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Structural Properties of Human Chorionic Gonadotropin (hCG) Affected by Ultrasonic Irradiation: An *in Vitro* Study

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Human chorionic gonadotropin (hCG) is a glycoprotein hormone that is an essential biomarker in oncology and pregnancy. The objective of this research was to examine the effect of ultrasonic irradiation (40 kHz) in various times of exposure (10 to 60 min) on the structure of hCG. The UV-Vis and near-UV CD data illustrated that ultrasonic irradiation could induce alterations in the tertiary structure of hCG and these conformational variations were irreversible. The ultrasonic-induced variations were observed in the intrinsic fluorescence emission. Furthermore, after long periods of exposure, ANS affinity to hCG increased considerably. A transition to the random coil was observed in far-UV CD data. Ultrasonic irradiation could increase the negative surface charge on hCG. The effect of ultrasonic time revealed an initial increment and an eventual reduction in hCG size. After 60 min exposure, some new bands were observed at the SDS-PAGE profile of hCG. Overall, our *in vitro* experiments demonstrated that the sensitive balance between various noncovalent interactions in the structure of hCG could be easily disrupted after ultrasonic treatments. Results from this study are useful to achieve a better understanding of the physicochemical effects of ultrasonic irradiation on proteins. Besides, can help to determine safe limits for people particularly pregnant women.

Highlights: Our *in vitro* experiments showed that ultrasonic treatments of hCG result in time-dependent structural variations. Results from this study are useful to achieve a better understanding of the physicochemical effects of ultrasonic irradiation on peptide hormones. Besides, the findings can help to determine safe limits for people particularly pregnant women.

Keywords: Human chorionic gonadotropin (hCG), Tryptophan-free protein, Ultrasonic irradiation, β-Structure protein, SDS-PAGE profile

INTRODUCTION

Ultrasound, a mechanical energy form, is acoustic radiation at frequencies beyond the human hearing limit and can be transmitted into the human body. The impact of ultrasound is associated with heating, turbulence, shear stresses, dynamic agitation, and cavitation. The ultrasound application, at frequencies between 20 and 1 GHz, causes physical and chemical variations in a viscous medium through the collapse of cavities and cyclic generation [1-3]. Inevitably, the ultrasonic radiation interaction with living material disturbs the structure and features of biomolecules in a way that generally depends on the power and duration of exposure. The impacts of ultrasound can be divided into non-thermal and thermal mechanisms. However, the non-thermal biological impacts of ultrasonic radiation have still puzzled researchers. Despite several applications of ultrasound in the food industry [4,5], medical field [6], and pharmaceutical field [7,8], the details of ultrasound-induced disruption in protein structure remain unidentified. Several investigations on soluble proteins, such as whey protein [9], bovine serum albumin (BSA) [10], and integral membrane protein [3], have been performed. In all cases, sonication could denature proteins, and proteins' secondary and

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Fig. 1. The crystal structure of hCG, obtained from Protein Data Bank ID code 1HRP. Helix (Pink); β-strand (Yellow); N-acetyl-D-glucosamine (NAG) molecule (blue).

tertiary structures were altered.

On the other hand, numerous recent investigates have shown that the public is unknowingly subjected to very high-frequency sound (11.2-17.8 kHz) and ultrasound (>17.8 kHz) signals in the air in public places [11]. Recently, there has been an increment in the number of systems that operate in very high frequency/ultrasound signals in public spaces. Hence, ultrasonic pollution is one of the important problems in the world and lots of people are at risk of being affected by ultrasonic pollution. There is sufficient evidence that noise can induce ischemic heart disease, sleep disturbance, and hearing impairment [12]. Recently, Daiber *et al.* demonstrated that environmental noise can induce oxidative stress and cardiovascular disease [13]. Noise exposure can increase the production of free radicals and the levels of malondialdehyde and catalase [14]. Noise pollution can also alter the DNA structure [15]. More importantly, a high level of noise pollution can act as a common source of stress on pregnant women making a diversity of psychological and physiological variations [12].

Bendokiene *et al.* demonstrated an association between hypertension amongst reproductive-aged women and the residential road traffic noise [16].

Human chorionic gonadotropin (hCG) is a peptide hormone and the most acidic protein in humans that belongs to the gonadotropin hormone family [17]. This heterodimer protein has 237 amino acids (Fig. 1). Some of the variants of this placental hormone are highly sialylated glycoproteins [18,19]. hCG has the longest circulating half-life in human blood (about 36 h). This glycoprotein consists of two noncovalently joined subunits (α and β) [20]. The α-subunit has 92 residues, and the β-subunit includes 145 residues. The α-subunit is N-glycosylated at Asn_{52} and Asn_{78} and the β-subunit is N-glycosylated at Asn_{52} and Asn_{78}. Previous studies have established that there are multiple variants of this hormone in urine and blood: free hCG, beta core fragment (hCGβcf), nicked hCG (hCGn), β-subunit (hCGβ) and nicked beta subunit (hCGβn) [21]. hCG has an extremely wide range of biological functions. This peptide hormone displays an essential role

in maintaining pregnancy by adjusting the level of progesterone and estrogen. Additionally, during pregnancy hCG plays a key role in fetal growth and improves the anti-macrophage inhibitory factor. hCG detection is also effective in trophoblastic disease evaluation [22]. More interestingly, the hCG receptors are detected in the hypothalamus and hippocampus of the mother [23]. This glycoprotein hormone regulates local immune cell numbers and forces them to adopt a distinctive phenotype to protect and maintain the fetus [24].

Although there have been lots of efforts to identify the effects of ultrasonic irradiation on human health, there are few efforts carried out to identify the physicochemical mechanism of ultrasonic on protein structure. It should be also noted that in previous studies, the impacts of noise stress and noise pollution on the plasma level of gonadotropin hormones have been studied experimentally *in vivo*. However, there are a few reports about the impacts of ultrasonic on hCG structure *in vitro* from a physicochemical point of view. Furthermore, as mention earlier, the details of ultrasound-induced disruption in protein structure remain unidentified. As a result, due to the vital function of hCG, the objective of this study is to examine the possible effects of simulated ultrasonic irradiation (40 kHz) in various times of exposure (from 10 to 60 min) on hCG structure. This study is useful to achieve a better understanding of the physicochemical effects of ultrasonic irradiation on peptides and protein structures as an important class of bio-macromolecules. Besides, the findings obtained from this investigation can help to discover safe limits for people, particularly for pregnant women.

