A proposition for long-term biocompatibility test of dental materials in vitro

Masaaki NAKAMURA, Hideki KODA and Haruyuki KAWAHARA

Department of Biomaterials Osaka Dental University 1-47, Kyobashi, Higashiku, Osaka 540, Japan
(Head: Prof. H. KAWAHARA)

Received on May 31, 1983

The importance of establishing a testing method for long-term biocompatibility of dental materials cannot be overemphasized, since the materials remain in the living system for a long period of time once inserted. Unfortunately, the current testing methods lay greater stress on shorter testing periods than on longer ones. In addition, none of them include testing in an environment simulating the oral cavity. The present study incorporated to some degrees these two essential factors, i.e. long-term test and oral simulation. Standardization of each step was made and satisfactory results were obtained.

Key Words: Biocompatibility Test, Dental Materials, Cell Colony Formation

INTRODUCTION

The biocompatibility tests for dental materials consist of two main methods, i.e. biocompatibility test in vitro and in vivo based on animal experiments as steps to be performed before the clinical usage test. The authors have been investigating in vitro tests for many years in view of its potential superiority.1-10) Once inserted, however, these materials remain in the living system for a long period of time, so that it is very important to know the long-term biological properties of these materials. At present, no long-term biocompatibility test in vitro has been established yet. In order to elucidate and compare the changes in cytotoxicity of each material seen in the course of time, the authors have been attempting to establish a long-term biocompatibility test in vitro.11-22) The project that the authors have been studying is outlined below.

MATERIALS AND METHODS

Standardization was made of each step of the cell culture method, specimen preparation, data analysis, and data arrangements.

1. Cell culture method

Strain HeLa S3 (Puck et al., 1956)23) of the epithelial cells, one of the most commonly used strain cells, was selected. Although methods for checking cell growth include the cell nucleus counting method, agar plating method, and cell colony forming method, the latter method is the most suitable for the present purpose. The reason is that not only are the obtained data of the same quality as the other methods, but also it is possible to automate the colony counting system because colony formation occurs two-dimensionally on the
2. Specimen preparation

Three types of dental materials were employed: 1) Type mouldable to test specimens; This commonly used type of material is easily mouldable by means of mixing, casting or polymerization. 2) Type unmouldable to the shape and size of test specimens; This type of material is already moulded, including artificial teeth, dental implants, and root canal filling material. 3) Liquid or pasty type; This type is also unmouldable to the shape and size of test specimens, including some impression materials and root canal medicaments. In order to establish the methodology, the first type was used.

In vitro experiments showed that the experimental results are different depending on the size of test specimens. An overall view and study of the experimental results showed that the surface area of one cm$^2$ was the most adequate.$^{1-22}$ The specimen was shaped

---

**Figure 1** a: Specimen moulds. A stainless steel split mould (Rear) and a glass tube (Front, left) are shown. A material was filled into the tube and shaped cylindrically (Front, right).

b: The specimens ready for use. The specimens with bars are for the experiments either for dynamic extraction with a suspended specimen or for static extraction (Left and center). They were used for tests after coating of the bar with a histologic wax, which was known to be non-cytotoxic. Also shown are cylindrically shaped specimens for the dynamic extraction tests with a freely moving specimen (Right).
cylindrically so as to easily mould the specimens and for smooth preparation. In the case of restorative materials, the specimens were made by directly filling into the specimen mould and setting (Figure 1). When casting or polymerization is required, a wax pattern is first formed and specimens were then moulded by a predetermined method. Then, these specimens were completed to the shapes of the specimens suitable for the three different extraction methods which will be described later.

