easier for PRF experiment. However, economical and ethical issues prohibit the use of large numbers of large animals for these experiments.

In addition, the authors claimed that true Choukroun's PRF could fill a large defect without the need for filling materials in humans.[2] However, there are few references pertaining to Choukroun's PRF only as a bone-filling material. Most references involved the use of Choukroun's PRF for soft tissue augmentation.[2] The use of fibrin-based material only as a filling material for large bone defects remains controversial.[7,8]

In summary, the failure to produce true Choukroun's PRF from the rabbit is largely due to the low technical quality of the procedure. We can give 6 detailed technical tips as follows: 1) Select New Zealand white rabbits; 2) use sedation before sampling; 3) if possible, use a vessel dilatant; 4) consider an EDTA-pretreated conical tube to prevent immediate blood adherence to the tube wall; 5) use arterial blood if the sampling amount exceeds 5 mL; and 6) do not use a vacuum tube such as a glass tube or glass-coated tube that may be designed for humans, because it may collapse the rabbit's fragile vessel and may also have a low success rate. The required amount of PRF in each experimental design must be tailored to the defect size; this will determine the required amount of blood sampling.

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Platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) in human cell cultures: Growth factor release and contradictory results

To the Editor:

We recently read an article in *OOOOE* about platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) in human cell cultures,[1] and that article raised significant questions regarding methodology and conclusions. Because Dr. Gassling was not able to clarify his methodology when we contacted him, we think it is important to share this discussion with the scientific community and the readers of *OOOOE*.

In their work, Gassling et al. tried to evaluate the growth factor release of a PRP and a PRF (described as the Choukroun's PRF) in contact with 3 different kinds of human cell cultures (cell bank lineages of fibroblasts, osteoblasts, and osteoblast-derived osteosarcoma cells [Saos-2]). The objectives of this work were obviously debatable. Indeed, with this methodology, the authors could not evaluate the true growth factor content of the tested products (because cells are consuming growth factors for their own development), and they did not evaluate the cell behavior in contact with the platelet preparations: no evaluation of the cytotoxicity of these products, no evaluation of the cell proliferation or differentiation patterns, no scanning electron microscope evaluation of the cell cultures. Moreover, we know that the PRF leukocytes[2] massively interact with the cells in culture, and that primary cultures from the same donor as for the PRF/PRP should be preferred to cell bank lineages to avoid immune cross-reactions.[3,4] The methodology of Gassling et al. was thus flawed and the results from such a study intrinsically difficult to interpret. However, Gassling et al. drew the conclusion that PRP application in cell cultures leads to higher levels of growth factors than PRF application. These statements must be thoroughly discussed.

Contradictory results

The methodology used by Gassling et al. generated contradictory results. For transforming growth factor (TGF) β1 and platelet-derived growth factor (PDGF) AB, the values obtained in the PRP group were quite low but not shocking, because the PRP samples were not activated by thrombin or batroxobin during injection in the cell culture.[5] In such conditions, the PRF growth factor contents were logically expected to be much higher than the PRP amounts, because most platelets were activated in the PRF clots (this is the definition of PRF).[2,6,7] Surprisingly, Gassling et al. reported the contrary.

However, the major concern is that the reported amounts of growth factors in the PRF group were always close to 0. These values are highly disturbing and contradictory to all published data about PRF. In the first article about the growth factor content of PRF,[8] we demonstrated that an intact PRF membrane slowly released in 7 days on average 273.4 ng TGF-β1 and 50.3 ng PDGF-AB. This is an enormous amount of these growth factors. In comparison, Gassling et al. quantified on average values 1,000 times lower: for TGF-β1, 198 pg/mL with Saos-2 cells, 468 pg/mL with osteoblasts, 106 pg/mL with fibroblasts; for PDGF-AB, 443 pg/mL with Saos-2 cells, 193 pg/mL with osteoblasts, 215 pg/mL with fibroblasts. And several times, Gassling et al. reported that the minimum quantities of these growth factors in the PRF group were simply nil! Considering that TGF-β1 is a very important cytokine and that cells in culture also produce this growth factor to interact together, this nil value raises legitimate questions.

