ARS Vacation Studentship Report 2008

Title of project
Is pentraxin 3 regulated by antioxidants under conditions of sepsis?

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Background (not more than 500 words)
Pentraxins are a group of acute phase proteins which are produced in response to inflammatory conditions *in vivo* and which play a key role in the humoral innate immune system. [1,2,3] Routinely used in the diagnosis and monitoring of inflammation and infection, C reactive protein is a classical short pentraxin.[1, 2, 4] CRP is produced in the liver in response to inflammation and is the main inducer of interleukin 6, together with serum amyloid P component (SAP), another member of the short pentraxin protein group.[3, 4,5, 6]

Recently discovered pentraxin 3 (PTX3) is the first member of the long pentraxin family group.[4, 5] Unlike CRP and SAP, PTX3 is expressed in the tissues following exposure to pro-inflammatory stimuli including specific microbial constituents, TNFα and IL-1β.[1, 3, 4, 5, 6] Studies have revealed that PTX3 levels correlate well with disease severity in the septic patient.[5, 6] However, the validity of using PTX3 as a routine marker in intensive care patients has yet to be elucidated.[4]

Oxidative stress has consistently been demonstrated in patients with severe sepsis and acts as a trigger for PTX3 up-regulation in endothelial cells, fibroblasts and macrophages.[6] During oxidative stress, loss of essential protective antioxidants occurs, free radicals are formed and overwhelming degrees of tissue injury result.[7] A greater understanding of oxygen radical-mediated mechanisms will hopefully enable the development of new therapies for use in critical care patients with the aim of improving clinical outcomes in this group.[7]

Consequently, the aim of this project was to treat human endothelial cells with a range of antioxidants with and without bacterial cell components or proinflammatory cytokines and to determine the subsequent expression of PTX3 using enzyme linked immunosorbent assay (ELISA). To date, the regulation of PTX3 within a generated model of sepsis has yet to be defined.

Report on research undertaken (not more than 1000 words)

Use the following headings: Methods used, Results and Discussion.
Figures may be provided on separate sheets.

Methods used

Human umbilical vein endothelial cells (HUVEC) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% heat inactivated fetal calf serum, 50μg/ml gentamicin and amphotericin B. Cells were then treated with a range of antioxidants; 1μM mito Q, 1μM triphenylphosphonium (TPP), 100M trolox, 25mM N-acetyl cysteine (NAC), 1μM idebenone and 100μM dehydroascorbic acid (DHA), ± 2μg/ml lipopolysaccharide (LPS) and 20μg/ml peptidoglycan G (PEPG), 10ng/ml tumour necrosis factor (TNFα), 20ng/ml interleukin 1β (IL-1β) or 10ng/ml TNFα and 20ng/ml IL-1β. Controls consisted of phosphate buffered saline (PBS) or ethanol. Treated cells were incubated for 24 hours at 37°C. Condition media was then removed and stored at –80°C for further experimentation.

Pentraxin 3 expression from conditioned media was determined using enzyme immunosorbent assay (ELISA). Briefly, 96 well ELISA plates were coated with 100μl of 700ng/ml monoclonal antibody (MAb) to PTX3 in coating buffer (15mM sodium carbonate buffer, pH 9.6) and incubated overnight at 4°C.

After incubation, plates were washed with washing buffer (PBS + 0.05% Tween 20) and blocked with 200μl of 10% dry milk in washing buffer for 1 hour at 37°C. Plates were washed as stated above and 100μl of either recombinant human PTX3 standard (75pg/ml to 2.4ng/ml) or conditioned media, undiluted for unstimulated and 1/5 dilution for stimulated cells (in duplicate) were added to each well. Following 2 hours incubation at 37°C, plates were washed and 100μl of 25ng/ml biotinylated polyclonal antibody (PAb) to PTX3 were added to all wells.

