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# The effectivity of bovine colostrum and Mesenchymal Stem Cell (MSC) on the improvement of Alkaline Phosphatase (ALP) and Takeda G-Protein Coupled Receptor-5 (TGR5) level in post-hepatectomy Wistar rats

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## ABSTRACT

**Background:** The factor affecting the liver's healing process after resection in liver malignancy is liver fibrosis. Bovine Colostrum (BC), as one of the anti-fibrosis products of the liver, improves hepatic fibrosis and reduces hepatocyte damage caused by carbon tetrachloride (CCl4). Meanwhile, Mesenchymal Stem Cell (MSC) is a new therapeutic source after resection. MSC is able to differentiate into specific cells in the healing process. This study aims to determine the effects of BC, MSC, and a combination of both in terms of increasing Alkaline Phosphatase (ALP) and Takeda G-Protein Coupled Receptor-5 (TGR5) levels in Wistar rats post hepatectomy 50% with liver fibrosis.

**Methods:** This study is an experimental study with a randomized control trial design. Subjects were 25 Wistar rats (Rattus norvegicus) which were divided into 5 groups: Sham (surgery only), K (Control), K1 (CCL4 +BC), K2 (CCL4 + MSC), and K3 (CCL4 + MSC +BC). Blood was also taken to assess ALP and TGR5 at Day-3, Day-7, and Day-10 of treatment. Data were analyzed using SPSS version 25 for Windows.

**Results:** A significant differences are found in ALP in the Sham group with K2 ($p = 0.014$) and the K2 group with K1 ($p=0.026$) on day 10. Significant differences in TGR5 are found in the Sham and K2 groups ($p=0.009$), control with K2 ($p=0.014$), K2 with K1 ($p=0.007$) on day 3. On day 7 in the control group with the combination ($p=0.013$). The test results showed that TGR5 on day 3 had a moderate significant strong correlation on ALP levels on day 10 ($r=0.596$; $p=0.014$).

**Conclusion:** The combination of BC and MSC was not better than the administration of BC or MSC only in increasing ALP and TGR5 levels in rats with liver fibrosis after hepatectomy 50%. Based on this study, the administration of MSC is recommended.

**Keywords:** Bovine Colostrum, MSC, Cholangiocytes, ALP, TGR5.
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## INTRODUCTION

Liver resection is the primary choice of treatment for liver malignancy. The health service database in France shows that there were 28,708 hepatectomies between 2007 and 2010.[1] In addition, the results of a previous review showed that the number of laparoscopic liver resections in the world had reached 6,000 cases and this number continues to increase.[2] Early research found that patients with hepatitis B-induced hepatocellular carcinoma with an Ishak histopathological score of 6 (cirrhosis of the liver and nodular tissue) had a poor prognosis and high recurrence rate. About 27% of patients had recurrences and 14% died after 2 years of partial hepatectomy. In addition, about 32% of patients had recurrences and 27% died after 5 years of partial hepatectomy.[2]

The factor affecting the liver healing process after hepatectomy is hepatic fibrosis. In general, the wound healing process consists of three phases, inflammation, fibrogenesis and remodeling. However, the liver healing process is done with the fibrosis regression process.[3] One of the anti-fibrosis products of the liver is bovine colostrum (BC). Dong Hyun Sinn et al. found that BC has a total antioxidant capacity (TAC) which consists of enzymes or non- enzymes such as superoxide dismutase, glutathione peroxidase, catalase, vitamin E, vitamin C and beta carotene. Apart from having TAC, BC has a healing growth factor for the liver.[4]

On the other hand, mesenchymal stem cells (MSC) is a new therapeutic source

after hepatectomy. MSC is obtained from the bone marrow, stromal tissue, adipose tissue, placenta, and umbilical cord. This cell can differentiate into specific cells in the healing process, hepatocyte-like cells, which produce paracrine growth factors regulating the proliferation of cholangiocytes and cholangiocytes-like cells.[5]

Cholangiocytes are a small part of parenchyma cells, and they have an important role in regulating bile acid overload after partial hepatectomy through Takeda G-Protein Coupled Receptor-5 (TGR5) activation. The protection of bile acid overload could reduce liver damage, leading to more advanced liver regeneration. During the regeneration, extreme hemodynamic changes of liver enzymes occur.[6]

Based on those mentioned above, this study aims to evaluate the effects of BC, MSC, and its combination on improving ALP and TGR5 levels in Wistar rats post hepatectomy 50% with liver fibrosis. The combination of MSC and BC provides a synergistic effect in the healing process of liver fibrosis. There were no previous studies that discuss the effect of the combination of BC and MSC. Combination therapy after hepatectomy is expected to prevent recurrences from reducing mortality and morbidity in patients after hepatectomy.

