# AN IMMUNOHISTOCHEMICAL ANALYSIS OF TGF-ß1, AS A PREDICTOR OF DELAYED WOUND HEALING IN EPISIOTOMY SURGERY OF SPRAGUE DAWLEY RATS

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## ABSTRACT

*This animal experiment was carried out in the Laboratory of Inter University Center (PAU), Food and Nutrition Department, Gadjah Mada University, Yogyakarta, to prove an assumption that stress in pregnant women inhibited the delivery-wound healing process after birth. A number of 20 adult female Sprague-Dawley rats, averagely 3 months old, weighing about 300 g, were used for the experiment. Before experiment was started, all experimental animals were housed and maintained for adaptation in 2 weeks, with standard food and water ad libitum in accordance with the guidelines from Gadjah Mada University School of Veterinary Medicine, Yogyakarta. To make those rats pregnant, a male sprague-Dawley rat was put in each stable at a male - female ratio of 1 : 5. After pregnancy occurred, those rats were randomly assigned in two groups of 10 rats each. Group I was control without any treatment given. Group II were subjected to stress by putting them one by one into a box with external dimensions of 30 x 30 x 51 cm, provided a sound abating enclosure, and a transparent access door that permitted observation during treatment. They were subsequently treated by an ultrasound wave produced by an ultrasonic processor with the dose of 6.6 KHz and duration of 60 seconds. Nearby the delivery phase, stress was induced to all samples of group II, one by one according to the protocol. After those rats were stressed, they were subjected to episiotomy incision under general anesthesia on their perineal region from vulva to anus about 1 cm in length and its depth was limited to subcutis layer. The episiotomy wound was then closed by 3 simple suture with cotton no. 000. General anesthesia was performed by combination of ketamine/xylazine, intramuscularly. The dose of ketamine was 90 mg/kg BW, and xylazine was 10 mg/kg BW. Samples of control group were subjected only to an episiotomy incision under general anesthesia with the same procedures as that for group II. On day 3 after episiotomy, 10 rats of both groups, 5 rats each, a biopsy was performed on the site of episiotomy wound (vulva), then those rats were killed. From the biopsy tissues, stained with hematoxylin and eosin, histopathologic preparations were made to count for fibroblast cells. From the same biopsy tissues, but without staining, immunohistochemistry preparations were also made to examine TGF-ß1 cytokines found in the rat tissue. In the rest of samples, i.e., 10 rats, biopsy was also performed with the same procedures as that done on day 3. Then, histopathologic preparations were made for fibroblast count, and also immunohistochemistry preparations for TGF-ß1 examination. Results: Statistical analysis using multivariate test showed that there was statistical difference ($p < 0.05$) in fibroblasts count between two groups, and there were also TGF-ß1 in both groups but were statistically different between control and treated group. TGF-ß1 in treated group was lesser both in day 3 and day 5 of examination. Conclusions: From statistical analysis of results, it could be concluded that stressed Sprague-Dawley's rats may experience delayed episiotomy healing, as it was shown by less TGF-ß1 in rat's tissues of treated group. It could also be concluded that TGF-ß1 cytokine act as a predictor for wound healing.*

***Keywords**: Stress, episiotomy, ketamine, immunochemistry, fibroblast and TGF-ß1*

## INTRODUCTION

Delayed wound healing process after delivery remains a problem, as it causes have never been investigated. Various factors may involve in delayed wound healing process. However, in such case, delayed process may likely result from stress during pregnancy that lasts until the progress of labor and delivery process. Tension experienced during delivery, complicated with intense pain during labor and delivery, is responded by the body as stress (Niven, 1992), which at certain level it may reduce the defense of the body (Adler, 1999), thereby it induces delayed wound healing through a mechanism that is also still uncertain.

