A Study on a Root Canal Filling, Containing Thymol in High Concentration

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Safety evaluation was made on Biological canal paste (BCP), one of newly developed endodontic pastes used for Ultrasonic Bio-Mechanical Obturation System. BCP was characteristic of a high content of thymol to show extreme antimicrobial action. The basic biological tests on cytotoxicity, systemic toxicity, mutagenicity and dermal sensitization were carried out in this study. On potency of cell growth inhibition *in vitro*, BCP was as same as Canals-N[®] and much less than Canals[®]. As neither death nor toxic syndrome in mice was found from administration of BCP in oral maximal dose of 1.0 mg/kg, it was estimated that LD₅₀ of BCP should be not less than the value of 'slightly toxic' classified by Deichmann. BCP showed negative results in mutagenicity examined by Ames test, and also in delayed type allergenicity by Adjuvant and patch test. From the above it was suggested that BCP was possibly safe so far as it was used properly for dental therapy.

Key words: Biological canal paste/Thymol/Toxicity

INTRODUCTION

Volatile oils such as clove oil, eucalyptus oil, mentha oil, and cinnamic oil are made of each original aromatic vegetable and called essential oils. They have been used for medical drugs since more than 400 years ago^{1,2)}. Some essential oils and also chemicals contained in them were introduced to dental therapy, and they played important roles showing various effects of disinfection, anti-inflammation and cyto-activation, as well as aromatic effect^{1,3-5)}.

Especially thymol, a main constituent of thyme oil, mosla oil, *etc.*, is to be more appreciated for its excellent sterilizing action⁶⁾. A newly developed root filling technique with application of thymol preparations was named Ultrasonic Bio-Mechanical Obturation System and introduced to clinical practice, resulting in a considerably good clinical

progress^{7,8)}. Two endodontic pastes of respective formula containing thymol in a high concentration were used there; one paste was a pulp capping named Biological canal paste (BCP), and the other was a root canal filling named Mechanical canal paste (MCP)⁷⁾. Intention of the composition of BCP was to bring the lesion a better calcification, which was given not only by a proper stimulation but also by a remarkable and lasting sterilization.

No study was made on their toxicity for clinical application of the thymol-rich pastes. Matters of concern had been their therapeutic effect and physical properties to be improved in manipulation, obturation, endurance and so on. An attempt was made in this paper to evaluate safety of BCP on the basis of our studies of cytotoxicity *in vitro*, single dose toxicity, mutagenicity and dermal sensitization.

MATERIALS AND METHODS

I Test compound

Biological canal paste (BCP, Nihon Shikayaku Co. Ltd., Japan) was a test sample used for all experiments. Composition of BCP⁷⁾ is shown in Table 1.

II Cytotoxicity test

Two commercial root canal fillings were tested as positive control of the test: BCP was compared with an eugenol-containing paste, Canals[®] (Showa Yakuhin Kako, Japan) and an eugenol-free paste, Canals-N[®] (Showa Yakuhin Kako, Japan), on inhibition of cell growth.

Mouse L-929 cells (NCTC clone 929, Dainippon Pharmaceutical, Japan) were grown in plastic tissue culture flasks (Falcon, USA) with Eagle's minimum essential medium (MEM-1, Nissui Pharmaceutical, Japan) supplemented with 3 % fetal bovine serum (FBS, Difco, USA) 0.17 % tryptose phosphate broth (Difco, USA) and 2 % L-glutamine (Wako Pure Chemical, Japan).

Immediately after mixing of powder and base to extract the elution during harding, BCP

Table 1 Composition of BCP

Component		%
Powder	Calcium hydroxide	30.00
	Barium sulfate	25.00
	Bithmuth oxycarbonate	25.00
	Zinc oxide	20.00
Base	MCT*	63.00
	Yellow bees wax	15.00
	Rosin	6.00
	Thymol	16.00

*MCT: middle chain fatty acid triglyceride

Powder and base are mixed just before application in the ratio of 0.85 g to 0.5 ml

paste was added to culture medium and stirred well for 2 minutes by an automatic mixer.

