Comparison of Human Cytokine Array, Cytokine Bead Arrays, and Protein ELISA in determining cytokine production in media supernatant from in vitro strain of human dermal fibroblasts.

Aric Anloague
Dr. Jonathan Lowery Ph.D.

Abstract

This experiment analyzes three different techniques used in literature to detect cytokine release from cells *in vitro*: the Human Cytokine Array (HCA) by R&D Systems, the BD Cytometric Bead Array (CBA) assay, and protein enzyme-linked immunosorbent assay (ELISA). The model used to test these assays was a human dermal fibroblast cell culture that was subjected to strain profiles by the Flexcell FX-6000 that include a repetitive motion injury, a profile with the injury strain as well as a strain representing myofascial release, and a control that was not strained. Each of these techniques analyzed the conditioned media of the cultures collected 96 hours after the strain profile was completed. The HCA was proficient at analyzing a large amount of cytokines at once, though was less sensitive than the other assays. The CBA measured six cytokines at once and showed high sensitivity in detecting differing concentrations in the conditioned media. The ELISA could only measure one cytokine at a time, but also showed high sensitivity in detecting differing concentrations in the conditioned media. This experiment also looked at the cost and amount of sample used. This experiment also considers price, amount of sample used and duration of the assay to determine ideal situations to use each technique.

Introduction

The purpose of this review is to determine the ideal technique for analyzing cytokine release from fibroblasts in cell culture. The three techniques that will be compared are the Human Cytokine Array (HCA) by R&D Systems, the BD Sciences Cytokine Bead Array (CBA), and protein enzyme-linked immunosorbent assay (ELISA). Cytokines, both pro- and anti-inflammatory, play distinct roles in the wound healing by initiating processes such as acting as a white blood cell mobilization, stem cell mobilization, and regulation and initiation of the inflammation response. Measuring cytokine release is beneficial to researchers for many reasons including, using them as potential biomarkers, determining immune system health, understanding the inflammatory response, understanding the response to a treatment, etc.

Each of these techniques use a slightly different method of detection. The HCA uses a membrane that is premarked with antibodies for 36 different cytokines in duplicate. The membranes are incubated with sample and detection antibodies that precipitate a dark color when a cytokine is present—darker intensities suggest higher concentration of cytokine, though concentrations are only relative. The CBA uses different beads each labeled with different antibodies for the cytokines of interest. The beads are allowed to incubate with the samples and a detector antibody labeled with different fluorophores that are then analyzed using flow cytometry. If there is more cytokine present, then more beads will be detected by the flow cytometer. Comparing the number of beads detected in the unknowns to a standard curve allows this procedure to develop concentrations of cytokines present in each sample. The ELISA protocols vary depending on the secondary antibody visualization used. In this experiment, a 96-well plate is coated with the antibody for the desired cytokine, and the sample is incubated with a secondary

antibody conjugated with biotin. Following the incubation, streptavidin HRP is added to precipitate a color and it is developed using TMB chromagen, and the color intensity is identified and compared to a standard curve to develop concentrations of cytokines present. These different detection methods may result in different sensitivities of sample and affect the accuracy of acquisition.

In order to test the pros and cons of each of these techniques, a model was needed that not only used a cell type that releases cytokines in an injured/strained state, but also a method that could produce an injury-like state. Over a series of experiments, Standley, Meltzer *et al.* established a fibroblast model that put cultured fibroblast cells through a repetitive motion-like strain and a strain mimicking myofascial release [1-6]. During these strains, the fibroblasts release cytokines into the media in which they are cultured. This conditioned media is what is used to measure the cytokines released and is relatively easy to analyze by all three of the techniques.

Previously using these models, Standley and Meltzer showed using the HCA and ELISA that IL-1α, IL-1β, IL-2, IL-3, IL-6, and IL-16 increase substantially increase following a repetitive motion strain and showed a significant reduction of IL-6 alone with a treatment they deemed a counterstrain [3]. In a later study, they showed that with their MFR treatment, using the HCA technique, only GROα showed a significant change with increased expression [4]. Standley and Meltzer did not use the CBA in any of their studies. This experiment attempts to validate the findings of Meltzer and Standley to validate the effectiveness of each technique, and it takes into consideration the price of the assay, amount of sample used per assay, and duration of each assay to suggest the ideal use for each technique.