MATERIALS AND METHODS

Materials

Highly purified hCG (5000 I.U., white, pyrogen-free powder, freeze-dried, and sterile) obtained from the urine of pregnant women, were acquired from Karma Pharmatech GmbH (Germany). ANS (8-anilino-1-naphthalene sulfonic acid), Coomassie brilliant blue, sodium dodecyl sulfate (SDS), 2-mercaptoethanol, NaH_2PO_4 and Na_2HPO_4 were purchased from Sigma-Aldrich Chemical Co. (USA) and CinnaGen Co. (Iran), respectively. Deionized double-distilled water (with the resistance of 18.3 MΩ) was consumed through all measurements. Experiments were obtained in phosphate buffer (PB) pH 7.4 (0.1 M).

Instruments

The thermo scientific barnstead NANO pure water purification system (USA), the varian cary 100 bio UV-Vis spectrophotometer (USA), the AVIV 215 circular dichroism spectrometer (USA), the agilent cary eclipse fluorescence spectrophotometer (USA), the dynamic light scattering (DLS), Brookhaven Instruments Corporation, Holtsville (USA) and the Malvern Zetasizer Nano ZS (UK) were applied. The ultrasonic trials were performed in the WiseClean, digital ultrasonic cleaner-set WUC-D10H (South Korea) with a high frequency of 40 kHz and power consumption of 665 W.

Sample Preparation

25 μM hCG in PB (pH 7.4, 0.1 M) was put into small shielded tubes 1 cm × 1 cm (width and height). All tubes were filled with hCG solution, checking that no air bubbles remained in the tubes. The samples were divided into two groups: the control (reference) group as well as the experimental group. The control group (25 μM hCG in PB) was kept in small tubes and placed outside the ultrasonic apparatus and did not receive any treatment. The experimental group (25 μM hCG in PB) was placed in the ultrasonic apparatus. The ultrasonic irradiation time was altered from 10 to 60 min at 10 min intervals using a 40 kHz.

UV-Vis (Ultraviolet-Visible) Absorption Measurements

The UV-Vis absorption spectra of the untreated and the experimental groups were scanned at a wavelength between 200-450 nm. In the beginning, the system was baselined with buffer solutions, and afterward, spectra of hCG solution (8 μM) were recorded. At first, the UV-Vis spectroscopic data of the control group were explored. Subsequently, the spectroscopic properties of hCG in the ultrasonic condition were scanned. To keep the temperature at 37 ºC, each measurement was achieved in a thermostated conventional quartz cell.

Intrinsic and Extrinsic Fluorescence Measurements

The fluorescence of tyrosine residues of reference hCG (the control group) was examined. Subsequently, the emission properties of hCG immediately after 10 to 60 min treatment in the ultrasonic irradiation (the experimental group) were studied in the wavelength range between 293 and 360 nm *via* an excitation wavelength of 275 nm. Afterward, the accessibility of hydrophobic domains of the reference and the experimental group was determined by ANS fluorescence study in the wavelength range between 400-600 nm *via* an excitation wavelength of 350 nm. Samples including 30 μM ANS and 8 μM hCG in 0.1 M PB (pH 7.4) were prepared. To keep the temperature at 37 ºC, Protherms bath model NTB-211 temperature controller was utilized and a cuvette with a 1 cm path length, a 10 nm excitation slit, and a 10 nm emission slit was applied in each measurement.

Circular Dichroism (CD) Measurements

The near-UV CD and far-UV CD spectra of the reference and the experimental groups at 37 ºC were recorded from 250 to 305 nm and 200 to 250 nm, respectively. Far-UV CD spectra were scanned in a quartz cell (0.1 cm path length) with a 20 nm min^{-1} scan speed and a 0.2 nm resolution, on the other hand; near-UV CD spectra were taken with a 1 cm path length quartz cell. The concentration of hCG in the experiments for the far-UV region was 8 μM in PB and the concentration of protein in the experiments for the near-UV region was 25 μM in PB. *Via* subtracting the proper baseline, each of the CD spectra was corrected. The CD spectra deconvolution software (CDNN, version 2.1) was applied to deconvolute all CD-spectra.

Dynamic Light Scattering (DLS) and Zeta Potential Measurements

The hydrodynamic diameter of the control and the experimental groups were acquired at 633 nm and a fixed 90º scattering angle. Before measuring, all samples were filtered *via* nylon filters (0.20 μm). The zeta potentials of the reference and the experimental groups (8 μM hCG in PB) were also achieved. With the data from 5 runs, the average values of hydrodynamic diameter and zeta potential were determined.

Gel Electrophoresis

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed to evaluate the effect of ultrasonic on hCG. The assay was performed consistent with the standard protocol [25]. The stacking (4% acrylamide, pH 6.8) and separating (12% acrylamide, pH 8.8) gels were run at a constant voltage of 100 V. The chambers of electrophoresis contain SDS (0.1%) and Tris-glycine buffer (pH 8.3). The hCG solutions were diluted with a buffer containing bromophenol blue (1%), 25% Tris-HCl (pH 6.8, 1 M), SDS (2%), and glycerol (25%) and then heated at 95 ºC for 5 min. The gels were stained with Coomassie blue (0.25%) dissolved in glacial acetic acid (10%), water, and methanol (45%) solution. The detaining solution contained water, methanol, and glacial acetic acid (8:1:1).

