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INCREASED CHEMICAL ACETYLATION OF PEPTIDES AND PROTEINS AFTER DAILY INGESTION OF DIACETYL ANALYZED BY NANO-LC-MS/MS (Q-TOF)

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Abstract

Background: Acetylation alters several protein properties including molecular weight, stability, enzymatic activity, protein-protein interactions, and other biological functions. Our previous findings demonstrating that diacetyl/peroxynitrite can acetylate L-lysine, L-histidine, and albumin *in vitro* led us to investigate whether diacetyl-treated rats suffer protein acetylation as well.

Methods: Wistar rats were administered diacetyl daily for 4 weeks, after which they were sacrificed, and their lung proteins were extracted to be analysed by Nano-LC-MS/MS (Q-TOF). A C18 reversed-phase column and gradient elution with formic acid/acetonitrile solutions from 2 to 50% over 150 min were used to separate the proteins. Protein detection was performed using a microTOF-Q II (QTOF) equipped with captive source and an electrospray-ionization source. The data from mass spectrometry were processed using a Compass 1.7 and analyzed using Protein Scape, software that uses Mascot algorithms to perform protein searches.

Results: A set of 3162 acetylated peptides derived from 351 acetylated proteins in the diacetyl-treated group was identified. Among them, 23 targeted proteins were significantly more acetylated in the diacetyl-treated group than in the PBS control. Protein acetylation of the group treated with 540 mg/kg/day of diacetyl was corroborated by Western blotting analysis.

Conclusions: These data support our hypothesis that diacetyl exposure in animals may lead to the generation of acetyl radicals, compounds that attach to proteins, affecting their functions and triggering adverse health problems.

Key words: Radical acetylation**,** 2,3-butanedione, diacetyl, food additive, lung diseases, proteomics.

1. Introduction

Diacetyl is a flavoring commonly used in foodstuffs, as it lends a buttery flavor to products such as popcorn, coffee blends, cakes, cookies, wines and other goods. [1-6] It is a volatile α-dicarbonyl and a highly electrophilic compound [3] approved worldwide for use by food industries, despite ongoing health concerns dating back to 1986 [7], when the first cases of bronquiolitis obliterans involving diacetyl emerged. [5, 8, 9]

Recently, we reported that the reaction of peroxynitrite with α-dicarbonyls, namely diacetyl and methylglyoxal, in aerated phosphate buffer pH 7.4 results in the acetylation of free amino acids, peptides and proteins added to the reaction mixture. [10-13] This reaction is initiated by nucleophilic addition of peroxynitrite to the carbonyl group of the α-dicarbonyl compound yielding a peroxynitroso adduct, whose homolysis yields acetyl radicals. Dissolved molecular oxygen adds to the radical to ultimately produce acetate from diacetyl or acetate and formate from methylglyoxal. [11-12] Formyl radical intermediate generated by methylglyoxal/peroxynitrite was shown to add to the α-amino group of L-lysine-containing synthesized tetrapeptides. [13] On the other hand, diacetyl/peroxynitrite-generated acetyl radicals have proven been proven to attack both the α- and ε-amino groups of free and blocked L-Lys, L-Lys-containing peptides and serum albumin. [10] These findings have raised the hypothesis that radical acetylation of proteins contributes to transacetylase–promoted post-translational protein modifications at sites where both methylglyoxal or diacetyl and peroxynitrite are present. [10-13] From these facts, the competition of chemical (induced by diacetyl) and enzymatic (occurring naturally in organisms) acetylation can be inferred, with the former process contributing to the increase of total protein acetylation.

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Another source of acetylation *in vivo* is found through the action of acetyltransferases. These enzymes reversibly catalyze the transfer of the acetyl group from acetyl-CoA to the ε-amino group of protein lysine residues [14], a process promoted by lysine acetyltransferase and lysine deacetylase [15-17] at the N-terminus during the synthesis of proteins. Protein acetylation is highly and more conserved in eukaryotes and prokaryotes than phosphorylation, but it is less common than phosphorylation and ubiquitination. [18] Acetylation can reportedly alter the protein function, size, enzymatic activity, stability, protein-protein interactions and other protein properties. When acetyltransferase is deregulated, and lysine acetylation is increased, modifications may occur in genes and in the regulatory machinery, resulting in the manifestation of tumours in cells. [14] On the other hand, ATP-dependent acetylation has recently been reported to play a role in many cellular processes such as catalytic activity, immune responses and metabolic processes, including the generation of precursors of "energy-rich" metabolites such as acetylphosphate (acP). AcP-dependent acetylation tends to govern the translation of nucleotides, purine and pyrimidine metabolism and degradation of RNA. [19]

In this work, we use proteomic and western blotting techniques to investigate if diacetyl is also capable of leading to increases *in vivo* protein acetylation. Based on our findings, we support the hypothesis that diacetyl exposure in animals may lead to increases in protein acetylation, which may affect protein functions and trigger adverse health problems.

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2. Methods

Animal treatment

All animals were fed *ad libitum* and kept in a cabinet at 50–70% humidity, at a temperature of 19–26 °C in a cycle of 12 hours light/12 hours dark. This study adheres to the guidelines established by the Brazilian College of Animal Experimentation (COBEA) and was approved by the Ethical Committee of the School Medicine of the Federal University of São Paulo (UNIFESP, protocol no. 1949-11).

Eight-to-twelve-week-old male Wistar rats (250–300 g) were divided into two groups (6 animals each). The control group received phosphate-buffered saline (PBS), while the treated group received 540 mg/kg/day of diacetyl (Cat B8530-7, Sigma Aldrich, USA) dissolved in PBS. Both groups were dosed using gavage.

After 4 weeks of treatment, the animals were anesthetized with ketamine and xylazine (Sigma Aldrich, USA) and sacrificed. The lung tissue was collected and immediately frozen in liquid nitrogen and stored at –80°C.

Preparation of lung extracts

Tissue preparation

Frozen lungs were ground into a fine powder in liquid nitrogen using a mortar and pestle. The homogenization process was used to avoid the activation of proteases and prevents protein degradation. The sample was lyophilized prior to analysis in order to remove residual water and stabilize the sample for handling at room temperature, thereby facilitating the weighing process and preparation of the sample.

Protein extraction optimization

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Due to the wide range of proteins and interfering substances in the final extracts, the samples were obtained in the following three steps prior to proteomics analysis: tissue disaggregation and cell homogenization; protein extraction from the biological matrix; and protein precipitation and solubilisation in a urea buffer.

Three methods of protein extraction were tested to quantify the amount of protein in the lysates before and after precipitation (Table 1). Thirteen milligrams of lyophilized lung suspended in one mL of extraction buffer were used.

The lung powder was suspended and shaken for 1 hour at 4°C. After centrifugation (10 min, 5,000 x RPM, 4°C), 200 µL of the supernatant was mixed with 800 µL of DTT solution in cold acetone (2 mg/mL) and incubated overnight at –20°C. Afterwards, the samples were centrifuged (10 min, 16,000 x RPM, 4°C), and the sediments were washed 4 times with the DTT solution, dried in vacuum and solubilized in urea buffer (7 mol L^{-1} urea, 2 mol L^{-1} thiourea, 4% CHAPS). The total protein concentration was determined by the Bradford method. [20]

Tryptic Digestion

Prior to tryptic digestion, polypropylene microtubes were individually filled with an extract aliquot containing 250 µg of the lung protein.. The samples were reduced with Dithiothreitol (DTT) solution until achieving a final concentration of 5 mmol L^{-1}, and they were then incubated for 25 minutes at 56 °C. To achieve alkylation in the samples, iodoacetoamide (IAA) was added until reaching a final concentration of 14 mmol L^{-1}. The samples were then incubated for 30 min at room temperature and protected from light. Afterward, they were diluted until the concentration of urea was reduced to 1600 mmol L^{-1}, and a $CaCl_2$ solution was added until reaching a final concentration of 1 mmol L^{-1} of $CaCl_2$.