Then the medium with BCP was filtered ($0.45\mu\text{m}$, Millipore, Japan), and diluted in the original medium to various concentrations of BCP. Cells were seeded in culture dishes ($35\text{ mm } \phi$, Falcon, USA), and 5 hours after incubation, the medium was exchanged to the fresh one with BCP. After 7 day cultivation, adherent cells were fixed to dish and stained with 0.1 M crystal violet in 0.1 M citric acid. Inhibition of cell growth was measured with Monocelator (Olympus, Japan) in comparison with control growth in BCP-free medium. Calculation of the percentage of inhibition was based on the control cell adhesion¹¹⁾.

III Single dose toxicity test^{10, 12)}

ICR strain specific pathogen free male mice (4 weeks old, Charles River Japan, Inc.) were used. Test was started after 1 week prebreeding. Mice were bred in plastic cages in a barrier sustained room. Food and water were available ad libitum.

Mice were forcibly given BCP orally in dose of 1.0 g/kg (160 mg thymol/kg) with sonde. Freshly mixed BCP paste was diluted with olive oil. The control group was given olive oil in dose of 0.1 ml/10 g body weight instead of BCP. The experimental period was continued for 14 days after single administration. Mortality and general signs were observed and body weights were measured daily. Dietary consumption (D.C.) and water consumption (W.C.) were measured once a day per group through the experiment period and divided the number of mice. Dietary efficiency was calculated from the amount of gain in weight divided by amount of D.C. Gross observation on organs and tissues of all mice was performed at the end of the experimental period. Liver and kidneys were excised from all animals for weighing and for histopathological examination. After wet organ weights were measured in absolute value and then ratio to body weight was calculated, organs were prepared by the routine method of histological studies. Any other organ found macroscopic changes at necroscopy was also given histopathological examinations.

IV Mutagenicity test¹³⁾

Mutation assay was carried out by preincubation method of modified Ames test by Yahagi (1975)^{14, 15)}. The *Salmonella typhimurium* tester strains TA98 and TA100 (IFO14193 and IFO14194, Institute for Fermentation Osaka, Japan) were used to detect the induction of frameshift mutations and base pair substitutions in the presence of metabolically activating S9-mix (Oriental Yeast, Japan). Dimethyl sulfoxide (DMSO, Infinity pure reagent grade, Wako Pure Chemical, Japan) was used as a negative control and 2-aminoanthracene (Wako Pure Chemical, Japan) was used as a positive control. Freshly mixed BCP was diluted in physiological saline and filtered ($0.45\mu\text{m}$, Millipore, Japan) as described in cytotoxicity test. From preparatory experiment, BCP was tested in the concentrations from 0.01 to 100 mg/plate.

V Dermal sensitization test

The adjuvant and patch test was carried out as described by Sato *et al.*¹⁶⁾ and Katuta *et*

Table 2 Evaluation of skin reaction (scale for scoring)

(1) Erythema formation		(2) Edema formation	
None	0	None	0
Very slight	1	Slight	1
Well defined	2	Moderate	2
Moderate to severe	3	Severe	3
Severe erythema to slight eschar formation	4		

$$\text{Mean response (MR)} = \frac{\sum_{i=1}^n [(1)+(2)]}{\text{Total number of animals}}$$

*al.*¹⁷⁾ with use of clean male guinea pigs of Hartley strain (Kyudo Co. Japan), weighing c.a. 350 g.

At the first stage of induction, on the first day, the shoulder region of animals was shaved and emulsified Freund's complete adjuvant was injected intradermally at the 4 corners, and lattice abrasion was made on the injection site. The BCP paste was applied in 50 % to an adhesive plaster for patch test (Torii Pharmaceuticals Inc.) and covered with surgical drape (Steri-Drape, 3M) to fix in place for 24 hours. Similar procedures of abrasion and application of patches to the same sites were repeated on the following two days.

At the second stage of induction, on the 8 day, 10 % of sodium lauryl sulfate in yellow petrolatum was applied. On the 9 day, the BCP paste at the same concentration used in the first stage of induction was spread on a piece of filter paper. The filter was applied on the same shoulder region with plastic adhesive tape (Blenderm, 3M) and left for 48 hours.

At the challenge stage, on the 22 day, after shaving flank region, BCP paste was applied on the skin with open patch. The concentrations of sample were 2.5, 10, 25 and 50 %. Acetone was used for a vehicle of BCP and then also for a negative control of the test. Positive control was 0.0001–0.1 % Sudan I (Wako, Japan) in acetone. The site of challenge reaction was evaluated at 24 and 48 hours after application, in accordance with the scale for scoring of skin reaction (Table 2) and the total erythema and edema scores show the extent of reaction.

