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原花青素减轻热应激诱导的 牛卵母细胞氧化应激

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摘要:【目的】旨在探究体外成熟液中添加原花青素 B2(PB2)对热应激牛卵母细胞的保护机制, 为揭示热应激对牛卵母细胞质量和胚胎体外培养发育能力的影响提供科学依据。【方法】以 6~8 岁淘汰荷斯坦牛为试验对象, 屠宰场共采集牛卵巢 103 对。实验室抽吸卵巢表面卵泡内卵丘-卵母细胞复合体(COCs), 将 COCs 随机分成 3 组进行体外成熟: 对照组(38.5 °C 培养 24 h); 热应激组(41 °C 培养 8 h 转入 38.5 °C 培养箱继续培养 16 h); 原花青素组(25 μmol/L PB2, 41 °C 培养 8 h 转入 38.5 °C 培养箱继续培养 16 h)。体外成熟培养 24 h, COCs 经透明质酸酶消化后, 在体视显微镜下统计各组卵母细胞成熟率; 使用化学试剂对卵母细胞进行孤雌激活, 体外培养 48 h 统计卵裂率, 168 h 统计囊胚率; 利用荧光染色法检测卵母细胞活性氧(ROS)、还原型谷胱甘肽(GSH)活性, 以及囊胚细胞总数; 利用实时荧光定量 PCR(qPCR)检测各组卵母细胞抗氧化基因(*SOD1*、*SOD2*、*CAT*)和成熟相关基因(*GSS*、*GPX1*、*CAS6*)相对表达量。【结果】(1)COCs 体外成熟 24 h, 与热应激组相比, 原花青素组提高了卵母细胞成熟率, 但两者之间差异不显著($P>0.05$); (2)孤雌激活后, 与热应激组相比, 原花青素组卵裂率和囊胚细胞总数显著提高($P<0.05$), 囊胚率差异不显著($P>0.05$); (3)与热应激组相比, 原花青素组 ROS 活性极显著降低($P<0.01$), GSH 活性显著提高、成熟基因和抗氧化基因表达量显著升高($P<0.05$)。【结论】在体外成熟培养液中添加 25 μmol/L 原花青素 B2, 可以有效缓解热应激对牛卵母细胞造成的氧化应激, 增强胚胎发育潜力, 并提高其质量。

关键词:原花青素; 卵母细胞; 热应激; 氧化应激

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Reduction of heat stress-induced oxidative stress in bovine oocytes by Proanthocyanidins

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Abstract: [Objective] The objective of this study is to explore the protective mechanisms of proanthocyanidin B2 (PB2) when incorporated into the maturation medium of bovine oocytes under heat stress conditions. This investigation aims to provide a scientific foundation for enhancing the quality of bovine oocytes exposed to high temperatures and improving their capacity for embryonic development in vitro. [Method] A total of 103 pairs of ovaries were collected from 6–8 years old Holstein cattle. The cumulus oocyte complex (COCs) in ovarian follicles were aspirated in the laboratory and randomly divided into 3 groups for in vitro maturation: control group (cultured at 38.5 °C for 24 h); heat stress group (cultured at 41 °C for 8h and transferred to 38.5 °C incubator for 16 h); proanthocyanidin group (25 μmol/L PB2, cultured at 41 °C for 8h and transferred to 38.5 °C incubator for 16 h). After the oocytes were treated by hyaluronidase, the oocyte maturation rate of each group was measured under stereomicroscope. After parthenogenetic activation of oocytes by chemical agent, the cleavage rate and blastocyst rate were recorded at 48 h and 168 h, respectively. The ROS and GSH of oocytes and the total number of blastocysts were measured by fluorescence staining. The expression levels of antioxidant genes (*SOD1*, *SOD2*, *CAT*) and maturation genes (*GSS*, *GPX1*, *CAS6*) were detected by quantitative real-time PCR (qPCR). [Result] (1) COCs matured in vitro for 24 h. Compared with the heat stress group, the proanthocyanidin group increased the oocyte maturation rate, but there was no significant difference between the two groups ($P>0.05$); (2) After parthenogenesis activation, compared with heat stress group, the cleavage rate of proanthocyanidin group was significantly increased ($P<0.05$), the blastocyst rate was not significantly different ($P>0.05$), but the total number of blastocyst cells was significantly increased ($P<0.05$); (3) After 24h maturation in vitro, compared with heat stress group, ROS content in proanthocyanidins group was significantly decreased ($P<0.01$), GSH content was significantly increased ($P<0.05$), the expression levels of maturation genes and antioxidant genes were significantly increased ($P<0.05$). [Conclusion] By adding proanthocyanidin B2 to in vitro maturation medium, oxidative stress of bovine oocytes induced by heat stress can be effectively alleviated, thus improving the maturation process of oocytes in vitro and enhancing the development potential of embryos as well as embryo quality.

