JBR Journal of Interdisciplinary Medicine and Dental Science

Cox et al., J Interdiscipl Med Dent Sci 2016, 4:2
DOI: 10.4172/2376-032X.1000194

Review Open Access

A Timeline of Biological Assessments: Our Nonspatial Continuum

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Received date: April 4, 2016; **Accepted date:** April 13, 2016; **Published date:** April 20, 2016

Abstract

Much of our dental history is recognized through oral tradition, which has led to myths and misconceptions regarding the efficacy of early restorative agents. Dental biomaterial history has tended to report only certain aspects research in a few textbooks that are expensive and not readily available to the larger dental audience. Our purpose is to provide a detailed peer-reviewed document, which provides a chronological account of our *in vitro* and *in vitro* biological continuum. This document follows a published historical chronological timeline of biomaterial testing literature beginning with its little known inception in 1779. Many of the early dental restorative agents evolved due to their anodyne capacity to alleviate tooth pain and provide a modest bacteriostatic capacity. Those that were successful were modified as temporary cavity filling agents and evolved to more permanent fillings. Unfortunately, many of the early agents e.g. antimony, arsenic, asbestos, canthrides, formalin, mercury, mustard, phenol to name a few were toxic to the tooth and supporting periodontal tissues as well as failing to support lost tooth structure. The National Institute of Dental Research required biomaterial testing in the late 1940's. Even today, the agency permits many pre-1950 agents via the grandfather clause for commercial inclusion and clinical use, while requiring all new post-1958 agents to pass both *in-vitro* and in vivo testing hurdles. We routinely place restorative agents that infiltrate in graded interphases to interdiffuse into vital enamel, dentine and even cementum by forming a unique biomimetic substrate that mimics the color and opacity of the human tooth. Our biological continuum is still evolving with technologies that will continue to change our clinical future. Our profession has been the global benefactor of dynamic change. Unfortunately, our biomaterials testing status quo is not acceptable as many toxic agents e.g. formalin still remain in our clinics.

Keywords: Bacteriometic; Biocompatibility; *in-vitro*; *in-vivo*; Pulp; Seal

The Value of Written History

In the public domain, oral tradition has been handed down through generations that intend to convey practical and moral accounts of collective wisdoms as to how we should conduct ourselves.

Some humans study history from a desire to enhance the understanding of their heritage. Science history stems from a compilation of written accounts by individuals that have become part of our scientific record. In his 1895 inaugural lecture, Lord Acton stated, "science is deposited like the grains of gold in the sand of a river; and the knowledge of the past, the record of truths revealed by experience, is eminently practical, as an instrument of action and a power that goes to making the future" [1].

Euclid's Elements were written circa 300 BC while he lived in Alexandria Egypt-consisting of 13-books-described as the most influential textbook ever written by one individual. It proved instrumental in the development of scientific thought, logic and methodology-one of the first mathematical works to be printed on a press, only surpassed by the Bible in printed editions [2] Abraham Lincoln kept a copy of Euclid's Elements in his saddlebag and studied it by lamplight. "You never can make a lawyer if you do not understand what demonstrate means . . . I left Springfield, went home to my father's house, and stayed there till I could give any proposition in the six books of Euclid at sight" [3].

Following the Biological Timeline of Dental Material Testing

When did biological testing of dental products and their components become a concern and part of the scientific record? What was the 1st U.S. agency to establish biological testing? When did it originate? Is each chemical in the assemblage of our professional and OTC dental products biologically acceptable? If not, why do some toxic agents still remain in the composition of certain of today's dental products? Which biological test standards came first-*in-vitro* (bench-top) or *in-vivo* (animal)? Now that we have your attention, we'll attempt to answer these questions in the following text.

The origins the National Institutes of Health (NIH) and the National Institutes of Dental Research (NIDR) are traced to the US Marine Hospital Service (MHS) of 1779 that provided medical care for merchant seamen. By 1880, European scientists had established that person-to-person contact of bacteria caused cholera and that mosquito borne blood bites transmitted malaria [4,5]. As a result of these and other findings, the MHS was charged with expanded clinical duties at Ellis Island to inspect immigrants for infectious diseases. Joseph Kinyoun-a MHS physician/microbiologist at the MHS Hygiene Laboratory Staten Island-was able to identify bacteria in sick immigrants for diagnosis of infectious diseases (e.g. cholera) and to

quarantine them to prevent the spread of disease amongst the general population [6].

Perhaps the first dental restorative agent to arouse professional scrutiny was the introduction of dental amalgam during the American colonial era. Circa 1834, Edward and Moses Crawcour travelled from Europe to tour the colonies to introduce their quicksilver paste to colonial dentists. With no formal dental training, they rapidly became recognized as frauds with no ethical scruples. Lacking clinical cavity preparation and restorative standards, many clinicians experienced poor amalgam outcomes.

Without amalgam standards, clinicians would file tiny flakes of silver from the edges of coins and triturate them with mercury in a mortar and pestle for a few moments. Some placed the mix in a cloth pad to squeeze out excess mercury and a few even manipulated the mix in their palm and thumb to determine its plasticity before plugging it into the tooth. In those decades-before Black-mercury became an easy blame for failed amalgam restorations.

Many charlatans and untrained colonial clinicians attempted a crude effort to remove cavity debris and to plug the defect with amalgam, without paying attention to periodontal health, tooth contact and anatomical contour. A few weeks post insertion often resulted in gingival and pulp pathology. An outcome observation of that era showed the damaging effect of amalgam was improper clinical insertion [7]. It wasn't until the late 1800's when Black gave his independent research on principles of cavity preparation and amalgam composition to the dental profession [8]. He made no personal patent claims for his research. His time, talent and financial research support was entirely his own-there was no government funded dental research of those decades.

Through the late 1500's to the mid-1800's there were several different theories regarding the cause of human caries. For a comprehensive review of caries literature, we refer the reader to the 2010 article by Ruby [9]. By the early 1900's, only a few clinical colleagues treated caries as a bacterial disease or thought about caries prevention. Until then, most clinicians of the young profession were mainly concerned with the restoration of carious lesions.

In 1888, Kuehns was one of the 1st to report on correlation of fluoride and mottled enamel to reduced caries. He observed a rural family with mottled brown-spot enamel, which he attributed to excess fluoride intake [10]. Research by Eager and McKay also associated fluoride to reduced caries [11].

Authorities Search for Answers

Thousands of young men were rejected from military service during WW-II due to extremely poor oral and dental health. The armed forces health service branches convincingly lobbied the US Congress to create an agency to study and provide oral health standards for incoming military recruits. On June 24th 1948, President Truman signed legislation for the formation of the NIDR as the 3rd arm of the NIH. Its formation was a consequence of lobbying efforts of the armed forces to learn why so many young men were rejected from service. The NIDR was charged to identify the cause of caries and pyorrhea and to seek preventive agents and treatments that would benefit society at large [12]. In 1958 President Eisenhower signed legislation to fund construction of the NIDR facilities in Bethesda MD that became reality in the 1960's [13]. Research directives were immediately implemented, which enabled Dean to establish water fluoridation studies in various US communities and to identify it's effects on prevention of tooth decay [14].