Plates were incubated for a further hour at 37°C and washed. Streptavidin-horseradish peroxidase (100μl of 1.0mg/ml diluted 1/4000) were added to each well and plates incubated as before for 40 minutes. After incubation, plates were washed and 100μl/ well of chromogen substrate added. The reaction was stopped using 50μl 2N H_2SO_4 following the development of sufficient colour. Plates were then read at 450nm and 540nm in an automatic ELISA plate reader.

Acid phosphatase assays were also carried out to determine cell viability. 96 well plates were treated with antioxidants and stimuli as stated above and incubated at 37°C for 24 hours. Condition media were removed and plates were washed with 200μl/ well of PBS, twice. The latter was removed and 100μl of acid phosphatase buffer (0.1M sodium acetate and .01% V/V triton X-100 containing 5 mM P-nitrophenylphosphate) were added to each well. Plates were then incubated for 1 hour at 37°C. The reaction was stopped by the addition of 15μl of 1M NaOH to each well and plates were read at 405nm in an automatic plate reader.

HUVEC were also treated with 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0 and 3.0 μM mito Q or TPP and ethanol as control. ELISA to determine PTX3 secretion was then performed as above.

Results

Results showed that cell viabilities were >95% in all experiments performed. Data analysis confirmed a significant difference in PTX3 secretion when comparing stimulated and unstimulated samples in all experiments (Kruskal-Wallis $p<0.0001$). Results showed that HUVEC treated with antioxidants alone did not induce the expression of PTX3 except for cells exposed to mito Q and TPP ($p=0.0286$). Cells stimulated with LPS/ PEPG or IL-1β produced significant increases of PTX3 expression at a similar level. (Figures 1 and 2) Results illustrated a greater rise in PTX3 when HUVEC were treated with either TNFα or TNFα and IL-1β. (Figures 3 and 4)

Cells treated with LPS/ PEPG showed no reduction in PTX3 when compared to relevant standards. However, an increase in pentraxin secretion was observed in cells treated with trolox, NAC and DHA but this was not found to be statistically significant. (Figure 1)

Results demonstrated that cells treated with antioxidants and TNFα also showed no decrease in PTX3 secretion except in stimulated samples treated with mito Q and TPP. When compared to the relevant ethanol control, the reduction in PTX3 production due to mito Q involvement was not significant ($p=0.2$), however, the result for TPP was statistically different ($p=0.0286$). There was also a significant difference between the levels of PTX3 found in the stimulated mito Q and TPP samples. Significant differences in PTX3 production were also observed between stimulated samples treated with mito Q and trolox and also mito Q and DHA. In addition, a significant difference in PTX3 secretion was found to exist between the stimulated TPP sample and the stimulated samples treated with trolox, NAC idebenone and DHA. (Figure 2)

For cells treated with IL-1β there was no significant reduction in PTX3 levels in any of the stimulated samples. By comparison, results for cells stimulated with both TNFα and IL-1β showed a significant reduction in PTX3 levels when treated with ethanol and NAC when compared to the control. However when results for mito Q, TPP, trolox and idebenone were compared with the relevant ethanol control, no significant decrease in PTX3 production was found. (Figure 3) In samples stimulated with TNFα and IL-1β and treated with ethanol, mito Q, TPP, trolox and idebenone, PTX3 was suppressed to a similar level in all samples. Although NAC also reduced PTX3 secretion this was to a lesser extent with a statistically significant difference found between NAC and ethanol, mito Q, TPP and idebenone ($p=0.0286$). (Figure 4)

Results of acid phosphatase assays for the titration experiment of mito Q and TPP, showed that cell viabilities exceeded 95% except when cells were treated with 3.0 μM, which reduced cell viability to 40%. Subsequent ELISA was performed using 0 to 2.0 μM mito Q and TPP. Results showed that for cells stimulated by LPS/ PEPG and treated with varying concentrations of mito Q and TPP, higher levels of PTX3 were observed at all concentrations compared to the ethanol controls. The concentration of PTX3 peaked at 0.75 μM mito Q and 1.0 μM TPP before becoming reduced at higher concentrations of treatments. (Figure 5) A similar pattern of PTX3 secretion at higher concentrations of mito Q and TPP was also seen in HUVEC stimulated by TNFα. (Figure 6) Results for cells stimulated with IL-1β showed a rise in PTX3

