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Evaluating the Antioxidant Effects of Human Hair Protein Extracts

Hui Ying Lai ^{1,2 #}, Shuai Wang ^{2 #}, Vaishali Singh ^{2 #}, Luong T. H. Nguyen ²,
Kee Woei Ng ^{1,2 *}

¹ *Nanyang Environment & Water Research Institute (Environmental Chemistry and Materials Centre), Interdisciplinary Graduate School, Nanyang Technological University, Singapore*

² *School of Materials Science and Engineering, Nanyang Technological University, Singapore*

[#] *Equal contribution*

^{*} *Corresponding author*

Associate Professor Kee Woei Ng

School of Materials Science and Engineering

Nanyang Technological University

50 Nanyang Avenue, Singapore 639798

Tel: +65 6513 8294

Fax: +65 6790 9081

Email: kwng@ntu.edu.sg

Abstract

The intrinsically high cysteine content in human hair keratins and keratin associated proteins confer hair its outstanding mechanical strength through the formation of strong intermolecular disulphide bonds. In addition, these proteins offer the potential to be exploited as potent antioxidants. This report presents our findings on the antioxidant effects of human hair protein extracts and their consequent protective role against oxidative stress in human dermal fibroblast cultures. Protein extracts were obtained from human hair using sodium sulphide as the reducing agent, and characterized using SDS-PAGE, Western blotting, MALDI-ToF mass spectrometry and amino acid analysis. Cysteine was found to account for 11.2 mole % in the extracted fractions. By measuring DPPH radical scavenging activity, the hair protein fractions were shown to possess significant antioxidant ability ($IC_{50} = 16.22 \mu M$). As a supplement in cell culture media, the extracts protected human dermal fibroblasts from H_2O_2 induced oxidative stress, which was demonstrated by the maintenance of cell viability and reduced ROS production. Besides offering mechanical support as a scaffolding material, the unique antioxidizing ability of human hair protein extracts may also be exploited in biomedical applications.

Keywords: keratin, keratin associated proteins (KAPs), antioxidant, biomaterial, human hair, fibroblast

1. Introduction

Keratin is a class of intermediate filament structural proteins that can be found in the epidermis and epidermal appendages of vertebrates such as wool, nails, feathers, hooves, and hair. Together with keratin associated proteins (KAPs), they confer unique mechanical properties found in the epidermal appendages. As biomaterials, these proteins exhibit tremendous potential as regenerative substrates due to their inherent biocompatibility, biodegradability and bioactivity [1-4]. Keratins and KAPs derived from human hair offer several advantages over its animal counterparts due to its potentially lower immunogenicity and the possibility to personalise autologous material platforms depending on the patient's needs. Abundant and readily available supplies of human hair make this an economical source of human-derived bioactive biomaterials. To this end, keratin-based coatings, films, hydrogels, sponges, and fibres have been explored in various tissue engineering and regenerative medicine applications [5-10].

With increasing interests in human hair extracts as functional materials, other intrinsic properties and potential applications are being studied. Various studies on chicken feather keratin hydrolysates have demonstrated antioxidant and antibacterial activities [11, 12]. A recent work conducted by Wan *et al.* identified a novel antioxidant peptide from chicken feather keratin which exhibited free radical scavenging and Fe^{2+} chelation properties [13]. However, there have been no reports about the antioxidant activity of keratin extracts obtained from human hair thus far. The antioxidizing capability of a protein is usually derived from rich amounts of reductive or free radical scavenging amino acid side chains [14]. Amino acid analysis of extracted human hair proteins revealed that these generally contain 9-10% cysteine residues, which are the highest among known human proteins [5, 15]. When extraction is done using a reductive method, the oxidized dimers of cysteines are reduced to

their monomers containing active free thiol (-SH) groups [8, 16, 17], conferring keratin monomers and KAPs antioxidizing potential. Although the cysteine amount (14 mole %) present in chicken feather is similar to that in human hair, the keratin composition of chicken feather mainly consists of low molecular weight fractions of around 20 kDa [18]. In addition, feather keratin is reported to contain more beta-keratin, which is similar to the hard keratin which can only be found in the cuticle of human hair. Due to such differences, the antioxidant capacity of human hair extracted keratins is therefore worthy of exploration.

