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The presence of modified nucleosides in extracellular fluids leads to the specific incorporation of 5-chlorocytidine into RNA and modulates the transcription and translation.

--Manuscript Draft--

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Cover Letter

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Original title: The presence of modified nucleosides in extracellular fluids leads to the specific incorporation of 5-chlorocytidine into RNA and modulates the transcription and translation.

Dear Prof. Dhalla,

Dear Editor-in-Chief,

We have the pleasure to submit a revised version of our manuscript entitled “The presence of modified nucleosides in extracellular fluids leads to the specific incorporation of 5-chlorocytidine into RNA and modulates the transcription and translation”.

Please find hereafter our point-by-point answers to the comments and suggestions of the referee. The manuscript has been carefully revised according to their comments and the modifications have been directly introduced in the revised version. Concerning the references, we also used the zip file from the website in order to encode references via endnote.

Prof. Pierre Van Antwerpen

(Corresponding author)

General comments:

The referee states that the manuscript shows an exposure of 2 model cell types, immortalized endothelial cells and primary epithelial prostatic cells, to chlorinated nucleosides results in the selective incorporation of 5-chlorocytidine into the RNA, which may influence the translation yield. It is shown that all the chlorinated compounds penetrate into the cells, yet only 5-chlorocytidine is incorporated to a significant extent into RNA and not DNA. The extent of incorporation differs between cells type, which may reflect a limitation of the study, whereby the length of incubation of each cell type with the chlorinated nucleosides is different, which will affect the extent of incorporation and / or turnover.

This general comment is more specifically discussed in the 'specific comments' stated below by the referee. We suggest looking at the point-by-point answer to the specific comments

Specific comments:

1) In respect to the ability of HOCl or MPO to chlorinate RNA / DNA within the cells, as the authors indicate, these experiments were performed in the presence of cell culture media and serum, which will have quenched the HOCl prior to it reacting with the cells. It is important to indicate the concentration of active chlorine species present on addition of HOCl to DMEM / serum in the absence of cells.

A: The HOCl solution was obtained after dilution from a stock solution of sodium hypochlorite (12 % chlorine; 150-182 g/L active chlorine according the manufacturer). The concentration was calculated by measuring extemporary the absorption of the diluted solution at 292 nm using 350 M^{-1} cm^{-1} as extinction coefficient. Based on this calculation, the volume of HOCl solution was added in the culture medium to obtain a final concentration of 300 µM which corresponds to 0.06 g/L of active chlorine species.

This value has been added to the manuscript in section 'Chlorination of nucleic acid in endothelial cells by HOCl or by MPO/H_2O_2/Cl^- (MPO system) between brackets.

2) The cell density and actual incubation time of the cells with chlorinated nucleosides for each cell type should be indicated - time taken to reach 20-50% confluence does not allow for easy comparison, and would be critical to the extent of incorporation and possibly removal by repair mechanisms.

A : The referee is right when complaining about the incubation time and clarification. Immortalized endothelial cells grow faster (24-48 h) than primary culture of prostatic cells (3-18 days). As mentioned in the manuscript, incorporation of ClCyt in RNA does not appear in prostatic cells unless the modified medium was changed 24h before while after 24 or 48 h of incubation time with endothelial cells, the incorporation was detected (table 2). This phenomenon could be explained by the half-life of mRNA which is between 5 and 15 h and the depletion of ClCyt cytoplasmic pool.
*In this context, it is difficult to compare both cellular models and consequently the phrase: "The ClCyt incorporation was 1.7 times higher for epithelial prostatic cells in comparison with endothelial cells (**see Table 2**)" in section **Cellular penetration and incorporation of modified nucleosides** has been removed.*

In order to clarify this point and to also answer to the point 4, we transform the figure 2 into table 2 where are written down the incubation times. The text has been also modified accordingly.

3) The authors report that RNA instability could account for lack of 5-chlorocytidine incorporation if fresh chlorinated nucleoside-containing media is not applied to the cells the day before lysis. An alternative is that deamination occurs, resulting in 5-chlorouracil - this ionizes poorly and is not usually identified by LC-MS.

A: In preliminary study, we firstly optimized the acquisition and quantification of 5-Cl(d)Uracil, because those modified nucleotides were expected according literature. The acquisition could be done in negative mode by LC-MSMS. Secondly, we oxidized DNA and mRNA by HOCl and the MPO system in order to select the nucleotides of interest. Despite our method was able to detect 5-Cl(d)Uracil, we were unable to detect them after direct oxidation and enzymatic hydrolysis. It remains unclear whether the deamination occurs on the nucleic acid or after release of the 5-

ClCyt in biological fluids, spontaneously or enzymatically. Moreover treatment by acid hydrolysis (prior to GC-MS) could be a cause of deamination of ClCyt in ClUracil; but we do not use this kind of treatment. Actually, we did not detect them after mRNA extraction and enzymatic hydrolysis. However, we suggest adding a sentence in the manuscript to take this possible phenomenon into account.

4) Figure 2 shows 3 figures each with only one bar showing 5-chlorocytidine incorporation. These data could be better displayed in a Table.

A: This modification has been applied and table 2 created. The figures were renumbered accordingly.

5) Expression of incorporation data as a ratio of modified / unmodified nucleosides makes the data difficult to compare back to previous studies, or studies where cells are exposed to HOCl / MPO systems as the more common way of expressing modification of nucleosides is as pmol / mol parent.

A: We agree with the referee. Actually, authors used different units expressing modifications of nucleosides:
Masuda et al. (2001) used numbers of lesions per 10^5 (d)Gua.
Badouard et al. (2005) used numbers of lesions per 10^6 cells.
Jiang et al. 2003 used mol ClUra/10^6 mol Thy.
Whiteman et al. (1999) used nmol/mg DNA.
…
We choose to calculate the ratio ClCyt/Cyt, first, to normalize results according to the amount of nucleic acid extracted which is provided by Cyt amount and to better compare different conditions. We could suggest to express this also in ppm and add this in table 2.

ORIGINAL ARTICLE

The presence of modified nucleosides in extracellular fluids leads to the specific incorporation of 5-chlorocytidine into RNA and modulates the transcription and translation.

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Abbreviated title: Specific incorporation of chlorocytidine into RNA.

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Keywords: myeloperoxidase; RNA; plasma; 5-chlorocytidine; transcription/translation

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Abstract

Myeloperoxidase (MPO) is able to promote several kinds of damage and is involved in mechanisms leading to various diseases such as atherosclerosis or cancers. An example of these damages is the chlorination of nucleic acids, which is considered as a specific marker of the MPO activity.

Aims: Since 5-chlorocytidine has been recently shown in healthy donor plasmas, this study aimed at discovering if these circulating modified nucleosides could be incorporated into RNA and DNA and if their presence impacts the ability of enzymes involved in the incorporation, transcription and translation processes.

Results: Experimentations, which were carried out *in vitro* with endothelial and prostatic cells, showed a large penetration of all chloronucleosides but an exclusive incorporation of 5-chlorocytidine into RNA. However, no incorporation into DNA was observed. This specific incorporation is accompanied by an important reduction of translation yield. Although, *in vitro*, DNA polymerase processed in the presence of chloronucleosides but more slowly than in control conditions, ribonucleotide reductase could not reduce chloronucleotides prior to the replication. This reduction seems to be a limiting step, protecting DNA from chloronucleoside incorporation.

Conclusion: This study shows the capacity of transcription enzyme to specifically incorporate 5-chlorocytidine into RNA and the loss of capacity -complete or partial- of different enzymes, involved in replication, transcription or translation, in the presence of chloronucleosides. Questions remain about the long-term impact of such specific incorporation in the RNA and such decrease of protein production on the cell viability and function.

Introduction

Although acute inflammatory reaction is a beneficial host defense mechanism, an inadequate resolution and uncontrolled inflammatory reactions can lead to chronic inflammatory syndromes and also to cancer development [1,2]. Indeed, a link between inflammation, oxidative stress and carcinogenesis (cancer of stomach, liver, prostate, pancreas, …) has been established for a long time [3-5]. Chronic inflammation induces alteration of various biochemical processes: (i) an imbalance of the cellular redox regulation toward oxidative stress, generating cellular ROS/RNS, (ii) an increase of DNA damages, inactivation of DNA repair mechanisms and induction of genomic instability, (iii) a stimulation of cell proliferation, angiogenesis and metastasis by kinases and transcription factors activation and by tumor suppressor genes inactivation, (iv) a deregulation of cellular epigenetic control in gene expression and (v) an inappropriate epithelial-to-mesenchymal transition [1]. All these alterations are involved in carcinogenesis and can be illustrated by so many concrete examples. Among factors identified into these altered processes, an enzyme is often cited, myeloperoxidase (MPO) [1].

This hemic enzyme is mainly contained in azurophilic granules of neutrophil [6]. MPO is able to produce hypochlorous acid (HOCl) in the presence of chloride anions (Cl^-) and hydrogen peroxide (the MPO/H_2O_2/Cl^- system) conferring an antimicrobial action to it [2,7,8]. However, MPO is also present in extracellular fluids after degranulation of neutrophils (oxidative stress) or associated with neutrophil extracellular trap [9,10]. When MPO diffuses, its oxidative products, such as hypochlorous acid, are able to react with different targets, as proteins, lipids, DNA and RNA [11-13]. Several chlorinated DNA/RNA products from MPO activity have been shown [14-16]. Henderson et al. (1999) suggested that HOCl is an intermediate in 5-chloro-2'-deoxycytidine production by the MPO/H_2O_2/Cl^- system. Furthermore, Stanley et al. (2010) showed that the cellular genetic material can be chlorinated

by HOCl and N-chloramines, producing 8-chloro-2'-deoxyguanosine (CldGua), 5-chlorocytidine (ClCyt), 5-chloro-2'-deoxycytidine (CldCyt), 8-chloro-2'-deoxyadenosine (CldAdo) in DNA and RNA. Masuda et al. (2001) showed a major production of CldGua and ClCyt in isolated RNA and DNA.

