

邻苯二甲酸二(2-乙基己基)酯对 离体人成熟精子功能的影响

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摘要 该文研究邻苯二甲酸二(2-乙基己基)酯[di(2-ethylhexyl) phthalate, DEHP]在体外对人成熟精子功能的影响。用0, 0.1, 1, 10, 100 $\mu\text{mol/L}$ DEHP处理离体人成熟精子后, 利用伊红苯胺黑染色、计算机辅助精子分析系统、精子穿甲基纤维素实验、金霉素染色等方法检测精子存活率、运动、超活化、获能、顶体反应等生理功能。结果显示, DEHP在短时间内不影响精子存活率, 但是抑制精子运动、超活化以及孕酮诱导的获能和顶体反应。推测DEHP体外急性染毒会抑制人成熟精子功能。

关键词 邻苯二甲酸二(2-乙基己基)酯; 人成熟精子; 运动; 超活化; 顶体反应

Effect of Di(2-Ethylhexyl) Phthalate on the Functions of Human Spermatozoa *In Vitro*

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Abstract The aim of this study was to explore the effect of di(2-ethylhexyl) phthalate (DEHP) on the functions of human spermatozoa *in vitro*. Human ejaculated spermatozoa *in vivo* were exposed to DEHP at different concentrations (0, 0.1, 1, 10, 100 $\mu\text{mol/L}$). The viability, motility, hyperactivation and acrosome reaction (AR) of DEHP-treated spermatozoa were assessed by eosin-nigrosin staining, computer-aided sperm analysis, penetration of the artificial viscous medium and chlortetracycline staining. The results indicated that DEHP did not affect the viability of human spermatozoa, but it significantly inhibited the motility, hyperactivation and progesterone induced capacitation and AR of human spermatozoa. These results implied that *in vitro* exposure to DEHP inhibits the function of human spermatozoa in a short time.

Keywords di(2-ethylhexyl) phthalate; human spermatozoa; motility; hyperactivation; acrosome reaction

邻苯二甲酸二(2-乙基己基)酯[di(2-ethylhexyl) phthalate, DEHP]是典型的邻苯二甲酸的酯化衍生物(phthalate esters, PAEs), 在工业生产中作为塑化剂

广泛用于医疗器材、生活用品、化妆品等领域^[1]。DEHP与塑料分子以氢键或范德华力连接, 因此很容易通过物理方式脱离塑料进入大气、食品、饮用水

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中,也可以在塑料生产或燃烧过程中通过烟尘的沉降释放到环境中,最终进入人体^[2]。虽然DEHP急性毒性较低,但长期暴露于其中会导致肝、心、肺以及生殖发育系统的毒性,因此被广泛关注^[3-8]。

研究表明,PAEs进入人或动物体内会有类似雌激素的作用,从而干扰内分泌,因此是一种潜在的环境雌激素污染物^[9]。DEHP作为应用最为普遍的PAEs,其雄性生殖毒害作用早在1945年就有报道^[10]。大量啮齿类动物实验结果显示,DEHP不仅扰乱雄性动物睾酮的分泌,而且影响睾丸的正常发育,造成睾丸畸形和损伤,影响精子发生^[4-6,11-13]。此外,DEHP同样具有雌性生殖毒害作用。它能干扰雌性动物正常内分泌,改变雌二醇、孕酮、促黄体生成素水平及卵泡刺激素的分泌量,扰乱雌性动物正常排卵^[14-16]。目前已发现的DEHP雄性生殖毒性作用机制可能为:DEHP破坏睾丸中支持细胞细胞膜,影响支持细胞正常功能,无法为生殖细胞提供物质和代谢支持^[5,17];DEHP还导致生殖细胞氧化应激,提早凋亡^[12];此外,DEHP还激活过氧化物酶体增殖物激活受体(peroxisome proliferator-activated receptors, PPARs),改变与睾丸发育、精子发生以及睾酮生物合成相关基因的表达^[18-20]。DEHP同样具有雌性生殖毒性,它作用于卵巢,通过激活PPARs,抑制雌二醇合成过程中相关酶活性,激活氧化应激,积累活性氧物质造成卵巢损伤^[14,21]。

