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Title: INFLUENCE OF PLATELET-RICH PLASMA ON THE HISTOLOGICAL CHARACTERISTICS OF THE AUTOLOGOUS FAT GRAFT ON THE UPPER LIP OF RABBITS

Short Title:

Article Type: Original Articles

Keywords: Platelet-rich plasma; fat graft

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Abstract: Objective: In this study has been evaluated the influence of platelet-rich plasma (PRP) on the histological characteristics of the autologous fat graft on the upper lip of rabbits.
Material and methods: Nine New Zealand white rabbits were used. Eight rabbits underwent fat harvest from the groin fat pads using a modified Coleman technique. One side of the upper lip was infiltrated with autologous fat, and the other side with fat and PRP. Four of the infiltrated rabbits were euthanized 8 weeks after the lip augmentation, and the other four infiltrated rabbits plus one control case were euthanized 12 weeks after the procedure. Coronal sections of both upper lips were analyzed microscopically to evaluate the quality of the fat graft, the inflammatory reaction, the presence of oil cysts, the degree of fibrosis, and the neovascularization.
Results: The infiltration of adipose tissue plus PRP presented less inflammatory reaction ($p<0.05$) and less oil cysts ($p<0.05$) than the infiltration of adipose tissue without PRP.
Conclusions: The infiltration of fat tissue plus PRP generates a lower inflammatory reaction and a lower formation of oil cysts than the infiltration of isolated fat. The PRP increases the maintenance of the transplanted fat cells.

Suggested Reviewers:

Opposed Reviewers:

Response to Reviewers: Reviewer #1: This study attempts to evaluate the influence of platelet-rich plasma (PRP) on autologous fat grafting of the upper lip of rabbits. One side of the upper lip was infiltrated with autologous fat, and the other side with fat and PRP. Rabbits were euthanized at 8 weeks and 12 weeks after lip augmentation. Histological evaluation of the quality of the fat graft, the inflammatory reaction, the presence of oil cysts was made.

In considering the objectives of this study, namely improving long-term viability of autologous fat grafting, we are left with questions that dispute the conclusions drawn by the authors:

1. Can true assessment of fat survival be made at 8 or even 12 weeks following injection when we observe clinically that resorption is often maximally demonstrated between 4 and 6 months?

Answer:

Firstly, we have to remind that the metabolism of the rabbits is much faster for all kind of cells than the metabolism of human beings. For example, while in humans bone remodeling takes between 6 and 9 months, in rabbits it takes place in 6 weeks. Attending this fact, we suggest an example of study published in pubmed literature where the histological evaluation is made after 6 weeks postop.

* Effect of combining platelet-rich plasma with anorganic bovine bone on vertical bone regeneration: early healing assessment in rabbit calvariae. Torres J, Tamimi F, Tresguerres IF, Alkhraisat MH, Khraisat A, Blanco L, Lopez-Cabarcos E. Int J Oral Maxillofac Implants. 2010 Jan-Feb;25(1):123-9.

This article, published in the IJOMI in 2010 and written by one of the authors of the present paper, reports histological evaluation of the effects of PRP on calvarial bone of rabbits made 6 weeks after the procedure. Although this study is regarding to bone tissue, we present some papers that study the effect of PRP at different stages of healing in fat grafts.

Secondly, it has been demonstrated that the maximum effect of the platelet-rich plasma, and of the growth factors on themselves, takes place during the first week of the process of wound healing or of the process of biointegration of a transplanted tissue. It is widely exposed in the following review:

* Physiol Rev. 2003 Jul;83(3):835-70. Regulation of wound healing by growth factors and cytokines. Werner S, Grose R.

In the same order of demonstration in the field of fat transplantation we also have the following papers:

* Ann Plast Surg. 2010 Jul;65(1):101-6. Platelet-rich plasma (PRP) promotes survival of fat-grafts in rats. Nakamura S, Ishihara M, Takikawa M, Murakami K, Kishimoto S, Nakamura S, Yanagibayashi S, Kubo S, Yamamoto N, Kiyosawa T.

This study evaluated the effects of platelet-rich plasma (PRP) on resorption and adipocyte survival in autologous fat-graft of rats prepared with isogenous PRP. Fat grafts prepared without PRP (control group) became united to the tissue adjacent to the implantation site and were significantly resorbed from 30 days. On the other hand, fat grafts prepared with PRP (PRP group) demonstrated little resorption from 30 to 120 days and appeared pink, had a soft, supple feel, and were easily compressible. Histologic sections of grafts in the control and PRP groups at 10 days exhibited similar consolidation of the grafted tissue, which contained morphologically normal adipocytes with different degrees of granulation and capillary formation. From 20 days normal adipocytes were obviously decreased in the control group, while the PRP group demonstrated increased granulation tissue and capillary formation and good maintenance of normal adipocytes for at least 120 days.

The effect of the PRP on the mesenchymal tissues has an early effect. It's also true that this effect increases cell survival on time. The effect of PRP on fat transplanted cells maintenance takes place in the first week of their contact.

* Rossatti B. Revascularization and phagocytosis in free fat autografts: an experimental study. Br J Plast Surg 1960;13:35-41.
* Saunders MC. Survival of autologous fat grafts in humans and mice. Connect Tissue Res 1981;8:85-91.

2. The study would indicate less inflammation (especially early on) but this does not translate long term into less fibrosis and increase volume of viable cells. This may relate to the small numbers tested or to length of time assessed. Either way, it is impossible to draw conclusions.

The statistically significant differences between the fat graft with and without addition of PRP are only for the parameters: "inflammatory reaction" and "presence of oil cysts". Regarding to the degree of fibrosis, PRP group showed a considerable decrease in the presence of fibrosis compared with isolated fat group at 8 weeks although did not reach statistical significance, while minor differences were observed at 12 weeks.

Now it has been corrected.

Corrected text:
CONCLUSIONS

Fat grafting means a variable but almost constant degree of swelling due to the technique and the many passes that it requires. Histologic examination of the fat graft suggests an early inflammatory response to the injected fat, much higher when isolated fat is infiltrated than when it is mixed with PRP. There is a sequestration of nonviable tissue due to the inflammatory reaction, what is pathologically expressed as oil cysts. The transplanted fat with PRP shows a statistically significant decreasing of this early inflammatory reaction and a decrease of the presence of oil cysts. By the mechanisms of decreasing the inflammatory reaction and decreasing the cellular necrosis of the transplanted fat cells, PRP increases the cellular maintenance of the transplanted fatty tissue, promoting the biointegration and proliferation of the fat cells in the new soft tissue where are infiltrated.

3. Less oil cysts supposedly translates to less necrotic tissue with less fibrosis. This is not demonstrated - whether this becomes apparent long term is still unknown.

Thus the conclusion that PRP is as an 'enhancer of the fat graft that promotes the viability and the maintenance of the volume of the fat infiltrated that makes possible to avoid the overcorrection of the defect' cannot be extrapolated from this study.

I would therefore recommend that the study only be accepted if revised to a pilot study, stating that definitive conclusions cannot be made until increased numbers and length of study time are available.

Answer:
The text has been corrected attending to the comments of the reviewer.
In the corrected text the conclusions of the evaluation of the results of the study have been reported in a concise and strict way. The conclusions are that the PRP decreases the inflammatory reaction on the transplanted fat graft, and it also decreases the presence of oil cysts (oil cysts are a well-known histological sign of cellular necrosis).
We agree with the reviewer that with the results of this study cannot be extrapolated that PRP decreases the presence of fibrosis. But the decreasing of the inflammatory reaction and the decreasing of cellular necrosis on the early stage of fat infiltration can be concluded of this study. We agree with the reviewer that the very long term presence of cellular necrosis and cellular resorption cannot be extrapolated from our study, but the results show the effect of PRP on early stages of fatty tissue transplantation, that is the main stage where PRP and its growth factors act, as it has been documented in the first comment for this reviewer.
Adding PRP on fat grafting doesn't avoid the fat resorption totally, but at least it has a demonstrated effect on decreasing the inflammatory reaction that is always associated to the lipoinfiltration technique. So, it decreases the cellular necrosis due to the inflammatory reaction associated to the procedure on the early stages of biointegration of the transplantated tissue during the first days after the procedure.

This study shows the histological characteristics that can be evaluated by hematoxylin/eosin staining. To evaluate the exact cellular maintenance of the transplanted fatty cells, other stain techniques should had been performed (like Sudan III). Anyway, we agree with the reviewer that it cannot be extrapolated that PRP makes possible to avoid the overcorrection of the defect when lipofilling procedures are performed, but we can conclude that the addition of PRP helps to avoid the cellular necrosis due to the early inflammatory reaction on filling soft tissues with fat.
It would be interesting to evaluate a more long-term effect of the addition of PRP on fat grafting. We have based our investigation on the literature of the biology of PRP (mainly on the bibliographic references 3-4-5-6, but also on other references), and it shows that the effect of the growth factors takes place during the first week of contact with the cells in wound healing. Our investigation shows this early effect. The literature about Growth Factors also suggests an increasing of the revascularization and growing capacity of the cells where they act, what we have not been able to demonstrate with our study. It would require specific lipid staining, stereologic evaluation, and, as the reviewer suggests, an increased length of study time. It can be the purpose of a different investigation that the investigation presented in our paper.

About the number of rabbits used for the presented investigation, we consider that this number of rabbits (nine) has been enough to demonstrate that PRP decreases the inflammatory reaction and the presence of oil cysts even if $n<30$, showing a statistically significant value of "p-value" with the non-parametric test using for the evaluation of the results.

Reviewer #2: The paper evaluated the influence of PRP on the histological characteristics of the autologous fat graft on the upper lip of rabbits, it is an interesting issue in Plastic Surgery, it is an original aspect of the problem, but the paper has many problems and needs major revisions prior to a resubmission.
The methods as they were presented here are not reproducible. Scientific method should be performed by any research only by reading the paper, so all information has to be pointed.

1.)Animal experimental scientific paper has to show the number of approval of the Ethics Committee of the Institution?
Prior to beginning the "in vivo" animal study the protocol was approved by the ethical committee for animal experiments of the Rey Juan Carlos University (URJC). Experiments were conducted in accordance with the guidelines laid down by the European Communities Council Directive of 24 November 1986 (86/609/EEC) and adequate measurements were taken to minimize pain and discomfort in the animals.

2.)Weight and age of animals?
Nine healthy 13 week-old male New Zealand rabbits weighting between 2.5-3 kg were used.

3.)What kind of installations, feedings, care, analgesia post-operative used in animals?

The animals were accommodated in the official stable for animal assays of the URJC at 22-24 ºC with 55-70 % humidity, light cycles of 12 hour, and air renewal 15 times per hour. The rabbits were fed with a Panlab® (Barcelona, Spain) diet while drinking was permitted ad libitum.

