已形成，如小鼠约在 6 日龄，故 37 ℃的体外培养温度可能对精原干细胞自身形成无影响[108]，还可去除已分化的各级生精细胞[109]，因此现在多采用 37 ℃培养。有研究表明 37 ℃不会降低小鼠精原干细胞生物学活性，在 32～37 ℃均可进行培养[110]；但培养温度为 34 ℃可以提高小鼠精原干细胞增殖数量和存活时间，培养效果优于 30 和 37 ℃[111]。然而在大鼠上，37 ℃下培养生精细胞会抑制 A 型精原细胞的增殖[112]。

3　小　结

精原干细胞分离培养技术在小鼠上的研究已经比较成熟，而在大动物上还存在发展的空间。动物精原干细胞分离方法在分离效率、经济成本等方面各有优劣，亟需寻找一种既高效又经济的分离方法。虽然小鼠的精原干细胞长期培养体系已经建立，但大动物精原干细胞在体外培养维持的时间还比较短，而人精原干细胞可在体外长期培养但还不具备成分明确的培养体系。

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