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Since it is variably defined, the definition of stress remains controversial. Walter Cannon ND defined stress as physiological reaction against stressor, while stressor is all conditions perceived as threatening either physical and physiological integrity of the body (cited from Carlson,1994b; Black,1995). Lazarus and Folkman (1994) suggested that the definition of stressor depends on cognitive assessment and level of adaptation of an individual to his environment (Taylor, 1991a; Sarafino,1994a). Ganong (1995) defined stress as a stimulation that is disturbing and increasing cortisol secretion. Such increase of glucocorticoid level in short term provides an advantageous effect, but in longer term it may be distressing and hazardous. Glucocorticoid increase in stress individuals inhibits inflammatory response and fibroblast activity, resulting in the inhibition of growth factor (Ganong, 1995). Black (1995) stated that stress is a disharmonious condition or a threatened-homeostatic condition, excited by a psychological, environmental, or physiological stressor.

Stress is directed towards mind and emotion that affecting central nervous system and immune system and activating two-way system in between. Stress occurs through HPA (Hypothalamic-Pituitary-Adrenal) axis, and subsequently initiates reaction cascades in whole body. Goleman (1995) stated that stress in human and animal involves hippocampus and amygdala, structures that locate in medial temporal lobes of the brain. Stimulation in amygdala and hippocampus may render the hypophysis to release Adreno-Corticotropin Hormon (ACTH), which subsequently may activate adrenal tissue, thereby the adrenal cortex releases glucocorticoid or cortisol hormones that finally enter the circulation and distribute to whole body, affecting the activities of several vital organs (Ganiswarna, 1995). A large number of studies had proved that cortisol reduced immune response of the body (McCance KL and Huether SE, 1994).

The process of wound healing is a complex phenomenon involving a number of process, including inflammatory process induction, parenchymal cells regeneration, migration and proliferation of either parenchymal as well as connective tissue, ECM protein synthesis, remodeling of connective tissue and parenchymal components and collagenization and acquization of wound strength (Cotran, 1996). Histologically, delayed wound healing process can be observed by the inhibition of fibroplastic and collagenation process starting from day 8 post-injury. Delayed fibroplastic and collagenation process may result from several factors, such as inhibited various growth factors, e.g., PDGF, FGF, TGF-ß, and cytokines (IL-1 and IL-4) secreted by leukocyte and fibroblast in wounded sites where healing process is undergoing (Cotran et al, 1996).

Transforming Growth Factor-ß (TGF-ß) was initially discovered as a cofactor, together with TGF-α, which is produced by platelet, lymphocyte T, macrophage, fibroblast in bone, and placenta. TGF-ß has an antiproliferative characteristic and acts as negative regulator of immunity and hematopoiesis. The antiproliferative effect of TGF-ß may be induced in various cell types, such as epithelial cells, endothelial cells, smooth muscle cells, fetal hepatic cells, initial progenitor myeloid cells, as well as T and B lymphocyte cells (Stites and Terr, 1991). TGF-ß may inhibit T-cell dependent polyclonal antibody production and inhibit the induction of NK cells activity as well as induce the activation of killer cells by IL-1, and increase, but also subsequently reduce, the level of antibody. Primary activity is related to fibroplasia and immunosuppression, while the predominant influence is on wound healing and bone 'remodeling' (Cotran RS, Kumar V, Collins T, 1999).

According to Stites and Terr (1991), TGF-ß1 has a chemotactic and mitogenic effect towards fibrobrlast, able to increase collagen and fibronectin, so that it is entirely able to stimulate the growth of tissue. In severe stress, total number of secreted ACTH will be excessive, beyond that needed to produce maximal cortisol release. In such condition, according to Selye (1983), an individual has been at the stage of "exhaustion", and wound healing process is severely inhibited.

## MATERIALS AND METHODS

This study used 20 adult female Sprague Dawley rats, with the age of averagely 3 months, with body weight of about 300 grams. This study was carried out at the Laboratory of Inter University Center (PAU), Food and Nutrition Department, Gadjah Mada University, Yogyakarta. Before the experiment was started, all experimental animals were housed and maintained for adaptation in 2 weeks, with standard food and water ad libitum.

