Original article

Changes type III collagen expression in human uterosacral ligaments of uterine prolapse

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Abstract: There are some controversies regarding the content of type III collagen fibers in uterosacral ligaments in pelvic organ prolapse (POP). The role of these fibers are still unclear. This study aims to compare the content of type III collagen fibers in uterosacral ligaments (USL) in patients with or without POP.
This is a cross-sectional analytic study conducted in 23 women with POP from Jakarta Indonesia. The control group were 23 women without POP. The study took place in Dr. Hasan Sadikin Hospital during May-October 2011.
The type III collagen fiber content in USL of women with POP was 50% higher than those in women without POP (p=0.036). In conclusion, the type III collagen fibers content in USL of women with POP is more dense compared with those without POP.

Key words: Type III collagen fibers; Pelvic organ prolapse; Uterosacral ligaments.

INTRODUCTION

Gabriel et al[1] found that there were strong immunohistochemistry reactions of type III collagen fibers in uterosacral ligament (USL) in women with pelvic organ prolapse (POP) compared with type I and type II collagen fibers. Jackson[2] also found that there was reduction of collagen amount with predominantly immature collagen content in women with POP and cystocele, compared with those without POP.

In women with POP, there were several changes in cell transcription program in USL, leading to changes in matrix production, mechanical properties, cell shape, inflammatory reaction, and healing process. Furthermore, the immature collagen content also relatively higher.[3,4]

The study regarding type III collagen fibers in USL of women with POP has been widely conducted worldwide, but mainly in Caucasians. The data from Asian women is still scarce. This is the first study comparing type III collagen fiber content in USL in Indonesian women.

MATERIAL AND METHODS

This is a cross-sectional analytic study, conducted in Dr. Hasan Sadikin Hospital Bandung during May-October 2011. Subjects were women with grade 2, 3 and 4 POP, while the control group was women without POP or with POP grade 1. Women with pelvic organ malignancy, intraabdominal tumors, and previous history of pelvic surgery were excluded from this study. Tissue samples were taken from the uterus after total abdominal or vaginal hysterectomy. To obtain tissue samples, the distal portion of USL (approximately 1 cm from its attachment from cervix) was cut (approximately 0.5 cm^3). Samples were then immersed in formaldehyde solution before being sent to the Department of Pathology for immunohistochemistry examination. The immunohistochemistry assay was done using type III collagen fiber staining (Abcam ab7778 rabbit polyclonal to collagen III). To interpret the immunohistochemistry assay, these guides were used: for determining type III collagen fibers distribution: +1 if distributed less than 20%, +2 if distributed 20-50%, +3 if distributed 50-80%, and +4 if distributed more than 80%. For immunostaining intensity: 0 if no staining, +1 if weakly stained, +2 if moderately stained, and +3 if strongly stained. After determining distribution and intensity, the HistoScore were calculated using the formula: distribution x (intensity+1), and the value range were 1-16.

Data were analyzed using SPSS program version 16.0.

RESULTS

The study was conducted during a six-month period, from May-October 2011. During that period, all subjects fulfilled inclusion criteria were enrolled to this study. There were 23 women with POP, and 23 women without POP or with POP grade 1 served as control group. The characteristics of subjects may be seen in table 1. There were significant differences in age and menopausal status between POP group and control group ($p<0.05$), while parity and BMI were not significantly different ($p>0.05$). No subject had received hormone replacement therapy. Most of POP subjects had grade 3 POP (62.5%), followed by grade 2 (26.1%), and grade 4 (8.7%). There were no grade 1 POP subjects in this study.

TABLE 1. – Subject characteristics (n=46)

Notes: +) Unpaired t test
++) Chi square
+++) Mann Whitney U

Characteristics	Pop (n=23)	Control (n=23)	p
1. Age Mean (SD):	50.2 (9.2)	62.4 (7.8)	0.001^{+}
2. Menopausal status			
Premenopause:	2 (4.3%)	12 (26.1%)	0.001^{++}
Postmenopause:	21 (45.7%)	11 (23.9%)	
3. Parity Median:	4	4	0.645^{++}
Range:	2-12	0-8	
4. BMI (kg/m^2) Median:	21.21	22.97	0.095^{+++}
Range:	16.65-31.18	20-27.77	

The comparison of distribution and intensity of type III collagen fibers between POP and control group may be seen in figure 2a, 2b. The median of type III collagen fibers distribution was significantly higher in POP group ($p<0.05$). The intensity of type III collagen fibers was higher in POP group as well but not significantly different with the control group ($p>0.05$).

