

Beagle 犬卵母细胞体外成熟研究进展

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【摘要】 国内实验 Beagle 犬种质资源保存利用及制备基因修饰人类疾病动物模型, 要求有充足的 Beagle 犬胚胎。目前诱导排卵技术在犬上效果不明显, 体内获取犬成熟卵母细胞有困难。同时, 尽管科研人员对犬卵母细胞体外成熟培养进行了多方面的尝试, 但尚未获得突破, 成熟率低, 严重制约了其在种质资源保存、基因修饰模型制备及生物医学研究中的应用。本文梳理了不同犬龄及生殖周期、卵母细胞形态与体积及脂滴在 Beagle 犬卵母细胞体外成熟中的影响, 以为其体外成熟寻找新的突破口。

【关键词】 犬; 疾病模型; 卵母细胞; 体外成熟

【中图分类号】 Q95-33 **【文献标识码】** A **【文章编号】** 1005-4847(2017) 01-0107-04

Doi:10.3969/j.issn.1005-4847.2017.01.020

Research progress of in vitro maturation of Beagle dog oocytes

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【Abstract】 Sufficient embryos are needed for the preservation of Beagle dogs germplasm resources and the preparation of gene-modified human disease animal models. Up to now, the induced ovulation technique has no effect on dogs, it is hard to obtain mature oocytes in vivo, although the scientists try a lot in many aspects, but still could not make a breakthrough. The in vitro maturation rate is too low to support the preservation of germplasm resources, application in gene-modified disease models and biomedical research. Aiming to provide useful information on breakthrough in dog oocytes maturation, this review will summarize the effect of different age and reproductive stage, different morphology and size of the oocytes and lipid droplet on the in vitro maturation of dog oocytes.

【Key words】 Beagle dogs; Disease model; Oocytes; in vitro maturation

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Beagle 犬是国际公认的新药安评与研发首选用犬, 作为国家犬类实验动物种子中心, 对国内优质 Beagle 犬种质资源或者濒危犬种进行保护利用, 具有重要意义。目前国内 Beagle 犬只有活体保种单一形式, 同时经多代的繁衍后, 未免会对其遗传稳定性造成影响, 而 Beagle 犬种子体外保存是解决上述问题的最好方法之一。近年来基因修饰技术进步很快, 我国也已成功实现世界首例基因敲除犬, 但该技术及体外保种要求有充足的实验材料(受精

卵), 目前成熟卵母细胞一是从自然发情排卵的母犬输卵管中手术获取, 二是从卵巢获取卵丘-卵母细胞复合体(cumulus oocytes-complexes, COCs)再进行体外成熟(in vitro maturation, IVM)。如方法一获得成熟卵母细胞, 其成本很高且非常困难, 而方法二是比较适宜的选择, 因此需要急切解决犬 COCs 体外成熟、受精等技术壁垒, 以尽快应用于保种及疾病模型研究。

[基金项目] 广州市“珠江科技新星”项目(201610010144); 广州市科技基础条件平台建设项目(201605040005)。

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1 不同培养体系对犬卵丘-卵母细胞体外成熟的影响

犬是非季节性单次发情动物,分为发情前期、发情期、发情后期及乏情期。母犬一年发情 1~2 次,犬发情间期平均 7 个月,母犬排卵虽然主要集中在发情期前 1~3 d 内,但在发情期的 7 d 内随时都可发生排卵,排卵的确切时间难以掌握^[1]。且犬排出的卵母细胞处于生发泡期 (germinal vesicle, GV),需在输卵管内完成成熟过程(48~72 h),因此犬排出的卵母细胞在 2~3 d 后,才具有受精能力,再次启动减数分裂过程。多年来科研人员尝试了各种各样的卵母细胞体外成熟培养体系^[2],如往培养液添加促性腺激素 (FSH, LG, PMSG, HCG) 的^[3-5]、甾类(雌二醇、孕酮)的^[1,6]、不同类型血清的^[6,7]、生长因子的 (IGF-1, EGF)^[8]、各种蛋白源的^[9]、透明质酸酶的^[10]、抗氧化剂的^[7,11]、细胞周期抑制剂的^[12]、模拟输卵管内环境与犬输卵管上皮细胞共培养的^[13,14],甚至将卵母细胞注入体外培养的输卵管内等等^[15],但无论采用何种基础培养液及添加各种生物活性物质,仍只有 20% 左右的犬卵母细胞能成功发育至 MII 阶段^[16]。事实上,大概 60% 的 COCs 取出后就已停止发育,大概 25% 的卵母细胞退化,只有少量卵母细胞能成功发育至 MII 阶段,但因质量太差,其继续体外受精及发育能力亦会大大降低^[17,18]。诸多研究表明,目前对犬卵母细胞体外成熟培养体系是不适宜的,到底 COCs 在体外缺少了何种因子的刺激,致其大部分停止发育? 研究者们必须另辟途径,寻找突破口。