Those rats were then rendered to be pregnant by putting a male sprague-Dawley rat in each stable at a male - female ratio of 1 : 5. After pregnancy occurred, those rats were randomly assigned in two groups of 10 rats each. Group I was control that received no treatment, so that they were at stable condition (stress negative), while Group II was treatment group that was subjected to stress by assigning those rats one by one into a box with sound abating enclosure and an access door on top of the box, which was No.630-0427, from sonicator machine Vibra-Cell, model VC 600, High Intensity Ultrasonic Liquid Processor, made in USA. The rats were exposed to ultrasonic wave with capacity of 6.6 kHz for 1 minute. Nearby the delivery phase, approximately at the gestation day 18 and 19, when there were signs that they were about to give birth, the rats were anesthesized with intramuscular general anesthesia using the combination of ketamin-selazin with each dose of 90 mg/kg BW and 2 mg/kg BW. Hairs around the vulva were shaved, cleaned and disinfected using iodine-tincture, and episiotomy surgery was done from the vulva to perineum as long as 1 cm in average. The wound was closed using sterile cotton no. 000 with 3 single simple sutures. Blood in the operating wound was dried, disinfected with iodine tincture, and antibiotics of Pencillin oil 0.15 cc was applied intramuscularly (clinical dose 7 mgr/kg BW), and the animals were returned to the cage. At day 3 post-episiotomy, 5 rats from each group was randomly taken, and biopsy was made from tissue at episiotomy site (vulva). Biopsy results were immersed in formalin 10%. At day 5 post-episiotomy, biopsy was made to the

remaining rats in both groups, and the results were preserved in formalin 10%, and rats were then sacrificed. Histopathological preparations were subsequently made from fixed biopsy tissue using Hematoxylin and Eosin (HE) staining, and tissue incision preparations were made without staining to perform immunohistochemical analysis for the presence of TGF-ß1 in tissue. In histopathological preparations with HE staining, fibroblast was counted using the standard method of Pathology Laboratory, Gadjah Mada University School of Veterinary Medicine. For immunohistochemical analysis, immunohistochemical preparations were made using streptavidin-biotin method as follows:

Fixed incised tissues containing paraffin were deparaffinized, rehydrated, and washed with PBS for 10 minutes, and incubated with 3% H2O2 solution in absolute methanol for 10 minutes. Preparations were rewashed with PBS for 10 minutes, and given with streptavidin-peroxidase conjugate for 5 minutes. Preparations were rewashed again with PBS for 10 minutes, and given with chromogen substrate for 5 minutes at room temperature. Preparations were washed with distillate water for 10 minutes, given with counterstain for 3 minutes, rewashed cleanly and given with mounting media solution and finally covered with covering glass.

The analysis of fibroblast count was carried out using factorial pattern statistics and measured at the degree of error of 5%. Immunohistochemical analysis for the presence of TGFß-1 was undertaken by observing the presence of specific color (red brick) on the tissue under microscope observation. Results of this examination were qualitative.

## RESULTS AND DISCUSSION

### Data description

A number of twenty Sprague-Dawley rats were used as samples. Those rats were randomly divided into two groups, 10 in control group (without treatment) and 10 in treatment group (receiving stress induced from Sonicator at the capacity of 6.6 kHerz for 1 minute). To balance both groups, sample selection was undertaken by means of the following parameters: body weight, rats originated from one breeding, and having similar characteristics.

Table 1. Bodyweight of selected experimental animals as samples in Laboratory of Inter University Center (PAU), Food and Nutrition Department, Gadjah Mada University, Yogyakarta, 2001

| Group | Number | Bodyweight (gr) | Group | Number | Bodyweight (gr) |
|---|---|---|---|---|---|
| I (Control) | 1 | 294 | II (Treatment) | 1 | 288 |
| | 2 | 298 | | 2 | 294 |
| | 3 | 294 | | 3 | 287 |
| | 4 | 288 | | 4 | 292 |
| | 5 | 300 | | 5 | 304 |
| | 6 | 297 | | 6 | 297 |
| | 7 | 298 | | 7 | 298 |
| | 8 | 300 | | 8 | 302 |
| | 9 | 299 | | 9 | 297 |
| | 10 | 302 | | 10 | 296 |

Source: primary data, year 2001

Table 1 shows that body weight of experimental animals in treatment and control groups are almost similar. Rats were generally obtained from one breeding, so that they had similar characteristics.