Keywords: proanthocyanidin; oocytes; heat stress; oxidative stress

【研究意义】动物遭受热应激时,采食量减少、生长速度减慢、繁殖性能下降、免疫功能紊乱,对畜牧业生产造成严重的经济损失。热应激对牛繁殖性能的影响主要表现在降低公畜精液品质,引发母畜发情异常、受精率降低、受胎率下降、流产率提高等方面^[1-2]。热应激对牛繁殖性能的影响是限制养牛业发展的重要瓶颈,已受到国内外畜牧业广泛关注。【前人研究进展】牛遭受高温刺激时卵巢上卵泡内的生化特性发生改变,导致卵母细胞质量降低,发育能力受阻。在卵泡生长或卵母细胞成熟期间,高温会导致卵母细胞早期老化并损害其功能。在生发泡破裂(germinal vesicle breakdown, GVBD)阶段,高温导致核和细胞质成熟减弱,纺锤体形成异常,此外,高温还干扰卵母细胞线粒体功能, DNA断裂,导致胚胎质量下降。热应激造成羊卵母细胞在生发泡破裂阶段发育停滞,染色质结构异常^[3]。牛卵母细胞在热应激条件下,体外成熟受阻,早期胚胎发育潜力降低^[4]。猪卵母细胞在热应激条件下,卵母细胞线粒体功能受损,大量活性氧物质(reactive oxygen species, ROS)产生,卵母细胞发生氧化应激,卵母细胞结构和发育受损,导致早期胚胎发育受损、死亡^[5-6]。为克服热应激对卵母细胞线粒体以及胚胎发育的影响,研究人员尝试在成熟液中添加各种试剂。Ross等^[7]在成熟液中添加胰岛素样生长因子1 (insulin-like growth factor 1, IGF-I),将卵巢卵泡内卵丘-卵母细胞复合体(cumulus oocyte complex, COCs)置于41 °C高温条件下,持续14 h,发现IGF-I增加了线粒体膜电位活性、减少了卵母细胞中ROS的产生,并降低了囊胚内细胞团

(inner cell mass, ICM)中细胞凋亡的百分比。Li等^[8]在猪卵母细胞成熟液中添加褪黑素减少了热应激卵母细胞的ROS的产生,提高了卵母细胞受精能力,改善了热休克卵母细胞的氧化状态。Nabenishi等^[5]在牛卵母细胞成熟液中加入半胱氨酸恢复了核成熟、提高了囊胚率和GSH含量。

【本研究切入点】原花青素(proanthocyanidin)是一种天然多酚类化合物,广泛存在于植物中,包括水果、蔬菜、坚果等^[2]。在原花青素的各种异构体中,原花青素B2(proanthocyanidin B2, PB2)是主要的二聚体,来源最广泛^[9]。PB2具有多种生物活性,包括显著的抗氧化、抗炎、抗毒性和抗肿瘤作用^[10-11]。有研究^[12]表明,原花青素B2(PB2)能促进糖尿病小鼠卵母细胞的成熟进程,恢复成熟期卵母细胞老化引起的发育停滞。PB2通过增强线粒体膜电位活性,降低ROS产生,提高羊卵母细胞发育能力^[13]。PB2还可以通过调节*Nrf2/ARE*信号通路保护小鼠大脑皮层神经元免受氯氰菊酯诱导的氧化损伤^[14]。金磊^[15]前期初步研究发现:在牛体外成熟液中添加25 $\mu\text{mol/L}$ 的PB2显著提高了卵母细胞成熟率,提高了胚胎发育能力。然而,天然有效的抗氧化剂PB2对热应激牛卵母细胞的作用机制尚不清晰。【拟解决的关键问题】在荷斯坦奶牛卵母细胞体外成熟液中添加25 $\mu\text{mol/L}$ PB2,探究其对热应激诱导氧化应激过程中,对卵母细胞成熟保护机制,为体外胚胎工厂化生产提供科学参考或理论依据。