Establishing Biocompatibility Testing Standards

George Charpy, a noted French chemical engineer of the late 1800's, wrote that "development and analysis of materials testing of any future agency should establish any new material as judged only by performance tests . . . give a meticulous and precise description of all tests; avoid all vagueness that permits or necessitates any arbitrary decisions on the part of the personnel doing the testing; specify the relation between the precision of the testing and the precision required in the results of the tests; and state how the data are to be recorded" [15].

To solve the testing conundrum, the NIDR and Food and Drug Association (FDA) agencies were required to identify biological compatibility and safety of pre-1948 as well as new dental restorative materials. To provide a reasonable answer to the safety issue of toxicity, the NIDR and FDA committees created a "grandfather clause" that granted acceptable use of all pre-1948 dental restoratives that had been in clinical use since the emergence of US dentistry in the late-1700's and for new U. S. Class I and Class II materials and devices.

In the 1950's, there were no operational biocompatibility research guidelines that existed. The question was: what should be the NIDR recommended standards to use on all new dental agents destined for clinical use? Since there were hundreds of historical dental agents, an almost impossible research task existed to rapidly test the biocompatibility of agents that had been around for 150-years-let alone new agents. If In their group decision, the committee's supposition was that if the pre-1948 agents had been in clinical use and clinically acceptable, then they must be biologically satisfactory. Consequently, 1948 was the starting point for biocompatibility testing-any agent developed after 1948 would be subject to new testing standards [16]. And if the mechanical properties of new agents were equal to or better than pre-1948 products, they were then submitted to an independent research laboratory for biological testing.

Many of today's clinical restoratives and devices have successfully passed the 1st test gate-an *in-vitro* cell culture biocompatibility test against positive and negative controls. The 2nd test gate for the restorative agent is submitted for *in-vivo* biological evaluation by implanting the agent into the soft tissues of small animals (rat, mouse, and rabbit). If successful, the agent is next submitted to a 3rd *in-vivo* usage test into facial cavities of non-human primate teeth at 3-time periods to evaluate the histologic response in non-exposed and exposed Class-V-cavities. Following compliance through the 3-test gates, it is then submitted for the human clinical testing under defined conditions [17].

Material Sensitivity Causing Irritation

Several commercial dental technic laboratories may use nickel-sensitizing metals to fabricate restoratives, which have a lower cost than noble metals. In addition, some company's produce agents that contain aldehydes, phenols, ketones, which are known to be toxic when evaluated by current FDA and International Organization for Standardization (ISO) *in-vitro* and *in-vivo* biocompatibility tests.

To informed clinicians and patients, the question remains, can FDA, ISO, NIH, NIDCR and the ADA continue to permit sales and use of these agents in humans? It may be explained: most international dental

groups retain their own grandfather clause or they rely on the US 510-K document that permits use of toxic aldehydes, formocresol or phenols-since they are certified as germicidal-even though they dehydrate, burn and also denature vital tissues if not properly applied. Ironically, some of today's dental restoratives contain these and other known carcinogens, even though they were grandfathered before the FDA had developed biological testing standards.

Certain crowns and bridges are fabricated with non-noble nickel or beryllium, which are known to sensitize vital tissues, causing redness and swelling that often leads to chronic itching and a skin rash in the mouth, face, arms, legs and torso of humans. If severe oral sensitization persists, the main clinical recourse is to remove the offending restoration.

As discussed above, pre-1948 NIDR grandfathered agents, are not held accountable by today's biocompatibility standards, even though some would fail the initial *in-vitro* tests if tested today. Biocompatibility data show that cell sensitivity, irritation and toxicity may lead to necrosis when placed on vital tissues. Fortunately for today's patients, most countries follow international harmonized biocompatibility standards for clinical use. For patient safeguard, new products must 1st pass material function and suitability tests-followed by *in-vitro* and *in-vivo* tests and if they harmonize with International tests-they may proceed to the dental marketplace. However, since a new product contains grandfathered glutaraldehyde or phenol agents, they are not held accountable to the same long-term functional and biocompatible standards as a new chemistry, so they may proceed to the commercial marketplace as some component was grandfathered before biological tests were required.

Caveat emptor-buyer beware. When a new restorative agent is brought to your clinical attention-you should request from the company, all Material Safety Data Sheets (MSDS) data, which supports the new agents long-term clinical safety for patient use-otherwise you may inadvertently place a new restorative agent with minimal clinical benefit that may cause irritation and lead to possible failure.

Which Biological Tests Came First? *In-vitro* or *in-vivo*

Origins of the scientific method may be traced to Anton von Leeuwenhoek, who gained notoriety for developing the microscope in the late 1600's. Without a formal doctoral thesis, he was perhaps the 1st to use common sense methodology, wherein he defined questioning steps that led to the development of his own scientific methodology-in part the basis of today's research methodology [18].

Any colleague who plans a research project should understand the scientific method. It involves development of some personal interest, after which they formulate a working hypothesis. They should next formulate a NULL hypothesis-a non-relationship between 2-measurable ideas or observations. Next, they should collect relevant literature to understand what previous research has been carried out-to prevent reinvention of the wheel.

Before computers, research colleagues often spent days in medical and dental libraries to collect notes on methodologies and read publications related to their field of interest. This process was often the most challenging in the development of any study. Today, literature is easily accumulated from the www using only a few key words, with the Information easily gathered in just a few minutes.

In 1926, Souder at the US National Bureau of Standards (NBS) framed specifications for dental amalgam using previous published research by Black [19]. The ADA-established in 1859 and reorganized in 1922-adopted laboratory and clinical testing as their controlled research discipline in 1930, using Charpy's advice to establish the Council of Dental Therapeutics to oversee the evaluation of dental products. Since then, other groups have attempted to provide worldwide harmonization of testing and certification standards. Over 100-countries are members of the 1952 ISO formulary that promotes harmonization of dental materials biocompatibility test standards. It is ironic that even though the US has membership in the ISO, it still holds fast to their FDA regulations, which remain independent of ISO guidelines-at the same time both standards reach similar end results [20].

In 1948, the Federation Dentaire Internationale (FDI est. 1900), The American Association of Dental Schools (AADS est. 1923), The International Association for Dental Research (IADR est. 1920), The Dental Manufacturers of America (DMA est. 1932) and the Food and Drug Administration (FDA est. 1968) each contributing to the development of dental material specifications.