All rabbits were anesthetized with an intramuscular dose of 0.75 mg/kg ketamine (Imalgene 1000®, Rhone Merieux, France) and 0.25 mg/kg xilacine (Rompun®, Bayer, Leverkusen, Germany).

Post operative antibiotics were administered, Terramicina® (Pfizer, Spain), in water (4g/l) for 3 days. Also analgesics (0.1ml/kg) were administred Buprex® (Shering-Plough, Madrid, Spain)

4.)Animals stayed all procedure under anesthesia, from blood collection, PRP preparation, lipoaspiration, fat sedimentation, preparation of fat grat plus PRP and the injection of fat on the lip?

All surgery stage procedures except blood collection were done under the following antesthesia protocol: (intramuscular dose of 0.75 mg/kg ketamine (Imalgene 1000®, Rhone Merieux, France) and 0.25 mg/kg xilacine (Rompun®, Bayer, Leverkusen, Germany).

5.)Describe more about aqueous component drained.

The aqueous componend drained was mainly de infiltration solution. After the sedimentation of the fat, it was decanted.

6.)To obtain blood from animals, they are under anesthesia?
No.

7.)From 4 ml blood collected, which final volume obtained?

10 cc of whole blood was withdrawn via ear venous aspiration into 5 cc test tubes and mixed with a 3.8 % sodium citrate solution at a ratio of 1 cc sodium citrate solution to 5 cc whole blood, achieving anticoagulation through calcium binding. The blood was then centrifuged following the technique described by Anitua9-10 with a Nahita® centrifuge (Navarra, Spain) into three basic components; red blood cells, PRP, and platelet poor plasma (PPP). Because of differential densities, the red blood cell layer forms at the lowest level, the PRP layer in the middle, and the PPP layer at the top. A pipette (Gilson, France) was used to separate each layer, from the less dense to the more dense; therefore PPP was separated first (about 1.25 cc) and PRP second (about 1.25 cc), leaving the residual red blood cells (about 2.25 cc).

8.)What were the dose of drugs of anesthesia?

Post operative antibiotics were administered, Terramicina® (Pfizer, Spain), in water (4g/l) for 3 days. Also analgesics (0.1ml/kg) were administred Buprex® (Shering-Plough, Madrid, Spain)

9.)What the size of groin area was infiltrated with 30cc, and the depth of this infiltration?

Both whole groin fat pads were totally infiltrated with the solution. Previously to the presented investigation, we performed laparotomy dissection of other rabbits used in another investigation to check the anatomy of groin fat pads.

Needle was no more than 5mm depth for the infiltration of the solution.

Groin fat pads are easily reachable with a superficial infiltration.

10.)What was the sedimentation time of the far lipoaspirated?

Sedimentation time was around 10-15min. The aqueous component of the fat lipoaspirated was quickly separated of the fat phase just by sedimentation.

11.)The experiments were made as a double-blind experiment?

Neither researcher that injected fat on the lip nor the pathologist that read the images knew what group were from the sample?

Yes, this is really a double-blind experimental study.

12.)Why at 8 weeks evaluation there were no control group?

The same control case euthanized at 12 weeks can be used as control case for the group biopsied at 8 weeks, cause it was a virgin specimen.

13.)How the animals were submitted to euthanasia?

Sodium barbiturate (pentobarbital) (Dolethal®; Vetoquinol, Lure, France): 1cc/Kg intravenous.

14.)It is appropriate to a good scientific contribution of knowledge that the two different kind of treatment could be applied to a small area, superior lip in each half, without any block to this two different treatment not to be mixed? It will be more appropriate to make each different treatment in a different animal?

We consider that the split mouth design is the better study design in order to evaluate differences into the same intrinsic characteristics of an animal. Regarding the block of this two different treatments not to be mixed, is guaranteed because of the split lip of rabbits that block in a natural way both treatments

Most experimental animal studies evaluating fat graft maintenance evaluate fat blocks. Some prominent studies also used Coleman technique. We wanted to reproduce the usual clinical practice, by evaluating the maintenance of the fat grafted by a Coleman technique.

Previously to perform the investigation, literature review was strictly made. In the literature we find the following main animal studies evaluating fat maintenance:

* Karacaolgu E. "The role of recipient sites in fat-graft survival: experimental study". Annals of Plastic Surgery 2005.
  - In this study groin fat pats are transplanted as a block to the gonial aspect of the mandible of New Zealand rabbits.
* De Giacomo C. "Assessment of collagen deposits after implant of fascia lata and fat in the vocal folds of rabbits: histomorphometric study". Rev Bras Otorrinolaringol 2005.
  - In this study cervical fat is infiltrated in small diposits in the vocal folds of rats.
* Altman JI. "Demineralized bone matrix and fat autograft in a rabbit model of frontal sinus obliteration". Otolaryngology-Head and Neck Surgery 2007.
  - In this study dorsal fat blocks by direct excision are used to obliterate frontal sinus in rabbits.
* Brucker M. "Long-term fate of transplanted autologous fat in novel rabbit facial model". Plast Reconstr Surg 2007.
  - In this study fat from groin fat pads harvested by a modified Coleman technique is infiltrated in the upper lip of rabbits.

* Palma PC. "Effect of purified collagen on lipograft survival: experimental basis for periurethral lipoinjections". J Endourol 2003.
 In this study free fat tissue was transplanted to the preauricular region.
* Eppley BL. "Autologous facial fat transplantation: improved graft maintenance by microbead bioactivation". J Oral Maxillofac Surg 1992. Free fat tissue transplanted with the addition of Fibroblastic Growth Factor.
* Eppley BL. "A physicochemical approach to improving free fat graft survival: preliminary observations". Aesthetic Plastic Surgery 1991.
* Karaçal N. "The effect of fibrin glue on fat graft survival". J Plast Reconstr Aesthet Surg 2007. Groin fat pads of rats were transplanted to subdermal pockets with the addition or not of PRP.
* Nakamura S. "Platelet-rich plasma promotes survival of fat-grafts in rats". Ann Plast Surg 2010. Free fat with the addition of PRP.
* Hong SJ. "Enhancing the viability of fat grafts using new transfer medium containing insulin and beta-FGF in autologous fat transplantation". J Plast Reconstr Aesthet Surg 2010. Blocks of fat from the groin fat pads transplanted to the dorsal region of New Zealand rabbits.
* Pires Fraga MF. "Increased survival of free grafts with PRP in rabbits". J Plast Reconstr Aesthet Surg 2010. Fat blocks harvested from the scapular region and transplanted to the retroauricular region of rabbits, with the addition of PRP.

15.)Why in this study were not realized bio-integration and cell proliferation evaluation?

Our study was made with the aim of doing a histological general evaluation of the main parameters of the transplanted fat cells. We evaluated the samples under optical microscopy. Immunohistochemical techniques were not at our disposal to evaluate biointegration, cell proliferation, or other parameters that require more complex techniques based on biomarkers.

16.)The experiments with animals were made in a aseptic way? From figure 2 there were not.

Clorhexidine was used as chemical antiseptic on the abdominal region before starting the procedure, as well as in the perioral region of each animal.

17.)Were there any complications, as infection, animal lost?
There were no complications. No infections appeared in the specimens presented in the investigation.

18.)How was evaluated the fat viability? Objective way?

Fat viability was evaluated together with two pathologist doctors. Evaluation of fat viability was based on the previous histological parameters evaluated and graded (oil cysts, fibrosis), as well as in the anatomical disposition observed under magnification. Anyway, it has been added in the discussion this parameter should be better evaluated by specific lipid staining or stereologic measurement.

19.)The conclusions were not wrote as conclusions, but as Discussion, and should be rewrite.
It has been rewritten.

20.)References list didn´t follow Vancouver' style.

We have followed the rules dictated in the following sites:
www.icmje.org
www.nlm.nih.gov/bsd/uniform_requirements.html
www.metodo.uab.es/enlaces/2006%20Requisitos%20de%20Uniformidad.pdf

21.)Only 5 papers appear in PubMed check using PRP and fat graft, and in the Discussion in this paper was not used the paper of Por YC, Yeow VK, Louri N, Lim TK, Kee I, Song IC; J Plast Reconstr Aesthet Surg. 2009 Aug;62(8):1030-4. Epub 2008 Jun 11. Platelet-rich plasma has no effect on increasing free fat graft survival in the nude mouse.

This article of Por has been added to the references and mentioned in the discussion as suggested by reviewer.

INFLUENCE OF PLATELET-RICH PLASMA ON THE HISTOLOGICAL CHARACTERISTICS OF THE AUTOLOGOUS FAT GRAFT ON THE UPPER LIP OF RABBITS

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Objective: In this study has been evaluated the influence of platelet-rich plasma (PRP) on the histological characteristics of the autologous fat graft on the upper lip of rabbits.

Material and methods: Nine New Zealand white rabbits were used. Eight rabbits underwent fat harvest from the groin fat pads using a modified Coleman technique. One side of the upper lip was infiltrated with autologous fat, and the other side with fat and PRP. Four of the infiltrated rabbits were euthanized 8 weeks after the lip augmentation, and the other four infiltrated rabbits plus one control case were euthanized 12 weeks after the procedure. Coronal sections of both upper lips were analyzed microscopically to evaluate the quality of the fat graft, the inflammatory reaction, the presence of oil cysts, the degree of fibrosis, and the neovascularization.

Results: The infiltration of adipose tissue plus PRP presented less inflammatory reaction ($p<0.05$) and less oil cysts ($p<0.05$) than the infiltration of adipose tissue without PRP.

Conclusions: The infiltration of fat tissue plus PRP generates a lower inflammatory reaction and a lower formation of oil cysts than the infiltration of isolated fat. The PRP increases the maintenance of the transplanted fat cells.

Keywords: fat graft; platelet-rich plasma

INTRODUCTION

The loss of volume is a well-known sign of aging and it is perceived as a non aesthetic characteristic of human face. Fat injection is widely used to fill up the soft tissue defects to restore the facial volume. The fat graft has the positive features of an autologous graft, presenting a high immunological tolerance when it is infiltrated. The major drawback of fat infiltration is its variable degree of maintenance, with a resorption oscillating from 0% to 100% [1-2]. Several modifications in the various methods of harvesting, treatment and placement of the fat have been described with the aim of keeping the volume of infiltrated fat tissue as longer as possible. Recently, the investigations about the maintenance of the fat graft are looking for a way to improve the long-term outcomes by stimulating the adipose cells. In this order of research, several agents added to the fat graft, like insulin, isolated growth factors, or beta-bloquers have been studied and evaluated. Platelet-rich plasma (PRP) is presented as an enhancer of the adipose cell survival when it is infiltrated with the fat tissue.

