Dear Editor and Members of the Editorial Board:

Thank you very much for giving us the opportunity to revise our manuscript "Long-pulse gastric electrical stimulation protects the interstitial cells of Cajal in diabetic rats via the IGF-1 signaling pathway". We are extraordinarily appreciative to the reviewers and the editor for reviewing our manuscript and providing valuable feedback. We have already used these comments to improve our manuscript. Changes in the text are highlighted in red. Our point-by-point responses to the reviewers' comments are detailed below:

Reviewer #1 (00498408)

Major points:

1. Title: the word "regeneration" should be removed, as in the paper the Authors did not demonstrate the regeneration of ICC. An English editing is highly recommended. There are several grammar errors and some sentences need to be rephrased

Response: Thank you very much for your positive response and constructive suggestions, which are very helpful for improving our work. According to the suggestion, we used "protects" instead of "promotes the regeneration of" in the paper. Besides, our manuscript has been edited by two independent native-English speaking scientists.

2. In the Introduction, the new role of stem cells and their targeting in

gastrointestinal disorders in diabetes should be mentioned, given recently published studies that demonstrate the importance of the IGF-I/IGFBP3 axis in gastrointestinal complications of diabetes and their role on stem cells. Therefore, the following References should be added in support: - Circulating IGF-I and IGFBP3 Levels Control Human Colonic Stem Cell Function and Are Disrupted in Diabetic Enteropathy. Cell Stem Cell 2015 2015 Oct 1;17(4):486-98.

Response: We appreciate the reviewer's comments very much, and revisions have been done as suggested. We added the reference and additional text in Introduction section to illustrate the importance of IGF-1 more clearly.

Reference:

D'Addio F, La Rosa S, Maestroni A, Jung P, Orsenigo E, Ben Nasr M, Tezza S, Bassi R, Finzi G, Marando A, Vergani A, Frego R, Albarello L, Andolfo A, Manuguerra R, Viale E, Staudacher C, Corradi D, Batlle E, Breault D, Secchi A, Folli F, Fiorina P. Circulating IGF-I and IGFBP3 Levels Control Human Colonic Stem Cell Function and Are Disrupted in Diabetic Enteropathy. Cell stem cell 2015; 17(4): 486-498 [PMID: 26431183 DOI: 10.1016/j.stem.2015.07.010]

3. The term "regeneration" throughout the text should be removed as the Authors never showed a regeneration of ICC with GES. Despite this, a co-localization of Ki67 and c-kit to identify whether there is a proliferation of c-kit+ ICC will be supportive.

Response: Thanks for your suggestions. Ki67 is a type of nucleoprotein that is associated with rRNA transcription, and reflects the proliferation of cells. Our previous study (Chen Y et al.,2013) clearly demonstrated that the proliferation of ICCs (evaluated using double immunolabeling for c-kit and Ki67) in different layers of the gastric wall was enhanced after electrical stimulation. Similarly, the

proliferation of intramuscular ICCs (also immunolabeled for c-kit and Ki67) in the colon was up-regulated in diabetic constipation rats after electrical stimulation(Xu J et al.,2013). The present results reveal that the expression of the c-kit protein and the number of ICCs were increased after GES treatment.

Reference:

Chen Y, Xu JJ, Liu S, Hou XH. Electroacupuncture at ST36 ameliorates gastric emptying and rescues networks of interstitial cells of Cajal in the stomach of diabetic rats. PloS one 2013; 8(12): e83904 [PMID: 24391842 PMCID: Pmc3877115 DOI: 10.1371/journal.pone.0083904]

Xu J, Chen Y, Liu S, Hou X. Electroacupuncture regulates apoptosis/proliferation of intramuscular interstitial cells of cajal and restores colonic motility in diabetic constipation rats. Evidence-based complementary and alternative medicine : eCAM 2013; 2013: 584179 [PMID: 24348706 PMCID: Pmc3852313 DOI: 10.1155/2013/584179]

4. The Authors should speculate more on the reasons why GES2 works better on gastric emptying as compared to GES1 and GES3

Response: According to your suggestion, we have added content in Discussion section to speculate more on the reasons why GES2 works better.

