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Deciphering the Role of TGF-β1 in Altering Collagen I and Collagen III in the New Zealand Rabbit's (Oryctolagus cuniculus) Urethral Wall in Urethral Stricture Development

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ABSTRACT

Background: Presently, there's a lack of standardization in animal models used for studying urethral stricture. Transforming Growth Factor Beta 1 (TGF-β1) is known to regulate the deposition of extracellular matrix in both normal and pathological conditions. This factor holds promise as a potential model for simulating urethral stricture. **Objective:** This study aims to investigate the impact of Transforming Growth Factor Beta 1 (TGF-β1) on Collagen I and Collagen III within the urethral wall of New Zealand Rabbits (Oryctolagus cuniculus) in the context of developing urethral stricture in animal models. **Methods:** We conducted genuine laboratory experiments using Male New Zealand rabbits (Oryctolagus cuniculus), which were categorized into five groups: control, placebo, and three treatment groups (TGF-β1 injections of 1 µg, 2 µg, 4 µg). After a duration of 6 weeks, we conducted urethrography, histopathological analysis, and assessed the formation of collagen I and collagen III within the urethral wall. **Results:** Elevating the dosage of TGF-β1 led to a reduction in the average urethral lumen diameter of rabbits (29.3% in the 2µg group and 34% in the 4µg group) compared to the control group. In fact, three rabbits experienced a decrease of ≤ 50% in their urethral lumen diameter. As the doses of TGF-β1 increased, we observed significant increases in the density of collagen I, and collagen III in both the periluminal and peripheral regions of the urethral spongiosum. Additionally, there was a tendency for the collagen I/collagen III ratio to decrease in the periluminal region, with collagen III density surpassing that of collagen I. In the peripheral spongiosa area, notable mean differences were observed between the control group, 1T, and 2T groups, with collagen I density tending to be higher than that of collagen III. Furthermore, the percentage of urethral lumen diameter exhibited a robust negative correlation with periluminal collagen I density ($r = -0.672$, $p = 0.001$), peripheral spongiosa collagen I density ($r = -0.603$, $p = 0.005$), periluminal collagen III density ($r = -0.717$, $p = 0.001$), and an exceptionally strong negative correlation with collagen III density of peripheral spongiosa ($r = -0.804$, $p = 0.000$). **Conclusion:** TGF-β1 exerts an influence on altering the composition of collagen I and collagen III within the urethral wall of rabbits, leading to a reduction in the diameter of the urethral lumen. Further research is warranted to determine the optimal dose of TGF-β1 required to induce urethral stricture effectively.

Keywords: TGF-β1, Urethral Stricture, Collagen I, Collagen III.

1. BACKGROUND

Brachial Urethral stricture remains a challenging urologic disorder to address effectively. Commonly employed treatments for anterior partial urethral strictures, such as internal urethrotomy and urethral dilatation, exhibit a high failure rate (40-60%) attributed to the formation of spongiofibrotic tissue post-procedure (1). Consequently, research into the pathogenesis of urethral stricture and the development of strategies to prevent its recurrence is gaining momentum (2). Nevertheless, the establishment of a standardized

animal model that can accurately replicate the aberrations in wound healing and fibrotic processes associated with urethral stricture remains elusive (3,4). Transforming Growth Factor Beta 1 (TGF-β1), a polypeptide cytokine, plays a pivotal role in regulating extracellular matrix deposition, both in normal tissue repair and in pathological fibrosis. TGF-β1 is recognized as a key factor in the development of urethral stricture models (4,5).

In previous research, a novel animal model utilizing mice and the application of the profibrotic agent TGF-β1 was introduced to simulate the fibrotic processes involved in urethral strictures (4-6). This study observed an increase in collagen deposition (7,8). However, the diagnosis of urethral stricture was solely based on histopathological findings and protein expression, without an assessment of the distribution of periluminal and peripheral spongy collagen (9). Moreover, this mouse model offered only limited urethral caliber, making it challenging to conduct additional urethral manipulations, including urethrography and endoscopy (14).

