Format for ANSWERING REVIEWERS

Dear Editor,

Please find enclosed the edited manuscript in Word format as requested. (file name: ESPS_MS13411R1_ACL_MSC.docx, ESPS Submission number 13411).

Title: Mesenchymal stem cells and collagen patches for anterior cruciate ligament repair

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Name of Journal: *World Journal of Stem Cells*

ESPS Manuscript NO: 13411

We thank you and the four reviewers on the valuable comments to help to improve considerably our manuscript. We also thank the editor to invite us for a revision of the manuscript.

The current version of the manuscript comprises 4,850 words, 2 tables, 8 figures and 40 references. Figs 7-8 were improved according to the comments of the four reviewers.

The images can be downloaded as EPS files as EPS files or Photoshop files with all layers editable in the provided download links. These files can be edited with any vector software.

We also provided the text as a Microsoft Word .docx file as requested. We also improved the language and the formatting of the manuscript to be matched the style required for publication in WJSC.

The manuscript was sent to a proofreading agency (Proofit, inc. CA, USA) to improve its language and grammar.

We paid attention to fulfill all of the reviewer's comments. However, the request of reviewer #4 02615988 to replace the cell activity resazurin method with an alternative method we could not fulfill since all of the experiments would need to be repeated as this assay uses live cells and cannot be done on fixed samples. We revised the manuscript text and marked the changes in color according to the four reviewer's comments in the manuscript.

Thank you again for publishing our manuscript in the *World Journal of Stem Cells.*

Sincerely yours,

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Reviewer #1 (00462683)

1) The authors suggest that collagen patches that have proven beneficial for repairing cartilage and meniscus may be suitable to repair the anterior cruciate ligament (ACL). Although the results are very preliminary, are very encouraging.

Answer: We thank the reviewer for this statement. We agree that these results are promising. In the nearer future we will focus on combining cell adherence with cell stretching experiments. This publication is intended to provide for the first time primary cell culture data from TC isolated from human ACL and to explore the regenerative potential of seeding these cells onto these commercially available collagen scaffolds.

2) Figure 9 shows as TC, when seeded on NC patch, overexpress collagen-2, unlike what is observed when they are grown on CG patch. How do you interpret this result? The combined use of MSC and TC provides some benefit to using only TC?. Although the results are preliminary, the authors could give their opinion.

Answer: This result for TC is non-relevant as it was found to be non-significant (P = 0.9797, the mean deviation from 1.0 was only -0.02319. However, we re-analyzed the statistics of the gene expression data and found that TC grown on culture inserts significantly down-regulated collagen type 2 (col2). For TC on NC patches we found a significant down-regulation of tenomodulin (TNMD) in terms of an autochthonous TC phenotype may be seen as a sign of dedifferentiation. The combined culture of hMSC with TC led to the significant up-regulation of collagen type 3, a result, which may be interpreted as a first sign of differentiation of MSC in the presence of TC with cell-cell contact. It has been shown by several groups that increase of TC atypical gene expression such as production of proteoglycans, e.g. aggrecan or collagen type (see Stoll [2010] J Orthop Res 28(9):1170-1177) is not preferred. In the light of these results, we may conclude that already after 7 days of 3D culture of NC patches, CG seem to be superior over NC for TC. We added a short paragraph starting on page 12 line 29.

3) Minor comments: Page 5, line 19: Define DIS Some errors or confusion in the naming of the figures and tables in the text should be corrected: Page 16, lines 29 and 31: Results in the text refer to Figure 9, and not to Figure 6

Answer: We carefully went through the text and figure legends. We adjusted the formatting to match the requirement of the journal WJSC. We also defined all the abbreviations in the figure legends as requested by the editor.

Page 17, lines 18 and 19: Table 2 must be changed by Table 1

Answer: This has been changed.

Page 18: Lines 3-4: Figure 5 must be replaced by Figure 7

Answer: This has been changed.