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The enzymes trypsin and LysC endoproinase were added in the ratio of 1:50 of (enzyme: substrate). The samples were incubated for 18h at 37 °C. The enzyme reaction was stopped by adding TFA (trifluoroacetic acid) at the final concentration of 0.4%. The samples were centrifuged at 2500 rpm for 10 minutes at room temperature, and the pellet was discarded. The sample was evaporated until the volume was reduced to approximately 50 µl using a vacuum concentrator (Speed Vacuum, Thermo Fisher Scientific, USA). Finally, 50 µl of 0.5% trifluoroacetic acid (TFA) were added thereto. Detergents were removed from the sample using a Pierce detergent removal spin column (Pierce, # 87776), which was used according to the manufacturer's specifications; the samples were filtered through a 22 µm PVDF syringe filter stocked at 4 °C for mass spectrometry analysis.

NanoLC–ESI/MS/MS analyses

In this study, on average 6 biological replicates and two replicate techniques were used. However, due to technical problems, we used 11 replicates of the control group and 10 replicates of the group treated with 540 mg/kg/day of diacetyl. Each trypsinized sample was dissolved with 100 µL of a mixture of water/acetonitrile/TFA (949:50:1 v/v). All analyses were performed using a Nano-UHPLC Advance (Bruker Daltonics, Germany) equipped with a pump, an auto sampler, and a thermostatically controlled column compartment. A C18 reversed-phase column (Magic C18 AQ, Michrom, P/N: CP3/61271/00, USA), particle size 3 µm, internal diameter 0.1 mm, length 100 mm was used. The column temperature was kept at 40°C. Samples were separated using a gradient mobile phase consisting of (A) formic acid/ACN/H_2O (1:20:979) and (B) formic acid/ACN/H_2O (1:950:50) in a gradient elution from 2 to 50 % over 150 min, as a graph in SM1. The flow rate was set at 0.500 µL/min, and the injection volume was 5 µL. Detections were performed using a micrOTOF-Q II (Bruker Daltonics, USA), an

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accurate mass instrument equipped with captive source (Bruker Daltonics, USA) and an electrospray-ionization source (ESI). The mass spectrometer was running in positive mode, with the desolvation temperature at 180 °C and the nebulizer set at 500 V and 0.4 bars. All the operations, acquisition, and analysis of data were controlled by Hystar software Version 1.7 (Bruker Daltonics, USA). For MS/MS analyses, five precursor ions were automatically selected to undergo collision and fragmentation with argon gas (≥ 2 L/min). Mass spectra were collected between 50 to 3,000 *m/z,* and calibration was performed at the beginning of every day using the Tune-Mix ESI-G (Agilent Technologies, EUA). The collision energy was 12 eV, collision RF 600 Vpp, transfer time 140 µs, and pre-pulse storage 14 µs. MS/MS parameters were three precursor ions, absolute threshold 2000 cts, smart exclusion 5X, excluded after three spectra, and released after 1 min. The tune parameters were Funnel 1RF 300 Vpp, Funnel 2 RF 400 Vpp, hexapole RF 400 Vpp, quadrupole ion energy 6.0 eV, and low mass 300 m/z. The TOF (time of flight) conditions included the following: repetition rate 5 KHz, sample rate 2 Ghz, flight tube 8600 V, reflector 1700 V, detector source 1700 V, and detector TOF 2140 V. Argon was used as a collision gas at a pressure of 2×10^{-6} mbar, and the collision energy values were 10–200 eV.

Bioinformatic Analysis

Data deconvolution and database search

Data from mass spectrometry were processed using a Compass 1.7 for OTOF (Bruker Daltonics, USA) and deconvoluted to generate a file compatible with Mascot. This file was analyzed using Protein Scape (Bruker Daltonics, USA), a program that uses Mascot algorithms to perform the search. The database used was Swissprot, an annotated protein sequence database. The taxonomy was *rattus*, and the enzyme was trypsin with two missed cleavages. The fixed modification was carbamidomethylation, and the

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variable modifications were oxidation of methionine and acetylation of lysine and arginine. Mass tolerance modification was 150 ppm to 1 Da. Mascot analysis of all proteins ($p < 0.05$) used a minimum score of 35.

Protein Network Analysis

The protein-protein interaction analysis was performed using Cytoscape 3.3.0 software (http://www.cytoscape.org/) [21], and the protein interaction network was obtained from the STRING 8.2 database (http://string-db.org/). [22] STRING 8.2 uses the metric of "confidence score" to define the confidence of the interactions. We selected only the interactions with proteins identified in our analyses.

Orthologs Analysis

Orthologs were subjected to Gene Ontology (GO) term analysis based on PANTHER classification online tools (http://pantherdb.org/). To determine the biochemical functions of acetylated proteins detected in the lungs of the group treated with diacetyl, GO was performed using IDs with the *Rattus norvegicus* genome found in the Uniprot database. This particular database was chosen as the reference database for the output report of biologicals process, proteins class, cellular components, pathways and molecular functions. [23] These analyses were performed to acquire insights of the acetylation involved in the functions and pathways of proteins.

Analysis of Sequence Model Around Acetylated Lysine

The software motif-x was employed to determine specific sequences of amino acid (15 amino acids upstream and downstream of the acetylation site) in all protein sequences acquired from NanoLC-MS/MS analysis. The entire database (IPI Rat Proteome) was used as a background database parameter, and the significance was 0.000001. [24, 25]

Western blotting

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Samples containing 25 µg of proteins from lung homogenate were subjected to 12% SDS–PAGE electrophoresis and electroblotted onto a nitrocellulose membrane (Millipore, USA). Following the blocking and washing steps, the membranes were incubated with the primary Acetylated-Lysine antibody (Cell Signalling, USA) and anti-rabbit IgG HRP-linked (Cell Signalling, USA) as a secondary antibody. The membranes were then detected using a chemiluminescence kit "Pierce ECL Plus Western Blotting Substrate" (Thermo Scientific, USA) and chemiluminescence software (GeneGnome System/Gene Tools Software, Syngene, UK).

3. Results

Protein extraction optimization

We performed three different protein extraction methods to determine which one yielded the most consistent results when reproduced. That method was then adopted, allowing for increased accuracy in the estimation of protein amounts from the lysate extracts. Figure 1 shows a comparison of the amount of proteins between lung lysate and the solution of precipitate lung proteins resuspended.

MS/MS analyses of diacetyl-promoted acetylation of rat lung proteins

In this work, qualitative proteomic analysis was used, specifically the *bottom up* technique. The lung extracts were analyzed by NanoLC-MS/MS, and significant differences were shown among the protein profiles in the control and diacetyl-treated groups. Acetylation was set in the search engine as a variable modification, and overall, the analyses showed 10,302 peptides identified as belonging to 603 proteins in lung tissue. A set of 327 acetylated proteins in the control group and 351 proteins in the group

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236 treated with 540 mg/kg/day of diacetyl were detected. This increase in acetylation can
237 occur either enzymatically or chemically, a phenomenon presently demonstrated in this
238 work.

239 Among all identified proteins, 93 are common in both groups, and 23 target proteins,
240 presumptively chemically acetylated, were identified on the basis of the presence of at
241 least one common peptide that was neither acetylated nor caused to undergo more
242 acetlyation in the group treated with diacetyl. Thus, proteins of the control group were
243 found to have the same peptide sequence but without acetylation. The table 2 display the
244 identified proteins have a statistically significant score

245 The protein analysis revels some peptides in common in both groups, among these
246 peptides we observed some of them that have, the post translational modification,
247 acetylation more abundant in group treated with 2,3-butanedione than in control group.
248 The proteins that exhibited this pattern of acetylation described above we called them as
249 target proteins, that proteins and their respectives peptides (are described in Table 3,
250 which provides target proteins identification and their respectives peptide scores in both
251 groups and descriptions of the peptide acetylation positions. The acetylation ratio from
252 target proteins cited in table 3 is available in table 4, these ratio was calculated in order
253 to more effectively visualize the increase in acetylation.