## METHODS

This study includes 25 healthy and active male Wistar rats, 5 months old, 200-250 grams weight, were adapted and given standard feed (AIN-76A) and treated in the Molecular Biology Laboratory, Universitas Islam Sultan Agung, Semarang, Indonesia. About 25 male Wistar rats were adapted a week and divided into 5 groups randomly. There were several groups in this study such as Sham group (surgery only), Control group I (oral NaCl), treatment group I (received intraperitoneal CCL4 injection 2 µL/gram twice a week for 7 weeks, performed liver resection 50 % and given BC 15µL/gram orally with milk powder from goodhealth milk every day for 10 days), treatment group II (treatment intraperitoneal CCL4 injection 2 µL/gram twice a week for 7 weeks, performed 50% liver resection and administered 1000,000 MSC cells for 10 days), and treatment group III (intraperitoneal CCL4 injection 2 µL/gram twice a week for 7 weeks, performed 50% liver resection and administration of 1,000,000 MSC cells and accompanied by colostrum bovine 15µL/gram orally with milk powder from goodhealth milk every day for 10 days). In the 8th week, ALP and TGR5 were measured on Day-3,7, and 10.

MSCs were derived from the Umbilical cord (UC) of pregnant single Sprague-Dawley (SD) rats. UC's blood vessels were removed before it was transferred to a T25 culture flask which contained complete Dulbecco's Modified Eagle's medium (DMEM) (Sigma-Aldrich, Louis St, MO) mixed with 10% Fetal Bovine Serum (FBS) (Gibco™ Invitrogen, NY, USA) and 100 IU/mL penicillin/streptomycin (Sigma-Aldrich). The cells were incubated in a humidified atmosphere containing 5% $CO_2$ at 37°C. The medium was changed every 3 days. When these cells have reached 80% confluency, they were passaged with trypsin. This experiment used cells from the 4*th* passage. This study has obtained ethical approval from the authorized Institutional Review Board of the Ethics Committee of the Medical Department.

TGR5 levels were measured using the ELISA method. The reagent used was the Rat Gpbar1 ELISA kit from Wuhan Fine Biotech Co., Ltd. The sample used was hepatic tissue. The liver tissue was used in buffered PBS (0.1M; pH=7.4) with a ratio of 1 gram of tissue and 9 ml of PBS to remove residual blood. Destruction of cells is accomplished by the freeze-thaw cycle method. Then, the sample was centrifuged for 5 minutes at 5,000 g to take the supernatant. The protein concentration obtained should not be more than 0.3 mg.

Measurement of ALP levels refers to the International Federation of Clinical Chemistry (IFCC) methods. 2.5 ml of solution VI and 0.05 ml of reagent water were used as blank. 0.05 serum added to 2.5 ml of VI solutions in the container. Solution VI contains 2A2M1P 0.35 mmol/l, 4NPP 16 mmol/l, HEDTA 2 mmol/l, Magnesium sulfate 2 mmol/l, and Zinc Sulfate 1 mmol/l. Spectrophotometric at a wavelength of 405 nm.

All data were presented as mean ± standard deviation with differences between groups analyzed by One-Way ANOVA Post hoc LSD for parametric test and Kruskal-Wallis Post hoc Mann-Whitney for the nonparametric test. A p-value less than 0.05 is statistically considered significant. Data were analyzed using SPSS version 25 for Windows.

## RESULTS

On day 7, the combination group had the highest ALP levels (42.40±23.90 U/L), followed by the control group (38.80±25.30 U/L). The lowest ALP level was found in the BC group (24.20±9.52 U/L). The Kruskal-Wallis test showed no significant difference in ALP level observations on day 7 (p=0.552) (Figure 1).