RESULTS AND DISCUSSION

UV-Visible Absorption Studies

To provide evidence for the structural effects of ultrasonic irradiation on hCG, UV-Vis spectroscopy measurements (one of the most constructive accessories for exploring the structural variation of bio-macromolecules) were carried out. The UV-Vis absorption spectra of hCG with and without ultrasonic irradiation are demonstrated in Fig. 2A. As revealed in this figure, this glycoprotein hormone has two main maximum absorption peaks (λ_{max}): one at 214 nm region related to the $n\rightarrow\pi^*$ transition of C=O (which is referred to peptide linkage) and at 276 nm region caused by transitions of $\pi\rightarrow\pi^*$ of the aromatic residues [26,27]. After exposing hCG to ultrasonic irradiation, alterations in λ_{max} at 276 nm region took place. As displayed in this figure, after 10 to 40 min of ultrasonic irradiation, it made an increment in the maximum absorption at 276 nm. These enhancements in λ_{max} are indeed due to the disorders at the microenvironment of hCG [28]. Additionally, the absorption spectra showed that more side chains of aromatic residues of hCG were exposed to the solvent (inset of Fig. 2A) [20]. Our observation correlates with the results presented by Vera *et al*. who showed that quinoa proteins subjected to ultrasound treatment had a noteworthy alteration in λ_{max} and this result may point out a higher aromatic residue exposure and a greater degree of unfolding

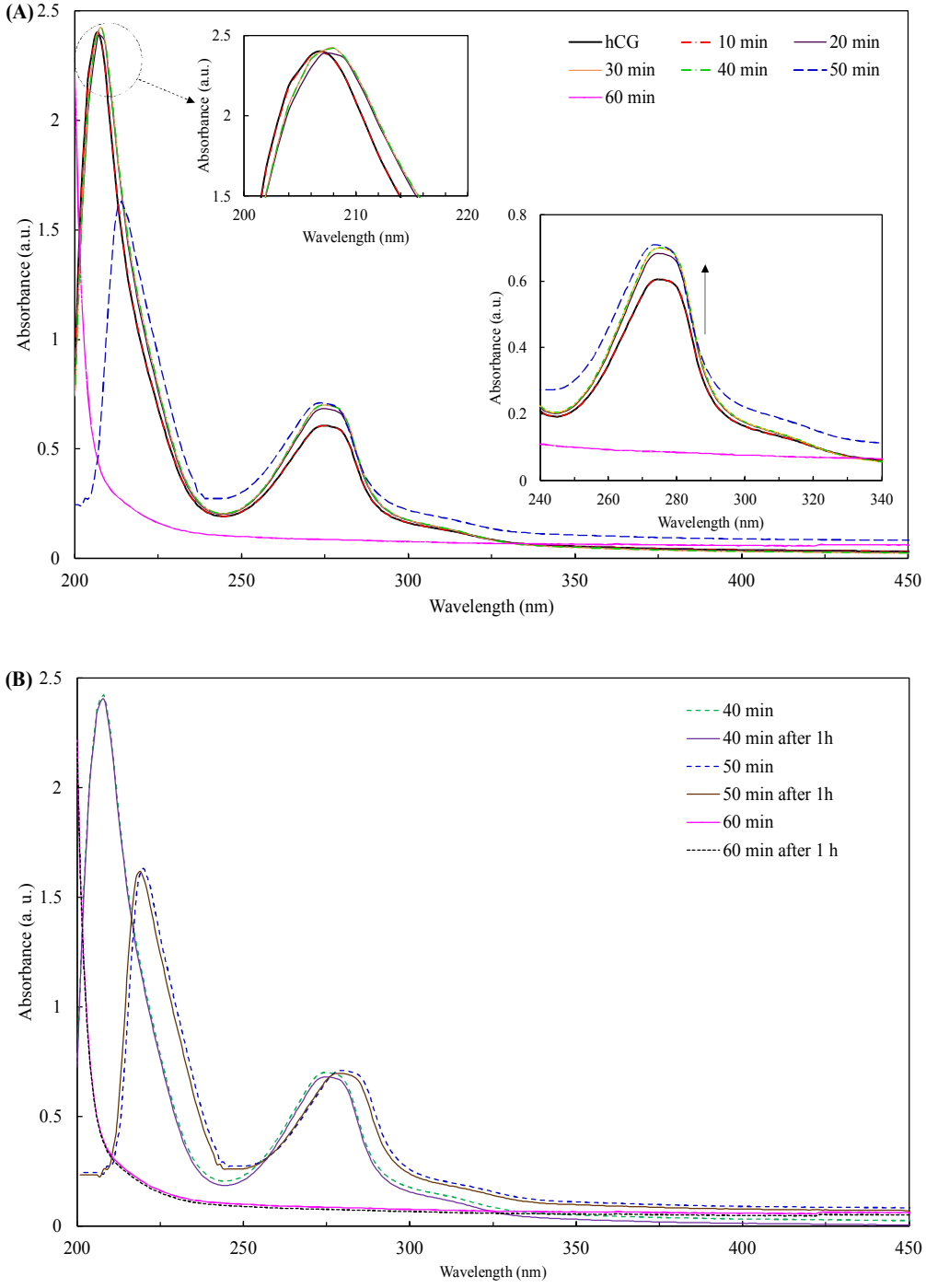

Fig. 2. (A) UV-Vis spectra of 8 μM hCG in PB without and immediately after ultrasonic irradiation at 37 ºC. Upper inset: magnification of the λ_{max} at 214. Lower inset: the UV-Vis spectra of hCG before and immediately after exposing to ultrasonic irradiation in the range of 240-340 nm. (B) UV-Vis spectra of hCG immediately and 1 h after exposing to ultrasonic irradiation.