Table 2. Results of differential test on the bodyweight of experimental animals in control and treatment group in the Laboratory of Inter University Center (PAU), Food and Nutrition Department, Gadjah Mada University, Yogyakarta, 2001

| Variable | Group | Mean | t | df | p | Notes |
|---|---|---|---|---|---|---|
| Bodyweight (gr) | Treatment | 295.50 | -0.697 | 18 | 0.495 | Not significant |
| | Control | 297.00 | | | | |

Table 2 demonstrates that results of differential test on the bodyweight of experimental animals in both groups reveals no significant difference ($p > 0.05$). Table 3 shows average fibroblast count in each group (treatment and control) observed. Table 4 indicates that results of differential test to fibroblast count in both groups reveal significant difference ($p < 0.05$).

Table 3. Results of fibroblast count in treatment and control groups in the Laboratory of Inter University Center (PAU), Food and Nutrition Department, Gadjah Mada University, Yogyakarta, 2001

| Rep. | Control | | Treatment | |
|---|---|---|---|---|
| | Day 3 | Day 5 | Day 3 | Day 5 |
| 1 | 15 | 30 | 6 | 10 |
| 2 | 8 | 30 | 10 | 20 |
| 3 | 20 | 12 | 20 | 10 |
| 4 | 15 | 35 | 8 | 10 |
| 5 | 17 | 24 | 10 | 23 |
| ∑ | 59 | 136 | 54 | 64 |
| x ± Sd | 11.8 ± 5.7 | 27.2 ± 8.9 | 10.8 ± 5.4 | 12.8 ± 6.7 |

Source: primary data, year 2001
Note: unit in cell count

Mean of fibroblast count in treatment group was lower than that in control group, with the ratio of 12.60 : 12.70, indicating that the probability of wound healing in treatment (stress) group was slower than that in control (non-stress) group.

Table 4. Results of differential test on fibroblast count in treatment and control group in the Laboratory of Inter University Center (PAU), Food and Nutrition Department, Gadjah Mada University, Yogyakarta, 2001

| Variable | Group | Mean | t | df | p | Notes |
|---|---|---|---|---|---|---|
| Fibroblast content | Treatment | 12.70 | -2346 | 18 | 0.031 | Significant |
| | Control | 20.60 | | | | |

Source: primary data

Table 5. Results of immunochemistry analysis of TGF-ß1 using Streptavidin-biotin method to vulval tissue at day 3 and 5 post-episiotomy in the Laboratory of Inter University Center (PAU), Food and Nutrition Department, Gadjah Mada University, Yogyakarta, 2001.

| Day | Control | Results | Treatment | Results |
|---|---|---|---|---|
| | No. of rats | | No. of Rats | |
| 3 | 4 | ++ | 12 | - |
| | 30 | ++ | 11 | - |
| | 21 | +++ | 1 | - |
| | 18 | + | 22 | - |
| | 28 | ++ | 23 | + |
| 5 | 3 | ++ | 7 | - |
| | 15 | +++ | 9 | - |
| | 13 | + | 14 | + |
| | 6 | + | 5 | - |
| | 8 | + | 10 | - |

Source: primary data, 2001
Notes: + = mild positive, ++ = moderate positive, +++ = severe positive

Table 6. Results of differential test on Transforming Growth Factor-ß1 (TGF-ß1) in treatment and control group in the Laboratory of Inter University Center (PAU), Food and Nutrition Department, Gadjah Mada University, Yogyakarta, 2001.

| Variable | Group | Mean | t | df | P | Note |
|---|---|---|---|---|---|---|
| TGF-ß1 Content | Treatment | 0.20 | -5.657 | 18 | 0.001 | Significant |
| | Group | 1.80 | | | | |

Table 5 shows that control group contains higher TGF-ß1 than does treatment group. Due to the influence of TGF-ß1, produced fibroblast count is higher in control group than in treatment group. Table 6 reveals that significant difference was found between TGF-ß1 content in treatment group and that in control group, in which the content in treatment group was lower than that in control group, with the ratio of 0.20 : 1.80.