Huang等^[22]发现,长期接触DEHP的聚氯乙烯加工厂工人的精液中DEHP含量升高,且影响精子质量。其他的研究组也发现,少弱精不育症病人体内含有较高浓度的DEHP^[23-26],这说明DEHP在体内影响人精子发生,降低精子质量^[27]。人成熟精子是男性生殖能力的最终体现者,精子正常功能(运动、超活化、获能、顶体反应等)是保证顺利完成受精过程的关键因素^[28]。但是,DEHP对人精子超活化、获能、顶体反应等功能的影响尚不清楚。因此,本研究通过在体外用不同浓度DEHP处理人成熟精子,检测其对成熟精子生理功能的影响,为进一步系统地阐明DEHP的雄(男)性生殖毒害作用及其机制提供理论基础。

1 材料与方法

1.1 主要试剂

DEHP(母液用二甲亚砜配制)、盐酸金霉素、孕酮和甲基纤维素等分析纯化学试剂均购自Sigma

公司; Percoll分层液购自GE公司; HTF培养基购自Millipore公司; 精子活力伊红苯胺黑染色试剂盒购自上海酶联生物科技有限公司。

1.2 精液标本采集

按照WHO标准从南昌大学第二附属医院门诊就诊的男性中筛选正常活力[总活力 $\geq 50\%$, 前向运动a级 $\geq 25\%$ 或(a+b)级 $\geq 50\%$]的精液标本,标本提供者年龄25~40岁,无心、肝、肾疾病,禁欲3~5 d,取样前48 h禁酒和咖啡等对精子有影响的食物或药物,手淫取精。

1.3 精子存活率和运动能力检测

10例正常人精液样本,每例分成5组(90 μL /组),移入5个1.5 mL离心管中,分别加入10 μL 含有不同浓度DEHP的HTF培养基,使得DEHP的终浓度分别为0, 0.1, 1, 10, 100 $\mu\text{mol/L}$,其中,0 $\mu\text{mol/L}$ 为加入0.1%二甲亚砜的对照(下同)。混匀后于37 $^{\circ}\text{C}$ 、5% CO_2 的细胞培养箱中孵育,分别于1, 12, 24 h三个时间点取各管精子进行活力和运动力检测。精子存活率和运动能力检测参照文献^[29]。

1.4 精子超活化检测

正常人精液样本经percoll密度梯度离心纯化后,用HTF培养基重悬后,分成5组(90 μL /组),移入5个1.5 mL离心管中,分别加入10 μL 含有不同浓度DEHP的HTF培养基,使得DEHP的终浓度分别为0, 0.1, 1, 10, 100 $\mu\text{mol/L}$ 。混匀后于37 $^{\circ}\text{C}$ 、5% CO_2 的细胞培养箱中孵育2 h后,分别插入装有1%(w/v)甲基纤维素溶液的毛细管,于37 $^{\circ}\text{C}$ 、5% CO_2 的细胞培养箱中继续孵育1 h后,取出毛细管,用徕卡DM2500显微镜观察毛细管1 cm和2 cm处精子数。具体操作详见参考文献^[30]。

1.5 精子获能和顶体反应检测

正常人精液样本经percoll密度梯度离心纯化后,用HTF培养基重悬。纯化后的精子样本分成2组,自发顶体反应组和诱发顶体反应组:每组分别取5个1.5 mL离心管,分别加入48 μL 纯化精子样本和1 μL 含有不同浓度DEHP的HTF培养基至终浓度分别为0, 0.1, 1, 10, 100 $\mu\text{mol/L}$,混匀后于37 $^{\circ}\text{C}$ 、5% CO_2 的细胞培养箱中获能3.5 h后,分别在自发顶体反应组各管中加入1 μL HTF和在诱发顶体反应组各管中加入1 μL 含有20 $\mu\text{mol/L}$ 孕酮的培养基,混匀后继续孵育0.5 h。精子获能和顶体反应通过金霉素(chlortetracycline hydrochloride, CTC)染色后,用徕卡DM2500

正置荧光显微镜观察和拍照。具体操作详见参考文献[29]。

1.6 数据统计

实验数据以 $\text{mean} \pm \text{SEM}$ 形式表示, 显著性差异分析用SPSS 11.5软件, 采用 t 检验进行数据分析。 $P < 0.05$ 为差异具有显著性, $P < 0.001$ 、 $P < 0.0001$ 均为差异极显著。