2 不同犬龄及生殖周期对卵丘-卵母细胞体外成熟的影响

犬卵巢功能随着年龄的增加而不断下降,在国家犬类实验动物种子中心,虽然也存在 6 岁以上的种母犬怀孕分娩情况,但其胎均产仔数明显要比 6 岁以下的低,且出现病、弱仔机率上升。经比较,从 6 岁以上母犬卵巢获得的 COCs 数显著低于 6 岁以下正常母犬(未发表数据)。而 Hewitt 等^[19]也发现供体母犬的年龄与 COCs 的获取数呈现负相关,COCs 的平均获取数随供体年龄的增长每年下降 4.7 枚(Hewitt *et al.*, 1998)。Lopes 等^[20]对取自 6 岁以下和 7 岁以上母犬的卵母细胞体外培养比较,发现 6 岁以下母犬所取的卵母细胞在体外培养成熟的潜力更高。

有学者研究在不同生殖周期所取的 COCs 是否会对体外成熟率有影响,但结果不相一致。Rodrigues 等^[21]认为 COCs 的体外成熟不受母犬生殖阶段的影响,在不同生殖周期采集的 COCs 对其减数分裂的恢复没有显著差异。但更多的研究认为生殖周期是 COCs 体外成熟的重要影响因素,供体母犬发情期卵巢的内环境含有高浓度的雌激素、孕酮及一些未知因子,有利于其后的体外成熟^[22]。卵泡期的犬 COCs 恢复减数分裂和达到 MI、MII 的能力比乏情期的高^[23],Oh 等^[24]认为适当生殖周期的卵母细胞对其减数分裂的恢复有重要影响。尽管如此,Yamada 等^[25]采集经诱导超数排卵后的母犬排卵前 COCs,其体外成熟率仍只有 32%,对照组为 12%,表明虽然生殖周期对 COCs 体外成熟存在一定的影响,但并不是关键因素之一。

3 卵丘-卵母细胞形态及体积与体外成熟的关系

卵丘细胞与卵母细胞紧密连接形成 COCs,卵丘细胞通过缝隙连接 (Gap junctions, GJs) 与卵母细胞间发生营养物质、离子以及 cAMP 等调节小分子的交换,从排卵前到排卵后,卵丘细胞与卵母细胞间发生着复杂的信号传递,从而对卵母细胞的发育实现分子水平的调控。但对于犬类动物 GJs 的研究仅限于形态学上的研究。

为了提高 COCs 体外成熟率,学者们都遵循一个形态学标准,即胞质颜色深暗而均一,直径 > 100 μm ,有两层以上完整的颗粒细胞层^[27]。对于为何以这个标准选取,也是经过验证的。Lopes 等^[28]发现根据上述标准选取的犬卵母细胞核凋亡的比例低于 15%。Otoi 等^[29]发现直径小于 100 μm 的 COCs 成熟率仅为 4%~10%,Songsasen 等^[30]发现仅有一层颗粒细胞的卵母细胞在培养 48 h 后退化。Hewitt 等^[19]也发现直径大于 100 μm 的 COCs 发育至 MI、MII 阶段的比例为 20%,而直径 < 100 μm 的 COCs 其比例仅为 4%~10%。这个标准在牛卵母细胞上也同样适用,可根据其胞质颜色判断细胞的发育潜能^[26]。上述研究表明卵母细胞体积大小、胞质颜色等对其核体外成熟有一定的影响。