On day 10, all groups had elevated ALP levels compared to day 7. The highest ALP levels were in the MSC group (1,439.00±639.85 U/L) and the lowest ALP levels were in the control group (108.00±120.21 U/L). The One-Way ANOVA test showed no significant difference in the observation of ALP levels on day 10 (p=0.068) (Figure 1).

On day 10, the sham group had lower mean ALP levels than the control and treatment groups. A significant difference in the sham group was found only in the sham group with MSC (p=0.014). In the treatment group observation, MSCs had higher ALP levels than the combination group and BC. A significant difference was found in the MSC group with BC (p=0.026) (Figure 1).

On day 3, the highest TGR5 concentration was the MSC group (2,282.67±204.18) and followed by the combination group (2014.33±86.99). The lowest TGR5 level was found in the BC group (1,224.30±749.05). The results of the Kruskal-Wallis analysis showed a significant difference in the TGR5 concentration on day-3 (p=0.026) (Figure 2).

On day 3, a significant difference was found in the sham and MSC groups (p=0.009). A significant difference was also found in the control group with MSC (p=0.014). In the treatment group observation, the highest mean concentrations of TGR5 levels were MSC, combination, and BC. Significant differences were found in the MSC and BC (p=0.007) (Figure 2).

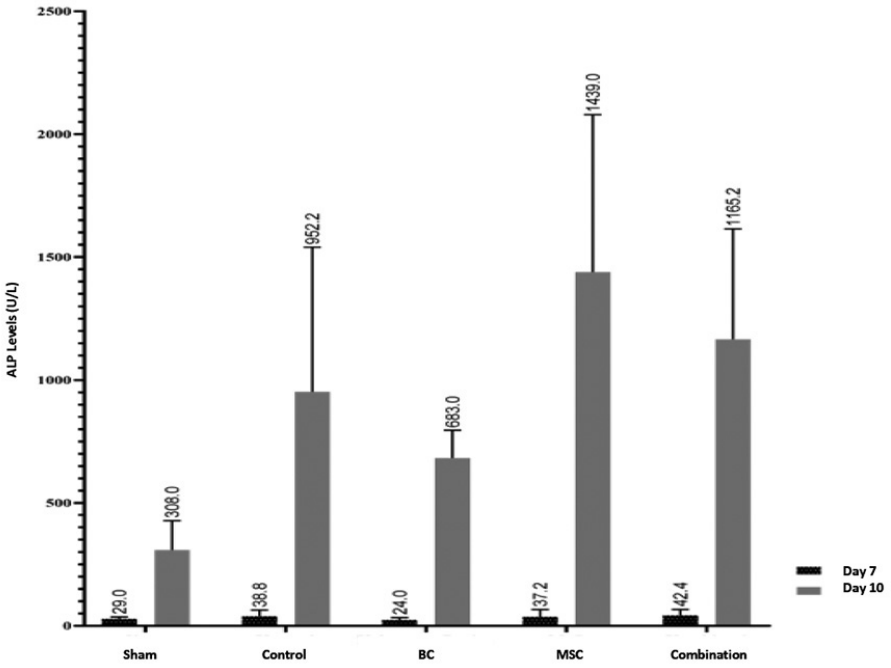


**Figure 1.** ALP levels in each group on Day 7 and 10

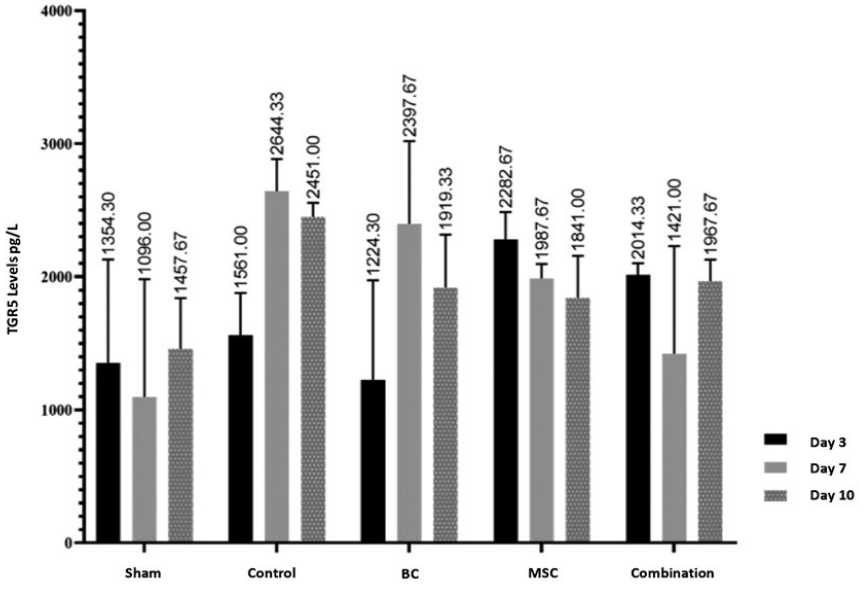


**Figure 2.** TGR5 levels in each group on Day 3, 7, and 10

**Table 1. Correlation test of TGR5 and ALP levels**

| Variables | ALP (Day 7) | | ALP (Day 10) | |
|---|---|---|---|---|
| | r | p | r | p |
| TGR5 (Day 3) | 0.183[a] | 0.330 | 0.596[a] | 0.014* |
| TGR5 (Day 7) | -0.150[a] | 0.528 | -0.025[a] | 0.922 |
| TGR5 (Day 10) | 0.909[a] | 0.721 | 0.077[b] | 0.763 |

TGR5: Takeda G-Protein Coupled Receptor-5; r: Coefficient Correlation; ALP: Alkaline Phosphatase; [a]: Spearman Correlation Test; [b]: Pearson Correlation Test; *Statistically significant if p-value less than 0.05

