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Validation of oxidative stress assay for schizophrenia

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ABSTRACT

Accumulating evidence implicates oxidative stress in a range of diseases, yet no objective measurement has emerged that characterizes the global nature of oxidative stress. Previously, we reported a measurement that employs the moderately strong oxidant iridium (Ir) to probe the oxidative damage in a serum sample and reported that in a small study ($N = 15$) the Ir-reducing capacity assay could distinguish schizophrenia from healthy control groups based on their levels of oxidative stress. Here, we used a larger sample size to evaluate the Ir-reducing capacity assay to assess its ability to discriminate the schizophrenia ($N = 73$) and healthy control groups ($N = 45$). Each serum sample was measured (in triplicate) at three different times that were separated by several weeks. The Intraclass Correlation Coefficient (ICC = 0.69) for these repeated measurements indicates the assay detects stable components in the sample (i.e., it is not detecting transient reactive species or air-oxidizable serum components). Correlations between the Ir-reducing capacity assay and independently-measured total serum protein levels ($r = +0.74$, $p < 2.2 \times 10^{-16}$) suggest the assay is detecting information in the protein pool. For cross-validation of the discrimination ability, we used machine learning and receiver operating characteristic (ROC) analysis. After adjusting for potential confounders (age and smoking status), an area under the curve (AUC) of ROC curve was calculated to be 0.89 ($p = 9.3 \times 10^{-5}$). In conclusion, this validation indicates the Ir-reducing capacity assay provides a simple global measure of oxidative stress, and further supports the hypothesis that oxidative stress is linked with schizophrenia.

1. Introduction

Oxidative stress is commonly viewed as an imbalance between oxidative and antioxidant activities (Finkel and Holbrook, 2000; Reuter et al., 2010; Sies, 2018; Valko et al., 2007) and is implicated in the development of various diseases including cancer, diabetes, cardiovascular, neurological, and pulmonary diseases (Dalle-Donne et al., 2006; Reuter et al., 2010). Also, emerging evidence suggests oxidative stress, redox dysregulation and inflammation also impairs normal brain development (Leza et al., 2015; Sawa and Sedlak, 2016; Schiavone and Trabace, 2017), and may be linked to the pathophysiology of schizophrenia (Conus et al., 2018; Do et al., 2015, 2009; Hardingham and Do, 2016). In fact, it has been suggested that measurements of oxidative stress and inflammation could be valuable markers for studying schizophrenia (Chan et al., 2015a, 2015b; Davison et al., 2018; Flatow et al., 2013; Fraguas et al., 2017; Lai et al., 2016; Leza et al., 2015; Schiavone and Trabace, 2017; Tomasik et al., 2016; Yang et al., 2013) and potentially serve as targets for antioxidant treatment (Conus et al., 2018; Do et al., 2015, 2009; Hardingham and Do, 2016; Lavoie et al., 2007; O'donnell et al., 2014).

Traditional approaches to identify signatures of oxidative stress focus on molecular biomarker(s) that are typically measured using chemically specific analytical methods (e.g., HPLC, mass spectroscopy and immunoanalysis) (Bloomer and Fisher-Wellman, 2008; Dalle-Donne et al., 2006; Flatow et al., 2013; Frustaci et al., 2012; Gagné, 2014; Ho et al., 2013). Because oxidative stress appears to operate at a systems-level, a wide variety of candidate molecular biomarkers have been considered including the reactive oxidants believed to be responsible for damage (e.g., ROS and RNS) (Bitanihirwe and Woo, 2011; Flatow et al., 2013; Frustaci et al., 2012), the endogenously generated protective antioxidants (e.g., GSH and ascorbic acid) (Conus et al., 2018; Do et al., 2009; Hardingham and Do, 2016; Monin et al., 2014; O'donnell et al., 2014; Reddy et al., 2003; Yao et al., 2006, 1998b, 1998a; Zhang et al., 2012), proteins involved in inflammation and antioxidant protection (e.g., cytokines and defense enzymes) (Dalle-Donne et al., 2006; Fraguas et al., 2017; Jeffries et al., 2018; Khanna et al., 2015; Leza et al., 2015; Schiavone and Trabace, 2017) or the damage associated with oxidative stress (e.g., lipid peroxidation and protein carbonylation)