Methods

Fibroblast Culture/Flexcell protocol: Primary human dermal fibroblasts were purchased from ATCC (#PCS-201-012) and according to the suggestions of ATCC. Mechanical stimulation was performed on a Flexcell FX-6000 (located in the Indiana Center for Musculoskeletal Health core lab) according to the methods described by Metzler, Standley *et al* [6]. Cells were seeded on flexible collagen I-coated membranes and strained to mimic a repetitive motion strain in an 8 hour cycle with 1.6 second bouts of deformation increasing at 22%/second starting at a baseline strain of 10% and a maximum of 16.6%, followed by decreasing strain to baseline at 22%/second—these cells represent the Injury strain. Cells that represent the Myofascial Release (MFR) strain underwent the same 8 hour protocol as the injury strain, followed by a 3 hour rest period. After the rest period, these cells were subjected to a single 60 second bout of stretch at 6% beyond resting length at a loading rate of 3% followed by release at 1.5%/second until return to resting length. The control group did not undergo either of these strain protocols. Conditioned media was collected 96 hours after the strain protocols from each sample and stored at -80°C. These protocols were run 3 times on three different dates and are labeled as Run #1- Run #3. Each Run had 1 control group, 2 Injury Strains, and 2 Injury + MFR Strains.

Human Cytokine Array: The collected conditioned media from each strain profile were simultaneously analyzed for CCL1/I-309, CCL2/MCP-1, MIP-1α/MIP-1β, CCL5/RANTES, CD40 Ligand/TNFSF5, Complement Component C5/C5a, CXCL1/GROα CXCL10/IP-10, CXCL11/I-TAC, CXCL12/SDF-1, G-CSF, GM-CSF, ICAM-1/CD54, IFN-γ, IL-1β/IL-1F2, IL-1ra/IL-1F3, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p70, IL-13, IL-16, IL-17A, IL-17E, IL-18/IL-1F4, IL-21, IL-27, IL-32α, MIF, Serpin E1/PAI-1, TNF-α, and TREM-1 using the Proteome Profiler Human Cytokine Array from R&D Systems. Each run was analyzed on separately. The techniques were performed following the instructions provided by the manufacturer. Signal density was analyzed with the Licor C-Digit.

Human Pro-inflammatory Cytokine Cytometric Bead Array: The collected conditioned media from each strain profile in each run were simultaneously analyzed for IL-8, IL-1β, IL-6, IL-10, TNF, and IL-12p70 using the Human Pro-Inflammatory Cytokine Cytometric Bead Array kit from BD Biosciences. All runs were analyzed simultaneously. The techniques were performed following the instructions provided by the manufacturer with the BD Accuri Flow Cytometer. Data were analyzed with paired T-tests between each of the strain profiles.

Enzyme-linked Immunosorbent Assay: The collected conditioned media from and each strain profile in each run were analyzed using a separate human ELISA kit for IL-6 and for IL-8 from ThermoFisher Scientific to determine the concentration of these respective proteins. All runs were analyzed simultaneously. The ELISA methods were performed following instructions provided by the manufacturer. Data were analyzed with paired T-tests between each of the strain profiles.

Results

Human Cytokine Array: Of the thirty-six cytokines tested for, only six were detected: CXCL1/GROα, CXCL12/SDF-1, IL-6, IL-8, MIF, and Serpin E1/PAI-1. The relative signal of each cytokine for each sample was compared to the average of the signal density of the reference spots for each sample. There was no detection of CXCL1/GROα in Run #3, no detection of CXCL12/SDF-1 in Run #1, and no detection of IL-6 in Run #1 and Run #3 (fig. 1). No statistical analysis was performed on the data because there was not enough samples that showed detection.

Human Pro-inflammatory Cytokine Cytometric Bead Array: Of the six cytokines tested for, only IL-6 and IL-8 were detected. The concentrations of IL-6 and IL-8 varied from run to run (fig. 2), so the fold change between strain profiles was calculated, and the averages from all runs were taken (fig. 4). For IL-6, there was a significant increase in expression in the Injury group compared to the control ($p=0.0145$; 95% CI=1.762 to 5.644), and then a significant decrease in the Injury + MFR group compared to injury ($p=0.0241$; 95% CI=-3.136 to -0.5975). The Injury + MFR group for IL-6 still showed increased expression when compared to the control ($p=0.0297$; 95% CI= 0.4425 to 3.231).

For IL-8, there was an observed increase in expression in the Injury group when compared to the control (p=0.0782; 95% CI=-0.4753 to 3.875), and a significant decrease in the Injury + MFR group when compared to the Injury group (p=0.0429; 95% CI=-2.285 to -0.940). The Injury + MFR group approached the expression value of the control group with no significant difference between the two groups (p=0.306; 95% CI= -1.098 to 2.118).

Enzyme-Linked Immunosorbent Assay (ELISA): The ELISAs similarly showed varied concentrations between runs for both IL-6 and IL-8 (fig. 3), so the fold change between strain profiles was calculated and the averages from all runs were taken (fig. 4). For IL-6, there was a increase in expression in the Injury group when compared to the control (p=0.0057; 95% CI= 1.464 TO 2.889), and a decrease in the Injury + MFR group when compared to the Injury group (p=0.0709; 95% CI=-1.973 TO 0.181). The Injury + MFR group still remained higher than the control (p=0.0293; 95% CI=0.3171 to 2.250).