5. The choice of focusing on M-SCF instead of S-SCF should be better supported. A clear experiment could be assessing the S-SCF peripheral levels in the different treatment groups. Also, expression of M-SCF should be confirmed with another method (other than WB) such as immunofluorescence, immunohistochemistry, RT-PCR.

Response: Thanks for your constructive suggestions. SCF is the ligand for c-kit and it exists as both a soluble form (S-SCF) and a transmembrane form (M-SCF). Based on the results of previous studies, M-SCF is deemed to be more important to the

differentiation, survival and phenotype of mature ICCs (Rich A et al.), and S-SCF is necessary for the proliferation of ICC progenitor cells (Bardsley MR et al.; Lorincz A et al.). In the gastric wall, the proportion of progenitor cells in the total population of ICCs is rather small in adult rats. Therefore, we focus on M-SCF in the present study.

Reference:

Rich A, Miller SM, Gibbons SJ, Malysz J, Szurszewski JH, Farrugia G. Local presentation of Steel factor increases expression of c-kit immunoreactive interstitial cells of Cajal in culture. American journal of physiology Gastrointestinal and liver physiology 2003; 284(2): G313-320 [PMID: 12388202 DOI: 10.1152/ajpgi.00093.2002]

Bardsley MR, Horvath VJ, Asuzu DT, Lorincz A, Redelman D, Hayashi Y, Popko LN, Young DL, Lomberk GA, Urrutia RA, Farrugia G, Rubin BP, Ordog T. Kitlow stem cells cause resistance to Kit/platelet-derived growth factor alpha inhibitors in murine gastrointestinal stromal tumors. Gastroenterology 2010; 139(3): 942-952 [PMID: 20621681 PMCID: Pmc2933938 DOI: 10.1053/j.gastro.2010.05.083]

Lorincz A, Redelman D, Horvath VJ, Bardsley MR, Chen H, Ordog T. Progenitors of interstitial cells of cajal in the postnatal murine stomach. Gastroenterology 2008; 134(4): 1083-1093 [PMID: 18395089 PMCID: Pmc2435491 DOI: 10.1053/j.gastro.2008.01.036]

6. Finally, the role of IGF1/IGF1R in protecting ICC should be addressed more in details with other experiments (using for example an anti-IGF1R to block the pathway in vitro/in vivo).

Response: Thanks for your constructive suggestion. In previous studies, IGF-1 signaling pathway was proved to protect ICCs in diabetic models. Horváth VJ et al. were the first to demonstrate that IGF-1 signaling is responsible for maintaining the ICC network *in vitro*. In their study, murine gastric muscles were cultured in hyperglycemic basal media with or without IGF-1, and slow waves and the expression of c-kit were significantly reduced in tissues grown in basal media without IGF-1, while IGF-1 supplementation entirely prevented this effect. Moreover, the protective

effect conferred to ICCs by IGF-1 was paralleled by the up-regulation of SCF expression. These results convincingly confirmed the protective role of IGF-1. Moreover, it seems difficult to apply IGF-1 antagonist in our long-period *in vivo* study in rats, but your constructive suggestion inspires us to use IGF-1 antagonist in our future study about acute GES.

Reference:

Horvath VJ, Vittal H, Lorincz A, Chen H, Almeida-Porada G, Redelman D, Ordog T. Reduced stem cell factor links smooth myopathy and loss of interstitial cells of cajal in murine diabetic gastroparesis. Gastroenterology 2006; 130(3): 759-770 [PMID: 16530517 DOI: 10.1053/j.gastro.2005.12.027]

Horvath VJ, Vittal H, Ordog T. Reduced insulin and IGF-I signaling, not hyperglycemia, underlies the diabetes-associated depletion of interstitial cells of Cajal in the murine stomach. Diabetes 2005; 54(5): 1528-1533 [PMID: 15855342]

7. The relevance of the GES treatment in gastric emptying should be also confirmed by treating diabetic rats with a conventional therapy (e.g. drug, device), thus demonstrating that GES has a more powerful effect. This will definitely improve the quality of the paper.