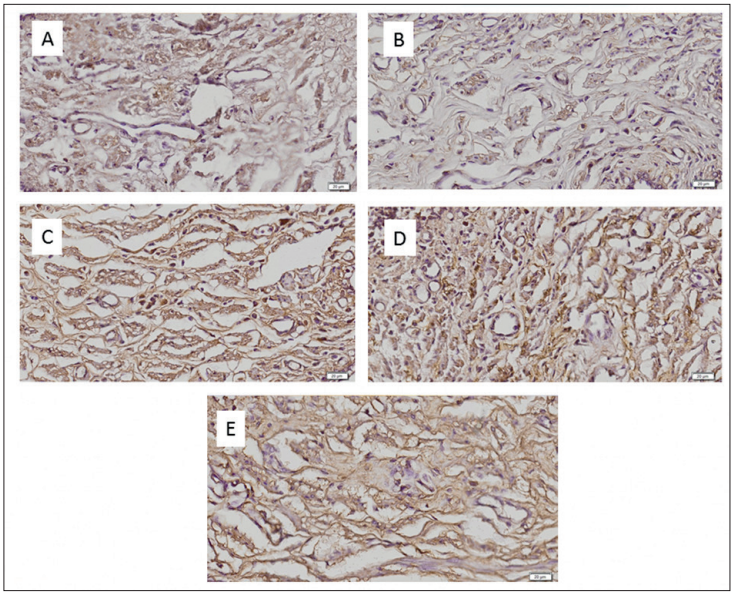

Figure 1. Collagen I Expression (Immunohistochemical Staining) in Periluminal Urethral Tissue (400x). This figure presents cross-sections of collagen I expression at a magnification of 400x in different treatment groups: (A) Control, (B) Sham, (C) TGF-β 1 mcg, (D) TGF-β 2 mcg, and (E) TGF-β 4 mcg. Notice the increased expression depicted in chocolate coloration across all treatment groups.

Building upon the success of TGF-β1 in inducing urethral stricture fibrosis in mice, we have adapted this approach to a larger experimental animal, the New Zealand rabbit, given its larger urethral caliber (14). We aspire for this model to evolve into an ideal representation for urethral stricture research in the future. Our evaluation focuses on intensifying the fibrosis process by assessing periluminal and peripheral spongiosa collagen type I and collagen type III within the injection site, with the aim of establishing an animal model exhibiting a histopathological pattern akin to that observed in humans.

This research study has obtained ethical research approval from the ethics committees of the Saiful Anwar Hospital Faculty of Medicine at Universitas Brawijaya (registration number: 400/251/K.3/302/2019).

2. OBJECTIVE

This study aims to investigate the impact of Transforming Growth Factor Beta 1 (TGF-β1) on Collagen I and Collagen III within the urethral wall of New Zealand Rabbits (Oryctolagus cuniculus) in the context of developing urethral stricture in animal models.

3. MATERIAL AND METHODS

Animals:

A total of twenty male New Zealand rabbits (Oryctolagus cuniculus) were sourced from a standardized animal research breeding center and used for preconditioning before the experiment. These rabbits were housed in individual cages with a 12-hour reversed cycle lighting schedule and had unrestricted access to food and water. Anesthesia for surgical procedures was administered through intramuscular ketamine (75 mg/kg) and xylazine (50 mg/kg). Carbon dioxide asphyxia was employed as a means of euthanasia before assessing urethral histology.

Study Design:

The principal objective of this study was to assess the impact of local TGF-β1 injections on spongiofibrosis within the rabbit's urethral wall. The rabbits were randomly categorized into five groups: control, placebo, and three treatment groups receiving TGF-β1 injections of 1 μg (1T), 2 μg (2T), and 4 μg (4T). Rabbits designated as Placebo/Sham (n = 4) received a 1 μL saline injection in the spongious urethra. The remaining rabbits received injections of 1 μg, 2 μg, and 4 μg of TGF-β1 in 100-μl buffered saline, followed by urethral incisions (as shown in the illustration). Six weeks later, while under sedation, the rabbits underwent a urethrography examination. Following this, the rabbits were promptly euthanized, and penile and urethral tissues were collected for histological analysis. Subsequently, investigations into collagen I and collagen III expressions were conducted in the urethral wall, both in the periluminal and peripheral spongiosa regions. All data collected from these various assessments were analyzed by investigators who were blinded to the group allocations.