And so, when did dental material biocompatibility testing begin? Dental literature suggests that biocompatibility testing was fostered, by growing interest in poor root canal treatment outcomes. In 1901, Hunter of London UK spoke at McGill University in Canada, where he publically condemned root canal treatment for causing dental infection, septic gastritis and many other systemic infections [21]. His concept was accelerated in 1918 by Billings who promoted the concept of focal infection and necrosis as being caused by septic root canal treatment. Billings claimed root canal treatment was responsible for causing heart disease, arthritis, anemia and most systemic diseases. His recommendation was simply a call for complete extraction of all teeth-as a general cure. That practice provided an economical windfall for many dentists by complete extraction of teeth of young adults, followed by the fabrication of dentures to remedy the issue of decayed teeth [22]. From discussions with aged edentulous adults who lived during the focal infection era, we learned that many patients had their teeth extracted in a "preventive attempt" to avoid systemic infections. In reality, their treatment often left them with poor fitting dentures that were generally no better than the possibility of decayed teeth and periodontal disease, in which they were told they would suffer.

Root Canal Treatment finally Gains Respectability to Advance *in-vivo* testing

Hunter and Billings damned vital pulp capping that led to root canal treatment and repeated clinical visits for additional treatment and ultimately extraction resulted in "bad press" for clinicians attempted such procedures. In 1936 Fish and MacLean, published clinical data, demonstrating that sterile root canal procedures promoted a new era of respectability by clinicians and the general population [23].

Additional research by Gottlieb of the University of Vienna reported on histological biocompatibility reactions in animals, which supported sterile clinical procedures [24]. In 1931, Rickert and Dixon from the US presented their study "Implantation of a hollow polyethylene tube" at the 8th Int. Paris Dent Congress. They filled tubes with restorative agents and placed them in the connective tissues of various laboratory animals and evaluated the histological effects at specific time periods [25]. Their report inspired hundreds of research studies on dental material biocompatibility by Grossman [26], Ingle [27], Hyakusoku [28], Nicholls [29], Rowe [30], Torneck [31], Seltzer et al. [32] and many others [33]. Chapter-13 of the 1975 ADA publication Guide to

Dental Materials and Devices committee-chaired by Stanley-provided 225-references that critically reviewed Endodontic Instruments, Devices and Materials [34]. They reported the "real strength of *in-vivo* studies" provided a dynamic physiological component of a vital milieu of arterial and venous circulation, neurotrophic influences, hormones, enzymes, connective and inflammatory cell modulation, which yet today-remains outside *in-vitro* capabilities.

In-vitro Biocompatibility Research as an Alternative Analysis

A number of *in-vitro* technologies were proposed in the early 1900's to evaluate the use of vital cells in an artificial media. However, *in-vitro* tests were restricted as to what could be placed into the medium to control such variables as serum composition, incubation temperature, ideal pH and agitation.

The 1959 dental literature records that Kawahara et al. from Osaka University published the first *in-vitro* biocompatibility tests on a silver-tin amalgam [35]. Other parallel studies by Maizumi et al. [36], Keresztesi et al. [37], Leirskar et al. [38] and Helgeland et al. [39] reported on the *in-vitro* biocompatibility of dental materials by tissue culture.

Comparing dated timelines of dental materials biocompatibility research-*in-vivo* studies were first reported. However, *in-vitro* research has since increased in its sophistication and model types. Today, many cell lines have been developed for studying dental and medical materials and devices. Differing opinions discuss the importance of using primary cell lines from mice, rats, rabbits or humans. Should we use primary organ cultures, or primary human adult diploid cells with 46-chromosomes, or should we use cell lines with an unusual number of chromosomes?

Silicate cement was the 1st restorative agent to promote *in-vivo* biocompatibility testing. A 1936 *in-vivo* study by Manley was first to report biocompatibility research on silicate, 19-years before Kawahara published his *in-vitro* research [40].

Tooth-colored silicate was 1st used by Thomas Fletcher in 1873 England [41]. However, it never became popular until modified with fluoride as Ascher's Artificial Enamel by Schoenbeck of Germany in 1907 [42]. In 1925, several commercial cements were available to the US dental market; zinc oxide powder used eugenol as its mixing solvent became a popular zinc oxide eugenol (ZnOE) pulp protectant against tooth sensitivity. Silicate and zinc phosphate cements both used phosphoric acid (H_3PO_4) as the liquid solvent. After placement-due to a long gel-set phase-the silicate surface was covered with petrolatum to provide an air-inhibited layer as it passed thru a 24-hour reaction. This allowed the patient to leave the clinic and return after several days for final finishing and polishing [43]. But many patients avoided this return trip, due to added chair time and expense. Unfinished silicates easily stained with tea, coffee, red wine, foods and the cavity margins soon became ditched leaving exposed enamel for easy microleakage into the restorative interface. Non-fluoridated silicate became demonized due the H_3PO_4 etching of the smear layer and tubule plugs-during the 24-hour gel-set stage-allowing fluid flow along the restoration interface and into the open dentine tubules-causing dentine hypersensitivity and patient tooth pain, especially when drinking iced beverages or eating ice-cream [44]. Non-fluoridated silicates permitted bacterial microleakage, leading to recurrent caries that resulted in pulp inflammation and necrosis. Whereas, fluoridated silicates and glass ionomers are known to recharge with fluoride from toothpaste and other agents to prevent recurrent caries [45]. Clinical data show that 50-year old fluro-silicate restorations show no recurrent caries. Clinicians have been reported to state at meetings, "If it isn't broken, leave it alone", That clinical observation speaks volumes for the preventive effect of fluoride-long before todays "new found" clinical outcome analysis-the supposed new research mantra-then again, long-practicing clinicians have said for decades that their clinical outcomes have always been their true reality. Is the issue of clinical outcome analysis really new?

Myths or Misconception? Do Acids Kill the Vital Dental Pulp?

In 1936, Manley was first to publish *in-vivo* pulp biocompatibility data. He placed a number of class V dental restorative materials in dog teeth, reporting that vital pulps restored with silicate, copper-oxyphosphate and amalgam became inflamed and necrotic due to the phosphoric acid solvent. Conversely, he postulated that cavities restored with ZnOE were "pulp protected" as no inflammation was observed [46]. Today's research has shown that eugenol is both sedative and germicidal. Manley's 1936 article was the 1st publication to condemn cements with H_3PO_4 by causing pulp inflammation. His 1943 publication on human teeth planned for orthodontic extraction, showed severe pulp pathology, which he reasoned was due to acid cements-those same patients reported cold hypersensitivity. Even Gurley and van Huysen supported-quite incorrectly-that Manley's concept of toxicity was due to phosphoric acid. Today, Manleys acid theory of pulp death is incorrect [47]. We find it remarkable that in 1927, Crowell-a chemist at the U.S. National Bureau of Standards-speculated that bacteria were responsible to cause pulp inflammation and not H_3PO_4 acid [48]. In 1961, Roydhouse stated "pH is a measure of hydrogen-ion concentration in solutions, it is not suitable for describing some property of solids. . .Presence of a large buffering agent such as the calcium hydroxyapatite of enamel and dentine and the addition of an acid or alkali alters the pH but little, until the buffer is used up. . .since dentine and enamel are great reservoirs of a high pH alkaline buffer, their high pH will rapidly neutralize most acids, above all, a small drop of H_3PO_4, before reaching the pulp. . .The [alkaline] nature of tooth material makes it doubtful that acid could be the sole cause of pulp degeneration beneath unlined silicate restorations" [49].