4) Page 18: Lines 9 and 10-11: Figure 7 must be replaced by Figure 8

   Answer: This has been changed.

5) Figure 7: Given the order of appearance of the results in the text, it would be more convenient than the panel B of Figure 7 was actually the panel A.

   Answer: We changed the order of panels in figure 7 in the revised version as suggested.

6) Data presented in Figure 8 (ratio of OHP/GAG) could be included as panel C in Figure 7

   Answer: We added Figure 8 as panel C to Figure 7 and renamed the figures, figure legends and the references within the text.

Reviewer # 2 (01000364)

This paper by Horovitz et al. compared two different collagen patches seeding with either bone marrow mesenchymal stem cells or primary tenocytes for possible anterior cruciate ligament repair. Chongrogide is composed of porcine collagen I and III as a bilayer membrane. Novocart is a biphasic, three-dimensional collagen-based matrix from bovine. Both of collagen sponges have been used for cartilage repair. ACL injury is a common disease and the repair of ACL remains a challenge due to its limited self-regenerative ability. Despite of the progression of reconstructive techniques for ACL repair, an ideal biological scaffold for ACL reconstruction is needed. Thus, this study is of importance. However, the following concerns should be addressed.

1) Overall, the written need to be improved. For example, in the abstract, the first two sentences of methods are background information.

Answer: We corrected the manuscript and checked carefully for the language and grammar throughout the manuscript. We gave the final version to a certified proofreading agency (see attached receipt).

2) Figure 4, in the chondrogide (CG) scaffold, the number of MSCs is low on both Day 1 and 7. Both TC and MSC decreased in the 1:1 premix. This should be described in the result section on page 12. Is the low number due to z scan level? Is the magnificent the same for the SEM of Novocart scaffold?

Answer: We thank the reviewer for pointing out this question. Yes, the magnification and scanning window was identical for both scaffolds, which was 200 µm stacks, which were projected in the z-axis as a single image. This information is provided in the figure legend. We also added a sentence describing these results of a low cell density on CG.

3) Figure 5 shows no difference in DNA content but figure 4 shows less MSCs on day 7. Is this discrepancy due to the large amount of DNA content in scaffold itself?

Answer: Thank you for pointing out this discrepancy. This is a possibility. However, it is also likely to be a donor-specific phenomenon of the cells seeded on the scaffold for cLSM microscopy, which possibly does not represent the standard for MSCs. We added discussion on this part on page 15 line 8.

4) Figure 6 conflicts with figure 4 if the cell number of MSCs decreased on day 7 is true. In the method part, it states that the resorufine signal is proportional to the number of living cells and corresponds to the cell metabolic activity. If the number of MSCs on day 7 is decreased, would the fluorescence intensity be decreased? Please explain.

Answer: This is indeed an apparent conflict in the data. We have tested the resazurin red assay now on a number of different primary cells and cell lines in our lab and yes, the signal is linearly correlated to the number of cells under a certain culture conditions, e.g. with and without fetal calf serum or cultured in hypoxia or normoxia. We think, that the conflict in the data is a donor-specific phenomenon of the microscopy data and does not represent the mean of the 5 co-culture repeats. We added a statement in the discussion on page 16 line 16.

5) Figure 7, the GAG content of premix is lower than other groups on day 7. Please explain.

Answer: This could be the result of a positive interaction on the side of the MSC that start differentiating towards a more tenogenic phenotype. We did not monitor the gene expression profile of these mixed cultures since we cannot distinguish the RNA from the two cell populations in the "premix" situation. We added a sentence in the discussion explaining this result: "However, interpreting the results of the RT-PCR data demonstrating a significant increase of col3 in presence of TC on culture inserts together with the significantly increased OHP/GAG ratio of "premix" of TC and MSC on the 3D patches (Fig 7C) could be interpreted as an early differentiation of MSCs shutting down gene expression of GAG and increasing collagen expression."

