Biopolymer poly hydroxybutyrate-hydroxyvalerate membrane*

Chen Jian-hong, Tang Qian, Wu Jian, Liang Huan-you

Abstract

BACKGROUND: Poly hydroxybutyrate-hydroxyvalerate (PHBV) has been used to construct bioprosthetic heart valve. It remains unclear whether it can be used as membrane for guided bone regeneration.

OBJECTIVE: To investigate the biocompatibility of PHBV membrane and evaluate its efficiency of promoting bone regeneration *in vivo*.

METHODS: Effects of 100%, 75%, 50%, 25% PHBV extract solution on relative growth rate of dog bone marrow mesenchymal stem cells were measured by MTT method and cytotoxicity of the biomaterials was evaluated. Bone defects were made on distal bilateral tibias and treated with PHBV membrane; the proximal bilateral tibias undergoing reduction of periosteal flap and were used as control.

RESULTS AND CONCLUSION: The toxicity gradation of PHBV membranes was grade 0−1. That is, they were not toxic to growth and proliferation of bone marrow mesenchymal stem cells. New bone regeneration was observed in the defects covered with PHBV membranes at week 2 post-surgery. The defects covered with PHBV membranes were filled with mature bone at week 12 post-surgery. The bone repair in experimental groups was earlier and better than that in control groups. Results demonstrated that PHBV membrane, which has no cytotoxicity to mesenchymal stem cells in a wide range of extract concentration, could be a promising biopolymer membrane for guided bone regeneration.

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INTRODUCTION

Shape and quantity of regenerated bones depends on existence and maintenance of space beneath the membrane. Reduced or collapsed space greatly affects bone regeneration. Therefore, selection of membrane materials is very important for guided bone regeneration[1-2]. Poly hydroxybutyrate-hydroxyvalerate (PHBV) is a high molecular polymer of prokaryotic microbe in cells under unbalanced nutrition[3-4]. It has good biocompatibility and biodegradation, and two forms of degradation: hydrolysis and biodegradation in vivo. The degraded product, b-hydroxybutyric acid, finally transforms into CO_2 and H_2O, not leading to physiologic reaction[5-6]. As a novel degradable biomaterial, PHBV has a promising application in medicine[7-8]. It has been used to construct bioprosthetic heart valve[9]. But very little data are available regarding the application as membrane for guided bone regeneration. Cooperating with Institute of Biomaterials, School of Material Science, South China University, the present study developed novel PHBV degradable membrane, and the cytotoxicity and guided bone regeneration capacity were evaluated to investigate the feasibility of constructing membrane for guided bone regeneration.

MATERIALS AND METHODS

Design

A randomized, controlled, animal experiment.

Time and setting

This study was performed at the Central Laboratory and Animal Experimental Center, Third Hospital of Sun Yat-sen University, from May 2005 to December 2006.

Materials

PHBV membrane was provided by Institute of Biomaterials, School of Material Science, South China University. DMEM, fetal bovine serum, trypsin, and MTT were purchased from Jingmei Company. Medical superclean bench, CO_2 incubator, desk centrifuge, ELISA reader, inverted phase contrast microscope were provided by the Central Laboratory, Third Hospital of Sun Yat-sen University. Pathological microscope was provided by Pathology Department of Third Hospital of Sun Yat-sen University. Healthy hybrid dogs, aged 1 year, weighing 10−12 kg, were purchased from Guangzhou Shima Laboratory Animal Factory.

Methods

Primary culture and passage of bone marrow-derived mesenchymal stem cells (BMSCs)

Under sterile condition, heparinized (1 mL heparin) syringe was used to draw 5 mL bone marrow from iliac bone of the dog and primary cultured using whole bone marrow culture method (direct attachment)[10-12], followed by surface antigen identification. The bone marrow was dilated through 100-mesh steel web, mixed with DMEM, centrifuged at 1 000 r/min for 5 minutes, mixed with DMEM containing 10% PAA serum at a ratio of 1: 3 in 50-mL culture flask after discarding the supernatant and incubated with 5%CO_2 at 37 °C for primary culture. The culture solution was replaced after 24 hours, and cells were suspended using PBS for twice, mixed with 5 mL DMEM containing 10% PAA serum. The culture solution was replaced at the next day. After 10−12 days, cells confluent to 80% in a single layer were trypsinized (0.25%) and passaged at a

ratio of 1: 2. Cell growth curve was drawn using active cells of passages 2–4.