1 材料与方法

1.1 试验材料

牛卵巢采自焦作市伊赛肉牛屠宰场,品种为荷斯坦奶牛,共屠宰106头年龄为6~8岁的健康牛,采集到可用卵巢103对。卵巢保存在37 $^{\circ}\text{C}$ 含有100 mg/L青霉素和50 mg/L链霉素的生理盐水中,4 h内送回实验室。抽吸卵泡液前用生理盐水清洗卵巢3遍,用擦手纸将卵巢表面多余的水分吸干净。用带有12号针头的注射器抽吸卵巢表面直径为3~8 mm卵泡的卵泡液。卵泡液被转移到90 mm培养皿中,于体视镜下用捡卵针挑选含3层以上卵丘细胞且胞质均匀的COCs进行体外成熟^[16]。

1.2 试验设计

COCs随机分成3组体外成熟24 h:(1)对照组(Conrtol):38.5 $^{\circ}\text{C}$,培养24 h;(2)热应激组(HS):41 $^{\circ}\text{C}$ 培养8 h,之后在38.5 $^{\circ}\text{C}$ 中继续培养16 h。(3)原花青素组(HS+PB2):在含有原花青素(25 $\mu\text{mol/L}$)的成熟液中,41 $^{\circ}\text{C}$ 培养8 h,转移到38.5 $^{\circ}\text{C}$ 中继续培养16 h。用透明质酸酶消化卵丘细胞,统计各组成熟率,检测卵母细胞ROS、GSH及相关基因表达,孤雌激活后统计各组胚胎发育能力。每次试验重复3次,每组COCs个数90枚。

1.3 测定项目与方法

1.3.1 卵母细胞体外成熟

COCs放入体外成熟液中置于饱和湿度、38.5 $^{\circ}\text{C}$ 、5% CO_2 培养箱中培养24 h。成熟液由90% TCM-199(Gibco, LifeTechnologies Corporation)、10%FBS(Gibco, LifeTechnologies Corporation)、E2 1 $\mu\text{g/mL}$ (SIGMA-ALDRICH, Co.)、FSH 10 $\mu\text{g/mL}$ (Animal Health Canada Inc.)、LH 10 $\mu\text{g/mL}$ (中国科学院畜牧研究所)组成。将培养24 h的COCs转移到0.1%透明质酸酶TCM-199溶液中消化1 min,期间用移液枪轻轻吹打,促使卵丘细胞脱落。将脱去卵丘细胞的卵母细胞快速转移到38.5 $^{\circ}\text{C}$ 含10% FBS的TCM-199中终止消化,清洗3次,在体视镜下挑选带有第一极体的卵母细胞,此时卵母细胞处于第2次减数分裂中期(metaphase of meiosis II, MII),以第一极体的排出为卵母细胞成熟的标志,统计卵母细胞成熟率。

1.3.2 ROS、GSH活性测定

ROS的测定方法参照张航等^[17],GSH测定方法参照Lee等^[18],每组各取10枚卵母细胞置于100 μL 染色工作液中,饱和湿度、38.5、5% CO_2 培养箱内温育30 min,每隔5 min轻轻晃动,使细胞中的ROS与DCFH-DA探针(上海碧云天生物技术有限公司)或者细胞中GSH与TrackerBlue CMF2HC染料(invitrogen,赛默飞世尔科技公司)充分接触;用含1%PVA的PBS洗涤3遍,除去多余的探针或染料;将卵母细胞置于倒置荧光显微镜(Carl Zeiss AG)下拍摄荧光图像,荧光越强则其含量越高,用Image-J v1.53软件分析图像荧光密度值。

1.3.3 卵母细胞总RNA提取和反转录

总RNA的提取使用微量RNA提取试剂盒(赛默飞世尔科技公司),按照说明书进行操作,将10个带

有第一极体的卵母细胞放入 Extraction Buffer 中,42 ℃ 孵育 30 min,然后加入 100 μL 70% 乙醇混合,转入纯化柱,2 200 r/min 离心 2 min,再加入 100 μL Wash Buffer,8 000 r/min 离心 1 min 重复 2 遍。将纯化柱转移到新的 0.5 mL 微离心管中,添加 10 μL DEPC 水,室温静置 1 min,16 000 r/min 离心 1min,丢弃纯化柱,将微离心管收集到的 RNA,直接使用反转录试剂盒(QIAGEN)逆转录 cDNA;使用定量试剂盒(QIAGEN)定量分析^[19]。

