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邻苯二甲酸二丁酯降低雄激素浓度导致尿道下裂发生机制研究 *

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摘要 目的：验证邻苯二甲酸二丁酯（DBP）是通过降低血清雄激素水平导致自噬异常激活，同时探讨 DBP 致子代大鼠尿道下裂发生的具体机制。**方法**：将孕鼠随机分为 DBP 染毒组与对照组，并于妊娠期 14-18 天通过灌胃的方式，分别用 DBP（750 mg/kg/ 天）饲养 DBP 染毒，用等量花生油饲养对照组。依照此方法成功构建了子代新生大鼠尿道下裂模型。采集子鼠生殖结节（GT）用福尔马林保存，用免疫组织化学（IHC）染色观察生殖结节组织中自噬水平，即 LC3B 及 Beclin1 表达水平；在子鼠麻醉后采集血液标本，用放射免疫分析方法观测子鼠血清睾酮水平。在原代大鼠尿路上皮细胞（PUECs）基础上，用 Western 印迹方法检测有无双氢睾酮（DHT）对 PUECs 中 LC3I、LC3II 及 Beclin1 表达水平影响。**结果**：DBP 染毒组尿道下裂发生率为 42.3%，对照组子代无尿道下裂。DBP 染毒组子代 GT 组织中自噬表达较对照组明显增加。DBP 染毒组（n=10）较对照组中血清睾酮水平有明显差异（n=10）（$P<0.05$）。体外研究表明 DHT 缺乏组 Beclin1 及 LC3 蛋白转化率水平较对照组升高。**结论**：孕期暴露于 DBP 可以诱发子代尿道下裂发生，这可能是由于 DBP 降低子鼠雄激素水平促使自噬发生导致的，然而该疾病的机制仍需要进一步研究。

关键词：邻苯二甲酸二丁酯；尿道下裂；雄激素；自噬
中图分类号：R-33；R695 **文献标识码**：A **文章编号**：1673-6273（2020）04-629-05

Reduced Androgen Level Induces Autophagy Leading Hypospadias Caused by Di-n-butyl Phthalate (DBP)*

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ABSTRACT Objective: To verify that reducing levels of serum androgen could induce abnormal activation of autophagy by di-n-butyl phthalate (DBP) during maternal exposure and further investigate the specific mechanism of DBP-induced hypospadias in male offspring. **Methods:** Pregnant rats were randomly divided into the DBP-treated group and the control group, the former were intragastrically treated with DBP at 750mg per kilogram of the body weight per day on the 14-18 days during gestation while the latter with the equivalent peanut oil. The hypothalamic model of the offspring newborn rats was successfully constructed according to the methods above. The expression level of autophagy markers, including LC3B and Beclin1, in the genital tubercle (GT) was measured by immunohistochemistry (IHC) staining after GT was harvested from male offspring and stored in Formalin. The level of serum testosterone in the rats were measured by radioimmunoassay with blood samples collected after anesthesia in offspring. The expression levels of LC3I, LC3II and Beclin1 were detected by Western blot in primary urethral epithelial cells (PUECs) under treatment with or without dihydrotestosterone. **Results:** The rate of hypospadias in the DBP-treated group was 42.3%, while no hypospadias was observed in the control group. The autophagy expression was increased apparently in GT tissues of DBP-treated groups while compared to the control group. The levels of serum testosterone in the DBP group (n=10) were significantly different from those in the control group (n=10)($P<0.05$). In vitro studies, the Beclin1 and ratio of LC3II/LC3I in the DHT-deficient group were higher than those in the control group. **Conclusions:** Maternal Exposure to DBP can induce hypospadias, which may be due to DBP-induced autophagy by reducing serum androgen in offspring. However, the mechanism of this disease still needs further research.

