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第25卷 第5期 西北农业大学学报 Vol. 25 No. 5
1997年10月 Acta Univ. Agric. Boreali-occidentalis Oct. 1997

乳中环核苷酸提取方法研究

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摘 要 根据环核苷酸理化性质及乳的特点，采用酶法从乳汁中提取环核苷酸。研究表明，该方法条件温和、工艺合理、操作方便。经放射免疫测定提取浓缩液浓度为cAMP 20 μmol·L^{-1}，cGMP 0.2 μmol·L^{-1}。

关键词 乳，环核苷酸，酶法
中图分类号 Q524.03

自从1957、1963年cAMP和cGMP先后被发现以来[1,2]，环核苷酸在生命活动中重要的调节作用已为大家公认。虽然cAMP和cGMP在基础理论研究方面有较大进展，但环核苷酸提取方法报道甚少，仅章翰等[3]对单个核细胞内cAMP提取方法有报道。目前从乳汁中提取环核苷酸尚无报道。本研究根据环核苷酸理化性质及乳的特点，采用酶法从羊乳中提取环核苷酸，旨在探讨工艺合理、简便易行的提取方法。

1 材料与方法

1.1 主要材料

羊奶，西北农业大学羊场。$CaCl_2$，NaCl和二甲苯，西安化学试剂厂。胃蛋白酶，四川开县生物制品厂。2,5-二苯基恶唑(PPO)和P-双-[2-(5-苯基恶唑基)]苯(POPOP)，上海试剂一厂。^{3}H-cAMP，标准cAMP，蛋白激酶，活性炭，牛血清白蛋白，^{3}H-cGMP，标准cGMP，cGMP抗体，NaAc及微孔滤膜等均由中国原子能院整套提供。LS-9800液闪仪，美国Beckman公司，D_t-15旋涡混匀仪，江苏国华仪器厂。水浴箱，微量加样器等。

1.2 提取方法

1.2.1 乳中环核苷酸提取工艺流程 乳 —(纱布过滤)→ 清洁乳 —(除去乳脂)→ 脱脂乳 —(酶处理)→ 凝乳 —(过滤分离)→ 初滤液 —(75℃提取过滤)→ 滤液 —(75℃浓缩)→ 浓缩液 —(冷却、除乳糖)→ 淡黄色液体

1.2.2 原料乳的前处理 先将鲜乳用纱布过滤，除去羊毛、泡沫等杂质。检查乳质，剔除乳房炎乳，因乳房炎乳带有大量病菌，直接影响环核苷酸的数量和质量。将清洁乳放置几小时，让乳脂上浮，然后除去乳脂肪。由于冻结乳酪蛋白物理状态改变，解冻时不易被凝乳酶作用，易产生絮状物凝结，不易产生凝块。所以原料乳不宜冷冻存放。

1.2.3 酶处理及过滤 向清洁乳中加入占乳总量0.01%～0.02% $CaCl_2$，用生理盐水将粉状酶(酶量多少根据效价而定)溶解后预混，再加入乳中搅拌，更利于酶与乳的充分接触。将加入酶的乳放在37℃温箱或水浴中，保持数小时，使乳凝固，乳清排出。过滤乳清

收稿日期 1997-05-20
课题来源 陕西省"八五"攻关资助项目及西北农业大学青年基金资助项目
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得到初滤液。

1.2.4 *初滤液75℃再提取及蛋白质检查* 乳中蛋白质主要为酪蛋白，约占总蛋白的4/5，已被酶凝固而除去。乳清蛋白，酶不能使其凝固而留在提取液中。乳清蛋白以胶体状态存在，对热不稳定，初滤液加温到75℃时，蛋白质遇热变性而沉淀。取上清液1滴，放入黑色比色板孔中，加1滴20%磺酰水杨酸，如出现白色沉淀，证明还有蛋白质存在，继续除去。直到无白色沉淀为止，过滤提取液。