When tissue injuries occur, reactive oxygen species (ROS) produced in mitochondria are released into surrounding tissues. The ROS participate in the modulation of inflammatory response and elimination of micro-organisms and neoplastic cells; however, excessive ROS curb normal cell functions by damaging cellular proteins, lipids, DNA, and carbohydrates [14, 19-21], which will lead to adverse effects such as mutagenesis or even carcinogenesis [22-24]. In this study, we extracted total proteins from human hair using a reducing agent and investigated their antioxidizing potential by evaluating free radical scavenging activity and protective effects on human dermal fibroblasts (HDFs) against external oxidative stress. To the best of our knowledge, this is the first study to evaluate the antioxidizing activity of human hair extracts using a cell culture model with the intention to further explore this phenomenon for high end biomedical applications.

2. Material and Methods

Materials

Hydrogen peroxide (H₂O₂), sodium sulphide (Na₂S), N-Ethylmaleimide (NEM) and dialysis tubings (molecular weight cut-off 12,400 Da) were purchased from Sigma Aldrich. Dulbecco's modified Eagle's medium (DMEM), L-glutamine, sodium pyruvate, 0.25%

trypsin, PicoGreen DNA quantification kit, antibody-antimycotic mixture, secondary goat anti-mouse horseradish-peroxidase conjugated (HRP) antibody, CM-H₂DCFDA, SimplyBlue SafeStain solution, precast 4-12% Bis-TRIS NuPAGE gels, lithium dodecyl sulphate sample buffer, sample reducing agent, MOPS SDS running buffer, NuPAGE antioxidant, and iBlot gel transfer stacks were purchased from Life Technologies. Primary mouse polyclonal antibody against total human hair keratins (clone AE13, #ab16113) was obtained from Abcam. The Super Signal West Pico chemiluminescent substrate was purchased from Pierce.

Protein Extraction

Total proteins from human hair were extracted using a previously described method [8, 17]. In short, random hair samples collected from local hair salons were first washed with soap, followed by 70% ethanol, rinsed extensively with deionized (DI) water, and subsequently air-dried. The cleaned hair was delipidized by being soaked in a mixture of chloroform and methanol (2:1 v/v) for 24 hr. The delipidized hair was then air-dried and cut into 1-cm-long fragments for protein extraction. Delipidized hair fragments (50 g) were immersed in 1 L of 0.125 M Na₂S solution and incubated at 40 °C for 4 hr. The resulting mixture was filtered and exhaustively dialyzed against 2 L of DI water using cellulose tubing. The dialysis step was repeated six times. To block free thiol groups, 100 mM NEM was mixed with the protein extracts in a ratio of 1:10. The mixture was shaken for 4 hr at room temperature for the reaction to occur. Subsequently, the NEM was removed by dialyzing the solution against DI water using a membrane with a MWCO of 10 kDa.

SDS-PAGE

Samples were prepared by mixing 10 µl protein extract solutions (1 mg/ml) with 5 µl LDS loading buffer and 2 µl sample reducing agent. The volumes of each sample mixture were then adjusted to 22 µl by adding DI water. Proteins were denatured by heating the mixture at 90 °C for 15 min, followed by loading 20 µl of each sample into each well of 10-well 4-12% Bis-Tris NuPAGE Novex gels. Electrophoretic separation was performed at a constant voltage of 120 V for 90 min. After staining with Coomassie blue solution for 60 min at room temperature, the gels were washed overnight in DI water and imaged using a gel documentation system (ImageQuant LAS 4000 mini).

Western Blotting

Procedures of protein electrophoresis were carried out as described above, with the exception of protein amounts loaded into each well which was decreased to 1 µg for Western blotting. After electrophoresis, the proteins were transferred onto a nitrocellulose membrane using the iBlot Dry Blotting System at 20 V for 6 min. The membrane was then kept in blocking buffer, made up of 5% non-fat milk dissolved in PBS containing 0.05% Tween-20 (PBST), under constant shaking for 30 min. Incubation with primary antibodies against total human hair keratin (Abcam; 1:1000 diluted in blocking buffer) was done for 1 hr at room temperature. Unbound primary antibodies were rinsed off with PBST, and the membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1:4000 in blocking buffer) for 30 min. Chemiluminescence signals were developed using the Super Signal West Pico chemiluminescence substrate and visualized using a luminescent image analyzer (ImageQuant LAS4000 mini).