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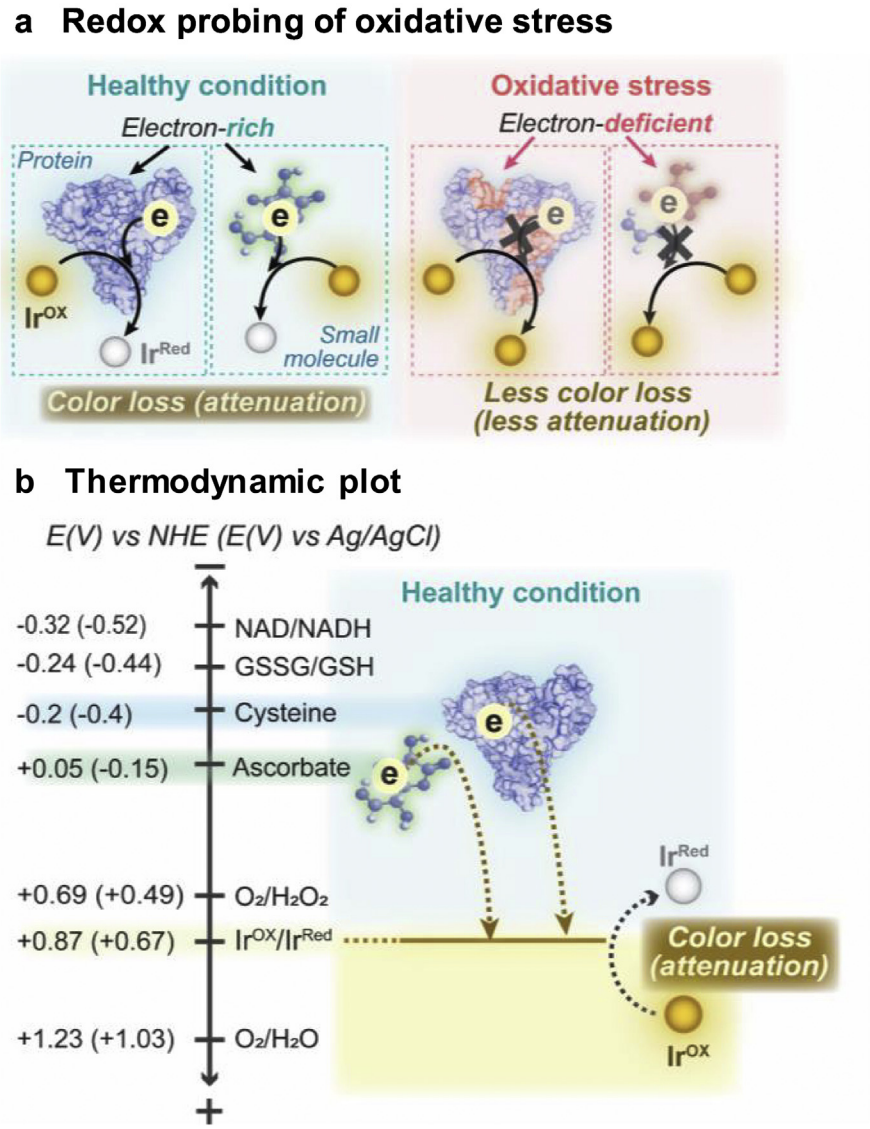
(Berlett, 1997; Davison et al., 2018; Javitt and Kantrowitz, 2009; Koga et al., 2016; Stadtman and Levine, 2003; Valko et al., 2007; Yang et al., 2013). An alternative approach is to generate a single global measurement of oxidative stress (e.g., Total Antioxidant Status) (Deepa et al., 2013; Ghiselli et al., 2000; Huang et al., 2005; Rice-Evans and Miller, 1994; Woodford and Whitehead, 1998). While many of these measurement approaches have shown promising results on small sample sizes, validation on larger sample sizes has been challenging, and no single (or set) of measurements has emerged as an accepted marker of oxidative stress (Dalle-Donne et al., 2006; Frustaci et al., 2012; Ho et al., 2013).

Recently, we reported a new global measurement for oxidative stress that uses the moderately strong oxidant K_2IrCl_6(IV) (designated Ir^{OX}) to probe a sample (e.g., a serum sample) for "historical" chemical information of oxidative stress (Kim et al., 2017). This method is not based on a hypothesis of what specific chemical(s) should be measured, but rather is designed to detect chemical features indicative of an imbalance between oxidative and reductive activities. Specifically, Scheme 1a illustrates that the Ir^{OX} mediator diffuses into the sample where it can accept electrons from a wide range of chemical species and such oxidation-reduction reactions convert the yellow-colored Ir^{OX} to the colorless reduced form of this mediator (Ir^{Red}). Attenuation of the yellow color can be readily detected spectrophotometrically and provides a quantitative measurement of the sample's "Ir-reducing capacity" which is inversely related to oxidative stress. As suggested in Scheme 1b, the Ir-reducing capacity assay is not specific to an individual chemical species but rather measures electron-donating activities from a wide range of physiological reductants (e.g., glutathione, ascorbate and proteins) and thus provides a global measurement (Polidori et al., 2001; Taverna et al., 2013). Importantly, this measurement is especially sensitive to thiols which are likely sites of oxidative damage (e.g., the cysteine residues of proteins) (Berlett, 1997; Dalle-Donne et al., 2006; Davies, 2016; Ho et al., 2013; Kim et al., 2017; Stadtman, 1993; Stadtman and Berlett, 1997; Stadtman and Levine, 2003). In initial studies with a small sample size ($N = 15$), we demonstrated that the Ir-reducing capacity assay detected a significantly higher level of oxidative stress for persons diagnosed with schizophrenia compared to the healthy control group (Kim et al., 2017).



Scheme 1. The redox-mediator (K_2IrCl_6, Ir^{OX}) is used to probe for reducing capacities of biochemical components in serum through the attenuation of optical signal. (a) The redox mediator (Ir^{OX}) accepts electrons from physiological reductants (e.g., protein (albumin) and small molecule (ascorbate)) and then is reduced to Ir^{Red} thus attenuating the Ir^{OX} optical signal. However, the oxidatively damaged reductants can donate less electron to Ir^{OX}, which results in less attenuation of Ir^{OX}. (b) A thermodynamic plot shows that the electron transfer from various reductants to Ir^{OX} is thermodynamically favorable because the redox potential of Ir^{OX} is more oxidative than other biological redox molecules.