The research of modified nucleobases in genetic material is an important aspect, especially as a sustained inactivation of the DNA repair pathways, caused by inflammation, contributes to cancer development. For instance, Gungor et al. (2007) highlighted MPO itself (from activated neutrophils) inhibits Nucleotide Excision Repair (NER) pathway in human alveolar epithelial cells using an *in vitro* inflammatory simulation [17] but this inhibition mechanism has not been confirmed *in vivo* yet [18]. Although MPO is mainly expressed in neutrophils and to a lesser extent in monocytes [6], aberrant MPO expression was observed in several diseases in non-myeloid cells, as epithelial ovarian cancer cells [19]. Recently, Roumeguere et al. (2012) localized for the first time MPO into secretory prostatic epithelial cells of patients suffering from prostatic lesions (cancer, prostatic intraepithelial neoplasia PIN, prostatitis and benign prostatic hyperplasia BPH) [20]. Now, acute inflammation in response to bacteria (bacterial prostatitis) induces a prostate epithelial cell proliferation by loss of key regulator (NKX3.1) expression and an increase in Gleason grade. This study highlighted a potential link between inflammation and neoplasia [21,3]. Nowadays, MPO role is not clearly involved in some diseases but it is important to assess its activity and consequences of chlorinated product generation.

Although its origin has not been known (in situ by oxidation, via circulating MPO or from degradation and elimination), ClCyt has been shown for the first time in plasma of healthy donors [22]. While into urines of diabetic patients, 8-halogenated dGua have been highlighted by Asahi et al. (2010). Given that circulating chlorinated nucleosides have never been studied, this project aimed at measuring the impact of the presence of these modified nucleosides in

extracellular fluids on replication, transcription and translation. For this reason, we determine if circulating chlorinated nucleosides could penetrate into cells and then incorporate into RNA and/or DNA. The ability of enzymes involved in the incorporation, transcription and translation processes were also tested in the presence of chlorinated nucleosides.

Material and methods

Materials and reagents

Ammonium acetate, LC/MS-grade methanol, hydrolysis enzymes, RIPA buffer, dithiothreitol (DTT), deoxy-nucleotides triphosphate (dNTP) mix for PCR, primers for PCR (66F and 255R), (2'-deoxy)cytidine ((d)Cyt), (2'-deoxy)guanosine ((d)Gua), 8-oxo-(2'-deoxy)guanosine (oxo(d)G) and internal standards (5-fluorocytidine and Labeled dGua) were purchased from Sigma Aldrich (Steinheim, Germany). Sodium hypochlorite (150-182 g/L active chlorine) was purchased from Carl Roth (Karlsruhe, Germany). Chlorinated nucleosides (Cl(d)Gua and Cl(d)Cyt) were obtained from BioLog Life Science Institute (Bremen, Germany). Protease inhibitors (cOmplete™ ULTRA Tablets) were purchased from Roche (Mannheim, Germany). RNeasy Mini Kit and DNeasy Blood & Tissue Kit were from Qiagen (Hilden, Germany). Human recombinant MPO (hrMPO; 121 U/mg) was produced as described by N. Moguilevsky [23]. HBSS, DMEM, DMEM-added antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL), human prostate epithelial cells PrEG and their medium PrEGM were purchased from Lonza (Verviers, Belgium). Additives for DMEM (10 % FBS, 2 % of a 50x concentrated HAT and 1 % of a 100x concentrated solution of non-essential amino acids) were obtained from GIBCO (Thermo Fisher Scientific). Endothelial cells EA.hy926 are an immortalized endothelial cell line derived from human umbilical veins HUVEC. Plasma samples were prepared from blood drown from healthy donors and dialyzed patients. This study is suitable to the Declaration of Helsinki and its protocol was approved by the Ethics Committee of the ISPPC ("Intercommunale de Santé Publique du Pays de Charleroi") Hospital. The transcription kit called mMessage mMachine T7 Ambion®, Phusion GC buffer and Phusion DNA polymerase were obtained from Thermo Fisher Scientific (Waltham, MA, United States). Rabbit reticulocyte lysates, amino acid solution (without methionine) and RNasine were purchased from Promega (Leiden, Netherlands). Radiolabeled methionine 35S-

Met was from PekinElmer (Waltham, MA, United States). SYBR green was obtained from Eurogentec (Seraing, Belgium).

Chlorination of nucleic acid in endothelial cells by HOCl or by MPO/H_2O_2/Cl^- (MPO system)

Endothelial cells (EA.hy926) were divided in wells and incubated at 37°C until 100% confluence point (1.10^6 of cells/well). Thereafter cells were incubated in triplicate with or without HOCl (300 µM; 0.06 g/L active chlorine species) in DMEM for 30 minutes or with or without hrMPO (200 ng/mL) and H_2O_2 (100 µM) in DMEM for 24h. DNA, RNA and cytoplasmic pools were extracted, hydrolyzed and analyzed by LC/MSMS.

All detailed protocols of sample handling (extraction, purification and hydrolysis) and LC/MSMS analysis have been previously published and validated in [22].

Extraction of cytoplasmic pool, RNA and DNA:

Briefly, cells were washed twice with Hank's Balanced Salt solution (HBSS) prior to extraction procedure. DNA and RNA were extracted and purified from cultured cells using respectively DNeasy and RNeasy kits and cytoplasmic pools using RIPA buffer and ACN precipitation (protein elimination).

Enzymatic hydrolysis or dephosphorylation and nucleoside analysis by LC/MSMS:

Enzymatic hydrolysis was performed for extracted DNA and RNA and dephosphorylation for free nucleotides from cytoplasm, as described in [22].

Briefly, DNA and RNA were digested into nucleosides in the presence of internal standards (Labeled dGua ($^{15}N_5$) and 5-fluorocytidine) using nuclease P1, PDE II, PDE I, alkaline phosphatase and appropriated buffers. Free nucleotides from cytoplasmic pool were dephosphorylated using alkaline phosphatase and appropriated buffers.

Thereafter, all samples were dried by vacuum centrifuge, dissolved in 50 µL aqueous mobile phase and 10 µL were injected into LC/MSMS (in dynamic MRM positive mode). Briefly, the analyses were performed using a LC/MS system from Agilent Technologies (Santa Clara, CA, USA): an Agilent 1290 Infinity Binary – UHPLC system fitted to a mass spectrometer Agilent Jet Stream electrospray ionization source (AJS) - Triple Quadrupole (QqQ) 6490 series. Nucleoside separation were performed at 4°C on Poroshell 120, EC-C18, 2.1 x 100 mm, 2.7 µm column, preceded by a Poroshell 120, EC-C18, 2.1 x 5 mm, 2.7 µm guard column, using an ammonium acetate 10 mM in water pH 5/methanol gradient. All these LC and MS parameters were detailed and validated in a previous article [22].

Plasma analysis

Two ml of blood were collected and centrifuged 10 min at 3000 g 4°C. Plasma is stored at -80 °C until analysis to avoid any further nucleotide modifications. Plasma (200 µL) from 18 healthy donors (aged 37 years old +/-10; 10 men, 8 women, non-smoker) and 19 elderly dialyzed patients before dialysis session (aged 77 years old +/- 12; 12 men, 7 women; 5 smokers) were purified by protein precipitation, which was performed by adding ACN (1800 µL) and centrifuged for 40 minutes at 11960 × g. Supernatant (1800 µL) was collected and evaporated by vacuum centrifuge, prior to be dephosphorylated and analyzed by LC/MSMS, as described and validated in [22].

Cellular penetration and incorporation of modified nucleosides

Briefly, prostatic and endothelial cells were grown in presence or absence of modified nucleosides before that cytoplasmic pool, RNA and DNA were extracted to be analyzed to determine cellular penetration and RNA or DNA incorporation.

Experimentations were carried out with endothelial EA.hy926 and epithelial prostatic cells PrEC in quadruplicate and were incubated in wells with the normal medium (DMEM or PrEGM, respectively) until 20 and 50 % of confluence. Thereafter, control cells were incubated in the presence of normal medium whereas treated cells were incubated in the presence of modified appropriated medium (supplemented with 500 nM standard solutions of Cl(d)Cyt, Cl(d)Gua and oxo(d)G). Consequently, utilization of modified medium for treated cells was applied from 20 or 50 % of confluence to 100 %. Given that growth speed depends on cellular types, medium was changed each day for endothelial cells and each three days for prostatic cells. When cells were at 100 % of confluence (~ 1.10^6 cells), they were washed thanks to HBSS and DNA, RNA and free nucleotides from cytoplasm were extracted, purified, hydrolyzed and injected in LC/MSMS, as described previously. Free nucleosides from cytoplasm were used to estimate penetration into cells whereas DNA and RNA were used to estimate incorporation into DNA or RNA.

In vitro **transcription and translation**

Briefly, nucleotides were partially chlorinated and used to perform plasmid transcription and mRNA translation. Transcription and translation yields were estimated by quantification of mRNA and proteins, respectively, and RNA composition was also determined by LC/MSMS.