Preparation of PHBV membrane extract solution

PHBV membrane was sterilized using ethylene oxide, and 100% standard concentration of extract solution was prepared according to ISO standard[13-14]. Briefly, according to a ratio of 6 cm2 samples and 1 mL leaching medium, the membrane material was soaked into complete culture solution in 5% CO_2 at 37 ℃ for 2 days. The extract solution was extracted and stored sterilely at 4 ℃.

Toxicity gradation of PHBV membranes using MTT[15-16]

According to growth curve, BMSCs at a density of 4×10^3 per well were seeded in 96-well culture plate. Four experimental groups and one negative control group were set. In the four experimental groups, 100%, 75%, 50% and 25% diluted material leaching liquor were respectively added, 200 L per well, and negative control was added culture solution. There were eight parallel wells in each group. MTT assay was performed at 1, 3, 5 days after culture to determine relative growth rate (RGR) of BMSCs. Five-grade scoring system for toxicity was used: grade 0: RGR≥100%; grade 1: RGR≥80%; grade 2: RGR≥50%; grade 3: RGR≥30%; grade 4: RGR≥0.

Model establishment of bone defects[17-18]

Eight dogs were anesthetized by intramuscular injection with 3% pentobarbital sodium (1 mL/kg). Respiratory frequency was monitored, and anaesthetic was further administrated if necessary. The anesthetized dog was placed at the operating-table in supine position with lower limbs abduction. The skin of medial middle-segment of the tibia was cut open, middle tibia, 5.0 cm×1.5 cm, was exposed and two round bone defects, 0.5 cm×0.5 cm, were made, breaking through the medullary canal, with 2 cm interval. There were four bone defects in bilateral tibias in total.

Grouping

Left and right proximal bone defects were used as control group, which were not treated except reduction of periosteal flap. The left and right distal bone defects were used as experimental groups, which were covered with PHBV membrane with 2 mm of the margin of the membrane over the defect region. The bilateral margins were inserted subperiosteum and fixed, and the turnup periosteal flap was reduced and covered PHBV membrane. The periosteum of left and right tibia operation area was apposition sutured, and the skin was sutured layer by layer. During operation, the animals were treated with 2 g chlormycetin added in 5% glucose solution (250 mL), and intramuscular penicillin (80×10^4 U) was administrated, twice a day for 4 days to prevent infection.

Sample processing and observation

Two animals were sacrificed at 2, 4, 8, 12 weeks, respectively. Bilateral tibias were harvested, fixed in 2.5% glutaral for 24 hours. Implanted PHBV membrane was removed. Bone growth in defect region was observed. After X-ray actinogram, the tibia was cut into samples of 10 mm ×10 mm ×10 mm, containing the defects. The samples were split from the center of the defect region, half of which was used for pathological section and the other was stored.

Main outcome measures

PHBV membrane cytotoxicity and effects of guided bone regeneration.

Statistical analysis

Results were analyzed using SPSS 13.0 software by *t*-test.

RESULTS

Growth curve of BMSCs

Growth curve revealed that cultured cells rapidly proliferated from day 3, peaked at days 5, 6 became stable at day 6, and gradually decreased thereafter (Figure 1).

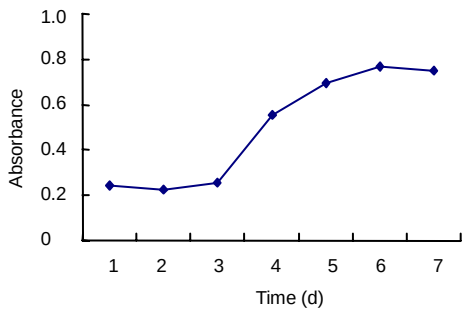


Figure 1 Standard curve of bone marrow mesenchymal stem cells using MTT method

PHBV membrane cytotoxicity results (Table 1).