Meticulous *in-vivo* human tooth pulp studies by Brännström et al. demonstrated-beyond any doubt-that pulp inflammation was due to bacterial infection and not from acid toxicity [50]. The Achilles heel of Manley's study was-he failed to apply controlled bacterial stains to micro-slides of dentine and inflamed pulps. He speculated that the rubber dam provided a complete bacterial barrier during treatment. He was totally unaware that microleakage of bacteria through the restorative interface post placement caused pulp inflammation. Today, well-defined pulp biology usage tests are required by ISO to include properly controlled bacterial stains as part of any *in-vivo* test [51].

In-vivo Biocompatibility Test Models

Implantation tests vary with the animal model. Any dental agent is mixed per directions, immediately placed into sterile polyethylene tubes and implanted into connective tissues of mice, rats or rabbits for prescribed times. Tests generally used control agents, such as polyethylene tubes filled with sterile bone wax or ZnOE agents as per Torneck's model [52]. Reports were mixed as some publications reported that silicate alone caused slight tissue reactions while others

reported more severe tissue responses. Unfortunately, results from different studies were often downplayed or excluded due to obscurities in distinguishing tissue damage from the placement surgery when compared to the various tested agents. Some modifications of surgical tissue implantation have been suggested and actually implemented, but there still remain a number of strong opinions as to just why any one specific test is supposedly more reliable than another-it is difficult to standardize subjective or biased opinions of individual researchers.

Some researchers used gingival tissue responses in an attempt to evaluate any dental material that lies in direct contact to the gingival-agent interface. When measuring the gingival response to amalgam, silicate or resin restorations, their histological responses are often judged as similar. As a consequence, it has been suggested that mechanical surface defects-smoothness, roughness and plaque biofilm-are more responsible for a severe gingival response than the biocompatibility of the dental agent itself.

Hypersensitivity patch tests were used by Bergman et al. in 1977, reporting a number of observations in patients demonstrating severe gingival reactions, ranging from slight erythema to strong vesiculation due to cobalt, nickel and cadmium containing alloys that are used in some pediatric crowns and solders [53]. Many patients today are increasingly becoming subject to nickel hypersensitivity-even more important-hypersensitivity is a greater concern to laboratory technicians and office personnel who are in daily contact with nickel, beryllium and other fine particles. Those patients with crowns containing high beryllium content often report severe hemorrhagic gingiva when in direct contact with the final restoration. The only clinical recourse is to remove the entire restoration and replace it with a non-irritating adhesive composite or ceramic restoration. It is important that clinicians, office personnel, technicians and patients be made aware of the metal composition of the final restoration content. Clinicians should validate that each patient does not have any *in-vivo* hypersensitivity to known metal irritants such as nickel, beryllium or copper.

Evolution of International *in-vivo* Testing Specifications

In the late 1800's, the US National Dental Association Research Institute had focused its effort to support the evaluation of biological and preventive aspects of new dental materials, but their financial support for biological research testing was limited, especially when compared to the large volumes of new data that were being produced on the physical and mechanical aspects of amalgam. After lengthy efforts to publish their amalgam data Souder et al. finally could realize that peer review colleagues accepted their data, which was published in the 1920 Dental Cosmos [54].

In the early 1900's, many dental agents were natural resins, waxes, essential oils, alginates and zinc cements used for dentures. In the last half of the 1900's, dental procedures moved from extractions and dentures towards operative procedures with enamel and dentine interdiffusion of interphase hybridization with adhesive polymers. In a dynamic sense, progress of harmonization testing remains increasingly strategic to developing new standards for biocompatibility testing of restorative products.

In the early 1900's, most researchers were content to use their own *in-vivo* biocompatibility test models-consequently, most *in-vivo* studies were of individual design and not well suited for harmonization, since their test bore little correlation to other *in-vivo* studies. As harmonization progressed, new dental agents were placed in the teeth of several animals of a similar age, tooth-type, maxillary and mandibular quadrants and compared against 2-controls. In early *in-vivo* pulp biology studies, silicate and ZnOE were selected to serve as positive and negative biological controls with 3-specific placement intervals (short, intermediate and long-term) with new agent histology compared to at least 2-unknown double-blind controls.

Early peer reviewed pulp publications established standards, which most organizations now follow. *In-vivo* primate placement in class-V cavities became the accepted 3rd biocompatibility test-gate with the particular agents placed under the same clinical conditions as in humans [55-64].

By 1978, the ADA published the 8th edition of Guidelines to Dental Materials and Devices "Recommended Standard Practices for the Biological Evaluation of Dental Materials", which were intended guidelines for the testing of new products [64]. With the great rush of new adhesive restorative systems-it should be noted that phenol, acetone, ethyl alcohol, formocresol and glutaraldehyde are toxic and mutagenic in various concentrations and in time of direct contact. They had been grandfathered in the 1948 mandate. If glutaraldehyde were brought to the dental marketplace as a new dental agent, it would fail to pass the 1st *in-vitro* biocompatibility test-gate. Thus glutaraldehyde would not be approved to advance to the 2nd *in-vivo* or 3rd primate usage test-gate and to the 4th human clinical test path.

In 1978, the FDI adopted Technical Commission Report #204 "Recommended Standard Practices for Biological Evaluation of Dental Materials" compiled by the FDI working group. It contained hundreds of pages of laboratory tests proscribing the biological evaluation of dental materials. That document was recognized as the 1st modest attempt to establish uniform testing procedures-adopted "with the intention that any particular dental material should not be subjected to all suggested tests. . .the manufacturer could select the most important test they felt appropriate". The reader is referred to recent ADA / FDI / ISO documents for the most current revisions that are found on the web [65].

In 1997, ISO published their 2nd edition of: 10993-1 Biological Evaluation of Medical Devices, composed of 16-detailed sections [66]. It is a revised document, by which all member bodies-composed of manufacturers, academics, researchers-had met over several years, in an attempt to harmonize the many different standards from various countries such as Germany, England, New Zealand, Australia, Japan, US and many other countries. That final document is a detailed compilation of hundreds of pages that cover general as well as specific detailed biocompatibility tests that must be followed, before the agent passes to the next challenge. Paradoxically, even though the U.S. is an active member of the ISO Harmonization Group process and has even hosted several ISO meetings, the U.S. still remains independent in many of the ISO harmonizing tests.

When any dental product is found to comply with final official ADA specifications, then the commercial name may be placed on the List of Certified Dental Materials and Devices. Following such an announcement, the manufacturer may then use the seal in their promotional materials and labeling that the product complies with official certification and to allow them to display the ADA Seal of Certification on their product.

From the 1950's until now, researchers have added their own personal modifications to different *in-vitro* tests. One of the earliest *in-vitro* tests was the hanging drop method, which could be carried out

quite economically on glass slides. But, without coordinating harmonization, some countries began to promote their own *in-vitro* test, each of which had its own supposed particular advantage and possible disadvantage. The challenge during the 1960's was that not any one international group had taken a firm leadership role, as many countries were developing and promoting their own biological testing standards.