Key words: Dibutyl phthalate; Hypospadias; Androgen; Autophagy
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前言

尿道下裂是因前尿道发育不全导致尿道开口畸形的一种疾病,是阴茎中最常见的先天性畸形,发生率约为 0.3%到 0.7%[1,2]。尿道下裂的确切机制尚不清楚，但环境内分泌干扰物(EEDs)被认为是导致该先天性疾病的潜在原因。DBP 是 EEDs 典型代表,通常存在于聚氯乙烯塑料产品中,包括儿童玩具、药品、食品生产包装中。已经有大量实验证实 DBP 会影响生殖道发育,这其中包括尿道下裂[3]。

自噬在肿瘤发生发展、胚胎发育中起重要作用[4,5]。有研究发现自噬与雄激素信号通路关系密切:雄激素剥夺能明显影响前列腺上皮细胞中自噬发生[6,7]。DBP 可诱导癌细胞产生自噬,但 DBP 诱导的自噬是否影响尿道下裂发生尚未得到评估[8,9]。

孕期暴露于 DBP 可以成功建立子代尿道下裂模型[10-12]。但是其中具体的机制通路尚未明确揭示。我课题组本次研究发现 DBP 染毒组的子代大鼠 GT 组织存在自噬异常表达,并随后在 PUECs 中验证了雄激素缺乏可以诱导子代自噬水平升高。该研究结果可为 DBP 诱导的尿道下裂形成机制提供新的线索及干预方法。

1 材料和方法

1.1 动物和暴露设计

SD 大鼠由上海实验动物中心提供,并给予常规饲料、无限制饮水。于适宜昼夜交替、温度、湿度下饲养。取未妊娠过的成年雌鼠 20 只,成年雄鼠 40 只,按雌: 雄 = 1:2 合笼,于次日下午 4 点寻找阴栓,找到阴栓当日记为妊娠期(gestation day,GD)第 0 天。将成功交配的雌性大鼠随机分配成对照组和 DBP 染毒组各 10 只,每天记录体重并分别饲养。将 DBP(99.5%,Sigma Chemical Co.,St.Louis,MO,USA)溶解在花生油中。孕鼠于 GD 14-18 天给予 DBP(750 mg/kg/ 天)。对照组仅接受花生油。动物建模参见文献[13]。DBP 染毒组 52 只雄性大鼠有 22 只存在尿道下裂。对照组 42 只雄性大鼠未发现尿道下裂。各选取 10 只用于样本采集和检测。大鼠于麻醉收集血液样品,并用二氧化碳使动物安乐死。获得的生殖器结节组织于液氮中冷冻保存以用于 Western 印迹,或储存在福尔马林中用于 IHC 染色。

1.2 抗体和试剂

LC3 抗体(＃12741)购自 Cell Signaling Technology(Danvers,MA,USA)。Beclin-1 抗体(ab62472)和 GAPDH(ab8245)购自 Abcam (Cambridge,UK)。DHT (A8380) 购自 Sigma-Aldrich(Burlington, MA,US)。DBP 溶于食用油(1:3)中并以胃饲法给药,剂量为 750 mg / kg/ 天。将 DBP 溶解于 DMSO 中至 10 μmol/ L 以用于体外研究。

1.3 细胞培养

PUECs 来源于产后 7 天(PND 7)上的胎鼠尿道组织。原代细胞建立过程由 PriCells Biopharmaceutical Company (中国武汉)辅助建立。PUECs 培养在 PriCcells 上皮细胞培养基中,其中补充有 10 %胎牛血清(Gibco,Foster City,CA)和 1 %青霉素 / 链霉素(HyClone)。细胞培养液为碳吸附胎牛血清与无酚红培养基去激素置配。DHT 缺乏组为空白去激素培养液,对照组为空白去激素培养液中添加 10 nM DHT 模拟正常雄激素环境。

1.4 免疫组化

GT 组织用福尔马林固定、石蜡包埋并切片(5 μm),再用苏木精和伊红(H&E)染色。经光学显微镜以鉴定尿道下裂。按照我们既往报道的方法进行 IHC 分析[13]。在连续乙醇稀释中脱蜡再水化后,于微波炉中修复抗原 20 分钟。然后进行洗涤,封闭和用抗体孵育。一抗是 Beclin-1 抗体(1:200,ab62472,Abcam),LC3 抗体(1:200,＃12741,CST)。