[29]. This data also correlates with those presented by He *et al.* who showed that as the ultrasonic irradiation time increases, the λ_{max} of bovine serum albumin (BSA) increases obviously [30]. It should be noted that no obvious alterations in λ_{max} at 214 nm after 10 to 40 min exposing hCG to ultrasonic irradiation were detected. However, after 50 min of ultrasonic irradiation, it made a reduction in the λ_{max} at 214 nm but still made an increment in the λ_{max} at 276 nm. This phenomenon could be assigned to a perturbation of α-helix induced via ultrasonic irradiation [31]. More importantly, the absorption spectra revealed that 60 min exposure to ultrasonic irradiation has an extremely high effect on the hCG structure and the λ_{max} was vanished completely. Subsequently, ultrasonic irradiation can cause time-response changes in the tertiary structure of hCG. Generally, the biophysical effects of ultrasonic irradiation can be divided into non-thermal and thermal mechanisms. As a general rule, the bio-macromolecules cannot absorb the acoustic energy directly, so this kind of energy affects bio-macromolecules indirectly by the non-thermal mechanism known as the cavitation phenomenon. This phenomenon increases free radicals in solution [30]. Accordingly, due to the cavitation effect, water molecules can produce some free radicals in solution which can initiate changes in the tertiary structure of hCG. It should be noted that the UV-Vis spectrum of hCG immediately after exposure to ultrasonic irradiation was not considerably different from hCG sample 1 h after 40-, 50- and 60-min exposure (Fig. 2B). Therefore, the time difference in assaying did not account for the variations detected. Hence, the ultrasonic-induced conformational modifications were irreversible.

Steady-state Fluorescence Emission Studies

Fluorescence spectroscopy is one of the most powerful electromagnetic spectroscopies to examine protein folding and dynamics. There are three intrinsic fluorophores for almost all proteins: Tyr (tyrosine), Trp (tryptophan) and Phe (phenylalanine). However, hCG contains 7 Tyr residues and does not have any Trp residues [32]. Thus, fluorescence spectra of Tyr residues in hCG were applied to discover hCG conformational variations under ultrasonic irradiation. It should be mentioned that the application of Phe or Tyr fluorescence emission is limited to tryptophan-free protein [33]. Tyr residue is regarded as a simple fluorophore and seems to be relatively insensitive to the protein local environment. However, if there are no proton acceptors in the local environment of protein and the phenol side chain is shielded from a solvent, intermolecular and intramolecular interactions can reduce the quantum yield of Tyr [34]. It has been also published that the phenol hydroxyl group ionization causes the fluorescence quantum yield reduction. Tyr fluorescence emission in hCG has a maximum intensity ($\lambda_{max,em}$) around 303 nm [34]. Figure 3A displays fluorescence spectra of hCG with and without ultrasonic irradiation at 37 ºC. As displayed in this figure, after 10 min of ultrasonic irradiation, it made a very slight reduction in the fluorescence emission of Tyr residues (~2%) with no shift. However, 20 min treatment in the ultrasonic irradiation had a greater influence on hCG than 10 min exposure to ultrasonic irradiation (~48%) and longer periods (>20 min) made the fluorescence vanish completely. Consequently, according to our data, the ultrasonic-induced alterations were identified in the intrinsic fluorescence emission of Tyr residues in hCG. Such a manner suggests that ultrasonic condition induced variation in fluorescence emission of Tyr residues *via* varying the position or structure of Tyr residues of hCG [20]. Similarly, He *et al.* and Jiang *et al.* indicated that the $\lambda_{max,em}$ of BSA [30] and black bean protein [35] reduce noticeably compared with those samples without ultrasonic irradiation. As mentioned earlier, hCG is a tryptophan-free glycoprotein, therefore any reduction in the fluorescence emission of this protein is owing mostly to Tyr and Phe residues. Consequently, based on our results and previous results [30,35] we can propose that the cavitation effect of ultrasonic irradiation can induce the aromatic residues oxidation, therefore, can cause hCG unfolding.

The hydrophobic fluorescent probes can be consumed to recognize the protein surface hydrophobicity. It is well accepted that the hydrophobic interactions are very crucial for conserving the conformation, stability, as well as function of proteins [9]. ANS, as an organic substance, is generally utilized for discovering the tertiary/quaternary structure of protein molecules because ANS fluorescent only after associated with the hydrophobic surface of protein [36]. Figure 3B revealed that the surface hydrophobicity of hCG is low. After 10 min treatment with