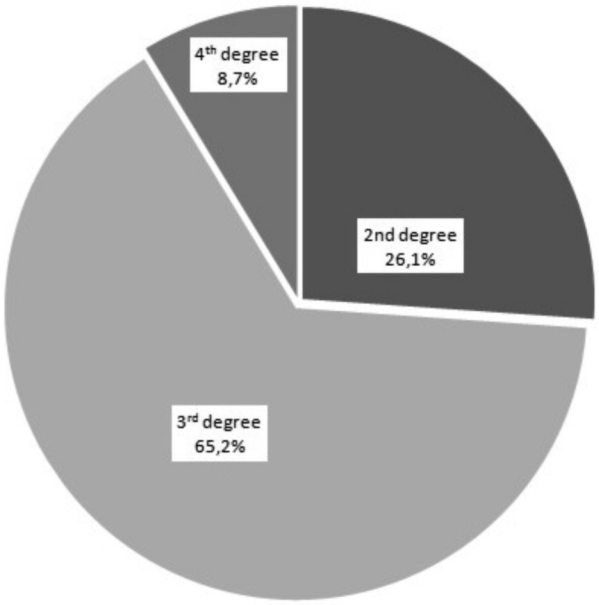

Figure 1. - Prolapse Organ Pelvic distribution.

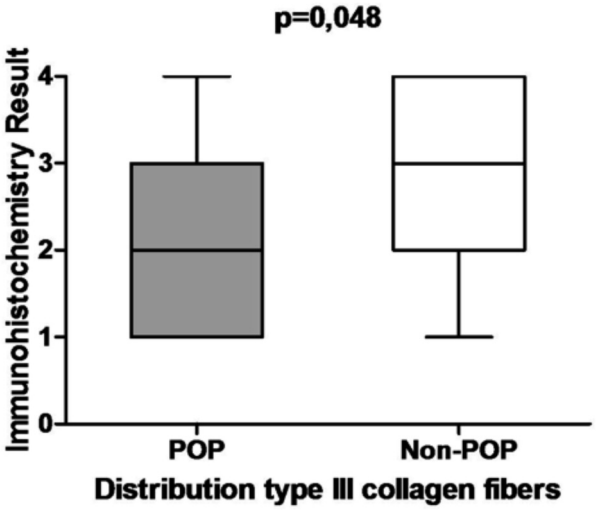

Figure 2a. - Comparison of distribution of type III collagen fibers in women with and without POP. Immunohistochemistry result: +1 (< 20%), +2 (20-50%), +3 (50-80%), and +4 (>80%).
Statistical analysis using Mann-Whitney U nonparametric test.

The comparison of Histo Score between two groups may be seen in figure 3. There was a significant difference of Histo Score between POP and control group (median 12, range 3-16 vs median 8, range 1-16, p<0.05). There was no significant correlation between degree of POP and type III collagen fiber content (p>0.05). Furthermore, there were no significant correlations between age and menopausal status to type III collagen fiber content, as seen in table 3 and figure 4.

DISCUSSION

Although the exact pathophysiology of POP is still unknown, there are some risk factor contributing in the occurence of POP, i.e pregnancy, vaginal delivery, age, elevation of intraabdominal pressure, menopause, hypoestrogenic status, trauma, genetic factor, race, muskuloskeletal disorders, chronic debilitating illness, smoking, and previous history of surgery. From previous studies, mechanical and