1.5 蛋白质测定和 Western 印迹

使用具有 10× 蛋白酶抑制剂混合物(Thermo Scientific)的 RIPA 提取试剂提取总蛋白质。通过 BCA 蛋白质测定试剂盒测定蛋白质浓度。于聚丙烯酰胺凝胶 SDS-PAGE 进行电泳。然后将凝胶的蛋白质转移到硝酸纤维素膜(Bio-Rad)上。随后于 5%脱脂奶粉中封闭,并在 Beclin-1 抗体(1:1000,ab62472,Abcam),LC3(1:1000,#12741,CST)4℃下孵育。经 TBST 洗涤三次后,将膜与合适二抗一起温育 1 小时。使用 Supersignal West Pico 化学发光底物(Pierce)显现蛋白质印迹的结果。

1.6 放射免疫分析

选用 PND 7 的大鼠血清测定睾酮浓度。麻醉后自心脏收集血样。将血液中在 4℃下以 3000 rpm 离心 15 分钟以分离血清。按照我们既往报道的方法使用直接放射免疫测定试剂盒计算每只幼崽的睾酮浓度,重复三次,取算术平均浓度为最终睾酮浓度[13]。

1.7 统计分析

所有数据均以平均值± 标准偏差(SD)表示。每个实验组的治疗条件相同。 Mann-Whitney U 检验或独立样本 t 检验用于评估差异的显着性。使用 SPSS v.20 软件(IBM,Inc.)进行统计学分析。*P* 值 <0.05 被认为具有统计学意义。在 PND 7 时,收集每只幼鼠的生殖器结节组织用于 IHC 及 Western 印迹。大鼠相关实验及体外实验均重复至少三次。

2 结果

2.1 孕期暴露于 DBP 成功构建子代雄鼠尿道下裂模型

GD20 期 DBP 暴露组(203± 5.3 g)与对照组(204± 6.8 g)孕鼠体重无明显差异。对照组及 DBP 暴露组均无子代雄鼠死亡。DBP 暴露组子代雄鼠体重 (6.3± 0.3 g) 与对照组体重(6.4± 0.9 g)无明显差异。与对照组中 55 只子代均为正常雄性大鼠相比，实验组 52 只中有 22 只尿道下裂的雄性大鼠(42.3%)。所有雄性子鼠畸形尿道口均开口于腹侧并伴有明显阴茎缩短,这同先前发现一样,子代尿道下裂大鼠均具有明显该病特征[14]。

2.2 DBP 诱导子鼠 GT 组织中自噬发生

LC3B 及 Beclin1 是重要的自噬蛋白,LC3B 表达高低及 Beclin1 转化率可以反映自噬活性[15]。通过 IHC 分析 GT 组织得到 DBP 染毒组 LC3B 水平较对照组升高,Beclin1 水平较 DBP 染毒组降低(图 1A 和 B)。这表明尿道下裂组织中自噬水平相对较高。

2.3 DBP 致使子代血清睾酮浓度下降

通过放射免疫分析测血清睾酮，对照组中血清睾酮水平(n=10)较 DBP 染毒组(n=10)有明显差异(*P*＜0.05)。这说明 DBP 引起子代尿道下裂与血清睾酮水平下降关系紧密。