1.2.5 *浓缩滤液及除去乳糖* 滤液继续置75℃浓缩，提取液中乳糖呈溶液状态存在。乳糖晶粒大小为10～20 nm，是一种双糖，比重在20℃时为1.5453[4]。将提取液浓缩到占初滤液体积1/25～1/30时，冷却浓缩液，边搅拌边冷却至20℃，保持数小时，然后放入4℃的冰箱过夜，形成乳糖结晶于容器底部，取上清液离心，弃去沉淀得到液体环核苷酸。

1.3 **cAMP和cGMP浓度测定**

1.3.1 *cAMP浓度测定*[5] 采用蛋白质竞争结合分析法，按中国原子能研究院cAMP测定方法操作，测定范围每管为0.25～16 pmol.蛋白激酶结合率约50%，吸附剂用牛血清白蛋白和活性炭。放射性测定用LS-9800液闪仪，以cAMP不同浓度为横坐标，Co/Cx为纵坐标绘制标准曲线。根据标准曲线查cAMP浓度，乘以样品稀释倍数得样品cAMP $\mu mol \cdot L^{-1}$.

1.3.2 *cGMP浓度测定*[6] 采用放射免疫测定法，按中国原子能研究院cGMP测定方法操作，测定范围每管在0.25～4 pmol.放射性测定用LS-9800液闪仪，以标准cGMP不同浓度为横坐标，Co/Cx为纵坐标绘制标准曲线，根据标准曲线查cGMP浓度，乘以样品稀释倍数得样品cGMP $\mu mol \cdot L^{-1}$.

2 结 果

2.1 **感观评定**

提取液为淡黄色液体，无混浊，呈均匀半透明状。气味芳香，无任何副作用。

2.2 **浓度测定**

样品测定前用活性炭脱色，稀释后用于测定cAMP浓度。取1 mL脱色液于小试管内，在60℃水浴上蒸干。干渣加适量的NaAc溶液溶解，离心。取上清液40 μL直接测定cGMP浓度。经多次平行测定，提取液浓度为cAMP 20 $\mu mol \cdot L^{-1}$，cGMP 0.2 $\mu mol \cdot L^{-1}$.

3 讨 论

3.1 **提取理论基础**

环核苷酸普遍存在于哺乳动物体内多种组织、细胞及细胞外液中，是体内低分子活性物质，产生存在于细胞内并溢出细胞，使细胞外液含有cAMP和cGMP.环核苷酸的结构特点是单核苷酸AMP和GMP分子中的磷酸与核糖形成3′，5′位的环式结构，由于环式结构，有其相应的稳定性质。cAMP分子量为329.2，溶于水，对酸、热相当稳定。cGMP分子量为344.2，亦溶于水，对酸、碱、热相当稳定。动物组织中、小肠粘膜环核苷酸含量高[7]，但材料来源有限且只能利用一次。血浆、尿液环核苷酸含量低，利用效果差。而哺乳

动物羊奶中环核苷酸含量高[8],从中提取是一条有效途径,它好象一条生产流水线,动物体内cAMP和cGMP源源不断的溢出细胞,进入乳汁,再从乳汁中提取,所以乳是提取环核苷酸较为理想的天然材料。

3.2 本方法特点

乳中蛋白质主要以酪蛋白为主(4/5),如何除去酪蛋白,化学法采用HCl酸化法,调酪蛋白的等电点,让其沉淀。实践证明,加入HCl调等电点,酪蛋白沉淀颗粒小,固液相分离不清,似糊涂状,很难过滤分离,过滤时间长,滤液混浊,提取效果差。采用高氯酸、三氯醋酸等试剂处理,影响因素多,操作繁琐,出汁量少,提取效果差。本方法对乳中蛋白质处理采用两步,第一步在温和条件下,用凝乳酶将酪蛋白凝固除去,其中凝固分为两个阶段,先是酪蛋白在凝乳酶作用下转化成副酪蛋白,后是副酪蛋白在Ca^{2+}作用下成网状结构,凝固收缩,促使乳清和小分子cAMP和cGMP进入提取液。凝乳保持适宜硬度,提取液澄清,容易过滤。第二步对乳清蛋白在75℃下再提取,乳清蛋白遇热变性,细胞膜间隙增大,细胞内耐热小分子cAMP和cGMP得以逸出。乳糖采用低温结晶法除去,也是本法一个特点。