Are We There Yet? Has Biocompatibility Harmonization Been Achieved?

IADR Pulp Biology Group (PBG) records show that Cotton [67] was the driving force to standardize Biological Testing Standards. Cotton was Chairman of the U.S. Naval Medical Research Institute Dental Sciences Department in Bethesda MD-his foresight and leadership at the 1975 IADR meeting in London culminated in formalization of the IADR PBG. As the PBG Charter President, Cotton immediately brought together a diverse group of dental researchers- Kawahara from Japan; Tronstad from Sweden; Avery, Langeland and Spångberg from U.S.; Klötzer from Germany; and other world colleagues. In just 2-years, Cotton organized the 1st international PBG Symposium on Methodology and Criteria in the Evaluation of Biologic Effects of Dental Materials; held at the 1977 IADR meeting in Copenhagen Denmark [67]. At that International Symposium, Cotton challenged the PBG membership to read the Presidents Science advisory committee: B-4 Recommendation [68]. It stated that "full but critical use should be made of safety and efficacy data from other countries when the data tended to support, as well as when they tend to negate a conclusion of either safety-in-use or efficacy". Cotton's closing comments were: "We must not be frozen into standard protocols, likewise our minds must not be frozen to old and outdated ideas on testing . . . Adaptability and change are paramount." IADR-PBG records detail the strong leadership of Cotton [69]. However, it is ironic that the IADR PBG Group-the brainchild of Cotton-has never recognized Cotton with their annual Pulp Biology Award for his many accomplishments that paved the road for ISO standardization guidelines-perhaps he will receive that recognition someday a largely overdue honor that is more than justly deserved.

The Biocompatibility Testing Stage was Finally Established

Kaare Langeland-a charter member and driving force of the young PBG-stated "Until passage of the Medical Device Bill by the U.S. Congress in 1976 . . . testing of biologic effects of dental materials . . . has not been a serious effort". Langeland noted "the U.S. ADA and the National Bureau of Standards (NBS) had directed most of its resources on physical properties . . . no similar support has existed in the biologic field". Langeland pointed out that "the relevant NIH section . . . was pure and free of any speaker on the biologic properties of the involved materials . . . the great majority . . . 90% were old known investigators of physical properties". Langland's [35] final comments remain prophetic even today when he stated "biological testing is extremely expensive, particularly the full scale usage tests . . . the purpose of my presentation is to lead into a discussion of possible correlation present available screening tests and usage tests for local toxicity". Today, almost 4-decades after Langland's [35] challenge, his remarks ring clear-have we finally settled the harmonization concerns of biocompatibility testing?

The 1977 Pulp Biology Symposium: Materials Screening Standards

Leif Tronstad reported that a number of *in-vitro* tissue culture tests to assess material cytotoxicity had been developed by various colleagues in just one decade. Even though some meaningful *in-vitro* data had been obtained, many tests were time-consuming, expensive and needed elaborate equipment that tended to be difficult for many laboratories to acquire. By 1969, L. Spångberg had developed a radiolabelled chromium release method with the supposed advantage by which various stages of mix of any agent could be tested [70]. However due to strict governmental regulations, many laboratories remain inadequately equipped for proper handling and their disposal of radioactive agents. In 1977 J. Autian described an agar overlay tissue culture test, which depended on the capacity of the test material to diffuse through an *in-vitro* agar medium to supposedly exert its toxic influence in the system [71]. In 1979, Tronstad L et al. proposed improvements to the *in-vitro* Millipore filter test method that was designed to assess and rank the toxicity of the tested material against controls [72].

Following PBG committee meetings that had evaluated the development of different tests-they suggested "*in-vitro* tests of meaning" should depend on direct cell-to-material contact with an intervening agent. The PBG working group settled upon a simple Millipore filter test method with minimal testing devices and equipment and more important with cell-to-material contact having been established with Millipore filters. An elaborate human epithelial (HeLa) cell test was established as a monolayer on Millipore filter discs of 0.45 to 8μm dimensions, incubated and immediately placed on an agar plate with the filter disc on top. The dental material to be tested was mixed per directions and immediately placed into a sterile glass ring. After setting had occurred, it was placed on the filter-agar overlay and incubated for 2-hours, after which the glass test rings with attached cells were further incubated for several hours in oxidative enzyme systems to assess for cell damage to the monolayer cells using specific stains. Zones of enzyme inhibition for each tested agent were observed to measure the cell inhibition toxicity of the agent and compared to the suggested positive and negative control agents.

Current *in-vitro* Cytotoxicity Biocompatibility Tests: 2009

Since 1999, the ISO working committees (BS EN ISO–10993-5) for the Biological Evaluation of Medical Devices have met each year and reviewed data to create realistic harmonization codes for the testing of any new dental device or material and to update the 10993-5:1999 document. That revision was published in July 2009-a hardcopy is available to ISO members for £ 85.00 and £170.00 for non-members that consists of 20-parts under the general title of Biological evaluation of medical devices-Part-5 deals specifically to any Tests for *in-vitro* cytotoxicity. Since Professor Kawahara's 1955 publication, many models have been proposed, the committee has noted that due to the general applicability of *in-vitro* cytotoxicity tests and their widespread use in evaluating a rather large range of materials and devices, the real purpose of the ISO-10993-5 is only to provide a roadmap or scheme for *in-vitro* testing, which requires decisions to be made in a series of steps-rather than to specify any one particular test. In this way, the selection process should lead the company and testing organization to the most appropriate test for their new device or agent.

The committee identified 3-tests: an extract test; a direct contact test and an indirect contact test. The choice of any one or more of these 3-depends upon the nature of the sample that will be evaluated, the potential site of material or device placement in the organism and the nature of the use of the same. The intention of the ISO 109993-5:2009 is to leave open the choice of the test type, allowing the strategy to make available a battery of tests, which reflects the approach of many organizations or groups that suggest or require *in-vitro* tests. Once decided, the details of material or device preparation; the preparation of the cultured cells and the manner in which the cells are exposed to the samples or extracts are then determined. At the end of the exposure test, the evaluation of the presence and extent of the *in-vitro* cytotoxic effect is determined.

The 10993-5:2009 committee redefined *in-vitro* cytotoxicity test methods, so endpoints could be grouped into the following categories of testing evaluation. 1) Morphological assessment of cell damage. 2) Measurements of cell damage. 3) Measurements of cell growth. 4) Measurements of specific aspects of cellular metabolism. An investigator must realize that there are several means of producing results in each of these 4-categories and so they must be aware how the tests fall into certain categories as well as to employ both positive and negative agents for test controls. In addition, the investigators should also compare their material/device to similar products. For guidelines of quantitative test protocols, the ISO-10993-5: 2009 committee has also provided 24-annexes for guidance of interpretation of the results [73].