For IL-8, there was no statistically significant changes between groups, however, the observed expression of the Injury group appears higher than the control (p=0.150; 95% CI=-3.037 to 9.903), and expression appears to decrease towards the values of the control in the Injury + MFR group when compared to the Injury group (p=0.319; 95% CI=-5.495 to 2.923). There was also no significant difference between the Injury + MFR and control (p=0.2043; 95% CI=-2.825 to 7.119)

In order to compare the effectiveness of the CBA and ELISAs, X-Y plots for IL-6 and for IL-8 were generated comparing the concentrations of each sample for each run (fig. 5). Linear regression for IL-6 showed the best fit line with a slope of 0.807 and Y-intercept at 5.678 and an R^2 value of 0.945. Linear regression for IL-8 showed the best fit line with a slope of 0.665 and Y-intercept at 5.254 and an R^2 value of 0.732.

Discussion

The three techniques discussed vary in the cytokines analyzed per run, duration of the assay, amount of sample used, monetary cost, and sensitivity. Comparing these differences can help determine which assay should be used depending on the needs of the experiment. The human cytokine array (HCA) and the cytokine bead array (CBA) both analyze the amount of cytokines provided by the kits used, 36 and 6 respectively. The HCA took approximately 24 hours to measure all 36 cytokines, and the CBA took approximately 5 hours to measure the 6 cytokines. The Enzyme-linked Immunosorbent Assay (ELISA) only measures one cytokine at a time, and takes approximately 4 hours per cytokine. The duration of the techniques between the HCA and CBA are comparable when compared to the number of cytokines analyzed, but the ELISA takes significantly longer.

When sample supply is limited, the volume of sample used per run is important. The HCA uses 1mL of each sample per run, the CBA uses 50μL of each sample per run, and the ELISA varies from kit to kit. For the IL-6 kit, 100μL of each sample per run was used and the IL-8 kit used 50μL per run. Because of the number of cytokines and volume of sample analyzed per run,

the CBA technique is the most efficient. The ELISA is comparable, but the volume of sample is required for each cytokine analyzed, whereas **the CBA uses that volume for analysis of 6 cytokines at once.**

The monetary cost of the techniques are important to consider. The cost depends on the goals of the experiment. The ELISA is the cheapest option at approximately $479.00/kit if analysis of only one cytokine is desired. The HCA kit provides four membranes per kit, which is enough for four samples, at a cost of $519.00/kit. The CBA kit costs approximately $1364.00/kit, but the ELISA and the CBA both can run up to ninety-six samples at once including the standards needed for generating the standard curve. **If there are a low number of samples, the HCA is more cost efficient, but if more samples are in need of analysis, the CBA and ELISA become more cost efficient depending on the desired number of cytokines to be analyzed.**

The HCA assay is the most beneficial when uncertain which cytokines may be detected because of the number of cytokines detected simultaneously. It can be a very costly experiment in terms of amount of sample used per run and cost per sample analyzed at $519.00 for four samples. Additionally, the HCA showed no significant differences in cytokine expression between groups (fig. 1) and IL-6 and IL-8 were below the detection limit, even though it was shown that they were present in the conditioned media by the CBA and ELISA, suggesting a low sensitivity in detection for the HCA. It may be beneficial to **use the HCA as a preliminary test on only a few of the samples to determine what may be detected, and then a different assay such as the CBA or ELISA for detection of concentration depending on the goals of the experiment.**

The CBA is efficient, cost effective, and accurate when testing multiple samples for multiple cytokines. As previously stated, it is capable of running 96 samples at once, including the standards for the standard curve, and considering **the CBA kits cost $1364.00 each, this equates to approximately $14.21 per sample, which is much more cost effective than the HCA, which is $129.75 per sample.**

Only the CBA and ELISAs measure exact concentrations of cytokines. They both advertise a detection range that goes as low as 1pg/mL and depending on the kit, they can go as high as 5,000pg/mL. The lower limit was pushed in this experiment with the lowest concentrations for IL-6 appearing in the control groups of Run #2 and Run #3 at 2.73pg/mL and 2.86pg/mL respectively for the CBA, and 5.97pg/mL and 5.19pg/mL respectively for the ELISA. The X-Y plots in figure 5 compare the concentrations detected between the CBA and the ELISAs. The detected concentrations were more similar to each other between assays in IL-6 than in IL-8 because the best-fit line for IL-6 has a slope of 0.807, which is closer to the ideal slope of 1 than IL-8, which has a slope of 0.665. The R^2 values for IL-6 and IL-8 were 0.945 and 0.732 respectively, again suggesting a better fit line in the IL-6 data. These data show the variability that can occur between the cytokine concentration detection between the assays.

In this experiment, the CBA showed significant increase in fold change for both IL-6 and IL-8 in the Injury groups compared to the control. These trends were similar in the ELISAs, though the increase observed in the Injury group, when compared to the control, was not statistically significant. Similarly, the CBA showed significantly lower levels of IL-6 and IL-8 in the Injury +

MFR group when compared to the Injury group, and the ELISAs, again, showed similar trends, though not statistically significant. These trends suggest that the CBA has a higher sensitivity than the ELISAs making it the more efficient assay.