3.3 有关酶处理的条件

酶的本质是蛋白质,主要影响因素有温度、pH、激活剂等。温度是酶促反应的重要因素之一,当温度低于25℃时,凝乳很慢,随温度增加,凝乳随之加快,在37℃时酶的活性最大,凝乳最快,因而相对其它温度需要较少量的酶。正常乳的酸度为1.44～1.62 $g \cdot kg^{-1}$,在乳酸菌作用下酸度升高。除正确掌握时间外,可用乳酸将原料乳酸度调至1.98 $g \cdot kg^{-1}$左右,使酶产生最大效益,有利于凝乳聚集沉淀。原料乳因季节变化等原因,可引起乳中可溶性钙减少,影响酶的活性,加入适量的$CaCl_2$,使凝乳保持适宜的硬度,有利于澄清和过滤。酶用生理盐水溶解,NaCl作为酶的激活剂,使酶活性增强。

4 小　结

1)酶法提取环核苷酸,采用较为温和条件,经酶处理后乳酪蛋白凝固收缩,促使乳清及小分子cAMP和cGMP进入提取液,且凝乳保持适宜的硬度,提取液澄清,出汁量多,容易过滤。

2)初滤液经75℃再提取,乳清蛋白遇热变性,使细胞膜间隙增大,细胞内耐热小分子cAMP和cGMP得以逸出。

3)该提取方法,工艺合理,操作方便,浓缩滤液条件还需进一步改进。

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Extraction Method of Cyclic Nucleotieds of Milk

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Abstract Based on physical and chemical properties of cyclic nucleotydes and the speciality of milk, cyclic nucleotides in milk were extracted with enzyme method. The study showed that the method was moderate in condition, reasonable in technique and easy in operation. The extracted fluid concentration of cAMP and cGMP were, by radioimmunoassay detction, 20 $\mu mol \cdot L^{-1}$ and 0.2 $\mu mol \cdot L^{-1}$ respectively.

Key words milk, cyclic nucleotides, extraction method

第25卷 第5期 西北农业大学学报 Vol. 25 No. 5
1997年10月 Acta Univ. Agric. Boreali-occidentalis Oct. 1997

Sex Ratio of Different Developmental Stages in Vitro Produced Bovine Embryos

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Abstract The objective of this study was to determine whether the bovine male embryos develop faster in vitro than female embryos. The sex of embryos were determined with PCR (Polymerase chain reaction) technique. On day 5 (in vitro insemination = day 0), the embryos were divided by developmental stages into 3 groups: 16 cells, more than 16 cells and morula. In 3 groups, the percentages of males were respectively 32%, 53% and 63%. On day 7 (in vitro insemination = day 0), the embryos were classified by developmental stages into 4 groups: morulae, early blastocysts, blastocysts and full-expanding blastocysts. After sexing, the percentages of males were 28%, 38%, 60% and 70%, respectively. The study demonstrated that IVF bovine male embryos develop faster in vitro than female embryos.

Key words bovine, embryo, in vitro development, male, female

A general review of the methods in sexual control of animals, currently the separation of X- or Y-chromosome-bearing spermatozoa[1] and sex determination of preimplatantion embryos[2] are being explored two ways. However, more recently, some data showed that male embryos develop at a faster rate in vivo and in vitro than female embryos. Avery *et al*[3] found that the percentages of males in bovine from the 3 developmental groups were 78%, 27% and 11% respectively. Also similar studies on mouse[2,4] and bovine[5] embryos have been reported. However, Berg *et al*[6] indicated that no differences in the sex ratio between fast- and slow-developing embryos could be found by transferred to recipients. For exploring a simple, noninvasive method to embryo sexing, the objective in this study is to reveal the effect of sex on bovine embryonic growth rate in vitro.