4 脂滴形态对卵丘-卵母细胞体外成熟的影响

脂滴 (lipid droplets, LD) 是富集在动物脂肪组织中的动态细胞器,是由磷脂单分子层、游离胆固

醇和特殊蛋白覆盖在核心的中性脂质组成,控制着体脂的贮存。蛋白质组学研究表明:LD 参与脂类代谢和运输、细胞内物质交换、信号转导及细胞骨架构成。犬、猪、牛等卵母细胞和胚胎细胞内以脂滴的形式储存大量的内源性脂质,因此胞质颜色深暗,这些脂质为卵母细胞及早期胚胎发育供能^[31]。LD 在猪卵母细胞中的研究相对较多,猪、马卵母细胞成熟过程中脂滴形态和含量是一个动态变化过程^[32-34],且其脂质代谢改变会干扰单精受精和胚胎发育^[35]。

而关于犬卵母细胞 LD 的研究鲜有报道。2012 年 Apparicio 等^[36]通过基质辅助激光解吸电离(matrix-assisted laser desorption mass spectrometry, MALDI-MS)法首次对犬和猫卵母细胞里 LD 的化学构成进行研究,为改进其体外培养及冷冻保存技术提供参考。2016 年 Ariu 等^[37]首次分析了犬 COCs 体积及生殖阶段与 LD 分布的关系,结果表明在卵泡期,大部分卵母细胞 LD 呈现不规则分布,但在黄体期及乏情期,LD 主要在核周边分布,且不管犬处于哪个生殖阶段,直径大于 120 μm 的卵母细胞 LD 含量要显著高于直径在 110 ~ 120 μm 之间的卵母细胞。犬卵母细胞含有大量 LD,推测其在卵母细胞体外成熟过程中有重要作用,需进一步展开研究以探明其如何影响卵母细胞的发育过程。

5 展望

2005 年,公布的犬的全基因组序列分析,发现犬基因组与人的相似性达 95.7%,且有数百种遗传疾病与人类相似^[38],是研究人类疾病机理机制的重要模型。但从 2005 年^[39]首例克隆犬的诞生到 2015 年首例基因敲除犬的出生^[40],已过去 10 年,有关犬辅助生殖技术远远滞后于小鼠、猪等动物的研究,尤其是卵母细胞体外成熟培养效率停滞不前,究其原因,一是其独特的生殖生理,发情排卵时间难以把握,GV 期及成熟卵母细胞获取有困难;二是犬 COCs 现行体外成熟培养技术体系尚未满足启动其恢复减数分裂的要求,提示需要从分子机制来展开研究。犬卵母细胞体内成熟调节机制是什么? 卵丘细胞、脂滴究竟通过什么通路影响着卵母细胞的成熟? 输卵管内存在哪些关键因子促进卵母细胞减数分裂的恢复? 等等,这些问题,都亟待解决。

参 考 文 献

[1] Kim MK, Fibrianto YH, Oh HJ. Effects of estradiol-17 β and

progesterone supplementation on in vitro nuclear maturation of canine oocytes[J]. *Theriogenology*,2005,63:1342-1353.

[2] Chastant-Maillard S, Viaris DLC, Chebrout M, et al. The canine oocyte: uncommon features of in vivo and in vitro maturation[J]. *Reprod Fertil Dev*,2011,23(3):391-402.

[3] Lee SR, Kim MO, Kim SH, et al. Effect of conditioned medium of mouse embryonic fibroblasts produced from EC-SOD transgenic mice in nuclear maturation of canine oocytes in vitro[J]. *Anim Reprod Sci*,2007,99(1-2):106-116.

[4] Otoi T, Shimizu R, Naoi H, et al. Meiotic competence of canine oocytes embedded in collagen gel[J]. *Reprod Domest Anim*, 2006,41(1):17-21.