Response: Thanks for your constructive suggestions. Gastroparesis is a chronic disorder that not only causes gastric symptoms but also leads to dystrophia. Treatment for gastroparesis is challenging, and results have been disappointing over the past decades. The regular medical therapies for this condition includes dietary modifications, the administration of prokinetic agents and/or other drugs. GES is usually an alternative therapy for refractory gastroparesis patients who are not responsive to drugs or unable to undergo chronic pharmacotherapy due to serious

side-effect. But your constructive suggestions inspire us to do relative work and systematic comparation between GES and traditional therapy in our further research.

Reference:

Chen Y, Xu J, Liu S, Hou X. Electroacupuncture at ST36 increases contraction of the gastric antrum and improves the SCF/c-kit pathway in diabetic rats. The American journal of Chinese medicine 2013; 41(6): 1233-1249 [PMID: 24228598 DOI: 10.1142/s0192415x13500833]
Chen Y, Xu JJ, Liu S, Hou XH. Electroacupuncture at ST36 ameliorates gastric emptying and rescues networks of interstitial cells of Cajal in the stomach of diabetic rats. PloS one 2013; 8(12): e83904 [PMID: 24391842 PMCID: Pmc3877115 DOI: 10.1371/journal.pone.0083904]

8. Figure 2 should be redrawn because the actual bar-graphs are difficult to recognize and results do not appear immediately understandable. Figure 5 C: in the labeling of WB gel it is indicated SCF. Is this M-SCF?

Response: Thanks for your kind reminds. We are very sorry for we did not make Figure 2 easy to understand. So we have already redrawn Figure 2 and make the actual bar-graphs more clear and the results easy to understand in the revised manuscript. In Figure 5C, the labeling of WB gel should be M-SCF and we have corrected it in the revised manuscript.

Minor points:

Several grammar mistakes are present throughout the text and need to be corrected. Spell out GES in Introduction section would be helpful.

Response: Thanks for your suggestions.Our manuscript has been edited by two independent native-English speaking scientists and we have added new content to spell out GES in Introduction section.

Reviewer #2 (03254149)

1. Results. Weight and blood glucose level: Is there any clinical significance in a slight increase of body weight by GES (Fig. 2A)?

Response: Thank you very much for your positive response and constructive suggestions, which were very helpful for improving our work. In long-standing diabetic gastroparesis patients, dystrophy is a common and serious problem caused by the interrupted gastrointestinal motility. Our results suggested that long-pulse GES could regulate gastric emptying and increase body weight in diabetic rats, possibly indicating an improvement in nutrition intake, but further study are needed to confirm this speculation.

2. Results. Weight and blood glucose level: I don't understand why blood glucose level are similar unnaturally at 0W, 2W, 4W and 6W (Fig. 2B). In general, blood glucose level is increased with time after injection of STZ.

Response: To our knowledge, two methods are commonly applied to induce diabetic models with STZ: a single injection of a large dose (immediate model) and multiple injections of small doses (delayed model). The former method is associated with the direct damage to β cells, which leads to continuous hyperglycemia, while the latter method is associated with the chronic damage to β cells and T lymphocyte-mediated immunoreaction that may possibly lead to increased blood glucose increasing over time. The method we adopted to induce a diabetic model was the former of these two,

which may cause blood glucose to be maintained at a high level after STZ injection.

Reference:

Djordjevic PB, Lalic NM, Jotic A, Paunovic I, Lalic K, Raketic N, Nikolic D, Zamaklar M, Rajkovic N, Lukic L, Dimitrijevic-Sreckovic V, Dragasevic M, Nikolic D, Markovic I. Human fetal islet transplantation in type 1 diabetic patients: comparison of metabolic effects between single and multiple implantation regimens. Transplantation proceedings 2004; 36(9): 2869-2873 [PMID: 15621171 DOI: 10.1016/j.transproceed.2004.09.050]

Herold KC, Bluestone JA, Montag AG, Parihar A, Wiegner A, Gress RE, Hirsch R. Prevention of autoimmune diabetes with nonactivating anti-CD3 monoclonal antibody. Diabetes 1992; 41(3): 385-391 [PMID: 1532369]

Liberatore RR, Jr., Barbosa SF, Alkimin M, Bellinati-Pires R, Florido MP, Isaac L, Kirschfink M, Grumach AS. Is immunity in diabetic patients influencing the susceptibility to infections? Immunoglobulins, complement and phagocytic function in children and adolescents with type 1 diabetes mellitus. Pediatric diabetes 2005; 6(4): 206-212 [PMID: 16390389 DOI: 10.1111/j.1399-543X.2005.00136.x]

3. Results. Electron microscopy: line 6-7: The authors should describe how enteric nerves were destroyed.

Response: Thanks for your suggestion. The intercellular spaces were dilated, gap junctions between ICCs and enteric nerves were reduced and exudation of fibrin was occasionally observed. We also added above descriptions in revised manuscript. In present study, we focused on ICCs and therefore paid less attention on enteric nerves.

