The authors would like to thank the reviewers for their time and their valuable comments concerning our manuscript entitled "**Animal models applied to acute-on-chronic liver failure: Are new models required to understand the human condition?**" which we are resubmitting for consideration of publication.

As you can see, we have followed all reviewer suggestions. The critiques were addressed. I hope this manuscript will now be considered suitable for publication.

Answer Reviewers:

Reviewer #1

1. The authors carefully showed the mechanism of ACLF due to alcohol. It is necessary to discuss more about the mechanism due to toxicity and drugs. In Southeast Asian countries, the prevalence of HBV is high, ACLF often occurs when patients are being treated for chronic hepatitis B and they decide to stop taking the medicines for various reasons. Currently, the covid epidemic is serious, patients cannot go to the doctors, treatment interruptions are frequent and ACLF occurs. The authors should mention more about these issues.

   Response: We agree with your excellent suggestions. Therefore, we add a paragraph citing about the mechanism due to toxicity and drugs. We highlight the high prevalence of hepatitis B virus-related acute-on-chronic liver failure (HBV-ACLF) in Asia and the factors that may contribute to the high mortality of these patients, bellow:
   "In contrast, the reactivation of hepatitis B virus (HBV) is the leading cause of ACLF in the Asian region, with high prevalence where HBV-related acute-on-chronic liver failure (HBV-ACLF) accounts for over 70% of ACLF[17,18]. However, early diagnosis and prevention measures through long-term HBV infection suppression with antiviral agents (such as lamivudine, tenofovir, entecavir or telbuvidine) or sustained eradication of hepatitis C virus (HCV) infection in patients with compensated or decompensated cirrhosis can decrease mortality and prevent the development of HBV-ACLF and HCV-related acute-on-chronic liver failure (HCV-ACLF) in this region[19,20]. Patients with chronic hepatitis B or HBV-related cirrhosis are at risk of developing ACLF, with multi-organ failure and high short-term mortality[21]. HBV reactivation could be either a spontaneous setting of treatment cessation[22] or due to intensive chemotherapy or immunosuppressive therapy[23,24], treatment related[25] or reactivation of the occult HBV

infection by rituximab (anti-CD20)-based chemotherapy[26–28] or immune restoration after highly active antiretroviral therapy for HIV[29,30]. Similarly, HCV infection reactivation has also been reported, mainly following immune suppressive therapy[31,32] . Drugs such as anti-tuberculosis drugs, methotrexate and antiretroviral drugs in HIV/AIDS-infected individuals have been implicated in triggering liver injury, particularly in the setting of underlying chronic liver disease due to HBV or HCV[31,33–35]. Drugs are seen as a precipitating factor in ACLF, although but databases on concerning drugs as an acute insult leading to ACLF are extremely scarce. This factor limits the study and knowledge of the effects of certain drugs and medications in ACLF development. This indicates the need for further data and assessments concerning models on hepatic injury caused by different herbal and medicinal preparations in cirrhosis patients[8]. "

Furthermore, in view of the global importance of the pandemic resulting from the infection by the new coronavirus disease 2019 (COVID-19) we did a brief discussion of the possible relationship between the development of ACLF and the severe acute respiratory syndrome coronavirus 2 infection (SARS-CoV-2). However, little is known about the events that can lead to SARS-CoV-2 infection as a precipitating factor for ACLF:

"Another important issue concerning the knowledge and studies of the events that lead to ACLF development in humans is directly related to the current pandemic scenario caused by the new coronavirus disease 2019 (COVID-19). The hallmark of ACLF is excessive systemic inflammation, and patients with ACLF exhibit higher levels of inflammatory markers and pro-inflammatory cytokines—IL-6, IL-1β, and IL-8. Systemic inflammation inducers can be exogenous or endogenous and viruses have been described previously as triggering inflammation[13] . A cytokine storm has been reported in patients with COVID-19, characterized by increased IL-2, IL-7, G-SCF and TNF-α[44]. Thus, it is believed that the excessive inflammatory response associated with COVID-19 can serve as a trigger for ACLF in patients with underlying chronic liver disease, which could justify the increase in liver disease patient deaths[45]. However, other mechanisms may also contribute to ACLF

development in COVID-19 patients, such as hypoxic changes and iatrogenic causes such as drugs and ventilation, exacerbating underlying liver disease[46,47]. A case study reported the development of ACLF precipitated by severe acute respiratory syndrome coronavirus-2 infection (SARS-CoV-2) in a patient with HBV-related cirrhosis without no previous anti-viral treatment. The authors suggest that the SARS-CoV-2 infection induced systemic inflammatory response syndrome (SIRS), and the resultant immune dysregulation could have precipitated ACLF, in turn. Since the patient had not been on nucleoside analogs (NA) treatment for HBV prior to admission, it is possible that the ACLF was caused by HBV flare in a context of uncontrolled inflammation and dysbalance of innate and adaptive immune responses triggered by the SARS-CoV-2 infection[48]. This highlights the importance of the treatment in patients with HBV and other chronic liver disease in the current pandemic status worldwide. Nevertheless, long-term follow-up clinical studies are required to explore the potential relationship between ACLF development in COVID-19 patients[45]."