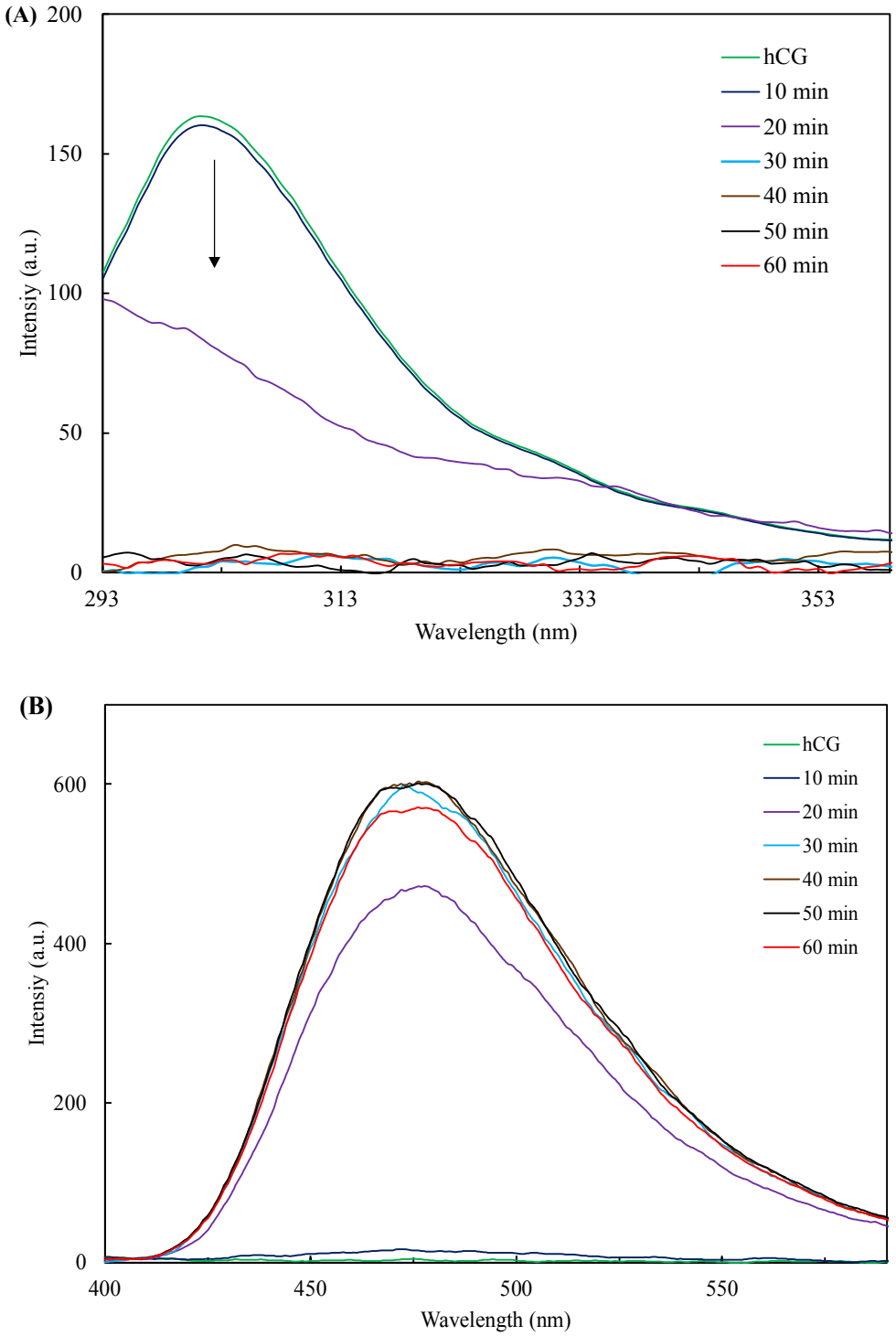

Fig. 3. (A) The spectra of intrinsic fluorescence intensity of 8 µM hCG in PB with and without ultrasonic irradiation at 37 ºC. (B) ANS binding at 350 nm (excitation wavelength): 8 µM hCG in PB with and without ultrasonic irradiation at 37 ºC.

ultrasonic irradiation, very low ANS affinity was observed for hCG, too. However, after longer periods (>20 min) treatment with ultrasonic irradiation, ANS affinity to hCG increased considerably. This data revealed the hydrophobic core formation in the hCG after treatment with ultrasonic irradiation. The alteration in hydrophobicity as a function of ultrasonic irradiation time are extremely noteworthy, presumably because of the unfolding of hCG. Nevertheless, as shown in Fig. 3B, the surface hydrophobicity reduced after sonication for 60 min, which is a symbol of protein aggregation. This result is in consistent with the data from increasing surface hydrophobicity of whey protein concentrate for up to 5 min of sonication and decreasing its surface hydrophobicity for more than 5 min [9]. It has been published that the exposure of hydrophobic groups can lead to the change in the tertiary structure of proteins [37] which is in good agreement with UV absorption data as mentioned above. As mention earlier, the cavitation phenomenon can affect water molecules and produce some free radicals in solution. It is widely accepted that water molecules play a key role in the folding and self-assembly of proteins [38]. As a result, it could be hypothesized from our observations and previous results [30,35] that ultrasonic irradiation can change the structure of water molecules, consequently, the structure of hCG modifies. However, it should be emphasized that further experiments must be done to conclude the underlying mechanisms. In consequence, the obtained extrinsic and intrinsic fluorescence data specified that ultrasonic irradiation initiates structural changes in hCG.

CD Studies

CD spectroscopy can measure any alternation in the secondary/tertiary structure of macromolecules, typically proteins [39]. With the purpose of considering the impact of ultrasonic irradiation on the hCG structure, the far-UV CD method was employed, and the CD spectra are shown in Fig. 4A. As displayed, the far-UV CD spectrum of hCG was detected via the presence of a remarkable negative band at around 208 nm wavelength. Our observations are coincident with those published by Fralish *et al.* [40]. Compared with the control sample, far-UV CD studies of hCG subjected to ultrasonic displayed significant changes in its secondary structure particularly after 60 min (Fig. 4A). Subsequently, the content of the hCG secondary structure was calculated. hCG consists of 11.97% α-helix, 35.01% β-sheet and 16.48% β-turn (Table 1). Accordingly, hCG is primarily a β-structure protein [40]. Ultrasonic irradiation after 10 min made an insignificant change in the secondary structure content of hCG, conversely, longer periods (>20 min) made an obvious alternation in the secondary structure content of hCG. Consequently, as the ultrasonic exposure time increased, the α-helix content reduced and the content of the β-sheet barely altered, and at the same time, the content of the random coil structure increased. Obviously, after 60 min exposure, the secondary structure of hCG was changed clearly and a transition to the random coil appeared. Interestingly, our data correlate with those of Li *et al.* [37] who have shown that ultrasonic irradiation at high power condition (>600 W) can increase a transition from α-helix to the random coil in the secondary structure of alcalase hydrolysates. The protein secondary structure depends on the interactions between various parts of the protein along with the local sequence of amino acids, thus it is probable that ultrasonic irradiation disrupts these interactions. The previous study also displayed that the decrease in α-helix content of protein might be owing to the hydrophobic surface exposure [37]. Hence, ultrasound irradiation could initiate the interactions breakdown that stabilizes the structure of hCG, for example, electrostatic interactions and/or hydrogen bonds. This phenomenon can lead to the protein unfolding [29], and the hydrophobic patches could be exposed to the surface of hCG. This conclusion is consistent with our observation from the ANS fluorescence studies. It should be mentioned that the far-UV CD spectrum of hCG immediately after exposure to ultrasonic irradiation was not considerably different from hCG sample 1 h after 40-, 50- and 60-min exposure (inset of Fig. 4A). Therefore, the time difference in assaying did not account for the variations detected.