4. Results. Electron microscopy: line 10: What is the definition of 'minor damage'?

Response: We are sorry for the confusion caused by this description. Minor damage means that the structure of the cytomembrane is relatively complete, organelles are abundant, there is slight cytoplasmic depletion, slightly swollen endoplasmic reticulum and mitochondria and limited vacuolization, there are few denuded

ribosomes and a small number of lysosomes, and there is little heterochromatin. We have added a definition for ‘minor damage’ to the Results section of the revised manuscript according to your helpful comment.

5. Results. Electron microscopy: The authors should mark the position of enteric nerves, ICC and SMCs in Fig. 3. The damaged parts also should be marked.

Response: Thanks for your suggestions. Figure 3 was revised accordingly.

6. Results. Gastric emptying: It is known that diabetic patients suffer from gastroparesis caused by abnormalities in the extrinsic innervation to the stomach and loss of excitatory or inhibitory neurotransmitters at the level of the enteric nervous system. Therefore, an almost complete improvement of gastric emptying by GES2 (Fig. 4) is very interesting. The authors should study immunoreactivity for PGP9.5, nNOS and vesicular acetylcholine transporter (vAChT) in DM + GES2 group rats.

Response: Thank you very much for your constructive suggestion. PGP9.5 is a neuronal-specific protein that is usually used as a marker for the total population of neurons. The different neurotransmitters that are expressed by neuron convey chemical information about these neurons. Enteric Nervous System (ENS) neurons include excitatory neurons (such as those expressing choline acetyl transferase (CHAT)) and inhibitory neurons (such as those expressing neuronal nitric oxide synthase (nNOS)). In a previous study (Du F et al.), we showed that chronic electrical stimulation significantly increased the mRNA expression levels of PGP9.5, nNOS and

CHAT in the colon of diabetic rats. In addition, the protein expression levels of PGP9.5, nNOS and CHAT measured using immunofluorescence and Western blot analysis were also increased after chronic electric stimulation. These results suggest that chronic electrical stimulation also promotes the regeneration of enteric neurons in diabetic rats, further contributing to an improved gastrointestinal motility. In the present study, the main emphasis is the mechanism underlying the protection effects on ICC induced by GES, we may study the role of ENS in GES in the future.

Reference:

Du F, Liu S. Electroacupuncture with high frequency at acupoint ST-36 induces regeneration of lost enteric neurons in diabetic rats via GDNF and PI3K/AKT signal pathway. American journal of physiology Regulatory, integrative and comparative physiology 2015; 309(2): R109-118 [PMID: 25972459 DOI: 10.1152/ajpregu.00396.2014]

7. Results. Immunofluorescence studies: I could not find any structural features of ICC in Fig. 6. The authors should present clear immunohistochemical data. The authors also should quantitative analysis of ICC distribution as has been reported by Horváth et al. (Diabetes. 2005; 54(5):1528-33) to estimate the effects of GES on ICC.

Response: Thanks for your suggestions. Embedding the figures into the manuscript may reduce their resolution and definition. We offer our sincere apologies for any inconvenience. We have up-loaded clearer figures with the revised manuscript.

8. The authors should refer the following paper to discuss protection of ICC structure by GES. 'Mitsui R, Komuro T. Distribution and ultrastructure of

interstitial cells of Cajal in the gastric antrum of wild-type and Ws/Ws rats. Anat Embryol. 2003; 206(6):453-60. [PMID: 12700899]'.

Response: Thanks for your suggestion. We added this reference in Discussion section to discuss protection of ICC structure by GES.