Having observed the results of investigation on fibroblast and TGF-ß1 to prove promoted episiotomy wound healing in Sprague-Dawley rats, it can be discussed as follows:

1. Stress response through HPA axis results in an increase of glucocorticoid secretion. Such an increase induces delayed inflammatory response and fibroblast activity, and suppress growth factor (Ganong, 1955). Interleukin-1 stimulates cell to produce procollagenase and prostromelycin to synthesize collagen. In stress individuals, IL-1 activity is inhibited, thereby inhibiting fibroplasia process, resulting in delayed wound healing (Cotran et al, 1996). Results of this study revealed that fibroblast count in treatment group (stress rats), compared to that in control group (non-stress rats) was significantly different ($p < 0.05$), in which treatment group had a lower fibroblast count.

2. Results of immunohistochemical analysis on tissue biopsy from episiotomy site (vulva), on day 3 and 5 using Streptavidine-Biotin method revealed that a remarkable number of red-brick cells were found in tissue from control group (TGF-ß1) positive, while those cells in tissue from treatment group were in far lower number. The difference was highly significant ($p < 0.001$). Results of this analysis proved that rats in treatment group (stress rats) contained less TGF-ß1 compared to those in control group.

3. Transforming Growth Factor ß-1, a growth factor having chemotactic and mitogenic effect on fibroblast, increases collagen and fibronectin, so that it can stimulate tissue growth (Stites dan Terr, 1991). In addition, TGF-ß1 also has an effect to inhibit endothelial proliferation, epithelial cells, smooth muscle cells, lymphocyte T and B, hemopoietic stem cells, and suppress the growth of NK cells and Killer cells (Cotran et al. 1996).

Since TGF-ß1 stimulates the growth of fibroblast and other growth factors, such as PDGF and FGF, it may stimulate the inititation of wound healing process at molecular level and at initial tissue level. Complete wound healing that involves all types of healing tissue occurring thereafter was not investigated in this study.

As a conclusion, delayed episiotomy wound healing can be detected by the presence or absence of TGF-ß1 in the tissue of experimental rats, so that TGF-ß1 is a predictor of delivery wound healing process.

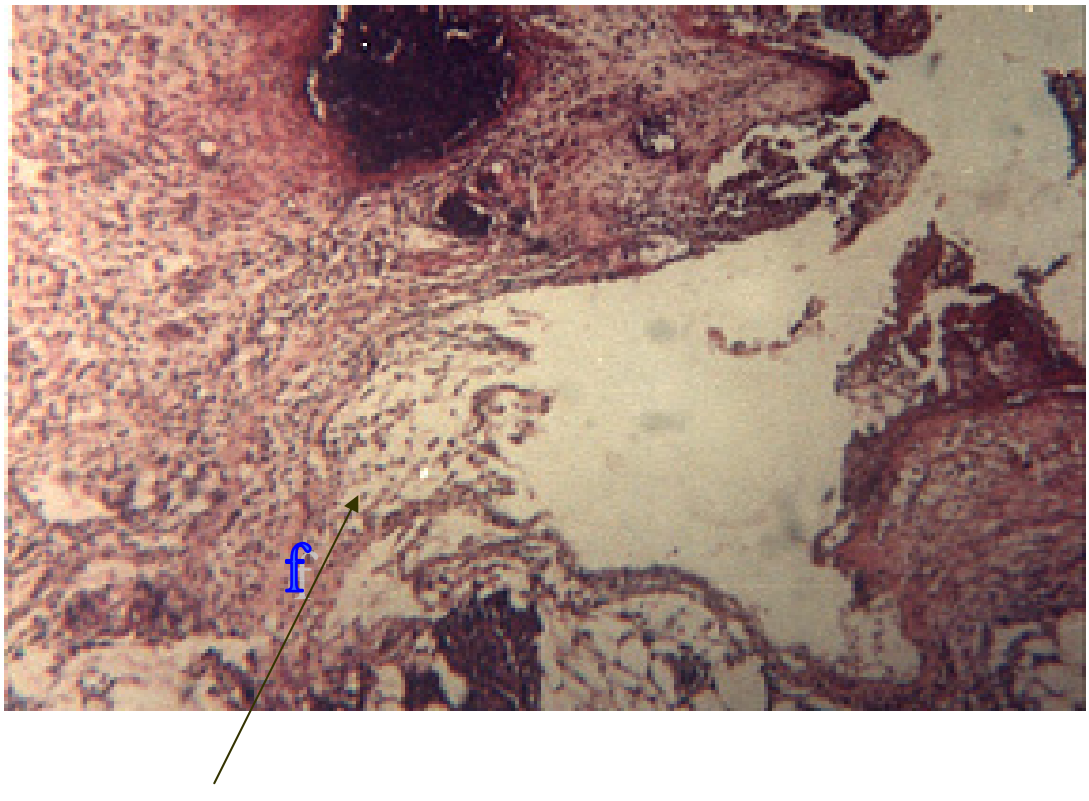


Figure 1. Histopathological picture of vulval tissue post-episiotomy, TGF-ß1 negative (Immunochemistry, 200 x).