Table 1 Porosity, density and compressive Young's modulus of the scaffolds (n=5)

Group	Time (d)	*A* value ($\bar{x}\pm s$)	RGR	Toxicity grade
A	1	0.397±0.065	99.37	1
	3	0.409±0.102	98.26	1
	5	0.794±0.125	98.91	1
B	1	0.400±0.065	100.06	0
	3	0.410±0.082	98.50	1
	5	0.796±0.073	99.19	1
C	1	0.400±0.045	100.06	0
	3	0.415±0.072	99.76	1
	5	0.796±0.079	99.19	1
D	1	0.400±0.042	100.00	0
	3	0.416±0.068	99.88	1
	5	0.801±0.081	99.84	1
E	1	0.400±0.043		
	3	0.418±0.045		
	5	0.805±0.053		

A: 100% standard extract group; B: 75% standard extract group; C: 50% standard extract group; D: 25% standard extract group; E: control group; RGR: relative growth rate

As shown in Table 1, the toxicity of experimental groups was graded ranging 0-1. With increasing extract concentration, absorbance (*A*) value was decreased, but the difference was not significant ($P > 0.05$). Effects of 100% standard extract on BMSCs growth were similar to 25% standard extract ($P > 0.05$). With increasing culture time, the quantity of BMSCs in each experimental group was increased. BMSCs proliferation

(*A* value) was similar between experimental groups and control group ($P > 0.05$).

Influence of PHBV membrane on bone regeneration

All animals normally took food from the second day after operation. The incision healed at 1 week postoperatively, free of injection.

Gross observation of samples

(1)Experimental groups: at 2 weeks postoperatively, connective tissue out of membrane was free of inflammatory reaction, and did not grow into the membrane; the surface of defects was smooth, connected with original bone, with no membrane displacement or collapse; the space beneath membrane remained well; dark red tissues fully filled the bone defects, with force of resistance and gravel-like feeling during probing. At 4 weeks, the surface of defects was smooth, and the defects were fully filled with tissues; the force of resistance was remained during probing. At 8 weeks, the surface of defects was smooth, and newly generated bone was observed, connected with surrounding normal bones. At 12 weeks, the bone defects were not evident, and the margins of defect surface were connected, with similar density to normal bone tissues. (2)Control group: at 2 weeks postoperatively, the surface of defects was concave, and soft tissues grew into defects region; the bone wound was not decreased; granulation tissue filled the defects; little force of resistance was felt during probing. At 4 weeks, soft tissues remained in the defects, and little bone generated in the defects; fiber tissues occupied the center of the defects. At 8 weeks, the surface of defects was concave; most of the defects was soft, with holes there. At 12 weeks, introcession was remained in the defects, and cortical bone was not continuous, with ingrowth of soft tissues.

X-ray results (Figures 2, 3)

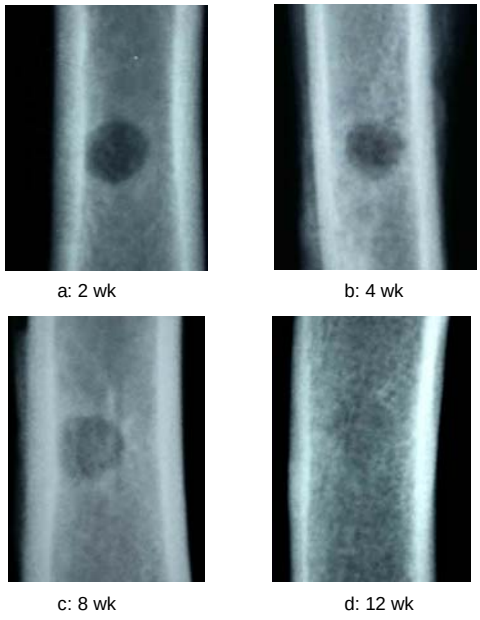


Figure 2 X-ray changes in bone defects at 2, 4, 8 12 wk after implantation of poly hydroxybutyrate-hydroxyvalerate membrane