1 Materials and Methods

1.1 In vitro embryo production

1.1.1 Media preparation Oocytes were aspirated and washed using TCM-Hepes buffer supplemented with 2% heat-treated cow serum (c.s) and 250 IU/mL heparin

收稿日期 1997-06-04
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(aspirated medium). Oocytes were cultured in a TCM-Bicarbonate medium supplemented with 16 IU/mL Suigonan-opl (hCG) and 15% c. s (IVM medium). Spermatozoa were capacitated in CR1-Bicarbonate medium supplemented 30 mg · L^{-1} heparin (IVF medium). Embryos were cultured in CR1-Bicarbonate medium supplemented with 2% c. s (IVC medium).

1. 1. 2 Culture of oocytes Bovine ovaries were obtained at a local slaughterhouse. Oocyte were aspirated and washed twice in aspirated medium and once in IVM medium, then oocytes were cultured in IVM medium (30～40 oocytes in 500 μL drops) under sterile paraffin oil at 39 C in 5% CO_2 and 95% N_2 for 22～24 h.

1. 1. 3 In vitro fertilization Frozen semem which divided from a single bull was thawed in water at 35～37℃ for 1 min, and then washed twice by centrifugation at 700 g for 10 min. After maturation culture, matured oocytes moved into IVF medium with treated spermatozoa and cultured under sterile paraffin oil at 39 ℃ in 5% CO_2, 95% N_2 and 100% humidity for 22～24 h.

1. 1. 4 Embryo culture After in vitro fertilization, presumptive zygotes were denuded from cumulus cells by vortex mixing. Naked zygotes were cultured with IVC medium under paraffin oil at 39 ℃ in 5%·CO_2 and 95% N_2 for 4 and 6 d.

1. 2 Embryo classification

On day 5 (in vitro insemination=day 0), the embryos were morphologically evaluated and divided by developmental stages into 3 groups: 16 cells, more than 16 cells and morula. On day 7 (in vitro insemination = day 0), the embryos were divided into 4 groups: morula, early bastocysts, blastocysts and full-expanding blastocysts.

1. 3 Preparation of embryos for PCR-amplification

Embryos were washed twice in pure PBS and once in 1×PCR buffer. Embryos were spliced into 2 parts (A and B samples) under an inverted Olympus microscope, individually, into separate vials containing 10 μL of 1×PCR buffer. At that time, 1 μL proteinase K was added(10 g · L^{-1}). After centrifuging briefly, each vial was covered with 30 μL of light paraffin oil on the reaction mixtures to prevent evaparation. The embryos were kept at 56℃ for 1 hour for digestion. Prior to PCR amplification, each vial was incubated at a temperature of 98 ℃ for 15 min to denature the proteinase K.

1. 4 Polymerase chain reaction

The PCR reaction were carried out in a total 25 μL reaction buffer. Amplification were performed in 10 mmol · L^{-1} Tris-HCl(pH8. 4), 50 mmol · L^{-1} KCl, 1. 5 mmol · L^{-1} $MgCl_2$, 0. 01% gelatine, 5 mmol · L^{-1} of each dNTP(dATP, dCTP, dGTP and dTTP) and 0. 25 IU of Tag DNA polymerase. BRY. 4a-specific primes were added in a volume of 10 pmol to A samples, and 10 pmol of BRY. 4a-specific and 2. 5 pmol of bovine satellite 1. 709 specific primers were added to B samples. A negative control, male and female coutrols (male and female genomic DNA), were included to detect any

validity of the reagents. Tab. 1 shows the details concerning sequences and products of both the Y-specific and bovine satellite primers used in the PCR sexing assay.

