Answer to the comments

Journal: World Journal of Hepatology
Manuscript Number: 36450
Title: Bioengineered humanized livers as better three-dimensional drug testing model system
Column: Basic Study
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Reviewer 1: (02904354)

In the paper, there are lots of grammar errors, which preclude my understanding. For examples, the sentence "However, the major hitches remains like the donor wise phenotypic variations and sourcing limitation." was wrong in grammar. The sentence "The mechanical strength of acellularized liver scaffolds after sterilization as compared with the native liver." was wrong in grammar. The sentence "Hence neglecting several crucial stimuli that allow control over the cellular organization and function." was wrong in grammar. The sentence "Mixed tensile strength, suture retention strength and compressive strength analysis of was analyzed according to the protocol described earlier" was wrong in grammar.

As per the suggestions, grammatical and sentence formatting errors has been revised and highlighted in blue colour throughout the manuscript.

Reviewer 2: (02861124)

The submitted manuscript deals with a very interesting piece of work on developing an ex vivo bioengineered humanized livers model to more efficiently screen the absorption, distribution, metabolism, excretion and toxicity of several pipeline drugs, and closely mimics the pre-clinical in vivo systems. Overall the work is well conceived, planned, executed and written. However, the manuscript suffers a poor language control that hampers the flow and readability. The major concern is the mixing of parts of methodology with results, and the too lengthy discussion. My comments/suggestions are highlighted in the attached manuscript file. I would recommend re-submission of the revised manuscript for further consideration.

Language, flow and readability of the manuscript have been revised throughout the manuscript. Mixing of parts of methodology and results have been removed and revised completely. The comments/suggestions highlighted in the manuscript have

been revised at each level and highlighted in blue. The manuscript has been revised thoroughly as per the suggestion.

Reviewer 3: (02861012)

In this manuscript Vishwakarma and co-authors describe their work on creating bioengineered humanized livers as better three-dimensional drug testing model system. The authors present convincing data that bioengineered livers are a feasible approach with repopulated acellular scaffolds being functionally active able to produce key molecules such as albumin. The work is very interesting however there are still some gaps that should be answered:

1. The authors do not give any information on the size/grams of livers they used to isolate human hepatic progenitor cells. How many cells were the authors able to isolate?

Human fetal liver of gestation aged 10-12 week were used to harvest intact whole liver. Whole liver was perfused in situ before harvesting from the fetal abdomen. Following to perfusion whole liver was subjected to mechanical dissociation which was further followed for enzymatic digestion and filtration through cell strainer of 40µm to obtain the single cell suspension as described in our earlier studies. The details of liver weight and number of cells isolated from them are as follows (**Vali et al. 2014**):

S. No.	Gestation wk/gender	Crown rump Length (cm)	Weight of liver separated (gm)	Cell isolation after density separation
1	10/F	6.5	1.4	4.02×10^7
2	10/M	6.2	1.8	4.11×10^7
3	11/M	7.4	1.8	3.92×10^7
4	11/F	8.7	2.0	3.97×10^7
5	12/F	8.7	2.4	3.8×10^7
6	12/F	8.7	2.1	3.92×10^7

2. The authors mentioned (page 9) that human HPCs were characterised for the expression of liver specific pluripotent markers etc; they need to show this data.

We have done extensive work since last two decades in isolation and characterization of Human fetal hepatic progenitor cells (hHPCs) (**Habibullah et al. 1994; Khan et al. 2008; Khan et al. 2010; Vali et al. 2014**). Few data of characterization are as follows:

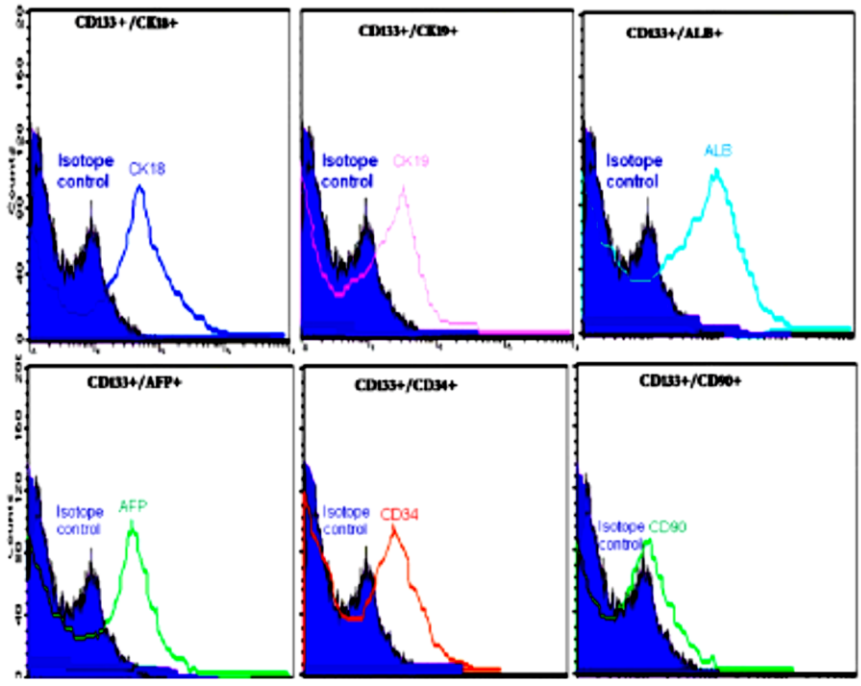

Figure 2: FACS analysis of CD133+ve cells express markers against epithelial, endothelial and MSCs markers. The overlaid histograms of analyzed markers with their unstained controls (filled blue). Only labeled cells are displaying a fluorescence value at least one log unit greater than the fluorescence measured at the population peak for unlabeled control cells (total liver cells) counted as positive for expression. [X-axis: relative log fluorescence. Y-axis: % counts].