Amino Acid Analysis

Amino acid analysis of the freeze-dried protein samples was conducted using the AccQTag Ultra chemistry kit on a Waters Acquity UPLC (First BASE Laboratories Sdn Bhd) to obtain amino acid compositions.

MALDI-ToF Analysis

Mass spectra were collected using the linear mode of AXIMA Performance (Shimadzu) MALDI-ToF mass spectrometer equipped with an N₂ laser (337 nm) at a laser power of 130 W. Protein samples were mixed with 3,5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid) matrix at volumetric ratios of 1:1 on a stainless-steel target plate and dried thoroughly at room temperature before analysis. Representative spectra were obtained by averaging 200 spectra within the mass range of 3,500-16,000 Da, and presented in smooth gauss mode of 200.

Free Radical Scavenging Activity

The free radical scavenging activities of ascorbic acid, L-cysteine, and hair proteins were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH), as described by Blois, with modifications [25]. Briefly, 100 µl of serial dilutions of each sample and 100 µl of water were added into a 96-well plate. These were first measured for background absorbance at 517 nm using a microplate reader (INFINITE F200, TECAN) before being mixed with 100 µl of freshly prepared 200 µM DPPH in a buffered solution of 60% v/v methanol and 40% v/v pH 5.5 acetate buffer. The resulting mixture was protected from light and incubated at room temperature for 30 min. The absorbance of the samples was again measured at 517 nm using

the microplate reader and the percentage of scavenging activity was calculated using Equation 1.

$$\text{Scavenging activity percentage (\%)} = \frac{1 - (A_{\text{sample}} - A_{\text{sample background}})}{A_{\text{control}} - A_{\text{control background}}} \times 100 \quad (1)$$

where,

A_{sample} = Absorbance of samples mixed with DPPH at 517 nm

$A_{\text{sample background}}$ = Absorbance of samples only at 517 nm

A_{control} = Absorbance of water mixed with DPPH at 517 nm

$A_{\text{control background}}$ = Absorbance of water only at 517 nm

Results obtained were mean values from two independent experiments.

Cell Culture

For maintenance, HDFs were cultured in high glucose DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS). Cells were incubated at 37 °C with 5% CO₂ and were subcultured at a ratio of 1:4 every two days using 0.25% trypsin. Prior to seeding, growing phase HDFs were harvested by trypsinization, and counted using a hemocytometer.

Oxidative Stress Induction and Protection

To evaluate the protective role of the hair proteins against oxidative stress, cells were treated with H₂O₂, in the absence of FBS, and analysed with PicoGreen and ROS assays as described. After seeding at a density of 30,000 cells/cm² in 24-well plates for one day, cells were exposed to freshly prepared cell culture media containing 0/10/100/500 µM H₂O₂ with either

hair proteins (1 mg/ml), blocked hair proteins (1 mg/ml), or only DMEM (negative control). 24 hr after H₂O₂ treatment, cell numbers in each group were determined by quantifying their double strand DNA (dsDNA) using the PicoGreen assay. Briefly, 100 µl of 0.25 % trypsin was added into each well. After 10 min incubation at 37 °C, all the cells were detached from the 24-well plate, before each well was added with 400 µl cell lysis buffer (0.005% SDS) and incubated at room temperature for at least 4 hr. After that, 100 µl of dsDNA standards or cell lysate samples was mixed with 100 µl PicoGreen working solution in a 96-well plate. Florescent readings were measured at excitation and emission wavelengths of 480 nm and 520 nm, using a microplate reader. Relative dsDNA amount was calculated based on the dsDNA calibration and directly correlated to cell numbers with the usage of a standard curve.

For the ROS assay, cells were seeded at a density of 5,000 cells/cm² in a 96-well plate. After seeding for one day, cells were treated with different media as mentioned above. After 2 hr incubation, the cells were washed with PBS and loaded with 10 µM CM-H₂DCFDA dye for 45 min. Thereafter, samples were rinsed again in PBS to remove excess dye, before fluorescence readings were measured at excitation and emission wavelengths of 492 nm and 527 nm, respectively, using a microplate reader. The ROS readings were normalized against cell numbers obtained by quantifying dsDNA using the PicoGreen assay at 2 hr post-treatment.