The trends in the CBA and ELISAs are supported by the findings of Standley and Meltzer, who also showed that in IL-6, there is an increase in a strain representing an injury when compared to the control, and there is a decrease in the strain representing either myofascial release or counterstrain [1, 3-5]. These studies did not show significant changes in IL-8, but the trends match the data observed in both the CBA and ELISAs, again validating the techniques performed in this experiment. The studies mention several other cytokines that were detected that were not detected by the HCA or CBA in this experiment, but that difference could be due to the overall lower concentrations of cytokine release in Run #2 and Run #3, and it could be based on the amount of time between the final strain profile and collection of the media, which in this experiment was 96 hours.

Overall, each of the different techniques has its merits and flaws. **The HCA is a beneficial technique to use when it is unknown which cytokines may be detected**. Its sensitivity is relatively low and has difficulty detecting low concentrations of cytokines. There were several samples that showed no detection of IL-6 and IL-8, which were clearly present based on the findings of the other two assays. It is also costly per sample tested, so it may be useful in a preliminary nature, identifying the cytokines released, but then using CBA or ELISA to determine the concentrations present.

The ELISA is useful when detection of only one to two cytokines is needed because it becomes more costly and time consuming with more. It has the benefit of being able to test ninety-six samples at once while using relatively low amount of sample for each test, but, again, if more cytokines are tested for, more sample will be used. Additionally, the ELISA is fairly sensitive in determining concentrations of samples with the ability to detect concentrations as low as 5pg/mL.

The CBA is the most versatile when multiple cytokines are to be analyzed at once. Its efficiency is the best of the three techniques in terms of time to complete the assay and amount of sample used per assay. While one kit is more expensive than one kit of each of the other runs, it more than makes up for the cost when considering the cost per sample tested and the amount of cytokines analyzed at once. The CBA is very sensitive in determining concentrations of samples with the ability to detect concentrations as low as 2 pg/mL.

To strengthen the findings of this study, the procedures could be repeated with different cell types including different tissues or different animal models. Different types of sample such as serum or donor plasma could be analyzed instead of media supernatant. The findings are limited by the concentrations of cytokines released as a result of the strain profiles. Finding a model that releases higher amounts of cytokines may resolve the low to no detection issue that occurred in the HCA. Ultimately, each assay has their place in research and it is important to use the technique that best fits the experimental design.

References

1. Meltzer, K.R. and P.R. Standley, Modeled repetitive motion strain and indirect osteopathic manipulative techniques in regulation of human fibroblast proliferation and interleukin secretion. J Am Osteopath Assoc, 2007. 107(12): p. 527-36.

2. Eagan, T.S., K.R. Meltzer, and P.R. Standley, Importance of strain direction in regulating human fibroblast proliferation and cytokine secretion: a useful in vitro model for soft tissue injury and manual medicine treatments. J Manipulative Physiol Ther, 2007. 30(8): p. 584-92.

3. Standley, P.R. and K. Meltzer, In vitro modeling of repetitive motion strain and manual medicine treatments: potential roles for pro- and anti-inflammatory cytokines. J Bodyw Mov Ther, 2008. 12(3): p. 201-3.

4. Meltzer, K.R., et al., In vitro modeling of repetitive motion injury and myofascial release. J Bodyw Mov Ther, 2010. 14(2): p. 162-71.

5. Dodd, J.G., et al., In Vitro Biophysical Strain Model for Understanding Mechanisms of Osteopathic Manipulative Treatment. J Am Osteopath Assoc, 2006. 106(3): p.157-66

6. Zein-Hammoud, M., Standley, P.R., Modeled Osteopathic Manipulative Treatments: A Review of Their in Vitro Effects on Fibroblast Tissue Preparations. J. Am Osteopath Assoc, 2015. 155(8): p. 490-502.