Nucleotide chlorination:

Nucleotides triphosphate (NTP) mix from transcription kit (15 mM of each NTP) were chlorinated using HOCl (47 mM) in triplicate for 1h at 37 °C. HOCl concentration was estimated by spectrophotometry at 292 nm and calculated using 363.5 $M^{-1}cm^{-1}$ as the molar extinction coefficient. Controls were performed using Milli-Q water instead of HOCl. To neutralize the remaining HOCl, an excess of methionine (12.5 mM in this case) was added for

1h at 37 °C. This quantity of methionine was estimated by measurement of potential remaining HOCl by the taurine assay, as previously described by Van Antwerpen et al. [24]. Proportions of NTP and chloronucleotides triphosphate (ClNTP) were estimated using NTP calibration curves and an LC/MS system from Agilent Technologies (Santa Clara, CA, USA): an Agilent 1290 Infinity Binary - UHPLC system fitted to a mass spectrometer Agilent 6520 series electrospray ion source (ESI) – quadrupole Time-Of-Flight (QTOF). Nucleotides were separated using an X Bridge® BEH Amide column (Waters, 2.1 x 150 mm, 2.5 µm particle size) at 5 °C and a gradient of ACN/Ammonium bicarbonate 50 mM pH 6.0, as detailed in supplementary data (**Table S1**). This analysis was performed in positive mode in MS scan (100 – 950 m/z). Source parameters were: positive ion mode (ESI +); gas temperature of 300 °C; gas flow of 10 L/min; nebulizer pressure of 50 psig; capillary voltage of 4500 V and fragmentor 150 V.

Transcription, RNA quantification and RNA analysis:

Plasmid pcDNA3.1-apoL1-V5-His_6 (50 ng/µL) was transcribed in three independent experiments in the presence of normal NTP or ClNTP (7.5 mM), thanks to T7 enzyme mix and transcription buffer, following the manufacturer protocol (from mMessage mMachine® T7 kit). The transcription was operated at 37 °C for 2h. Thereafter, RNAs were purified by: (i) DNA digestion by adding DNAse (95 mU/µL) and incubation at 37 °C for 15 min, (ii) Stopping the reaction by adding ammonium acetate (0.5 M), (iii) Protein elimination by adding phenol/ chloroform 1:1 (v:v), mixing, centrifugation at 12000 × g for 10 min and supernatant harvesting (iv) Phenol elimination by adding chloroform/isoamyl alcohol (Ready red), mixing and centrifugation, (v) Single nucleotides elimination and RNA precipitation by adding isopropanol (2:1) to supernatant overnight at -20 °C and centrifugation at 12000 × g at 4°C for 10 min, (vi) precipitate, containing RNA, was washed with ethanol 75 % and

centrifuged and (vii) precipitate was dried and redissolved in 10 µL of water. After purification, the produced RNA was quantified by UV measurement using Nanodrop®. The incorporation of ClNTP in mRNA was measured after enzymatic hydrolysis [25] before being analyzed by LC/MSMS as previously described [22]. Another part of these purified RNAs was translated, as detailed in the following paragraph.

Translation and protein quantification:

Transcribed RNA produced in the presence of normal NTP or ClNTP (20 ng/µL) as described in the previous paragraph were translated in rabbit reticulocyte lysates in the presence of radiolabeled methionine ^{35}S-Met (142 µM), other amino acids (23 µM) and RNasine (0.92 U/µL). The translation was operated in three independent experiments at 30 °C for 1h. RNase (20 ng/µL) was added and incubation was proceeded for 15 min at 37 °C. Thereafter, the translation efficiency was quantified by measuring the amounts of synthesized radiolabeled protein, after purification on SDS-polyacrylamide gel. Each solution containing translated protein was diluted in Laemmli buffer (1/10) and purified on 4-15 % SDS-polyacrylamide gel. Gel was washed with 100 mL of TCA 10%/CH_3COOH 10%/CH_3OH 30% for 30 min, with 100 mL of water for 30 min and with 100 mL of sodium salicylate 1 M for 30 min. Finally, gel was dried on Whatman paper for 1h30 at 80 °C and placed on radiographic film for 2h. The band intensities were measured by optic density after scan and compared.

DNA polymerase activity

Deoxynucleotide chlorination:

Solution of dNTP mix (10 mM of each dNTP) were chlorinated using HOCl (27 mM) in quadruplicate for 1h at 37 °C. Controls were performed using Milli-Q water instead of HOCl. Quantification of remaining HOCl was estimated by the taurine assay, as described above,

and methionine was added in adequate quantity to neutralize remaining HOCl (4.3 mM) and incubated for 1h at 37 °C. Proportions of dNTP and chloro-deoxynucleotides triphosphate (ClddNTP) were estimated using an LC/MS system (QTOF) and dNTP calibration curves, as described above.

DNA polymerase activity: qPCR:

In four independent experiments, plasmid pcDNA3.1-apoL6-66-255 (0.25 ng/µL) was mixed with Primer 66F (5'ACGTACCATATGGACGAAAGCCACCAA3') (5 µM), Primer 255R (5'GTACGTCTCGAGACTTCCAGTCCTCCCAGA3') (5 µM), dNTP or chlorinated dNTP (0.2 mM), an intercalating fluorescent dye SYBR Green (30 µM), Phusion DNA polymerase (20 mU/µL), and Phusion GC buffer 5x. Then qPCR was performed on CFX96 Touch™ Real-Time qPCR Detection System (from Bio-rad Laboratories, Temse, Belgium) and 42 cycles were performed as follows: (i) initialization step at 98 °C for 30 sec, (ii) 42 successive cycles including denaturation step at 98 °C for 10 sec, hybridization step at 56 °C for 20 sec, elongation step at 72 °C for 30 sec, and (iii) ending phase of final elongation at 72 °C for 10 min.

DNA samples were purified by precipitation using ammonium acetate (2.5 M) and 3 volumes of ethanol 100 % was added (overnight at -20°C), prior to centrifugation 10 min at 13 500 × g. Then supernatants were removed, precipitates were washed with ethanol 75 % (500 µL), centrifuged, dried and redissolved in hydrolysis buffer. Purified samples were hydrolyzed and analyzed by LC/MSMS, as previously described [22].

Ribonucleotide reductase activity

Cytoplasmic fraction extraction and purification:

Given that ribonucleotide reductase is a cytoplasmic enzyme, EA.hy926 endothelial cells (± 10.10^6 cells) were washed twice with HBSS and cytoplasm was extracted after cell lysis using RIPA buffer (0.5 mL/1.10^6 cells) and protease inhibitor (50 µL/1.10^6 cells). Lysates were collected and centrifuged for 10 minutes at 200 × g at 4 °C. Then supernatant (4 mL) was retrieved and ultrafiltrated using a 5 kDa filter and sodium phosphate buffer 0.025 M pH 7.0 (10 volumes), prior to be lyophilized. Enzymatic fraction was redissolved in 1 mL of water.

CDP/CTP chlorination:

Solution of 150 µM of cytidine diphosphate (CDP) and cytidine triphosphate (CTP) were prepared and chlorinated using HOCl (150 µM) for 1h at 37°C. Controls were performed using Milli-Q water instead of HOCl. The taurine assay was performed to estimate the excess amount of HOCl, as described above. Proportions of chloro-cytidine diphosphate (ClCDP) and chloro-cytidine triphosphate (ClCTP) were estimated using an LC/MS (QTOF), as described above.

CDP/CTP reductase activity:

The CDP/CTP reductase activity was adapted from [26,27] and was assayed using CDP, ClCDP, CTP, ClCTP, CDP/CTP mix or ClCDP/ClCTP mix (3 µM), DTT (6 mM), ATP (2 mM), magnesium acetate (4 mM) and enzymatic fraction. DTT and ATP were dissolved in sodium phosphate buffer (0.1 mM, pH 7.0), enzymatic fraction was diluted 100 times and the reaction is operated in the same buffer. Several blanks were performed: (i) one blank in which reagents (CDP or CTP or chlorinated derivatives) were replaced by sodium phosphate buffer and (ii) 6 blanks (one per each condition) in which enzymatic fraction was replaced by sodium phosphate buffer. Thereafter all samples were incubated at 37 °C for 0 (T0), 24 (T24) or 48 hours (T48). All experimental conditions were performed in quadruplicate.

Results

Chlorination of endothelial cells by HOCl or by MPO/H_2O_2/Cl^- (MPO system)

In these experiments, EA.hy926 endothelial cells cultured in culture medium DMEM (with serum 10%) were used as a cellular model. Results of these experiments showed no effect of HOCl on nucleic acids of endothelial cells, in DNA or RNA as well as in cytoplasmic pools. Moreover, no chlorinated nucleoside was detected in DNA or RNA in the presence of the MPO/H_2O_2/Cl^- system.

Plasma analysis

Give these latter results, we focused on plasma analyses from volunteers and hemodialyzed patients in the view to estimate the chlorinated nucleosides homeostasis.

Analysis of plasma from volunteers and elderly patients encountering hemodialysis showed a significant increase for the levels of Cyt (2.9 fold), ClCyt (5.8 fold) and oxoG (2.71 fold) in patient plasmas than in volunteer plasmas (**Table 1**). These nucleosides seem to accumulate into plasma of hemodialyzed patients while levels of guanosine did not show any difference between both groups. Nevertheless, ratios of modified/unmodified nucleosides (%) showed an increase of oxoG/Gua and ClCyt/Cyt ratios in patient plasmas but this increase is only significant for oxoG/Gua ratio. Accumulation of Cyt into plasmas of hemodialyzed patients could explain the ClCyt ratios at the limit of the signification ($p = 0.06$).