Here, we evaluated the Ir-reducing capacity assay using a larger sample size ($N = 73$ for schizophrenia group; $N = 45$ for healthy control group) and employed machine learning methods to cross-validate this assay. Our results validate that the Ir-reducing capacity assay can discriminate the schizophrenia and control groups based on their levels of oxidative stress (AUC = 0.89, $p = 9.3 \times 10^{-5}$). We envision that the Ir-reducing capacity assay could provide a useful global measurement to assist in clarifying the role of oxidative stress in disease. Importantly, the speed (1 h), simplicity and convenience of this assay suggests it could be broadly used by researchers and potentially even clinicians to understand and manage oxidative stress. Finally, it should be possible to enhance the discriminating capabilities of the Ir-reducing capacity assay by coupling this measurement with powerful information processing strategies capable of accelerating the discovery of characteristic signatures of oxidative stress (Kang et al., 2018).

2. Materials and methods

2.1. Chemicals

The following were purchased from Sigma-Aldrich: K_2IrCl_6 (IV), and phosphate buffered saline (PBS, pH 7.4). The water (>18 MΩ) used in this study was obtained from a Super Q water system (Millipore). A stock solution of 10 mM K_2IrCl_6 (Ir^{OX}) was prepared in PBS (pH 7.4).

2.2. Ir-reducing capacity measurement

An Ir-reducing capacity assay was performed to measure level of oxidative stress in serum sample as previously reported (Kim et al., 2017). Before the measurement, an aliquot of serum frozen at −80 °C was thawed in ice bath. In one 96 well microplate, 10 serum samples and 1 reference solution were simultaneously assayed. For the assay of serum sample, serum was 20-fold diluted with 0.1 M PBS and 6 μL of diluted serum was added into a 96 well plate containing 279 μL of 0.1 M PBS. After that, 15 μL of 10 mM K_2IrCl_6 (Ir^{OX}) solution was added to each well (this procedure results in an overall 1000-fold dilution of serum and 0.5 mM of Ir^{OX}). For reference solution, 15 μL of 10 mM K_2IrCl_6 solution was added to each cell containing 285 μL of PBS without serum sample. After 1-h incubation, absorbances of Ir^{OX}-mixed samples and Ir^{OX} (reference) were measured at 490 nm with a standard absorbance microplate reader (iMark, Bio-Rad Laboratories, Inc.). Ir-reducing capacity is estimated by calculating the attenuation ratio of optical signals as described in eq. 1.

$$Ir-reducing\ capacity\ (\%) = \left(1 - \frac{Abs_{(Ir(OX),Sample)}}{Abs_{(Ir(OX),Reference)}}\right) \times 100 \quad (1)$$

Three independent measurements of 118 serum samples were performed over a 45-day period. At each measurement time, each sample was assayed in triplicate, and the triplicate measurements were averaged and converted to the Ir-reducing capacity.

2.3. Study participants

Recruitment of people to participate in a clinical study designed to collect blood samples occurred between May 2015 and August 2016. Blood samples were collected from the Maryland Psychiatric Research Center, University of Maryland School of Medicine. We recruited 2 populations, people with a DSM-IV Diagnosis of Schizophrenia or Schizoaffective Disorder and a population of individuals without a major psychiatric diagnosis. All participants completed data collection procedures in a single 1–2 h study visit. Additionally, participants provided detailed clinical information. Blood samples (45 mL) were collected from individuals who had not been fasting using 6 tubes of BD Vacutainers and centrifuged at 3000 rpm. The resulting supernatant was removed using disposable plastic 1 mL pipettes. It was apportioned into 1 mL aliquots and stored at −80 °C in a freezer before analysis.

The study was approved by the University of Maryland (UMB) Institutional Review Board (IRB) approvals also granted from the State of Maryland Department of Health IRB and approval by University of Maryland College Park as exempt for deidentified blood samples. More detailed information is described in Supplementary material.

2.4. Statistical analysis

Statistical analyses were performed with R version 3.4.3. The continuous variables are reported as mean values and standard errors, and kruskal tests are applied to assess the significance of differences between groups. Categorical variables were reported as number of cases (percentage) and compared using the chi-square test. The intraclass correlation coefficient (ICC) is calculated using the 'irr' package based on an absolute-agreement, one-way random effect analysis of variance (ANOVA) model (Bartko, 1966; Shrout and Fleiss, 1979). Spearman's correlation coefficients are calculated to examine the relationship between our measurements (Ir-reducing capacities) and independent biochemical analyses of serum components. A linear regression model is employed to adjust confounding variables. After the adjustment, we assess the significance of difference of Ir-reducing capacity among groups. We further examine whether Ir-reducing capacity assay data can predict clinical status using machine learning methods (Max Kuhn Contributions form Jed Wing et al., 2008). Generalized Boosted Model (GBM) is used and the model performance is assessed through 5-fold cross validation of the training set (75% of 118 data) and validation with the test set (25% of 118 data). ROC (Receiver Operating Characteristic) metric and corresponding p-values are employed to evaluate the prediction performance for each fold (Robin et al., 2011).