Moreover, several independent studies have confirmed our statements. In an article published in the same *OOOOE* issue as the Gassling et al. article, Su et al.[9] from Taiwan quantified in the PRF releasate that the cumulative mean growth factor quantities were 52.73 ng PDGF-AB, 72.21 ng TGF-β1, and 249.16 ng insulin-like growth factor (IGF) 1 in the 5 hours after PRF preparation. In another article, in a small PRF obtained with only 8.5 mL of blood, He et al.[10] from China quantified a cumulative mean slow release of TGF-β1 >150 ng and of PDGF-AB >50 ng in only 7 days, and PRF was still releasing significant amounts of those growth factors (at the ng scale) after 28 days.

The analysis of the literature clearly shows that Gassling et al.'s data are problematic. Gassling et al. claimed that a possible explanation for the low growth factor contents that they quantified in the PRF group could be related to a lack of a suitable standardization of PRF preparation. This statement is not correct: As already shown, the cell content, clot architecture, and growth factor content of PRF are easily standardized,[2,8] but it requires knowing how to correctly handle the PRF protocol. The explanation of the Gassling et al. data has therefore to be found in their methodology, in the way PRP and PRF were tested.

Testing PRF . . . without using PRF?

Gassling et al. performed the cell cultures in 24-well Nunclon polystyrene plates (Nunc, Roskilde, Denmark). These plates were designed with a culture area of 1.9 cm^2 per well, for a working volume of 1 mL per well. Gassling et al. claimed to introduce 1.5 mL PRF into each well to use equivalent volumes of PRP and PRF in the test groups, because they were not able to compare platelet numbers in PRP and PRF. Trying to compare 1.5 mL unactivated platelet liquid suspension (PRP) with 1.5 mL activated leukocyte-platelet fibrin solid biomaterial (PRF) is obviously a debatable approach, but this is not the main issue. If we assume that injecting 1.5 mL of liquid PRP into a 1-mL well is not a problem, we think that introducing 1.5 mL of PRF into a 1-mL well raises legitimate questions.

PRF is not a liquid suspension of unactivated platelets.[6] PRF is a solid fibrin-based dense biomaterial (Fig. 1)[11] with a very specific 3-dimensional architecture (thick polymerized fibrin strands), cell content, and distribution (>95% of the platelets and >50% of the leukocytes from the initial blood harvest).[2] In such conditions, what does "1.5 mL" of such a biomaterial mean? Were the authors only using a fragment of PRF, and if so, which part of this clot? And was it "1.5 mL" of clot or membrane? These are crucial items of information, because leukocytes and platelet aggregates are concentrated in specific parts of the clot (Fig. 1),[2] and the initial exudate of the PRF clot is also rich in growth factors.[7,8,12]

Consequently, there is another logical question: how to introduce a 1.5-mL fibrin bulk into a 1-mL culture well without damaging all of the cells? The PRF membrane remains a very dense and strong fibrin biomaterial in culture conditions for several weeks, with or without the contact of cells, as it has already been proven by several studies.[3,4,8] It therefore means that the PRF plugs used by Gassling et al. were squeezing the cultivated cells during the whole experiment.

Last but not least, the authors claimed to collect the culture media from the wells for analysis, but how to add and retrieve culture medium in a 1-mL well filled with a 1.5-mL fibrin plug ? Did Gassling et al. discard the 1.5-mL PRF volume when changing the culture medium ? The PRF biomaterial releases nanograms of TGF-β1 for at least 28 days; the few