Cellular penetration and incorporation of modified nucleosides

As chlorinated nucleosides (ClCyt) are present in plasma, we wondered whether circulating nucleosides were able to penetrate into cells and incorporate into DNA and RNA. In order to

address this question, we used two cellular models (immortalized endothelial cells and primary prostatic cells).

First, after incubation with the two cell types with modified medium (medium supplemented with standard solutions of modified nucleosides), cytoplasmic pool analyses of (Cl/Ox)NTPs showed a penetration of all chlorinated and oxidized nucleosides, for all experimental conditions (**Fig. 1** and **Fig. S1**). Overall ratios of penetrated chloronucleosides were between 0.008 and 0.8 % for endothelial cells and between 1.0 and 93 % for prostatic cells. Therefore, penetration of chloronucleotides was 40 to 227 times higher for epithelial prostatic cells (primo-culture) than for endothelial cells (immortalized cells). Indeed, as shown in **Fig. 1a**, ratios of CldG, ClCyt, ClG and CldCyt were respectively 40, 120, 125 and 227 times higher in prostatic cells.

Concerning the incorporation, DNA analyses showed no incorporation of CldCyt while ClCyt is detected in mRNA with ratios between 0.013 % (130 ppm) and 0.12 % (1179 ppm), depending on the kind of cells (**see Table 2**). ~~The ClCyt incorporation was 1.7 times higher for epithelial prostatic cells in comparison with endothelial cells (**see Table 2**).~~ Given that primo-cultures grow more slowly than immortalized cells, exposition to modified medium was longer for prostatic cells, involving a higher accumulation of ClCyt in cytoplasm and mRNA. Nevertheless, if modified medium was not changed the day before cellular lysis, no chlorinated mRNA and no accumulation in cytoplasm were observed, except for Cl(d)Gua (see results for prostatic cells 50% → 100%, **Fig. 1b**). mRNA instability could explain these results. Indeed, RNA is continually renewed and it allows us to hypothesize that all accumulated ClCyt into cytoplasmic pool (thanks to penetration phenomenon) were incorporated into RNA, before that the chlorinated RNA was eliminated and renewed. Moreover, ClCyt could be deaminated into chlorouracil, leading to a ClCyt misdetection. It

remains unclear whether deamination occurs on the nucleic acid, in biologic fluids or during the sample treatment [28,29]. As we were unable to detect them in nucleic acid during preliminary study, this deamination process cannot be discarded.

Finally, it is noteworthy that oxoG and oxodG are present into RNA and DNA for both control cells and treated cells (see **Fig. S2** in Supplementary data). Given that no significant difference exists, it could be explained by an oxidative stress sustained by cells during incubation or by an oxidation process during the sample handling.

In vitro transcription and translation

The penetration and specific incorporation of ClCyt into RNA prompted us to test the impact of their presence on transcription and translation yields.

Nucleotide chlorination:

Quantification of chlorinated NTP in mixes by LC/MS showed a ratio of 3.4, 1.7 and 2.6 % of ClCTP and 4.9, 2.7 and 3.8 % of ClGTP for batch 1, 2 and 3, respectively, ratios being calculated as follows (example of ClCTP): $AUC_{\text{ClCTP from ClNTP}}/AUC_{\text{(CTP+ ClCTP) from ClNTP}} * 100$. GTP was easily chlorinated compared to CTP (1.49 fold).

Transcription, RNA quantification and RNA analysis:

Plasmid transcription in the presence of synthetized ClNTP showed a significant decrease of RNA production in comparison with controls (1.7 fold), as shown in **Fig. 2**. Moreover, the analysis of these produced RNAs by LC/MSMS showed a significant incorporation of modified nucleotides compared to control. Although proportions of ClGTP were higher than ClCTP in the nucleotide mix (4 ± 1% vs 2.6 ± 0.9 %), ratios of ClCyt were significantly higher than ratios of ClGua into RNA in the presence of ClNTP (**Fig. 3**). These results show a

preferential incorporation of ClCyt in comparison with ClGua. Moreover, ratios of ClCyt in RNA (5.7 ± 0.9 % of ClCyt/Cyt) are 2.1 times higher than ratios of ClCTP in ClNTP mix (2.7 ± 0.9 % of ClCTP/CTP), which shows that ClCTP is preferred to CTP during the transcription.

These results showed that the presence of ClNTP during the transcription lead to a decrease of transcription yield and to a preferential incorporation of ClCTP compared to CTP or ClGTP. It also confirms the results observed on the cellular models where a specific incorporation of ClCyt into RNA was already observed.

Translation and protein quantification:

RNA translation highlighted an important decrease of protein production when RNAs are modified in the presence of ClNTP (3.8 fold in comparison with control RNA), as shown in **Fig. 4**. These results showed that the presence of ClNTP has a bigger impact on translation than transcription yield.

DNA polymerase activity

Quantification of chlorinated NTP in mixes by LC/MS showed a ratio of 17.2, 19.5, 31.1 and 15.0 % of CldCTP and 8.3, 8.8, 9.6 and 7.9 % of CldGTP for batch 1, 2, 3 and 4, respectively.

As DNA seems not to be affected by HOCl treatment and in the presence of chloronucleotides in the cultured medium, it was interesting to analyze the activity of DNA polymerase in the presence of CldNTP.

In the presence of the CldNTP, the replication yield was significantly lower than controls: 13.6 ± 0.3 vs 12.8 ± 0.2 cycles to reach the threshold level of DNA (Ct, **Fig. 5**). Analysis of produced DNA showed an incorporation of CldCTP and other modified nucleotides (**Fig. 6**,

$p<0.01$, Welsh's t-test) during the DNA amplification, indicating that DNA polymerase is able to take over the chlorinated nucleotides, but its timeliness seemed to be reduced. Moreover, the incorporation of CldCyt and oxodGua are significantly higher compare to CldGua. These results are not consistent with the absence of incorporation into DNA observed with the incubation of ClCyt in the cellular models.

Considering that the phosphorylation of nucleosides and the reduction of nucleotides are potentially limiting steps in the incorporation of nucleosides into DNA and demonstrating that the incubation of cells with ClCyt leads to an incorporation into RNA (including a phosphorylation step), we hypothesized that the limiting reaction would be the reduction of the chloronucleotide into chlorodeoxynucleotide before their incorporation into DNA. For this reason, we tested the ribonucleotide reductase activity.

Ribonucleotide reductase activity

The function of the ribonuclease reductase is to reduce the nucleoside di- or triphosphate (NTP or NDP) in their corresponding deoxy-NTP or NDP (dNTP or dNDP) in cytoplasm, prior to be used for DNA replication into nucleus.

Quantification of chlorinated CDP and CTP by LC/MS showed a ratio of 80 and 93 % of ClCDP and ClCTP, respectively. Ratios were calculated as follows: $AUC_{ClCDP}/AUC_{(ClCDP + CDP)} * 100$ and $AUC_{ClCTP}/AUC_{(ClCTP + CTP)} * 100$, respectively.

Although reductase activity was proved in samples for Cyt and illustrated by an increase of dCyt quantity with incubation time (see **Fig. 7a**), results showed no clear reductase activity for ClCyt in the presence of the enzyme (see **Fig. 7b**). Indeed, a peak of CldCyt was detected in each sample (even in all controls) but ClCyt could not be reduced in CldCyt by ribonucleotide reductase.

Discussion

Chlorination of endothelial cells by HOCl or by MPO/H_2O_2/Cl^- (MPO system)

Although many authors highlighted chlorination of isolated DNA or RNA by HOCl, MPO system or chloramines [16,14,15,30], only few experimentations have been carried out on various cells. Indeed Badouard's [31], Stanley's [15] and our previous study [22] showed chlorination of (d)Cyt by HOCl or N-acetyl-lysine chloramines (in leukemic cells, aortic smooth muscles cells and endothelial cells, respectively) in the presence of HBSS or PBS, unlike results obtained in this present study (in the presence of DMEM with 10 % of serum). Results were totally different depending on the used medium (DMEM or HBSS); DMEM and its additives (in particular serum 10%) seem to block chlorination of cytoplasmic pool, DNA and RNA, contrary to (less physiologic) HBSS medium.

For Hawkins et al., in a cellular system, HOCl-induced damage to genetic material may be mediated by reaction of pre-formed protein chloramines rather than, or in addition to direct reaction with HOCl [32,33]. Given that protein abundance and their high rate constant for reaction with oxidative products, HOCl reacts faster with proteins, as plasma/cytoplasmic proteins and histones (via non-covalent ionic interactions with Lys and Arg residues), than with DNA in nucleosome [15,34-36]. It could explain why DNA, RNA and cytoplasmic pool were not reached by HOCl in a "complex" medium such as DMEM which contains serum proteins.

Plasma analysis

Since *in vivo* studies showed chlorination of guanosines (in human urine from diabetic patients and liver), uracils (in inflammatory fluids and atherosclerotic lesions) and cytidines (in plasma from volunteers) [37-39,22,10] and since chlorinated nucleosides do not come

from punctual action of HOCl or MPO system on cells (in complete culture medium), we analyzed plasma, which is in direct contact with those cells. Concerning levels of ClCyt found in plasma from volunteers, results obtained in this study converged to our previous results: a median range of 0.82 – 1.88 nM against an average of 1.0 ± 0.2 nM [22]. Moreover, the level of ClCyt is significantly higher in elderly patients with cardiovascular risk and submitted to repeated oxidative stress during the hemodialysis session. Unfortunately, there is a lack of data to compare those concentrations in plasma with literature.