Near-UV CD is an appropriate technique to observe any variations in proteins' tertiary structure. The near-UV CD spectrum of protein depends not only on the micro-environment of the aromatic amino acids side chain (related to the $\pi \rightarrow \pi^*$ transition) but also on the disulfide bonds (related to the $n \rightarrow \sigma^*$ transition) and even the non-protein cofactors [39]. hCG, As reported contains 6 Phe residues, 7 Tyr residues, and 11 disulfide bonds [32]. The CD spectra of hCG with and without ultrasonic irradiation are demonstrated in Fig. 4B. There are two notable minima at

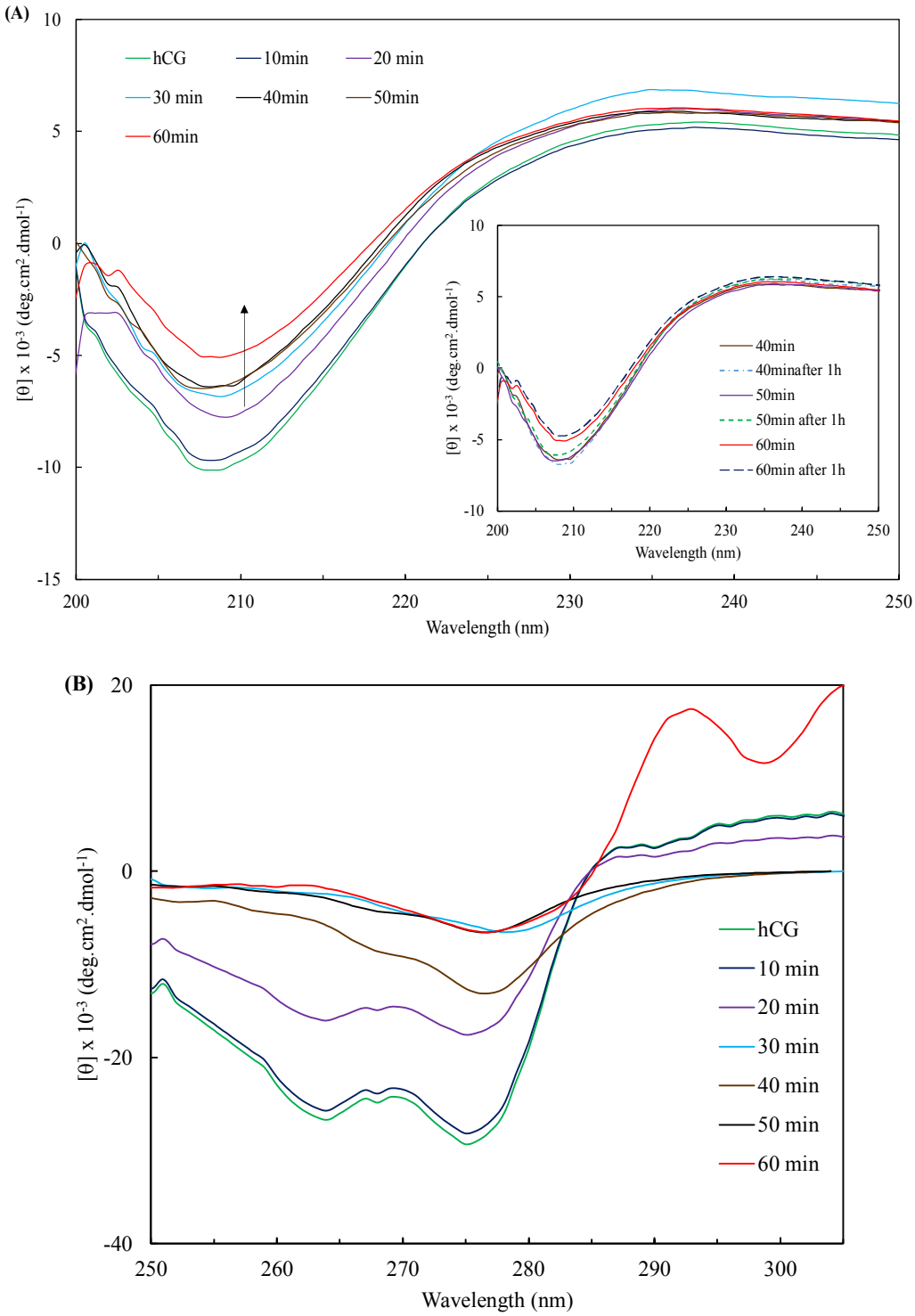

Fig. 4. (A) The spectra of the far-UV CD of 8 μM hCG in PB with and without ultrasonic irradiation at 37 ºC. Inset: far-UV CD spectra of hCG immediately and 1 h after exposing to ultrasonic irradiation. (B) The spectra of the near-UV CD of 25 μM hCG in PB with and without ultrasonic irradiation at 37 ºC.

Table 1. Content of the Secondary Structure of 8 μM hCG in PB with and without Ultrasonic Irradiation Estimated from CD Spectra in the far-UV Region (200-260 nm) at 37 °C

	α-Helix (%)	β-Sheet (%)	β-turn (%)	Random coil (%)
hCG (untreated)	11.97	35.01	16.48	36.54
After 10 min	11.91	34.98	16.44	36.67
After 20 min	11.11	34.71	16.34	37.84
After 30 min	10.90	34.18	16.33	38.59
After 40 min	10.90	33.97	16.33	38.80
After 50 min	10.88	33.96	16.32	38.84
After 60 min	10.65	32.71	16.31	40.33

262 nm and 268 nm in the hCG near-UV CD spectrum which are attributed to Phe residues [39]. After 20 min of ultrasonic irradiation, it made a reduction in this remarkable minimum. Also, 30 min treatment in the ultrasonic irradiation had a greater impact on this peak. Besides, for longer periods (>30 min), fine structure and loss of CD signal took place at this wavelength. The near-UV CD spectrum of hCG also showed a remarkable minimum between 275-285 nm, which is attributable to the phenolic group of Tyr residues. It should be mentioned that the near-UV CD spectrum of disulfide bonds arises around 260 nm wavelength and is mostly quite weak. After exposing hCG to ultrasonic irradiation (>30 min), the amount of the peak at this region reduced. Indeed, the reduction in the asymmetry of the hCG tertiary structure caused these variations. This observation reveals good agreement with UV-Vis absorption as well as fluorescence emission data as mentioned above.