VI Statistical analysis

Data were presented as the mean $\pm$ S.E.M. from 3 to 5 animals or 2 to 9 samples per group as indicated in the respective tables. Statistical analysis were performed using Student's t-test if appropriate or Welch's t-test. P values of <0.05 were considered as significant.

All animal experiments were performed with the approval of The Animal Care Committee of Kyushu Dental College.

RESULTS

I Cytotoxicity test

Cytotoxicity of BCP, Canals-N[®] and Canals[®] is shown as the percentage of inhibition of cell growth on dish bottom (Fig. 1). Fifty percent inhibitory concentration (IC₅₀) was 6.9 mg/ml for BCP, 6.2 g/ml for Canals-N[®] and 1.0 mg/ml for Canals[®]. No significant difference was found between BCP and Canals-N[®]. BCP was almost as same as Canals-N[®] and much less than Canals[®] on cytotoxicity potency.

II Single dose toxicity test

No mouse died and showed abnormal behaviors and appearances such as piloerection through the test period. There was no significant difference in body weight gain between BCP and control groups (Table 3). D.C. and W.C. were calculated in amount of per day and per body, and comparison was made on the mean value of each period between BCP and control groups. Animals given BCP showed no significant difference from the control in D.C. by period (Table 4) and in W.C. (Table 5) in any period of the observation.

Only relative organ weight of liver in BCP group decreased ($p < 0.05$), but on the other organ weights, no significant difference was seen from control (Table 6). In histopathological examination of liver and kidney, inflammatory infiltrating cells were seen partially and slightly in both groups.

Fig. 1 Effect of BCP on the growth of L cells. The percentage of inhibition was based on control cell growth, values are mean $\pm$ S.D. (n=5)

Table 3 Change in body weight of male mice administered BCP in oral dose of 1.0 g/kg

		BCP	Control
Number of mice at start		5	5
Death during experimental period		0	0
Number of mice at termination		5	5
Body weight.(B.W.,g $\pm$ S.D.) [Ratio to start(%)]	At start	27.3 $\pm$ 0.8	28.0 $\pm$ 0.9
	On 7th day	31.4 $\pm$ 1.6[115]	31.5 $\pm$ 1.1[113]
	At termination	34.0 $\pm$ 1.9[125]	33.8 $\pm$ 1.0[121]
Gained B.W.,(g $\pm$ S.D.) [Ratio to control(%)]	1st ~7th day	4.1 $\pm$ 1.0[117]	3.5 $\pm$ 0.5
	8th ~14th day	2.6 $\pm$ 0.7[87]	2.0 $\pm$ 0.6
	1st ~14th day	6.7 $\pm$ 1.4[116]	5.8 $\pm$ 0.6

Termination; 14th day after administration

No significant difference is from control.

Table 4 Change in dietary consumption and dietary efficiency of male mice administered BCP

		Dietary consumption (g/body)		Dietary efficiency (%)
		For period	Average for 1 day(mean±S.D.)	W.G./D.C.
BCP	From start to 7th day	36.6	5.2±0.2	11.2
	From 8th day to termination	34.7	5.0±0.1	7.5
	During experimental period	71.3	5.1±0.2	9.4
Control	From start to 7th day	34.4	4.9±0.2	10.2
	From 8th day to termination	32.0	4.6±0.2	6.3
	During experimental period	66.4	4.7±0.3	8.7

Termination; 14th day after administration

W.G.: Weight gain (g/body)

D.C.: Dietary consumption (g/body)

Table 5 Change in water consumption of male mice on oral administration of BCP

(g/body)

		For period	Average for 1 day(mean±S.D.)
BCP	From start to 7th day	45.1	6.4±0.4
	From 8th day to termination	42.1	6.0±0.1
	During experimental period	87.2	6.2±0.4
Control	From start to 7th day	51.6	7.4±0.6
	From 8th day to termination	44.2	6.3±0.4
	During experimental period	95.8	6.8±0.7

Termination; 14th day after administration

Table 6 Absolute and relative organ weight in male mice on oral administration of BCP

(g/body)

		Absolute organ weight (g)			Relative organ weight (%)		
No. of mice		Liver	Kidney		Liver	Kidney	
			Right	Left		Right	Left
BCP	5	1.80±0.16	0.25±0.03	0.25±0.03	5.27±0.22*	0.73±0.08	0.73±0.10
Control	5	1.95±0.17	0.26±0.01	0.27±0.02	5.77±0.33	0.77±0.03	0.78±0.05

Values are mean ±S.D.. *: Statistical difference is from control (p<0.05).