Effect of chloronucleotides on transcription, translation and replication

Although all chlorinated (deoxy)nucleosides were able to penetrate into cells from extracellular fluids, penetrated proportions were variables for each nucleoside, according to cellular type and incubation time. Indeed, prostatic primo cultures are more sensitive to chloronucleoside penetration than endothelial immortalized cultures. In this context, only ClCyt is incorporated into RNA, regardless of cellular type, while DNA does not seem to be affected by the presence of chlorinated (deoxy)nucleosides into culture medium, even if these nucleosides are able to penetrate into cells. This specific incorporation of ClCyt into RNA is relevant as it is the only chloronucleoside found in human plasma while no chlorodesoxynucleosides were detected [22]. This specific incorporation is accompanied by an important reduction of translation yield. Some questions remain about the long-term impact of such incorporation into RNA and such decrease of protein production on the cell viability and function.

This specific incorporation of ClCyt proved that the essential phosphorylation step (chloronucleosides → chloronucleotides) before to be incorporated into RNA remains effective despite the modification. Steps of ribonucleotide reduction and of DNA

polymerization were also essential in the nucleoside incorporation process. Although DNA polymerase was able to use chlorinated nucleotides as substrates, it processed more slowly than in control conditions. Moreover, ribonucleotide reductase could not reduce chlorinated nucleotides into chlorinated deoxynucleotides before to enter into nucleus for the replication process. This limiting reaction could explain why ClCyt is specifically incorporated into RNA but not into DNA. However, it is probably not the only limiting factor for DNA incorporation; thus, the low CldCyt / dCyt ratio could also prevent incorporation, as well as inefficient processing by the polymerase in the presence of chlorinated nucleosides/nucleotides. Another limiting factor may be some cellular mechanisms which prevent that 5-substituted cytosine residues are metabolized to triphosphates prior to be used by DNA polymerase, reducing their DNA incorporation [40-42].

Valinluck and Sowers have shown in few *in vitro* studies that 5-halogenated cytosines were recognized by cells as 5-methyl cytosine residues and can thus mimic endogenous methylation of DNA; causing an inappropriate de novo methylation with gene silencing [40]. Moreover, these chlorinated residues increase probability of mispairing with adenines and thus miscoding as a thymidine (C → T mutation) [43]. Sassa et al. highlighted that 8-halogenated guanosines are miscoded by one of three DNA polymerases, potentially causing mutations [44]. For this reason, these halogenated damage could lead to the heritable changes in gene expression (methylation patterns or mutations) as observed in human cancer (as prostate tumors) [45], these damage could represent an important link between the inflammation and cancer development. For this reason, it is positive that no chlorinated damage -caused by the presence of HOCl, MPO system or chloronucleosides in extracellular medium- was detected in DNA, especially as DNA repair glycosylases present no activity against CldCyt [46,47] and CldGua [44].

It is noteworthy that the concentrations of ClCyt used in the *in vitro* experiments are higher than those found in plasma of patients (500 nM vs 6 nM). As ClCyt is probably produced during a local inflammation process where MPO is produced and active, it could be interesting to measure the local concentration of ClCyt in tissue subjected to inflammation and compared this value to plasma concentration that constitutes a dilution compartment.

In conclusion, our approach showed that RNA synthesis and translation could be influenced by the presence of ClCyt in the extracellular fluids, together decreasing protein production, while DNA seems protected. Although DNA damage was not observed, cellular RNA or protein damage could lead to altered functions of important enzymes and proteins in reached tissues, thus contributing to the multifactorial carcinogenesis process [11].

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Tables

Table 1. Comparisons of modified nucleoside levels in plasma between healthy donors and hemodialyzed patients.

	Volunteers		Hemodialyzed patients		p-value
	Medians	**Range (25-75%)**	**Medians**	**Range (25-75%)**	
Cyt (nM)	215	171-284	617	433-733	< 0.001
ClCyt (nM)	1.12	0.82-1.88	6.5	2.6-10.5	< 0.001
Gua (nM)	1.08	0.8-1.4	1.02	0.89-2.08	0.45
oxoG (nM)	0	0-3.14	2.71	0.58-5.57	< 0.001
ClCyt/Cyt (%)	0.17	0.108-0.458	0.41	0.2-0.62	0.06
OxoG/Gua (%)	0	0-0.03	0.86	0.08-2.05	< 0.001

Plasmas from healthy donors (volunteers) and hemodialyzed patients were purified and dephosporylated, prior to be analyzed by LC/MSMS. Concentrations (expressed in nM) of each compounds (Cyt, ClCyt, Gua and oxoG) were calculated using calibration curves and ratios of ClCyt and oxoG were calculated as follows: $AUC_{\text{modified nucleosides}}/AUC_{\text{unmodified nucleosides}} * 100$. Data comparisons between volunteers and patients were performed using non-parametric Mann-Whitney test.

Table 2. Incorporation of ClCyt into RNA (n = 4).

	Endothelial cells		Prostatic cells	
Density cells **Incubation time**	20 → 100% 48 h	50 → 100% 24h	20 → 100% 18 days	50 → 100% 21 days
Incubation time after the last medium renewal	48 h	24 h	24 h	3 days
ClCyt/Cyt in mRNA	% ppm	% ppm	% ppm	% ppm
Controls	N.D.	N.D.	N.D.	N.D.
Modified medium	0.070 ± 0.009* 699 ± 86*	0.013 ± 0.002* 130 ± 21*	0.12 ± 0.01* 1179 ± 126*	N.D.

Endothelial cells and epithelial prostatic cells were incubated in the presence of normal medium (controls) whereas treated cells were incubated in the presence of modified medium (supplemented with 500 nM of standards of Cl(d)Cyt, Cl(d)Gua and oxo(d)G) at 20 % (**20 → 100%**) or 50% (**50 → 100%**) of confluence until they reached 100 % of confluence. When cells were at 100 % of confluence, DNA and RNA were extracted, enzymatically hydrolyzed and injected into LC/MSMS. Analyses showed no DNA incorporation (data not shown) but an exclusive incorporation of ClCyt into RNA. Data were expressed in ratio of modified/unmodified nucleosides in % or in ppm. Data comparisons between control conditions and modified medium conditions were performed using non parametric Mann-Whitney test. N.D.= Not detected. * Significant difference ($p < 0.05$).

Figure Legends

Fig. 1. Cellular penetration of chlorinated nucleosides (n = 4). Endothelial cells (**a** and **c**) and epithelial prostatic cells (**b** and **d**) were incubated in the presence of normal medium (controls) whereas treated cells were incubated in the presence of modified medium (supplemented with 500 nM of standards of Cl(d)Cyt, Cl(d)Gua and oxo(d)G) at 20 % (**a**,**b**) or 50% (**c**,**d**) of confluence until they reached 100 % of confluence. When cells were at 100 % of confluence, free nucleosides from cytoplasm were extracted, dephosphorylated and analyzed by LC/MSMS. Analyses showed various penetration efficiencies of all chlorinated/oxidized nucleosides. Data comparisons between control conditions and modified medium conditions were performed using non parametric Mann-Whitney test. N.D.= Not detected. * Significant difference ($p< 0.05$).

Fig. 2. Quantification of *in vitro* transcribed RNA in presence of NTP or ClNTP (n = 3). Plasmid pcDNA3.1-apoL1-V5-His_6 was transcribed in presence of NTP (controls, light grey histograms) or chlorinated NTP (dark grey histograms) and the *in vitro* transcribed RNA was quantified using Nanodrop®. Results showed a decrease of transcription yield (1.7 fold) in presence of ClNTP in comparison with controls. Data comparisons between control condition and ClNTP condition were performed using t-test. *** Significant difference ($p< 0.001$).

Fig. 3. Quantification of modified nucleosides in *in vitro* transcribed RNA in presence of NTP or ClNTP (n = 3). Plasmid pcDNA3.1-apoL1-V5-His_6 was transcribed in presence of NTP (controls, light grey histograms-not visible) or chlorinated NTP (dark grey histograms). The *in vitro* transcribed RNA was purified, enzymatically hydrolyzed and analyzed by LC/MSMS. Results showed a high presence of ClCyt, ClGua and a lesser quantity of oxoG into RNA when it was transcribed in presence of ClNTP. Data comparisons between control condition and ClNTP condition were performed using Welch's t-test (**$p< 0.01$, *$p<0.05$).

The One-way ANOVA showed a significant difference between groups (p<0.001); ratios of ClCyt in *in vitro* transcribed RNA are significantly higher compared to ClGua and oxoGua ratios and ratios of oxoGua are significantly higher compared to ClGua (*p<0.05, Holm-Sidak post hoc test for multiple comparisons).

Fig. 4. Quantification of proteins from *in vitro* modified RNA translation (n = 3). Equal quantities of *in vitro* transcribed RNA in presence of NTP (control RNA, light grey histograms) or chlorinated NTP (modified RNA, dark grey histograms) were translated in reticulocyte lysates in the presence of radiolabeled methionine (^{35}S-Met). The translation efficiency was estimated by measuring the amounts of synthesized radiolabeled protein. Results showed a significant difference between the two conditions. Data comparisons between control condition and chlorinated RNA condition were performed using t-test. * Significant difference (p< 0.001).

Fig. 5. DNA polymerase activity (n = 4). A template was amplified by qPCR in the presence of dNTP (light grey histograms) or CldNTP (dark grey histograms) and quantified using SYBR green. A larger number of cycles was necessary to reach the threshold level (Ct) in the presence of CldNTP, in comparison with controls. Data comparisons between control condition and CldNTP condition were performed using t-test. * Significant difference (p< 0.001).