254 Some peptides showed post-transductional modifications, and these peptides are listed in
255 Table 3. As expected, L-lysine appears to be the predominant acetylated amino acid in
256 the peptide sequence, although arginine and histidine residues were found to be
257 acetylated as well.

258 The increase in acetylation can be clearly seen in Table 4, which shows the increase in
259 the acetylation ratio in the peptides identified in both groups. The student's t-test was

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applied, and the difference was significant with $p < 0.0001$, demonstrating that there was a significant increase of the acetylation in these peptides.

Analysis of the distribution of acetylated proteins within the subcellular localization revealed that they were predominantly located in the cellular membrane and cytoplasm (53%). Nineteen percent are known to be present in the nucleus and 12% in the cytoskeleton, while 14% in different organelles, including mitochondria and endoplasmatic reticulum.

Protein Interaction analysis

Figure 2 consists of the acetylated protein network from treated group. This network represents this protein interaction. Nodes represent the proteins in the network, and each color represents a different situation in relation to protein acetylation, while the edges represent the interactions between the proteins.

Orthology analyses

In order to reveal the involved cellular and metabolic processes as well as the subcellular location of the differentially expressed proteins in acetylation level with 2,3-butanedione treatment, the GO-based analysis was conducted.

Analysis of the Molecular Function (Fig 3A) revealed catalytic activity (57%), followed by specific binding function (19%). The analyses of biological functions (Figure 3B) indicated some processes in which acetylated proteins are involved, including cellular processes (28.6%) and responses to stimulus (14.3%). The top three protein classes (Fig 3C) display hydrolase (19%), chaperone (14.3 %) and oxidoreductase (14.3 %) activities. The cellular component analyses (Fig 3D) demonstrated that acetylated

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proteins belong to macromolecular complexes (9.5%), cell organelles (9.5%), extracellular region (4.8%) and other cell parts (19%).

Motif analysis of proteins containing arginine-, lysine- and histidine-acetylated peptides

In order to characterize the possible specific sequence motifs surrounding acetylated arginine, lysine and histidine residues in peptides of lung samples , a logo sequence to compute the likelihood of amino acids at the positions surrounding the acetylation site was generated. Ten significantly enriched motifs were obtained from all the identified acetylated sites including *K, *R, *H (*K represents the acetylated lysine, *R represents the acetylated arginine and *H represents the acetylated histidine). As shown in Figure 4, logos with the highest scores were used and all motif analyses are available in SM 2-7. Figures 4A and 4B show the motif surrounding acetylated arginine in samples from the control and treated groups, respectively, and Figs 4C and 4D show the motif surrounding acetylated lysine. A number of reports have already demonstrated the occurrence of acetylation in arginine residue [26]. Figures 4E and 4F portray the motif surrounding acetylated histidine from control and groups treated with diacetyl respectively.

Western blotting

Western blotting experiments indicated that the acetylation level was significantly higher in the treated group as compared to the control group (Figure 5). Figure 5A shows an increase in acetylation in bands that correspond between 35-70 KDa in lanes 5,7 and 8 that were filled with samples from the group treated with 2,3-butanedione. The wells filled with samples from the control group (lanes: 2-4) did not display the acetylation band.

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306 Statistical analysis by the Student t-test revealed that mean values of protein intensities
307 and variances are significantly different, with p= 0.0091 for means and p= 0.0015 for
308 variance. This Western blotting experiment data confirms the result of LC-MS/MS
309 analysis, which revealed increases in protein acetylation from the group treated with
310 2,3-butanedione in comparison with the control group.

311

312 **Discussion**

313 **Protein extraction optimization**

314 Figure 1 shows the amount of proteins obtained from precipitated and resolubilizated
315 proteins using three buffers. Buffers 1 and 2 were slightly more efficient than buffer 3,
316 which led us to choose buffer 2 in all experiments.

317 **MS/MS analyses of diacetyl-promoted acetylation of rat lung proteins**

318 The results from tables 2 through 4 provide evidence of an increase in protein acetylation
319 in the group treated with diacetyl. Acetylation reportedly alters protein function, size,
320 enzymatic activity, stability, protein-protein interactions and other protein properties.
321 Some proteins regulate acetyltransferases and histone deacetylases and may induce
322 acetylation of other proteins. [27] When acetyltransferases are deregulated, and lysine
323 acetylation is increased, modifications may occur in genes and the regulatory machinery.
324 [14] These data show that diacetyl- triggered protein acetylation takes place in different
325 cell compartments and that it may be implicated in many cell functions.

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326

327 **Protein Interaction analysis**

328 The protein interaction analysis showed that some acetylated proteins are interconnected
329 and/or connected with other proteins. To exemplify this interaction, we can cite the
330 protein ASS1, found to be acetylated in the treated group, interacts with both ALS and
331 OTC. Present in our control group is the ALS enzyme, whose activity is regulated by
332 acetylation, according to www.uniprot.org. ASS1 interacts with OTC, which is present
333 only in the diacetyl-treated group. OTC, one of the enzymes of the urea cycle, acts by
334 detoxifying the excess of ammonium produced from amino acid catabolism and is
335 negatively regulated by lysine acetylation. [28]

336 Some acetylated proteins present in the network (figure 2) are involved in the cell redox
337 balance [14], in protein biosynthesis and has ATP and nucleotide binding activity,
338 maturation, structural maintenance and regulation of specific proteins [29], along with
339 cellular processes such as the basal metabolism, immunogenicity, cell cycle progression,
340 DNA repair and apoptosis. [30] Some proteins also induce anti-tumor immunity by
341 inhibiting angiogenesis and have antioxidant activity in neurons and the heart, protecting
342 against cell death. [31-32] Additionally, the proteins play a cytoprotective role being a
343 redox-responsive protein. [33]

344 The increase in chemical acetylation of lung proteins of diacetyl-treated rats described
345 here may be connected with the fact that diacetyl has been shown in vitro to generate
346 acetyl radicals upon reaction with peroxynitrite, and more slowly with hydrogen
347 peroxide. [13] The diacetyl/peroxynitrite system was then reported to promote
348 acetylation of isolated aminoacids, peptides and albumin. These data led us to postulate

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that post-translational chemical acetylation of proteins may contribute to enzymatic acetylation at sites where both diacetyl and peroxynitrite at inflammation are formed.

Orthology analyses

The Gene Ontology (GO) function analysis of the target proteins revel the distribution and function of these proteins as show inFigure 3. Protein acetylation regulates enzyme activities that mediate, for instance, the degradation of proteasomes and lysosomes by neutralizing the lysine residues in the active sites, thereby causing conformational changes. In addition to regulating the catalytic activity of metabolic enzymes, acetylation controls substrate accessibility, blocks substrate binding to the enzyme and modulates enzyme subcellular localization. [34]

The most crucial pathways are those related to the oxidative stress response (P00046), which causes cellular damage. In a normal functioning cell, several transcription factors respond to oxidative stress by modulating the expression of genes whose products relieve the altered redox status.

Motif analysis of proteins containing arginine-, lysine- and histidine-acetylated peptides

Figure 4 shows the possible motifs surround acetylated arginine, lysine and histidine. Despite lysine being the more common site of protein acetylation, some studies have demonstrated that arginine can be acetylated as well, triggering biological responses. [35-36] Acetylation in both lysine and histidine residue was previously demonstrated in vitro, [10-13] which reinforces our results about acetylation in these residues.