3. Extract preparation

These specimens were immerged in an extract for a definite period of time to extract the components. The extract was used for carrying out the tests. The same medium, MEM as that for the cell culture was employed as the extract. The composition of the extract is similar to that in the tissue fluid in a living system. Its use was a preparation for the tests in vitro under a simulated condition of oral environment in future. Four factors are involved in the extracting rate: Surface area of specimen, volume of extract, extraction period and extraction method. The volume of the extract was determined to be 10 ml.\(^1\)\(^{-23}\) As for the extraction period in vitro, it is impossible to obtain experimental data periodically by making these test factors to react for a long period of time in the same cell groups. However, because materials are exposed to the continuous passage of food or saliva in the actual oral environment, renewal of the extraction environment at a definite time interval, or an exchange of extract would simulate the conditions of the living system. Preliminary experiments were carried out for five classes of the extraction periods. Based on the relationship between the surface area of the specimen and the volume of the extraction or long-term test in vitro, a unit extraction period of two weeks was used. The extract was collected after two weeks and used for the test as the first extract, while the same specimen was immerged again in the new extract of the same volume for the same period (Figure 2). The liquid was used as the second extract. The same procedure was further repeated for a total of 20 weeks. The extract obtained for each period was tested.

4. Methods of extraction

The three extraction methods are as follows (Figure 3):

(1) To simulate a dynamic environment where orally inserted materials are held, a tightly sealed Erlenmeyer flask extracting the specimens in MEM was fixed in a gyrotory incubator and dynamic extraction of a freely moving specimen was carried out at 200 rpm and 37°C. Gyration at 200 rpm exerted on the specimen a great dynamic load including friction and collision with the vessel as well as extract flow.

![Figure 2 Test schedule. The specimen was immerged for a unit period of two weeks, after which extract was obtained and used for cell culture. The same specimen was used throughout the total testing period.](image-url)
Figure 3  The three extraction methods.  a:  Dynamic extraction with a freely moving specimen.
  b:  Dynamic extraction with a suspended specimen.  c:  Static extraction.

(2) Dynamic extraction of a suspended specimen; that is the specimen was suspended in
the middle of the extract and gyrated at 200 rpm.  Only the effect of the collision with the
vessel was excluded (1).

(3) Static extraction of a suspended specimen under a static condition where no dynamic
loads were applied.

5. Experimental method

1) Replicate culture

Four ml of cell suspension of a definite number of cells were poured into a plastic petri
dish and pre-stationary-cultured at 37°C for 24 hr in a 5% CO₂-95% air, humidified
environment.

2) Exchange of culture medium for extract

Then, the culture medium was exchanged for the above extract and the culture was
continued for an addition week.  At that time, in order to express easily the degrees of
various cytotoxicity, the extract obtained after each extraction period, was used as the
original extract and diluted with a fresh culture medium MEM and calf serum, so that
concentrations of the original extract became 25, 50, 75 and 91%, a total of four classes for
the tests.

3) Cell colony fixation and staining

Although the method depends on the immediate subsequent data analysis, homogeneous
staining is always obtained.  After being fixed with absolute methanol and double staining
using the May-Grunwald and Giemsa solutions, the specimens were rinsed, dried and
counted.

6. Data analysis

The routine counting of the colony-forming rate was carried out microscopically by
the researchers, so that it was very difficult to treat a number of the experimental cases, and
it takes not only much time, but also is inevitably accompanied by a risk of a dispersion of
the results.  No methods but the automated colony counting system which is the best
method for counting cellular proliferation (Figures 4 and 5).  As the cell colonies spread
two-dimensionally on the bottom surface of the petri dish, an unequaled condition can be
introduced into automated counting system. A computerised photo pattern analyzer permitted highly reproducible countings in a relatively short period of time. For the rate

![Cell colonies on the monitor screen. The dark dots on the monitor screen are the cell colonies.](image)

Figure 4 Cell colonies on the monitor screen. The dark dots on the monitor screen are the cell colonies.

