Safety Issues Associated With Platelet-Rich Fibrin Method

To the Editor:

In a recent series of articles[1-5] in the March 2006 issue of *OOOOE*, Dohan et al. describe a new autologous "platelet-rich fibrin concentrate (PRF): a second-generation platelet concentrate." On page e47, a "10-mL glass-coated plastic" blood collection tube is listed as an integral part of blood collection kit supplied by the Process company (Nice, France), and a picture of this tube is shown on page e41 in Fig. 3, *B*. I am formally communicating to you a very serious health hazard with the clinical use of plastic evacuated blood collection tubes with silica activators as advocated by the Dohan et al. articles.

These products, proposed by Dohan et al. for use in producing clinical therapeutics from the preparation of a platelet-rich fibrin matrix by the concurrent centrifugation and coagulation of a patient's blood, are designed and manufactured by Becton Dickinson for diagnostic use only. I wish to alert you and your readers that the practice, as described in these articles and through the sale of special "kits" through associated distributors, constitutes a severe safety and efficacy hazard to the patient for the following reasons:

1. Noncompliance to ISO 10993. This standard is generally used for biocompatibility of blood containers for clinical use and includes studies for cytotoxicity, mutagenicity, dermal irritation, hemolysis, and other appropriate parameters. The product proposed for use by Dohan et al. is an in vitro diagnostic product (IVDP) and not qualified for clinical use under ISO 10993.

2. Material hazard. The MSDS sheet from Becton Dickinson's web site (http://catalog.bd.com/ecat/msds/d01/vs60324-10.pdf) clearly states that the contents of the tube are an irritant and should not be allowed to contact human tissue.

3. Certainty of silica contact in the patient. The silica particles used in the BD product, although dense enough to sediment with the red blood cells, are sufficiently small for a fraction to remain in colloidal suspension in the buffy coat, fibrin, and platelet-poor plasma layers and will thus contaminate any therapeutic application to the patient.

4. Open-system architecture. The product's use as described in the papers is open to the environment and requires several manual manipulations to obtain the desired materials. This open system significantly adds to the potential of microbial and chemical contamination of the materials before application to the patient's wound site.

I have instructed our company's medical, legal, and regulatory staff to contact all distributors and to alert all of the authors of the articles to the serious hazards posed by the use of the IVDP product as proposed in these articles.

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Cytotoxicity analyses of Choukroun's platelet-rich fibrin (PRF) on a wide range of human cells: The answer to a commercial controversy

To the Editor:

Several important issues have been questioned following a reader's mail concerning the type of tubes used to produce platelet-rich fibrin (PRF) and the possible cytotoxicity of silica-releasing tubes (glass-coated plastic tubes) for the recipient organism. In fact, nu-

merous silica-based materials are used in dentistry, including as a direct bone-filling material. The present paper answers the questions step by step, then describes a cytotoxicity study carried on 4 different human cell types (gingival fibroblasts, keratinocytes, preadipocytes, and osteoblasts) placed in contact with PRF membranes for 12 hours, 24 hours, 3 days, and 7 days, respectively, of in vitro culture. Cell metabolic activity was evaluated with succinic dehydrogenase activity, which measures the mitochondrial respiration of cells. For this purpose, the methyltetrazolium (MTT) assay was used. The results show that PRF produced with glass-coated plastic tubes is not cytotoxic for these human cells and for some even seems to improve the mitochondrial respiration.

WHAT IS CHOUKROUN'S PRF?

Platelet-rich fibrin is a second-generation platelet concentrate which allows one to obtain, starting from an anticoagulant-free blood harvest, fibrin membranes enriched with platelets and growth factors. The PRF protocol was described for the first time in 2001 by Dr. Joseph Choukroun et al.[1] The protocol is not linked to a medical device nor to a specific machine: It is a general protocol, a simplified technique, free and openly accessible for all clinicians. It is not a blood-derived product, in contrast to the platelet-rich plasmas (PRPs) and fibrin glues[2]; to produce PRF, blood composition is not modified by the use of bovine thrombin, anticoagulants, or calcium chloride. Polymerization of PRF is performed according to a completely natural process, without any modifiers. Therefore, it is a totally autologous material prepared extemporaneously, in the same way as a bone harvest (chin, retromandibular line, illiac, parietal) or a palatal connective tissue harvest.