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370 **Western blotting**

371 Western blotting was used to confirm the increase of acetylation previously found by
372 NanoLc-ms/ms experiments. We used a specific acetylation antibody to detect bands
373 with a substantial increase in intensity in samples from the group treated with diacetyl
374 that reveals the protein acetylation increase. Figure 5 shows clear differences in
375 expression from lanes 2 to 4 from control group, and lanes 5, 7, and 8 from the group
376 treated with diacetyl. These results confirm the protein acetylation identified by NanoLc-
377 ms/ms analyses.

378 **5. CONCLUSIONS**

379 Altogether, our data strongly suggest that diacetyl gavage administered to rats may
380 constitute a source of acetyl radical that can attack and acetylate lung proteins. It is
381 tempting to hypothesize this is a contribution mechanism for the reported toxicity of
382 diacetyl to workers dealing with 'buttered' food who acquire bronquiolitis obliterans.

383 Herein, we first optimized extraction conditions for the lung proteins of Wistar rats, for
384 both rats in the control group and those treated with diacetyl (Fig.1). Afterwards, mass
385 spectrometry analysis and western blotting analyses were employed to identify and
386 quantify the proteins that are preferentially acetylated in the lung tissue, which were
387 principally modified in the diacetyl-treated animals.

388 Mass spectrometry analysis and western blotting analysis were employed to identify and
389 quantify the proteins that are preferentially acetylated in the lung tissue, which were
390 principally modified in the diacetyl-treated animals.

391 The proteins acetylated to different extents in the diacetyl-treated group were then
392 related to reported interactions with other key proteins and enzymes of cell homeostasis.

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393 Diacetyl treatment, apparently, modifies the lung protein profile. Twenty-three diverse
394 classes of proteins were found to undergo preferential acetylation (Fig 2). They are
395 present in different regions of the cell and are involved in different molecular and
396 biological processes. Our data indicate that the observed increased radical acetylation by
397 diacetyl occurs randomly (Fig 3).

398 In a comprehensive view, we found more peptides acetylated in the group treated with
399 diacetyl than the control group. The expected acetylation of lysine residues also occurred
400 in arginine and histidine, suggesting that unlike acetylase-driven acetylation of proteins,
401 radical acetylation occurs randomly, modifying residues of both the N-terminal, the C-
402 terminal and the side chain of basic amino acid residues (Fig 4). The Western blotting
403 analysis clearly demonstrated increased protein acetylation due to the daily intake of
404 diacetyl (Fig 5).

405 Our study is consistent with early *in vitro* studies that showed increases in protein
406 acetylation in the presence of 2,3-butanedione. The data reported here reinforce our
407 hypothesis that diacetyl exposure is capable of increasing protein acetylation *in vivo*, thus
408 raising a potential for diacetyl, a highly electrophilic α-dicarbonyl industrial xenobiotic,
409 to play a role in inflammatory bronquiolitis obliterans [37-38].

410

411 **DECLARATIONS**

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414 and Brazilian Innovation Agency (FINEP).

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AVAILABILITY OF DATA AND MATERIALS

Data generated and analyzed during this study are included in this published article (and in supplementary information files) or are available in the [PRIDE] repository, [https://www.ebi.ac.uk/pride/archive/]

AUTHORS' CONTRIBUTIONS

EJHB and NAA developed and framed the scientific questions. EJHB, EC and NAA designed the experiments. LDLJ, SBG and NAA carried out the proteomic studies, including sample preparation, mass spectrometry data acquisition and analysis of proteomics data. AMB, GBN and LF carried out the western blotting studies. All authors helped to draft the manuscript. LDLJ, GBN, AMB, LF and NAA analyzed the data. LDLJ, EJHB, RGL and NAA wrote the manuscript. LDLJ and NAA take full responsibility for the integrity of the data analysis. All listed authors were contributed to this research work, read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was approved by the Ethical Committee of the School Medicine of the Federal University of São Paulo (UNIFESP, protocol no. 1949-11).

The consent to participate is not applicable.

CONSENT FOR PUBLICATION

Not applicable.

438 ***COMPETING INTERESTS***

439 The authors declare that they have no competing interests.

440 **LIST OF ABBREVIATIONS**

441 ANXA2, Annexin-A2; ANXA5, Annexin-A5; ASS1, Argininosuccinate synthase;
442 BHMT1, Betaine-homocysteine S-methyltransferase-1; CALR, Calreticulin; CHAPS,3-
443 [(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ES1D, Carboxylesterase-
444 1D; DTT, Dithiothreitol; DNAH1, Dynein heavy chain-1; EF2K, Eukaryotic elongation
445 factor-2 kinase; HSP90B1, Endoplasmin; EPHA6, Ephrin type-A receptor; FABPL,
446 Fatty acid-binding protein; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase;
447 MGEA5, O-GlcNAcase; PARK7, Deglycase DJ-1; PARS2, Protein Probable proline-
448 tRNA ligase; PBS, Phosphate-buffered saline; PMSF, Phenylmethylsulfonyl fluoride;
449 SIPA1L1, Signal-induced proliferation-associated 1-like protein-1; STIP1, Stress-
450 induced-phosphoprotein-1; TUBB4B, Tubulin beta-4B chain; TFA, Trifluoroacetic
451 acid; UBR4, Ubiquitin-protein ligase; UGT2B, UDP-glucuronosyl- transferase-
452 2B2.

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Table 1(on next page)

Composition of the tested buffers.

Composition	**Buffer 1***	**Buffer 2****	**Buffer 3*****
Buffering agent	Tris 25 mM	Tris 25 mM	Tris 25 mM
Surfactant	CHAPS 2%	CHAPS4% TRITON 1%	CHAPS 4%
Protease inhibitors	Aprotinine, leupeptine, pepstatine, benzamidine and PMSF	Aprotinine, leupeptine, pepstatine, benzamidine and PMSF	Aprotinine, leupeptine, pepstatine, benzamidine and PMSF
Chaotropes			7 M Urea
			2 M Thiourea
Reducer			65 mM DTT

* Buffer 1 with power low solubility (few surfactants without chaotropes);** buffer 2 with median solubilizing power (more surfactants); ***buffer 3 with maximum solubilization power (presence of chaotropes, surfactants and reducers).

Table 2(on next page)

Target proteins

Target proteins, acetylated proteins in group treated with diacetyl but non-acetylated in control group.

Protein I.D.	Gene I.D.	Meta Score Control	Meta Score Treated	Peptides Control	Peptides Treated	*SC [%] Control	*SC [%] Treated
AL1A1_RAT	AL1A1	269.1	253.5	11	9	37.1	31.7
ANXA2_RAT	ANXA2	539.6	284.6	13	13	44.5	43.7
ANXA5_RAT	ANXA5	411	265	10	10	37.3	43.6
ASSY_RAT	ASS1	71	1030.1	3	21	20.6	64.3
BHMT1_RAT	BHMT1	172.3	834.8	7	24	30	63.1
CALR_RAT	CARL	277.1	607.2	8	13	31.7	49.5
CES1D_RAT	CES1D	598.6	571.1	15	16	38.8	50.8
DYH1_RAT	DNAHC1	1391.9	1603.7	77	89	23.5	27.7
EF2K_RAT	EEF2K	354	304.5	18	17	38.5	34.1
ENPL_RAT	HSP90B1	742.6	492.2	25	16	29.6	23.5
EPHA6_RAT	EPHA6	486.4	361.4	27	20	32.4	24.5
FABPL_RAT	FABPL	120.5	779.4	3	16	36.2	81.9
G3P_RAT	GAPDH	486.7	763	14	18	53.8	62.2
OGA_RAT	MEGEA5	308.5	353.6	16	19	26.7	31.6
PARK7_RAT	PARK7	173.3	187.5	4	10	28.6	51.3
PRC2A_RAT	PRRC2A	776.2	670.4	43	36	25.3	23.6
SI1L1_RAT	SI1L1	575.9	672.1	32	37	27.9	30.7
STIP1_RAT	STIP1	189.5	258.9	10	14	22.1	30
SYPM_RAT	PARS2	171.2	183.4	9	10	32.6	46.3
TBB4B_RAT	TUBB4B	801.7	463.1	20	15	52.1	48.8

TPP1_RAT	TPP1	99.3	109.3	5	6	9.9	11.7
UBR4_RAT	UBR4	1600.3	1813.1	90	103	24.2	30.2
UD2B2_RAT	UGT2B	390.2	705.3	21	25	44.7	59.1

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Table 3(on next page)

Peptides from acetylated proteins

Peptides from acetylated proteins in group treated with diacetyl but non-acetylated in control group.