Biology of the PRP

PRP is defined as a part of the plasma fraction obtained from autologous blood containing a platelet concentration over the usual levels of the normal blood. The biology of the PRP applied to the fat graft is based on the alpha granules of the platelets. There are approximately 50 to 80 alpha granules per platelet. The alpha granules contain

over 30 bioactive proteins known as growth factors (e.g. PDGF, TGF-β, IL-1, FGF, VEGF, EGF, IGF).

Platelet activation by calcium ion produces the degranulation of the alpha granules. Growth factors are transformed to a bioactive state and secreted to modulate their target cells. Growth factors bind to the transmembrane receptors present on the mesenchymal cells (mesenchymal stem cells, osteoblasts, fibroblasts, endothelial cells, epidermal cells, adipoblasts). Once bound to the transmembrane receptors, they act during the first days contacting their target cells, resulting in a biological stimulation and enhancement of cellular proliferation and biointegration of autotransplanted mesenchymal tissues that takes places in procedures like the autologous fat trasplantation[3-8].

MATERIALS AND METHODS

Prior to beginning the *in vivo* animal study the protocol was approved by the ethical committee for animal experiments of the Rey Juan Carlos University (URJC). Experiments were conducted in accordance with the guidelines laid down by the European Communities Council Directive of 24 November 1986 (86/609/EEC) and adequate measurements were taken to minimize pain and discomfort in the animals.

Nine healthy 13 week-old male New Zealand rabbits weighting between 2.5-3 kg were used. The animals were accommodated in the official stable for animal assays of the URJC at 22-24ºC with 55-70 % humidity, light cycles of 12 hour, and air renewal 15 times per hour. The rabbits were fed with a Panlab® (Barcelona, Spain) diet while drinking was permitted *ad libitum*.

Preparation of PRP

Immediately before surgery, 10cc of whole blood was withdrawn via ear venous aspiration into 5cc test tubes and mixed with a 3.8% sodium citrate solution at a ratio of 1cc sodium citrate solution to 5cc whole blood, achieving anticoagulation through calcium binding. The blood was then centrifuged following the technique described by Anitua[9-10] with a Nahita® centrifuge (Navarra, Spain) into three basic components; red blood cells (RBCs), PRP (sometimes referred to as "buffy coat"), and platelet poor plasma (PPP). Because of differential densities, the red blood cell layer forms at the lowest level, the PRP layer in the middle, and the PPP layer at the top. A pipette (Gilson, France) was used to separate each layer, from the less dense to the more dense; therefore PPP was separated first (about 1.25cc) and PRP second (about 1.25cc), leaving the residual red blood cells (about 2.25cc).

After preparation, PRP is stable, in the anticoagulated state, around 8 hours. We kept it in this condition during less than two hours in any case, and, just 1 or two minutes before doing the lip infiltration with fat, it was activated by 0,05mL of calcium chloride per each 1mL of PRP.

Surgical procedure

All rabbits were anesthetized with an intramuscular dose of 0.75 mg/kg ketamine (Imalgene 1000®, Rhone Merieux, France) and 0.25 mg/kg xilacine (Rompun®, Bayer, Leverkusen, Germany). Clorhexidine was used as chemical antiseptic before all procedures to the animals. The fat of the rabbit was harvested from the groin fat pads[11-12]. Fat pads were infiltrated with a solution of Ringer's lactate and 0.5% bupivacaine with 1:200,000 epinephrine. The solution was made of 40cc of Ringer's lactate and 20cc of bupivacaine; each groin fat pat was infiltrated with 30cc of the solution. Then fatty tissue was harvested through a small incision of 2mm placed in the skin between both groin fat pads. A lipoaspiration Coleman cannula 3mm wide and 15cm long attached to a 10mL syringe was used to harvest the fatty tissue (figure 1). After the lipoaspiration, the fatty tissue was separated of the nonviable components by decantation after sedimentation (waiting around 15 minutes to decantate the aqueous component). The refined fat was transferred to 1mL luer lock syringes through a 2mm diameter transfer.

An incision of approximately 2mm were placed in the oral commisure to insert the cannula. Different syringes of 1mL were used in the infiltration of each side of the upper lip of the rabbit: one syringe containing 0.4mL of fat plus 0.4mL of the aqueous component decanted during the refinement of the lipoaspirated tissue; and the other syringe containing 0.4mL of fat plus 0.4mL of activated PRP (50% of fat, and 50% of activated PRP). The random blinded infiltration was done in the right or left upper lip of the rabbit using a blunt Type I Coleman infiltration cannula 1.6mm wide and 9cm long (figure 2). The infiltration layer to insert the cannula and the fatty tissue was a subcutaneous and supramuscular plane[13]. The incisions used for the liposuction and for the lipoinfiltration were closed using a resorbable suture of 3/0 Vicryl Rapide. Post operative antibiotics were administered: Terramicina® (Pfizer, Spain), in water (4g/l) for 3 days. Also analgesics (0.1ml/kg) were administred: Buprex® (Shering-Plough, Madrid, Spain).

Four of the infiltrated rabbits were euthanized 8 weeks after the lip augmentation, and the other four infiltrated rabbits plus one control case were euthanized 12 weeks after the procedure. Rabbits were submitted to euthanasia based on pentobarbital (Dolethal®; Vetoquinol, Lure, France): 1cc/Kg intravenous. The specimens were fixed in formol during 48-72 hours in all cases, and embedded in paraffin. Coronal sections of both upper lips were analyzed microscopically with the collaboration of two pathologist doctors. Standard hematoxylin-eosin staining was performed for histological analysis. The characteristics evaluated were the following: the inflammatory reaction (lymphocytic infiltration, eosinophilic infiltration, and multinucleated giant cells), the presence of oil cysts, the degree of fibrosis, the neovascularization, and the viable fat.

RESULTS

For each hemilip, 3 sections chosen randomly were evaluated, and graded on degree of inflammatory reaction (0 to 8), oil cysts (0 to 8), fibrosis (0 to 8), neovascularization (0 to 8), and viable fat (0 to 8). The virgin rabbit upper lip is almost free of native adipose tissue; this facilitated histological examination and comparison of the graft tissue. Histological evaluation was done for each section and the mean was used for the statistical analysis. The investigation was done as a double-blind experiment for the researcher and for the pathologists. Data were analyzed using a Wilcoxon test, and results were considered to be significant at $p<0.05$ (table 1). The mean of the results is shown in the figure 5, showing a comparison of the histological characteristics that were evaluated at 8 and 12 weeks after lip augmentation with isolated fat versus fat plus PRP.

The examination of hemilips infiltrated with isolated fat shown an important inflammatory reaction, higher for the specimens analyzed at 8 weeks after infiltration (5.25/8) than for the specimens analyzed at 12 weeks after infiltration (2/8). On the other hand, hemilips infiltrated with fat plus PRP shown a notorious lower inflammatory reaction, being 0.25/8 at 8 weeks after infiltration, and 0/8 at 12 weeks after infiltration. The results show statistical significant difference between the lower inflammatory reaction of the hemilips infiltrated with fat plus PRP compared to the hemilips infiltrated with isolated fat at 8 weeks after infiltration (p-value 0.03599) and also at 12 weeks after infiltration (p-value 0.04853).

Oil cysts are a histological index of fat necrosis. Oil cysts can appear from blunt trauma or from non revascularization of the fatty tissue transferred. The same surgical trauma was caused in the lipoaugmentation of all hemilips because the same infiltration technique and surgical instruments were used. The statistical analysis shown statistical significant difference between the presence of oil cysts for the hemilips infiltrated with isolated fat (4.75/8) versus the hemilips infiltrated with fat plus PRP (0.5/8) at 8 weeks after infiltration (p-value 0.02021), and at 12 weeks after infiltration (2.25/8 for isolated fat and 0/8 for fat plus PRP, p-value 0.04853).

The degree of fibrosis was lower in the specimens infiltrated with fat plus PRP than for the specimens infiltrated with isolated fat, but the difference did not reach the statistical significance. At 8 weeks, it was 5.25/8 for the isolated fat, and 1.5/8 for the fat plus PRP (p-value 0.07959). At 12 weeks, it was 4.5/8 for the isolated fat, and 3/8 for the fat plus PRP (p-value 0.65317).

The revascularization and presence of vessels between the infiltrated fat cells did not show significant difference between the lips infiltrated with isolated fat and the lips infiltrated with flat plus PRP. For this histological characteristic the results were almost equal for both groups at 8 and at 12 weeks after infiltration.

The last histological characteristic presented in this study is the degree of viable fat. This parameter pretends to evaluate the quality and quantity of fat seen in the sections of each specimen. There were no statistical differences between groups for this

item, but it can be seen in the microscopically assisted pictures that the disposition of the cells kept a nicer and well-organized structure and less morphological distortion in the specimens infiltrated with fat mixed with PRP (figures 6-9).

DISCUSSION

The plasma rich in growth factors or PRP is a biological enhancer of the mesenchymal cells, and, among them, of the adipose cells. The use of PRP leads to the possibility to improve the histological characteristics of damaged or aged tissues. In this paper is presented an evaluation of the histological influence of PRP on the autologous fat graft. Significant refinements in the harvesting and injection of fat have been described over the past years in order to improve the quality of the fat used to fill up soft tissues with fat, and to improve the maintenance of the transplanted fatty cells.

Many studies and applications have been described in the medical literature to evaluate the effect of the PRP on different tissues. The PRP was firstly popularized in the field of oral and maxillofacial surgery. Fennis et al.[14-16] published several studies showing the promoting effect of the PRP on the bony healing in goats and humans, showing healing improvement even on irradiated bone graft used to fill up mandibular defects. Kim et al.[17-18] demonstrated a better osseointegration of dental titanium implants when demineralized bone powder was mixed with PRP. In plastic surgery, PRP has been used to improve and stimulate the tissue healing of several kind of flaps, and for the treatment of chronic ulcers[19-20]. In vascular surgery it has been used as well, for its stimulating effects on the endothelial cells. Aesthetic applications are recently using the PRP as injected mesotherapy pretending the biostimulation of the dermal fibroblasts.

Among the main animal experimental studies performed with the aim to evaluate the influence of different agents and factors on the survival rate of the transplanted autologous fatty tissue, not many of them use fatty tissue harvested by liposuction techniques. Eppley et al. evaluate the survival of free fatty tissue harvested by a modified Coleman technique and infiltrated adding Fibroblastic Growth Factor[21-22]. Brucker et al.[9], in 2007, publish their investigation about the survival of free fatty tissue harvested by a modified Coleman technique as well, evaluating the presence of fat cells after different periods of time. However, recent studies published in the second half of 2010, like the studies of Nakamura et al.[5], Hong et al.[23], and Pires et al.[24] about the effects of PRP on the maintenance of fat graft, report investigations performed using blocks of fatty tissue. Almost all the literature about effects of PRP on fat graft maintenance show a promoting effect of PRP on survival of fat cells[25-28], except the paper of Por et al.[29], exposing that PRP has not a statistically significant promoting effect on survival of fat graft in a study performed on rats.