The name "PRF" is protected by a copyright to make sure that our research work on this free and open-access protocol is not distorted by commercial companies interested in using the name.

THE CONTROVERSY AND THE ANSWER

Mr. O'Connell raises an interesting question concerning the type of tubes to be used preferentially to produce PRF. Indeed, in our first international paper on the topic,[3-7] we presented the standard protocol performed in the French dental offices, i.e., using glass-coated plastic tubes. However, the picture illustrated in that paper displayed a dry glass tube. In fact, the technique works with any type of dry glass tube (Terumo® Venoject® 10 mL) or glass-coated plastic tube (Terumo® Venosafe® 10 mL, Becton Dickinson Vacutainer® 10 mL or Greiner® Vacuette® 9 mL).

Platelet-rich fibrin was initially developed in dry glass tubes as early as 2000. In the medical field, the conventional PRF protocol has continued to be performed in these dry glass tubes, such as those available in the French hospitals. Most maxillofacial, ENT, and plastic surgeons, using PRF on a conventional basis, produce their PRF using a Coleman fat centrifuge adapted for this specific indication to standard glass collection tubes (Fig. 1).

Fig. 1. In Paris hospitals, PRF was originally produced using dry glass tubes, which means that these same tubes released silica. If all the glass containers contaminate their content, should we consider that it is a major public health problem? Obviously not.

In a dental office, the problem is different. French dentists are not initially educated to perform blood

harvests. Therefore, the use of plastic tubes has been recommended to avoid tube breaking and contaminations during these quite exceptional handlings in a dental office. However, with plastic tubes alone, PRF can not be obtained. The contact with silica is necessary to start the polymerization process: The silica behaves as a clot activator. To produce PRF, either dry glass tubes (because they obviously answer all the enforced standards) or glass-coated plastic tubes must be used.

The commercially available tubes are generally for diagnostic use only. Therefore, the question is to know whether we are allowed to use such tubes to produce the PRF membranes. We shall therefore answer step by step the remarks questioned.

1. Do the tubes used to procedure PRF have to comply with the ISO 10993 standard? First of all, we must define the reference standard: Is it the ISO 10993-4 or the ISO 10993-5?

The ISO 10993-4 (2002) provides general requirements for evaluating the interactions of medical devices with blood. A classification of medical and dental devices that are intended for use in contact with blood is described, based on the intended use and duration of contact as defined in ISO 10993-1. The basic principles which rule the evaluation of the interaction of devices with blood, the rationale for careful selection of tests according to specific categories, together with the principles and scientific basis of these tests, are defined. Detailed requirements for testing cannot be specified because of limitations in the knowledge and accuracy of tests for blood-interacting devices. In ISO 10993-4 (2002), the biologic evaluation was described in general terms, and therefore this standard may not necessarily provide sufficient guidance for testing methods for a specific device.

If one wishes to use the glass-coated plastic tubes under this standard, the problem is quite simple: It is impossible. The potential sanitary risk just cannot be demonstrated according to this standard. If the harvesting tubes are used for "in vitro use only," it is not because they are dangerous for human health, but just because the manufacturers did not anticipate the use of these tubes for something other than harvesting blood for analyses. Facing this problem of standards and classification, the simplest solution would be to use dry glass tubes; the ISO 10993 standard would then be necessarily respected, and the same PRF produced. However, may glass-coated plastic tubes be used without any sanitary or forensic risk?

The answer to this dilemna may be found in the French regulations. French legislation does not consider PRF to be a transfusion technique for blood-derived products (such as fibrin glues and PRPs), but as an autologous tissue graft (Fig. 2). Therefore, the ISO 10993-4 standard does not have to be applied to the harvesting technique and the PRF production. For security matters, PRF is ranked as an autologous biomaterial, and so we performed, as early as 2000, the relevant cytotoxicity tests based on the 10993-5 standard (1999). This aspect of the standard deals with the biologic evaluation of medical devices and defines tests for in vitro cytotoxicity. However it is important to recall that this standard can not be applied adequately to dental biomaterials, because it is too restrictive.[8] Indeed, numerous biomaterials used in dentistry demonstrate a well established cytotoxicity to various degrees.[9-11]