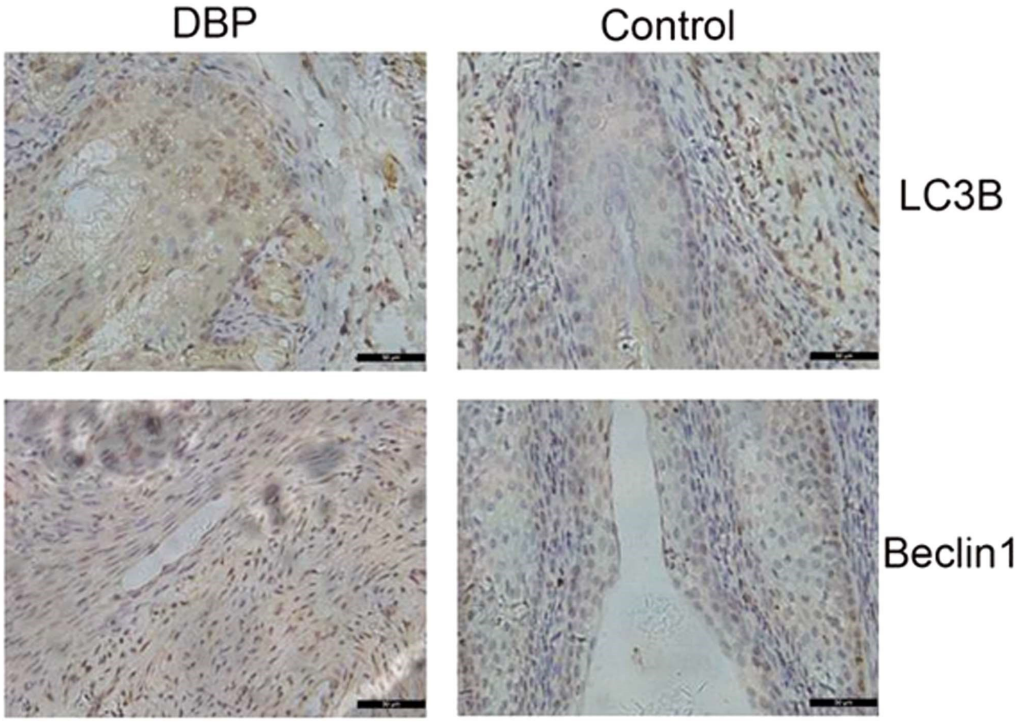

图 1 孕期暴露于 DBP 对于子鼠 GT 组织 LC3B 及 Beclin1 的影响(放大倍数 200×)

Fig.1 Effects of maternal exposure to DBP on expression of LC3B and Beclin1 in GT tissues of rats(original magnification 200×)

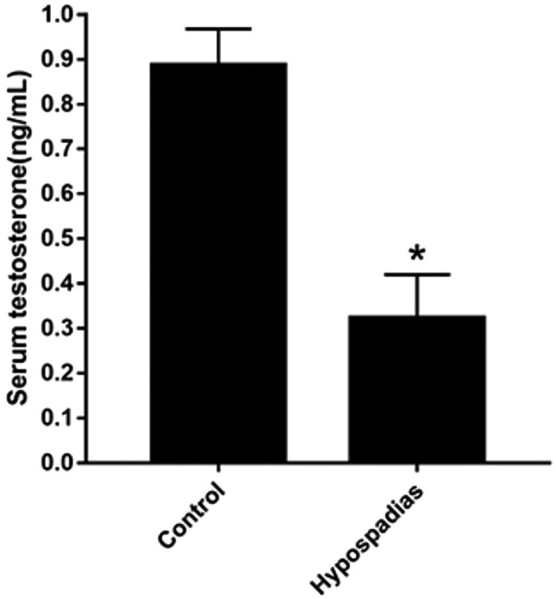

图 2 孕期暴露于 DBP 对于子代雄性大鼠血清睾酮的影响

(n=10,*P<0.05)

Fig.2 Effects of maternal exposure to DBP on serum testosterone in offspring male rats(n=10,*P<0.05)

2.4 雄激素缺乏促使自噬发生

采用 PUECs 细胞进行细胞实验以验证雄激素对于自噬的作用。Western 印迹显示 DHT(-)中 Beclin1 和 LC3 蛋白转化率(LC3II/LC3I)的表达水平升高。这表明雄激素缺乏在调节 DBP 诱导的自噬中起到关键作用。

3 讨论

尿道下裂是男性泌尿生殖系统常见疾病之一,主要表现为尿道口开口先天性畸形。其具体发病机制尚不清楚,目前认为其出现主要与内分泌、遗传、环境因素有关[16]。本次研究基于高表达的自噬与 DBP 致尿道下裂发展有关的假设,发现 DBP 可能是通过降低雄激素水平诱导自噬,进而导致尿道下裂发生。

目前研究发现自噬参与各种生理过程,包括细胞应激、损伤调节及免疫应答等[17,18]。既往研究发现,自噬参与了精子细胞顶体反应发生,并可通过促进精子线粒体自噬,使受精卵发生母系遗传[19,20],这暗示了自噬对于生殖及胚胎发育具有潜在影响。最新研究报道了邻苯二甲酸盐可以引起在睾丸组织 ATG5 和 Beclin1 表达升高[21]。该结果说明邻苯二甲酸盐类与自噬的发生有较强相关性。因此我们怀疑自噬与 DBP 引起的尿道下裂有关。本次研究中我们检测了尿道下裂子代生殖结节组织中的自噬标志物,发现自噬标志物在生殖结节中显着表达,因此有理由认为自噬与尿道下裂的发生有密切关系。但目前国内外鲜有自噬与尿道下裂发生间具体机制的报道,关于自噬如何进一步诱导尿道下裂发生仍需进一步研究。