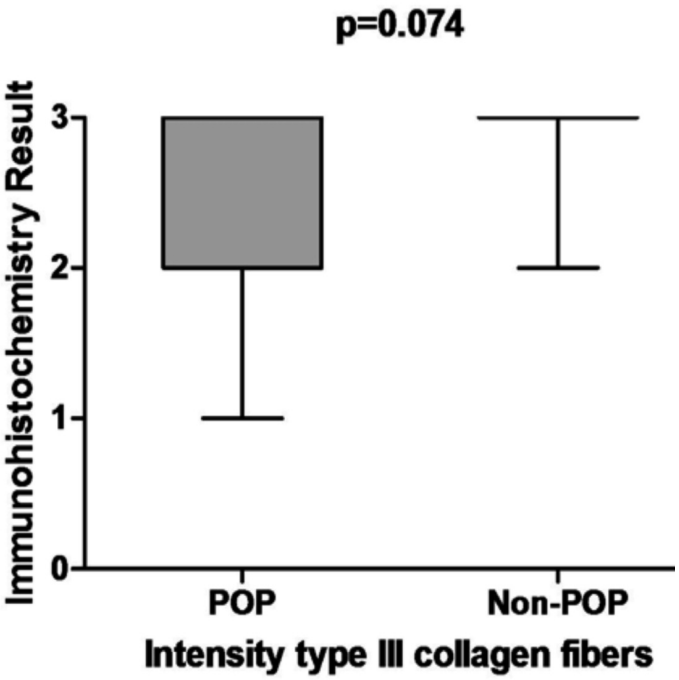

Figure 2b. - Comparison of intensity of type III collagen fibers in women with and without POP. Immunohistochemistry result: 0 (no staining), +1 (weakly stained), +2 (moderately stained), and +3 (strongly stained)
Statistical analysis using Mann-Whitney U nonparametric test.

metabolical changes in connective tissue may serve as predisposing factors for POP.[5-7] Furthermore, the reduction of collagen fiber amount as well as decreasing quality of those fibers also may contribute in POP.[8]

According to WHI study, there are higher risks in older women to develop POP (1.2 times higher in women aged 60-69 years, and 1.4 times higher in women aged 70-79 years compared with those whose aged 50-59 years). In a cross-sectional study of 21,449 menopausal women in Italy, the risk of POP in older women are higher compared to

Table 2. – Effect of degree of POP on the content of type III collagen fibers.

Notes: Kruskal Wallis test

Degree of POP	Mean Rank Histo Score of tipe III collagen	N	P
Degree 2	14.33	6	
Degree 3	10.93	15	0.535
Degree 4	13.00	2	

Table 3. – The influence of age on the content of type III collagen fibers.

Notes: Kruskal Wallis test

Age Classification (years)	Mean Rank Histo Score of tipe III collagen	N	P
30-39	15.25	2	0.547
40-49	27	10	
50-59	20	15	
60-69	25.58	13	
70-79	25.42	6	

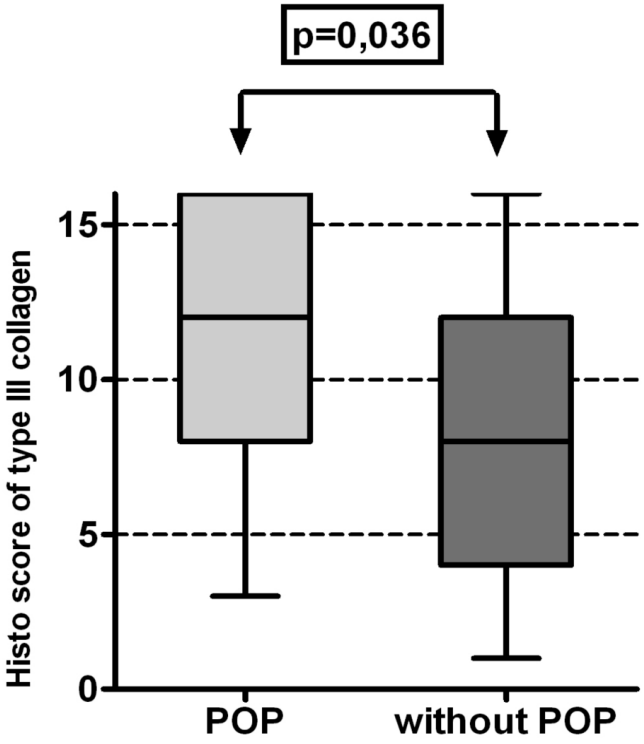

Figure 3. - Comparison of content of typeIIIcollagen fibers in women with and without POP.
Statistical analysis using Mann-Whitney U nonparametric test.

those in less than 51 years age group (1.3 times higher in 52-55 years age group, and 1.7 times higher in >56 years age group).[8] In this study, the mean of age was 50.2+9.2 years.

Women who experienced vaginal delivery have a higher risk of developing POP compared to nulliparas (8.4 and 10.9 times higher for twice and four or more deliveries respectively; 95% CI 4.7-33.8).8 According to Bradley et al.[9] women who experienced 1-2 times vaginal deliveries have 1.28 (0.49-3.32) cm vaginal descent; while in women who have 3-4 and more than 5 vaginal deliveries the vaginal descent was 2.35 (0.98-5.67) cm and 4.82(1.92-12.09) cm respectively. In this study, mean parity was 4 (range 2-12). In our group, high parity is a major factor for POP incidence. In contrast with this condition, in western countries POP may be found in the low parity group. Furthermore, we also found women with POP in low parity group as well.