On day 7, the control group and BC showed an increase in the concentration of TGR5 compared to day 3. Meanwhile, the sham, MSC, and combination groups decreased TGR5 concentrations compared to day 3. The highest TGR5 concentration was found in the control group (2,644.33±241.56) and followed by BC (1,096.00±886.31). The results of the Kruskal-Wallis statistical analysis showed significant differences (p=0.026) (Figure 2).

The sham group had a lower mean TGR5 concentration than the other groups. Significant differences were found in the sham group with the control (p=0.005) and the sham group with BC (p=0.021). The control group had a higher mean TGR5 concentration than the other groups. There was a significant difference in the control group with the combination (p=0.013). In the treatment group on day 7, the mean TGR5 concentration from the highest was BC, MSC, and combination, respectively. No significant difference was found between treatment groups (Figure 2).

On day 10, the sham and combination groups had an increased TGR5 concentration compared to day 7. Meanwhile, the control, BC, and MSC groups decreased the TGR5 concentration compared to the 7th day. The highest TGR5 concentration was found in the control group (2,451.00±106.30). Meanwhile, the lowest TGR5 level was found in the sham group (1,457.67±381.86). The One-Way ANOVA statistical analysis results showed a significant difference between groups (p=0.005) (Figure 2).

Based on Table 1, we found a correlation pattern between TGR5 levels on day 3 and ALP levels on day 10. The Spearman correlation statistical analysis results showed a moderately significant positive correlation (r=0.596; p=0.014) between TGR5 in Day 3 and ALP levels in Day 10 (Table 1).

## DISCUSSION

ALP levels on the 7th day of treatment were in the range 24.2±9.52 - 42.4±23.9 IU/L. The normal range of ALP is 44-147 IU/L.[7] This indicates that ALP levels have decreased on the 7th day of treatment. In previous findings, normal rats with 70%

liver resection experienced an increase in ALP levels on the 3rd day, reaching 307 U/L and decreased the next day to form a slope at 250 U/L.[8] The lower reduction in this study is might be because the rats had fibrosis due to CCl4 induction at 8 weeks. The mechanism of decreasing ALP levels in rats with liver fibrosis and hepatectomy is not well understood. However, hepatic fibrosis due to CCl4 induction could inhibit the regeneration process resulting in lower ALP levels.