Tab. 1　Details concerning the primers used in the PCR sexing assay

Template-sequence	Nucleotide position of the primers	Primer sequences	PCR product length (basepairs)
BRY. 4a (Redd et al, 1989)	1844-1872 2284-2312	5'-CAAGACCATACA-TATGTCATTATAGACAG-3'3'-CACAAAAA-CAAAATTTATGTACTTCATGT-5'	469
satellite 1. 709 (Skowronski et al, 1984)	2749-2770 2974-2994	5'-GCAGTGCATAAATATCAAAAGG-3'3'-CAAGGGATGTTGGAGGACTAG-5'	246

Amplifications were carried out in a Perkin-Elmer Thermal Cycler. All samples were denatured at 94℃ for 2. 3 min, followed by 50 cycles consisting of denaturation at 94 C for 30 s, annealing at 61 ℃ for 1 min, and extension at 72 ℃ for 1 min. After the last cycle, the samples were incubated for further 3 min at 72 ℃ to assure complete extension and then cooled to 35 ℃ for 10 s. The PCR products of all samples were then either analyzed immediately by agarose gel electrophoresis or stored in refrigerator until analyzed.

1. 5　Analysis of PCR products

Seven microliters of each sample were added to 1 μL of loading buffer containing glycerol, bromplenol blue, xylene cyanol and EDTA before loading on the gel. Electrophoresis was carried out in 2% agarose gel with 1×TBE buffer at 100 volts for 30 min, stained with ethidium bromide and visualized under ultraviolet light. The embryo was judged to be female if only a 246 basepair product from bovine satellite 1. 709 was visible in the sample. If a 469 basepair from BRY. 4a or a 246 and a 469 basepair were visible in the sample, the embryo was judged as a male(Fig.).

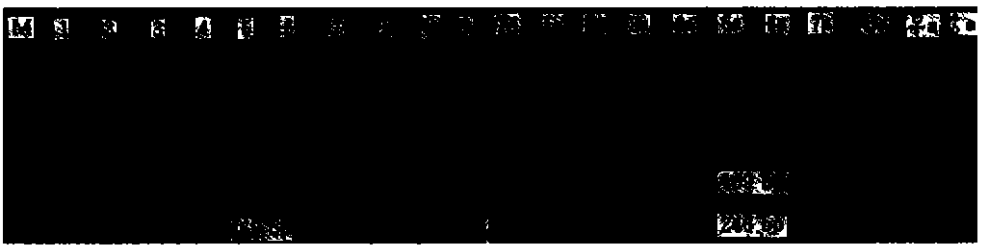

Fig.　Agarose gel electrophoresis after PCR

(M) PstI digested λ. 1-7 are embryo samples using the BRY. 4a primers. B, ♀ and ♂ are samples with respectively no, female and male bovine genomic DNA. The samples resolved on the agarose gel below are aliquots of the same samples as above amplified with an addititon of the satellite 1. 709 primerpair. Sample 1, 3 and 5 were assigned males, 2, 6 and 7 were assigned females, while sample 4 was not assigned.

2、6 and 7 were assigned females, while sample 4 was not assigned.

2 Statistical Analysis

To determine if there are differences in the developmental rate between male and female embryos, Chi-square analysis was used.

3 Results

On day 5 after insemination, a total of 110 embryos was submitted for sexing and 105 embryos had their sex determined (Tab. 2). Five embryos (1 16 cells, 2 more than 16 cells, 2 morula) either did not show amplification or were lost during the procedure. When embryos were collected from morulae developmental stage, approximately 63% of fast-developing embryos were males($P<0.05$). When embryos were collected from 16 cell developmental stage, approximately 68% of slow-developing embryos were females ($P<0.05$). The overall male percentage was 50% and it was no different from the expected 1 : 1 sex ratio($P>0.05$).

Data in Tab. 3 indicated that male embryos developed at a significantly faster rate than female embryos on day 7($P<0.05$). When embryos were collected from fast-developing stage, approximately 70% of embryos were males ($P<0.05$). When embryos were collected from slow-developing stage, approximately 72% of embryos were females ($P<0.05$). A total of 91 embryos was submitted for sexing and 87 embryos had their sex determined. Four embryos (3 early blastocysts, 1 blastocyst) either did not show amplification or were lost during the procedure. A total of 87 embryos was analyzed and displayed 1 : 1 sex ratio($P>0.05$).