Data analysis

The data underwent analysis using SPSS 20 and were presented as the standard deviation of the mean. For comparing multiple groups, we utilized one-way analysis of variance when the parametric assumptions were met; otherwise, the Kruskal-Wallis test was employed. Post hoc comparisons were carried out subsequently.

Furthermore, correlation analysis was conducted to examine the relationship between the percentage of urethral lumen and the density of collagen I and collagen III, both in the periluminal and peripheral spongiosa ar-

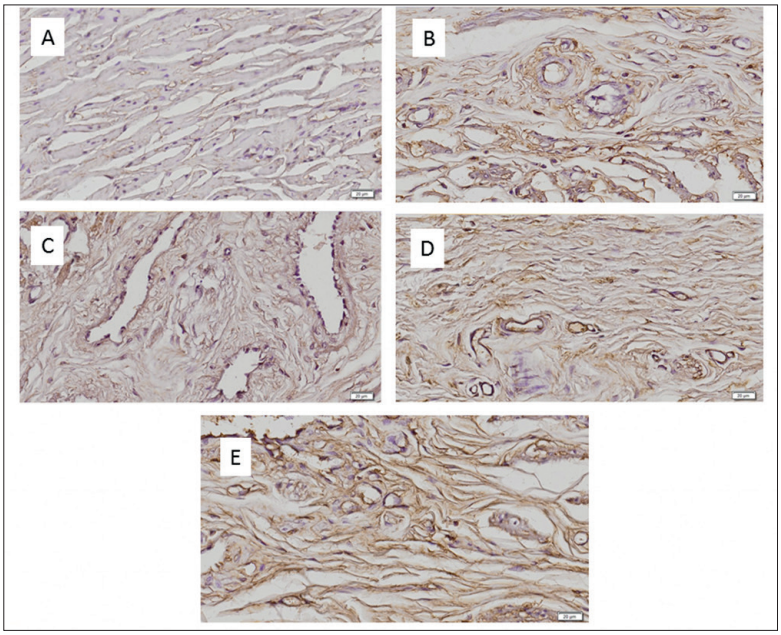

Figure 2 Collagen I Expression (Immunohistochemical Staining) in Peripheral Spongyosa Urethral Tissue (400x). This figure depicts cross-sections of collagen I expression at a magnification of 400x within various treatment groups: (A) Control, (B) Sham, (C) TGF-β 1 mcg, (D) TGF-β 2 mcg, and (E) TGF-β 4 mcg. Notably, there is an evident increase in chocolate-colored expression across all treatment groups.

eas. The threshold for statistical significance was set at $p < 0.05$.

4. RESULTS

Collagen I Density

Significant increments in collagen I density were observed in both the periluminal region and peripheral spongiosa of the urethral wall with escalating doses of TGF-β1. Within the periluminal urethral region, collagen I density exhibited a notable 1.4-fold increase in the 1T group ($p = 0.009$), a substantial 1.8-fold increase in the 2T group ($p = 0.004$), and a significant 1.7-fold rise in the 4T group ($p = 0.010$) compared to the control group. Meanwhile, in the peripheral spongiosa region, there was a substantial augmentation in the average collagen I density, with a 2.5-fold increase in the sham group ($p = 0.03$), a striking 5.8-fold elevation in the 1T group ($p = 0.014$), a remarkable 8.3-fold rise in the 2T group ($p = 0.003$), and a significant 8.6-fold increase in the 4T group ($p = 0.005$) as seen in Figures 1 and 2.