In this experiment, cell densities and incubation times for both assays were determined based on results of trial experiments (data not shown). Cells were seeded at a very high density (30,000 cells/cm²) for cell proliferation tests in anticipation of high cell death over a long period of time (24 hr), which therefore helped to prove the protective effect of human hair

proteins. On the other hand, ROS measurement was aimed to investigate the stress level experienced by viable cells, thus a shorter incubation time of 2 hr was adopted, during which cell numbers did not show statistically significant differences as presented in Figure S2 ($p > 0.05$). Within this short period of time, a lower cell seeding density (5,000 cells/cm²) was sufficient.

Statistical Analysis

Data were presented as mean $\pm$ standard deviation (SD). Statistical analysis was performed using Analysis of variance (ANOVA) and Student's t-test. Differences were considered statistically significant when $p < 0.05$.

3. Results and Discussion

3.1 Human Hair Extraction

The yield of keratin extraction using our protocol was approximately 20 wt%, based on calculation of the weight percentage of the resulting dry keratin powder compared to the starting weight of hair used for extraction. The morphologies of human hair before and after extraction were analysed using Scanning Electron Microscopy (SEM; Supplementary Material, Figure S1). Before extraction, the SEM image of human hair cross sections showed a fully-filled cortex, while surfaces were covered with smooth and well defined cuticle scales. After extraction, the hair cortex appeared to be deformed and collapsed, while the hair cuticle remained relatively intact. This contradiction is due to a higher cysteine content in hair cuticle as compared to hair cortex [26]. During the extraction process, proteins with lower cysteine content are likely to be extracted more efficiently. Although comparison of amino acids

compositions between the different hair components was not carried out in this study, cysteine content in hair extracts may vary according to the extraction cocktail [27-29].

3.2 Identification of keratins and KAPs

The contents of keratins and KAPs in human hair protein extracts were reported to be approximately 50% and 10%, respectively [30]. Coomassie blue stained SDS PAGE gel and Western blot images (Figure 1A) of our hair extracts showed the presence of acidic keratin monomers (~45 kDa), basic keratin monomers (~50 kDa), as well as keratin heterodimers (80-100kDa). Existence of multiple keratin subtypes [31, 32] was suggested by two strong bands in the acidic keratin region. Within the keratin dimer region (80-100 kDa), multiple bands indicating different heterodimer combinations were observed. Several bands representing the relatively low KAP content in the region below 20 kDa were also visible. Polyclonal mouse antibody (clone AE13) that recognizes total human hair keratins was used to identify the keratins in Western blotting. Specificity of the AE13 primary anti-keratin antibody was obtained from the data sheet specification from the supplier, which described the antibody to have higher affinity to acidic (Type I) keratins compared to basic (Type II) keratins. Our results concurred with this description. Besides that, the small molecular weight fraction proteins were recognized by the antibody, which were attributed to the presence of KAPs, and this was also consistent with the antibody supplier's indications. The presence of the KAP fraction, which was only visible in the Western blot due to non-specific binding of the AE13 antibody, but did not show up in the Coomassie blue stained gel, was further confirmed using MALDI-ToF mass spectrometry (Figure 1B). Collectively, it was concluded that the major components of human hair protein extracts were keratins and KAPs. In the process of functionalizing keratin-derived materials, the importance of KAPs has been

underestimated. It is still unclear how KAPs can affect the functional properties of keratin-based templates. Aligning with our hypothesis that high cysteine content of human hair protein extracts possesses potential antioxidant properties, KAPs, which are known to be richer in cysteine content as compared to keratin proteins [33-35], could play a vital role in the observed antioxidant activity even though their content by mass was relatively low in the extracted fractions.

Figure 1.