3. Results

Table 1 summarizes the characteristics of the healthy control group (HC, $N = 45$) and the persons with a diagnosis of schizophrenia (SCZ, $N = 73$). The schizophrenia group was further subdivided into those treated with clozapine (CLZ, $N = 49$) and those who were not receiving clozapine treatment at the time of the study (NoCLZ, $N = 24$). Table 1 shows that there is no significant difference between the healthy controls (HC) and schizophrenia groups (SCZ) in the variables of sex and race ($p > 0.05$). Comparisons among the three groups (HC, NoCLZ, CLZ), shows a difference is observed in the race variable ($p < 0.05$), showing that most of the schizophrenia group treated with clozapine were Caucasian and the group not treated with clozapine were mostly African-American. Significant differences between the HC and SCZ groups are observed in the variables of age, smoking and diseases ($p < 0.05$). It suggests that these variables can be considered as confounders and adjusted as covariates in the following analysis.

Since our goal is to develop a simple and reliable assay, we want to measure stable features of oxidative stress that are insensitive to air exposure, sample storage (at −80 °C), or the timing of serum collection, storage and measurement. To investigate the reliability of Ir-reducing capacity assay, we assayed the 118 samples at three different times that spanned 45 days. At each measurement time, a single aliquot for each of the 118 frozen serum samples (−80 °C) was thawed, assayed in triplicate, and the triplicate measurements were averaged and converted to the Ir-reducing capacity using Eq. 1. Each of the 118 data points in Fig. 1 represents the average reducing capacity from one serum sample as measured at three different times using different sample aliquots.

Table 1
Demographic information for healthy controls and schizophrenia group.

Variable	Healthy controls (HC)	Schizophrenia group (N = 73) (SCZ)		Test statistics	
	(N = 45)	NoCLZ (N = 24)	CLZ (N = 49)	HC vs SCZ	Among three groups
Age/years	36.2 ± 13.6	44.6 ± 10.9	42.3 ± 13.4	$\chi^2 = 8.45$, $p = 0.004$[1]	$\chi^2 = 9.12$, $p = 0.010$[1]
Sex/male	25 (56%)	14 (58%)	34 (69%)	$\chi^2 = 0.833$, $p = 0.361$[2]	$\chi^2 = 2.06$, $p = 0.357$[2]
Race				$\chi^2 = 13.3$, $p = 0.101$[2]	$\chi^2 = 29.9$, $p = 0.019$[2]
African American	20 (44%)	18 (75%)	17 (35%)		
Asian	3 (7%)	0 (0%)	0 (0%)		
Causcasian	17 (38%)	4 (17%)	29 (59%)		
Hispanic	2 (4%)	0 (0%)	0 (0%)		
Native American	0 (0%)	1 (4%)	0 (0%)		
Mixed	3 (7%)	1 (4%)	3 (6%)		
Smoking	8 (18%)	17 (71%)	23 (48%)	$\chi^2 = 14.8$, $p < 0.001$[2]	$\chi^2 = 19.8$, $p < 0.001$[2]
Diseases					
Hypertension	3 (7%)	10 (42%)	11 (22%)	$\chi^2 = 7.08$, $p = 0.008$[2]	$\chi^2 = 12.1$, $p = 0.002$[2]
Diabetes	0 (0%)	6 (21%)	9 (18%)	$\chi^2 = 8.04$, $p = 0.005$[2]	$\chi^2 = 9.89$, $p = 0.007$[2]

Tests used: [1] Kruskal test, [2]Chi-squared test.
Abbreviations: N, Number of non-missing values; HC, Healthy controls; SCZ, Overall schizophrenia groups; NoCLZ, Schizophrenia group not treated with clozapine; CLZ, Schizophrenia group treated with clozapine.

To assess the reliability of the Ir-reducing capacity assay, we calculated the intraclass correlation coefficient (ICC) (Bartko et al., 1966; Hallgren, 2012; Shrout and Fleiss, 1979) which assesses the absolute agreement between different acquisitions. The calculated ICC from our results was determined to be 0.69 which indicates a moderate agreement for three replicate measurements. While this ICC value (0.69) is insufficient for a clinical test, it does indicate that this simple assay is reproducible even after prolonged sample storage. Additionally, the Ir-reducing capacity assay is not affected by repeated freezing and defrosting of the samples (Fig. S1 of the Supplementary Materials). Importantly, all these results indicate that the assay is measuring a stable feature in the serum (i.e., it is not measuring unstable reactive species) and it is not sensitive to the presence of air (i.e., no precautions were made to exclude oxygen when drawing the blood, processing the serum or assaying the samples).

