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## The Effect of Mixed Liquor Administration on The Johnsen's Score and The Number of Sertoli Cells and Leydig Cells on The Wistar Strain White Rats (*Rattus norvegicus*)

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### Abstract

To analyze the effect of mixed liquor administration on the Johnsen's score and the number of Sertoli cells and Leydig cells on the Wistar strain white rats (*Rattus norvegicus*). A total of 28 rats were divided into 4 groups: C, P1, P2, and P3. Rats were given mixed liquor with different dose, namely P1 (1 ml), P2 (2 ml), and P3 (4 ml) each day for 14 days, then compared to C group which was given 4 ml of distilled water using an oral gavage. Mixed liquor consists of 20% ethanol and 4% methanol. The histopathological features were evaluated by the Johnsen's score, the number of Sertoli cells, and Leydig cells in cross-sectional preparation of rat testicular tissue with 400× magnification. Data were analyzed using the Kruskal-Wallis test and One-Way ANOVA test with a confidence level of  $p < 0.05$ . The P3 group had the lowest Johnsen's score and the number of Sertoli cells,  $6.442 \pm 0.293$  and  $5.942 \pm 0.674$ , respectively. A significant decrease in increased dose occurred in the Sertoli cell count but not in the Johnsen's score. Group P2 had the lowest number of Leydig cells,  $6.421 \pm 0.360$ . The administration of mixed liquor caused a decrease in Johnsen's score and the number of Sertoli cells and Leydig cells on the Wistar strain white rat (*Rattus norvegicus*).

**Keywords:** mixed liquor, spermatogenic cells, Sertoli cells, Leydig cells.

### Introduction

Alcoholic drinks or liquor are drinks that contain ethyl alcohol or ethanol ( $C_2H_5OH$ ) which are known to cause addiction.<sup>[1]</sup> Addiction that is satisfied continuously will cause a tolerance effect, that is a desire to increase the dose in order to get the same effect.<sup>[1,2]</sup> This type of mixed alcoholic drink is often called mixed liquor or *miras oplosan* in Indonesian. Ethanol, which is commonly consumed has negative effect on the male reproductive organs and is associated with several incidence of infertility.<sup>[3,4]</sup> A study by Fauziah proves that *arak bali*, Balinese wine can reduce the number of

spermatogenic cells and the size of the seminiferous tubules of mice.<sup>[5]</sup> In another study by Antari, *arak bali* also had an impact on lowering the quality of spermatozoa and testosterone levels.<sup>[6]</sup>

Methanol is one of the substances that is often added because it is effective to increase the effect of drunk.<sup>[7]</sup> Mixed liquor containing methanol is also reported to cause some death in several areas in Indonesia.<sup>[8,9]</sup> Ethanol is known to have an effect on male infertility, so the addition of methanol to mixed liquor may have more impact on male reproductive organs.<sup>[3,4,10]</sup>

Apart from the substance themselves, cell damage is also caused by metabolites of these substances.<sup>[11-14]</sup> The transformation of ethanol into acetaldehyde and free radicals is known to occur directly in the testes.<sup>[15,16]</sup> Cell damage by ethanol and methanol occurs due to increased levels of ROS, decreased GSH levels, and

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decreased ATP synthesis.<sup>[11-13]</sup> Therefore, this study aims to analyze the effect of mixed liquor administration on the Johnsen's score and the number of Sertoli cells and Leydig cells on the Wistar strain white rats (*Rattus norvegicus*).

**Materials and Methods**

This study is an experimental laboratory study with post-test only control group design. This study used 28 male Wistar rats (*Rattus norvegicus*) which were divided into four groups. The treatment given was administration of mixed liquor with an oral gavage for 14 days to determine its effect on the histopathological features of rats' testes.

Mixed liquor was given at different doses in each group, namely 1 ml (P1), 2 ml (P2), and 4 ml (P3). The composition of mixed liquor was 20% ethanol and 4% methanol. The composition was determined based on the results of gas chromatography tests of mixed liquor samples.<sup>[17]</sup> This study used 28 rats in each group, so the total volume of mixed liquor to be prepared for 14 days

was  $14 \times [7(1+2+4)] = 686$  ml. 1000 ml of mixed liquor was made to simplify calculations and anticipate spills. The mixed liquor was made by mixing 208.3 ml of 96% ethanol, 40.81 ml of 98% methanol, and 750.89 ml of distilled water. Assessment of the histopathological features was carried out by identifying spermatogenic cells, Sertoli cells, and Leydig cells referring to Pintus et al.<sup>[18]</sup> The data was analyzed by statistical software product and service solution 20 for Windows (SPSS 20).