Tab. 2 Sex ratio of bovine IVF embryos by stages of development on day 5

Stages of development	No. of embryos	Males (n)	Females (n)	Sex ratio	Males (%)
16 cells	31	10	21	1:2.1	32
More than 16 cells	36	19	17	1.1:1	53
Morula	38	24	14	1.7:1	63
Total	105	53	52	1:1	50

Tab. 3 Sex ratio of IVF bovine embryos by stages of development on day 7

Stages of development	No. of embryos	Males (n)	Females (n)	Sex ratio	Males (%)
Morula	18	5	13	1:2.6	28
Early blastocysts	21	8	13	1:1.6	38
Blastocysts	25	15	10	1.5:1	60
Full-expanding blastocysts	23	16	7	2.3:1	70
Total	87	44	43	1:1	51

4 Discussion

Earlier findings have demonstrated that the rate of embryonic development and sex ratio of embryos have a clear relationship[2,3,4], and IVF bovine embryos have a higher oocurrence of male among more advanced embryo stages and a higher oocurrence of female among less advanced embryo stages[7]. Itoh and Goto[5] reported that the percentages of males in bovine from the 3 developmental groups (fast development, intermediate development and slow development) were 77%, 40% and 24%, respectively. Seller and Perkins-Cole[4] found that the percentages of males in mouse in the 3 groups (fast development, intermediate development and slow development) were 94%, 45% and 25%, respectively. In this study, 105 and 87 embryos fertilized from a bull sperm could be classified into different developmental stages on day 5 and day 7, respectively, and the results showed that male embryos developed more rapidly than female embryos did. On day 5, the percentages of males from the 3 developmental groups were 63%, 53% and 32%, respectively. And from the 4 developmental groups on day 7, the percentages of males were 70%, 60%, 38% and 28%, respectively. The present results were consistent with the previous findings[3,4,5].

Although reports in several mammals including mouse[2,4] and bovine[3,5] have indicated that the development of male embryos occurs more rapidly than female embryos. Contrary to the results, no differences in embryo development between male and female embryos would be detected. Berg et al[6] found that IVF embryos transferred to recipients (on day 7 after insemination) and taken to term did not show differences in the sex ratios between fast and slow developing embryos. For this reason, it is likely that embryos may have already been degenerate at the time of transfer (slow development), before embryos were classified into different development stages. In addition, the sex of fetus aborted during pregnancy may be another reason.

The faster development of male embryos is not fully understood. It could be argued that male embryos develop faster than female embryos because the redused DNA content would allow faster completion of DNA replication. However, the findings of Burgoyne[8] did not support this. A genetic influence by the Y chromosome on in vitro metabolism may be important. The increased growth in male embryos may be a consequence of differential gene expression caused by the Y-chromosome. In addition, early embryonic development is a very complex process, in vitro-produced embryos can be affected by a range of different factors such as inorganic ions, buffers, gas composition, amino acids, growth factors, vitamins and macromolecules[9].

Acknowledgments: We want to thank Dr. Steven Smith and Mr. Calus for their technical assistance.

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体外生产的牛胚胎在不同发育期的性比率

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摘 要 本研究旨在证实牛体外培养的雄性胚胎生长发育是否快于雌性胚胎。胚胎的性别鉴定采用多聚酶链式反应(PCR)技术。在体外受精的第5天,胚胎根据发育期划分为三个组:16细胞期,多于16细胞期和桑椹期。在3个组中,雄性胚胎所占百分率分别是32%,53%和63%。在体外受精的第7天,胚胎根据发育期划分为4个组:桑椹期、早期囊胚期、囊胚期和扩张囊胚期。在性别鉴定后,4个组中雄性胚胎所占百分率分别是28%,38%,60%和70%。结果表明:牛早期胚胎在体外培养条件下,雄性发育快于雌性。

关键词 牛,胚胎,体外发育,雄性,雌性

中图分类号 S823.35,S814.6