The PRP mixed with fatty tissue with the aim of obtaining a higher maintenance when fat infiltration takes place is widely used among doctors that practice lipofilling procedures. However, there are few histological studies published about the effect of PRP on the histological characteristics on the autologous fat grafting. The study

presented in this article shows an evidence of the promoting effect of PRP on the fat maintenance when infiltrated in the upper lips of rabbits. The histological translation of the effect of PRP increasing maintenance of transplanted fatty tissue is shown as a lower inflammatory reaction and lower signs of necrosis of the adipose cells when fat is infiltrated together with PRP. The degree of inflammatory reaction and swelling is pathologically translated as a lower survival for the adipose cells. The studies of Man[30] and Adler[31] show the effect of PRP on decreasing the edema during wound healing. The reduction of the postoperative edema led to an improvement in maintenance of fat grafting[32-33].

Despite the safety and minimal complications associated with fat grafting, the main limitation that has not allowed the widespread acceptance of autologous fat grafting has been its unpredictable success due to a low maintenance of its volume. The investigation presented in the article shows the PRP as an enhancer of the fat graft maintenance by decreasing the inflammatory reaction, and decreasing the cellular necrosis due to the inflammatory reaction in the earlier stages of lipoinfiltration. Authors like Bendinelli[34] report the anti-inflammatory effect of PRP by a reduction of COX2 and CXCR4 gens expression. The evaluation of the degree of viable fat by hematoxilyn-eosin staining was based on the anatomical disposition of the fatty tissue under magnification, as well as on the previous evaluated histological parameters; anyway, the evaluation of this parameter would require different diagnostic techniques like immunohistochemical marking (like PKH26 marking), specific lipid staining (like Sudan III stain), or stereologic exams, that were not the aim of this study[35].

CONCLUSIONS

Fat grafting means a variable but almost constant degree of swelling due to the technique and the many passes that it requires. Histologic examination of the fat graft suggests an early inflammatory response to the injected fat, much higher when isolated fat is infiltrated than when it is mixed with PRP. There is a sequestration of nonviable tissue due to the inflammatory reaction, what is pathologically expressed as oil cysts. The transplanted fat with PRP shows a statistically significant decreasing of this early inflammatory reaction and a decrease of the presence of oil cysts. By the mechanisms of decreasing the inflammatory reaction and decreasing the cellular necrosis of the transplanted fat cells, PRP increases the cellular maintenance of the transplanted fatty tissue, promoting the biointegration and proliferation of the fat cells in the new soft tissue where are infiltrated. However in this pilot study PRP may decrease inflammatory reaction in fat grafting procedures, further studies are necessary in order to confirm these results.

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***Authors' Response to Reviewer Comments**

Reviewer #1: This study attempts to evaluate the influence of platelet-rich plasma (PRP) on autologous fat grafting of the upper lip of rabbits. One side of the upper lip was infiltrated with autologous fat, and the other side with fat and PRP. Rabbits were euthanized at 8 weeks and 12 weeks after lip augmentation. Histological evaluation of the quality of the fat graft, the inflammatory reaction, the presence of oil cysts was made.

In considering the objectives of this study, namely improving long-term viability of autologous fat grafting, we are left with questions that dispute the conclusions drawn by the authors:

1. Can true assessment of fat survival be made at 8 or even 12 weeks following injection when we observe clinically that resorption is often maximally demonstrated between 4 and 6 months?

Answer:

Firstly, we have to remind that the metabolism of the rabbits is much faster for all kind of cells than the metabolism of human beings. For example, while in humans bone remodeling takes between 6 and 9 months, in rabbits it takes place in 6 weeks. Attending this fact, we suggest an example of study published in pubmed literature where the histological evaluation is made after 6 weeks postop.

- **Effect of combining platelet-rich plasma with anorganic bovine bone on vertical bone regeneration: early healing assessment in rabbit calvariae**. Torres J, Tamimi F, Tresguerres IF, Alkhraisat MH, Khraisat A, Blanco L, Lopez-Cabarcos E. Int J Oral Maxillofac Implants. 2010 Jan-Feb;25(1):123-9.

This article, published in the IJOMI in 2010 and written by one of the authors of the present paper, reports histological evaluation of the effects of PRP on calvarial bone of rabbits made 6 weeks after the procedure. Although this study is regarding to bone tissue, we present some papers that study the effect of PRP at different stages of healing in fat grafts.

Secondly, it has been demonstrated that the maximum effect of the platelet-rich plasma, and of the growth factors on themselves, takes place during the first week of the process of wound healing or of the process of biointegration of a transplanted tissue. It is widely exposed in the following review:

- Physiol Rev. 2003 Jul;83(3):835-70. **Regulation of wound healing by growth factors and cytokines.** Werner S, Grose R.

In the same order of demonstration in the field of fat transplantation we also have the following papers:

- Ann Plast Surg. 2010 Jul;65(1):101-6. **Platelet-rich plasma (PRP) promotes survival of fat-grafts in rats.** Nakamura S, Ishihara M, Takikawa M, Murakami K, Kishimoto S, Nakamura S, Yanagibayashi S, Kubo S, Yamamoto N, Kiyosawa T.

> This study evaluated the effects of platelet-rich plasma (PRP) on resorption and adipocyte survival in autologous fat-graft of rats prepared with isogenous PRP. Fat grafts prepared without PRP (control group) became united to the tissue adjacent to the implantation site and were significantly resorbed from 30 days. On the other hand, fat grafts prepared with PRP (PRP group) demonstrated little resorption from 30 to 120 days and appeared pink, had a soft, supple feel, and were easily compressible. Histologic sections of grafts in the control and PRP groups at 10 days exhibited similar consolidation of the grafted tissue, which contained morphologically normal adipocytes with different degrees of granulation and capillary formation. From 20 days normal adipocytes were obviously decreased in the control group, while the PRP group demonstrated increased granulation tissue and capillary formation and good maintenance of normal adipocytes for at least 120 days.

The effect of the PRP on the mesenchymal tissues has an early effect. It's also true that this effect increases cell survival on time. The effect of PRP on fat transplanted cells maintenance takes place in the first week of their contact.

- Rossatti B. Revascularization and phagocytosis in free fat autografts: an experimental study. Br J Plast Surg 1960;13:35-41.
- Saunders MC. Survival of autologous fat grafts in humans and mice. Connect Tissue Res 1981;8:85-91.

2. The study would indicate less inflammation (especially early on) but this does not translate long term into less fibrosis and increase volume of viable cells. This may relate to the small numbers tested or to length of time assessed. Either way, it is impossible to draw conclusions.

The statistically significant differences between the fat graft with and without addition of PRP are only for the parameters: "inflammatory reaction" and "presence of oil cysts". Regarding to the degree of fibrosis, PRP group showed a considerable decrease in the presence of fibrosis compared with isolated fat group at 8 weeks although did not reach statistical significance, while minor differences were observed at 12 weeks.

Now it has been corrected.

Corrected text:
CONCLUSIONS

Fat grafting means a variable but almost constant degree of swelling due to the technique and the many passes that it requires. Histologic examination of the fat graft suggests an early inflammatory response to the injected fat, much higher when isolated fat is infiltrated than when it is mixed with PRP. There is a sequestration of nonviable tissue due to the inflammatory reaction, what is pathologically expressed as oil cysts. The transplanted fat with PRP shows a statistically significant decreasing of this early inflammatory reaction and a decrease of the presence of oil cysts. By the mechanisms of decreasing the inflammatory reaction and decreasing the cellular necrosis of the transplanted fat cells, PRP increases the cellular maintenance of the transplanted fatty

tissue, promoting the biointegration and proliferation of the fat cells in the new soft tissue where are infiltrated.

3. Less oil cysts supposedly translates to less necrotic tissue with less fibrosis. This is not demonstrated - whether this becomes apparent long term is still unknown.

Thus the conclusion that PRP is as an 'enhancer of the fat graft that promotes the viability and the maintenance of the volume of the fat infiltrated that makes possible to avoid the overcorrection of the defect' cannot be extrapolated from this study.

I would therefore recommend that the study only be accepted if revised to a pilot study, stating that definitive conclusions cannot be made until increased numbers and length of study time are available.

Answer:

The text has been corrected attending to the comments of the reviewer.

In the corrected text the conclusions of the evaluation of the results of the study have been reported in a concise and strict way. The conclusions are that the PRP decreases the inflammatory reaction on the transplanted fat graft, and it also decreases the presence of oil cysts (oil cysts are a well-known histological sign of cellular necrosis).

We agree with the reviewer that with the results of this study cannot be extrapolated that PRP decreases the presence of fibrosis. But the decreasing of the inflammatory reaction and the decreasing of cellular necrosis on the early stage of fat infiltration can be concluded of this study. We agree with the reviewer that the very long term presence of cellular necrosis and cellular resorption cannot be extrapolated from our study, but the results show the effect of PRP on early stages of fatty tissue transplantation, that is the main stage where PRP and its growth factors act, as it has been documented in the first comment for this reviewer.

Adding PRP on fat grafting doesn’t avoid the fat resorption totally, but at least it has a demonstrated effect on decreasing the inflammatory reaction that is always associated to the lipoinfiltration technique. So, it decreases the cellular necrosis due to the inflammatory reaction associated to the procedure on the early stages of biointegration of the transplantated tissue during the first days after the procedure.

This study shows the histological characteristics that can be evaluated by hematoxylin/eosin staining. To evaluate the exact cellular maintenance of the transplanted fatty cells, other stain techniques should had been performed (like Sudan III). Anyway, we agree with the reviewer that it cannot be extrapolated that PRP makes possible to avoid the overcorrection of the defect when lipofilling procedures are performed, but we can conclude that the addition of PRP helps to avoid the cellular necrosis due to the early inflammatory reaction on filling soft tissues with fat.

It would be interesting to evaluate a more long-term effect of the addition of PRP on fat grafting. We have based our investigation on the literature of the biology of PRP (mainly on the bibliographic references 3-4-5-6, but also on other references), and it shows that the effect of the growth factors takes place during the first week of contact with the cells in wound healing. Our investigation shows this early effect. The literature about Growth Factors also suggests an increasing of the revascularization and growing capacity of the cells where they act, what we have not been able to demonstrate with our

study. It would require specific lipid staining, stereologic evaluation, and, as the reviewer suggests, an increased length of study time. It can be the purpose of a different investigation that the investigation presented in our paper.