2 结果

2.1 DEHP不影响离体人成熟精子的存活率

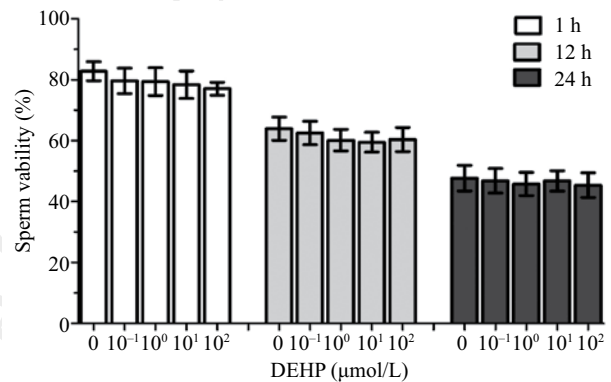
研究表明, DEHP具有较低的急性毒性和体外细胞毒性, 但是对离体人成熟精子的毒性尚不清楚。为了综合评价DEHP在体外对人成熟精子功能的影响, 本研究首先检测DEHP对离体人成熟精子是否具有毒性。结果表明, 与对照组相比, 0.1, 1, 10, 100 $\mu\text{mol/L}$ DEHP作用的精子, 其三个时间点(1, 12, 24 h)的存活率均无显著差异(图1)。这说明, 在短时间内(24 h), DEHP对离体人成熟精子不具有毒性。

2.2 DEHP抑制离体人成熟精子运动

人成熟精子的正常功能是男性生殖能力的保障, 而其运动能力是精子功能的直观表现。本实验的结果表明, 与对照组相比, 10, 100 $\mu\text{mol/L}$ DEHP显著抑制离体人精子三个时间点(1, 12, 24 h)的总运动率(total motility), 同时前向运动率(progressive motility)也随总运动率的降低而下降; 而1 $\mu\text{mol/L}$ DEHP在短时间(24 h)内并不会引起成熟精子运动的改变(图2)。

2.3 DEHP抑制人精子穿透甲基纤维素溶液能力

人精子穿透甲基纤维素实验能综合评价精子质量(畸形、运动以及数量), 也常用于检测精子超活化运动能力。鉴于上述DEHP能够抑制人精子运动能力的结果, 进一步检测DEHP对人精子穿透甲基纤维素溶液能力的影响, 综合评价其对精子质量的影响。结果显示, 10, 100 $\mu\text{mol/L}$ DEHP能够显著抑制人精子穿透甲基纤维素溶液能力, 而1 $\mu\text{mol/L}$ DEHP无效应(图3)。

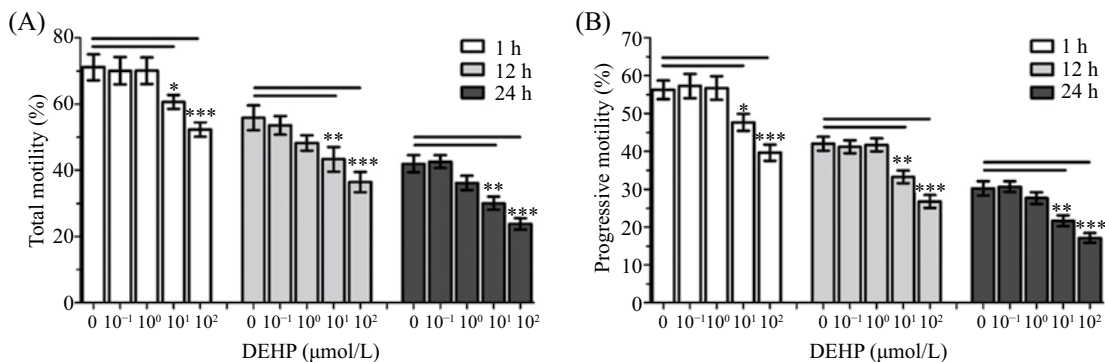


正常人精液样本经0.1, 1, 10, 100 $\mu\text{mol/L}$ 的DEHP处理1, 12, 24 h后, 分别用精子活力伊红苯胺黑染色试剂盒检测活力。n=10。

Human ejaculated sperm *in vitro* were exposed to DEHP at different concentrations (0.1, 1, 10, 100 $\mu\text{mol/L}$) for 1 h, 12 h and 24 h, respectively. Then, the viability were assessed by eosin-nigrosin staining. n=10.