levels compared to the ethanol controls with the addition of 0.25 μM of mito Q and TPP. An overall trend of a reduction in PTX3 at all subsequent concentrations of mito Q and TPP was then observed with a particularly marked fall in PTX3 in cells treated with 2.0 μM TPP. (Figure 7) Results demonstrated overall that for HUVEC stimulated with both TNFα and IL-1β, a greater degree of PTX3 suppression occurred at all concentrations of mito Q and TPP compared to cells stimulated with LPS/PEPG or IL-1β or TNFα and IL-1β. (Figure 8)

Discussion

Expressed by a variety of cells during oxidative stress, PTX3 creates an interesting foundation on which to base research regarding the treatment of critical care patients suffering from septic shock. It has previously been demonstrated that PTX3 secretion is triggered by exposure to particular microbial components and proinflammatory cytokines. Indeed, the results of this project confirm the latter finding as PTX3 levels rose significantly when HUVEC were stimulated with LPS/ PEPG, TNFα, IL-1β or TNFα and IL-1β combined. The greatest rises in PTX3 secretion were seen in the TNFα and TNFα and IL-1β experiments which may suggest that TNFα is acting as the predominant stimulus for PTX3 production by endothelial cells. In this study, it was hypothesized that PTX3 levels expressed by cells exposed to appropriate inflammatory stimuli would be reduced by antioxidant treatment. From results obtained however, antioxidants were found to have little effect on the secretion of PTX3 with the exception of cells stimulated by TNFα and treated with TPP and also cells stimulated with both TNFα and IL-1β and treated with ethanol and NAC. It must be noted that experiments were only repeated in quadruplicate due to the short time period of study and consequently, a greater number of repeats are required before any definite conclusions can be drawn from the results obtained.