About the number of rabbits used for the presented investigation, we consider that this number of rabbits (nine) has been enough to demonstrate that PRP decreases the inflammatory reaction and the presence of oil cysts even if $n<30$, showing a statistically significant value of “p-value” with the non-parametric test using for the evaluation of the results.

Reviewer #2: The paper evaluated the influence of PRP on the histological characteristics of the autologous fat graft on the upper lip of rabbits, it is an interesting issue in Plastic Surgery, it is an original aspect of the problem, but the paper has many problems and needs major revisions prior to a resubmission.
The methods as they were presented here are not reproducible. Scientific method should be performed by any research only by reading the paper, so all information has to be pointed.

1.)Animal experimental scientific paper has to show the number of approval of the Ethics Committee of the Institution?

Prior to beginning the "*in vivo*" animal study the protocol was approved by the ethical committee for animal experiments of the Rey Juan Carlos University (URJC). Experiments were conducted in accordance with the guidelines laid down by the European Communities Council Directive of 24 November 1986 (86/609/EEC) and adequate measurements were taken to minimize pain and discomfort in the animals.

2.)Weight and age of animals?

Nine healthy 13 week-old male New Zealand rabbits weighting between 2.5-3 kg were used.

3.)What kind of installations, feedings, care, analgesia post-operative used in animals?

The animals were accommodated in the official stable for animal assays of the URJC at 22-24 ºC with 55-70 % humidity, light cycles of 12 hour, and air renewal 15 times per hour. The rabbits were fed with a Panlab® (Barcelona, Spain) diet while drinking was permitted ad libitum.

All rabbits were anesthetized with an intramuscular dose of 0.75 mg/kg ketamine (Imalgene 1000®, Rhone Merieux, France) and 0.25 mg/kg xilacine (Rompun®, Bayer, Leverkusen, Germany).

Post operative antibiotics were administered, Terramicina® (Pfizer, Spain), in water (4g/l) for 3 days. Also analgesics (0.1ml/kg) were administred Buprex® (Shering-Plough, Madrid, Spain)

4.)Animals stayed all procedure under anesthesia, from blood collection, PRP preparation, lipoaspiration, fat sedimentation, preparation of fat grat plus PRP and the injection of fat on the lip?

All surgery stage procedures except blood collection were done under the following antesthesia protocol: (intramuscular dose of 0.75 mg/kg ketamine (Imalgene 1000®, Rhone Merieux, France) and 0.25 mg/kg xilacine (Rompun®, Bayer, Leverkusen, Germany).

5.)Describe more about aqueous component drained.

The aqueous componend drained was mainly de infiltration solution. After the sedimentation of the fat, it was decanted.

6.)To obtain blood from animals, they are under anesthesia?
No.

7.)From 4 ml blood collected, which final volume obtained?

10 cc of whole blood was withdrawn via ear venous aspiration into 5 cc test tubes and mixed with a 3.8 % sodium citrate solution at a ratio of 1 cc sodium citrate solution to 5 cc whole blood, achieving anticoagulation through calcium binding. The blood was then centrifuged following the technique described by Anitua[9-10] with a Nahita® centrifuge (Navarra, Spain) into three basic components; red blood cells, PRP, and platelet poor plasma (PPP). Because of differential densities, the red blood cell layer forms at the lowest level, the PRP layer in the middle, and the PPP layer at the top. A pipette (Gilson, France) was used to separate each layer, from the less dense to the more dense; therefore PPP was separated first (about 1.25 cc) and PRP second (about 1.25 cc), leaving the residual red blood cells (about 2.25 cc).

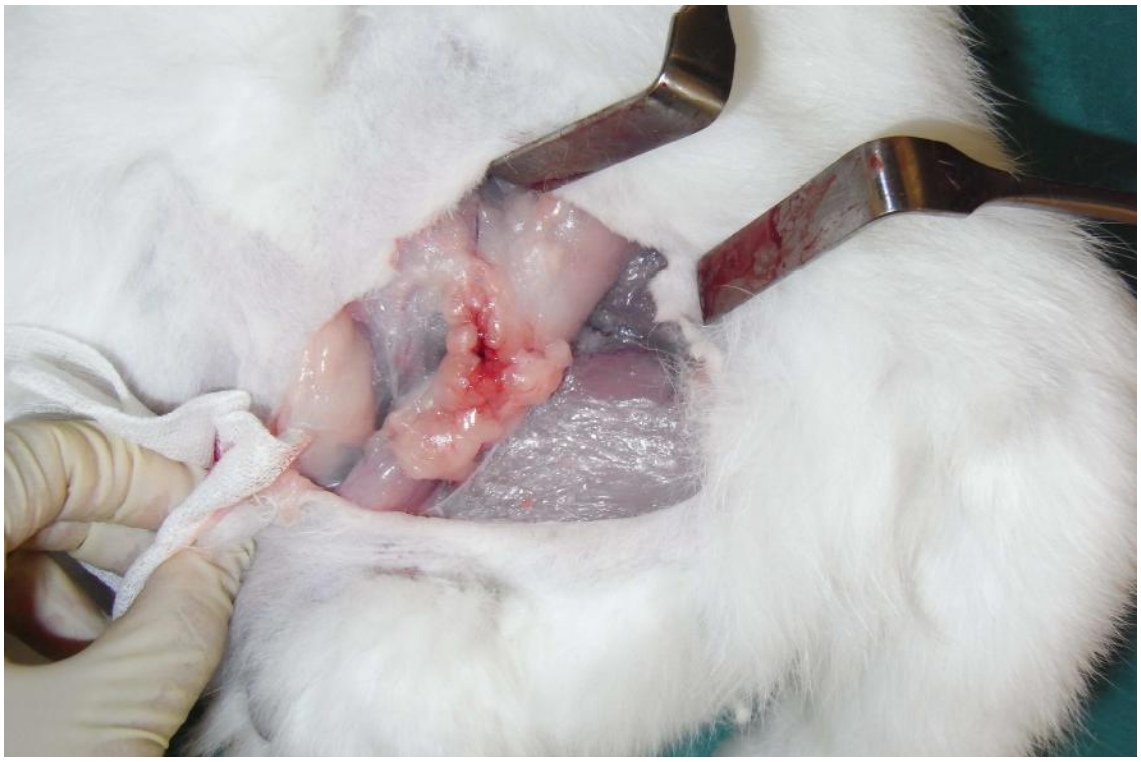
8.)What were the dose of drugs of anesthesia?

Post operative antibiotics were administered, Terramicina® (Pfizer, Spain), in water (4g/l) for 3 days. Also analgesics (0.1ml/kg) were administred Buprex® (Shering-Plough, Madrid, Spain)

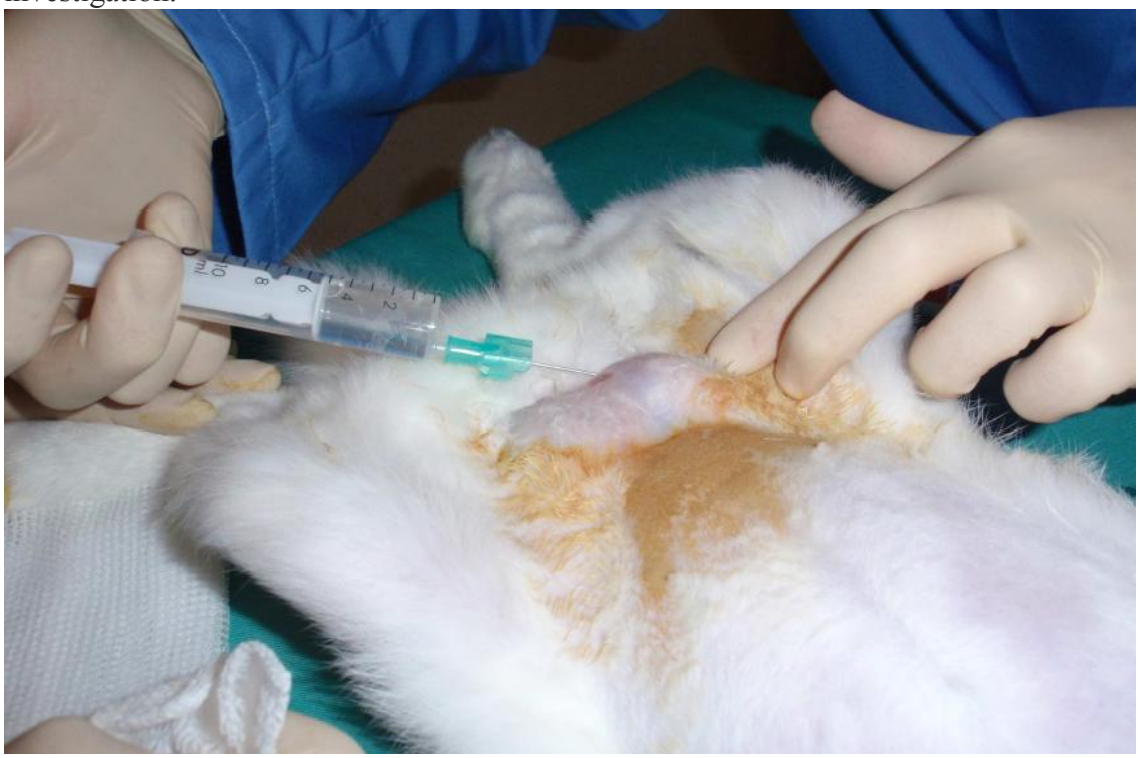
9.)What the size of groin area was infiltrated with 30cc, and the depth of this infiltration?

Both whole groin fat pads were totally infiltrated with the solution. Previously to the presented investigation, we performed laparotomy dissection of other rabbits used in another investigation to check the anatomy of groin fat pads.

Needle was no more than 5mm depth for the infiltration of the solution.



Dissection to check the fat pads. As it can be seen, they are easily reachable with a superficial infiltration. This specimen was not a specimen used in the presented investigation.



Infiltration with the wetting solution in one of the specimens of the investigation.

10.)What was the sedimentation time of the far lipoaspirated?
Sedimentation time was around 10-15min. The aqueous component of the fat lipoaspirated was quickly separated of the fat phase just by sedimentation.

11.)The experiments were made as a double-blind experiment?

Neither researcher that injected fat on the lip nor the pathologist that read the images knew what group were from the sample?

Yes, this is really a double-blind experimental study.

12.)Why at 8 weeks evaluation there were no control group?

The same control case euthanized at 12 weeks can be used as control case for the group biopsied at 8 weeks, cause it was a virgin specimen.

13.)How the animals were submitted to euthanasia?

Sodium barbiturate (pentobarbital) (Dolethal®; Vetoquinol, Lure, France): 1cc/Kg intravenous.