Relative organ weight(%)=Absolute organ weight(g)/body weight(g)×100

III Mutagenicity test

Numbers of His⁺ revertant colonies increased significantly in positive control (2-aminoanthracene) in both *S. typhimurium* TA100 (334 %) and TA98 strains (196 %)

Table 7 Mutagenicity of BCP in His⁺ revertant colonies of *Salmonella typhimurium* TA98 and TA100

	Concentration	No. of His ⁺ revertant colonies	
		TA98	TA100
BCP	100 mg/plate	— (n=2)	— (n=1)
	10 mg/plate	24 ± 6 (n=5)	195 ± 12 (n=5)
	1 mg/plate	36 ± 7 (n=5)	253 ± 46 (n=9)
	0.1 mg/plate	42 ± 8 (n=5)	254 ± 45 (n=9)
	0.01 mg/plate	38 ± 4 (n=5)	255 ± 18 (n=5)
2-Aminoanthracene (positive control)	0.5 µg/plate	196 ± 76 (n=5)*	334 ± 41 (n=5)*
DMSO (negative control)	100 µl/plate	37 ± 9 (n=5)	213 ± 25 (n=5)

No significant difference is found between 0.01~1 mg/plate of BCP and DMSO in each strain.

Values are mean±S.D., *; Statistical difference from control (p<0.05)

Table 8 Score of dermal sensitization of BCP in adjuvant and patch test

	Challenge conc. (%)	24 hrs			48 hrs		
		FR		MR	FR		MR
		Erythema	Edema		Erythema	Edema	
BCP	50	0/3	0/3	0.0	0/3	0/3	0.0
	25	0/3	0/3	0.0	0/3	0/3	0.0
	10	0/3	0/3	0.0	0/3	0/3	0.0
	2.5	0/3	0/3	0.0	0/3	0/3	0.0
Positive Control (Sudan I)	0.1	2/2	2/2	4.0	2/2	0/2	2.0
	0.01	2/2	2/2	2.0	2/2	0/2	1.5
	0.001	0/2	0/2	0.0	0/2	0/2	0.0
	0.0001	0/2	0/2	0.0	0/2	0/2	0.0
Negative Control (Acetone)	100	0/2	0/2	0.0	0/2	0/2	0.0

Challenge: Reaction-inducing

FR: Fraction response (=positive/total)

MR: Mean response

compared with the number of negative control. Any dose of BCP resulted in insignificant induction of His⁺ revertant colonies (Table 7).

From the above, no mutagenic effect of BCP was detected in both inductions of frameshift mutations in TA100 and base pair substitutions in TA98.

IV Dermal sensitization test

The result of the test was shown in Table 8. No positive reaction was found in any animal applied BCP of any concentration either 24 hrs or 48 hrs after challenge administration.

DISCUSSION

Firstly BCP was compared on cytotoxicity with Canals[®] and Canals-N[®], both of which were useful commercial products in endodontic practice. The base of Canals[®] is zinc-eugenol produced by chelating reaction between eugenol and zinc oxide⁴⁾. Occasionally, Canals[®] gave irritation to the teeth, and it is comprehensible that the adverse effect of irritation is mainly due to eugenol remaining unchanged in the base^{18,19)}. Canals-N[®], a non-eugenol type paste consisting of zinc oxide and a mixture of fatty acid and propylene glycol, was invented to get a less-irritative paste. Actually it was admitted that Canals-N[®] gave the patients less trouble of irritation and characteristic odor than a zinc-eugenol type paste such as Canals[®]^{18,19)}. Then the result of our cytotoxicity test coincided with degrees of adverse effect in clinical practice, because it was shown that cell growth inhibition *in vitro* by Canals-N[®] was much less than by Canals[®].