Protein I.D.	Gene I.D.	Peptide Sequence	Peptide Meta Score Control	Peptide Meta Score Treated	Acetylation Treated Group
AL1A1_RAT	ALDH1A1	-.MSSPAQPAVPAPLANLKIQHTK.I	15.4	16.1	7; 20; 22
ANXA2_RAT	ANXA2	K.ELPSAMKSALSGHLETVMLGLL K.T	15	21.7	3; 23
ANXA2_RAT	ANXA2	K.ELPSAMKSALSGHLETVMLGLL K.T	15	16.6	3; 23
ANXA2_RAT	ANXA2	K.GVDEVTIVNILTNR.S	71.9	18.2	14
ANXA2_RAT	ANXA2	K.SALSGHLETVMLGLLK.T	94	18.6	6
ANXA5_RAT	ANXA5	K.YMTISGFQIEETIDRETSGNLENL LLAVVK.S	16.4	16.7	15
ANXA5_RAT	ANXA5	K.YMTISGFQIEETIDRETSGNLENL LLAVVK.S	16.4	17.6	5; 30
ASSY_RAT	ASS1	R.GIYETPAGTILYHAHLDIEAFTM DR.E	39.8	16.1	13; 5
BHMT1_RAT	BHMT	R.IASGRPYNPSMSKPDAWGVTK.G	16.3	17.5	5
BHMT1_RAT	BHMT	R.IASGRPYNPSMSKPDAWGVTK.G	15.4	15.4	21; 30
CALR_RAT	CALR	K.HEQNIDCGGGYVK.L	33	85.7	13
CES1D_RAT	CES1D	K.GKVLGK.Y	24.4	15.1	2
CES1D_RAT	CES1D	R.SHRDAGAPTFMYEFEYRPSFVSA MRPK.T	18.5	22.7	2; 25
CES1D_RAT	CES1D	R.SHRDAGAPTFMYEFEYRPSFVSA MRPK.T	18.5	15.7	2; 7; 25
DYH1_RAT	DNAH1	R.SSLTRLASHMAEYECFQVELSK. N	19	16.7	5
EF2K_RAT	EEF2K	R.SGDLYTQAAEAAMEAMK.G	30.7	21.1	7
ENPL_RAT	HSP90B1	R.MMKLIINSLYK.N	18.8	16.1	1; 3
EPHA6_RAT	EPHA6	R.EASIMGQFDHPNIIRLEGVVTK.R	18.3	16.8	0; 5
EPHA6_RAT	EPHA6	K.SVTEFNGDTITNTMTLGDIVYK. R	28.2	50.8	22
FABPL_RAT	FABPL	K.SVTEFNGDTITNTMTLGDIVYK. R	16	36.2	22
FABPL_RAT	FABPL	K.YQVQSQENFEPFMK.A	28.2	33.9	4
G3P_RAT	GAPDH	K.RVIISAPSADAPMFVMGVNHEK. Y	18.6	23.1	1; 20; 22
G3P_RAT	GAPDH	K.RVIISAPSADAPMFVMGVNHEK. Y	18.6	15.8	20; 22
G3P_RAT	GAPDH	K.RVIISAPSADAPMFVMGVNHEK. Y	18.6	15.8	20; 22
G3P_RAT	GAPDH	K.RVIISAPSADAPMFVMGVNHEK. Y	18.6	23.1	1; 20; 22
OGA_RAT	MGEA5	K.LDQVSQFGCRSFALLFDDIDHN MCAADK.E	20	15.4	21; 28
PARK7_RAT	PARK7	K.GAEEMETVIPVDIMR	28.2	16.1	5; 6

Protein I.D.	Gene I.D.	Peptide Sequence	Peptide Meta Score Control	Peptide Meta Score Treated	Acetylation Treated Group
PRC2A_RAT	PRRC2A	K.ALYPGALGRPPPMPPMNFDPRW MMIPPYVDPR.L	17.9	30.3	9
PRC2A_RAT	PRRC2A	K.ALYPGALGRPPPMPPMNFDPRW MMIPPYVDPR.L	17.9	18.9	9
PRC2A_RAT	PRRC2A	K.ALYPGALGRPPPMPPMNFDPRW MMIPPYVDPR.L	17.9	16.6	21
PRC2A_RAT	PRRC2A	K.ALYPGALGRPPPMPPMNFDPRW MMIPPYVDPR.L	17.9	26.6	21; 32
PRC2A_RAT	PRRC2A	K.ALYPGALGRPPPMPPMNFDPRW MMIPPYVDPR.L	17.9	19.4	32
PRC2A_RAT	PRRC2A	K.ALYPGALGRPPPMPPMNFDPRW MMIPPYVDPR.L	17.9	15.1	9
PRC2A_RAT	PRRC2A	K.AVGTPGGNSGGAGPGISTMSRG DLSQR.A	18.4	22	21; 27
PRC2A_RAT	PRRC2A	R.ERSDSGGSSSEPFER.H	17.1	15.4	15
SI1L1_RAT	SIPA1L1	K.EKSKPYPGAELSSMGAIVWAVR. A	15.6	19.4	2
SI1L1_RAT	SIPA1L1	K.SLPLRRPSYTLGMK.S	19.5	16.8	5
STIP1_RAT	STIP1	R.RAMADPEVQQIMSDPAMR.L	20.7	18.9	1; 8
STIP1_RAT	STIP1	R.RAMADPEVQQIMSDPAMR.L	20.7	20.4	8
SYPM_RAT	PARS2	K.GIEVGHTFYLGTKYSSIFNAHFT NAHGESLLAEMGCYGLGVTR.I	17.8	15	21; 26
TBB4B_RAT	TUBB4B	R.INVYYNEATGGKYVPR.A	21.9	15.4	6; 12
TPP1_RAT	TPP1	R.EREPELAQLLVDQIYENAMIAAG LVDDPR.A	15.2	19.3	29
TPP1_RAT	TPP1	R.INTLQAIWMMDPK.D	15.9	15.1	3
UBR4_RAT	UBR4	K.ALGTLGMTTNEKGQVVTK.T	15.7	21.7	2
UBR4_RAT	UBR4	K.EKAAPPPPPPPPLESSPR.V	18.3	18.1	2; 9
UBR4_RAT	UBR4	K.EKEGESSGSQEDQLCTALVNQL NR.F	17.1	16.7	2; 24
UBR4_RAT	UBR4	K.FLSRPALPFILRLLR.G	15.1	30	5; 12
UBR4_RAT	UBR4	R.DNPEATQQMNDLIIGKVSTALK. G	28.8	17.2	6; 22
UBR4_RAT	UBR4	R.DNPEATQQMNDLIIGKVSTALK. G	17.3	21	22
UBR4_RAT	UBR4	R.MAGVMAQCGGLQCMLNRLAG VK.D	19.3	23.9	7
UBR4_RAT	UBR4	R.TGSTSSKEEDYESDAATIVQK.C	19.4	17.3	7; 21
UD2B2_RAT	UGT2B	K.EWDTFYSEILGRPTTVDETMSKV EIWLIR.S	15.2	16.8	12; 22