14.)It is appropriate to a good scientific contribution of knowledge that the two different kind of treatment could be applied to a small area, superior lip in each half, without any block to this two different treatment not to be mixed? It will be more appropriate to make each different treatment in a different animal?

We consider that the split mouth design is the better study design in order to evaluate differences into the same intrinsic characteristics of an animal. Regarding the block of this two different treatments not to be mixed, is guaranteed because of the split lip of rabbits that block in a natural way both treatments

Most experimental animal studies evaluating fat graft maintenance evaluate fat blocks. Some prominent studies also used Coleman technique. We wanted to reproduce the usual clinical practice, by evaluating the maintenance of the fat grafted by a Coleman technique.

Previously to perform the investigation, literature review was strictly made. In the literature we find the following main animal studies evaluating fat maintenance:

- Karacaolgu E. “The role of recipient sites in fat-graft survival: experimental study”. Annals of Plastic Surgery 2005.
    - In this study groin fat pats are transplanted as a block to the gonial aspect of the mandible of New Zealand rabbits.
- De Giacomo C. “Assessment of collagen deposits after implant of fascia lata and fat in the vocal folds of rabbits: histomorphometric study”. Rev Bras Otorrinolaringol 2005.
    - In this study cervical fat is infiltrated in small diposits in the vocal folds of rats.
- Altman JI. “Demineralized bone matrix and fat autograft in a rabbit model of frontal sinus obliteration”. Otolaryngology-Head and Neck Surgery 2007.

    - In this study dorsal fat blocks by direct excision are used to obliterate frontal sinus in rabbits.
- Brucker M. “Long-term fate of transplanted autologous fat in novel rabbit facial model”. Plast Reconstr Surg 2007.
    - In this study fat from groin fat pads harvested by a modified Coleman technique is infiltrated in the upper lip of rabbits.
- Palma PC. “Effect of purified collagen on lipograft survival: experimental basis for periurethral lipoinjections”. J Endourol 2003.
    - In this study free fat tissue was transplanted to the preauricular region.
- Eppley BL. “Autologous facial fat transplantation: improved graft maintenance by microbead bioactivation”. J Oral Maxillofac Surg 1992. Free fat tissue transplanted with the addition of Fibroblastic Growth Factor.
- Eppley BL. “A physicochemical approach to improving free fat graft survival: preliminary observations”. Aesthetic Plastic Surgery 1991.
- Karaçal N. “The effect of fibrin glue on fat graft survival”. J Plast Reconstr Aesthet Surg 2007. Groin fat pads of rats were transplanted to subdermal pockets with the addition or not of PRP.
- Nakamura S. “Platelet-rich plasma promotes survival of fat-grafts in rats”. Ann Plast Surg 2010. Free fat with the addition of PRP.
- Hong SJ. “Enhancing the viability of fat grafts using new transfer medium containing insulin and beta-FGF in autologous fat transplantation”. J Plast Reconstr Aesthet Surg 2010. Blocks of fat from the groin fat pads transplanted to the dorsal region of New Zealand rabbits.
- Pires Fraga MF. “Increased survival of free grafts with PRP in rabbits”. J Plast Reconstr Aesthet Surg 2010. Fat blocks harvested from the scapular region and transplanted to the retroauricular region of rabbits, with the addition of PRP.

15.)Why in this study were not realized bio-integration and cell proliferation evaluation?

Our study was made with the aim of doing a histological general evaluation of the main parameters of the transplanted fat cells. We evaluated the samples under optical microscopy. Immunohistochemical techniques were not at our disposal to evaluate biointegration, cell proliferation, or other parameters that require more complex techniques based on biomarkers.

16.)The experiments with animals were made in a aseptic way? From figure 2 there were not.

Clorhexidine was used as chemical antiseptic on the abdominal region before starting the procedure, as well as in the perioral region of each animal.

17.)Were there any complications, as infection, animal lost?
There were no complications. No infections appeared in the specimens presented in the investigation.

18.)How was evaluated the fat viability? Objective way?

Fat viability was evaluated together with two pathologist doctors. Evaluation of fat viability was based on the previous histological parameters evaluated and graded (oil cysts, fibrosis), as well as in the anatomical disposition observed under magnification. Anyway, it has been added in the discussion this parameter should be better evaluated by specific lipid staining or stereologic measurement.

19.)The conclusions were not wrote as conclusions, but as Discussion, and should be rewrite.
It has been rewritten.

20.)References list didn´t follow Vancouver' style.

Below we copy and paste references of an article published in *Aesthetic Plastic Surgery* 2004. In our paper, references have been listed in the same way as in this article, following Vancouver' rules.

Aesth. Plast. Surg. 28:334–339, 2004
DOI: 10.1007/s00266-004-3121-7

Aesthetic Plastic Surgery

The Fate of Intramuscularly Injected Fat Autografts: An Experimental Study in Rabbits

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We have followed the rules dictated in the following sites:

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www.nlm.nih.gov/bsd/uniform_requirements.html
www.metodo.uab.es/enlaces/2006%20Requisitos%20de%20Uniformidad.pdf

21.)Only 5 papers appear in PubMed check using PRP and fat graft, and in the Discussion in this paper was not used the paper of Por YC, Yeow VK, Louri N, Lim TK, Kee I, Song IC; J Plast Reconstr Aesthet Surg. 2009 Aug;62(8):1030-4. Epub 2008 Jun 11. Platelet-rich plasma has no effect on increasing free fat graft survival in the nude mouse.

This article of Por has been added to the references and mentioned in the discussion as suggested by reviewer.

Table

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	Inflammatory reaction	Oil Cysts	Fibrosis	Neovascularization	Viable Fat
Hemilips Isolated Fat 8 weeks	5.25/8	4.75/8	5.25/8	1.5/8	6.5/8
Hemilips Fat + PRP 8 weeks	0.25/8	0.5/8	1.5/8	2/8	7.5/8
Wilcoxon test					
Statistic	0.5	16	14.5	6	5.5
p-value	0.03599	0.02021	0.07959	0.65924	0.50499
Hemilips Isolated Fat 12 weeks	2/8	2.25/8	4.5/8	2.5/8	6/8
Hemilips Fat + PRP 12 weeks	0/8	0/8	3/8	2.75/8	8/8
Wilcoxon test					
Statistic	1	15	10	8.5	4
p-value	0.04853	0.04853	0.65317	1	0.18136

Table 1. Results and statistical analysis using a Wilcoxon test.

Figure
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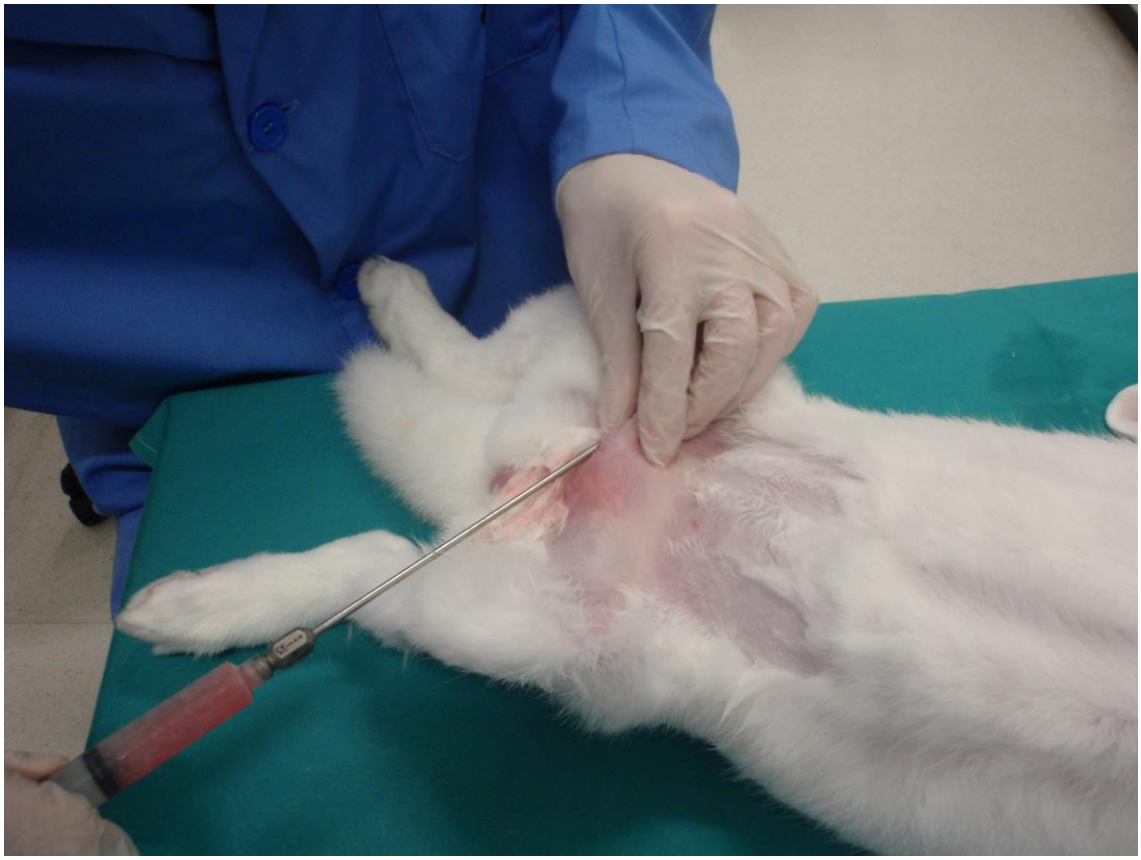

Fig.1. Harvesting of the fat tissue from the groin fat pads using a lipoaspiration Coleman cannula 3mm width and 15cm length with a blunt tip. A 10mL syringe is attached to the cannula for the aspiration of fatty tissue.