[5] Kim BS, Lee SR, Hyun BH, et al. Effects of gonadotropins on in vitro maturation and of electrical stimulation on parthenogenesis of canine oocytes[J]. *Reprod Domest Anim*,2010,45(1):13-18.

[6] Rodrigues BA, Rodrigues JL. Meiotic response of in vitro matured canine oocytes under different proteins and heterologous hormone supplementation[J]. *Reprod Domest Anim*,2003,38(1):58-62.

[7] Lee SR, Kim BS, Kim JW, et al. In vitro maturation, in vitro fertilization and embryonic development of canine oocytes[J]. *Zygote*,2007,15:347-355.

[8] Hatoya S, Sugiyama Y, Nishida H, et al. Canine oocyte maturation in culture: significance of estrogen and EGF receptor gene expression in cumulus cells[J]. *Theriogenology*,2009,71(4):560-567.

[9] Songsasen N, Yu I, Leibo SP. Nuclear maturation of canine oocytes cultured in protein-free media[J]. *Mol Reprod Dev*,2002,62(3):407-415.

[10] Rodrigues BA, Dos SL, Rodrigues JL. The effect of hyaluronan concentrations in hST-supplemented TCM 199 on in vitro nuclear maturation of bitch cumulus-oocyte complexes[J]. *Theriogenology*,2006,66(6-7):1673-1676.

[11] Hossein MS, Kim MK, Jang G, et al. Effects of thiol compounds on in vitro maturation of canine oocytes collected from different reproductive stages[J]. *Mol Reprod Dev*,2007,74(9):1213-1220.

[12] Hanna C, Menges S, Kraemeer D, et al. Synchronisation of canine germinal vesicle stage oocytes prior to in vitro maturation alters the kinetics of nuclear progression during subsequent resumption of meiosis[J]. *Reprod Fert Develop*,2008,20(5):606-614.

[13] Bogliolo L, Zedda MT, Ledda S, et al. Influence of co-culture with oviductal epithelial cells on in vitro maturation of canine oocytes[J]. *Reprod Nutr Dev*,2002,42(3):265-273.

[14] Vannucchi CI, de Oliveira CM, Marques MG, et al. In vitro canine oocyte nuclear maturation in homologous oviductal cell co-culture with hormone-supplemented media[J]. *Theriogenology*, 2006,66(6-7):1677-1681.

[15] Luvoni GC, Chigioni S, Allievi E, et al. Meiosis resumption of canine oocytes cultured in the isolated oviduct[J]. *Reprod Do-*