Collagen III density

The assessment of collagen III density revealed a consistent elevation in both the urethral periluminal region and peripheral spongiosa. In the periluminal area, there was a substantial 3-fold increase in density in the 1T group, a remarkable 4.5-fold rise in the 2T group, and a striking 5-fold augmentation in the 4T group ($p = 0.000$). In the peripheral spongiosa region, mean collagen III density exhibited an increase of 1.8 times in the sham group ($p = 0.005$), 1.7 times in the 1T group ($p = 0.009$), a notable 3.7-fold rise in the 2T group ($p = 0.000$), and a significant 3.2-fold elevation in the 4T group ($p = 0.000$) as seen in Figure 3 and 4.

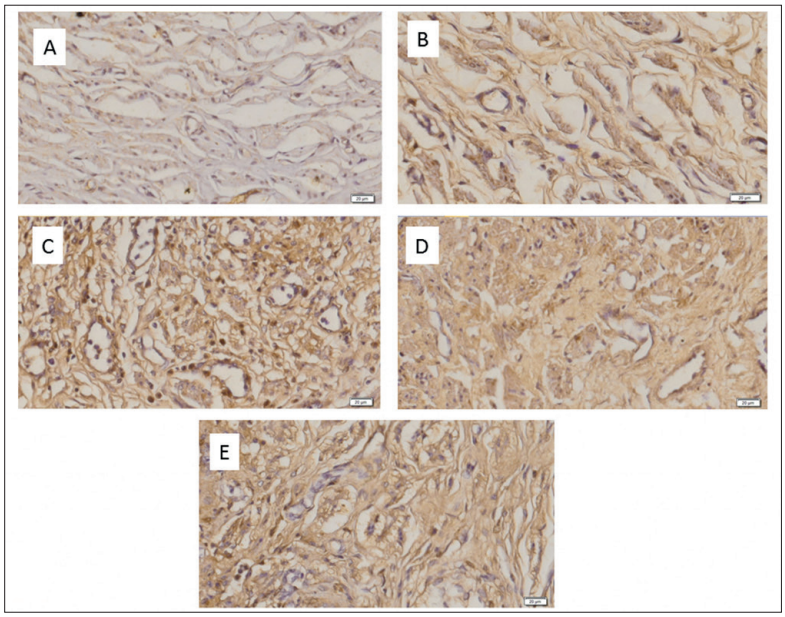

Figure 3. Collagen III Expression (Immunohistochemical Staining) in Periluminal Urethral Tissue (400x). This figure presents cross-sections of collagen III expression at a magnification of 400x within different treatment groups: (A) Control, (B) Sham, (C) TGF-β 1 mcg, (D) TGF-β 2 mcg, and (E) TGF-β 4 mcg. Notice the noticeable increase in brown-colored expression across all treatment groups.

Collagen I/III Ratio

The collagen I/III ratio in the periluminal region exhibited a significant downward trend, underscoring that the augmentation in collagen III surpassed that of collagen I in this particular area. Conversely, in the peripheral spongiosa region, significant mean differences were primarily observed between the control group and the 1T and 2T groups, hinting at a propensity for an increase in the collagen I/III ratio. This signifies a more pronounced upsurge in collagen I in the peripheral spongiosa region. The data encompassed the percentage of urethral diameter, along with the densities of collagen type I and collagen type III in both the periluminal urethral region and the peripheral spongiosa, as depicted in Table 1.

5. DISCUSSION

Radiological examination, such as urethrography, remains the prevailing diagnostic method for identifying urethral strictures (8). Urethral stricture is defined as a reduction in the urethral lumen diameter by 50% or more (1). This study demonstrated that TGF-β1 injection in the periluminal urethra led to a reduction in the urethral diameter within the treated area. Although only three subjects experienced a 50% reduction in urethral diameter, there was an observable trend