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Table 4(on next page)

Ratio of acetylation

Ratio of acetylation in both groups: control and treates with 2,3-butanedione

Protein I.D.	Gene I.D.	Peptide Sequence	Acetylation Ratio Control group	Acetylation ratio treated group
AL1A1_RAT	ALDH1A1	-.MSSPAQPAVPAPLANLKIQHTK.I	1	3
ANXA2_RAT	ANXA2	K.ELPSAMKSALSGHLETVMLGLLK.T	0.5	0.666666667
ANXA2_RAT	ANXA2	K.GVDEVTIVNILTNR.S	0	1
ANXA2_RAT	ANXA2	K.SALSGHLETVMLGLLK.T	0	1
ANXA5_RAT	ANXA5	K.YMTISGFQIEETIDRETSGNLENLLLAVVK.S	0	1
ASSY_RAT	ASS1	R.GIYETPAGTILYHAHLDIEAFTMDR.E	0	2
BHMT1_RAT	BHMT	R.IASGRPYNPSMSKPDAWGVTK.G	0	2
CALR_RAT	CALR	K.HEQNIDCGGGYVK.L	0	1
CES1D_RAT	CES1D	K.GKVLGK.Y	0	1
CES1D_RAT	CES1D	R.SHRDAGAPTFMYEFEYRPSFVSAMRPK.T	2	1.5
DYH1_RAT	DNAH1	R.SSLTRLASHMAEYECFQVELSK.N	0	1
EF2K_RAT	EEF2K	R.SGDLYTQAAEAAMEAMK.G	0	1
ENPL_RAT	HSP90B1	R.MMKLIINSLYK.N	0	2
EPHA6_RAT	EPHA6	R.EASIMGQFDHPNIIRLEGVVTK.R	0	2
FABPL_RAT	FABPL	K.SVTEFNGDTITNTMTLGDIVYK.R	0	1
FABPL_RAT	FABPL	K.YQVQSQENFEPFMK.A	0	1
G3P_RAT	GAPDH	K.RVIISAPSADAPMFVMGVNHEK.Y	1	1.5
OGA_RAT	MGEA5	K.LDQVSQFGCRSFALLFDDIDHNMCAADK.E	0	2
PARK7_RAT	PARK7	K.GAEEMETVIPVDIMR	1	0.5
PRC2A_RAT	PRRC2A	K.ALYPGALGRPPPMPPMNF	1	0.5

Protein I.D.	Gene I.D.	Peptide Sequence	Acetylation Ratio Control group	Acetylation ratio treated group
		DPRWMMIPPYVDPR.L		
PRC2A_RAT	PRRC2A	K.AVGTPGGNSGGAGPGISTMSRGDLSQR.A	0	2
PRC2A_RAT	PRRC2A	R.ERSDSGGSSSEPFER.H	0	1
SI1L1_RAT	SIPA1L1	K.EKSKPYPGAELSSMGAIVWAVR.A	0	1
SI1L1_RAT	SIPA1L1	K.SLPLRRPSYTLGMK.S	0	1
STIP1_RAT	STIP1	R.RAMADPEVQQIMSDPAMR.L	0	1
SYPM_RAT	PARS2	K.GIEVGHTFYLGTKYSSIFNAHFTNAHGESLLAEMGCYGLGVTR.I	1	2
TBB4B_RAT	TUBB4B	R.INVYYNEATGGKYVPR.A	0	2
TPP1_RAT	TPP1	R.EREPELAQLLVDQIYENAMIAAGLVDDPR.A	0	1
TPP1_RAT	TPP1	R.INTLQAIWMMDPK.D	0	1
UBR4_RAT	UBR4	K.ALGTLGMTTNEKGQVVTK.T	0	1
UBR4_RAT	UBR4	K.EKAAPPPPPPPPLESSPR.V	0	2
UBR4_RAT	UBR4	K.EKEGESSGSQEDQLCTALVNQLNR.F	1	1
UBR4_RAT	UBR4	K.FLSRPALPFILRLLR.G	0	2
UBR4_RAT	UBR4	R.DNPEATQQMNDLIIGKVSTALK.G	2	3
UBR4_RAT	UBR4	R.MAGVMAQCGGLQCMLNRLAGVK.D	0	1
UBR4_RAT	UBR4	R.TGSTSSKEEDYESDAATIVQK.C	0	2
UD2B2_RAT	UGT2B	K.EWDTFYSEILGRPTTVDETMSKVEIWLIR.S	0	2
		Mean	**0.283783784± 0.092 (27)***	**1.423423423± 0.104 (27)***

*** Mean ± Std. Error(N)**

Figure 1(on next page)

Comparison of the amount of total protein.

Comparison of the amount of total protein present in the lysate and the resolubilizated proteins (precipitate proteins resuspended) using lung tissue.

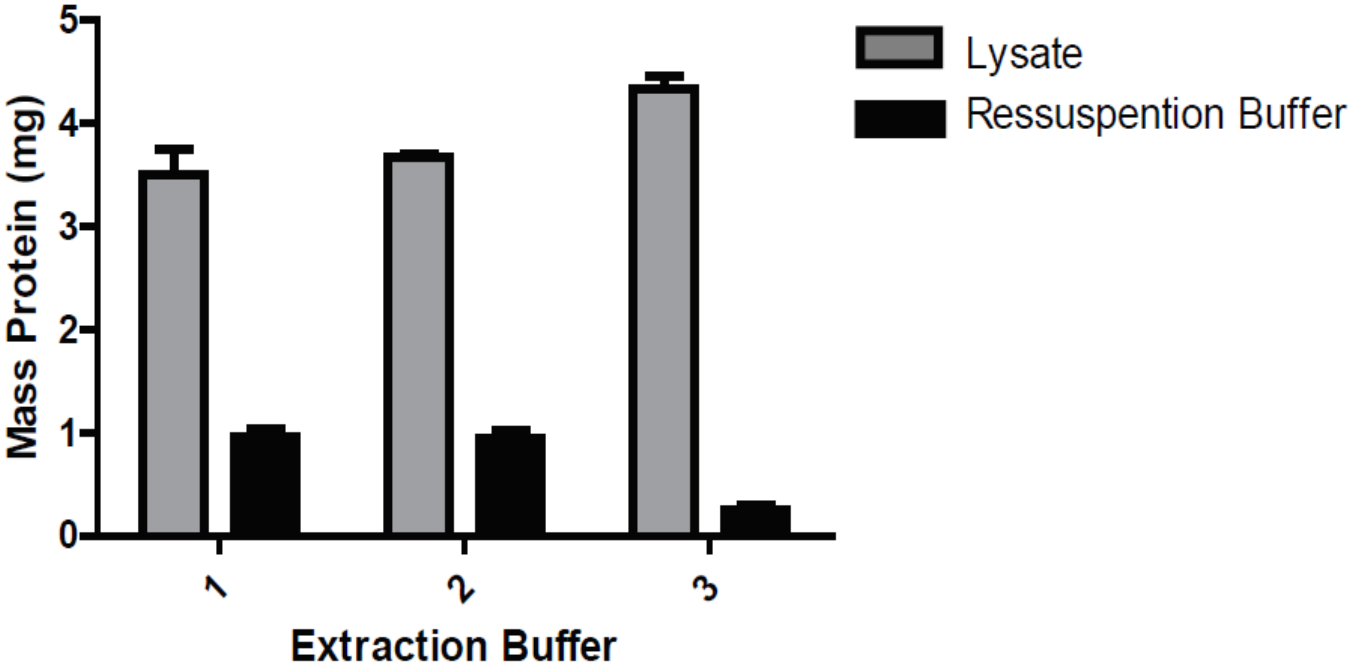

Figure 2(on next page)

Network of acetylated proteins in group treated with Diacetyl.