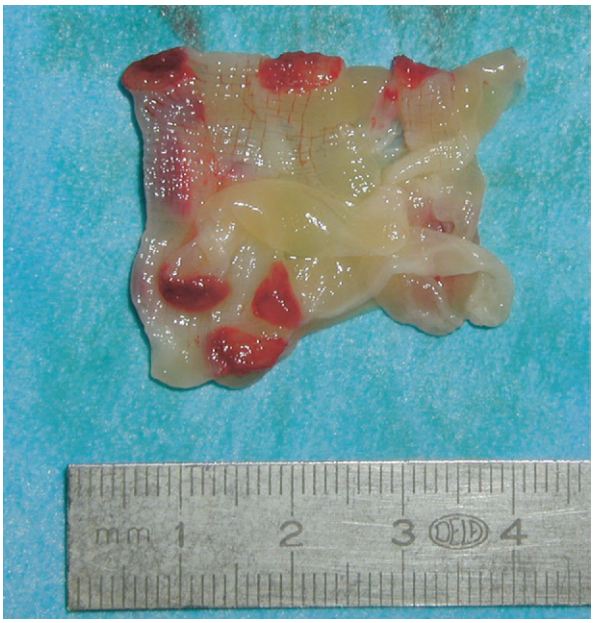

Fig. 2. PRF autologous filling graft for maxillofacial plastic surgery. The use of PRF membranes cannot be considered to be a transfusion technique, in contrast to the fibrin glues and other platelet concentrates. PRF is obviously an autologous graft tissue, without biochemical modification. The ISO 10993 standard can therefore not be adequately applied.

In summary, the ISO 10993 standard can not be adequately applied to PRF.

2. The Becton Dickinson technical file recalls that silica dust (aluminum silicate) is a recognized toxic agent but only when used at high concentrations and inhaled. It specifies as well that no primary irritant effect or sensitization due to silica has been established on the skin or the eyes. The toxic effects of silica (particularly phosphorus or aluminum silicate) have been observed for over a century in populations exposed to high concentrations of silica in the inhaled air (specifically for miners and in some industries).[12-14] The silica particles deposit heavily in the bronchi, inducing a severe intoxication of the weakest cells and

significant cell death. Prolonged exposure leads to overloaded cancer-free lung diseases[15,16] known as silicosis. It is a long-term chronic intoxication.[17-19] Very high levels of silica are required to induce a cell effect detectable in vitro.[20]

In dentistry, silica-based materials are in current use, because they belong to the dental biomaterials that offer the best tolerance with regard to living tissues, specifically bone. It must be recalled that most dental biomaterials are self-allergizing and toxic (resin composites, cements, etc.).[21-23]

3. Theoretically, patients treated with PRF could be placed in contact with silica particles. But this possible contamination by a few microparticles of silica powder does not provide any health hazard.

In dental surgery, silica particles are very often placed in contact with living tissues: All of our ceramics and many of our cement materials (e.g., glass ionomer) contain high levels of silica.[23-25] Indeed, we have numerous situations in which silica is in direct contact with bone and blood cells. During apical resections, glass ionomer is currently used as a filling material.[26] We may also mention the vitroceramic implants: Their surface contains a high level of silica, which allows fibrin nucleation and consequently osseointegration of these implants. In addition, some bone-grafting materials are bioglasses: Novabone Putty BioGlass, PerioGlas, etc. These products are approved by the FDA and are supposed to induce bone stimulation via ionic exchanges with bone cells. They are partially composed of calcium–phosphorus–sodium silicate. According to the companies who commercialize these biomaterials, the phosphorylated silicas are supposed to accelerate bone regeneration.

Finally, all glass tubes release silica. This is proved by the production of PRF using dry glass tubes: With dry plastic tubes, there is no fibrin clot and no PRF can be obtained. Therefore, the dry glass tube releases silica. This observation includes an obvious fact: all glass tubes used in medical devices (or simply to store drugs, such as perfusion phials or flasks) release a minimal quantity of silica, which is hardly detectable on the contacting walls. This small quantity is assimilated to a silica powder deposit which coats the plastic tubes mentioned in the paper. This is the reason why most manufacturers have replaced their dry glass tubes by glass-coated plastic tubes, which are equivalent from a biochemical point of view.

Should we consider that all of the drugs stored in glass tubes are toxic or carcinogenic?