1.3.4 卵母细胞孤雌激活

孤雌激活方法参考吴娇^[20]进行,将卵母细胞置于 5 μmol/L 离子霉素溶液(笠圣生物科技有限公司)中避光孵育 5 min,转入 2 mmol/L 6-二甲氨基嘌呤(上海源叶科技有限公司)的溶液中继续孵育 4 h,最后转移到 IVC(in vitro culture)培养液中,在 38.5 ℃、5% CO₂ 培养箱中培养 48 h 统计卵裂率,168 h 统计囊胚率。

1.3.5 囊胚细胞数计数

囊胚细胞计数参照万鹏程等^[21]方法操作,将囊胚放入 PBS/PVP 洗 3 次,转移到 10 μg/mL 的 Hoechst 33342 细胞蓝色荧光染料液(上海碧云天生物技术有限公司)中避光孵育 15 min,然后在 PBS/PVP 液滴中洗 3 次,吸取液体和囊胚置于载玻片上,盖上盖玻片,置于荧光显微镜(Carl Zeiss AG)下拍照,统计囊胚细胞数。有关指标的计算公式如下:

成熟率=MII 期卵母细胞数/卵母细胞总数×100%(1)

卵裂率=卵裂数/MII 期卵母细胞数×100%(2)

囊胚率=囊胚数/卵裂数×100%(3)

1.4 数据统计与分析

试验数据用 GraphPad Prism9.8 软件进行分析,显著性统计使用软件中的 ANOVA 模块进行单因素方差分析。所有试验均重复 3 次。

2 结果与分析

2.1 原花青素对热应激卵母细胞发育能力的影响

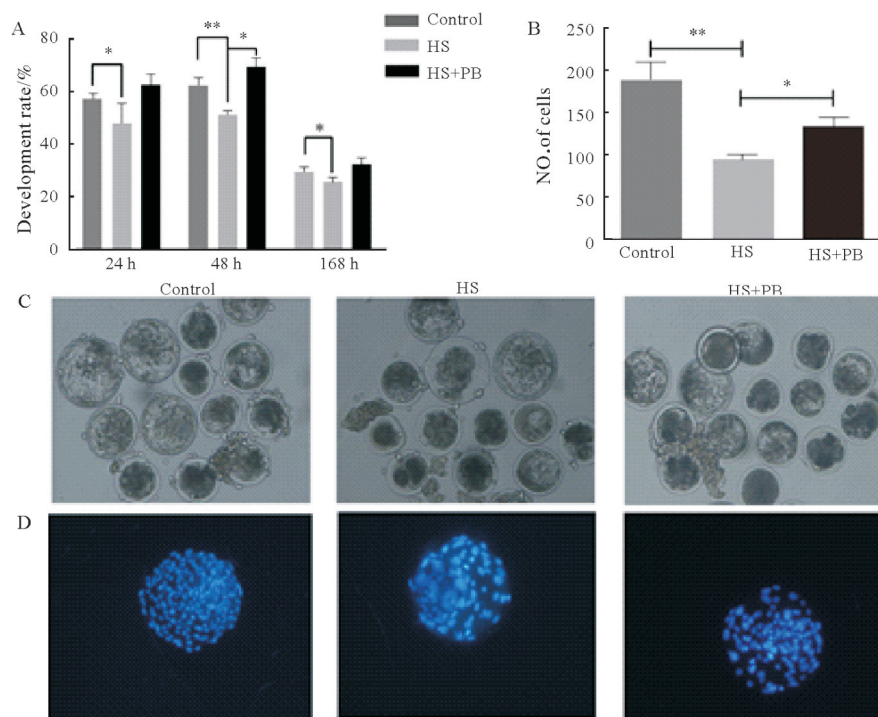
由表 1 和图 1 可知,热应激组卵母细胞成熟率和卵裂率显著低于对照组($P<0.05$),原花青素组卵母细胞成熟率高于热应激组,但两组之间差异不显著($P>0.05$)。与对照组相比,热应激组卵裂率显著降低($P<0.01$),囊胚率显著降低($P<0.05$);与热应激组比,原花青素组卵裂率显著提高($P<0.05$),但囊胚率差异不显著($P>0.05$)。囊胚细胞总数检测(表 1,图 1B,图 1D),热应激组囊胚细胞总数显著低于对照组($P<0.01$),原花青素组囊胚细胞数显著高于热应激组($P<0.05$)。结果表明,热应激抑制卵母细胞成熟,影响胚胎发育潜力;原花青素不仅能够逆转热应激对卵母细胞成熟的影响,而且还能够提高早期胚胎发育潜力,改善胚胎质量。