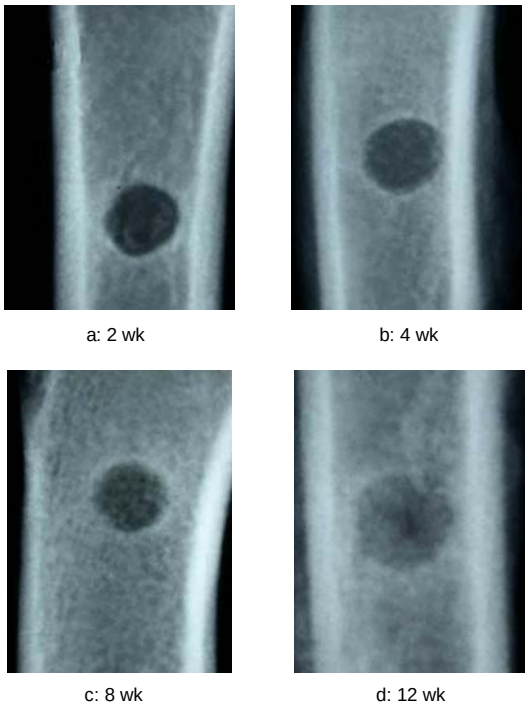


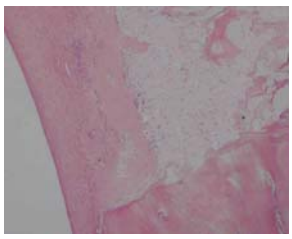
Figure 3 X-ray changes in bone defects of control group 2, 4, 8 12 weeks after operation

(1)Experimental groups: at 2 weeks postoperatively, the boundaries of bone defects were clear, and sparse bone trabecula was observed, appearing ground glass-like changes. At 4 weeks, mesh-like dense bone trabecula was observed in the bone defects. At 8 weeks, the density of defect margin was increased, with shadow of lamellar density enhancement. At 12 weeks, the bone density of newly generated was similar to normal bone, and fused with normal bone. But the defects were still visible (Figure 2). (2)Control group: at 2 weeks, bone defects presented with shading, with clear boundaries, but no osteogenesis. At 4 weeks, shading was observed at the defect region, and sparse bone trabecula was observed. At 8 weeks, the margin density of defects was increased, but lower than the experimental groups. Shading was visible in the defect region. At 12 weeks, the bone density in the defect region was increased, but significantly lower than the experimental groups (Figure 3).

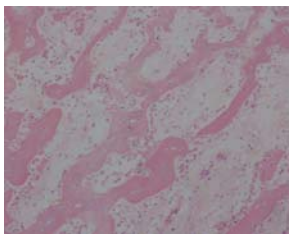
Pathological examination results

(1)Experimental group: at 2 weeks, the surface of bone defects was smooth and covered with periosteum of connective tissue, connected with periosteum of normal bone surface, but was significantly thickened. Bone matrix generated beneath the periosteum, mainly in a form of intramembranous ossification (Figure 4a). At 4 weeks, newly generated bone trabecula was tiny in the bone defects, and arranged in order (Figure 4b). At 8 weeks, the newly generated bone trabecula was thickened and fused, with lacune. The cell components were reduced. At 12 weeks, lamellar cortical bone formed in bone defects, and the cortical bone at the center was thin, with woven bone beneath the cortical bone. Bone construction remained incomplete (Figure 4c). (2)Control group: at 2 weeks, the soft tissues of bone defects were thick, with ingrowth of connective tissue and muscle tissue. At 4 weeks, a large amount of fibroplasias was

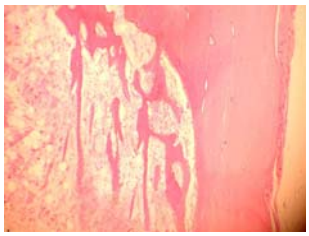
observed in bone defects, with little woven bone. At 8 weeks, the surface of bone defects was collapsed, and callus was composed of woven bone and fibrous bone. At 12 weeks, the collapse of defect surface was evident, and cortical bone formation was not obvious in bone defects (Figure 5).



a: Thickened periosteum and newly generated bone matrix at 2 wk (×100)



b: Thin and orderly bone trabecula at 4 wk (×100)



c: Bone cortex formation at bone defects with incomplete bone rebuilding (× 40)

Figure 4 Pathological changes in bone defects 2, 4, 12 wk after implantation of poly hydroxybutyrate-hydroxyvalerate membrane (Hematoxylin-eosin staining)

Figure 5 Evident collapse of bone defects surface in control group at 12 wk (Hematoxylin-eosin staining, ×40)

DISCUSSION

PHBV membrane cytotoxicity

Cytotoxicity of materials must be assessed before clinical application. Leaching liquor method can obain toxicity filter and simulate short-term effects of degraded products in human body to better reflect main cytotoxicity of solid materials[19-20]. RGR is an important parameter to evaluate material cytotoxicity.