Fig. 6. Quantification of modified nucleosides in *in vitro* amplified DNA (n = 4). A template was amplified by qPCR in the presence of dNTP (controls, light grey histograms) or chlorinated dNTP (dark grey histograms) and the *in vitro* amplified DNA was purified, enzymatically hydrolyzed and analyzed by LC/MSMS. Results showed the presence of CldCyt, oxodG and CldGua in decreasing amounts into DNA amplified in presence of CldNTP. Data comparisons between control condition and CldNTP condition were performed

using Welch's t-test (***$p< 0.001$). The Kruskal-Wallis test showed a significant difference between groups ($p<0.001$); the incorporation of CldCyt and oxodGua is significantly higher compared to CldGua (*$p<0.05$, Tukey post hoc test for multiple comparisons).

Fig. 7. CDP reductase activity (n = 4). Enzymatic fraction (Enz) was extracted from endothelial cells and used to estimate the ribonucleotide reductase activity at T0 (white histograms), T24 (light grey histograms) and T48 (dark grey histograms) of incubation in the presence of CDP, CTP (not shown), ClCDP, ClCTP (not shown) or mix (not shown). Results were expressed as ratio dCyt/total Cyt (%) (**a**) and as ratio CldCyt/ClCyt (%) (**b**). Results for data not shown (CTP, ClCTP and mixes) presented the same profile. Data comparisons between T0, T24 and T48 of each condition were performed using Kruskal-Wallis ANOVA on rank. * Significant difference ($p\leq 0.05$). Data comparisons between control conditions (CDP) and enzymatic conditions (CDP Enz) were performed using Kruskal-Wallis test. * Significant difference ($p<0.05$).

Figure 1

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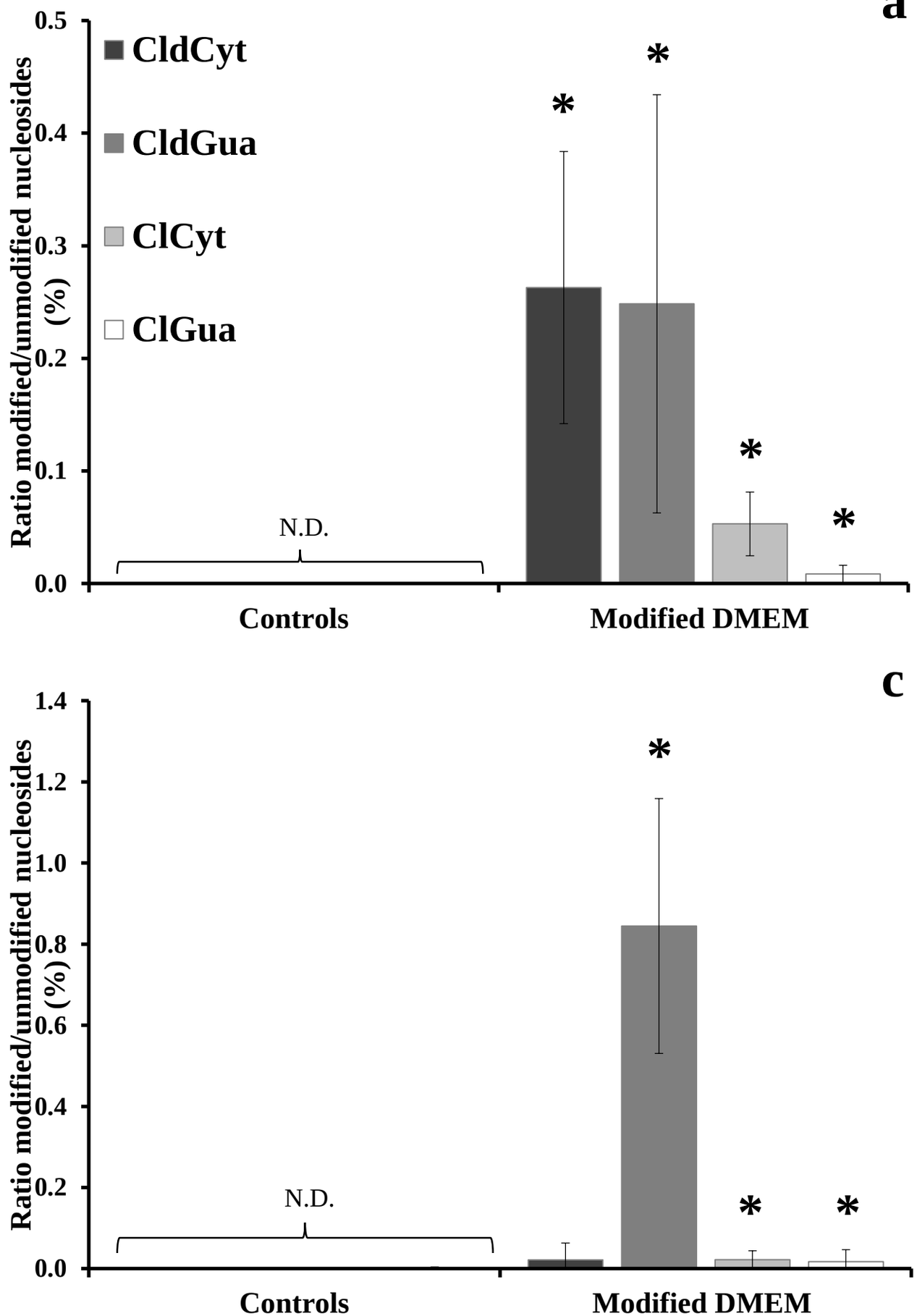
Figure 1