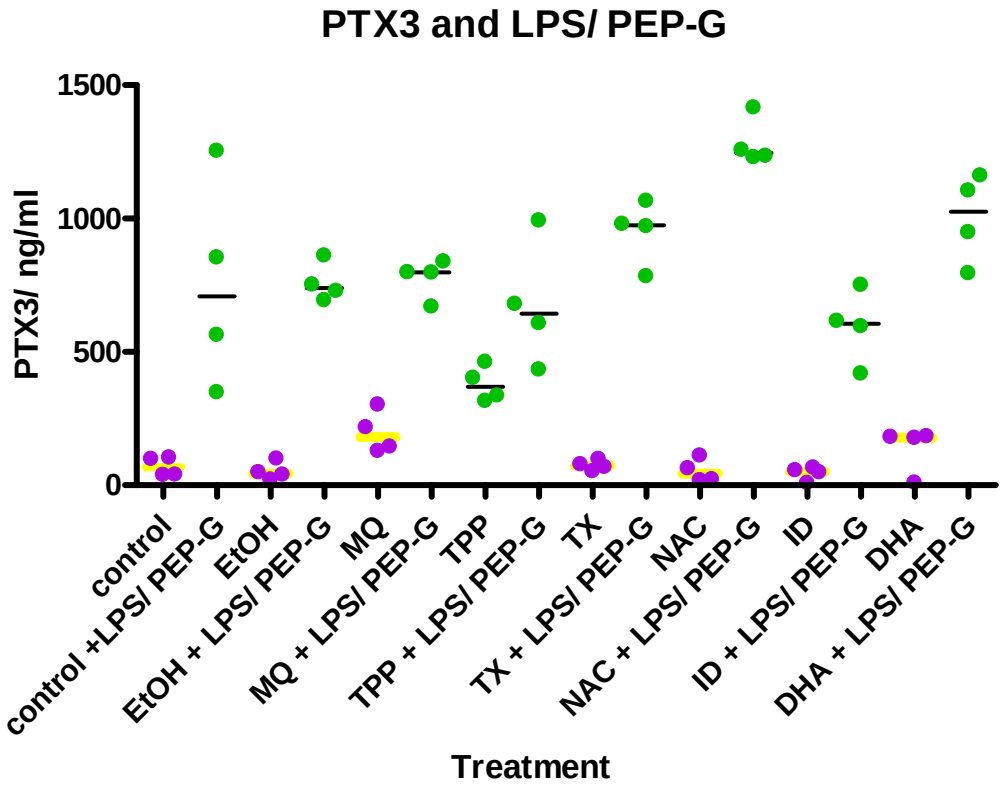


Figure 1

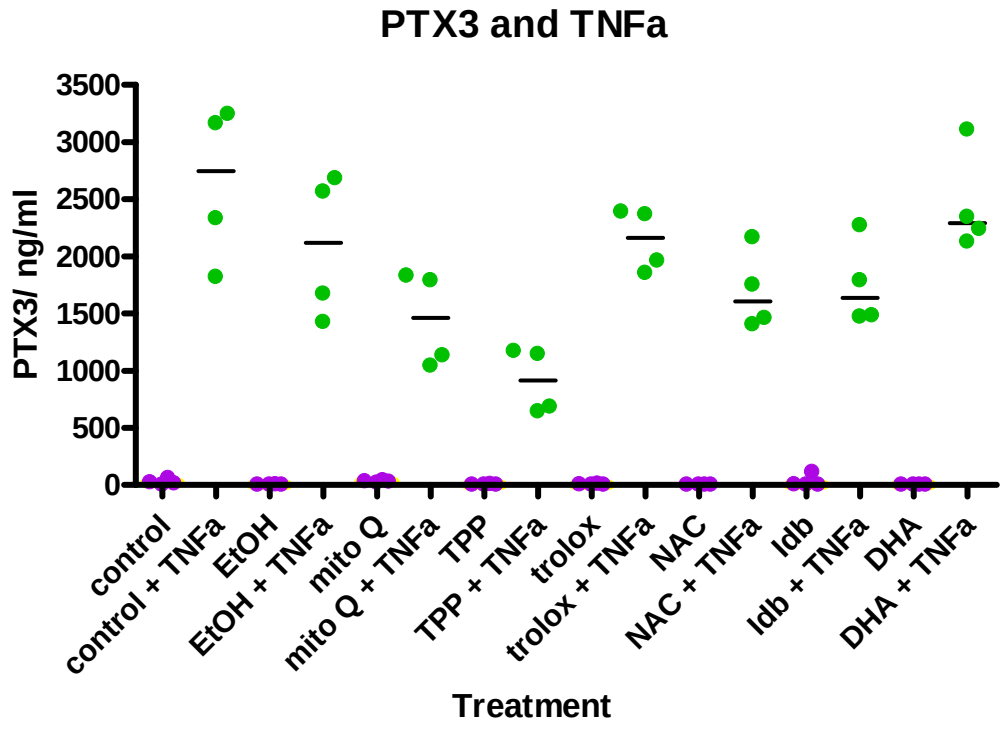
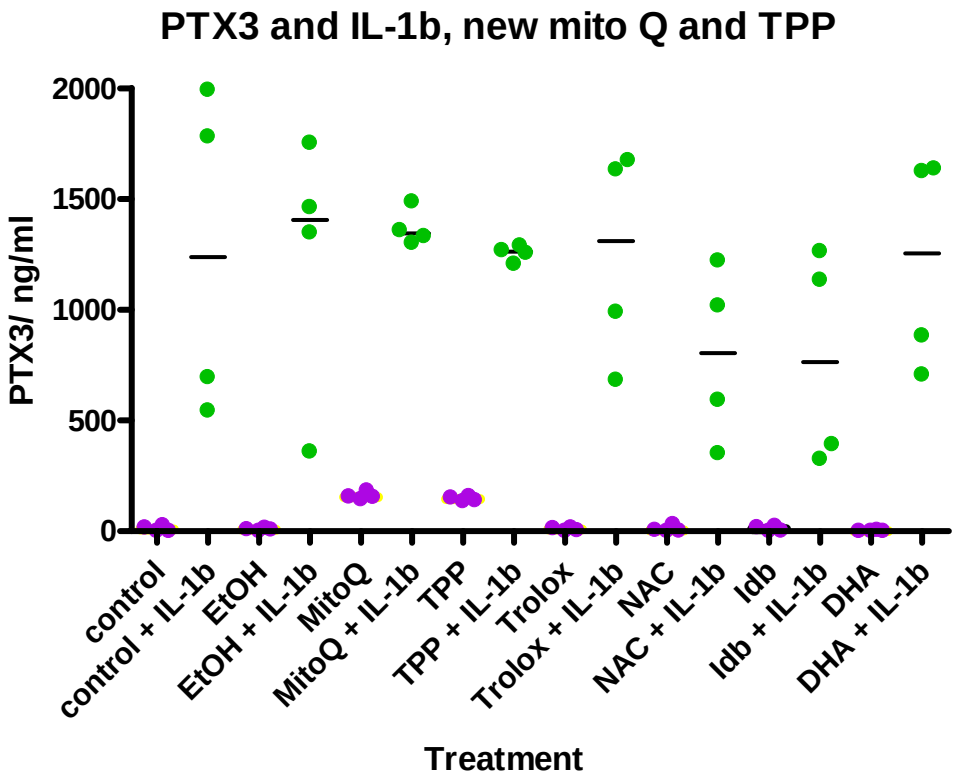