Figures

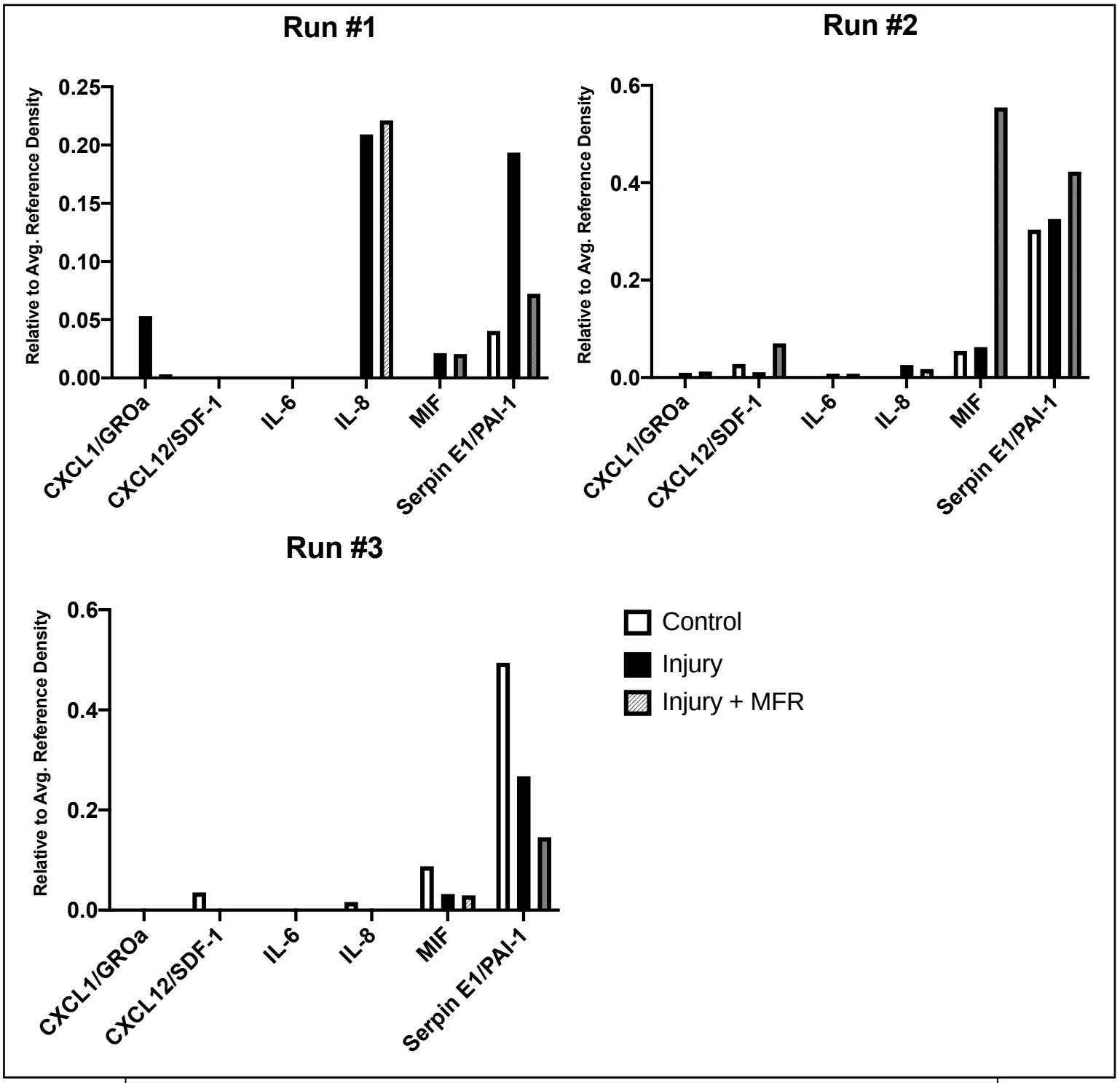

Fig. 1 Graphs displaying the cytokines detected in the Human Cytokine Array and their relative densities as compared to the average of the reference spots.