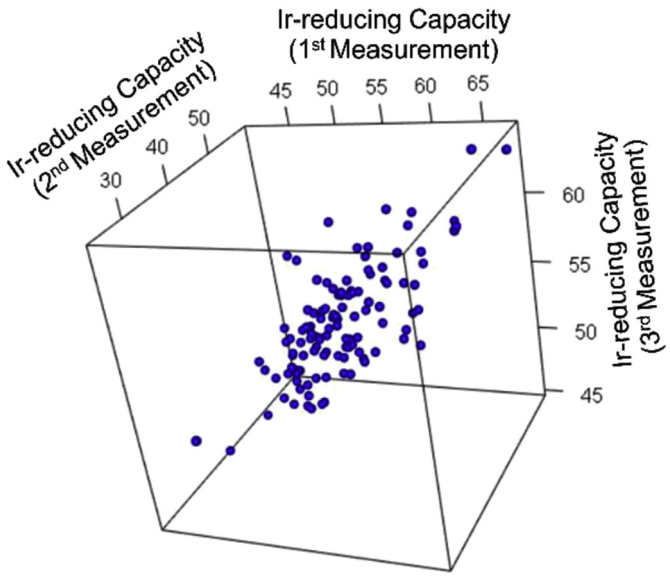


Fig. 1. Ir-Reducing capacity assay is moderately reliable for three repeated measurements. 3D plot of Ir-reducing capacities from three independent measurements of 118 serum samples that were performed over a 45-day period. For each measurement, a −80 °C frozen aliquot from each serum sample was thawed (one aliquot for each of the 118 samples), assayed in triplicate and the average of this triplicate for each sample forms the value for one axis (e.g., 1st Measurement). In separate weeks, measurements of the 118 samples were repeated to generate values for the 2nd and 3rd axes. The consistency among three independent measurements indicates that Ir-reducing assay is reliable and the redox information (i.e., the chemical components) being measured from the serum samples is stable over time.

Scheme 1 indicates that the Ir-reducing capacity assay is probing the serum components that can donate electrons to Ir^{OX}. It would be expected that measurements from the Ir-reducing capacity assay should be associated with serum levels for some of the more abundant reductants. To reveal such associations, we compared our measured Ir-reducing capacities to independently measured levels of several serum biochemicals. Fig. 2a shows the heat map for Spearman's correlation coefficients for the Ir-reducing capacity assay and levels for several serum biochemicals. Fig. 2b shows the levels of 5 serum biochemicals (and one ratio of serum components) are highly correlated with the Ir-reducing capacity ($p < 0.05$). Interestingly, most of these highly correlated biochemicals are serum proteins and Fig. 2c shows the correlation-cross plots between the Ir-reducing capacity and these 4 protein-related serum measurements. The Ir-reducing capacity assay showed a particularly strong positive correlation to total protein measurement ($r = +0.74$, $p < 2.2 \times 10^{-16}$). This correlation suggests to us that the Ir-reducing capacity assay is detecting the oxidative damage to proteins (e.g., oxidation of cysteine and lysine residues) presumably related to ROS related reactions (Stadtman, 1993; Stadtman and Levine, 2003).

The ability of Ir-reducing capacity assay to discriminate the schizophrenia group ($N = 73$) from the healthy controls ($N = 45$) is shown in Fig. 3a. The Ir-reducing capacity measured for the schizophrenia group (50 ± 3.0%) is lower than that for the healthy controls (53 ± 3.6%). This result is similar to that reported previously and indicates higher levels of oxidative stress for the schizophrenia group (Kim et al., 2017). A linear regression model was employed to control the effect of the confounding variables including age, smoking, and diseases and to analyze the discriminating ability of Ir-reducing assay (Lane et al., 2004; Pourhoseingholi et al., 2012; Wicherts et al., 2007). After this adjustment, this analysis shows that the difference in Ir-reducing capacity between the healthy control and schizophrenia groups is statistically significant ($p^* = 9.0 \times 10^{-8}$).

Fig. 3b compares the reducing capacity among the three groups; healthy controls (HC: 52.97 ± 3.58%), the schizophrenia group being treated without clozapine (NoCLZ: 51.44 ± 3.27%), and the schizophrenia group being treated with clozapine (CLZ: 48.76 ± 2.51%). Fig. 3b shows a statistically significant difference between healthy control and schizophrenia group not treated with clozapine (HC vs NoCLZ, $p^* = 0.03$), and between healthy control and schizophrenia group treated with clozapine (HC vs CLZ, $p^* = 3.6 \times 10^{-9}$). Interestingly, the clozapine-treated schizophrenia group (CLZ) showed the lowest Ir-reducing capacity or greatest level of oxidative stress.

In summary, the results in Fig. 3 validate in a larger sample size that the Ir-reducing capacity assay can discern the schizophrenia