Figure

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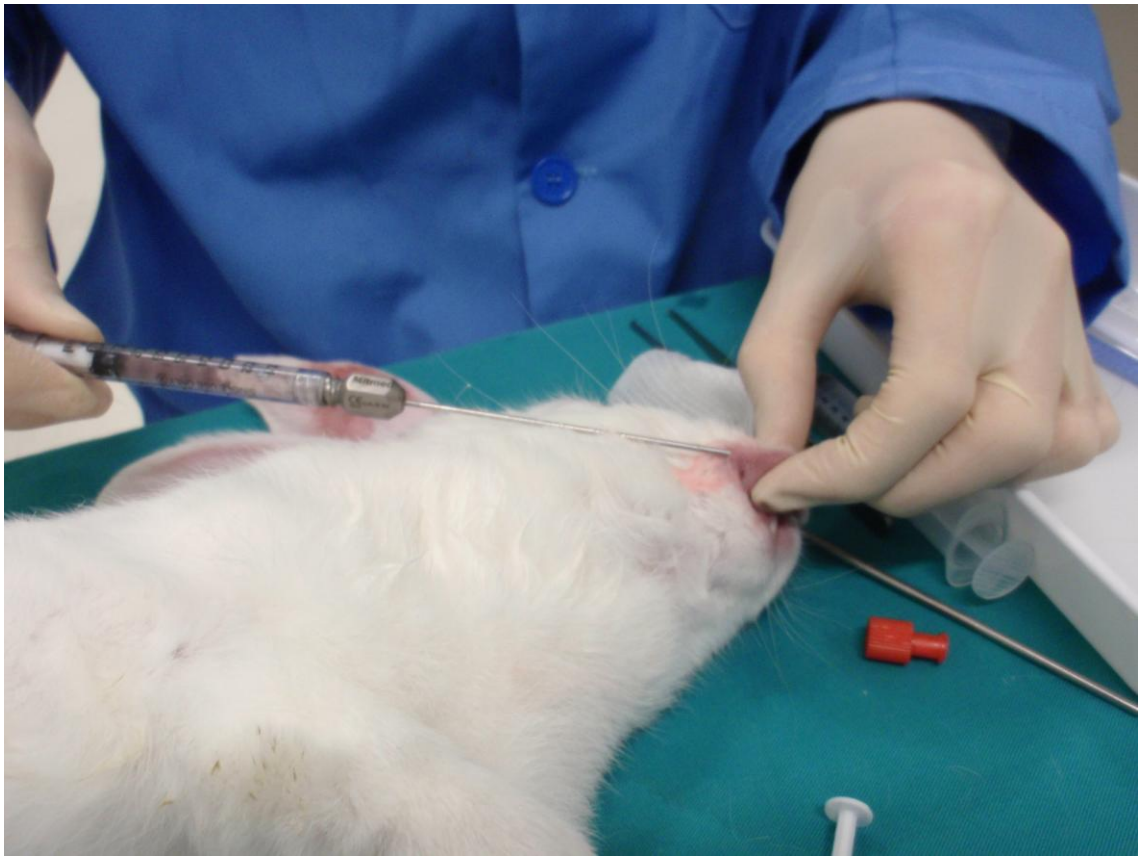

Fig.2.Lipoinfiltration of the right upper lip of the rabbit using a blunt Type I Coleman infiltration cannula and a 1mL syringe.

Figure

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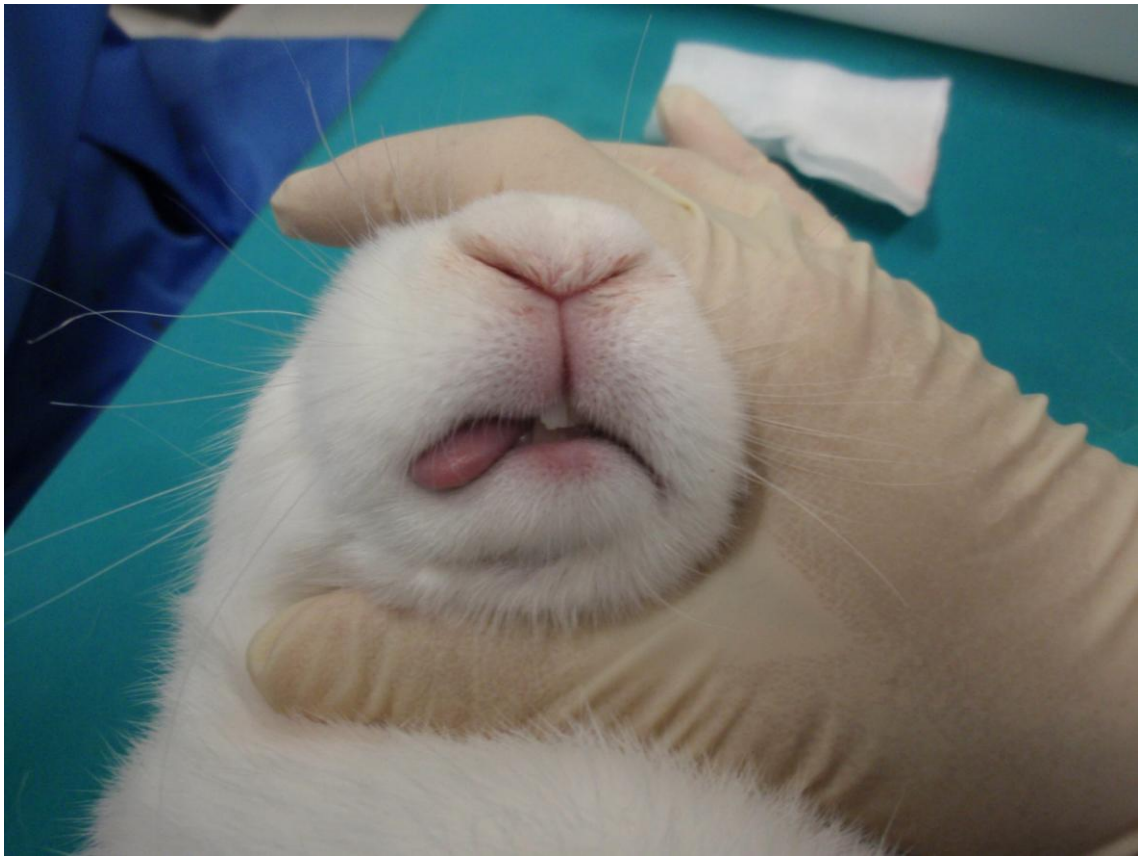

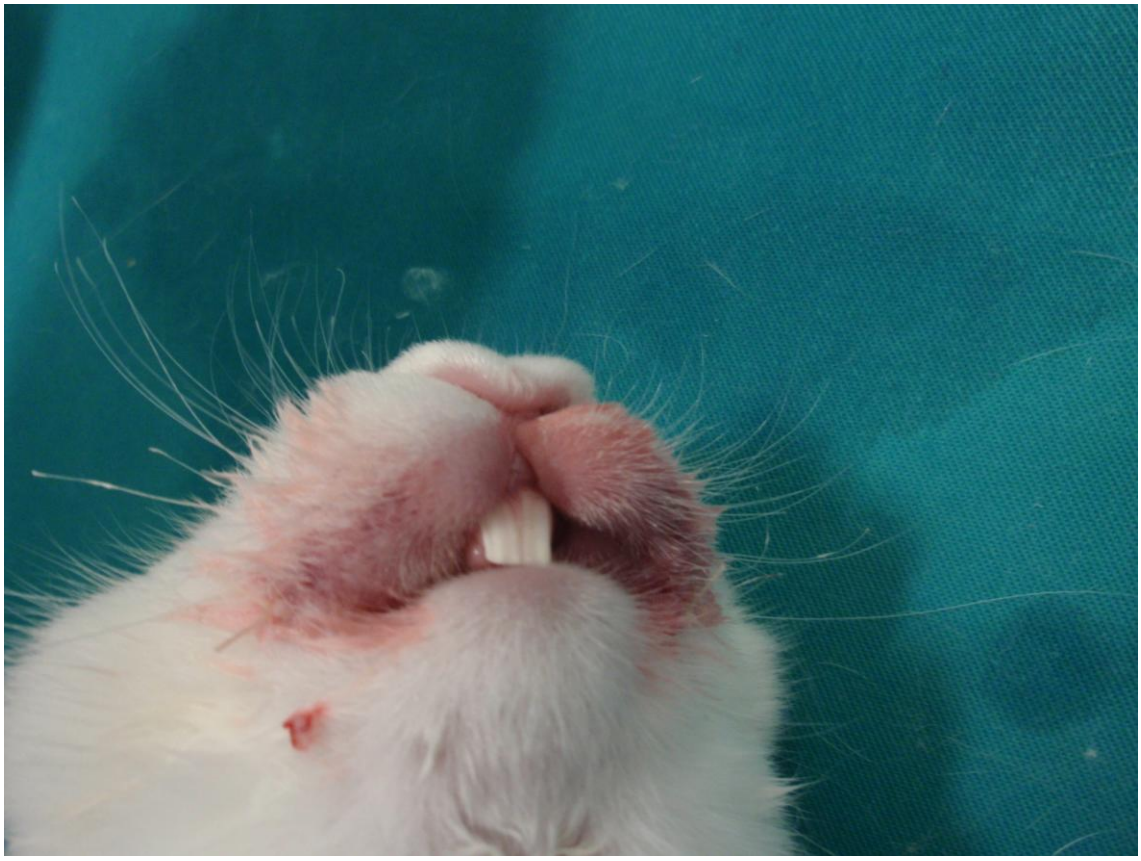

Fig.3 and 4. Before and after lip augmentation of the upper lip in one of the rabbits.

Figure

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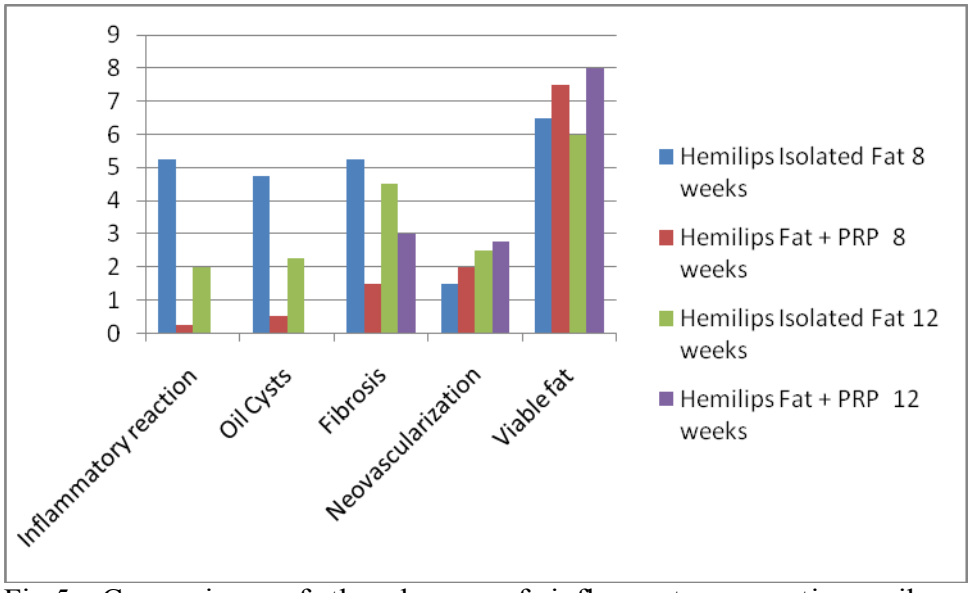

Fig.5. Comparison of the degree of inflammatory reaction, oil cysts, fibrosis, neovascularization, and viable fat at 8 and 12 weeks after lip augmentation with isolated fat and with fat plus PRP.