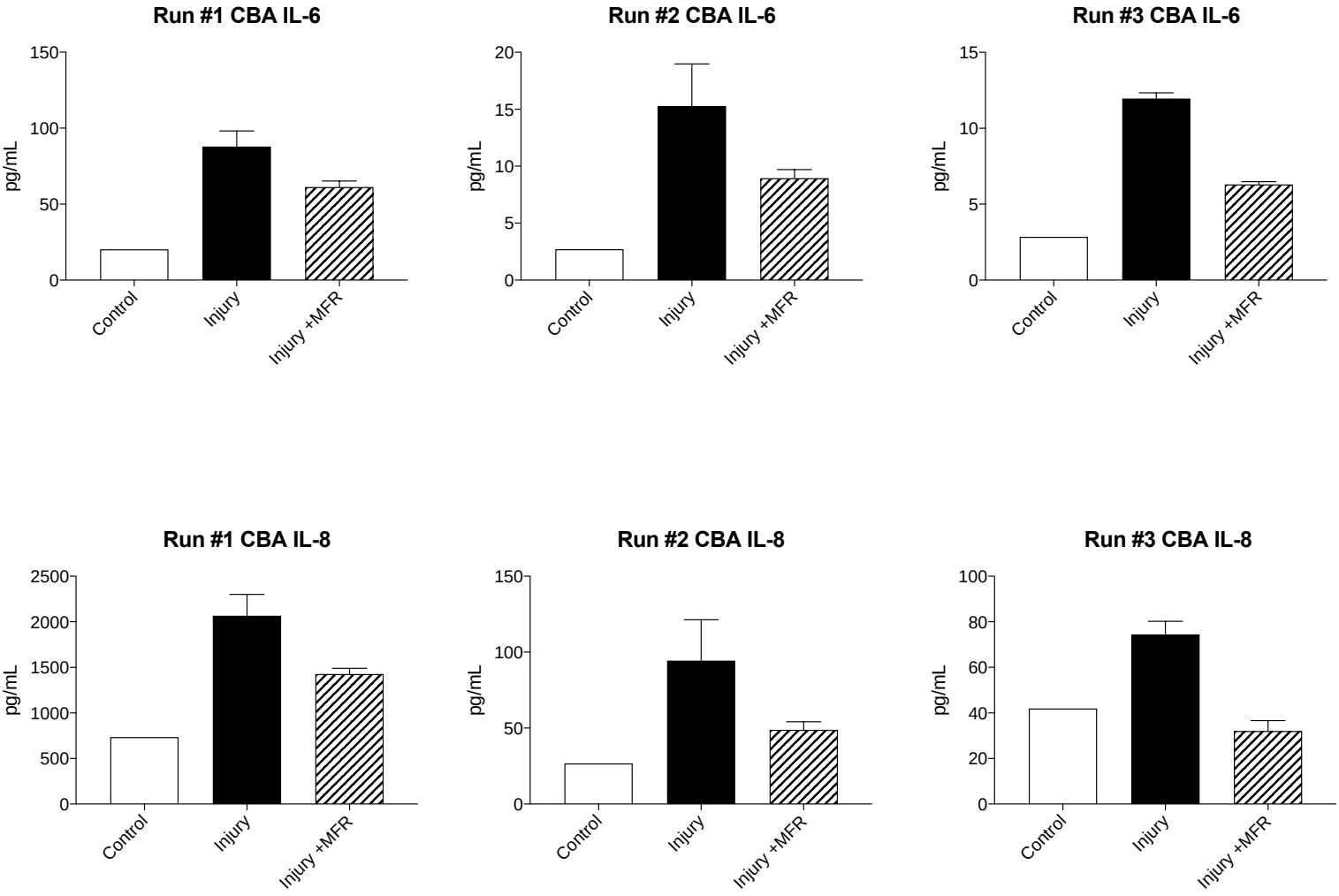

Fig. 2 Graphs displaying the concentrations of IL-6 and IL-8 for each run and strain profile as detected by the Cytokine Bead Array (CBA) assay. Data are expressed as mean concentrations ± SEM.