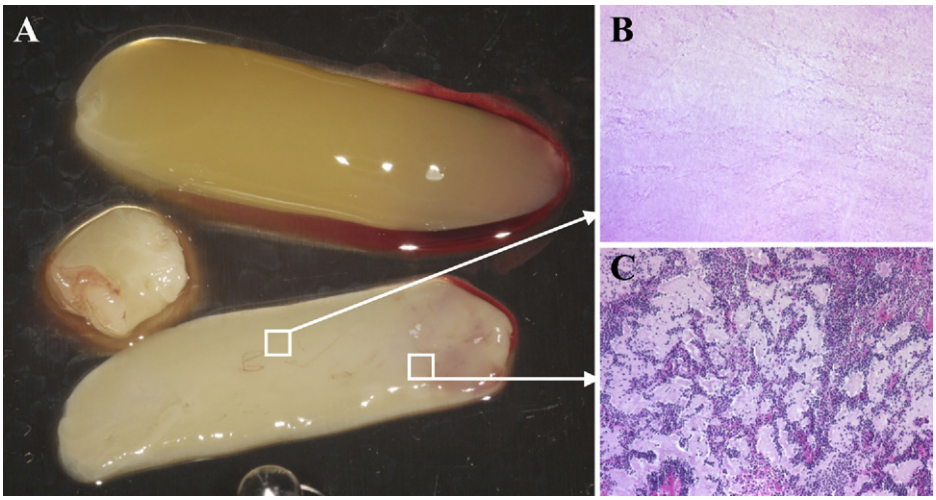

Fig. 1. Choukroun's PRF is a solid biomaterial (**A**) with a specific 3-dimensional architecture and cell distribution. The main body of the material is acellular and built with thick fibrin strands (**B,** hemalaun-eosin, magnification ×52), and leukocytes and platelet aggregates are concentrated in some areas (**C,** hemalaun-eosin, magnification ×52). PRF can be used as a clot (**A,** top), a condensed cylinder (**A,** middle), or a dense membrane (**A,** bottom). Gassling et al. claimed to have introduced 1.5mL of PRF within each 1mL culture well. What means <<1.5mL>> of PRF? How Gassling et al. were able to introduce 1.5mL of a dense fibrin bulk into a 1mL culture well where cultivated cells were supposed to live?

picograms quantified by Gassling et al. might thus be explained if the PRF was prematurely discarded.

Unfortunately for all of these legitimate questions, no photos of the experiments were provided in the article.

As a conclusion, we would like to thank the Editor-in-Chief of *OOOOE* for his very balanced and wise attitude, because the journal published simultaneously the articles of Gassling et al.[1] and of Su et al.,[9] i.e., 2 independent teams publishing exactly opposite results. The literature about platelet concentrates is full of misunderstadings and approximations, results are very difficult to sort and interpret even for specialists, and it is particularly important to keep the scientific debate opened.

We hope that Gassling et al. can answer all of our comments and help us to understand how they were able to conclude that Choukroun's PRF was releasing almost no growth factors, while 3 different independent teams published exactly the contrary at the very same moment.

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Platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) in human cell cultures: Response to letter of Dr. David Dohan Ehrenfest

In reply:

Thank you very much for forwarding the letter of Dr. Dohan Ehrenfest regarding our publication "Platelet-rich plasma and platelet-rich fibrin in human cell culture."[1,2] Before answering all of the questions raised in a step-by-step manner, we would like to make a few important remarks. The investigations were performed in our laboratory, which is renowned and certified by DIN-EN-ISO-9001:2000, under the guidance of head biologist Prof. Dr. Y. Açil., who has 25 years of scientific experience and has published over 80 international publications.

1. Dr. Dohan Ehrenfest stated that our experiments were performed with bank cell lineages. This is incorrect. The experimental design was performed using primary cell cultures (human fibroblasts and osteoblasts) from different patients according to the ethics guidelines of the Christian Albrechts University, Kiel. Only the Saos-2 cells were bank cells.
2. Concerning the measured cytokine concentrations, we are currently unable to explain the differences in our results from those mentioned in Dr. Dohan Ehrenfest's letter. However, it is to be expected that different results may depend on varying experimental conditions. For example, the preparation of PRF membranes by squeezing the PRF clot may impede the delivery of cytokines to a differing extent.
3. Dr. Dohan Ehrenfest assumes that 24-well cultivation plates (Nunclon; Nalge Nunc Corp., Roskilde, Denmark) have a working volume of 1 mL. This is correct, but the term "working volume," means the minimal volume that has to apply for in vitro experiments. Each well holds a maximum of 3 mL and thus an applied volume of 1.5 mL is possible.
4. In the present study, 1.5 mL PRF was prepared as follows. Several clots were transformed to membranes as described in the Material and Methods section. Then homogenizate was prepared by cutting membranes into small pieces. These pieces were transfered to a 1.5-mL cylinder to obtain the intended volume. This procedure ensures an equal distribution of all parts of the clot.
5. We believe that photos of every step of our experiments would overburden the journal's capacity. Moreover, *OOOOE* is a peer-reviewed journal and it must be assumed that the reviewers had carefully read our article and that there were no complaints.
6. Despite the fact that findings in the present investigation are not similar to those of other research groups, it should be mentioned that a recently published article reports a positive effect of PRF on periosteal cells compared with collagen membranes.[2]

In conclusion, we thank Dr. Dohan Ehrenfest and his colleagues for raising these questions concerning methodology, which may help to explain deviating results in the future.