2. In animal research, only ACLF due to toxicity has been studied, other reasons have not been done. This is a big obstacle; the authors should discuss this issue.

   Response: We have commented and discussed this, as suggested in the new topic entitled "**Disadvantages and Challenges for ACLF Animal Models", bellow:**

"**DISADVANTAGES AND CHALLENGES OF ACLF ANIMAL MODELS**

Due to the complexity of liver injury, the understanding of underlying liver disease mechanisms and their treatment has been limited by the lack of satisfactory animal models. Currently, no model has been able to completely capture the corresponding human acute and chronic liver disorder[52,61,85].

The ideal ACLF model should combine bacterial infection and high short-term mortality. As described previously, several existing ACLF models have been

developed by combining chronic and acute liver injury[72]. Chronic injury is most commonly induced by the injection of CCl_4 or via BDL surgery, whereas acute injury is induced by the injection of D-GaIN/LPS. The principle of these models is to reproduce the bi-factorial disease profile comprising chronic liver injury, which leads to the development of progressive liver fibrosis, and a precipitating event inducing further organ injury, resulting in ACLF and considerable mortality. This is not, however, entirely consistent with ACLF pathogenesis, and the surgery required for the BDL model is difficult. The clinical situation is often more complex, and different modulating factors may occur concurrently or sequentially[86]. Typically, 50% of patients develop bacterial infection as an ACLF complication, although the (initial) precipitating event was non-inflammatory[87].

The significant challenge to develop an ACLF model is the ability to unite all the clinical characteristics observed in humans, as this is a multifactorial disease with multiple precipitators and complications and, therefore, varying disease phenotypes and organ failures, making it almost impossible to develop a single experimental model capable of triggering all of the most important clinical features[86]. In a recent study by Xiang and coworkers (2020), the authors developed a new ACLF model that could sequentially reproduce three important clinical ACLF disease factors. To this end, a severe liver injury model was prepared by combining chronic injury (CCl_4 injection), acute hepatic insult (injection of a CCl_4 double dose), and systemic bacterial infection (i.p. injection of bacteria *Klebsiella pneumonia*). The findings indicate that this severe liver injury model developed acute-on-chronic liver injury, bacterial infection, multi-organ injury, and high mortality, some of the features of clinical ACLF. The authors highlight that the single bacterial infection step is crucial in inducing multi-organ failure in this model, as chronic-plus-acute liver injury did not drive the full course of ACLF in mice without bacterial infection[72]. In contrast, Schwarzkopf and coworkers developed a model combining chronic liver disease (CCl_4/EtOH or CYP2D6-linked adenovirus (ADV)-induced autoimmune hepatitis) with different precipitating events [two EtOH binges or

i.p. polymicrobial infection by cecal slurry (CS)]. After 7 weeks of CCl_4/EtOH, ACLF was induced with two alcohol binges (alcohol gavage with 31.5% Vol.) with an interval of 3 days between binges. Mice mortality was observed, as well as systemic inflammation and significant elevation of serum alanine aminotransferase (ALT) levels alongside other ACLF features[73]. According to the authors, polymicrobial sepsis by CS is closer to human infection-triggered ACLF than the *K. pneumonia* injection employed by Xiang[72,73]. These variabilities in current data also significantly interfere with the development of a standardized ACLF animal model. Furthermore, it is not yet possible to identify all ACLF precipitants, as over 40% of patients who develop ACLF exhibit no known precipitant, requiring further knowledge of ACLF activation events[22]."

Reviewer # 2

1. Authors made attempts to summary the animal models developed for investigating the pathogenesis and therapeutics for acute-on-chronic failure liver. Although several different approach-induced animal models were described in paper, these information seemed to talk in generalities, lacking deep-going things and novelty. Additional, the strength and drawback of different animal models should be highlighted. The overall challenges for cuttent animal models should be summarized and presented for requiring the new models.

   Response: Our main objective was to highlight the great importance of developing new experimental ACLF models in view of the scarce data in preclinical studies. The search for new models is of paramount importance so that it is possible to elucidate the mechanisms that lead to the development of ACLF. We believe that this central aim has been achieved, especially after the inclusion of new paragraphs in the revised text. Additional, we have included a topic discussing some disadvantages and challenges in developing ACLF models including two papers that developed the ACLF model also for bacterial infection associated with toxicity. The new topic entitled **"Disadvantages and Challenges for ACLF Animal Models"**, described above.

Reviewer # 3

1. Thank you for this review.

   Response: We appreciate your feedback.