**Results and Discussion**

The samples of this study were 28 adult male rats with 7 rats in each group. Observation of spermatogenic cells, Sertoli cells, and Leydig cells were made on both testes, so that each rat produced two data which were then averaged. The analysis was performed on 28 datas. The data of Johnsen's score was an ordinal data, so non-parametric test (Kruskal-Wallis) was chosen. The result showed that the data of Johnsen's score had significant difference ( $p=0.000$ ). Therefore, the analysis was continued with Mann-Whitney test.

**Table 1. Comparison of the Johnsen's score in each group.**

Group	N	Mean±SD	p-Value
C	7	8.350±0.165	0.000*
P1	7	7.550±0.104	
P2	7	7.478±0.152	
P3	7	6.442±0.293	

\* $p < 0.05$ , significantly different by statistic

**Table 2. Mann-Whitney test analysis for the comparison of the Johnsen's score and the number of Leydig cells.**

Comparison between Groups	C vs P1	C vs P2	C vs P3	P1 vs P2	P1 vs P3	P2 vs P3
p-value of Johnsen's score	0.002*	0.002*	0.002*	0.301	0.002*	0.002*
p-value of the number of Leydig cells	0.002*	0.002*	0.002*	0.083	0.442	0.025*

\* $p < 0.05$ , significantly different by statistic

**Table 3. Comparison of the number of Sertoli cells in each group.**

Group	N	Mean $\pm$ SD	Normality	p-Value	Homogeneity
C	7	11.542 $\pm$ 0.401	0.380	0.000*	0.291
P1	7	9.657 $\pm$ 0.504	0.552		
P2	7	7.557 $\pm$ 0.53	0.793		
P3	7	5.942 $\pm$ 0.674	0.361		

**Table 4. Post-hoc analysis for the comparison of the number of Sertoli cells.**

Comparison Between Groups	Mean Difference	CI95%		p-Value
		Lower Bound	Upper Bound	
C vs P1	1.885	1.094	2.677	0.000*
C vs P2	3.985	3.194	4.777	0.000*
C vs P3	5.600	4.808	6.391	0.000*
P1 vs P2	2.100	1.308	2.891	0.000*
P1 vs P3	3.714	2.922	4.505	0.000*
P2 vs P3	1.614	0.822	2.405	0.000*

\* $p < 0.05$ , significantly different by statistic

**Table 5. Comparison of the number of Leydig cells in each group.**

Group	N	Mean $\pm$ SD	Normality	p-Value
C	7	11.378 $\pm$ 0.297	0.006	0.000*
P1	7	6.907 $\pm$ 0.511	0.806	
P2	7	6.421 $\pm$ 0.360	0.608	
P3	7	7.150 $\pm$ 0.621	0.304	

\* $p < 0.05$ , significantly different by statistic

The Mann-Whitney test showed that the Johnsen's score of the control group (C) was significantly higher than

the group receiving mixed liquor with different doses: 1 ml (P1), 2 ml (P2), and 4 ml (P3) with the value of  $p=0.002$  on each compared group. So, all of the treatment groups (P1, P2, and P3) had significant reduction in Johnsen's score compared to the control group. Besides, the significant reduction was also found in the increasing dose from P1 group to P3 group (7.550 vs 6.442; 0.002); and from P2 group to P3 group (7.478 vs 6.442; 0.002), but there was no significant reduction from the P1 group to P2 group (7.550 vs 7.478; 0.301).

The data normality of the number of Sertoli cells was tested by Shapiro-Wilk test; the result showed that each group was normally distributed ( $p > 0.05$ ). The analysis was continued by the One-Way ANOVA test and showed a significant difference in the number of Sertoli cells ( $p=0.000$ ). The homogeneity test was also conducted to determine the selection of the post-hoc test method; the result showed that the data was homogeneous. Therefore, the analysis was continued with the post-hoc test.