The present study cultured BMSCs in vitro and the second to fourth passages were used to establish models. Those cultured cells grew and proliferated well and well simulated condition of materials implanted in bone tissues and allowed accurately assessment of cytotoxicity. In the present study, PHBV membrane materials were prepared into serial dilution extract solution at a terminal concentration of 100%, 75%, 50% and 25%. Results showed that the extract solution of PHBV membrane did not inhibit BMSCs growth or proliferation. There were no significant differences in cells growth between high and low concentration extract solution ($P > 0.05$). Moreover, the toxicity gradation of each concentration group at different stages ranged between 0 and 1. Those findings demonstrated that PHBV membrane ranging a extensive concentration has no cytotoxicity on BMSCs growth and proliferation, and can be used as biomaterials.

Osteogenesis beneath PHBV membrane

The PHBV membrane can well maintain regeneration space. Submembrane space was well maintained at 2, 4, 8, 12 weeks, with no space collapse. PHBV membrane guided bone regeneration since 2 weeks. Attached and aggregated BMSCs from periosteum, endosteum, bone stump, and bone marrow on the inner surface of PHBV membrane, form bone matrix. Significant differences were observed between experimental and control groups in appearance and quantity of newly generated bone. Coverage of PHBV membrane provided a stable space for bone regeneration and promoted ingrowth of bone tissues. Dense bone trabecula and osteoblast were observed in a early stage, and bone rebuilding completed early. However, in control group, the surface of bone defects was collapsed, and fibrous tissue and muscle fiber grew in bone defects; bone repair was slow, and callus was composed of woven bone and fibrous bone, with little newly generated bone. In conclusion, PHBV membrane has good biocompatibility, and can maintain space for bone regeneration and guide bone regeneration. It could become a promising natural membrane material for guided bone regeneration.

REFERENCES

[1] von Arx T, Broggini N, Jensen SS, et al. Membrane durability and tissue response of different bioresorbable barrier membranes: a histologic study in the rabbit calvarium. Int J Oral Maxillofac Implants.2005;20(6):843-853.
[2] Strietzel FP. Risks and complications of membrane-guided bone regeneration-A retrospective analysis. Mund Kiefer Gesichtschir. 2001;5(1):28-32.
[3] Iadevaia S, Mantzaris NV. Genetic network driven control of PHBV copolymer composition. J Biotechnol. 2006;122(1): 99-121.
[4] Feng L, Yoshie N, Asakawa N, et al. Comonomer-unit compositions, physical properties and biodegradability of bacterial copolyhydroxyalkanoates. Macromol Biosci. 2004; 4(3):186-198.
[5] Abou-Zeid DM, Muller RJ, Deckwer WD. Biodegradation of aliphatic homopolyesters and aliphatic-aromatic copolyesters by anaerobic microorganisms. Biomacromolecules. 2004;5(5): 1687-1697.
[6] Shishatskaya EI, Volova TG, Puzyr AP, et al. Tissue response to the implantation of biodegradable polyhydroxyalkanoate sutures. J Mater Sci Mater Med. 2004;15(6):719-728.
[7] Zinn M, Witholt B, Egli T. Occurrence, synthesis and medical application of bacterial polyhydroxyalkanoate. Adv Drug Deliv Rev. 2001;53(1):5-21.
[8] Chen GQ, Wu Q. The application of polyhydroxyalkanoates as tissue engineering materials. Biomaterials. 2005; 26(33): 6565-6578.
[9] Sodian R, Sperling JS, Martin DP, et al. Fabrication of a trileaflet heart valve scaffold from a polyhydroxyalkanoate biopolyester for use in tissue engineering. Tissue Eng. 2000;6(2):183-188.
[10] Kadiyala S, Young RG, Thiede MA, et al. Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro. Cell Transplant. 1997;6(2): 125-134.
[11] Arinzeh TL, Peter SJ, Archambault MP, et al. Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect. J Bone Joint Surg Am. 2003;85-A (10): 1927-1935.
[12] Pelled G, GT, Aslan H, Gazit Z, et al. Mesenchymal stem cells for bone gene therapy and tissue engineering. Curr Pharm Des.2002;8(21):1917-1928.
[13] United States Pharmacopeia Convention. Biological Tests/Biological Reactivity Tests, in vitro[S].USP27,2004.