6) A detailed description of gene change should be given in the result section.

Answer: we improved the results section by explaining the significant changes and trends of the gene expression data on page 15, lines 12-15.

7) Please discuss the advantages of the current strategy over the other tissue engineering techniques for ACL repair. 8. Biomechanical tests would add more value for the study.

Answer: We agree with the reviewer that biomechanical tests of the ACL and/or the collagen patches would add value to the manuscript. However, we are currently designing an experiment to apply strain-controlled loading onto these collagen patches but these novel results are still too preliminary to be reported here.

Reviewer # 3, 01237968

In this manuscript, the authors reported the effects of two different collagen scaffolds, Chondro-Gide (CG) and Novocart (NC) on anterior cruciate ligament (ACL)-derived tenocytes (TC) and bone marrow-derived mesenchymal stem cells (MSC). The results indicated that both TC and MSC adhered to CG and NC, and their mitochondrial activity increased after 7 days in culture. Meanwhile the number of TC was higher than that of MSC cultured on CG. Hydroxy-proline (OHP) and glycosaminoglycan (CAG) ratio of TC and MSC mixed culture was higher than that of TC or MSC alone. Quantitative RT-PCR analysis showed that the collagen scaffolds did not have significant effects on the expression of the ligament marker genes.

1) The authors tried to characterize the effects of CG and NC on TC and MSC, but the data as well as discussion are unfortunately preliminary and/or not informative. Most of the data including those of DNA content (Fig.5), OHP and CAG amounts (Fig.7) and the expression of the ligament marker genes (Fig. 9), failed to show significant differences between the controls and the CG or NC-treated cells.

Answer: We carefully went through the statistics of the RT-PCR data again. We found that relative gene expression revealed a significant down-regulation of TNMD for TC grown on NC scaffolds (P <0.0001) and for col2 in TC grown on culture inserts (P = 0.0121) relative to day 0 controls (Fig. 8A). Monolayer MSC significantly down-regulated MMP3 (P = 0.0065) after 7 days of culture, whereas MSC grown on culture inserts significantly up-regulated col3 (P = 0.006) and down-regulated MMP3 (P = 0.0065).

2) In fig.4, the number of TC and MSC cultured on CG was less than that on NC, and the number of MSC was less than that of TC, but reasons of the differences such as differential cell attachment onto the substrate, cell proliferation or cell survival were not shown.

   Answer: we can only speculate at this time why MSC did not like the CG so much as the TC seem to prefer the CG scaffold as well compared to NC. We would need to do additional experiments to confirm this result. This we plan to do in future experiments where we apply mechanical loading on the collagen patches.

3) In Fig.6, mitochondrial activity in TC and TC/MSC mixture cultured on CG significantly increased after 7 day in culture compared with that after 1 day. However, it is totally unclear how much CG was effective, because comparison of the activity in TC cultured on CG with that cultured in appropriate control condition was not shown. In addition, the authors claimed that ‘ patches promote adherence and proliferation of cells’ in page 2, line 30, but the data only showed mitochondrial activity.

   Answer: We agree with the reviewer that a comparison to a monolayer plastic control was not done. As we choose to digest the carrier with the embedded cells entirely with the addition of papain and then quantify from the digestion the amount of released DNA, total collagen content and glycosaminoglycan production there was no good possibility to compare this to a proper monolayer only control. It is also correct from the reviewer that the data of the resazurin red assay is showing cell activity rather than cell number.

4) In Fig. 8, OHP/GAG ratios in TC and MSC mixture significantly increased compared with those in TC or MSC alone, but meanings of the changes were not appropriately discussed. The authors claimed that ‘the cells are modifying the underlying matrix’ in page 3, line 4, but it is unclear what it means.