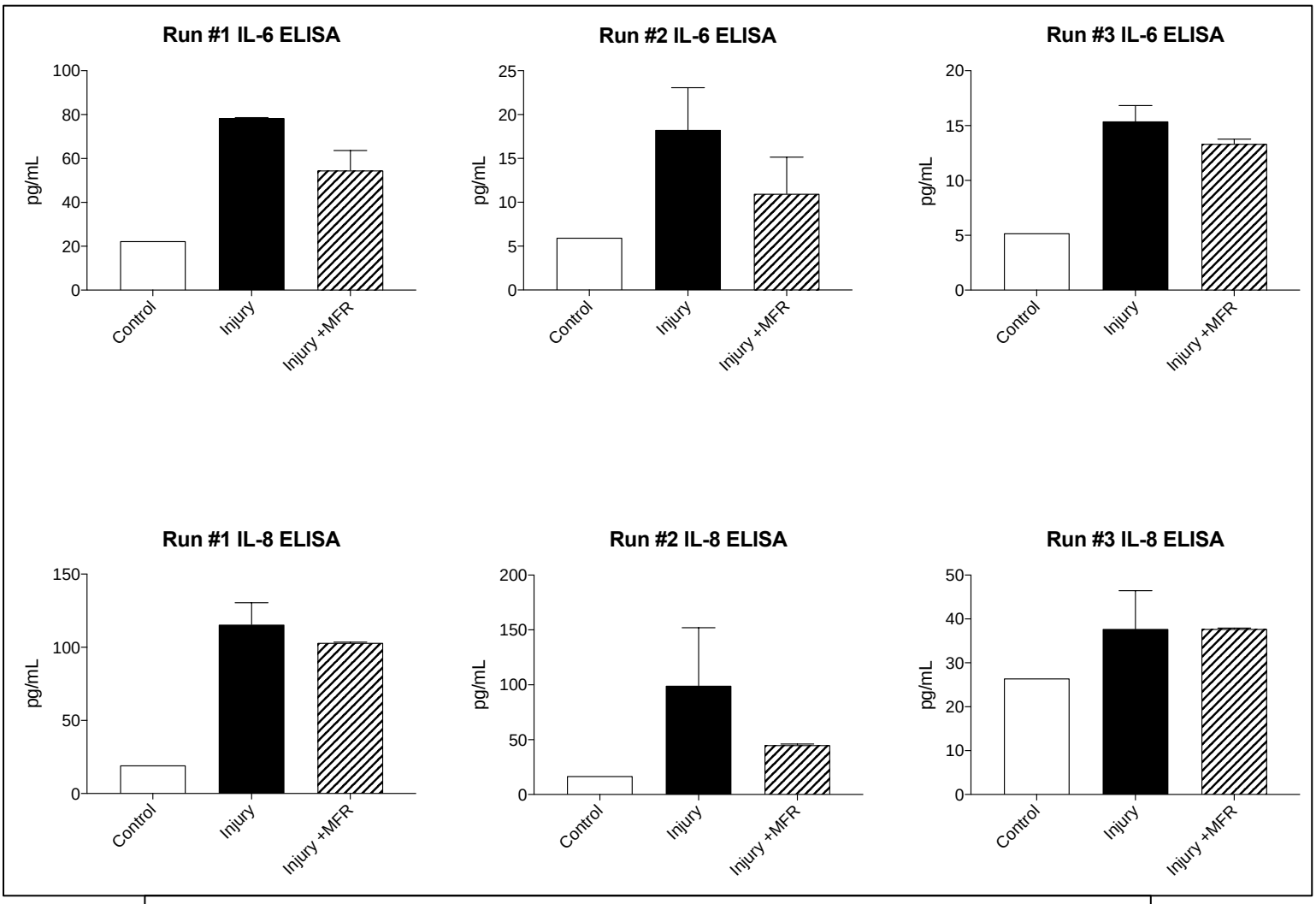

Fig. 3 Graphs displaying the concentrations of IL-6 and IL-8 for each run and strain profile as detected by ELISA. Data are expressed as mean concentrations ± SEM.

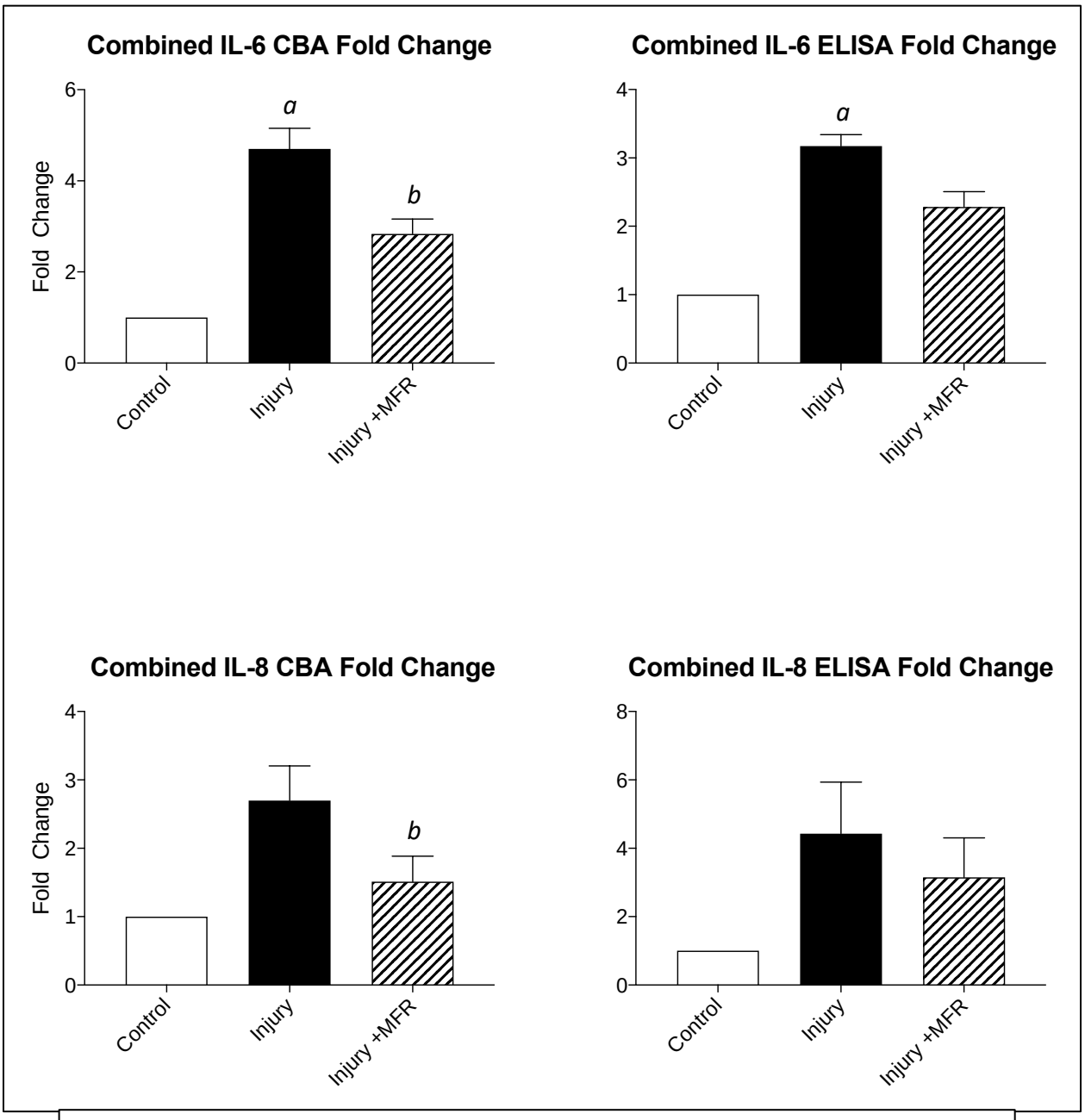

Fig. 4 Graphs displaying the average fold change of each run between each strain profile as compared to the controls for both the Cytokine Bead Array assay and ELISA. Data are expressed as mean fold change ± SEM and analyzed by paired T-tests. $p<0.05$: *a* compares to control; *b* compares to injury.