The post-hoc test showed that the number of Sertoli cells of the control group (C) was significantly higher than the group receiving mixed liquor with different doses with the value of  $p=0.000$  on each compared group. So, all of the treatment groups (P1, P2, and P3) had significant reduction in the number of Sertoli cells compared to the control group (C). Besides, the

significant reduction was also found in the increasing dose from P1 group to P2 group (9.657 vs 7.557; 0.000); from P1 group to P3 group (9.657 vs 5.942; 0.000); and from P2 group to P3 group (7.557 vs 5.942; 0.000).

The data normality of the number of Leydig cells was tested by Shapiro-Wilk test; the result showed that the control group (C) was not normally distributed ( $p=0.006$ ) and the remaining groups were normally distributed ( $p > 0.05$ ). The analysis was continued by the non-parametric test (Kruskal-Wallis) and showed a significant difference in the number of Leydig cells ( $p < 0.05$ ). Therefore, the analysis was continued with the Mann-Whitney test.

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fibroblasts after epithelial-stromal interactions. MMP is a major protease in degrading the extracellular matrix, which leads to cancer cell invasion and metastasis.<sup>7</sup>

EMMPRIN binds to Cyclophilin A (CypA). A previous study showed that the CypA-EMMPRIN interaction-initiated growth was signaling via a variety of pathways, including the MAPK, ERK1/2, and p38 signaling pathways that induce G1 to S transitions via cyclin D1 and p-RB in cholangiocarcinoma.<sup>8</sup> Correlation between EMMPRIN expression in cell carcinoma kidney has not been reported. Therefore, this study was conducted to analyze EMMPRIN expression with perirenal fat invasion status in renal cell carcinoma. This study aims to prove the role of EMMPRIN on the status of perirenal fat invasion in Clear Cell Renal Cell Carcinoma (CCRCC).

## Materials and Methods

### Preparation and Sample of the Study

This study's research design was an analytic observational study with a cross-sectional approach, which was carried out in the Anatomic Pathology Installation of Dr. Soetomo General Academic Hospital Surabaya, Indonesia. The study sample used 44 blocks of paraffin radical nephrectomy preparations for CCRCC patients at the Anatomical Pathology Institute of Dr. Soetomo General Academic Hospital, Surabaya, for the period January 2013-December 2018. The samples were divided into two groups based on perirenal fat invasion status. The parameter of assessment was the expression of EMMPRIN, which streaked positively on tumor cells. This study was approved by the Health Research Ethics Committee of Dr. Soetomo General Academic Hospital, Surabaya, Indonesia (Ethical Clearance No.1705 / KEPK / XII / 2019).

### Immunohistochemical Procedures

EMMPRIN expression in samples was observed using immunohistochemical staining. Paraffin blocks were cut 4  $\mu$ m, deparaffinized, and rehydrated with graded alcohol, then warmed with citrate buffer pH 6 for 20 minutes in the microwave. The primary

antibody, namely EMMPRIN (sc-71038, Santa Cruz Biotechnology, Inc.), was dripped by diluting 1: 250 at 40°C overnight. The secondary antibody is then dropped and incubated for 20 minutes. The final step, diaminobenzidine (DAB), was dripped, and counterstain was carried out with Meyer Hematoxylin.

### Immunohistochemical Staining Analysis

EMMPRIN expression was assessed using an Immunoreactive Score (IRS), which is the multiplication of the percentage of tumor cells stained (A) and the intensity of staining (B). The percentage is divided into a score of 0 = no positive tumor cells, score 1 = positive tumor cells < 10%, score 2 = positive tumor cells 10 - 50%, score 3 = positive tumor cells 51 - 80%, and score 4 = cells positive tumors > 80%. Intensity was divided into a score of 0 = colorless, score 1 = weak intensity, score 2 = moderate intensity, and score 3 = strong intensity. The IRS (AXB) was divided into four groups, namely negative (score 0), weak (score 1 - 3), moderate (score 4 - 8), and strong (score 9 - 12). EMMPRIN expression was observed in the membrane and cytoplasm of tumor cells.<sup>9</sup> EMMPRIN expression was observed using a binocular light microscope and evaluated by two pathologists.

### Statistical Analysis

The correlation between perirenal fat invasion status and EMMPRIN expression was tested by the Spearman correlation test. The test results are said to have a significant correlation if the *p*-value is <0.05.