3.3 Amino Acid Analysis

Amino acid analysis of freeze-dried extracted hair proteins showed 11.2 mole % cysteine content (Table 1). Other major amino acids include glutamine/ glutamic acid (13.6 mole %) and serine (11 mole %), which was consistent with previously reported results using different extraction methods [30]. The slight deviations may be due to differences in the extraction cocktail and natural diversity in human hair samples. As using individuals' hair samples could result in the amplification of individual differences in amino acid content and therefore skew results, we chose to use randomized hair samples in this study to normalize such differences. While they may not be the only contributors, cysteines, which contain the proton donor -SH group, may contribute to the antioxidant activity in hair protein extracts. Additionally, amino acids with aromatic (Trp, Tyr, Phe) or nucleophilic sulphur (Met) side chains are potentially oxidizable by easily abstracting hydrogen radicals or via other mechanisms such as antioxidant enzymes and transition metal binding complexes [14], further suggesting human hair protein extracts as potential antioxidant biomaterials.

Table 1. Amino acids composition (mol %) of human hair extracts

Amino acid	Present study	Literature [25]
	Mol %	Mol %
Alanine	4.7	6.2
Arginine	7.7	7.5
Aspartic acid/ Asparagine	6.3	7.9
Cysteine	11.2	18.6
Glutamic acid/Glutamine	13.6	15.8
Glycine	5.8	5.4
Histidine	0.9	0.9
Isoleucine	3	3.6
Leucine	7	9.2
Lysine	2.4	2.4
Methionine	0.5	0.6
Phenylalanine	1.8	2.0
Proline	8	5.4
Serine	11	9.3
Threonine	7.6	5.9
Tryptophan	0.5	0
Tyrosine	2.1	2.8
Valine	5.9	6.1

3.4 Antioxidant Potential of Extracted Human Hair Proteins

Scavenging of DPPH free radical is a sensitive antioxidant assay that has been widely used to survey the antioxidant activity of materials. The reaction of a material with DPPH radicals results in the conversion of the purple free radicals into yellow residues, which is quantified by measuring absorbance at 517 nm. In this study, it was hypothesized that the cysteine content within the human hair protein extracts could contribute to antioxidant properties due to their ability to release hydrogen atoms and form disulphide bonds. With this hypothesis, the DPPH assay fits the criterion in testing our sample. A previous study on antioxidant properties of chicken feather keratin extracts also utilized DPPH as the characterization assay [13]. Therefore, the DPPH assay was chosen. The capacity of human hair proteins to scavenge DPPH was demonstrated, as shown in Figure 2. The mean IC_{50} values ($n=2$) of ascorbic acid and L-cysteine standards were calculated as 8.67 μM and 8.38 μM , respectively, while that of the hair proteins was 16.22 μM , eluding to the potential of the extracted hair proteins as free radical scavengers capable of reacting with DPPH radicals, albeit being less potent than the controls used in this study. In comparison, Kouoh *et al.* measured an IC_{50} value of 97.74 μM for the antioxidant effects of bovine serum albumin (BSA) against H_2O_2 -induced ROS production in human neutrophil granulocytes [36], suggesting the potential superiority of our human hair keratin extracts as stronger antioxidants in mitigating H_2O_2 oxidative activity. Unfortunately, due to the incompatibility of NEM-blocked proteins with the DPPH assay, the IC_{50} value for this particular sample was not obtainable.

Figure 2.

The antioxidant potential of keratin extracts was further examined by culturing HDFs exposed to a broad range of H₂O₂ concentrations from 0-500 μ M in media containing hair proteins, as presented in Figure 3. The concentration of human hair proteins used in the cellular experiments was 1 mg/ml, or 20 μ M. This concentration was obtained based on the results of early trial experiments (data not shown) and it was in the same order of magnitude as the mean IC₅₀ value of human hair proteins measured by DPPH assay (16.22 μ M). When H₂O₂ concentrations were increased from 0 to 500 μ M, the number of HDFs cultured in hair proteins supplemented media did not change significantly ($p > 0.05$, Figure 3A) after 24 hr incubation. However, untreated cells (controls) and cells treated with blocked hair proteins recorded significant cell death with increasing H₂O₂ concentration up to 500 μ M ($p < 0.05$), thereby implying the positive protective effects from the hair proteins in solution, which was likely due to the antioxidizing influence of the free thiol groups. Normalized ROS generation measured against cell number quantified by dsDNA using PicoGreen Assay was shown in Figure 3B.

Figure 3.