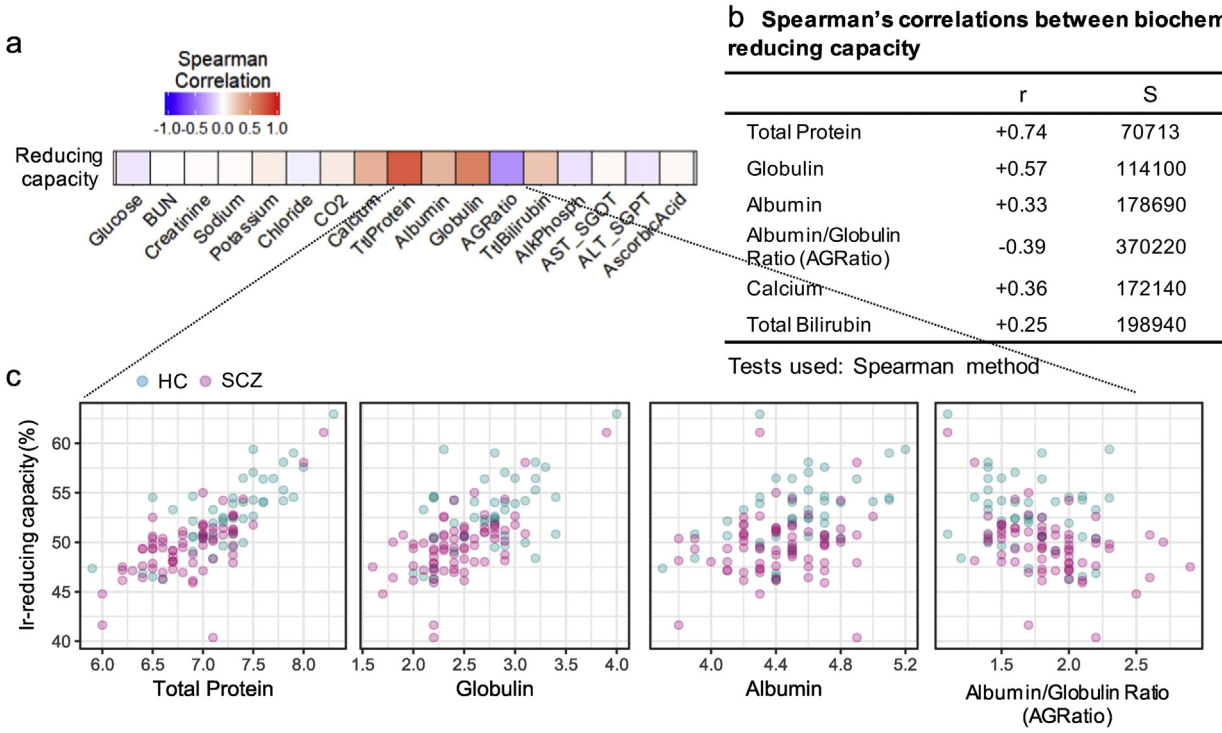


Fig. 2. Ir-Reducing capacity is positively correlated with total protein content in serum. (a) Heat map of Spearman correlation coefficients of Ir-reducing capacity and independent biochemical analyses of serum components. (b) Spearman's correlation coefficients of biochemicals that show statistically significant association between Ir-reducing capacity and serum content ($p < 0.05$). (c) Correlation-cross plots that show statistically significant association between Ir-reducing capacity and protein-related serum measurements.

group from the healthy control group based on their levels of oxidative stress.

To cross-validate the discrimination ability of Ir-reducing capacity assay, we used a machine learning approach (Bradley and Bradley, 1997; Chau et al., 2008; Liu et al., 2016; Schwarz et al., 2010; Yang et al., 2013). For this analysis, the data for 118 samples is split into the training and testing data sets. The model (GBM; Generalized Boosted Model) is 5-fold trained (fitted) on the training data set and the model performance for each fold is evaluated using ROC metric (Fawcett, 2006). In the meanwhile, the model is adjusted by confounders (age and smoking). After that, the optimized model is applied to the test data set.

Fig. 4 shows the ROC curve derived from the test data set after 5-fold cross validation processes. In Fig. 4, the calculated AUC (Area Under the ROC Curve) for Ir-reducing capacity assay was determined to 0.89 (95% confidence interval (CI): 0.78–1; $p = 9.3 \times 10^{-5}$). To find the optimal threshold point, "Yuden index" is employed (Fluss et al., 2005; Ruopp et al., 2008). At this threshold point (0.82), the accuracy (0.83), positive predictive value (PPV = 0.88) and negative predictive value (NPV = 0.75) are calculated and shown in Fig. 4. The ROC curves before the adjustment are shown in Figs. S2 and S3 of the Supplementary material. We note that our machine learning results are exploratory and of uncertain validity in the absence of a replication sample. Nevertheless, the

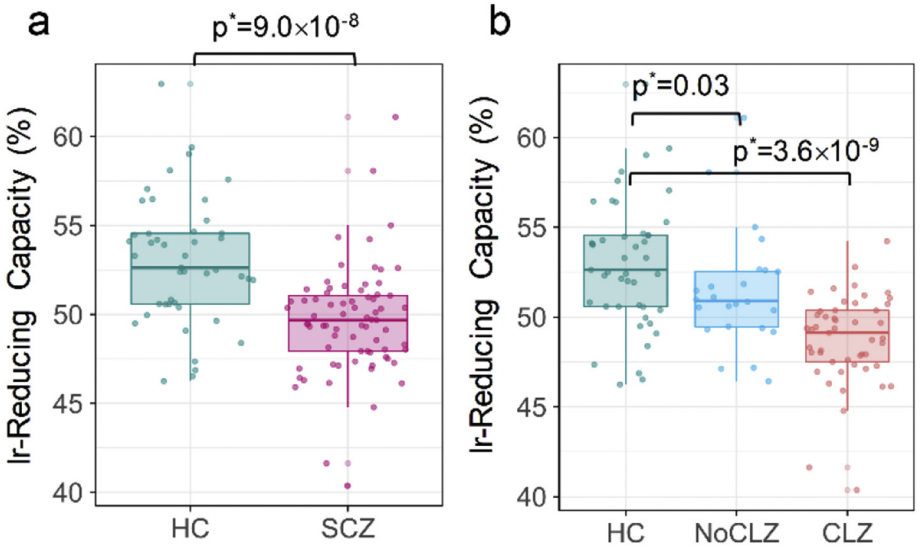


Fig. 3. Ir-Reducing capacity can discern the schizophrenia group from healthy controls. (a) The Ir-reducing capacity of the serum for healthy control ($N = 45$) and schizophrenia groups ($N = 73$). (b) The Ir-reducing capacity of the serum for healthy control (N = 45) and schizophrenia groups treated without clozapine (NoCLZ, $N = 24$) and with clozapine (CLZ, $N = 49$). (*p-value is obtained by applying the linear regression model adjusted with confounding variables (age, smoking and diseases)) (Fox, 2016).