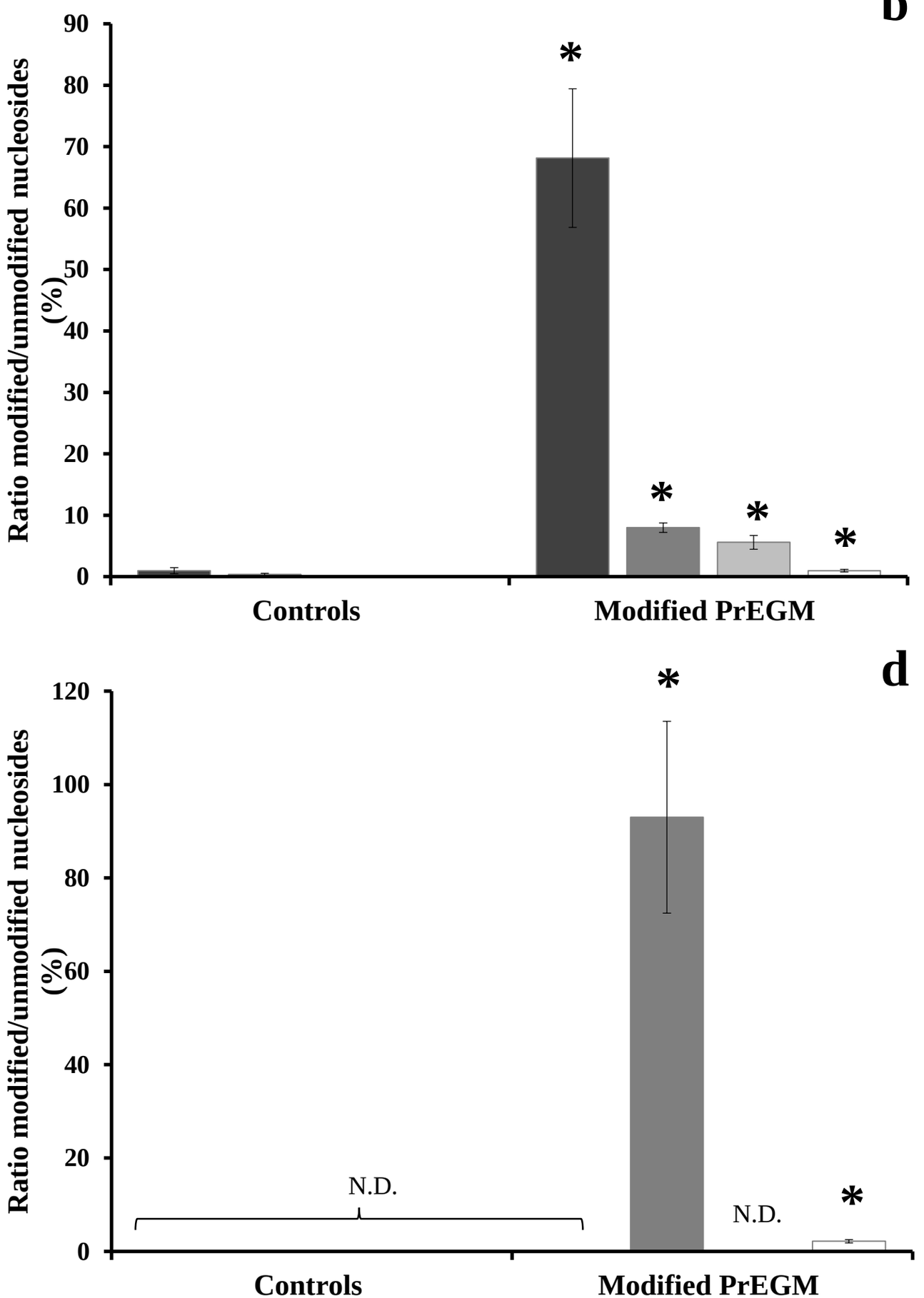


Figure 2

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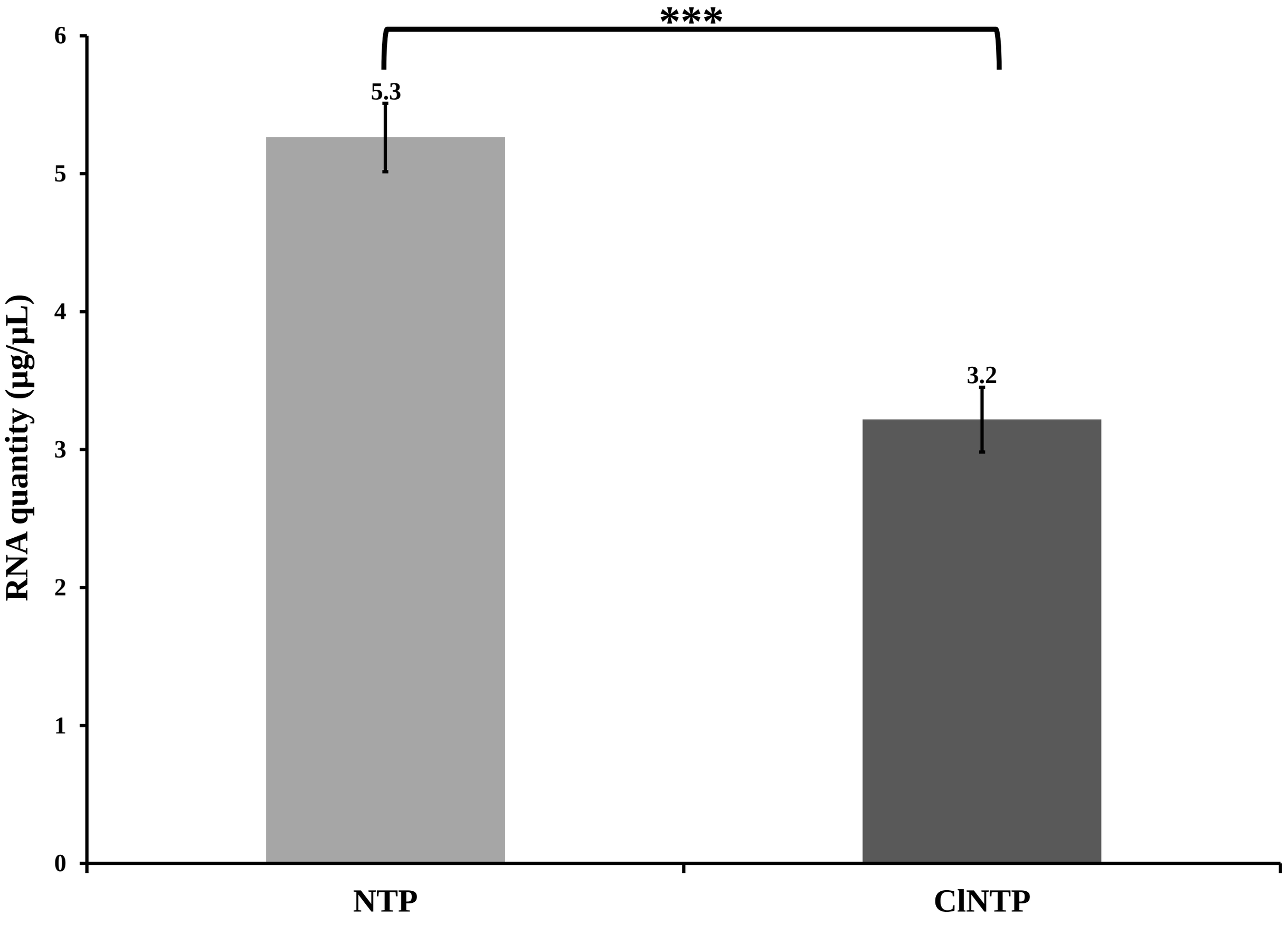

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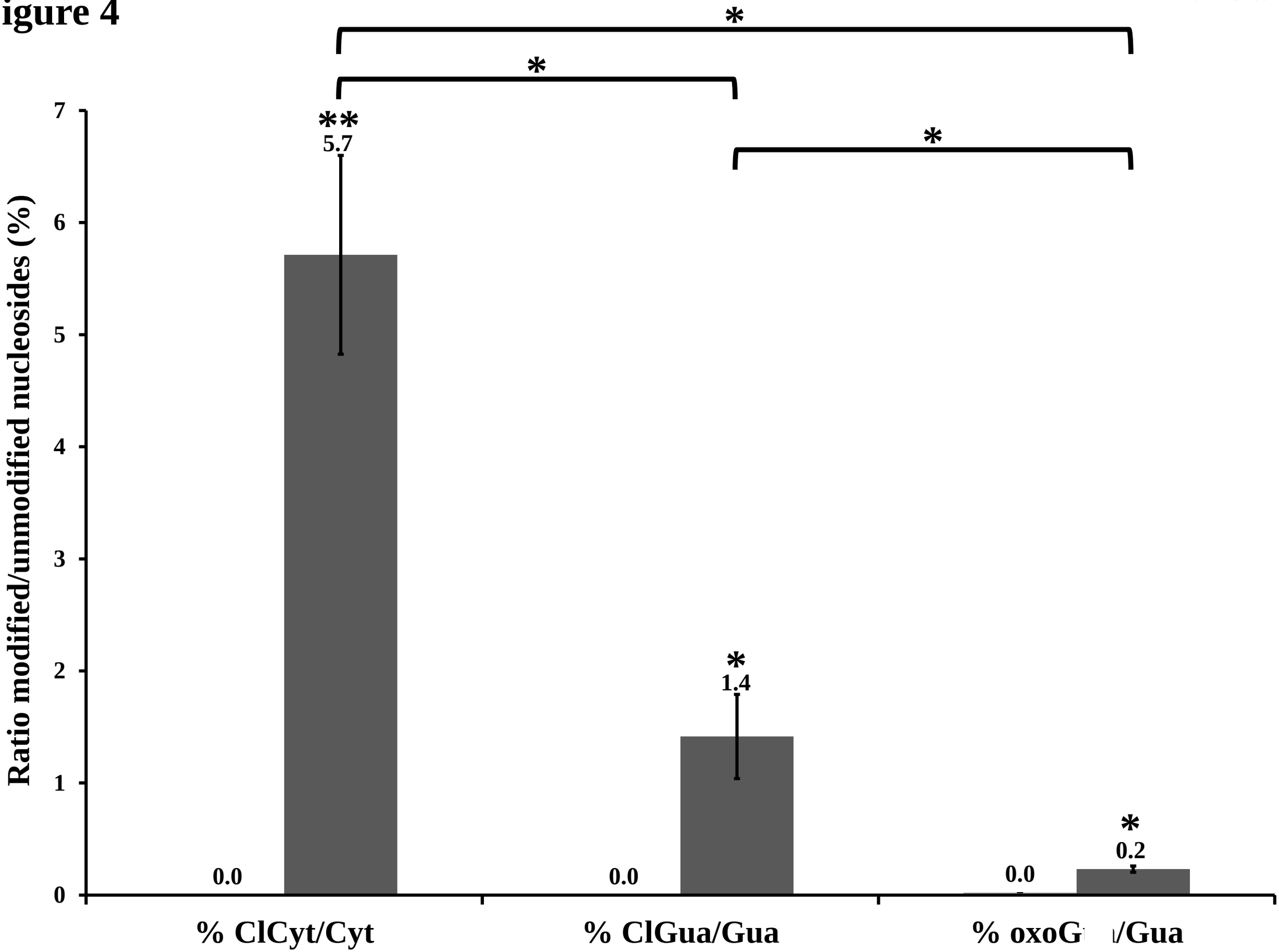

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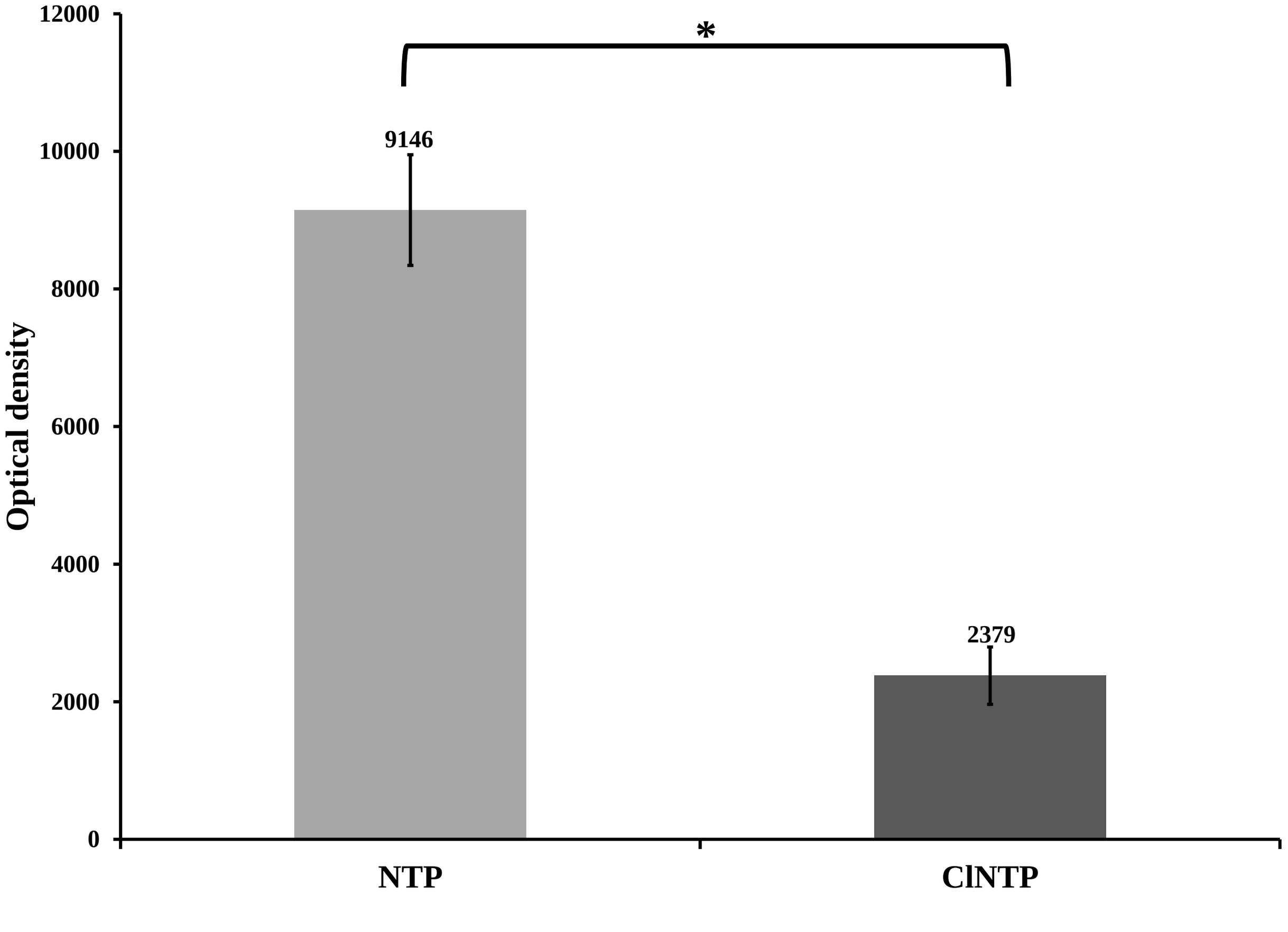