- mest Anim,2003,38(5):410-414.
- [16] Luvoni GC, Chigioni S, Allievi E, et al. Factors involved in vivo and in vitro maturation of canine oocyte[J]. Theriogenology,2005,63:41-59.
- [17] Viaris DLC, Reynaud K, Pechoux C, et al. Ultrastructural evaluation of in vitro-matured canine oocytes[J]. Reprod Fertil Dev, 2008,20(5):626-639.
- [18] Chebrou M, de Lesegno CV, Reynaud K, et al. Nuclear and cytoplasmic maturation of canine oocytes related to in vitro denudation[J]. Reprod Domest Anim,2009,44(Suppl 2):243-246.
- [19] Hewitt DA, England GC. The effect of oocyte size and bitch age upon oocyte nuclear maturation in vitro [J]. Theriogenology, 1998,49:957-966.
- [20] Lopes G, Sousa M, Luvoni G C, et al. Recovery rate, morphological quality and nuclear maturity of canine cumulus-oocyte complexes collected from anestrous or diestrous bitches of different ages[J]. Theriogenology,2007,68:821-825.
- [21] Rodrigues B, Rodrigues JL. Influence of reproductive status on in vitro oocyte maturation in dogs [J]. Theriogenology, 2003, 60(1):59-66.
- [22] Martins LR, Ponchiroli CB, Beier SL. Analysis of nuclear maturation in vitro matured oocytes from estrous and anestrous bitches [J]. Anim Reprod,2006,3:49-54.
- [23] Luvoni GC, Luciano AM, Modena S, et al. Influence of different stages of the oestrous cycle on cumulus-oocyte communications in canine oocytes; effects on the efficiency of in vitro maturation [J]. J Reprod Fertil. Suppl,2001,57:141-146.
- [24] Oh HJ, Fibrianto YH, Kim MK, et al. Effects of canine serum collected from dogs at different estrous cycle stages on in vitro nuclear maturation of canine oocytes[J]. Zygote,2005,13(3):227-232.
- [25] Yamada S, Shimazu Y, Kawano Y, et al. In vitro maturation and fertilization of preovulatory dog oocyte[J]. J Reprod Fertil Suppl,1993,47:227-229.
- [26] Nagano M, Katagiri S, Takahashi Y. Relationship between bovine oocyte morphology and in vitro developmental potential[J]. Zygote,2006,14(1):53-61.
- [27] Reynaud K, Saint-Dizier M, Chastant-Maillard S. In vitro maturation and fertilization of canine oocytes[J]. Methods Mol Biol, 2004,253:255-272.
- [28] Lopes G, Vandaele L, Rijsselaere T, et al. DNA fragmentation in canine immature Grade I cumulus-oocyte complexes[J]. Reprod Domest Anim,2010,45:275-281.
- [29] Otoi T, Ooka A, Murakami M, et al. Size distribution and meiotic competence of oocytes obtained from bitch ovaries at various stages of oestrous cycle[J]. Reprod Fert Develop,2001,13(2-3):151-155.
- [30] Songsasen N, Yu I, Gomez M, et al. Effects of meiosis-inhibiting agents and equine chorionic gonadotropin on nuclear maturation of canine oocytes[J]. Mol Reprod Dev,2003,65(4):435-445.
- [31] Dunning KR, Russell DL, Robker RL. Lipids and oocyte developmental competence; the role of fatty acids and beta-oxidation [J]. Reproduction,2014,148(1):R15-R27.
- [32] Kazuhiro K, Hans E, Pasisan T, et al. Morphological features of lipid droplet transition during porcine oocyte fertilisation and early embryonic development to blastocyst in vivo and in vitro[J]. Zygote,2002,10:355-366.
- [33] Prates EG, Marques CC, Baptista MC, et al. Fat area and lipid droplet morphology of porcine oocytes during in vitro maturation with trans-10, cis-12 conjugated linoleic acid and forskolin[J]. Animal,2013,7(4):602-609.
- [34] Ambruosi B, Lacalandra GM, Iorga AI, et al. Cytoplasmic lipid droplets and mitochondrial distribution in equine oocytes: Implications on oocyte maturation, fertilization and developmental competence after ICSI [J]. Theriogenology, 2009, 71(7):1093-1104.
- [35] Prates EG, Marques CC. Fatty acid composition of porcine cumulus oocyte complexes(COC) during maturation; effect of the lipid modulators trans-10, cis-12 conjugated linoleic acid (t10, c12 CLA) and forskolin[J]. In Vitro Cell Dev-An,2013,49(5):335-345.
- [36] Apparicio M, Ferreira C R, Tata A, et al. Chemical composition of lipids present in cat and dog oocyte by matrix-assisted desorption ionization mass spectrometry (MALDI-MS) [J]. Reprod Domest Anim,2012,47(Suppl 6):113-117.
- [37] Ariu F, Strina A, Murrone O, et al. Lipid droplet distribution of immature canine oocytes in relation to their size and the reproductive stage[J]. Anim Sci J,2016,87(1):147-150.
- [38] Lindblad-Toh K, Wade CM, Mikkelsen TS, et al. Genome sequence, comparative analysis and haplotype structure of the domestic dog[J]. Nature,2005,438(7069):803-819.
- [39] Lee BC, Kim MK, Jang G, et al. Dogs cloned from adult somatic cells[J]. Nature,2005,436(7051):641.
- [40] Zou Q, Wang X, Liu Y, et al. Generation of gene-target dogs using CRISPR Cas9 system[J]. J Mol Cell Biol,2015,7(6):580-583.