Figure 2



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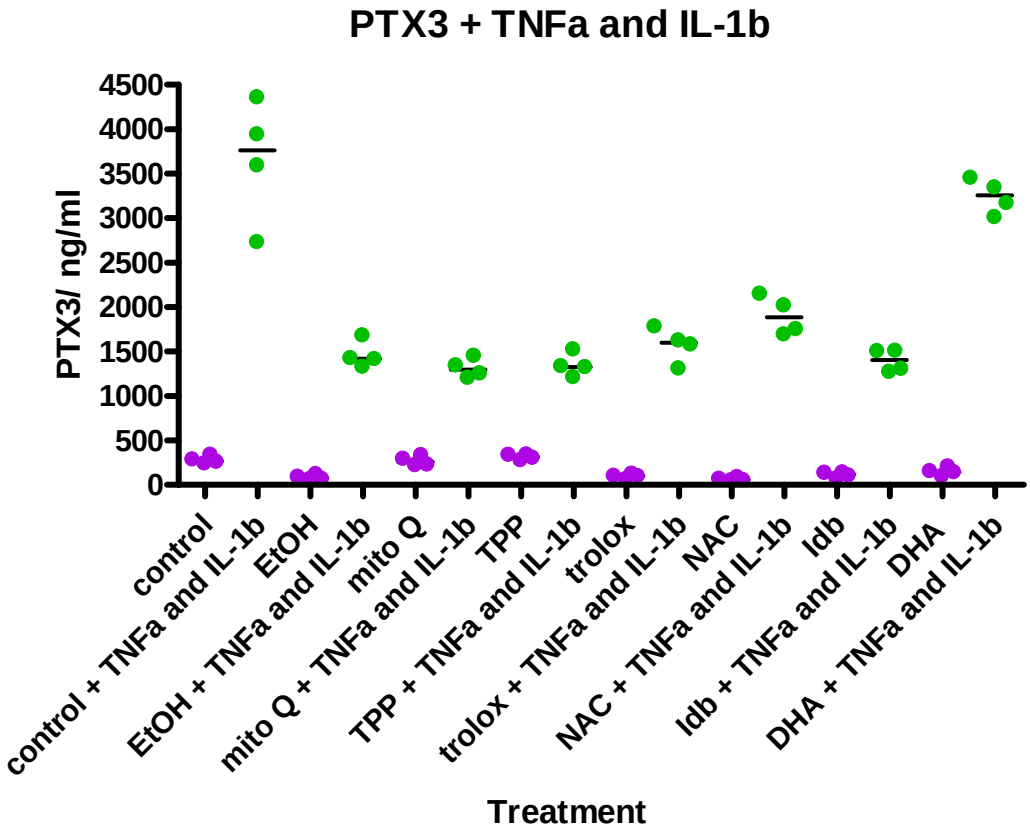