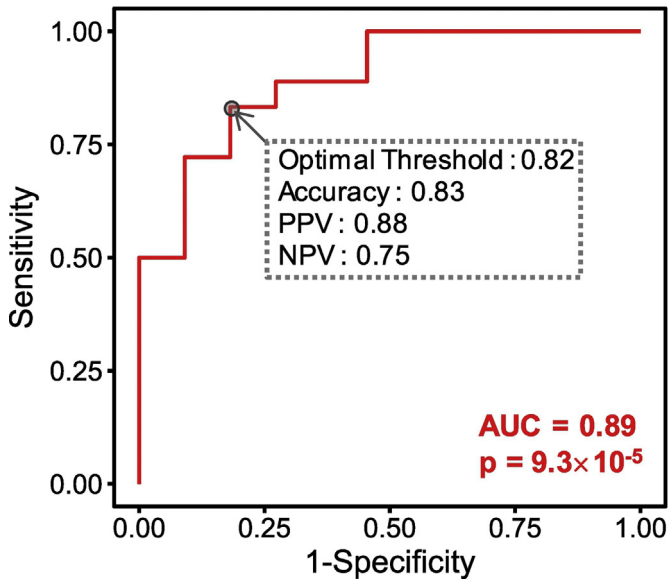


Fig. 4. The receiver operating characteristic (ROC) analyses demonstrates that Ir-reducing assay can discriminate the schizophrenia (SCZ) group from healthy controls (HC). ROC curve was drawn after 5-fold cross-validation. The area under the curve was 0.89 ($p = 9.3 \times 10^{-5}$) in the test set.

results of Fig. 4 demonstrate that Ir-reducing capacity assay possesses the ability to discriminate the schizophrenia from the healthy control groups and support the belief that oxidative stress may be linked to the pathophysiology of schizophrenia.

4. Discussion

Recent research suggests that oxidative stress, redox dysregulation and inflammation are involved in the pathophysiological mechanisms of schizophrenia (Bitanihirwe and Woo, 2011; Do et al., 2009; Fraguas et al., 2017; Koga et al., 2016; Leza et al., 2015; O'donnell et al., 2014; Schiavone and Trabace, 2017). Also, meta-analyses of a wide range of evidence indicates aberrant oxidative stress and inflammation changes occur in schizophrenia patients compared with control groups (Chan et al., 2015a; Chan et al., 2015b; Davison et al., 2018; Flatow et al., 2013; Kapur et al., 2012; Koga et al., 2016; Lai et al., 2016; Mondelli et al., 2015; Schiavone and Trabace, 2017; Schwarz et al., 2010; Tomasik et al., 2016; Watkins and Andrews, 2016; Weickert et al., 2013; Yang et al., 2013; Zhang et al., 2012). Previously, we reported that an Ir-reducing capacity assay is capable of providing a global measurement of oxidative stress, and we showed in a small study ($N = 15$) that this assay could discriminate persons diagnosed with schizophrenia from the healthy control group (Kim et al., 2017). Here, we use a larger sample size ($N = 118$) and validate that the Ir-reducing capacity assay can discriminate the schizophrenia and control groups based on their levels of oxidative stress (Kim et al., 2017). This result is consistent with the growing evidence that oxidative stress is linked to schizophrenia.

Oxidative stress is implicated in various diseases and there has been considerable effort to develop an appropriate measurement of oxidative stress. Because of the pleiotropic nature of oxidative stress, various candidate molecular biomarkers have been considered. Candidate biomarkers include the reactive species believed responsible for oxidative damage (Dalle-Donne et al., 2006; Frustaci et al., 2012; Ho et al., 2013; Valko et al., 2007), species involved in antioxidant protection (Dalle-Donne et al., 2006; Flatow et al., 2013; Ho et al., 2013; Kohen and Nyska, 2002; Maritim et al., 2003; Zhang et al., 2012), oxidatively damaged bioproducts,(Davies, 2016; Finkel and Holbrook, 2000; Kohen and Nyska, 2002; Stadtman, 2006) and markers of inflammatory activities (e.g., cytokines (Cascella et al., 2011; Chauhan and Chauhan, 2006; Davison et al., 2018; Kohen and Nyska, 2002; O'donnell et al., 2014; Tomasik et al., 2016), C-reactive protein (Dickerson et al., 2007; Fan et al., 2007; Fernandes et al., 2016; Singh and Chaudhuri, 2014), tumor necrosis factor α (TNFα) and interleukin 6 (IL-6)) (Fraguas et al., 2017; Leza et al., 2015; Mondelli et al., 2015; Schiavone and Trabace, 2017; Sies, 2018). The Ir-reducing capacity assay is different in that it does not measure a single molecular species but rather probes "chemical space" for global (i.e., systems level) information of oxidative stress. In some ways, the Ir-reducing capacity assay is analogous to the total antioxidant status measurement (Apak et al., 2016, 2004; Ghiselli et al., 2000; Huang et al., 2005), however the Ir-reduction reaction has high sensitivities for thiols (e.g., GSH) (Kim et al., 2017) which are targets of oxidative damage. Results reported here and elsewhere (Kang et al., 2018) suggest the Ir-reducing capacity assay is detecting information from the protein pool that is relevant to oxidative stress (Quinlan et al., 2005; Roche et al., 2008; Taverna et al., 2013). In particular, we show that results from the Ir-reducing capacity assay are correlated with independently-measured total serum protein levels ($r = +0.74$, $p < 2.2 \times 10^{-16}$). This correlation with protein level may be explained because proteins are the most abundant antioxidants in serum and could serve as a measurable pool for the accumulation of oxidative damage (Quinlan et al., 2005; Roche et al., 2008). Importantly, the measurement is stable since no special precautions were needed to exclude oxygen during sample preparation and analysis, and the measurement is unaffected by sample storage (for up to 2 years). Thus, the Ir-reducing capacity assay provides a global historical measure of oxidative stress (vs a molecularly specific measurement of a transient reactive or air-oxidizable species).