Increasing BMI also play a role on the incidence of POP. Women who are overweight (BMI 25-30 kg/m^2; OR 2.51, 95% CI 1.18-5.35) and obese (BMI> 30 kg/m^2; OR 2.56, 95% CI 1.23-5.35) are at high risk of developing POP.[8] Some studies suggest an association between POP and increased BMI, but other studies did not find a correlation between the POP and the increase in BMI, so the correlation of the two variables is still a controversy.[7] BMI in this study is still considered within normal limits, so the subject's BMI in this study did not include a risk factor for POP. The higher the BMI, the higher the risk of POP, but with the thought that obese women have higher estrogen level will reduce the risk of incidence of POP. The study specifically to determine the correlation between POP and BMI as confounding factors has not exist, therefore, the opinion is still a matter of controversy among researchers.

Most women with POP in this study were postmenopausal and had received no hormone replacement therapy. These are risk factors for POP.

Comparison of type III collagen in POP and without POP

Increased content of type III collagen fibers that play a role in tissue elasticity and elongation tissue will lead to decrease ratio in the content type I: III of collagen fibers, which will produce tissue laxity.[10]

The median histological score of type III collagen fibers in the uterosacral ligaments POP women in this study was 50% higher compared to women without POP ($p < 0.05$), the situation has been implicated in the occurrence of POP. The high content of type III collagen fibers to women with POP in this study was not sufficient evidence of an increasing in the content of type III collagen fibers because this study is cross-sectional.

The results in this study are in accordance to study conducted by Gabriel et al 1 who showed that the content of type III collagen fibers was significantly higher in uterosacral ligaments in patients with POP. They suggested that the content of smooth muscle in uterosacral ligaments about 20% and collagen type I in the uterosacral ligaments almost the same between postmenopausal women with and without POP, but the content of type III collagen was significantly increased for uterosacral ligaments in patients with POP. Suzme et al[11] found that the hydroxyproline levels decreased in uterosacral ligaments women with POP although in histopathology seen an increase in collagen density. Collagen synthesis in women with POP is increased in fibroblasts compared to control. It shows that the mRNA of collagen types I and III increased. The newly formed immature collagen is more susceptible to endogenous proteases and therefore is unlikely contribute to mature cross-linked collagen that confers strength and durability connective tissues.[4]

Connell et al[12] found that the expression of both collagen type I and collagen type III was significantly reduced 7.3- and 17-fold in the uterosacral ligaments of women with POP compared with controls, due to decrease in procolla-

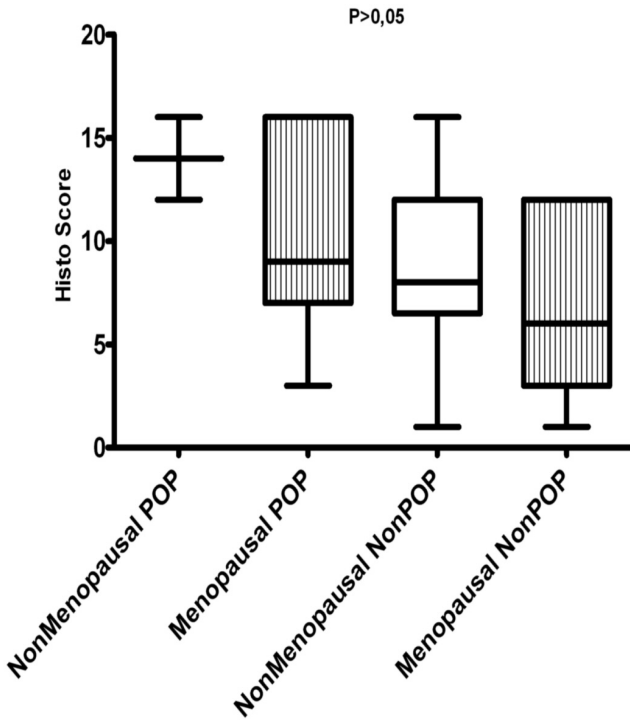

Figure 4. - Effect of menopausal status on the content of type III collagen fibers.
Statistical analysis using the Kruskal-Wallis nonparametric test (Dunn's multiple comparison test).

gen. These results were consistent with the finding of Suzme et al11, who showed that an increase in the diameter of collagen fibers in the uterosacral ligaments fewer in women with POP.