[14] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth.1983;65(1-2):55-63.
[15] Green LM, Reade JL, Ware CF. Rapid colorimetric assay for cell viability: application to the quantitation of cytotoxic and growth inhibitory lymphokines. J Immunol Meth. 1984;70(2):257-268.
[16] Ferrari M, Fornasiero MC, Isetta AM.MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. J Immunol Meth. 1990;131(2):165-172.
[17] Strietzel FP, Khongkhunthian P, Khattiya R, et al. Healing pattern of bone defects covered by different membrane types--a histologic study in the porcine mandible. J Biomed Mater Res B Appl Biomater. 2006;78(1):35-46.
[18] Nishimura I, Shimizu Y, Ooya K. Effects of cortical bone perforation on experimental guided bone regeneration. Clin Oral Implants Res. 2004;15(3): 293-300.
[19] Cao T, Saw TY, Heng BC, et al. Comparison of different test models for the assessment of cytotoxicity of composite resins. J Appl Toxicol. 2005;25(2): 101-108.
[20] Rosengren A, Faxius L, Tanaka N, et al. Comparison of implantation and cytotoxicity testing for initially toxic biomaterials. J Biomed Mater Res A. 2005;75(1): 115-122.

可降解聚羟基丁酸酯-羟基戊酸酯的共聚物膜*

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摘要

背景：聚羟基丁酸/羟基戊酸共聚酯已用于构建心脏生物瓣膜，但不知是否可作为骨再生引导膜？

目的：观察聚羟基丁酸/羟基戊酸共聚酯膜的生物相容性，并评价其成骨能力。

方法：采用 MTT 法检测 100%，75%，50%，25%聚羟基丁酸/羟基戊酸共聚酯材料浸提液对犬骨髓间充质干细胞相对增殖度的影响，并评价其细胞毒性；在犬胫骨缺损模型左右两侧远心端骨缺损上方覆盖聚羟基丁酸/羟基戊酸共聚酯膜作为实验组，在近心端仅以骨膜瓣复位作为对照组。

结果与结论：100%，75%，50%，25%聚羟基丁酸/羟基戊酸共聚酯膜的细胞毒性分级为 0~1 级，对骨髓间充质干细胞的生长及增殖无毒性；实验组术后第 2 周即可见新骨形成，12 周时骨缺损区已完全被新骨充填，骨修复质量明显优于对照组。表明聚羟基丁酸/羟基戊酸共聚酯膜具有良好的生物相容性，对骨髓间充质干细胞无明显毒性，引导成骨能力强。

关键词：聚羟基丁酸酯-羟基戊酸酯共聚物；骨髓间充质干细胞；生物相容性；骨缺损；引导骨组织再生
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本文创新性：以“聚羟基丁酸酯－羟基戊酸酯共聚物，引导组织再生”为关键词检索中国期刊全文数据库 2003/2011 文献，共检索到 20 篇，有研究将聚羟基丁酸/羟基戊酸共聚酯用于构建心脏生物瓣膜，但未见将其作为引导骨再生膜使用的报道。实验通过 MTT 实验和体内成骨实验检测聚羟基丁酸/羟基戊酸共聚酯膜材料的细胞毒性、生物相容性及成骨能力。

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2　表

表格按文中出现顺序标号(如表1，表2等)，有编号和标题。表格内容应有自明性，不应重复正文中的内容，应是正文内容的补充。确保表中内容和正文中引用内容一致。

每个表要有表注，提供详细而准确的信息，但不引文献。对表中出现的所有符号应作说明。将数据放在表中而非正文中常可缩减文章的长度。