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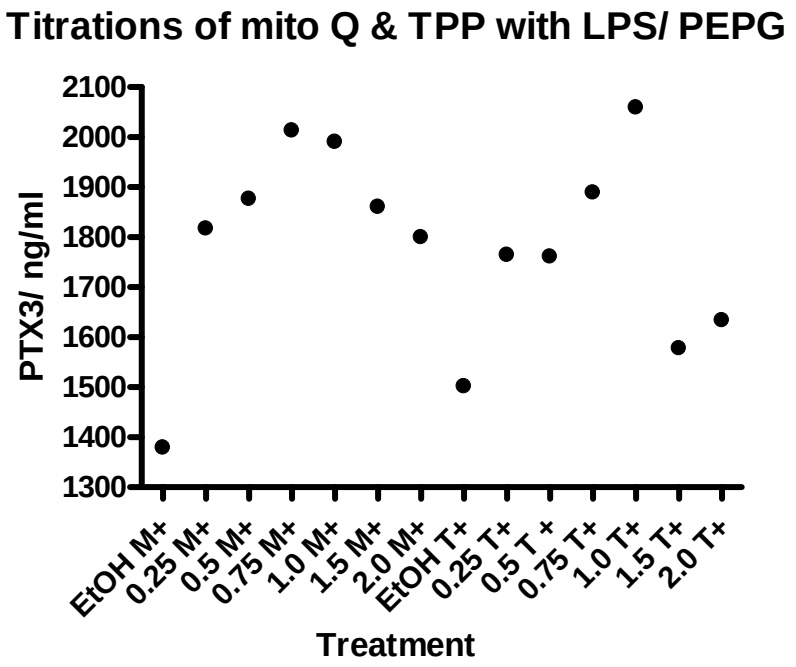

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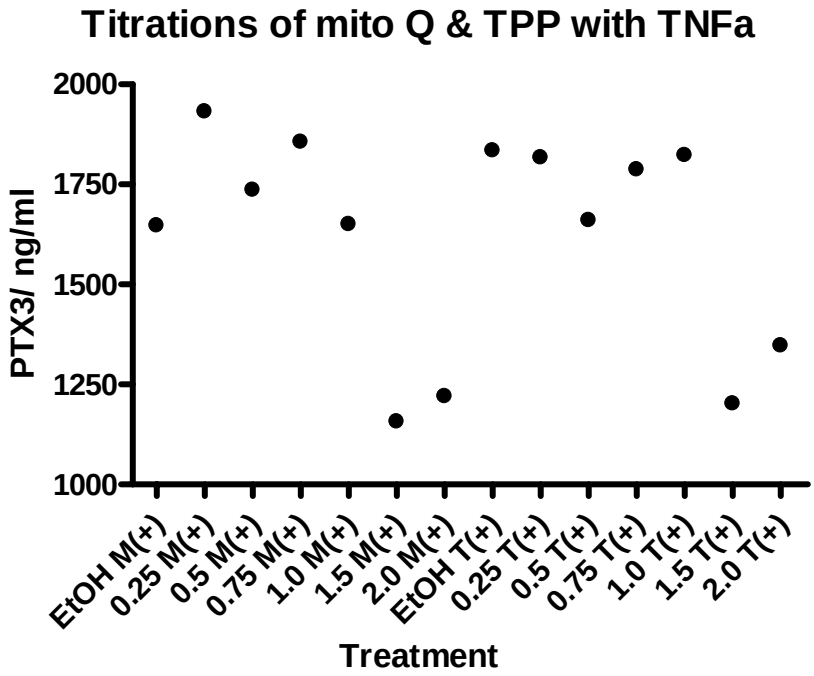
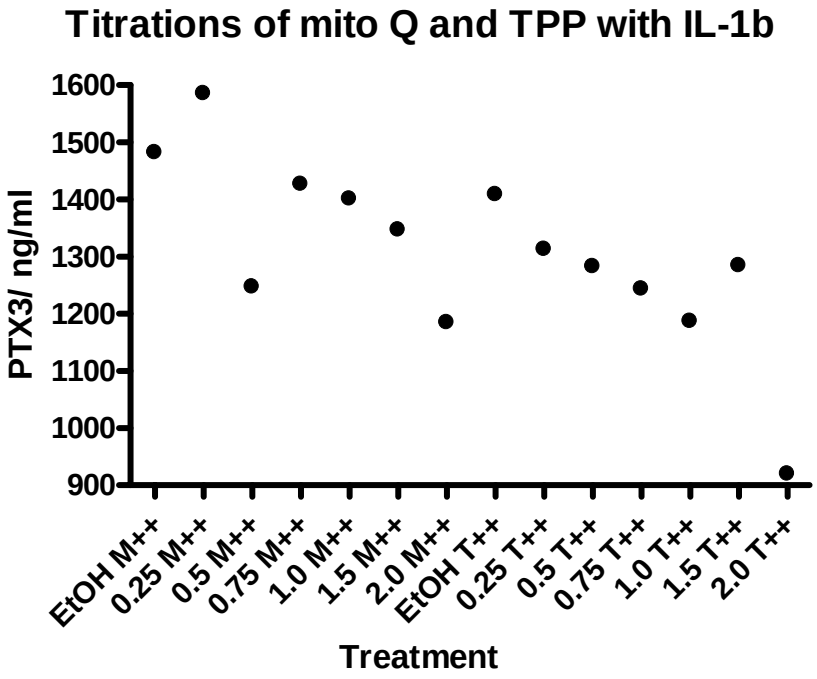