Markers	Percentage reactivity of stem cell markers			
	CD133+ve human fetal liver derived cells			
	10-13wk	14-16wk	17-18wk	19-20wk
SOX2	32 ± 10.8	30 ± 6.2	28 ± 4.5	27 ± 3.8
OCT4	36.7 ± 8.5	38.0 ± 6.4	42 ± 5.5	44.3 ± 5.7
CD90	11 ± 4.2	11.5 ± 2.1	10.4 ± 2.1	10.5 ± 2.2
CD45	0.6 ± 0.1	0.6 ± 0.2	~0.4	~0.3
AFP	12.7 ± 1.5	12.3 ± 1.1	11.8 ± 1.2	10.2 ± 1.5
CD49f	4.2 ± 0.9	4.7 ± 0.6	4.2 ± 0.5	4.8 ± 1.2
CD29	2.8 ± 0.3	2.9 ± 0.5	3.2 ± 0.6	2.9 ± 0.8

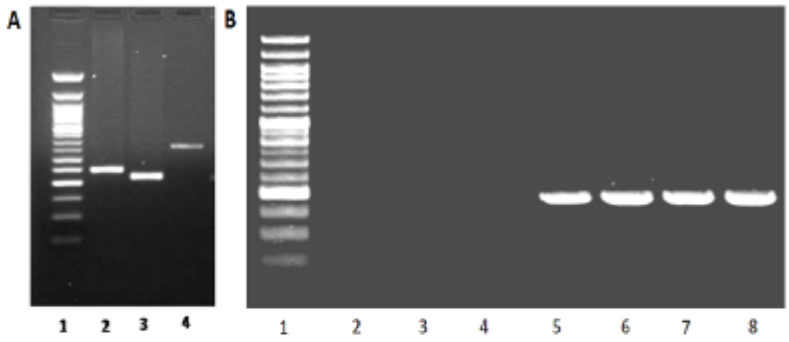

Figure 7: Agarose gel image shows differential gene expression analysis by reverse transcription polymerase chain reaction (A) gel image showed the expression of lane-2:CK19, lane-3: Albumin, Lane-4: AFP markers. Lane-1: molecular marker (100-500bp) (B) expression of albumin analyzed at day 2 (lane-2), day 5 (lane-3), day 10 (lane-4), day15 (lane-5), day20 (lane-6), day25 (lane-7) and day 30 (lane-8) by reverse transcription-polymerase chain reaction. Liver mRNAs and primary hepatocytes mRNA was used as controls.

3. The authors used 12 x 10^6 Epcam+ enriched hHPCs but it is unclear whether these cells were derived directly ex vivo or they were left to expand in vitro first.

EpCAM+ve cells were directly derived from the tissue and used after magnetic sorting followed by cell viability testing, cell counting and characterization.

4. It hasn't been clear how long overall can these humanized livers survive? The authors only show data up to day 7. How does this compare with the drug usage and metabolism in the human body?

This study was conducted only in 7days cultured cells within the humanized liver. We have followed the survival of these cells within the scaffold in culture up to 30 days and is in continuous follow up for further time points for long-term drug metabolism study.

5. The authors claim that their unique system allows for high-throughput studies. Can the authors be a bit more specific about this?

The word high throughput means the expedition of multiple factor analysis to identify several crucial molecular pathways and cellular response networks against multiple drugs in single humanized three-dimensional model system.

6. The authors have not described their data in comparison to what has been already described by the Pinzani group on liver bioengineering.

The work published by Pinzani and his group described repopulation of hepatic stellate cells (LX2), hepatocellular carcinoma (Sk-Hep-1) and hepatoblastoma (HepG2) which do not mimic with several crucial effects of human primary hepatic progenitor cells (used in our study). Hence, making realistic comparison with

Pinzani group work is not possible. Furthermore, Pinzani and his group is good for testing anti-cancer drug efficacy and safety but not the metabolism studies. Additionally, these cell lines lack several crucial molecular targets which are required for drug efficacy. Although their study falls in the similar direction, they have not demonstrated the use of repopulated human livers for drug testing. Hence, making any comparison with the efficacy of our model with their system is not possible.

Reviewer 4: (00068720)

The authors describe a way towards the development of suitable humanized preclinical model systems for pharmacological testing, which may reduce the cost and time duration of preclinical drug testing and further overcomes on the anatomical and physiological variations in xenogeneic systems. The data is interesting and relevant. In Results and Figure section, the authors indicate that One way and two way ANOVA was performed to identify the statistical significance among multiple groups, and the P value ($P<0.01$ or $P<0.001$ e.g.) has been marked, but how to compare between groups is not clear. The authors should discuss this point in more detail, and if possible, add some statistical parameters (F value, e.g.).

The Oneway ANOVA was performed to compare two groups as demonstrated in Figure 3C and 3D, Figure 5A and 5B using "Bonferroni's Multiple Comparison Test" to identify the significance between the groups. Whereas Two way ANOVA was performed to compare the multiple groups as demonstrated in Figure 6B wherein different groups of 2D cultured cells have been compared with the rate of drug metabolism in humanized liver. Column stats were also performed to identify mean and the standard error of mean for each variable.