表 1 原花青素对热应激卵母细胞发育能力的影响
Tab.1 Effect of PB2 on developmental ability of oocytes under heat stress

项目 Item	对照组 Control	热应激组(HS) Heat stress	热应激+原花青素组 Heat stress+PB2	P 值
卵母细胞数/枚 No. of oocytes	185	196	218	
成熟率/% Maturation rate	67.57±3.305 ^a	47.53±4.361 ^b	56.53±1.330 ^{ab}	0.035
卵裂数/% Cleage rate	70.60±1.400 ^A	51.25±1.250 ^B	62.85±2.350 ^{AC}	0.001
囊胚率/% Blastocyst rate	32.14±1.345 ^a	25.57±1.022 ^b	28.10±1.703 ^{ab}	0.020
囊胚细胞总数 No. of blastocyst cells	189.3±11.920 ^a	94.33±3.528 ^b	133.7±6.360 ^{ac}	0.028

同行数据上标相同字母表示差异不显著($P>0.05$),不同小写字母表示差异显著($P<0.05$),不同大写字母表示差异极显著($P<0.01$)。

The same letter of superscript indicates no significant difference($P>0.05$),different lowercase letters indicate significant difference($P<0.05$),and different uppercase letters indicate very significant difference($P<0.01$).



A.不同时间胚胎发育对比;B.囊胚细胞数分析;C.培养 168 h 胚胎发育状态;D.Hoechst 33342 染色胚胎状态,蓝色代表细胞总数;**表示差异显著 ($P < 0.01$),*表示差异显著 ($P < 0.05$)。

A.Comparison of embryonic developmental capacity at different times;B.Histogram of blastocyst count;C.Development of embryos cultured in vitro for 168 h;D.Hoechst33342 staining diagram,the blue represents the cell number;**indicated significant difference ($P < 0.01$),* indicated significant difference ($P < 0.05$).

图 1 原花青素对热应激卵母细胞发育能力的影响

Fig.1 Effect of PB2 on developmental ability of oocytes under heat stress

2.2 原花青素对热应激卵母细胞 ROS 和 GSH 活性的影响

由图 2A 和图 2C 可知,热应激组 ROS 活性显著高于对照组 ($P < 0.01$),原花青素组 ROS 活性显著低于热应激组,表明热应激诱导卵母细胞产生大量 ROS,PB2 能够抑制热应激诱导的 ROS 产生。