邻苯二甲酸酯类化合物(PAEs)作为塑料增塑剂广泛用于人们生活中,其对于内分泌系统的干扰及毒性作用近年来备受关注[22]。DBP 作为 PAEs 的一种,存在于各种消费产品当中,包括婴儿棉衣及化妆品当中[23,24]。DBP 主要通过抗雄激素活性以对生殖发育产生不利影响,而这一路经主要通过影响睾丸结构和功能,并最终使雄激素合成与分泌不足。雄激素在个体生长发育过程中的重要性已被公认,尤其是在雄性生殖系统生长、分化和发育中尤为重要[25-28]。最新的分子流行病学研究发现,与 ESR1 单核苷酸多态性相关的雌激素内分泌干扰物会导致尿道下裂的发生[29]。而 DBP 作为内分泌干扰物被认为可引起雄激素缺乏。本次研究我们采集子代大鼠的血液样本,发现 DBP 染毒组血清睾酮水平较对照组有显著降低,这与先前的研究结论一致。其他研究发现,雄激素缺乏会在与雄激素密切相关的其他组织(如前列腺)中引起自噬发生[7,30,31]。因此我们怀疑 DBP

可经抑制血清睾酮水平诱导自噬发生。为了证明这一假说,我们通过建立原代大鼠尿路上皮细胞(PUECs)模型,发现 DHT 缺乏导致自噬相关蛋白 Beclin1 和 LC3 表达相对增高,这表明至少在体外实验中,雄激素的缺乏可直接升高尿道组织中的自噬水平,但进一步的结果仍需要体内实验结果证实。

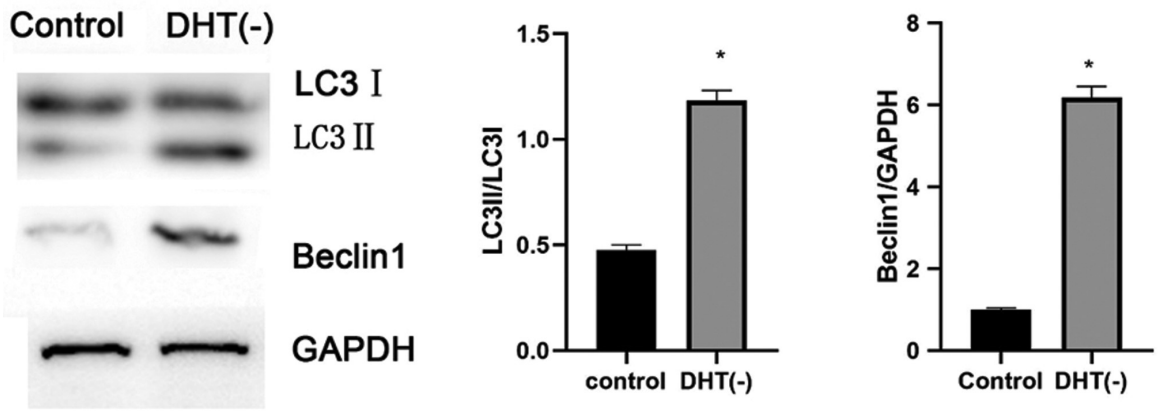

图 3 DHT 对 PUECs 细胞中 LC3I、LC3II 及 Beclin1 的影响(*P<0.05)

Fig.3 Effect of DHT on LC3I, LC3II and Beclin1 in PUECs(*P<0.05)

本次讨论也有明显的不足,因为条件所限,我们没有对具体的机制进行进一步研究。但我们研究发现,孕鼠经 DBP 作用后会引起子代雄激素水平下降,并导致子代 GT 组织中异常自噬发生,且该路径与尿道下裂发展具有较强相关性。我们首次提出了雄激素缺乏诱导自噬发生是 DBP 暴露下尿道下裂发生的可能机制之一,这使得通过针对雄激素缺乏、自噬的路径治疗由 DBP 引起的尿道下裂的潜在方法成为可能。本次发现还为母体因环境内分泌干扰化合物而引起子代的其他畸形的机制途径提供新的思路。

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