Confounding factors of age and menopausal state in this study were significant differences between the groups, but these factors did not influence to the content of type III collagen fibers in POP statisticaly. According to Kerkhof et al[3] and Jones et al[13] in patients with POP decrease resistance and weakening of the pelvic floor connective tissue, the circumstances associated with increasing age and the occurrence menopause. Increasing age and menopausal state would lead to hypoestrogenism. Chen[14] suggested that estrogen receptor alpha genotype associated with the incidence of POP. Hansen[15] states that estrogen replacement therapy increases the estradiol levels which influence tendon and ligament morphology and biomechanical properties in postmenopausal women. This is related to the smaller fibrils with a higher density. Relative stiffness was lower in estrogen replacement therapy users because lower proportion of immature collagen cross-links will reduce the potential strength of tendon and ligamentum.

Hormone replacement therapy will suppress the increase in content of type III collagen fibers, thus giving hormone replacement therapy can prevent POP.[10] The relationship between hormone replacement therapy and POP cannot be evaluated because all subjects in this study were not use of hormone replacement therapy.

Excessive stretching or tearing ATFP during vaginal delivery will contribute to the incidence of POP.[4] Reisenauer et al[16] found that the distribution of smooth muscle in uterosacral ligaments in patients POP was abnormal. Distribution of parity and BMI data both groups there was no significant difference so that does not affect the content of type III collagen fibers. Assumption of the greater degree of POP the higher the content of type III collagen fibers, according to this assumption resulting in statistical analysis to know the correlation, but the results of the statistical analysis of POP degree turns do not affect the content of type III collagen fibers.

CONCLUSION

The content of collagen type III patients with uterine prolapse is more dense than without uterine prolapse . We were not able to determine whether this was a primary contributor to the POP or a secondary manifestation of the POP itself.

REFERENCES

1. Gabriel B, Denschlag D, Göbel H, Fittkow C, Werner M, Gitsch G, et al. Uterosacral ligament in postmenopausal women with or without pelvic organ prolapse. Int Urogynecol J. 2005; 16:475-9.
2. Jackson SR, Avery NC, Tarlton JF, Eckford SD, Abrams P, Bailey AJ. Changes in metabolism of collagen in genitourinary prolapse. Lancet. 1996; 347:1658-61.
3. Kerkhof MH, Hendriks L, Brölmann HAM. Changes in connective tissue in patients with pelvic organ prolapse—a review of the current literature. Int Urogynecol J. 2009; 20:461-74.
4. Word RA, Pathi S, Schaffer JI. Pathophysiology of pelvic organ prolapse. Obstet Gynecol Clin N Am. 2009; 36:521-39.
5. Handa VL. Physiology and Pathophysiology of disorders of pelvic support. In: Bent AE, Cundiff GW, Swift SE, editors. Ostergard's Urogynecology and pelvic floor dysfunction. 6th ed. Philadelphia: Lippincott Williams & Wilkins, 2008; p. 417-21.
6. Rortveit G, Brown JS, Thom DH, Eeden SKVD, Creasman JM, Subak LL. Symptomatic pelvic organ prolapse: prevalence and risk factors in a population-based, racially diverse cohort. Obstet Gynecol. 2007; 109:1396-403.
7. Schaffer JI, Wai CY, Boreham MK. Etiology of pelvic organ prolapse. Clin Obstet Gynecol. 2005; 48(3):639-47.
8. Jelovsek JE, Maher C, Barber MD. Pelvic organ prolapse. Lancet. 2007; 369:1027-38.
9. Bradley CS, Zimmerman MB, Qi Y, Nygaard IE. Natural History of pelvic organ prolapse in postmenopausal women. Obstet Gynecol. 2007; 109:848-54.
10. Ewies AAA, Al-Azzawi F, Thompson J. Changes in extracellular matrix proteins in the cardinal ligaments of postmenopausal women with or without prolapse: a computerized immunohistomorphometric analysis. Hum Reprod. 2003; 18(10):2189-95.
11. Suzme R, Yalcin O, Gurdol F, Gungor F, Bilir A. Connective tissue alterations in women with pelvic organ prolapse and urinary incontinence. Acta Obstet Gynecol. 2007;86:882-8.
12. Connell KA, Guess MK, Chen H, Andikyan V, Bercik R, Taylor HS. HOXA11 is critical for development and maintenance of uterosacral ligaments and deficient in pelvic prolapse. J Clin Invest. 2008; 118:1050-5.
13. Jones NHJR, Healy JC, King LJ, Saini S, Shousha S, Allen-Mersh TG. Pelvic connective tissue resilience decreases with vaginal delivery, menopause and uterine prolapse. Br J Surg. 2003; 90:466-72.
14. Chen H-Y, Chung Y-W, Lin W-Y, Chen W-C, Tsai F-J, Tsai C-H. Estrogen receptor alpha polymorphism is associated with pelvic organ prolapse risk. Int Urogynecol J. 2008; 19:1159-63.
15. Hansen M, Kongsgaard M, Holm L, Skovgaard D, Magnusson SP, Qvortrup K, et al. Effect of estrogen on tendon collagen synthesis, tendon structural characteristics, and biomechanical properties in postmenopausal women. J Appl Physiol. 2009; 106: 1385-93.
16. Reisenauer C, Shiozawa T, Oppitz M, Busch C, Kirschniak A, Fehm T, et al. The role of smooth muscle in the pathogenesis of pelvic organ prolapse—an immunohistochemical and morphometric analysis of the cervical third of the uterosacral ligament. Int Urogynecol J. 2008; 19:383-9.