Dynamic Light Scattering (DLS) and Zeta Potential Studies

DLS is the greatest technique for monitoring the bio-macromolecules size. Accordingly, the DLS technique was exploited to find out the effects of ultrasonic irradiation on hCG size distribution. As demonstrated (Fig. 5), the peak diameter of native hCG is 4.1 nm. The effect of ultrasonic time revealed initial increment and eventual reduction in peak particle size. These alterations indicated that ultrasound in an aqueous system could promote interactions of hCG molecules and making some larger particles. Arzeni *et al.* [41] and Gülseren *et al.* [10] established that high-intensity ultrasonic irradiation increased the size of particles in egg white protein and BSA. Nevertheless, after 60 min treatment in ultrasonic irradiation, the peak diameter reduced. Ultrasonic processing for this long time possibly induced some larger particles dissociated into smaller ones. Ultrasound-caused protein dissociation has been reported earlier. The germin-like protein was reported to dissociate into smaller size after 40 min ultrasonic treatment [42]. It has been reported that at the air-liquid interface of ultrasonic-induced bubbles, proteins could be destabilized, causing the protein particles aggregation and homogenization [42]. Consequently, it could be concluded that the sensitive balance between various noncovalent interactions in the structure of hCG can be easily disrupted *via* ultrasonic irradiation, causing protein denaturation.

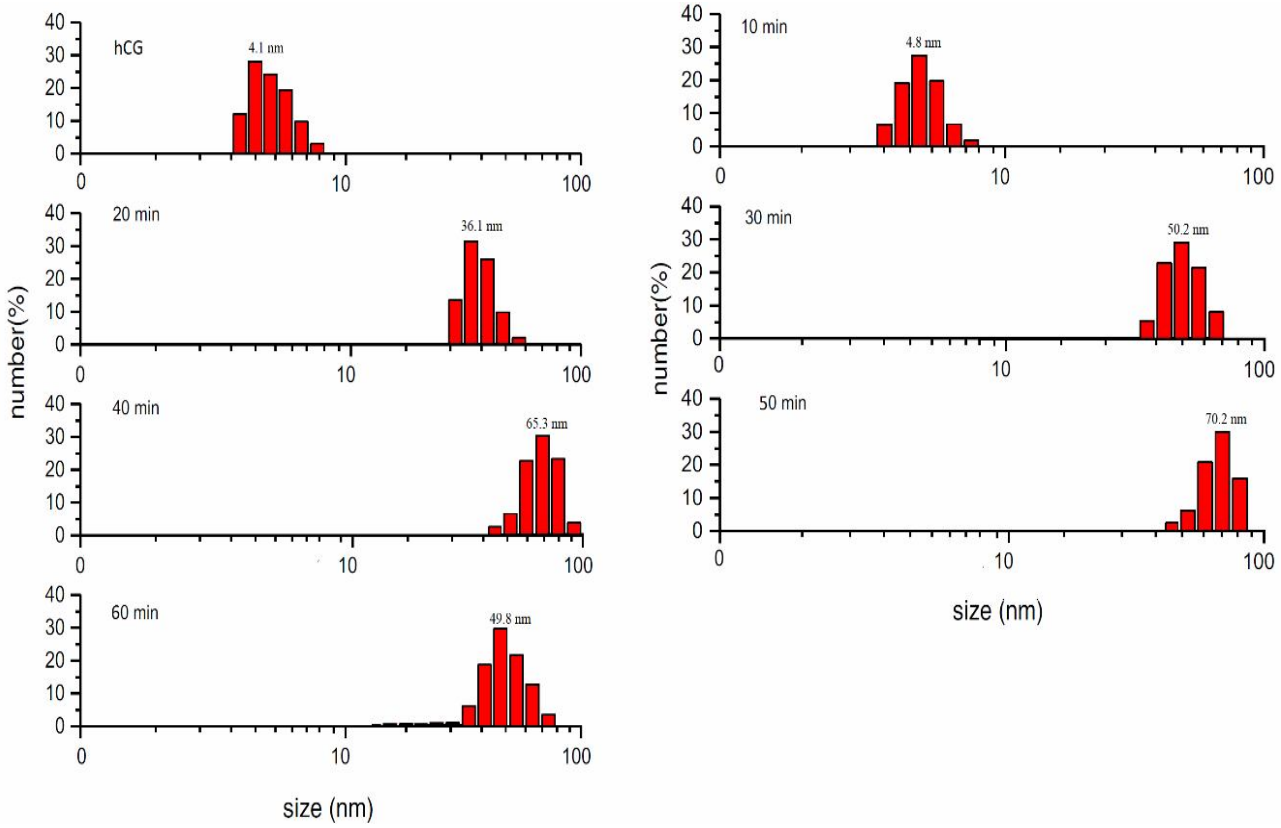

Fig. 5. The particle size distribution of 8 μM hCG in PB with and without ultrasonic irradiation.

Table 2. Zeta (ζ) Potential of 8 μM hCG in PB with and without Ultrasonic Irradiation

	Z-potential (mV)
hCG (untreated)	-16.42
After 10 min	-16.90
After 20 min	-19.34
After 30 min	-20.67
After 40 min	-23.28
After 50 min	-25.73
After 60 min	-30.32

Moreover, hCG aggregates formed by the non-covalent interactions could be removed with excessive ultrasonic duration, causing hCG particle breakage.