Even though the ISO committees have been diligent to reconcile biocompatibility-testing programs since the 1970's, there is still not any single comprehensive *in-vitro* test that mimics the actual *in-vivo* conditions found in the mouth. Brännström demonstrated-the elephant in the room-the dynamic dimension of bacterial microleakage plays the most prominent *in-vivo* role that is difficult to mimic *in-vitro* [74]. Consequently, an elusive correlation remains between *in-vitro* and *in-vivo* tests and the search to more closely simulate *in-vivo* circumstances are still advocated. As Cotton [67] stated in 1977 "Adaptability and change are paramount".

Is There a Definitive *In-Vitro* Biocompatibility Test Model?

If you have followed our chronological timeline of *in-vitro* versus *in-vivo* testing, you may realize that no simple or easily reproducible answer has yet presented itself-a proper scientific answer for biocompatibility testing is simply not easily reported by a simple yes or no response.

A number of *in-vivo* animal and human usage studies Kakahashi et al.[75], Brännström et al. [76], Bergenholtz [77,78] and Cox et al. [79-81] have demonstrated-beyond a doubt-that certain bacteria and their toxins can easily infiltrate into enamel lamella and restorative channels and rapidly penetrate down to the enamel-dentine-junction (EDJ). From this point, the bacteria and toxins can easily spread along the EDJ interface, where they penetrate into and through the dentine tubule complex. From this point, they may initiate a low-grade irritating response to the primary odontoblasts, in which the primary odontoblasts may respond by depositing a thin layer of reactionary dentine. However, if the insult persists as a chronic effect, the primary odontoblasts often die-whereby certain undifferentiated cells of the pulp proliferate to the dentine interface to form new odontoblastoid (like) cells, which rapidly proceed to deposit a new layer of reparative dentine directly adjacent to the bulk of the tooth's secondary dentine. However, if the bacterial insult is persistent, it may easily cause pulp inflammation and regional pulp necrosis occurs (Van Hassell) [82]. Such dynamic factors of microleakage and bacteria are extremely difficult to control and even more so, bacterial factors are difficult-if not impossible-to simulate in typical *in-vitro* bench top studies-the ISO committee is still waiting for such well controlled *in-vitro* tests to be repeated by "certified research laboratories".

Reflect on the following research data to assist your own interpretation: 1) *In-vivo* research data were first published in 1936 by Manley-implicating the H_3PO_4 component of silicate cement as the toxic factor to vital canine dental pulps. 2) It wasn't until 1955 that Kawahara's *in-vitro* tissue culture tests were published on silver-tin dental amalgam. 3) In 1968, Brännström demonstrated in controlled human studies-that silicate cement was non-toxic to vital pulp cells-bacteria were the causative agents for caries and pulp necrosis through microleakage along the restorative interface. 4) In 1977-after several years of personal travail with pulp biology research colleagues- Cotton [67] organized the 1st IADR PBG Symposium in Copenhagen, which strongly advocated for the creation of harmonized biocompatibility standards- Cotton was 1st to move towards harmonization of biocompatibility testing. 5) After years of meetings, the ISO committee published their 1st Book of Harmonized Biocompatibility testing standards in 1999. 6) In 2009, the ISO committee published revised testing standards that serve as suggested standards, which may be chosen for evaluation by the "developing group" for biocompatibility validation.

Are Toxic Agents Present in Todays Restorative Agents?

Yes. The facts remain-there are still certain toxic agents, which are minor and major components of restorative dental systems that range from irritating-to-mutagenic-to-carcinogenic and toxic when they contact vital tissues. For instance, research publications report that the toxic chemistry of certain polymers, aldehydes, formocresol, hydroxy ethyl methacrylate and bis-phenyl-A are known irritants, which range from mild-to-toxic with International test standards [83-87].

It remains somewhat curious that the infamous grandfather clause-since 1948-continues to permit many of the known irritational and toxic agents that are a minor component of "newly developed" dental restoratives. In addition to the various chemicals certain nickel, cadmium, zinc, copper metals are commonly found in many cosmetics, are also known to cause sensitizing reactions in humans. In 1991, Lucas discussed various degrees and side effects of biodegradation of dental metals [88]. Again, even with the continued ISO acceptance of these grandfathered irritating dental products-many worldwide dental agencies continue to permit their clinical use in humans-even though some are classified as germicidal [89]. Additional studies have documented that high concentrations of alcohols, aldehydes and acetone may rapidly dehydrate and denature vital cells, collagen fibers and other proteins especially when improperly placed by the clinician. Today, the dental marketplace continues to sell restoratives that contain glutaraldehyde, phenol and lead that are potentially toxic, not only to the patient but also more importantly to those office personnel who work in the clinic environment on a daily basis.

Can Researchers Have it Both Ways by Choosing Their Favorite *in-vitro* Test?

Reaching this point in the manuscript, we realize that the ISO–10993-5-2009 committee *in-vitro* tests recommendations are only suggestions. They serve as the first biocompatibility "test gate", especially when a new dental restorative product or device is developed for use in the oral cavity. Once the developing agency has passed their own personally chosen *in-vitro* biocompatibility test-they can then move to the next *in-vivo* biocompatibility-testing gate-again an animal system of their choice. Animal tests involve the surgical placement of the agent into the connective tissues of the tissues of rats or rabbits for certain periods of time along with positive and negative control agents such as ZnOE, silicate or ZnPh cements for evaluation of their histological responses.

Still, we are now confronted by the fact that initial *in-vitro* tests are not completely reliable to define possible false-positive or false-negative outcomes. For instance, if you challenge the *in-vitro* research laboratories who adhere to their "own suggested" *in-vitro* test and ask if they routinely employ known toxic control agents e.g. glutaraldehyde in their system-they may respond that they don't concern themselves with controls-since such agents (glutaraldehyde, phenol, acetone) have long been grandfathered as pre-1948 accepted dental agents. If you ask those same *in-vitro* researchers who sit on today's Pulp Biology committees to discuss and define testing models and challenge them with the critical issue of the biostability of the restorative interface and the bacterial microleakage and infection in their own *in-vitro* system, they quickly respond they "are only required to follow the suggested BS EN ISO–10993-5 standards. It seems contradictory that some of the same individuals who may sit on various ISO committees, can also define standards. Isn't it ironic that some researchers can have it both ways?

Does an Ideal Alternative *in-vitro* Biocompatibility Tests Exist?

So far, we have reviewed what are important issues of biocompatibility testing. So let us present possible solutions to what we see as a testing conundrum. Initially, any pulp biology research group should demonstrate more than a proficient knowledge of enamel, dentine, pulp and cementum tissues. More importantly, they must also recognize the dynamic biostability and bacteriometic seal dimension of oral bacteria and their toxins, which easily penetrate via microleakage from the tooth surface and through the restoration interface to the vital dentine and pulp.