![Automated cell colony counting system.](image)

Figure 5 Automated cell colony counting system. a: Pattern analyzer (Biotran III, New Brunswick, USA). b: Personal computer (MB-6890, Hitachi, Japan). c: Dissecting microscope (Nikon, Japan). d: Television camera (C141, New Brunswick, USA). e: Illuminating apparatus (890, Artek, Japan). f: Video-deck (CR-6600, Victor, Japan). g: Floppy disk player (MP-3630, Hitachi, Japan)
of cell colony area counted, the maximal value, mean value and minimal value of the counted values of each tested group were obtained at a confidence limit of 95%. Long-term changes in cytotoxicity were expressed via two-step treatment of these values as mentioned below.

1) Relative growth rate (RGR)

Each counted value for each test group in each period divided by the mean value for the control group in the same period, giving a relative growth rate. Since the test has four classes of concentration of the original extract, four values corresponding to each concentration were obtained for the relative growth rate. The potency of cytotoxicity of the tested specimens can be assessed according to the changes in the values of the relative growth rate for these four concentrations. In other words, the test specimen of potent cytotoxicity gives only a low relative growth rate at 25%, the lowest concentration of the original extract, and also gives relative growth rate at higher concentrations which is only slightly appreciable.

2) Cytotoxic scores

The above relative growth rate represents indirectly the toxicity and irritation that the test specimen exerts on the cells. However, because it is the cytotoxicity of each test specimen that the authors would like to know, possible conversion of the relative growth rate to scores representing immediately understandable cytotoxicity is very convenient. Hence, six grades of the cytotoxic score were provided. Relative growth rate, ≥100%=Score, 0 (No effects on the cells); RGR, 75%~99%=Score, 1; 50%~74%=Score, 2; 25%~49%=Score, 3; 1%~24%=Score, 4; 0%=Score, 5 (No cell growth).11-22)

RESULTS

The above tests were made for 52 weeks, first on the hardened specimens of four products from commercially available dental amalgam (Figure 6).10) The results obtained showed a distinct difference from the authors’ short-term data. Although the short-term data showed that the potent cytotoxicity in several hours after mixing disappeared after hardening,5,7,9) in the long-term dynamic extraction of a freely moving specimen, a potent cytotoxicity reappeared as a result of wear of the specimen due to friction between the vessel and the specimen. The long-term data obtained with amalgam in the dynamic environment were very interesting and important. They suggested that when various dynamic loads are applied after hardening of the materials, most dental materials, consisting of two or more components, can never be stable biologically, and proved substantially the necessity of the long-term biocompatibility tests in vitro which are closer to the oral environment. The value of each of the two other extraction methods was confirmed. As to the test period, the data on amalgam for 52 weeks showed a tendency that such a severe dynamic environment as 200rpm accelerated the dissolution of the components of the materials, thus a phenomenon that usually takes a long time to occur could be simulated in a short time. As a 20-week period was found to be sufficient for determining the overall tendency, this testing period was used for the subsequent experiment. Moreover, the tests for various metals, denture base resins (Figure 7),16) various composite resins (conventional, MFR, light-cured and for core use), dental cements and tissue conditioners elucidated the behavior of cytotoxicity after hardening. In addition, the application of the principle of the same test method will make it possible to make short-term tests up to approximately six weeks and also those
Biocompatibility reduced from cytotoxic score of dental amalgams. The materials used were Spherical-D (High copper type, Shofu, Kyoto, Japan), Dispersally (High copper type, Johnson-Johnson, USA), Spherical amalgam (Conventional type, Shofu, Kyoto, Japan) and Copper amalgam (Collected from the Manufacturer's Research Laboratory, Niimi, Japan). The four bars in each experimental week represented cytotoxic scores for four classes of the original extract concentrations, i.e. 25%, 50%, 75% and 91% from left to right. Also, six dots at the left side of the column of each experimental week correspond to cytotoxic scores; namely, the top dot represent Score 0, the second dot from the top; Score 1, etc.
Figure 7: Biocompatibility reduced from cytotoxic score of denture base resins. The materials used were Natural resin (No. 2, heat-curing type with colouring agents, Nissin, Kyoto, Japan), Natural resin (No. 1, heat-curing type with no colouring agents, Nissin), Polybase S (Fluid type resin, Nissin) and Orthodontic resin (Cold curing type, L.D. Caulk, USA). See key in Figure 6 Legend.