The ability of human hair proteins to reduce ROS levels in HDFs exposed to H₂O₂, in comparison with the untreated (control) and blocked hair protein treated cultures, was clearly shown at 100 μM of H₂O₂ ($p < 0.05$). This positive effect was less obvious at other concentrations of H₂O₂ tested. At H₂O₂ concentration of 500 μM, both cell numbers and ROS intensities of the untreated and human hair protein treated cells were comparable after 2 hr incubation (Figure S2 and Figure 3B). However, after 24 hr incubation at this H₂O₂ concentration, there was a significant level of cell death in the untreated sample compared to the hair protein treated sample (Figure 3A). As such, the untreated HDFs in this aggressive environment were likely to be dying or extremely unhealthy after 2 hr incubation, which could lead to false interpretation of the low ROS intensity of the untreated cells at 500 μM

H₂O₂. Future explorations into other cell physiological processes could be done to further clarify the potential antioxidizing role of hair proteins.

The most distinct characteristic of human hair protein extracts is their high cysteine content, which makes keratin and KAPs similar to small sulfhydryl containing molecules such as glutathione (GSH) and thioredoxin (TRX). These molecules have high cysteine contents and can be reversibly reduced or oxidized depending on the cellular environment [37-39]. Further studies are encouraged to quantify free-thiol groups in hair protein fractions and hair protein based material platforms obtained through different methods of extraction [8, 40-43], purification [30, 44, 45], and fabrication [1, 2, 6, 7, 17]. Subsequently, correlations between the amount of free-thiol groups and material platforms derived from hair proteins should be established and exploited. Recent studies have reported that coagulant activity which mediates healing process can be enhanced when dithiols were oxidized into disulphides [46, 47].

The current study emphasized the plausibility of human hair protein extracts as antioxidants, which has not been specifically evaluated previously. Studies to evaluate the feasibility of human hair proteins as biomaterials have been reported extensively by us and others, as described in the Introduction section. Our current findings therefore complement these previous functional studies of hair proteins, suggesting the potential of this material for further exploration.

4. Conclusion

Total proteins were extracted from human hair in reducing conditions. Coomassie blue staining, Western blotting and MALDI-ToF mass spectrometry analysis revealed the presence of acidic keratin monomers, basic keratin monomers, keratin heterodimers, and

KAPs in the extracts. Importantly, 11.2 mole % of cysteine was found in the extracts, which was suggested to have contributed significantly to the antioxidant activity of the hair proteins. These extracted protein fractions from human hair were proven to possess radical scavenging antioxidant ability using the DPPH scavenging activity assay. Moreover, when 1 mg/ml of hair extract was supplemented into the cell culture media, HDFs maintained their viability in the presence of different H₂O₂ concentrations (0-500 μ M). At 100 μ M H₂O₂, ROS intensity was significantly reduced in the hair proteins supplemented group compared to the blocked hair proteins supplemented group and untreated negative controls. The results of this study suggest the potential of using human hair extracted proteins as a source of biomaterial that is capable of effecting bioactive influences through its antioxidizing capacity.

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FIGURE CAPTIONS

Figure 1. Characterization of extracted human hair keratins. (A) Electrophoresis of total hair extract stained with Coomassie blue (left) and immunoblotted with a broad-spectrum antibody against hair cortex proteins for keratins (right), and (B) mass distribution of KAPs measured by MALDI-ToF mass spectrometry.

Figure 2. DPPH radical scavenging activity of A) hair proteins, B) ascorbic acid and C) L-cysteine standard. The IC₅₀ values of these samples calculated from Boltzmann model were 16.22, 8.67, and 8.38 μ M respectively. Calculation details can be found in Supplementary Material.

Figure 3. Relative cell numbers (A) and normalized ROS activity (B) of untreated HDF (negative control) and HDF cultured in hair proteins and blocked hair proteins supplemented media, in relation to increasing H₂O₂ concentrations. Results are reported as means $\pm$ SD (n=6). $p_n < 0.05$ ($p_1=5.46 \times 10^{-7}$, $p_2=5.50 \times 10^{-7}$, $p_3=5.94 \times 10^{-7}$, $p_4=1.47 \times 10^{-2}$, $p_5=2.32 \times 10^{-2}$, $p_6=9.18 \times 10^{-3}$, $p_7=6.59 \times 10^{-4}$, $p_8=7.6 \times 10^{-4}$)