图1 DEHP对人成熟精子活力的体外影响

Fig.1 The effect of DEHP on viability of human ejaculated sperm *in vitro*

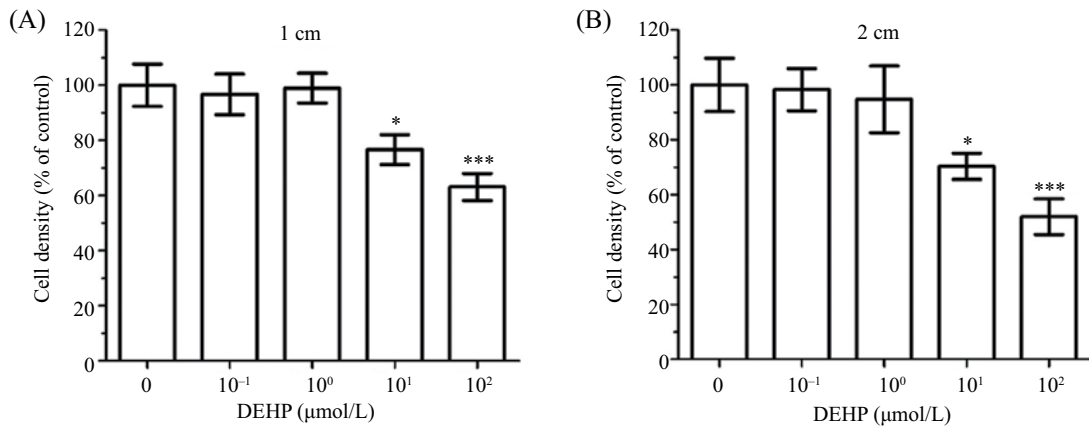


正常人精液样本经0.1, 1, 10, 100 $\mu\text{mol/L}$ 的DEHP处理1, 12, 24 h后, 分别用计算机辅助精液分析系统检测总活动率(A)和前向运动率(B)。n=10。 $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$, 与对照组(0 $\mu\text{mol/L}$)相比。

Human ejaculated sperm *in vitro* were exposed to DEHP at different concentrations (0.1, 1, 10 and 100 $\mu\text{mol/L}$) for 1 h, 12 h and 24 h, respectively. Then, the total motility (A) and progressive motility (B) were examined by computer-aided sperm analysis system. n=10. $*P < 0.05$, $**P < 0.001$, $***P < 0.0001$ compared with the control group (0 $\mu\text{mol/L}$).

图2 计算机辅助精液分析系统测定DEHP处理的人成熟精子运动参数

Fig.2 The motile parameters of DEHP-treated human ejaculated sperm were examined by computer-aided sperm analysis system

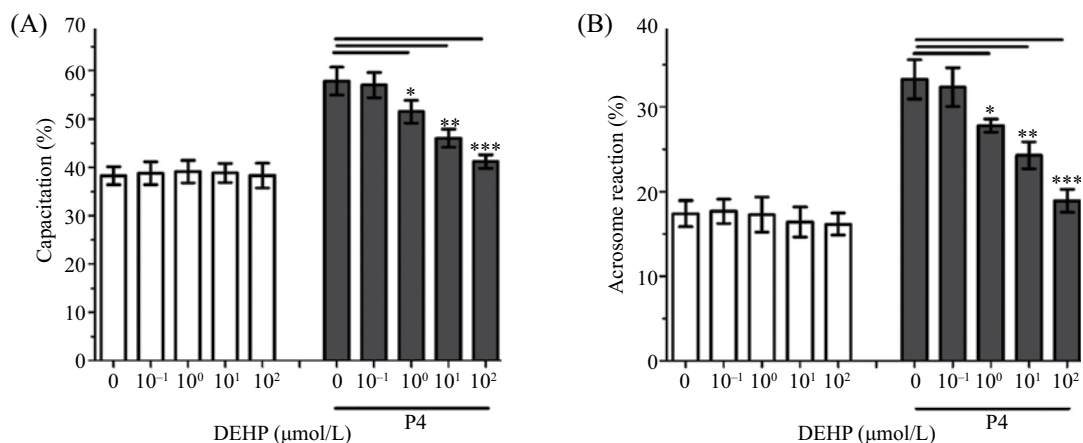


纯化的人精子经0.1, 1, 10, 100 μmol/L的DEHP处理后, 穿透1%甲基纤维素溶液。1 cm(A)和2 cm(B)处精子数与对照相比后的百分数评价其穿透甲基纤维素能力。n=10。*P<0.05, **P<0.001, ***P<0.000 1, 与对照组(0 μmol/L)相比。

Human ejaculated sperm *in vitro* were exposed to DEHP at different concentrations (0.1, 1, 10, 100 μmol/L), and then penetrated the 1% methylcellulose solution. Cell density (percentage of control) of 1 cm (A) and 2 cm (B) into methylcellulose was shown. n=10. *P<0.05, **P<0.001, ***P<0.000 1 compared with the control group (0 μmol/L).