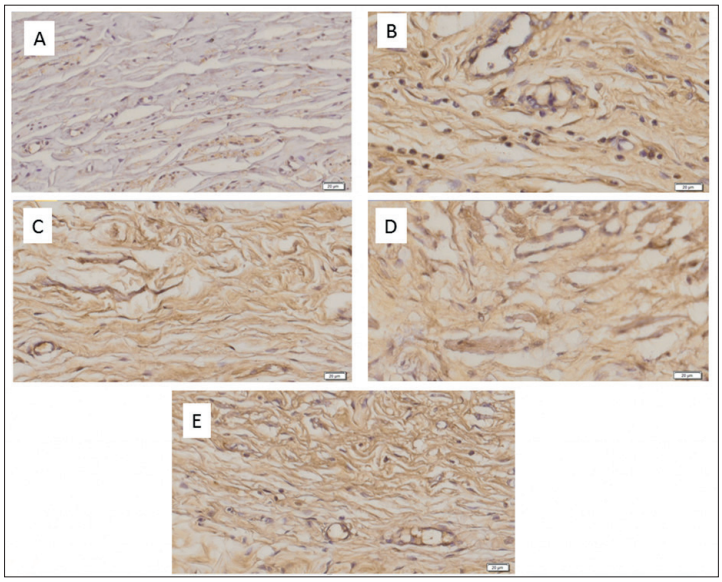

Figure 4. Collagen III Expression (Immunohistochemical Staining) in Peripheral Spongyosa Tissue (400x). This figure illustrates cross-sections of collagen III expression at a magnification of 400x across various treatment groups: (A) Control, (B) Sham, (C) TGF-β 1 mcg, (D) TGF-β 2 mcg, and (E) TGF-β 4 mcg. Notably, there is a distinct increase in chocolate-colored expression observed in all treatment groups.

collagen I predominated over collagen III, but their study did not specifically evaluate the periluminal and peripheral spongiosa areas. TGF-β1 injections are also known to increase collagen I and collagen III in mice (5). In their study, collagen III significantly increased with increasing TGF-β1 injection doses, while collagen I increased but not to a statistically significant degree. This phenomenon is likely related to the negative feedback mechanism of TGF-β1 response through the induction of Smad7 (15).

Significant increases in all components of periluminal urethral collagen are possible, as the stricture process itself involves an inflammatory process, stimulating profibrotic factors to deposit extracellular matrix in the tissue, ultimately leading to scar tissue formation (16). TGF-β1 is also known to act as a potent chemoattractant for immune cells (neutrophils and macrophages) during the inflammatory phase. It regulates immune cells and plays a role in the resolution of inflammation (17). TGF-β1 is involved in both normal homeostatic responses to tissue damage and pathological fibrotic processes. It stimulates fibroblasts

Dependent Variable		Treatment					Mean comparison analysis
		Control	Sham	1 µg	2 µg	4 µg	
Percentage of Urethral Diameter (%)		93.5±2.5	92.4±14.5	92.1±12.1	66.1±16.0	56.5±8.6	0.017*
Collagen Type I Density (%)	Periluminal	17.1±0.9	13.9±2.8	23.8±1.8	30.6±2.5	29.9±3.0	0.002*
	Peripheral Spongiosa	2.9±0.2	7.5±1.4	17.3±3.4	24.7±3.0	25.4±3.7	0.002*
Collagen Type III Density (%)	Periluminal	10.8±0.9	32.9±5.1	31.9±3.2	49.2±3.0	53.8±4.1	0.000**
	Peripheral Spongiosa	10.9±2.9	19.8±4.5	19.0±2.4	40.7±2.7	34.9±5.6	0.000**

Table 1. Urethral Diameter Percentage, Collagen Density (Collagen I and Collagen III), in Periluminal Urethra and Peripheral Spongiosa *Kruskal wallis test **One way ANOVA test

towards reduced urethral lumen diameter with increasing doses of TGF-β1.