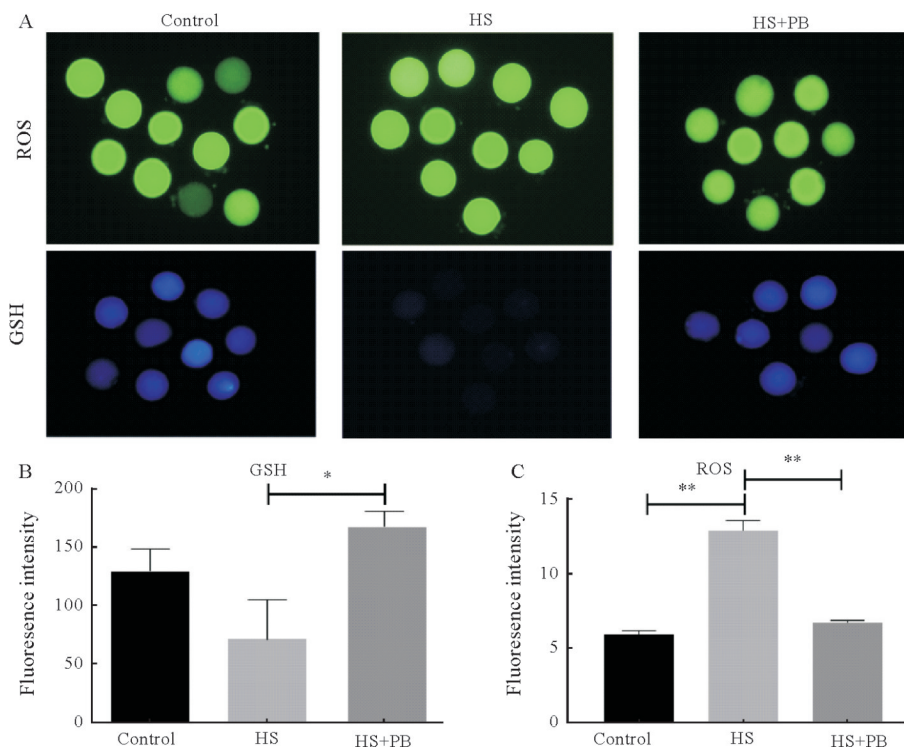


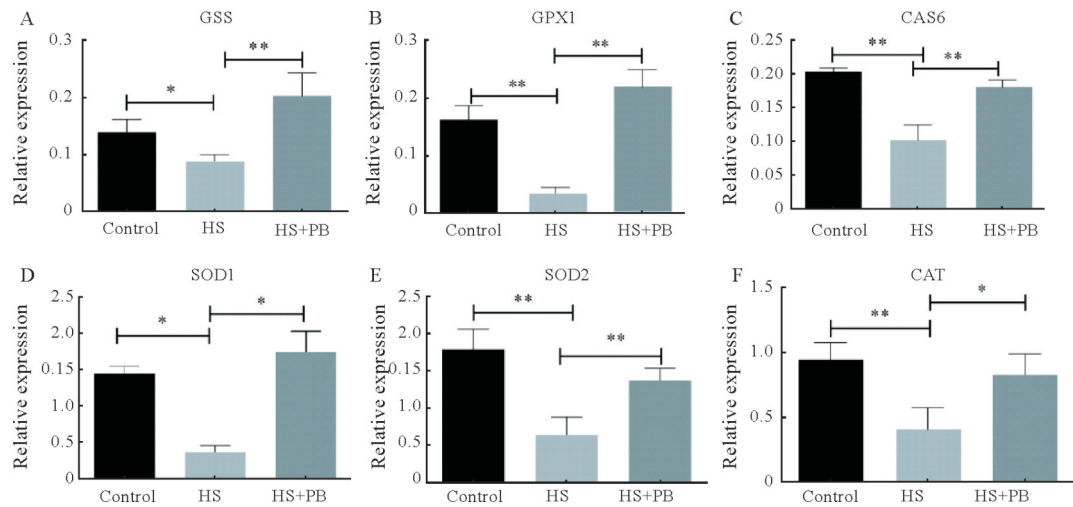
图 2 原花青素对热应激卵母细胞内 ROS 和 GSH 活性的影响

Fig.2 Effect of proanthocyanidins on ROS and GSH activity in oocytes under heat stress

由图 2A 和图 2B 可知,热应激组 GSH 活性低于对照组,两者之间差异不显著($P>0.05$),原花青素组 GSH 活性显著高于热应激组($P<0.05$),表明热应激降低了卵母细胞 GSH 活性,PB2 通过增强 GSH 活性缓解热应激对卵母细胞的影响。

2.3 原花青素对热应激卵母细胞相关基因表达的影响

定量 PCR 分析结果(图 3)显示,热应激组成熟基因(*GSS*、*GPX1*、*CAS6*,图 3A~C)和抗氧化基因(*SOD1*、*SOD2*、*CAT*,图 3D~F)的 mRNA 相对表达量与对照组相比显著下降($P<0.05$);原花青素组卵母细胞成熟基因和抗氧化基因的 mRNA 表达相对表达量显著高于热应激组($P<0.05$)。这表明原花青素通过增强卵母细胞抗氧化基因和细胞质成熟基因的表达,缓解热应激引起的氧化应激,提高卵母细胞的质量。



A.成熟基因:*GSS*、*GPX1*、*CAS6*,B.抗氧化基因:*SOD1*、*SOD2*、*CAT*。 **表示差异显著($P<0.01$),*表示差异显著($P<0.05$)。
A.Maturation related genes:*GSS*、*GPX1*、*CAS6* B.Antioxidant genes:*SOD1*、*SOD2*、*CAT*.**indicated significant difference($P<0.01$),* indicated significant difference($P<0.05$).