图3 DEHP对人成熟精子穿透甲基纤维素溶液能力的影响

Fig.3 The effect of DEHP on human sperm ability of penetration into viscous medium



纯化人精子经0.1, 1, 10, 100 μmol/L的EDPE处理后, 获能3.5 h, 自发获能(A)和顶体反应(B)以及20 μmol/L孕酮(P4)诱导的获能(A, P4)和顶体反应(B, P4)用金霉素(CTC)染色法检测。n=9。*P<0.05, **P<0.001, ***P<0.000 1, 与对照组(0 μmol/L)相比。

Human ejaculated sperm *in vitro* were exposed to DEHP at different concentrations (0.1, 1, 10, 100 μmol/L) and capacitated for 3.5 h, the spontaneous capacitation (A) and acrosome reaction (B) along with the 20 μmol/L progesterone (P4) induced capacitation (A) and acrosome reaction (B) of human sperm were examined by CTC staining. n=9. *P<0.05, **P<0.001, ***P<0.000 1 compared with the control group (0 μmol/L).

图4 DEHP对人成熟精子获能和顶体反应的影响

Fig.4 The effects of DEHP on capacitation and acrosome reaction of human sperm *in vitro*

2.4 DEHP抑制孕酮诱导的人精子获能和顶体反应

人精子进入女性生殖道后, 需经历获能和顶体反应来完成正常授精。获能和顶体反应是反映精子正常功能的重要指标^[19-20]。本实验的结果显示, DEHP对人精子自发顶体反应没有影响, 但显著抑制重要生理激素孕酮诱导的获能和顶体反应(图4A和图4B)。

3 讨论

研究表明, 在少弱精的男性不育病人精液中能检测到1 μmol/L甚至更高的DEHP^[25]。据此, 本研究

设置了0.1, 1, 10, 100 μmol/L四个不同浓度的DEHP, 在体外检测DEHP对人成熟精子生理功能的影响。结果显示, DEHP(0.1, 1, 10, 100 μmol/L)在短时间内(24 h)对人精子不造成毒性, 不降低人精子的存活率(图1)。但是, 10, 100 μmol/L DEHP显著降低精子运动(图2)以及超活化(图3)。正常情况下, 精液中DEHP的浓度小于1 μmol/L, 而在男性不育病人精液中能检测到1 μmol/L甚至更高的DEHP。但是, 这些浓度的检测都是在DEHP已经代谢稳定的情况下检测的, 大部分DEHP已经代谢为其代谢产物, 只有少

量以DEHP形式存在^[25],因而,10 $\mu\text{mol/L}$ DEHP很可能在其最初代谢过程中出现,并且维持数小时。本文结果表明,DEHP在几个小时以内影响精子功能,因此,10 $\mu\text{mol/L}$ DEHP对人精子功能的影响在一定程度上提示其男性生殖毒性。更重要的是,1 $\mu\text{mol/L}$ DEHP显著抑制重要生理激素孕酮诱导的精子获能和顶体反应(图4)。长期接触DEHP的女性,其生殖道中DEHP浓度高达2 $\mu\text{mol/L}$ ^[31-32],由此推测,DEHP抑制精子进入女性生殖道后的正常受精。DEHP抑制离体人成熟精子功能的机制可能与其体内生殖毒性不尽相同。首先,DEHP作用方式不同。DEHP进入体内后作用于睾丸中支持细胞和生殖细胞,导致其氧化应激,提早凋亡,改变与睾丸发育和精子发生以及睾酮生物合成相关基因的表达^[5,12,17,20,27]。然而,成熟精子是高度特化、转录沉默的细胞,DEHP对成熟精子功能的调控可能通过胞内信号转导来实现^[33]。其次,DEHP作用时间不同。DEHP进入体内要经过代谢和体液循环过程到达靶细胞后,再引起氧化应激,改变基因表达,诱导细胞凋亡。这些过程需要相对较长的时间^[27]。而DEHP通过胞内信号转导,在较短的时间内能抑制精子功能^[33]。最后,DEHP起作用的浓度不同。本研究结果表明,DEHP体外抑制人精子功能所需浓度比体内更高(图2~图4),这可能与其作用方式和时间有关。

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