The analysis of the collagen I/III ratio in the periluminal urethra revealed significant differences between the control group, the sham group, and the TGF-β1 injection groups at doses of 1 µg, 2 µg, and 4 µg ($p < 0.05$). Moreover, a significant mean difference was observed between the 1T and 4T dose groups ($p = 0.026$). In the control group, collagen I had a higher density than collagen III. Previous research has shown that in normal urethras, collagen I is approximately 1.9 times more abundant than collagen III, but in stricture tissue, this ratio increases to 4.8:1 (7). However, in our study, as the TGF-β1 injection dose increased, the density of collagen III in the periluminal urethra increased, resulting in a reduction of the collagen I/III ratio.

Study reported a 32.3% increase in total collagen in urethral stricture tissue, a finding consistent with our study, which showed a 33.2% increase in periluminal urethral collagen (7). Sangkum et al. (2015) found increased levels of collagen I and collagen III in urethral stricture patients (5). However, Sangkum reported that and myofibroblasts, which are primary producers of extracellular matrix, including collagen I and collagen III. Therefore, TGF-β1 is recognized as a profibrotic factor (17).

Comparatively, the mean density of both collagen types tends to be lower in the peripheral spongiosa region than in the periluminal area. This disparity is likely related to the location's greater distance from the urethral epithelial mucosa, resulting in less exposure to urine and a milder inflammatory process, which tends to reduce fibrosis. Urethral stricture pathophysiology is often associated with chronic inflammation, which can initiate with urine infiltration into the spongy tissue. Fibrosis and inflammation processes continue after urine extravasation into the spongy tissue (11). Urethral stricture is recognized as a progressive process involving both fibrosis and inflammation.

The density of collagen III, both in the control group and after TGF-β1 injection, surpassed that of collagen I. This may be attributed to TGF-β1's role as an inducer of collagen production (15). TGF-β1's activity, whether anti-inflammatory or profibrotic, depends on its cellular

source. TGF-β1 is a multifunctional cytokine that influences various biological pathways, including inflammation, wound healing, and fibrosis. The cellular source of TGF-β1 determines its molecular activity, with macrophage-derived TGF-β1 exhibiting more wound healing and profibrotic activity, while TGF-β1 secreted by regulatory CD4+ T cells can act as an anti-inflammatory agent (12).

Urethral stricture is a progressive condition characterized by changes in the composition of the extracellular matrix (ECM) within the urethral tissue and the corpora spongiosa (both periluminal and peripheral) (8). Elevated synthesis or reduced degradation of ECM components, particularly collagen, can lead to excessive collagen accumulation, initiating the fibrosis process (13). TGF-β1, as a profibrotic factor, is likely to play a pivotal role in this process. A significant accumulation of collagen in the periluminal urethra and peripheral spongiosa can result in compliance disturbances and urethral lumen constriction, ultimately leading to urethral strictures.

As in our previous publication, we noted that TGF-β1 played a role in altering the composition of total collagen in the rabbit's urethral wall, resulting in a decrease in urethral lumen diameter. For future research, it is essential to compare with higher doses of TGF-β1 (> 4 μg) to ascertain the effective dose of TGF-β1 in reducing urethral lumen diameter. Shortening the evaluation interval can also provide a more detailed understanding of the pattern of urethral histopathological changes.

6. CONCLUSION

In summary, this study highlights the potential role of Transforming Growth Factor Beta 1 (TGF-β1) in the development of urethral strictures. TGF-β1 injection into the periluminal urethra resulted in a notable reduction in urethral diameter, indicating its involvement in the pathogenesis of urethral strictures. While not all subjects exhibited a significant reduction, a clear dose-dependent trend in decreased urethral lumen diameter with increasing TGF-β1 doses was observed.

Furthermore, our analysis of collagen I/III ratios demonstrated significant differences between control and treatment groups, underscoring the influence of TGF-β1 on collagen composition in the urethral wall. Collagen III densities, in particular, exceeded those of collagen I, potentially attributable to TGF-β1's role as a collagen production inducer.

This research contributes to our understanding of the molecular mechanisms involved in urethral stricture formation and emphasizes the potential of TGF-β1 as a target for further investigations and therapeutic interventions. Future studies exploring higher TGF-β1 doses and shorter evaluation intervals may provide more insights into the evolving histopathological changes in urethral strictures, aiding in the development of more effective treatment strategies for this challenging urological condition.