4. Mr. O'Connell criticizes the fact that when collecting the PRF membranes, the harvesting tube is open, and therefore its blood content may be contaminated. This criticism illustrates a lack of knowledge of surgical protocols.

After centrifugation, PRF is fibrin matrix ready for use, and therefore a simple autologous biomaterial in the same way as a bone or an epithelial-connective tissue graft. Of course, one must be careful to avoid contamination, but the risks are the same as for any handling of autologous grafts during surgery. May we consider a chin bone or a palatal connective tissue graft as an "open-system architecture" offering major risks of contamination? Obviously, the answer is no.

In conclusion, we would like to thank Mr. O'Connell for questioning these interesting technical issues in his letter. It allowed us to provide more accurate information. And we are able to provide the cytotoxicity studies carried out on PRF using 4 different cell types, following relevant protocols related to the ISO 10993-5 standard.

CYTOTOXICITY STUDY: MATERIALS AND METHODS

Harvesting and cell lineage preparation

To perform humans cell cultures in the presence of PRF, we had to harvest tissue specimens from patients volunteering to undergo further blood collection for experimental purposes (to produce the PRF required for culture). Indeed, for requirements of immune compatibility, PRF membranes must come from the same donor as the cultivated cells. For this reason, the human harvests were performed on volunteer experimenters, healthy men aged 25 to 60 years. All of them accepted a small tissue harvest during a surgical treatment. A different volunteer was harvested for each tested cell type.

To obtain gingival fibroblasts, a 2-mm^2 gingival specimen was harvested on the alveolar ridge.

To obtain osteoblasts, a mandibular bone harvest was collected.

To obtain preadipocytes, 2 mL of fat tissue, originating from the inner face of the knee, were collected. The knee harvest, rich in slightly differentiated preadipocytes, allows one to obtain fat cells for culture purposes.[27,28]

To obtain keratinocytes, a 2-mm^2 piece of epiderm was collected in the area of the ear.

The explants were carried and stored in DMEM at +4°C, then placed in culture according to the explant technique. After the third confluence passage, the collected cell lineages were trypsinized, then freezed at −80°C.

Cell cultures

For each tested cell type, 8 culture plates (diameter 60 mm) were cultivated (20,000 cells per plate): 4

plates were used as controls (control group), and 4 received a PRF membrane (expressed from its serum), originating from the same donor as the explant (test group). The PRF was produced according to the protocol described above, using glass-coated plastic tubes (Becton Dickinson Vacutainer 10 mL). Then a plate of each group was removed for the MTT test at each experimental time: after 12 hours (H12), 24 hours (H24), 3 days (D3), and 7 days (D7), respectively.

All of the cell cultures were performed conventionally: incubation at 37°C and 5% of CO_2 culture in 4.5 g glucose DMEM (Cambrex ref. 12-709F), to which were added antibiotics (penicillin-streptomycin, Cambrex ref. 17-602F) at 1%, glutamine at 200 mmol/L (Cambrex ref. 17-605F), and fetal calf serum at 10% (Cambrex, ref. 14-801F). The culture medium was changed every 2 or 3 days, according to evaporation.

Testing procedure

At each experimental time, the cell suspension was diluted to the concentration of 5000 cells/mL. Then 200 μL of cell suspension were seeded into a 96-well tissue culture plate (Nunc, Wiesbaden, Germany), 20 wells for control group and 20 wells for test group. Cells were placed in the incubator for 24 hours to obtain a monolayer cell growth. After overnight attachment, each well was washed twice with sterile phosphate-buffered saline (PBS) solution and the MTT assays were performed. The experiments were repeated 3 times to ensure reproducibility.

MTT assay

This assay focuses on the ability of the mitochondrial dehydrogenated enzyme in living cells to convert the yellow water-soluble tetrazolium salt 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) into dark blue formazan crystals. This water-insoluble product is stored in the cytoplasm of the living cells. The amount of formazan formed is directly proportional to the mitochondrial enzyme.