Answer: We analyzed again the OHP / carrier and the GAG content / carrier and comparing these it seems clear that MSCs and TCs in co-culture produced less GAG than the other groups and contained more HOP (Figure 7A, B). This resulted in a significant increase of the HOP/GAG ratio. Possibly because MSCs started to differentiate towards a TC-like phenotype and produced less GAG and were stimulated by an increased collagen production. The same trend as we found on culture inserts, where we could analyze the gene expression of both cell populations. Unfortunately, we were not able to distinguish the RNA expression profiles this time but it would be interesting in a future experiment to analyze the transcriptome profiles of "premix" cell populations, which possibly needed to be separated again by cell sorting prior RT-PCR. We changed our interpretation to: " This suggests that the MSCs are changing in phenotype and possibly produce less GAG over time if in direct 1:1 co-culture."

5) In Fig.9, the expression of the tested genes was not significantly changed in TC and MSC among the tested cultured conditions, but the authors discussed the changes of the expression in page 3, line 5-6, and in page 15, lines 3-17, which is not appropriate.

   Answer: We carefully went through the statistics again and found significant deviations from the mean value of 1.0 (= baseline day 0 state of cells) indeed, which is possibly the fairest way to test for significance for relative RT-PCR gene expression data**.** Previously we only tested for Kruskal-Wallis among groups, which was not significant. We limited the discussion now on the significant changes in gene expression relevant for the TC phenotype on page 16 line 12.

6) Additional points; 1. page 3, line 9-10: A meaning of this sentence is not clear. 2. Fig.5: Control samples without cells had significant amounts of DNA, indicating that NC and CG contain DNA. However why NC and CG with cultured cells did not have more of DNA?

Answer: This is a good point. We quantified the DNA using two different fluorescent kits, one with Hoechst dye and a different dye using a different fluorescent spectrum, which was pico Green™ (life technologies, inc. Basel). We did not find any different results. We also digested samples of “material only control” with DNAseI to test for porcine DNA. And yes, we confirmed the existence of high DNA amounts in the samples. We think, the reason why we do not see a significant increase after 7 days is that the DNA produced of the cells seems minor with respect to the large amount of DNA already present in the collagen scaffolds. We added this point to the discussion.

7) Page 17, lines 27-30: It is unclear what the authors intend to mean, because MSC and TC were labeled with different fluorescent dyes and could be clearly distinguished.

Answer: we agree that the description of the cell morphology was misleading and we deleted the sentence: “It is likely that the cell morphology of the MSCs changed in a way that they became harder to identify even with CLSM…”

Reviewer # 4 02615988

Since Dynamic Intraligamentary Stabilization (DIS) approach in anterior cruciate ligament (ACL) repair has shown promising clinical benefits. This study aimed to study insight as to whether the collagen patches that are currently approved and marketed for cartilage and meniscus repair are also suitable for ACL repair, and elucidate the extent to which they impact and interact with cells. They tested two commercially available collagen scaffolds with regards to cell viability, adherence and proliferation of seeded tenocytes (TC), the primary cell type in ACL tissue. Overall this is a beneficial study, however, some concerns need to be addressed.

1) Methods section in Abstract is too long. The metabolic cell activity methods used in the manuscript is not clear and authors should use alternative methods to show metabolic activity of cells. To further support the

Answer: The resazurin red assay, which is known as Alamar Blue© from companies like Invitrogen (now life technologies inc., Basel) and also as Cell titre Blue© from Promega are highly established kits and methods (see also Xiao et al. [2010] Appl Biochem Biotechnol 162(7):1996-2007). These metabolic activity assays or also used as cytotoxicity assays are now multiple times evaluated and are used by many cell biologists. The advantage of this method over classical MTT and WST-8 is the obsolete requirement to kill the cells during the staining.

We cut down the methods part in the abstract as requested by the reviewer from initially 284 down to 162 words. We provide evidence at the protein level, which is the measurement of the total collagen content (HOP-proline assay).

2) Writing part of this manuscript also needs improvement

Answer: We let proof-read our manuscript by a certified proof-reading agency for academic material.