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INVITED COMMENT: Statistical Notes

The authors compare the content of collagen fibers from an histological examination between two groups of women with genital prolapse. The scales used to measure the results are ordinal and they had properly chosen to analyze the data with non parametric tests based on ranks. The use of rank based test should also be considered when data are from continuous variables.

The t-test is known to be the most efficient test to compare the means from two independent samples. This statement is not completely correct since to maintain its optimum properties it is required that the two samples are normally distributed.

The assumption of normality is an assumption therefore supposed true before the experiment. If it is tested before performing the t-test we introduce a multiplicity error, but especially with small sample sizes, most of the time a normality test will not refuse the hypothesis of normal distribution, without proving it.

Looking at the formula of the t statistic it is easy to see that the problem is related mainly to the standard deviation (s), when it is very large and/or it very differs between the two samples. In this case the denominator will be very large and the statistic very close to zero (not significant) although when m is large. As a result the confidence interval, calculated on the normal distribution, tends to be very wide.

A possible solution is to test the distribution of ranks between the two samples. Instead of considering the observed values all the observations are sorted and each value is substituted by its rank position.

Compared to observed values, the information about the distance between two values is lost but outliers can't make the variance grown abnormally. The more the we gain the possibility of comparing results from non metric scales like a Likert scale. In fact the distance between "agree" and "strongly agree" in not necessarily one neither it is the same distance of any other two adjacent position for example "neutral" and "disagree".

A rank based test will evaluate if in the two samples the splitted ranks are concentrated at the top/bottom or homogenously distributed.

The following example comes from an experiment on inhibition in lab animals of response to an allergic challenge.

There are only five observations per group: in the first one (A) the response is inhibited, in the other (B) it's not. Due to the allergic nature of response, observations in group B can be very different.

ID	Group	**obs.**	Rank
3	A	162.6	9
4	A	140.7	10
6	A	190.7	8
7	A	191.2	7
8	A	243.5	6
1	B	250.9	5
2	B	630.7	3
5	B	749.6	2
9	B	2347.3	1
10	B	591.9	4

In group A mean is 158.7±38.58 in group B is 914.1±822.36: it is clear that the inhibitor is working but due to the outlier (id=9) in group B the standard deviation is abnormal and the t-test is not significant ($p<0.08$).

If we censor the observation id=9 the group B mean is 555.8±214.04 and the t-test is significant ($p<0.006$). Not taking into account one value the difference between means dramatically decrease from 728.4 to 370.1 more than 50% but now it is significant. Adopting this approach the point is: the estimate of inhibition is 728.4 or 370.1?

The rank solution is preferable. In this case we have the best possible situation: the first five ranks are in group B ($p<0.03$).

Note that using this approach in general any value greater than the second rank value (in our example 749.6) will give the same result. The last problem is the best estimate of the effect. Using rank based analysis the median should be preferred, in our case 190.7 in group A and 630.7 in group B, the effect on medians is 440.

Talk to your doctor before you take any medicine.
Check a statistician before making an analysis.

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