Today, a great deal of research is focused on the mechanical bond strength testing of new restorative agents when submitted for clinical use. Something to consider, Nakabayashi reported that the general breaking (cohesive) capacity of normal human dentine is approximately 21-megapascals (MPa) [89,90]. On the other hand, the calculated modulus of enamel is up to 155 GPa and the thermal expansion is up to 2.3 X 10 [91]. In 1956, Craig et al. demonstrated that enamel and dentine have regional density extremes, which they demonstrated by Knoop indentation tests [92]. Ten Cate clearly demonstrated that enamel, dentine and pulp tissues are variable substrates in their developmental, morphological, physiological and aging phases-he stated that today's researchers should be responsible to be fully knowledgeable concerning all tooth substrates and to demonstrate proficiency in the test model before they begin their research studies [93].

Where Might We Focus New Research Issues?

As one searches the web to identify *in-vivo* and *in-vitro* studies of dental materials-it is clear that *in-vitro* publications far exceed *in-vivo* studies. We learned through our own decades of research experience-a well-defined and meticulous ISO *in-vivo* primate usage study takes several years of continuous effort-from inception to completion, as well as a strong funding base. In contrast, *in-vitro* tests are less time consuming and can be much less expensive to complete. As future ISO committees move towards harmonization of biocompatibility testing, they should consider to move beyond bond strength testing and to explore the development of tests that merge the more important biological issue of the biostability of a long-term bacteriometic seal-from both a biological as well as a complete mechanically "sealed" interface.

What Is a Bacteriometic Seal?

The term hermetic is from the blending of 2-ancient legends-the Greek god Hermes and the Egyptian god Thoth. Combined, they became personified as the mythological alchemist-Hermes-Trismegistus who allegedly possessed the magic ability to seal Pandora's treasure chest so no person or agent could gain entry to the contents without magical access. Perhaps Hermes was the first to understand the practical idea of "the seal is the deal". Century's later, in the Middle Ages and Renaissance eras, alchemists realized that they needed an airtight seal to improve the efficiency of their alchemical apparatus to condense and separate their novel mixtures into supposed magical potions. The 1939 JDR publication by Grossman was first to use the term HERMETIC seal of dental restorations [94]. From that usage, Ruby was first to think through and apply the importance of a bacteriometic seal in his cariology lectures with students and colleagues. Ruby's creation of the term bacteriometic seal brought together a unique meld of knowledge that stresses the importance of long-lasting biostability of a clinical seal against bacteria and their biological penalties-to maintain enduring pulp vitality against microleakage [95].

Is There a Connection Between Microleakage and Biocompatibility?

Koch wrote that Marcellus of Rome was credited as the first person to remove caries from a human tooth with a scalpel and to fill the cavity with gum mastic and ground pearl [96]. Fauchard et al. wrote that they would scoop debris out of cavities without any particular requirements and then fill the space with thin gold foil mats, which they condensed with a mallet. Gold was the more popular and successful filling agent than lead or tin foil-the latter 2-easily oxidized and stained the tooth tissues black [97].

American dentists from the mid 1700's through the early 1900's used gold foil as a clinically popular restorative agent that was condensed into prepared cavities, however gold was barely affordable to individuals who couldn't afford the expense. On the other hand, amalgam was more economical and the time devoted to its clinical placement was much less. The academics of the 1800's had no concept of material biocompatibility, however a few clinicians like Black [98] and Miller [99] were beginning to understand that the flora of the oral cavity played a direct relationship to cause caries. Clinicians like Webb were aware that the durability of the cavosurface seal along the interfacial margin was of primary importance for long-term clinical success [100]. Koch wrote, "the dental profession of the US may have

been conceived of in the rural village of Greenfield Ohio, but its parturition took place in Maryland." [101]. Harris [102] from Greenfield, Ohio along with Horace Hayden were initial forces for several dental firsts. They organized the first university accredited dental school at Baltimore MD; they organized the first American Journal of Dental Science Journal and they organized the first US Dental Society. Harris's 1855 US text Principles and Practice of Dental Surgery was the first to provide clinical dentistry with scientific documentation as to what had previously been considered an art form-his chapters on cavity preparation and filling materials are incredibly erudite for the young dental profession in the US of that era [102].

The Cavosurface Margin: It Leaks

Koch's 1910 text was apparently the first to comment "by 1855 all of the amalgam restorations made in America through a long period of years were leaky" [103]. It had become apparent that many clinicians were observing failed amalgams, which from their introduction to the US, commercial amalgams had no formulation standards. By the mid 1800's, due to the high cost of gold and the time required for proper clinical gold foil placement, silver amalgam had become the most common restorative agent for the general population. Certain clinicians began to realize that amalgams had specific failure issues-the most obvious were surface failure and fracture along its cavosurface interface. In 1861, Sir John Tomes of England reported that 6 out of 7 silver-tin amalgam restorations had shrunk-but his one low-copper amalgam remained sealed [104]. In 1895, Black realized that companies needed to produce a standard amalgam formulation, which would provide long-term clinical stability and prevent leakage along the restorative interface. Most pre-Black amalgam outcomes by the general clinicians demonstrated recurrent decay, pulp pathology and necrosis, which generally resulted in extraction. Black's research demonstrated that a steady application of 25-pounds pressure onto the amalgam surface caused it to flow and after several months the amalgam slowly spread and flattened without fracture or breaking from constant chewing pressure [105]. In 2005, J.W. Osborne validated Blacks 1895 amalgam flow data-demonstrating that creep was the apparent mechanism that self-sealed the more modern silver amalgams against microleagage-his data showed little to no corrosion products when placed in water or allowed to dry on the bench top [104].

In 1976, Kidd defined microleakage as the passage of bacteria, fluids, molecules or ions along the interface of a dental restoration and the wall of the cavity preparation [105]. In 1983, Lin demonstrated that low-copper amalgam had a greater potential for corrosion-ultimately reducing microleakage along the restorative interface [106]. Since the 1940's, dental amalgam has been manufactured under rigid standards and instructed to triturate and insert under proper clinical manipulation. Miles Markley demonstrated that with proper mixing and placement, amalgam restorations would last for many decades [45]. However, due to an almost phenomenological hyperbole regarding mercury toxicity and the rapid emergence of glass ionomers and adhesive restorations since the 1970's, caused a decline in amalgam placement. Even more ironic, it seems incredible-in light of today's adhesive technologies-that some dental schools are not teaching Fusuyama's concepts of minimal intervention, cavity design and restoration [107]. We think that G.V. Black would consider this as academic shortsightedness-failure to grasp the obvious.

Following Sir John Tomes observations on leaky cavosurface margins in the late 1800"s, Ames of Chicago delivered a paper stating; "If hand amalgamation was complete and no free copper remained after mixing, dentine would not stain-more importantly, it was realized as a permanent antiseptic to prevent recurrent decay after placement of the definitive restoration" [108]. Several U.S dentists published their clinical observations, that certain filling materials were able to create a bacterial barrier to the underlying dentine and vital pulp [109-113]. As we have discussed, Grossman [94] was first to write "the necessity for having a hermetic seal during root canal treatment is obvious". The question remains-is there a difference between a physical-mechanical seal and a bacterial-tight seal?