It was found that BCP was as cytotoxic as Canals-N[®], and much less than Canals[®]. Cytotoxicity ratio of BCP to Canals[®] was about 1/6 as estimated on the basis of IC₅₀ values. These results suggested that irritation of human tissue caused by BCP might not be more serious than by Canals-N[®] and much milder than by Canals[®]. Therefore, it was considered that adverse effect of BCP on human teeth would be within a permissible limit in the clinical practice.

Objective of single dose toxicity study was to elucidate toxicity of BCP given by enforced oral administration to mice. BCP was given in a dose of 1.0 g/kg, because we had confirmed through our preliminary test that possible dose in mice was maximal 1.0 g/kg for skilful procedure of oral administration.

It was reported that mean body weight of ICR-strain male mice was 28.0 g (S.D.1.1, n=30), 30.7 g (S.D.1.3, n=30) and 32.5 g (S.D.1.5, n=30) on 35th, 42nd and 49th day respectively²⁰⁾. The mice of BCP group showed rather better growth (Table 2). It was possible that better growth was brought about by olive oil used as a vehicle of BCP. There was no significant difference on the body weight gain between BCP and control groups. Not only on the body weight gain, but also on D.C., dietary efficacy, W.C., general condition and findings of necropsy, no significant difference was found between BCP and control groups. No change was seen in macroscopical comparison with the control, while a little decrease in relative liver weight was found in the BCP group. Also in histopathological examination no difference in liver as well as in kidney could be found between the BCP group and the control. Though a possibility of slight inflammation was shown by partial appearance of infiltrating cells in some specimens of both organs in the both groups, its cause was not clarified in this study.

It is estimated without doubt that oral LD₅₀ in mice was much higher than 1.0 g/kg, in which dose BCP caused neither death nor any toxic sign of the test animals as mentioned above. According to tabulation of toxicity classes by Deichmann *et.al*²¹⁾ chemicals or drugs with LD₅₀ of 0.5–5 g/kg were graduated into a class of “slightly toxic”. When calculated in

term of a human weighing 50 kg, BCP dose of 1.0 g/kg was 50 g which was too high amount to be accidentally taken. Fifty grams of BCP was equivalent to the amount of 200–500 folds as a dose of dental application into a human tooth. Therefore, it could be hardly considered that proper application of BCP in endodontic therapy gave the dental patients any systemic toxicity.

A mutagenicity test with use of microbe had been developed and demonstrated to be useful means for short-term screening of carcinogen. There was found a little possibility that BCP had mutagenicity, as the findings of the test on BCP were negative.

BCP even in high concentrations of 50 % showed negative results also in the dermal sensitization test, while BCP contained rosin, which showed positive effect alone in the same sensitization test¹⁷⁾. It was understandable that concentration of rosin in BCP paste was about 2.2 % as shown in Table 1, and it was not enough to cause sensitization, as rosin tested singularly showed a positive effect in concentration above 2.5 %¹⁷⁾.

CONCLUSION

BCP, a newly developed endodontic paste with thymol of exceptionally high content, was examined on its toxicities by the basic biological tests. Cytotoxicity of BCP was much less than that of Canals which had legal approval of dental use. BCP was clarified less than 'slightly toxic' on systemic toxicity and showed neither mutagenicity nor allergenicity. It was confirmed from the results that BCP was safe in so far as it was used properly for dental therapy.

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高濃度チモール含有根管充填材の基礎的研究

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抄 録

超音波バイオメカニカルオブチュレーションシステムに使用する新規開発根管充填材であり、優れた抗菌作用を発現するチモールを含有している Biological canal paste (BCP) の安全性評価を行った。生物学的基礎試験として、細胞毒性、全身毒性、変異原性、および感作性を調べた。BCP の細胞増殖阻止力は、Canals-N[®]と同程度で Canals[®]より小さかった。BCP の投与可能な最大量である 1.0 mg/kg のマウスへの投与により、死亡例はなく毒性症状も認められなかったことから、BCP の LD₅₀ は高く Deichmann による毒性分類表での「わずかに毒性がある」の範囲以上と考えられた。Ames 試験による変異原性試験およびモルモットを用いた感作性試験において、BCP は陰性であった。これらの結果から、BCP は歯科治療に用いる上では安全である可能性が示唆された。

キーワード：BCP/チモール/毒性