### Results and Discussion

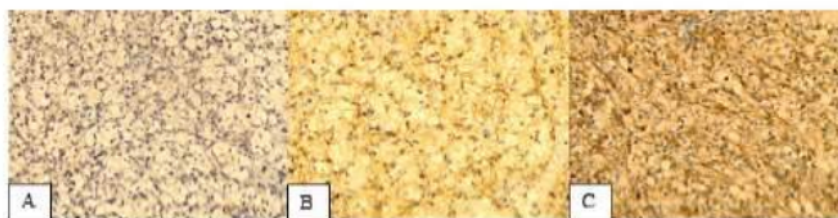
The patients' average age was 53.89 years with a male to female ratio of 2:1. In this study, clear cell, non-perirenal fat invasive (non-PFI) renal cell carcinoma was found in 59.1% (26/44) of cases, whereas clear cell, perirenal fat invasive (PFI) type renal cell carcinoma was only 40, 9% (18/44) of cases. The highest grade in this study was grade 3 (54.5%) cases (24/44). The clinicopathological characteristics of the patients are shown in Table 1.

**Table 1. The clinicopathological characteristics of the patient.**

Characteristics	n (%)
Age (years) #	
£ 40	2 (4.6)
41-50	13 (29.5)
51-60	17 (38.6)
61-70	11 (25.0)
>70	1 (2.3)
Gender	
Male	32 (72.7)
Female	12 (27.3)
Tumor Grade	
Grade 1	5 (11.4)
Grade 2	12 (27.3)
Grade 3	24 (54.5)
Grade 4	3 (6.8)
Perirenal Fat Invasion	
(pT1-2) / Non PFI	26 (59.1)
(pT2-4) / PFI	18 (40.9)

EMMPRIN expression in this study was stained on the membrane and cytoplasm of tumor cells (Figure 1). The results of this study indicated that EMMPRIN expression with a strong IRS score was more common in clear cell renal cell carcinoma with perirenal fat invasion,

namely 66.7% (Table 2). The Spearman correlation test results showed a significant correlation between perirenal fat invasion status and EMMPRIN expression ( $p < 0.05$ ) with a value of  $r = 0.352$  (Table 3). These results indicate that the higher the EMMPRIN expression is in line with the perirenal fat invasion status.



**Figure 1. EMMPRIN expression by immunohistochemical staining on clear cell renal cell carcinoma, magnification: 400×. A: EMMPRIN expression with weak intensity; B: Medium intensity EMMPRIN expression; C: EMMPRIN expression with strong intensity.**



Table 2. EMMPRIN expression on the status of perirenal fat invasion of Clear Cell renal cell carcinoma.

EMMPRIN Expression	Category	Non-invasive perirenal fat	Invasive perirenal fat	P
Percentage	<10%	0 (0%)	0 (0%)	
	11-50%	1 (3.8%)	0 (0%)	
	51-80%	21 (80.8%)	11 (61.1%)	
	>80%	4 (15.4%)	7 (38.9%)	
Intensity	Weak	3 (11.5%)	0 (0%)	
	Moderate	14 (53.9%)	7 (38.9%)	
	Strong	9 (34.6%)	11 (61.1%)	
IRS Score	Weak	4 (15.4%)	0 (0%)	0.019
	Moderate	13 (50%)	6 (33.3%)	
	Strong	9 (34.6%)	12 (66.7%)	

Table 3. The Spearman correlation test results of EMMPRIN expression with perirenal fat invasion status.

		EMMPRIN Expression
Perirenal fat invasion status	r	0.352
	p	0.019
	n	44

Most of the patients in this study were in the age range 51 - 60 years with 17 cases (38.6%), with a male to female ratio of 2:1 where the number of male cases was 32 cases (72.7%). This is in accordance with previous research which states that the highest incidence is found in the sixth and seventh decades of life and about 80% are between the ages of 40 - 69 years with the distribution of men more than women.<sup>10,11</sup>

The results of this study indicate that EMMPRIN expression is stronger in Clear Cell Renal Cell Carcinoma (CCRCC) with perirenal fat invasion, and there is a significant correlation which indicates that the higher EMMPRIN expression is in line with the occurrence of perirenal fat invasion in Clear Cell Renal Cell Carcinoma (CCRCC).