Network of acetylated proteins in group treated with Diacetyl. The green nodes are the proteins acetylated in group treated with Diacetyl but lack acetylation on treated groups. The red nodes are the proteins presents only in group treated with Diacetyl; the blue nodes are the proteins presents only in control group and the purple nodes are proteins present in both groups.

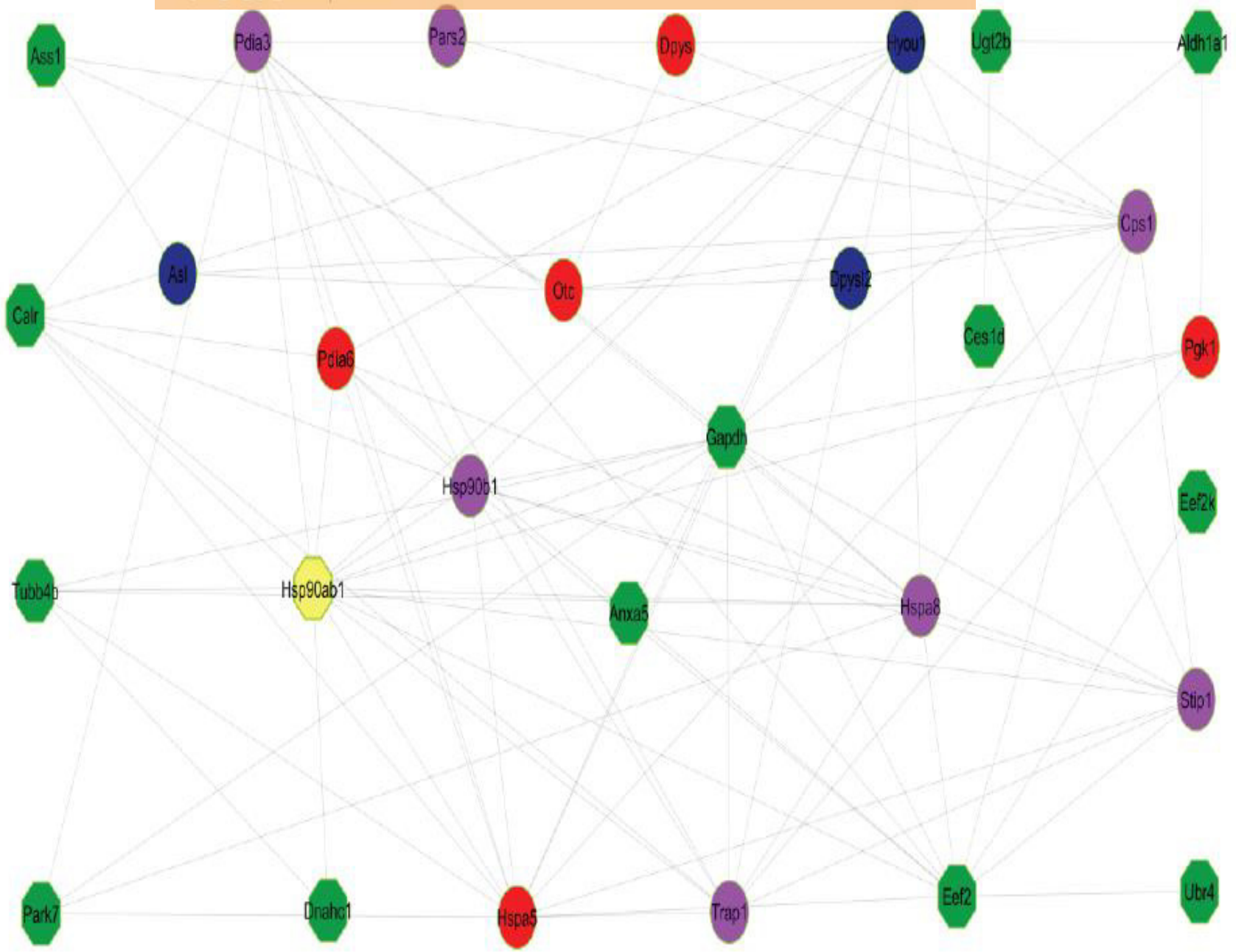

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Figure 3(on next page)

Orthology Analyses from more acetylated proteins in group treated with Diacetil than in control group.

Orthology Analyses from more acetylated proteins in group treated with Diacetil than in control group A) Molecular function. B) Biological process. C) Protein class D) Cellular component.

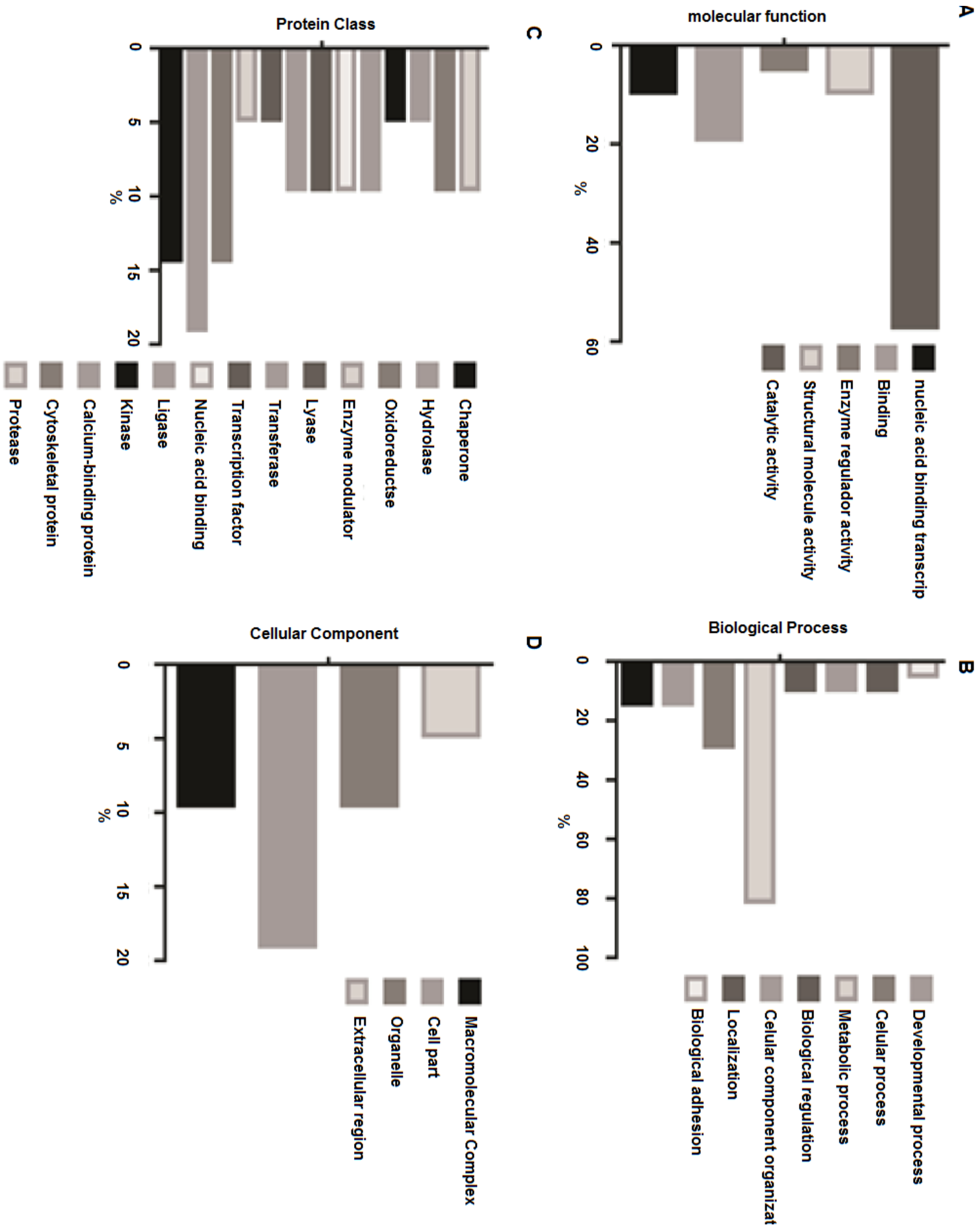

Figure 4(on next page)

Motif Analysis surrounding Arginine, Lysine and Histidine acetylated Peptides.