- **Ethics statement**: The welfare of the animals was ensured by adhering to the guidelines outlined in the eighth edition of the "Guide for the Care and Use of Laboratory Animals" (NRC 2011).
- **Author's contribution**: The author was involved on all steps of preparation this article including final proofreading.
- **Conflicts of Interest**: The authors have no conflicts of interest to disclose.
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Serum Thrombomodulin Level Can Predict Mortality in Patients With Sepsis?

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ABSTRACT

Background: Thrombomodulin (TM) is a type-1 trans-membrane glycoprotein on endothelial cells which is known to be involved in various biochemical pathways. TM can be detected in biological fluids such as blood and urine under many forms. Soluble thrombomodulin (sTM), consist of various particles of TM, is the predominant agent which is created by enzymatic or chemical catalysis of the whole protein under divergent conditions. TM plays a vital role in protein C system and is crucial in the pathogenesis of Sepsis. **Objective:** To identify the serum level of soluble thrombomodulin (sTM) in groups of patients: sepsis and septic shock including their survival and fatal in-hospital outcome; and validate the death prediction of serum sTM in patients with sepsis. **Methods:** This prospective observational study was conducted in 63 patients who were diagnosed with sepsis, septic shock according to Sepsis 3 criteria at the ICU Department of Hue Central Hospital, Vietnam, from 3/2022 to 3/2023. **Results:** Twenty participants developed septic shock (31.7%), morality within 28-days was 19 patients (30.2%), 22 patients complicated with acute kidney injury that necessitated renal replacement therapy (34.9%), 30 patients required mechanical ventilation (47.6%), the median length of ICU stay was 8 (3-28) days. Serum level of lactate and creatinine were significantly higher in septic shock group compared with sepsis and survival group ($p<0.05$). The median sTM level in septic shock group and fatal group were 4.68(3.38-6.46) ng/mL and 4.68 (1.69-6.46) ng/mL, respectively. These results were significantly higher than sepsis group [3.62 (1.51-1.94) ng/mL] and survival group [3.73 (1.51-5.9) ng/mL] ($p<0.05$). The death predictive power of DIC score, APACHE II score, creatinine, sTM and SOFA presented with AUC values of 0.723, 0.726, 0.777, 0.803 and 0.807, respectively. There were no significant difference of serum level IL-6 and PCT between survival and fatal group. The median DIC score in fatal group was 7 (3-7), which was significantly higher than survival group 4 (2-7) ($p= 0.001$). **Conclusion:** Sepsis is a common diagnosis among ICU settings which links the critically ill patients to higher complications and mortalities. Serum level of sTM in septic shock and fatal groups were significantly higher than sepsis and survival groups. sTM is a reliable marker and should be used in predict severity and mortality in sepsis patients.

Keywords: Sepsis, septic shock, thrombomodulin.

1. BACKGROUND

Thrombomodulin (TM) is a type-1 trans-membrane glycoprotein on endothelial cells which is known to be involved in various biochemical pathways (1, 2). TM can be detected in biological fluids such as blood and urine under many forms. Soluble thrombomodulin (sTM), consist of various particles of TM, is the predominant agent which is created by enzymatic or chemical catalysis of the whole protein under divergent conditions (3). TM plays a vital role in protein C system and is crucial in the pathogenesis of Sepsis (4).

The combination of TM and thrombin increases the activity of protein C up to 1000 times in comparison with thrombin alone (5,6). Activated protein C exerts anticoagulation antioxidative effects, prevents apoptosis and therefore helps reduce thrombosis and in protecting cells (7). In sepsis, the reduced expression of TM on endothelial cell leads to the compromised activation of protein C, which can facilitate inflammation and thrombosis. Also, TM contributes to the effective immune modulation, particularly in neutrophile adhesion, component activation and formation of cytokine (6). Another factor in pathogenesis of sepsis is the injury of endothelial cell caused by uncontrolled inflammatory reaction