Two hundered microliters MTT solution (0.5 mg/mL PBS) was added to each well. The plate was placed in a 37°C incubator for 4 hours. After the incubation period, the MTT solution was discarded from the wells and each well was washed twice with 200 μL PBS. Then 200 μL dimethyl sulfoxide (DMSO) was added into each well to dissolve the dark blue crystals formed in the presence of active mitochondria. The plates were agitated on the shaker for 30 minutes to enhance the dissolution of formazan. The spectrophotometric absorbance (optical density) was read at 540 nm with a microplate reader (Bio-Tek Instruments, Winooski, VT, USA), using DMSO as the blank. Detailed procedures for these measurements have been previously described by Mosmann.[29]

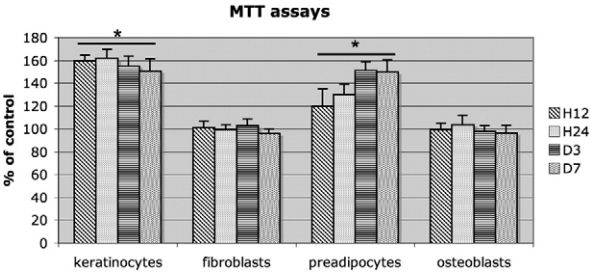

Fig. 3. MTT assay results. Viability of 4 cell types after in vitro culture in contact with PRF membranes during 4 experimental times: 12 hours (H12), 24 hours (H24), 3 days (D3), and 7 days (D7). Results of the test groups are expressed as percentages of the control and represent the mean ± SD (standard deviation). *$P < .05$ compared with control.

Statistical analysis

The mean optical density of the control group was set to represent a 100% viability. Results of the test groups were expressed as percentages of the control. Statistical analysis was performed by 1-way analysis of variance, and in case of significant difference the Tukey test was used. Statistical significance was assigned when $P < .05$.

RESULTS

This series of cytotoxicity tests confirms the total absence of cytotoxicity of PRF (Fig. 3). Even better, there is a significant difference between the test group and the control group: Given that the MTT test allows one to evaluate the mitochondrial respiration, we may consider that at least 2 cell types, keratinocytes and preadipocytes, placed in contact with PRF, "breathe" better than the control group cells.

As a conclusion, PRF produced in glass-coated plastic tubes represents absolutely no cytotoxicity risk. The contrary even seems true. This commercial controversy is over.

DISCUSSION

Evaluation of the biologic effects of dental materials is of great importance because their systematic side effects may take years before they appear.[21] The ISO 10993-5 standard is applicable for testing biocompatibility of medical devices with no specific indication for dental devices. Therefore, test designs are often clinically irrelevant for dental practice, and protocols closer to clinical conditions should be developed. For PRF, only 1 cytotoxicity study of the membrane considered as a biomaterial is possible.

Nevertheless, the search of a possible cytotoxiciy of PRF is not an easy protocol. Another protocol could have been used: leaving the culture medium in contact with the PRF membranes during 12 hours, then testing the contaminated medium on a monolayer of human cells placed in a 96-well tissue culture plate. We did not follow that protocol, because the membranes release many growth factors, and therefore the possible cytotoxicity of silica (or of PRF itself) would be totally annihilated by the massive effect of the cytokines. For this same reason, some other conventional tests (neutral red uptake and total nucleic acid content) are not relevant enough to test the cytotoxicity of PRF.

Anyhow, the cells cultivated on PRF present no sign of intoxication.

The PRF technique was initially developed using simple dry glass tubes, for which the issue of cytotoxicity obvioulsy does not exist. In the first international paper presenting the PRF technique, we insisted on the glass-coated plastic tubes. Indeed, the use of plastic tubes offers more security in terms of handling in a dental office, whereas glass tubes may prove to be dangerous in case of accident risks (breaking, cutting, and contamination). This evolution indeed applies in all fields, because the main manufacturers only sell plastic tubes now.

In conclusion, the silica microparticles coating these tubes represent a quite impossible risk of cytotoxicity, in contrast to bovine thrombin (used to prepare PRPs and fibrin glues),[30,31] which may generate immune side effects.[32-42] This is why Dr. Choukroun developed PRF, to avoid this thrombin-related risk.

In France and the rest of Europe, more than 2000 clinicians use PRF. After an investigation among the tube distributors for PRF, we have estimated that more than 80,000 tubes per year have been used in France in oral, maxillofacial, and orthopedic surgery. Which means that PRF has been used in at least 100,000 surgical protocols within 6 years. To this day, no accident claim has been recorded at the Material Drug Administration Department of the French Health Ministry.

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