Figure 6



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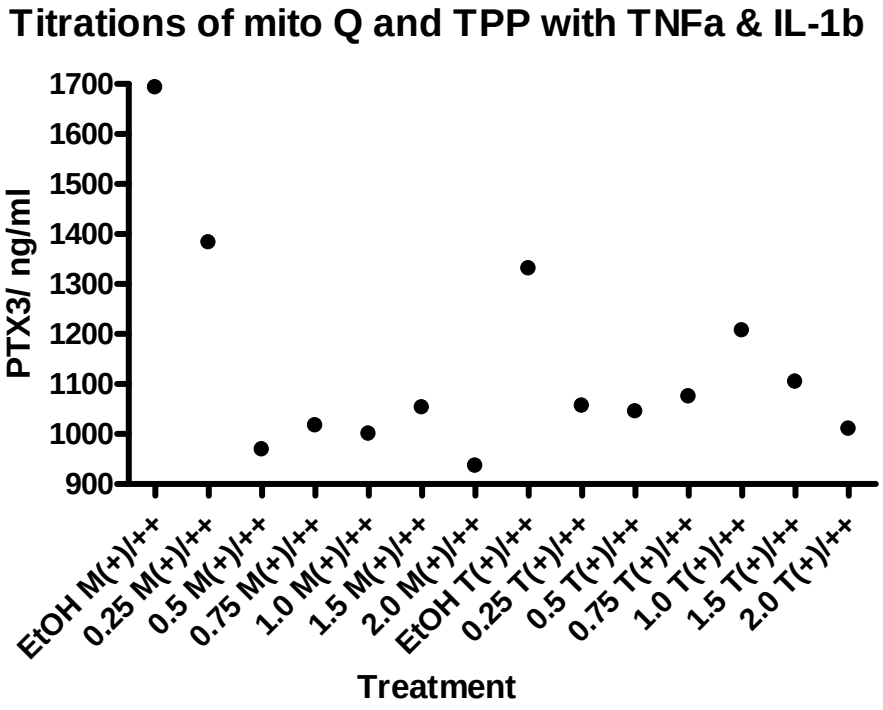

Figure 8

References

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