Reference:

Mitsui R, Komuro T. Distribution and ultrastructure of interstitial cells of Cajal in the gastric antrum of wild-type and Ws/Ws rats. Anatomy and embryology 2003; 206(6): 453-460 [PMID: 12700899 DOI: 10.1007/s00429-003-0323-8]

9. The authors should refer the following paper which demonstrates that application of IGF-1 prevent the loss of ICC network in mice stomach cultured with hyperglycemic media. 'Horváth VJ, Vittal H, Ord?g T. Reduced insulin and IGF-I signaling, not hyperglycemia, underlies the diabetes-associated depletion of interstitial cells of Cajal in the murine stomach. Diabetes. 2005; 54(5):1528-33. [PMID: 15855342]'. This paper showed the role of IGF-1 signaling in the maintenance of ICC for the first time. Thus, this paper will help the authors to write more constructive discussion.

Response: Thanks for your suggestion. We added this reference and new text in Discussion section to better discuss the role of IGF-1 signaling in the maintenance of ICC.

Reference:

Horvath VJ, Vittal H, Ordog T. Reduced insulin and IGF-I signaling, not hyperglycemia, underlies the diabetes-associated depletion of interstitial cells of Cajal in the murine stomach. Diabetes 2005; 54(5): 1528-1533 [PMID: 15855342]

Reviewer #3 (03476117)

Major points:

1. In general, the quality of the immunofluorescence staining is inadequate. More specifically, I am concerned about the specificity of the c-kit staining – In figure 6(A) there is an oval shaped structure stained on the right side of the image. To me this looks like a myenteric ganglion, which should not be c-kit positive. ICC are present at the level of the myenteric plexus but they surround myenteric ganglia, rather than being found within it (see references 1-4). A double label with a neuronal antibody, such as PGP9.5, is required to check that there is no overlap in their staining. However, I must admit that I am not 100% certain what the oval structure in the image is or even if it is actually at the level of the myenteric plexus. This is because these images have not been labeled. If the muscle layers, mucosa, serosa and other visible structures could be labeled it would be much easier for readers to interpret the images. To improve the immunohistochemical data, whole mounts of antral muscle need to be prepared in order to examine both intramuscular and myenteric ICC in control, diabetic and GES-treated animals. This will reveal valuable information about ICC changes.

Response: Thank you very much for your positive response and constructive suggestions, which are very helpful for improving our work. To our knowledge, the oval shaped structure in figure 6(A) may also be c-kit^{+} signal (indicates ICCs). However, the immunofluorescence in paraffin sections in this study only reflected two-dimensional structure of the gastric wall, and occasionally lead to overlapping

images occurs and influenced the ‘regular structure’. PGP9.5 is a neuronal-specific protein that is usually used as a marker for the total population of neurons. In a previous study (Du F et al.), we showed that chronic electrical stimulation significantly increased the mRNA expression levels of PGP9.5, nNOS and CHAT in the colon of diabetic rats. In addition, the protein expression levels of PGP9.5, nNOS and CHAT measured using immunofluorescence and Western blot analysis were also increased after chronic electric stimulation. Another study of us (Chen Y et al.) demonstrated that the expression of c-kit in each layer was apparently reduced in the DM group, but was up-regulated after electrical stimulation. Your constructive suggestion to prepare whole mounts of antral muscle is very helpful to examine ICCs in our further study about GES.

Reference:

Du F, Liu S. Electroacupuncture with high frequency at acupoint ST-36 induces regeneration of lost enteric neurons in diabetic rats via GDNF and PI3K/AKT signal pathway. American journal of physiology Regulatory, integrative and comparative physiology 2015; 309(2): R109-118 [PMID: 25972459 DOI: 10.1152/ajpregu.00396.2014]

Chen Y, Xu JJ, Liu S, Hou XH. Electroacupuncture at ST36 ameliorates gastric emptying and rescues networks of interstitial cells of Cajal in the stomach of diabetic rats. PloS one 2013; 8(12): e83904 [PMID: 24391842 PMCID: Pmc3877115 DOI: 10.1371/journal.pone.0083904]

2. It would be great if the authors could perhaps administer an IGF-1R antagonist to the diabetic rats before GES. According to their theory block of the IGF-1 signaling pathway should inhibit the recovery of ICC. This experiment would greatly strengthen the evidence for IGF1’s role. At the least the authors should make it clear that such an experiment (or something similar) will be required in the future to conclusively confirm their hypothesis.