Motif Analysis surrounding Arginine, Lysine and Histidine acetylated Peptides. A) Motif Analysis of control group, surrounding acetylated Arginine B) Motif Analysis of group treated with diacetyl, surrounding Arginine. C) Motif Analysis of control group, surrounding acetylated Lysine. D) Motif Analysis of group treated with diacetyl, surrounding Lysine. E) Motif Analysis of control group, surrounding acetylated Histidine. F) Motif Analysis of group treated with diacetyl, surrounding Histidine.

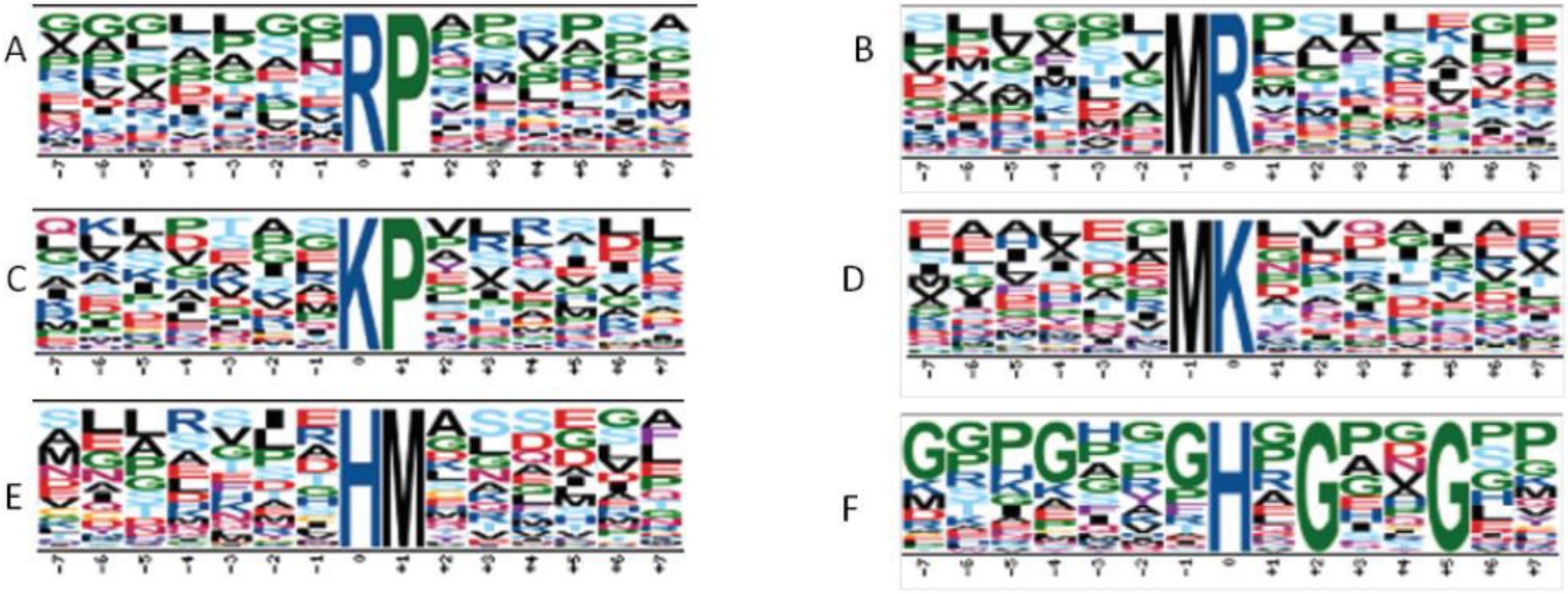

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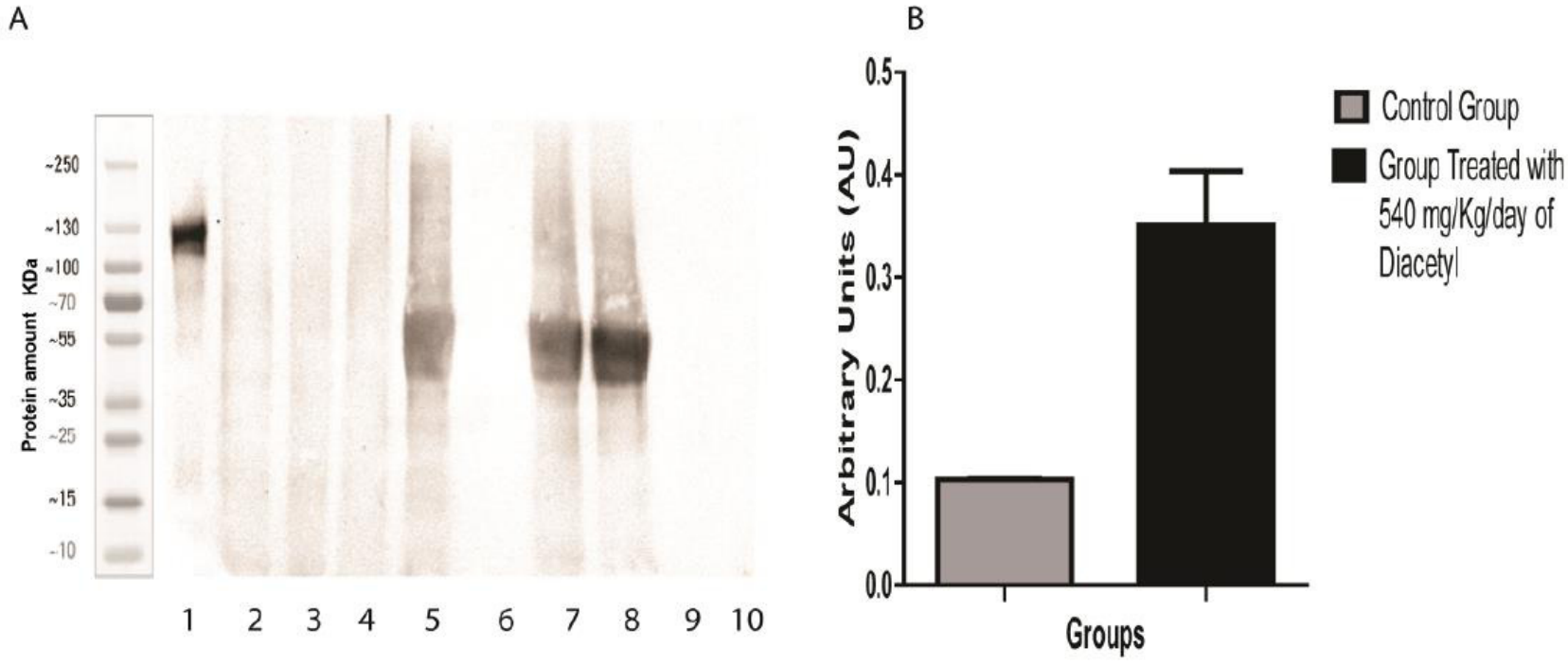
Figure 5(on next page)

Western blotting for acetylated proteins from lung samples.

Western blotting for acetylated proteins from lung samples. A) Western blotting 4 image: Lane 1: molecular weight; lanes 2–4: samples of the control group; lanes 5, 7, and 8: samples 5 of the diacetyl-treated group; and lanes 6, 9, and 10: sample buffer. B) Western blotting quantification



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