A physical term, zeta (ζ) potential value, is the most significant reference about the surface charge of bio-macromolecules [39]. The protein surface charge is dependent to the protein environment. The ζ-potential of hCG with and without ultrasonic irradiation are listed in Table 2. The ζ -potential of hCG was found to be around -16.42 mV. hCG is the most acidic glycoproteins with an isoelectric point (pI) value ranging from 3-7 and has almost 15 sialic acid residues per molecule [18], which was responsible for the obtained negative ζ-potential. Table 2 elucidates that ultrasonic irradiation caused a notable ζ-potential enhancement in hCG, besides, the ζ-potential variation between the hCG with and without ultrasonic irradiation became greater at longer ultra-sonication times. Consequently, hCG structural fluctuation is in combination with the surface charge alteration. As a result, ultrasonic irradiation can increment the extent of charged residues existing at the hCG surface. This result is consistent with a remarkable alteration in ANS access to the hCG hydrophobic sites; *i.e.*, ultrasonic irradiation prompted hCG structure alterations. Our results are coincident with those presented by Gulseren *et al.* [10] and Jiang *et al.* [35] who showed that the ζ-potential and surface hydrophobicity of patches of ultra-sonicated BSA and black-bean protein isolate enhanced compared to the native form of protein samples. This result could be associated with the fluctuation of water contents around hCG microenvironments. Consequently, ultrasonic irradiation could increase the negative surface charge on hCG and strengthen the inter-particle electrostatic repulsions.

Gel Electrophoresis Analysis

Electrophoretic protein patterns obtained through SDS-PAGE for ultrasound treated and untreated hCG are revealed in Fig. 6. As revealed in this figure, hCG presented two major bands representing the α subunit and the β subunit. hCG is a glycoprotein with multiple glycosylation sites, including four in the β-chain (MW 22.2) and two in the α-chain (MW 14.5 kDa), thus, the subunits do not run exactly on SDS-PAGE. This result was in accordance with the reports by Pong *et al.* [43]. No difference in the hCG fractions was distinguished between untreated and sonicated hCG after 40 and 50 min. The obtained results are in agreement with previous literature [44]. However, after 60 min treatment in ultrasonic irradiation, some new bands were observed. The appearance of these new bands might be due to protein hydrolysate. The results displayed that considerable fluctuations occurred to the hCG structure upon treatment with ultrasound after 60 min. This pheromone has been reported by other groups [45,46].

CONCLUSIONS

Nowadays, there are some pieces of evidence that ultrasound can alter the function of cells and their components such as proteins and enzymes. Although some previous studies disclosed that ultrasound can alter the physical, chemical, and biological properties of proteins, behind these works, there are some investigations exhibited that ultrasound had no significant effects on proteins [9]. Due to the vital action of hCG, investigating the effects of ultrasound on hCG are essential for discovering safe limits for people particularly for pregnant women. In this study, the UV-Vis studies and near-UV CD results verified that ultrasound (>30 min) can generate alternations in hCG structure. Additionally, the ultrasonic-induced conformational variations were irreversible. Besides, the ultrasonic-induced deviations were recognized in the intrinsic fluorescence emission of Tyr residues in hCG. Additionally, the alterations in hydrophobicity as a function of ultrasonic irradiation time are noteworthy. Our far-UV CD data demonstrated that as the ultrasonic exposure time increased, the α-helix content reduced, and the β-sheet content barely changed, and the random coil content structure increased. The DLS and ζ-potential surveys showed that ultrasonic (up to 50 min) can cause size and surface charge enhancement in hCG. Nevertheless, after 60 min treatment in ultrasonic irradiation, the peak diameter reduced. In the SDS-PAGE experiment, no difference in the hCG fractions was distinguished between untreated and sonicated hCG after 40 and 50 min. However, after 60 min treatment in ultrasonic irradiation, some new bands were observed. Consequently, our results, *i.e.*, significant loss of tertiary structure, higher ANS binding to the hydrophobic patches, and increment of the random coil content, suggest

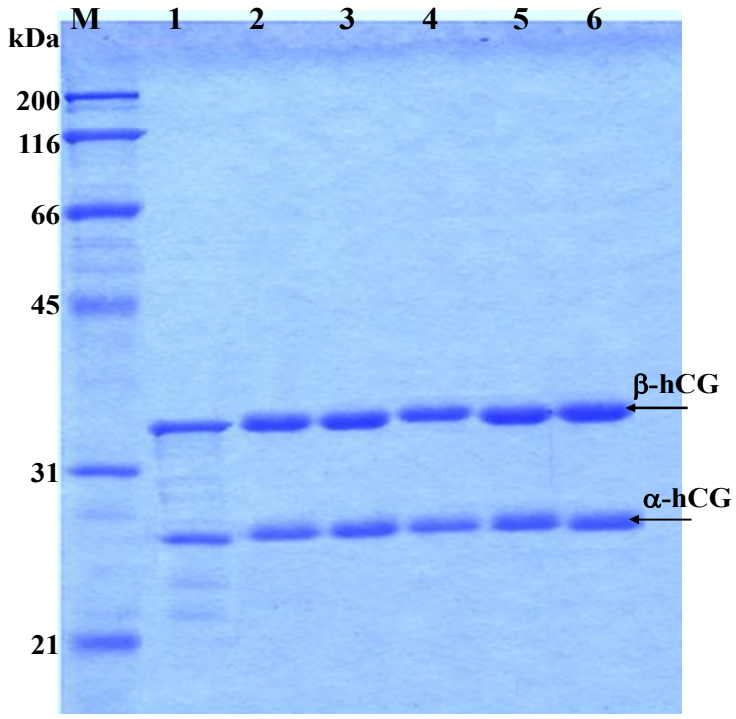

Fig. 6. SDS-PAGE profile of hCG with and without ultrasonic irradiation. Lanes (1), (3), and (5) represent hCG after exposing to ultrasonic irradiation for 60, 50 and 40 min, respectively. Lanes (2), (4) and (6) represent hCG before exposing to ultrasonic irradiation for 60, 50 and 40 min, respectively. M represents the molecular mass of the marker.

that hCG after exposure to ultrasonic radiation is unfolded. It would be possible that the sensitive balance between various noncovalent interactions in the structure of hCG can be easily disrupted *via* ultrasonic irradiation. Furthermore, after 60 min treatment in ultrasonic irradiation, hCG was hydrolyzed. Our *in vitro* experiments showed ultrasonic treatments of hCG resulted in time-dependent structural variations. However, the question of why some proteins were altered by ultrasound and some were not is still open.

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Declarations

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