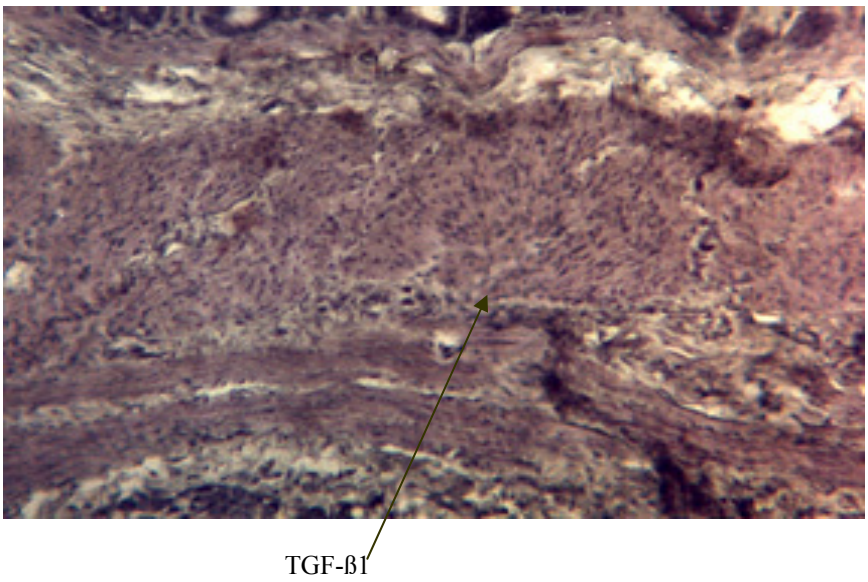


Figure 2. Histopathological picture of vulval tissue post-episiotomy in control group, TGF-ß1 positive (Immunochemistry, 100 x).

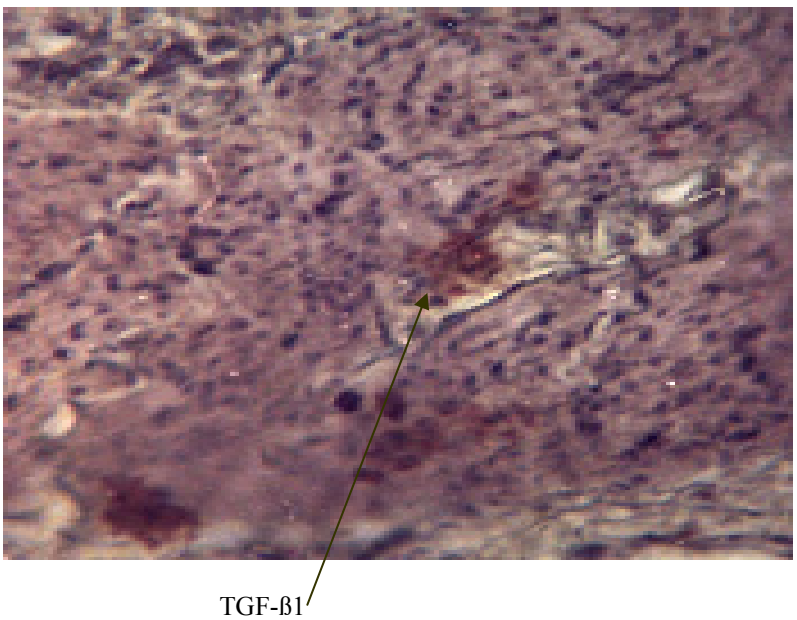


Figure 3. Histopathological picture of vulval tissue post-episiotomy in control group, TGF-ß1 positive (Immunochemistry, 200 x).

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