The Dynamic Nature of Microleakage

In 1947, Bartlestone demonstrated that open enamel channels readily permitted microleakage of radiolabelled agents from the oral surface into the vital pulp and vascular system within 15-minutes [114]. A few years later Nelson et al. used various research methods (e.g. dyes, stains, bacteria, radioactive isotopes and air pressure) to suggest that the restorative-enamel-dentine interface is an impermeable interface [115]. Research has shown that enamel has small lamella passageways that easily permit the seepage of oral fluids into the pulp. Going et al. reported that the extent of penetration depends on many factors e.g. molecular size, the cavity type, the seal of the restorative agent, the thermal environment between the cavity and restorative agent and the nature of the remaining smear debris layer after the tooth preparation is completed [116].

A 1939 study by Grossman [94] evaluated the marginal seal of 10-temporary filling restorative agents. One series of his tests used dyes, repeated over a maximum of 24-times. Of the 10-restorative agents, 6-showed definite dye microleakage and 1-gave variable results. Another series of dye and saliva tests showed 6-agents allowed no leakage, while 4-gave variable results. Grossman et al. speculated that the large molecular size of salivary proteins prevented dye passage through the restoration interface. His 2nd test series used Bacillus prodigious microörganisms to detect bacterial microleakage. Without exception, ZnOE was completely leak proof in all tests-much to Grossmans admitted surprise [117].

The issue of a bacteriometic seal against microleakage is now understood as a physical-mechanical barrier that forms a completely stable interfiffusion zone-1st reported in enamel by Gwinnett et al. [118]. In 1982, Nakabayashi demonstrated a stable hybridized layer of adhesive that had penetrated into the etched intertubular dentine [119]. Studies by Brännström, Bergenholtz demonstrated that microleakage of bacteria are the prime cause of pulp inflammation, due to failure of the restorative agent to provide a bacteriometic seal along the entire restorative interface [120]. Their studies appear to explain the absence of bacteria in Grossman's 1938 study, which evaluated eugenol. Today studies have shown that eugenol is bactericidal and so, it is excluded by the pre-grandfathered test-gate.

Cox et al. [80] suggested that the real issues of dental material biocompatibility were divided between material toxicity (e.g. gluteraldehyde) versus the long-term biostability of the hybridized interface to exclude the invasion of bacterial via microleakage. We suggest you read the classic studies by Manley, Zander, Massler, Langeland, with particular notice of the erudite publication by Kakahashi. *In-vivo* human studies by Brännström demonstrated that microleakage of bacterial factors through the restorative interface are the prime cause of pulp infection and necrosis [80].

Requisites for a Bacteriometic Seal

A series of clinical studies by Nyborg [121] reported that restoration of cavities with various calcium hydroxide Ca $(OH)_2$ agents was followed by a short period of pulp healing without any reported patient sensitivity. However, after several months placement, he observed pulp inflammation and necrosis in substantial numbers of his clinical cases, showing stained bacteria in the empty Ca $(OH)_2$ space-a consequence of microleakage. This is one of many clinical studies that cast doubt on the ability of agents to sustain a stable bacteriometic seal against microleakage and sustain long-term pulp vitality. Nyborg stated: "restorative procedures must be based upon long-term studies, supplemented by histo·pathological assessment" [121]. Barnes et al. reported 1-year clinical observations from their university patient records, which noted the loss of human pulp vitality-due to the loss of the Dycal™ base under amalgam restorations. They described the loss as "Disappearing Dycal™"-the consequence of microleakage along the unsealed restoration interface that simply permitted the Dycal™ "pulp protection" agent to dissolve over the year of placement and exit the restorative interface. As the Dycal™ base dissolved, opportunistic bacteria easily invaded along the restoration interface to recolonize and form a cariogenic biofilm that resulted in recurrent caries and pulp inflammation-in the absence of the Dycal™ "pulp protection" [122].

As a result of the Nyborg and Barnes articles, our pulp biology research group defined several *in-vivo* studies. The 1st study-published in 1982-was a 5-week vital pulp study in non-human primates. Following ISO guidelines, vital pulps were exposed at several intervals and direct pulp capped with Dycal™. Evaluation of serial-sectioned microslides showed pulp healing, reorganization of soft tissue and new dentine bridge formation directly adjacent to the Dycal™ at the exposure site in 99 of 120-serial sectioned teeth [123].

The second study was a 1 and 2-year proposal that combining histological and microbiological data, that demonstrated 86% of the pulps had initially formed new dentine bridges in 67% of the exposed and direct Dycal™ capped pulps. However, the dentine bridges showed tunnel defects through the entire bridge with stained Gram positive and negative microörganisms and recurring pulp inflammation that had invaded through the tunnels of the dentine bridges. Microbiological cultures of both aerobic and anaerobic mocroörganisms from the empty space-previously occupied by the $CaOH_2$ Dycal™ base-below the amalgam was due to the failed bacteriometic seal of the amalgams [124].

As we have shown, we realize that *in-vitro* tests are not refined enough to evaluate the same variables of an *in-vivo* study. In the consideration of a bacteriometic seal, the research colleague must knowledgeable of the 3-mineralized tissues and the non-mineralized vital pulp tissue. In order to prevent failure of any dental restoration we need to create a long-term bacteriometic seal along the entire restoration interface and its long-term ability to prevent bacteria and their toxins to penetrate into the vital pulp, where they easily cause inflammation and necrosis.

Remaining Challenges

Where will the biological testing future proceed? We have endeavored to present all of the available literature to develop our biological testing timeline of restorative materials-the beginnings are traced from Kinyoun of the Marine hospital of Ellis Island of 1779. *In-vivo* tests were first reported by Manley in 1936 [46]-followed by an *in-vitro* report of Kawahara in 1959 [36-38]. Since then, the scientific community has refined biological testing models to demonstrate that microleakage of microörganisms and their products through the restorative interface are of greater damage to vital pulp tissues than the pH of restorative agents [74-81]. Improved *in-vitro* tests have evolved to incorporate a dentine chamber that attempts to mimic the vital tooth, but lacks the complete physiological milieu of the vital dentine and tooth substrates. It remains for future research colleagues to develop more sophisticated biological tests that are within realistic limits of reproducibility. As Charpy wrote "development, analysis of materials testing of any future agency should establish any new material as judged only by performance tests . . . give a meticulous and precise description of all tests; avoid all vagueness that permits or obliges any arbitrary decisions on the part of the personnel doing the testing; specify the relation between the precision of the testing and the precision required in the results of the tests; and state how the data are to be recorded" [15]. As Cotton stated "We must not be frozen into standard protocols, likewise our minds must not be frozen to old and outdated ideas on testing . . . Adaptability and change are paramount". As Ruby has noted "the bacteriometic seal is the deal" [95].

Acknowledgements

Each author owes a collective note of thanks to all of our academic and clinical colleagues who have assisted and supported us in our formative research years and later throughout our chosen academic careers.

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