One obvious potential limitation is that the Ir-reducing capacity assay was designed to provide a global measure of oxidative stress, and as a result lacks specificity for an individual disease (i.e., this measurement may not be capable of distinguishing differences between the oxidative stress associated with schizophrenia from that associated with other diseases). Another obvious limitation is that the Ir-reducing capacity assay does not provide molecular details that could provide mechanistic insights of either oxidative stress or disease etiology. A further limitation of the Ir-reducing capacity assay is that it is expected to be susceptible to confounding factors associated with oxidative stress (e.g., age, diet, lifestyle, smoking status, and medication). Such confounding factors raise questions of what criteria are required to characterize a control as "healthy". In our statistical analysis, we adjusted for confounding factors to maximize the discerning ability of Ir-reducing capacity assay and observed statistical significance in discriminating ability (AUC = 0.89, $p = 9.3 \times 10^{-5}$). Within these limitations, we envision the Ir-reducing capacity assay could provide a simple objective chemically-based measure of oxidative stress analogous to global physical measurements that characterize health status (e.g., temperature and blood pressure). However, additional improvements and extensive validation would be required before this assay could be used for clinical analysis.

We should note three additional observations from this study. First, we failed to observe a significant correlation between the Ir-reducing capacity assay and symptom severity. In the previous study with a small size ($N = 15$), we observed that the Ir-reducing capacity assay was correlated to the severity of the anxious/depressive symptoms (Kim et al., 2017). This correlation was not observed here when a larger sample size was tested ($N = 118$) [Note: Fig. S4 of Supplementary material shows a very weak, statistically-insignificant correlation between symptom severity and the Ir-reducing capacity assay]. Potentially, the loss of a correlation to symptom severity might be because a larger sample size may have greater variation of participants both within and between groups (Davison et al., 2018). It also may be that the underlying

pathophysiology may not be solely discernible based on overt and sometimes subjectively rated and reported symptoms. The second interesting observation is that the clozapine-treated schizophrenia group showed greater levels of oxidative stress (Fig. 3b). We do not believe this is the result of assay-interference given the low concentrations of clozapine in serum and the 1000-fold dilution of serum for our assay. Possibly, the clozapine medication may contribute to oxidative stress (Bas et al., 2016; Newcomer, 2005; Polydoro et al., 2004; Reinke et al., 2004; Walss-Bass et al., 2008). However, we are unclear if this clozapine effect is based on medication-related or other factors pertinent to the study. For example, we measured Brief Psychiatric Rating Scale (BPRS) scores between the clozapine and non-clozapine groups and find that those treated with clozapine have more severe psychiatric symptoms. In fact, clozapine is a treatment that is reserved for those considered to have treatment-resistant schizophrenia (TRS) and this finding of higher oxidative stress in this group could relate to an illness subgroup or a more severe form of the illness. The results in Fig. 3b appear consistent with results that show that those with TRS may have more oxidative stress based on telomere damage (Yu et al., 2008) and genetic findings (Pinheiro et al., 2017). Nonetheless, future testing to ensure this is not a medication-related effect should be performed. The third interesting observation is that our serum measurement of oxidative stress is correlated to a diagnosis of a schizophrenia which suggests that information of oxidative stress in the Central Nervous System (CNS) can be measured in peripheral blood. This observation seems consistent with reports that blood-brain barrier (BBB) hyperpermeability occurs in schizophrenia associated with the oxidative stress and neuroinflammation (Axelsson et al., 1982; Najjar et al., 2017).

In summary, our results validate that the Ir-reducing capacity assay can discriminate persons diagnosed with schizophrenia from healthy controls based on their levels of oxidative stress. This work supports growing evidence for a link between oxidative stress and schizophrenia and may provide a simple objective measurement tool for researchers and potentially even clinicians. In the longer term, we envision this assay can be extended to an electrochemical format that provides simple real-time measurements and provides data in a convenient electronic format to enable advanced information processing methods to be applied to accelerate oxidative stress biomarker discovery and personalize patient care (Kang et al., 2018; Kim et al., 2017).

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Contributors

EK, DLK, and GFP designed the study and wrote the first draft of the manuscript. EK and ZK performed the assay. CK collected serum samples. EK and SC undertook the statistical analysis. All authors contributed to edit the manuscript and have approved the final manuscript.

Declaration of competing interest

All authors declare no conflicts of interest related to this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.schres.2019.07.057.

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