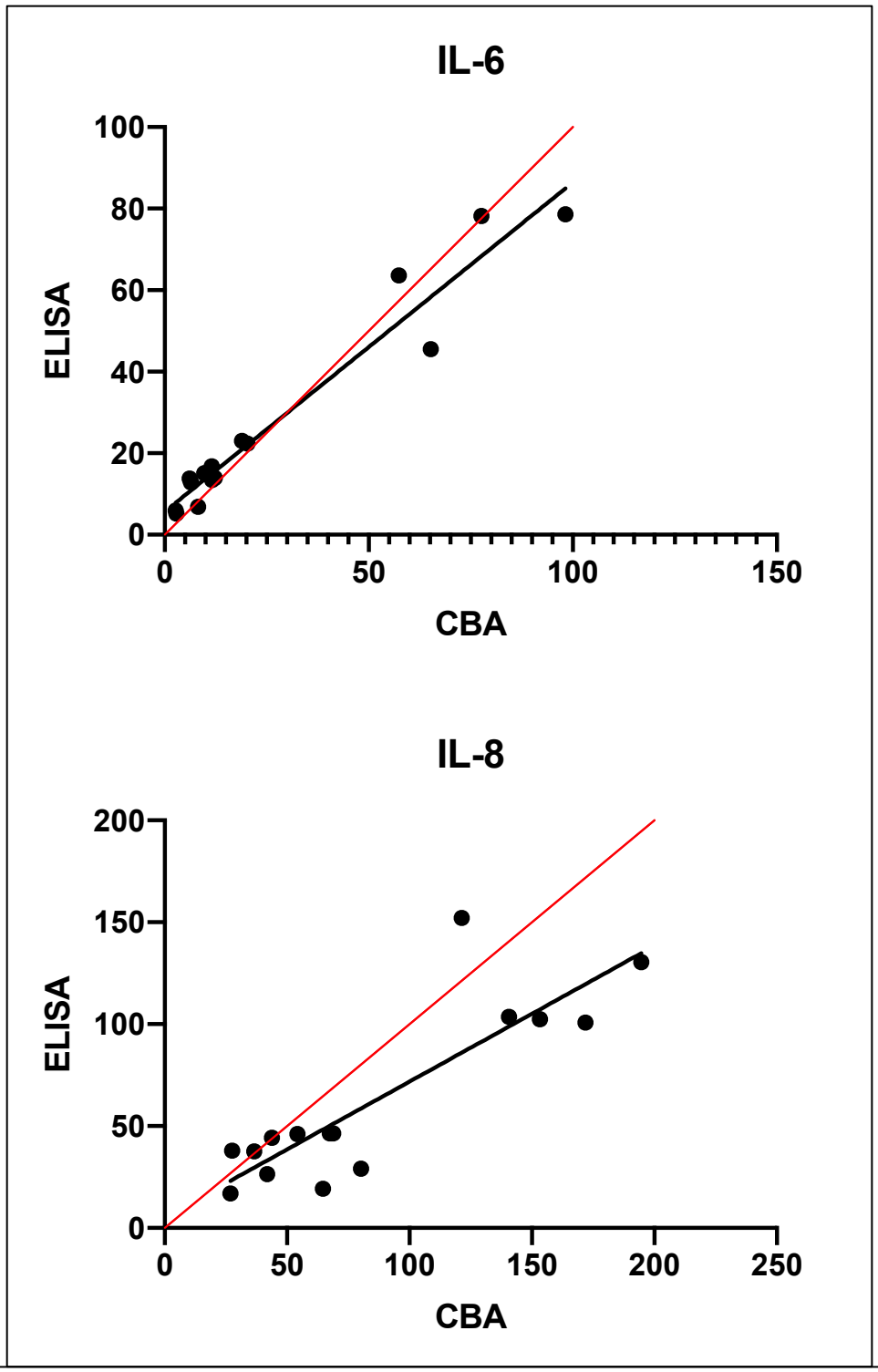

Fig. 5 Graphs displaying the plots of the concentrations (pg/mL) for each sample in pg/mL for the ELISA vs. the CBA. The black line represents the line of best-fit, and the red line represents the line of identity that would signify identical concentrations detected in the ELISA and CBA. The best fit line R^2 for IL-6= 0.945 and R^2 for IL-8= 0.732.