In this study, the drastic increase in ALP levels on day 10 might be due to hemodynamic changes of liver regeneration. In rats with partial hepatectomy, the increase in ALP was a marker of regeneration. The increase in ALP levels on the 10th day may also be due to the rise in the production of bile canaliculi cells which led to an increase in ALP levels in serum.[9] Thus, this study shows that in liver fibrosis and partial hepatectomy, the regeneration process is slower until the 7th day, and then the regeneration process begins to increase on the 10th day after treatment.

On TGR 5 observations, MSC had a significantly higher mean TGR5 level than BC, sham, and control on Day 3. After partial hepatectomy, the remaining liver will adapt to the excess bile acid, which led to bile acid intoxication.[10,11] Higher levels of TGR5 at baseline after liver resection treatment may protect the liver from damage due to excess bile acid. Nomenia research found that mice with TGR 5 knockout who underwent partial hepatectomy had more severe hepatocyte necrosis, prolonged cholestasis, and increased inflammatory response than normal mice with partial hepatectomy. In the absence of TGR 5 receptors, intrahepatic stasis resulting from abnormal hydrophobicity of bile acids and deficiency of bile acids in the urine can lead to excess bile acid levels, thus inhibiting liver regeneration.[12] In this study, the treatment and control groups showed higher TGR5 levels than the sham group.

TGR5 is expressed in the liver in non-parenchymal cells, such as Kupffer cells, sinusoidal endothelial cells, cholangiocytes, and mouse hepatocytes.[13-16] Activation of TGR5 reduces the liver inflammatory response by attenuating LPS-induced cytokine production via the classic TGR5-cAMP pathway and reduced NF-κB.[17] TGR5 signals NF-κB antagonists by decreasing IκBα phosphorylation, p65 nuclear translocation, and DNA binding activity of NF-κB.[17,18] Low TGR5 is known to be related to the inflammatory process that occurs in bile induced by bile acids. The mechanism of TGR 5 protects the liver from the cytotoxic effects of bile acids through the increased proliferation of non-ciliated cholangiocytes through inhibition of p-ERK-1/2 and inhibition of ciliated cholangiocyte cell proliferation through stimulation of p-ERK-1/2.[19] In the smooth muscle of the gallbladder, TGR5 signaling activates the cAMP-PKA pathway which causes hyperpolarization of smooth muscle by opening K-ATP channels thus inhibiting gallbladder contractility and increasing gallbladder filling.[20] In endothelial cell sinusoids, TGR5 activated AKT to increase NO production, serine phosphorylation of cystathionine γ-lyase and followed by hydrogen sulfide. In contrast, TGR 5 signaling shows inhibition of endothelin 1 (vasoconstrictor) expression and secretion. Thus, the effect of TGR 5 on sinusoids is a vasodilator so that it can increase microcirculation.[21,22]

Although MSC had a better TGR 5 receptor stimulation potential than BC on day 3, no significant difference was found between MSCs and the combination. MSC showed a better and significant difference in TGR5 compared to BC. On the 7th and 10th day observations, no significant differences were found between the treatment groups.

In this study, liver cell damage due to bile acid overload after partial hepatectomy was thought to be suppressed by TGR5 receptor expression from MSC and combination groups. This is based on the low levels of TGR 5 in the BC group on the 3rd day, followed by the low regeneration process in ALP levels on the 10th day. The correlation test results for TGR5 levels on the 3rd day with ALP levels on the 10th day showed significance with a moderate positive correlation. The high level of TGR5 on the 3rd day was due to the better induction of the proliferation of cholangiocyte cells which provides a protective effect against overload and hydrophobicity of bile acids.

On the 7th day, the control and BC groups increased TGR5 levels compared to the 3rd day. On the other hand, the MSC and combination groups showed a slight decrease in TGR5 levels on day 7. The control and BC groups had higher levels of TGR5 than the MSC group and the combination on day 7 was due to the slower regeneration process in the control and BC groups. On the 7th day, the control and BC groups were presumably still undergoing the regeneration process, so TGR5 was needed to protect the liver from damage caused by bile acid overload.