DISCUSSION

1. Automated colony counting system

The combination of the cell colony forming method, dynamic extraction and automated colony counting system accomplished the expected purpose. In particular, the introduction of the automated colony counting system resulted in epoch-making results for the analysis and arrangement of the data. The above system has indeed these favorable merits, but has its inherent weak points related to the apparatus, of which a great deal of care should be taken. Because the measurement by the photo pattern analyzer is based on the changes in transparency, it is necessary to confirm the presence of cell colonies and to eliminate all of the opaque objects other than the colonies. Uniform staining is indispensable also at the staining stage. Furthermore, there is a question of counting, resulting from the shapes of the cell colonies. When the two-cell colonies are adjacent or come into contact with each other, visual inspection and routine counting alone are far from satisfactory. The present method succeeded in solving the above question using mainly the data on the rate of the area represented by the cell colony within a unit area. A personal computer was additionally equipped to perform the counting, analysis, datum arrangement and tables preparation.

The authors would like to make improvements hereafter not only in the apparatus but also in the method so that a dynamic analysis of the kinetic cell proliferation without staining can be made.

2. Necessity for long-term biocompatibility test
In order to accomplish their function satisfactory, dental materials inserted in the oral cavity need to fulfill biological, physical, and chemical conditions. Of these conditions, with regard to the physical and chemical tests, material specifications have been established in America since 1926, and thereafter in other countries such as Japan, England, Australia, and Germany, or by such International Institutions as the FDI and ISO, the purpose of which is to standardize the quality elevation and control of materials. However, material specifications from the biological viewpoints have long been neglected until quite recently in spite of their undeniable necessity. It was only in 1980, that the Joint Working Group of ISO and FDI submitted temporary specifications for the biological test methods of dental materials (1980). These specifications consists of three test stages: the initial test, secondary test, and usage test, containing 10 items including the 3 in vitro tests in the initial test, 4 in the secondary test and 5 in the usage test, a total of 18 items (one overlapping between the secondary and usage tests). Out of these 18 testing items, as many as 15 items are for animal tests or tests for man. In the actual oral cavity, material specifications are closely associated also with general responses such as immune response in addition to the orally specific factors such as oral fluid, temperature fluctuation, pH fluctuation, oral faunas, drug ingestion and smoking, as well as biting force, contact with dissimilar restoration, food passage, and oral hygiene. It is probably extremely difficult to grasp exactly the effects of only experimental factors from the results obtained by the tests in vivo complicated with such various factors. Furthermore, the expense of test in vivo are high and the tests on humans can be performed only under very limiting conditions because the ethic problems involved. In view of these points, the authors have consistently insisted upon usefulness of the tests in vitro and investigated the possible cytotoxicity of the various dental materials. Of the biocompatibility tests for dental materials presented recently by the ISO/FDI, as few as three in vitro test methods have adopted temporarily those experimental techniques which are now in use in the field of the cell culture, histology and pharmacology, but the test period to follow up the cytotoxicity of the materials is at most 24 hr. Such a short term can not confirm the biological properties of the materials inserted in the living system over a long period of time.

As can be seen clearly from the above findings there is a serious defect in the biocompatibility test method of ISO/FDI; that is, the absence of a method of long-term test in vitro. Why do they depend entirely upon uncertain tests in vivo or limited tests on man and put no emphasis on certain tests in vitro? The authors wonder why emphasis has not be placed on tests in vitro. Namely, all of the specifications of tests for the physical, mechanical, and chemical properties of the materials adopt test methods in vitro, without any test methods in vivo. Only tests concerned with the biological properties have many tests in vivo.