图 3 原花青素对热应激卵母细胞成熟、抗氧化相关基因表达相对表达量的影响

Fig.3 Effect of proanthocyanidins on the expression of genes related to maturation and antioxidation of oocytes under heat stress

3 讨论

环境温度过高会导致机体产生一系列生理、病理变化^[22],引发的急性热应激易导致机体多种组织器官产生不同程度损伤^[23]。在雌性动物中,生殖功能障碍与环境温度过高引起的热应激密切相关。热应激对牛卵母细胞的影响主要体现在排卵、卵母细胞的发育和成熟过程中^[24]。热应激会增加卵母细胞氧化应激损伤的风险。ROS 活性是机体氧化状态的重要指标,高活性的 ROS 会引起蛋白质氧化、脂质过氧化和 DNA 损伤,导致细胞死亡^[25]。热应激通过诱导一系列与氧化应激有关的反应影响卵母细胞的成熟和胚胎发育,这些反应包括 ROS 积累和谷胱甘肽-谷胱甘肽过氧化物酶(glutathione-glutathione peroxidase, GSH/GPx)系统^[26],减少氧化应激引起的 ROS 积累可以促进胚胎发育。谷胱甘肽(GSH)是一种高效的抗氧化剂,能有效清除 ROS,保护细胞免受氧化损伤。而热应激引起卵母细胞内 GSH 活性降低,导致卵母细胞更易受氧化应激影响^[27]。本研究结果表明卵母细胞遭遇热应激后 ROS 活性升高, GSH 活性降低,抗氧化基因表达降低,与前人的研究结果一致。

原花青素 B2 是一种具有抗氧化和抗炎作用的多酚类黄酮化合物。本研究表明,原花青素 B2 能够逆转热应激对卵母细胞成熟的影响,能够降低热应激引起 ROS 活性,提高 GSH 含量,增强细胞抗氧化能力。原花青素 B2 通过诱导多种抗氧化基因和抗氧化酶活性发挥其强大的抗氧化潜能^[28]。本研究为原花青素 B2 在奶牛体内的潜在应用提供了一定的依据,以预防和减轻热应激的不良影响。CAT 和 SOD 是抗 ROS 诱导的氧化损伤的主要酶促抗氧化剂。具体来说, SOD 可以催化超氧自由基生成分子氧和过氧化氢, CAT 将 SOD 分解的过氧化氢分解为水和氧。热应激通过氧化失活提高 ROS,降低 CAT 和 SOD 活性。与本研究结果一致的是,热应激后卵母细胞 ROS 活性升高, GSH 活性降低, CAT 和 SOD 活性显著下降^[29]。原花青素 B2 预处理可缓解高温诱导的氧化状态。先前的研究^[30]也报道了原花青素 B2 的抗氧化作用,通

过提高卵母细胞中CAT和SOD的活性,改善游离脂肪酸诱导的氧化应激。本研究还检测了成熟相关基因(*GSS*、*GPX1*、*CAS6*)的表达活性,发现添加原花青素能够提高卵母细胞成熟相关基因的表达。热应激显著降低了胚胎的卵裂率和囊胚率,而添加原花青素组的卵裂率显著的高于热应激组,但囊胚率差异不显著。热应激损害牛卵母细胞的发育能力,降低了第7天和第8天的囊胚率^[31],这与本研究结果一致。牛胚胎对热应激的抵抗力与胚胎基因组的激活几乎同时产生。随着胚胎发育,胚胎对高温的抵抗力越来越强^[32]。这也就解释了热应激组卵裂率显著低于对照组和原花青素,而囊胚率与原花青素组差异不显著的原因。囊胚总细胞数代表囊胚的发育状态和质量,囊胚总细胞数越多,说明囊胚发育越好,质量越高。试验结果表明,热应激可显著降低孤雌胚胎囊胚总细胞数,而添加原花青素后,囊胚总细胞数显著增加,但仍显著低于对照组。添加外源抗氧化剂可以提高卵裂率与囊胚总细胞数^[33],与本试验结果一致,添加原花青素提高了囊胚总细胞数,缓解热应激对胚胎带来的损伤。

4 结 论

本研究探明了原花青素可以通过增加GSH含量、减少ROS积累、增加抗氧化基因和成熟相关基因的表达,改善热应激对胚胎的损伤,促进胚胎发育,提高胚胎卵裂率、囊胚率和囊胚细胞总数,为胚胎工厂化生产提供了解决思路。但是具体的原花青素是如何调控热应激损伤机制还有待进一步研究。本研究仅初步探究了原花青素对热应激后孤雌胚胎发育能力的影响,尚未对体外受精胚胎进行验证,下一步工作笔者将针对原花青素对牛热应激体外受精胚胎发育能力开展研究,以期揭示原花青素调控胚胎热应激损伤的机制。

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