MSCs are known to differentiate into specific liver healing processes, namely into hepatocyte-like cells in vitro.[23] Through this mechanism, hepatocyte-like cells will produce paracrine growth factor, which regulates the proliferation of cholangiocyte and cholangiocyte-like cells. The differentiation ability of MSC is because these cells have the ability of paracrinization. This paracrinization is associated with the ability of MSC to secrete certain molecules related to the regeneration process. The process of paracrinization through exosomes from MSC is also associated with the production of oval cells or atypical ductal cells (ADC), which act as progenitor cells during the liver regeneration process. A previous study also showed that MSC administration in the damaged liver tissue leads to the MSC migration to the injured areas for repairing and restoring the damaged liver structure and its function. It is due to the immunomodulatory effect of MSC and it releases secretome to enhance the regeneration of hepatocyte.[24]

BC is known to contain protective factors and can be a source of immunomodulatory molecules that affect immunological status. In a previous study, high concentrations of IL-1b, IL-6, TNF- and INF-g were detected in BC. These cytokines are known as inflammatory cytokines that synergistically mediate inflammation.[25] The study showed that in BC, TNF-α cytokines were more dominant than other cytokines. TNF-α as pro-inflammatory, proliferative, and apoptotic cytokines. TNF-α enhances the survival of Hematopoietic Stem Cells (HSC) and immunological activation associated

with liver fibrosis. TNF- α plays a role in chronic liver injury and inflammation, but the role of TNF-α in liver fibrosis is still controversial.[25]

A study showed that TGR5 also modulates signal transducer activation and activates transcription 3 (STAT3). STAT3 is a transcription factor that plays an important role in the inflammatory process in the liver. Expression of pro-inflammatory cytokines was increased in TGR5-deficient mice, including IL-1b, TNFa, IL-6, IFN-c, monocyte chemoattractant protein-1 (MCP-1) and Interferon-Inducible Protein-10 (IP-10).[26] This is related to the previous explanation that, on the 3rd day of TGR5 observation, the lowest TGR5 value was found in the BC group.

In addition, BC contains an antioxidant enzyme in the form of lactoperoxidase (LPO). LPO is a member of the peroxidase family and catalyzes the oxidation of thiocyanates to synthesize various antimicrobials.[27] In this study, the administration of BC therapy containing LPO to rats with liver cirrhosis will provide a protective effect against free radicals due to CCl4 toxicity. Other than that, it can provide benefits to prevent liver infections that are regenerating.

Based on the discussion above, MSC has the potential to help the liver regeneration process through the oval cell mechanism. With BC therapy, the content of growth factors, antioxidants, antimicrobials, and cytokines such as IL-6 is expected to help regenerate the liver and protect the liver from the formation of fibrotic tissue. The administration of combination therapy can produce a synergistic process between liver cell proliferation carried out by MSCs and stimulation of proliferation and anti-fibrotic protection by bovine colostrum. However, the administration of MSC itself has a better potential to induce cholangiocyte and liver regeneration than BC and combination due to an immunomodulatory effect of MSC. It enhances the regeneration of hepatocytes by release secretome.[28] Other than that, in combination therapy, BC has a growth factor product that leads to immunomodulator disruption from MSC.[29] Theoretically, BC might be effective against free radical from CCL4, but it is presumably less effective to protect the liver from bile acid overload. So, the administration of MSC without BC or combination is recommended in this study. The limitation of this study is that the indicator of liver regeneration in this study is still limited to ALP, so it is necessary to assess with broader indicators.

## CONCLUSION

The combination of BC and MSC does not improve better than the administration of BC or MSC itself in terms of increasing ALP and TGR5 levels in rats with liver fibrosis after hepatectomy 50%. The administration of MSC is recommended in this study. There is a relationship between increased ALP levels and increased TGR5 levels in rats with hepatic fibrosis after 50% hepatectomy. Further research is needed in the combination therapy as a liver regeneration process with broader indicators such as measurement of specific biochemical levels in the liver and liver histopathological examination to determine the particular cells that develop after the therapy.

## CONFLICT OF INTEREST

There is no competing interest regarding the manuscript

## ETHICS CONSIDERATION

The present study obtained approval for the animal experiment from the Ethical Committee for Health Research Universitas Islam Sultan Agung Semarang (approval number 337/X/2020/Komisi Bioetik date of release October 15th, 2020) prior to the study being conducted.

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## AUTHOR CONTRIBUTIONS

AE, BP contributed for conception, design, analysis and interpretation. BP, IR, AP, EP contributed for intellectual content and final approval.

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