Figure
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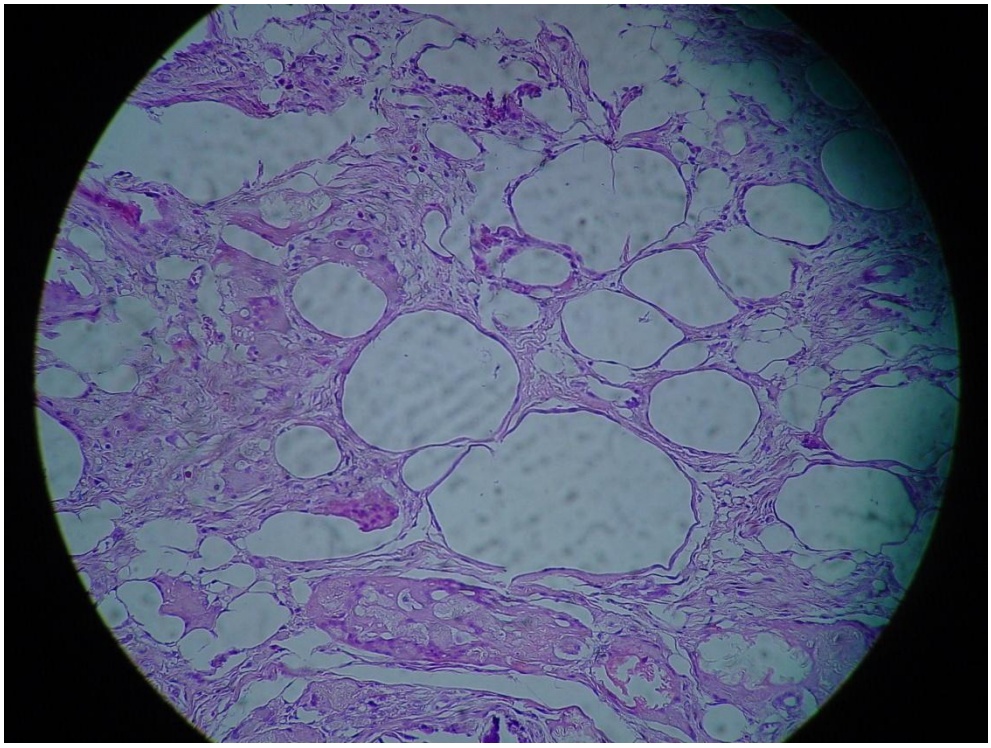

Fig.6. Section of specimen infiltrated with isolated fat at 8 weeks after infiltration under microscopic magnification (x20). Oil cysts can be seen in this field. We can also appreciate eosinofilic infiltration and some giant multinucleated cells as an expression of an important degree of inflammatory reaction.

Figure

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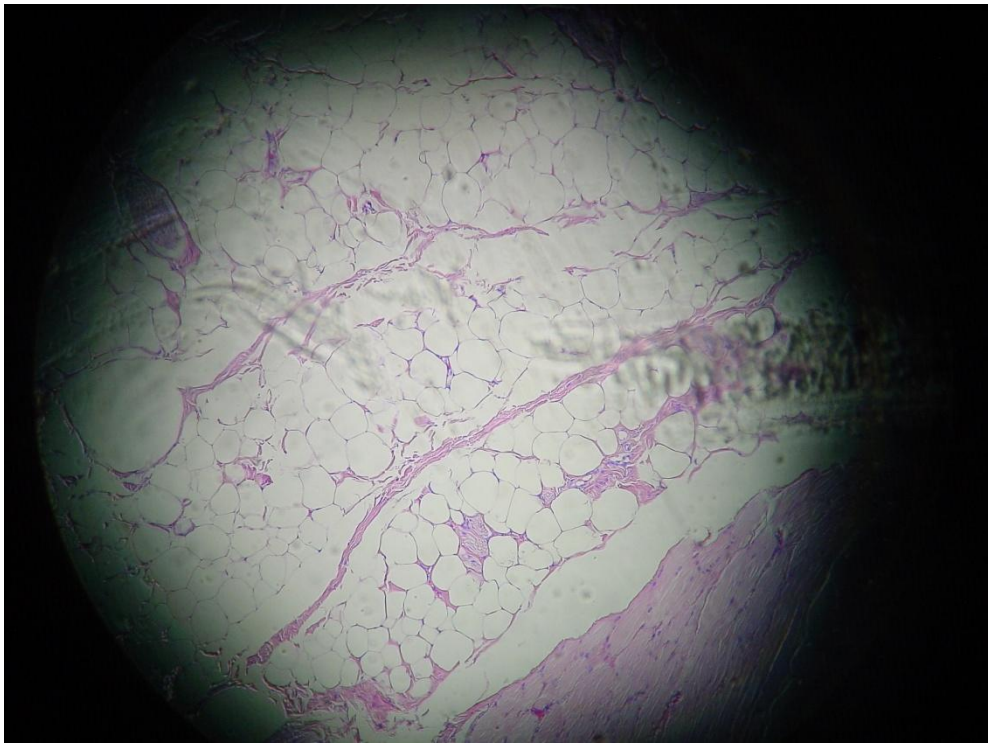

Fig.7.Section under microscopical magnification (x20) of an hemilip with fat plus PRP at 8 weeks after infiltration. There is no inflammatory infiltration, no important signs of necrosis or fibrosis, and a nice disposition of the fat cells. A nice viable fat tissue is perceived in this field.

Figure

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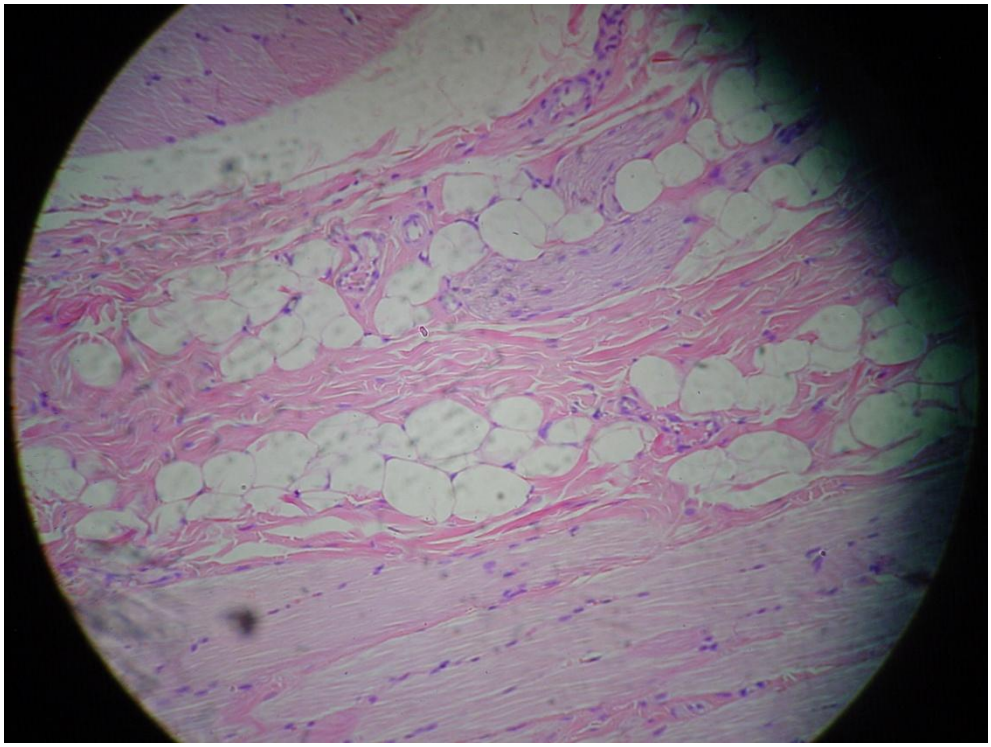

Fig.8.Section of lip at 12 weeks after infiltration of isolated fat tissue, under microscopical magnification (x20). No oil cysts appear in this field. A low inflammatory reaction appear in this image. It can be appreciated a 4/8 degree of fibrosis.

Figure

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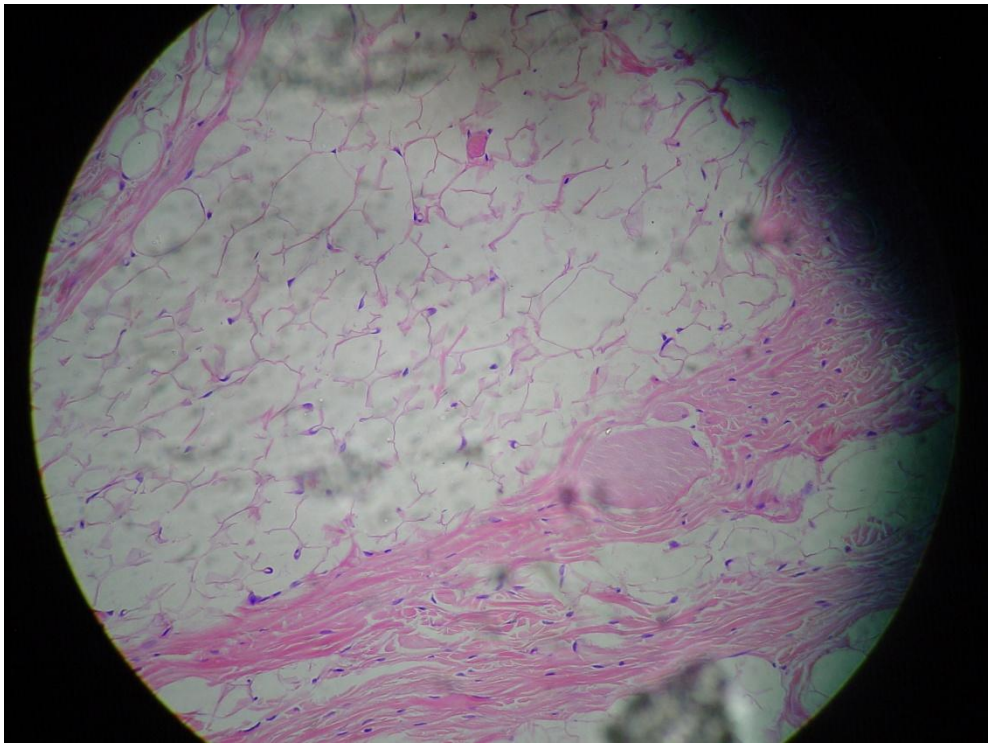

Fig.9.Section under magnification (x20) of specimen at 12 weeks after infiltration using fat with PRP. There is 3/8 degree of fibrosis. There is a proper disposition of the fat tissue.