The results of this study are in line with the research conducted by Zheng et al., which stated that the increased expression of EMMPRIN was significantly correlated with tumor size, depth of invasion, lymph vessel invasion, MMP 2, MMP 9, and tumor VEGF ( $p < 0.05$ ) in gastric carcinoma. This study showed that the abnormal expression of EMMPRIN can increase tumor cell invasion and angiogenesis by increasing the expression of MMP and VEGF in stromal fibroblasts and gastric carcinoma cells so that increased EMMPRIN expression could be used as an effective and objective marker in predicting invasion and prognosis in gastric carcinoma.<sup>12</sup> A study by Nakamura et al. also found that high EMMPRIN expression is a significant marker of poor prognosis in endometrial cancer. EMMPRIN affects the proliferation, migration, and invasion of tumor cells

through the expression of TGF- $\beta$ , EGF, VEGF, MMP-2, MMP-9. The binding between growth factors such as TGF beta, EGF, IGF, and TNF alpha with their receptors can activate cadherin E inhibiting factors such as Snail. The decrease in E-cadherin expression resulting in the loss of bonds between cells is an early stage of EMT.<sup>13</sup>

Various studies have shown that EMMPRIN plays an important role in the invasion and metastasis of various tumors, such as hepatocellular carcinoma, astrocytic glioma, retinoblastoma, and oral squamous cell carcinoma through increased MMP production. EMMPRIN has also been found to play a role in urothelial carcinoma invasion through the secretion of MMP2, MMP9, MMP14, and VEGF.<sup>14</sup>

The multifunctional role of EMMPRIN in advanced RCC is not only as an adhesion molecule involved in Cell-Matrix-Extracellular interactions (ECM) but also as a mediator for tumor invasion and angiogenesis through stimulation of VEGF production. Multivariate analysis showed a strong association between EMMPRIN and VEGF expression and poor prognosis in advanced RCC.<sup>6</sup> EMMPRIN expression was found to be significantly associated with increased tumor invasion. These observations strongly suggest that EMMPRIN may be actively involved in the growth, invasion, and metastasis of OSCC. In addition, measurement of EMMPRIN levels can help predict a patient's prognosis.<sup>15</sup>

EMMPRIN is a transmembrane glycoprotein belonging to the immunoglobulin superfamily that is highly expressed on the cell surface of various types of tumors, including kidney cancer.<sup>5</sup> EMMPRIN acts as a cellular adhesion molecule and induces the secretion of matrix metalloproteinases (MMPs) and the release of cytokines.<sup>16</sup> EMMPRIN stimulates cancer cells and fibroblasts peritumoral to secrete matrix metalloproteinases (MMPs), which are capable of lowering extracellular matrix protein (ECM), and EMMPRIN directly promotes tumor proliferation, invasion, and metastasis.<sup>17</sup>

EMMPRIN has been shown to be involved in the regulation of tumor cell invasion and metastasis. First, EMMPRIN combines with the alpha6beta1 integrin into the FAK P13K-Ca (2+) pathway and the MARK signal, which then produces interstitial collagenase (MMP-1), forming a CD147-MMP-1 complex on the surface

of tumor cells, thus modifying the pericellular cell matrix tumor to promote invasion. Second, EMMPRIN is a receptor for platelet GPVI and mediates platelet movement through the GPVI-EMMPRIN Combination, thereby increasing the potential for metastasis. High EMMPRIN expression can be used to determine the TNM stage, histopathological stage, metastases, and worse survival in patients with kidney cancer.<sup>18</sup>

Another study investigated the effects of EMMPRIN on prostate cancer proliferation. EMMPRIN is expressed on the cell surface of most tumor cells, which results in proliferation, invasion, metastasis, and angiogenesis of cancer cells. Previous studies have shown that EMMPRIN can increase prostate cancer invasion and metastasis. The study showed that the inhibition of the EMMPRIN gene had a significant effect on the prostate cancer cell cycle, where a decrease in EMMPRIN expression resulted in an increase in the G0/G1 phase and a significant decrease in the S and G2 phases, indicating the cessation of the G1 phase. The G1 phase, the cell cycle phase in which cells grow and synthesize mRNA and protein for DNA synthesis, is very important because it determines whether the cell is committed to division or escape the cell cycle. The study states that EMMPRIN suppresses the progression of cancer cells by resting the cell cycle in the G0/G1 phase of cancer by suppressing cyclin D1 expression, there by inhibiting cell proliferation.<sup>19,20</sup>

### Conclusion

In conclusion, EMMPRIN expression was significantly correlated to the perirenal fat invasion. EMMPRIN expression has an important role in the Clear Cell Renal Cell Carcinoma (CCRCC).

**Conflict of Interest:** The authors declare that they have no conflict of interest.

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