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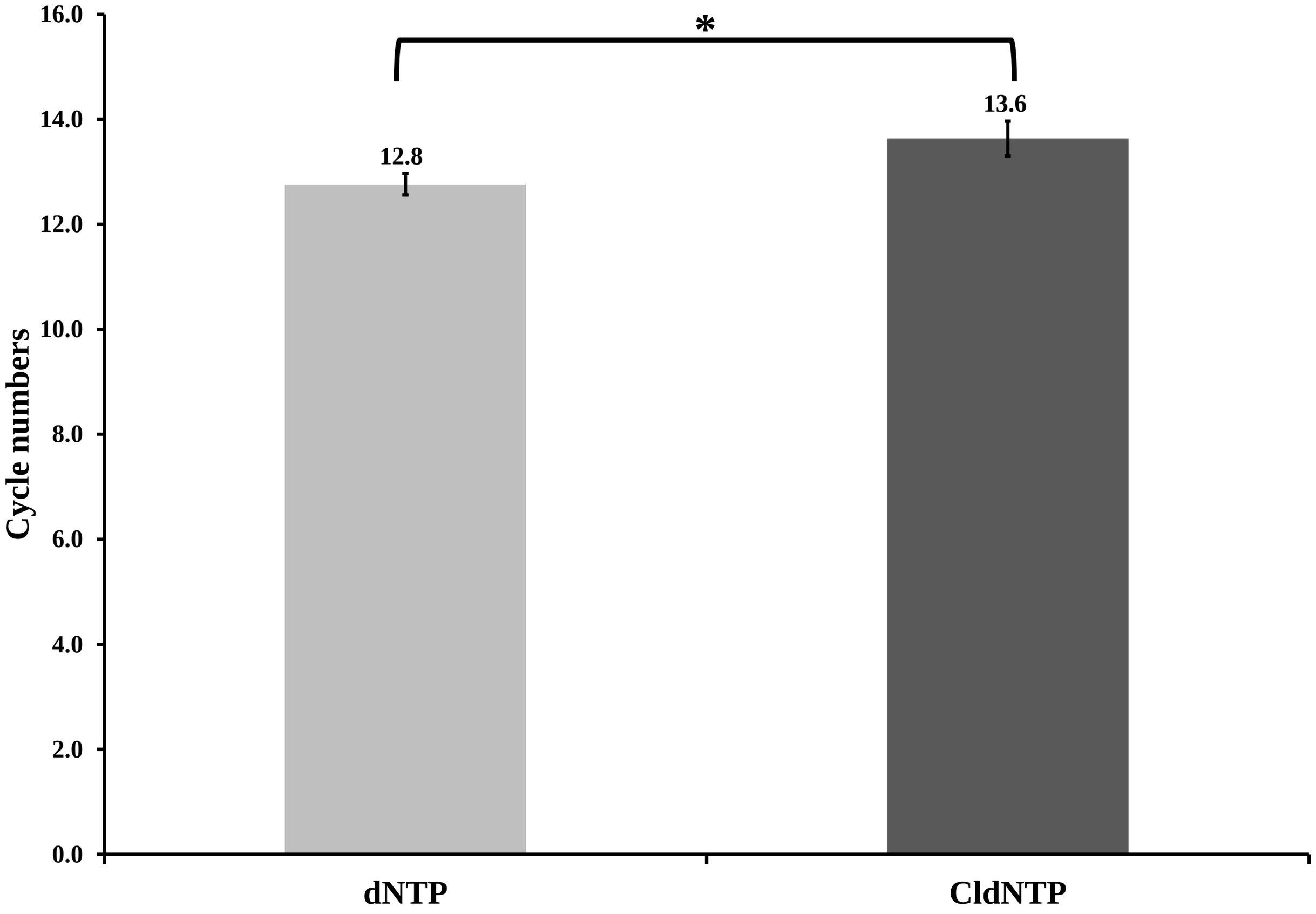

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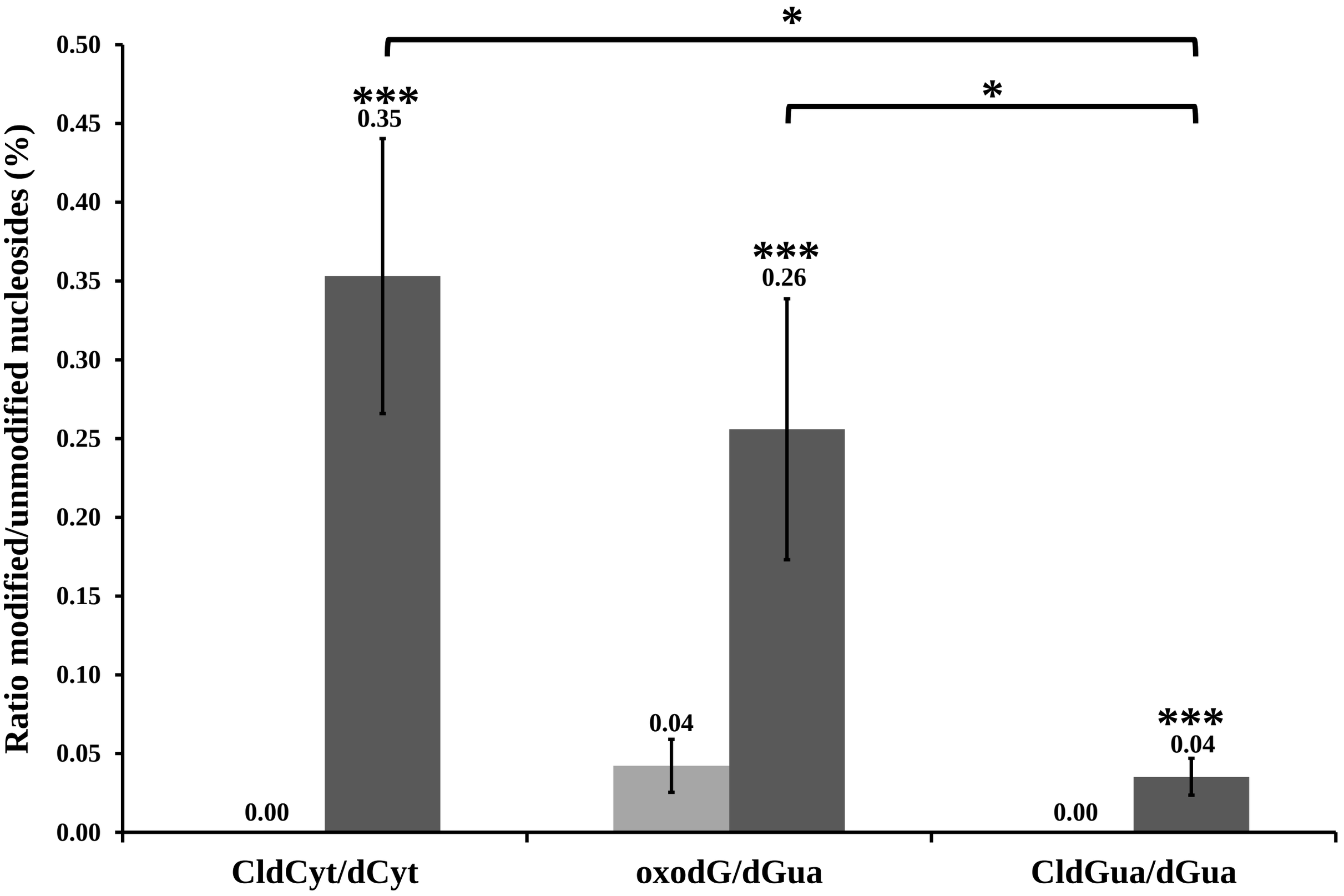

Figure 7

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Figure 8