Response: Thanks for your constructive suggestion. In previous studies, IGF-1 signaling pathway was shown to protect ICCs in diabetic models. Horváth VJ demonstrated that IGF-1 signal was responsible for the maintenance of ICC network *in vitro* for the first time. In their study, murine gastric muscles were cultured in hyperglycemic basal media with or without IGF-1, the slow waves and expression of c-kit was significantly reduced in basal media without IGF-1, and IGF-1 supplementation could entirely prevent these change. Besides, the protection of ICCs by IGF-1 was paralleled by the up-regulation of SCF expression. These results confirmed the protective role of IGF-1 convincingly. However, it seems difficult to apply IGF-1 antagonist in our long-period *in vivo* study in rats, but your constructive suggestion inspires us to use IGF-1 antagonist in our future study about acute GES.

Reference:

Horvath VJ, Vittal H, Ordog T. Reduced insulin and IGF-I signaling, not hyperglycemia, underlies the diabetes-associated depletion of interstitial cells of Cajal in the murine stomach. Diabetes 2005; 54(5): 1528-1533 [PMID: 15855342]

Horvath VJ, Vittal H, Lorincz A, Chen H, Almeida-Porada G, Redelman D, Ordog T. Reduced stem cell factor links smooth myopathy and loss of interstitial cells of cajal in murine diabetic gastroparesis. Gastroenterology 2006; 130(3): 759-770 [PMID: 16530517 DOI: 10.1053/j.gastro.2005.12.027]

3. In their discussion of the role of IGF-1 signaling the authors need to reference the paper by Horváth et al (5), which previously showed that IGF-1 levels could affect ICC

Response: Thanks for your suggestion. We added the reference and new text in Discussion section to better discuss the role of IGF-1 signaling in the maintenance of ICC.

Minor points:

1. Define GES in the abstract and introduction.

Response: Thanks for your suggestion. We defined GES in the abstract and introduction in the revised manuscript.

2. Define SD rats in the methods section.

Response: Thanks for your suggestion. We defined SD rats in the methods section.

3. It is unclear how the antral tissues were fixed for immunofluorescence. On page 6 it is stated that Zamboni's fixative was used, while on page 8 it is stated that 4% paraformaldehyde was used. Please clarify.

Response: Thanks for your kind remind. We offer our sincere apologies for this mistake. After a careful check, we actually used Zamboni's fixative in immunofluorescence studies. We have corrected it in the revised manuscript.

4. As far as I can see Figure 1 is not referenced anywhere in the body of the manuscript. It should be mentioned in the Material and Methods Section.

Response: We are sorry for any inconvenience. Figure 1 is referenced at "Gastric electrical stimulation" part in Materials and Methods section.

5. Figure 2 shows that the blood glucose is increased in the DM group compared

to control (for weeks 0-6). However, it is not made clear (in the figure legend or on the graph) whether the authors analyzed the difference in blood glucose between control and the GES groups.

Response: Thanks for your careful reading. In present study, we analyzed the difference in blood glucose between the control and DM group as well as the difference between the DM and GES groups. We did not analyze the difference between the control and GES groups for more than one conditions were changed.

6. In Figure 3 markers need to be placed on the images to indicate ICC, SMC, Enteric nerves, mitochondria, ER, etc. because currently it is difficult to interpret the images. This change will make it much easier to see what the authors are describing.

Response: Thanks for your suggestions. Figure 3 was revised accordingly.

7. Figures 6(E) and 6(F) have unusual staining patterns that need to be discussed. In 6(E) the c-kit staining appears to be increased throughout all layers of the section including the mucosa, etc. In contrast, 6(F) shows intense staining in three distinct bands.

Response: Thanks for your careful reading. Embedding the figures into the manuscript may reduce their resolution and definition. We offer our sincere apologies for any inconvenience. We have up-loaded clearer figures with the revised manuscript. For our paraffin sections, the angle of each section might not be exactly the same and

this may occasionally lead to overlap image. In addition, there was still some non-specificity staining though we had applied animal serum to reduce it.