3. An approach to a simulated condition of the oral environment

The goal of the test methods for materials is to achieve a simulated condition of the oral environment to which the authors have already attempted. Since obviously, all of the in vivo or clinical elements can be reproduced under such a simulated environment, the results obtained therein will provide important and suggestive information. The authors believe that the creation of such a simulated condition of the oral environment is not only of great benefit to test methods for materials but also brings much useful information to
each field of clinical dentistry. The method of the long-term biocompatibility test in vitro that the authors have presented this time is very primitive and incomplete in view of the points mentioned above, and may be called preliminary. Its underlying concept, however, aims at oral simulation, which can be applied not only for dental materials science but for the whole field of dentistry.

CONCLUSION

Naturally, materials to be used in the living system should be compatible therewith, indeed, but in actuality, there are no materials that fulfill all of the requirements. Though the authors have long been in pursuit of true biomaterials, the discovery of such materials is still far from their grasp. However, more efforts to develop these ideal materials should be made. The present long-term biocompatibility test does meet this purpose and will surely serve as a step for better oral simulation in the future.

ACKNOWLEDGMENT

The authors are grateful to Drs. K. Imai, J. Yokota, T. Hosohama, H. Kobayashi, S. Maehara, M. Izutani, H. Taguchi and Y. Ohgitani and Ms. Y. Ohta for their assistance throughout the present project.

REFERENCES

が必要であり、また各因子はすべて定量的変数ではなく、半定量的あるいは定性的な変数であることも多い。この意味において、枠の数理化理論を基に多変量解析を採用した。

その結果、多くの調査条件と寸法精度との関係が明らかにされ、それらの条件の背景を有するパラッキを規定している潜在因子を探索し、その因子の影響力を重みづけを行うことができた。

長期生物テスト法によるコンポジットレジンおよび充填用グラスアイオノマーセメントの細胞毒性（in vitro）

中村正明、川原春幸、今井弘一、友田 達、川田義典、光 司郎

大阪歯科大学歯科理工学教室

長期にわたるコンポジットレジンおよび充填用グラスアイオノマーセメントの硬化体の細胞毒性をしらべるために、HeLa S3 細胞を用いて細胞コロニー形成法を使って生物テスト（in vitro）を行った。規格サイズに作製された各実験試料を培養液 MEM に浸漬され、200 rpm の旋回投入浸漬の動的負荷が与えられた。この実験環境は臨床の場の in vitro におけるシミュレーションを目標するものである。そして、つきのような結果を得た。テストした新コンポジットレジン、TMM-SiO２レジンおよび従来のアダプティブは実験初期に弱い細胞毒性を示したものの、その後は実験終了時の20週後までの間にほとんど細胞毒性を示さなかった。これに反して、新コンポジットレジン、TMM-SiO２レジンおよびフジアイオノマーは実験初期に中等度の細胞毒性を示した。しかし、その後は他の 2 材料と同じくほとんど細胞毒性を示さなかった。

以上の大部分の実験期間を通じての安定した生物学的性質から、今回しらべた各材料は一定バイオマテリアルとしての適性を有していると言えよう。また、動的環境下での長期生物テストの必要性も明確にされた。

本研究の一部は文部省科学研究費補助の試験研究 (56870103) による。

In vitro における歯科材料の長期生物テスト法

中村正明、幸田秀樹、川原春幸

大阪歯科大学歯科理工学教室

長期にわたる in vitro での歯科材料の生物テスト法を確立するために、細胞コロニー形成法を活用して各種歯科材料をテストした。一定の大きさに作成したテスト材料成分を抽出した浸漬液で細胞コロニーを培養した後、コロニー形成度により各材料の細胞毒性を推定した。浸漬は動的ならびに静的環境下で 2 週間の単位期間ずつ行い、同一試料で合計 20 週間継続しその間の細胞毒性の消長を表わした。その結果、各種金属材料、床用レジン、各種充填材料、歯科用セメントや粘膜調整材などについ