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Mutagenicity of a bi-stranded clustered DNA lesion containing (5′*S*) or (5′*R*) 5′,8-cyclo-2′-deoxyAdenosine in *Escherichia coli* model

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Ionizing radiation induces DNA damage, including characteristic clusters and tandem lesions e.g., 5′,8-cyclo-2′-deoxyPurines (cdPus). Clustered DNA Lesions (CDL) defined as 2 or more lesions within 1-2 helical turns resulting from a single radiation track contribute to the harmful effects of radiation. Moreover, the presence of CDL and cdPus in human DNA may decrease the efficiency of the DNA repair mechanisms, which in consequence may lead to, e.g., carcinogenesis. This preliminary study showed the mutagenic potential of CDL containing dU on one strand and 5′,8-cyclo-2′-deoxyAdenosine (cdA) on a complementary strand separated by up to 4 bp. Mutagenicity was determined using *Escherichia coli* reporter assay and 40-mer model ds-oligonucleotides with CDL. Mutation frequencies were determined to be significantly higher for CDL than for single isolated lesions (cdA or dU placed only in one strand). The results demonstrated that the dU lesion located on the opposite DNA strand separated by 0 or 1 bp from cdA led to severe mutagenicity. The most frequent mutations observed comprised point deletions and transitions. Oligonucleotides with CDL containing ScdA/RcdA demonstrated even up to 100% mutation rate. Interestingly, increasing the distance between lesions within CDL to 4 bp led to full recovery of the correct sequence of ds-oligonucleotides, indicating an efficient repair process. The results obtained with the bacterial model are in agreement with previous *in vitro* studies on eukaryotic models. The high mutagenicity and/or inhibited repair process of clusters with lesions located in close proximity provides additional verification of the previously presented trends describing how the distance between cdPu and dU affects DNA repair processes.

Keywords: 5′,8-cyclo-2′-deoxyadenosine (cdA), clustered DNA lesion, DNA damage, DNA repair, mutagenicity, *Escherichia coli*

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Abbreviations: AP site, apurinic/apyrimidinic site; BER, Base Excision Repair; CDL, Clustered DNA Lesions; cdPus, 5′,8-cyclo-2′-deoxyPurines; cdA, 5′,8-cyclo-2′-deoxyAdenosine; ScdA, (5′*S*) 5′,8-cyclo-2′-deoxyAdenosine; RcdA, (5′*R*) 5′,8-cyclo-2′-deoxyAdenosine; DSB, double-strand break; IR, ionizing radiation; oligo, oligonucleotide

INTRODUCTION

Ionizing radiation (IR) induces the direct or indirect formation of various DNA damage. From the chemical point of view, the lesions produced by exogenous factors (e.g., ultraviolet, X-ray, γ-rays, etc.) and endogenous ones (e.g., estrogens, metabolites, respiratory cycles) are structurally identical. Radiation-induced lesions (e.g., during radiotherapy), such as Clustered DNA Lesions (CDL, 2 or more lesions within 1-2 helical turns created by the passage of a single radiation track) and/or 5',8-cyclo-2'-deoxyPurines (cdPus) can affect the spatial structure of the double helix and subsequently impair the repair of genetic material. CdPus being a unique DNA lesion have the additional covalent bond between C5' and C8 of the guanine or adenine moieties. Depending on C5' chirality (5'*S* or 5'*R* diastereomer) cdPus demonstrated different biological impact (Kuraoka *et al.*, 2000). Changes in the geometry of the DNA double helix structure are more likely to occur if rigid lesions such as cdPu appear (in comparison with the flexible lesions such as ^{oxo}dG) (Kusumoto *et al.*, 2002; Brooks, 2017). Subsequently, cdPus may interfere with repair and replication in *Escherichia coli* because in most cases polymerases cannot bypass its complex structure and Nucleotide Excision Repair (NER) is inefficient (Jasti *et al.*, 2011; David-Cordonniert *et al.*, 2000; Pednekar *et al.*, 2014). When CDL contains an apurinic/apyrimidinic site (AP site) and cdPu, the efficiency of proteins involved in the repair of the AP site through the Base Excision Repair (BER) machinery can decrease, as shown in our previous studies on nuclear and mitochondrial extracts of eukaryotic cells (Karwowski, 2019; Boguszewska *et al.*, 2021a; Kaźmierczak-Barańska *et al.*, 2021; Boguszewska *et al.*, 2021b; Karwowski *et al.*, 2014; Karwowski, 2021). The AP site located opposite to 5',8-cyclo-2'-deoxyAdenosine (cdA), or 1 nucleobase in the 5'-end direction was found to be a cause of the termination of ds-oligo repair, as polymerases cannot operate properly. Interestingly, when the distance between lesions increased to 7 or 10 nucleobases (in both directions), the repair process occurred quite efficiently, and the strand reconstitution was observed. The most problematic CDL ´variant´ included AP site placed 4 bases in the 5'-end direction from cdA (inefficient strand ligation). In contrast to above, the AP site settled 4 bases in the 3'-end direction from cdA resulting in its proper repair. Interestingly, the proper enzymatic action was observed when the distance between the lesions located on the same strand increased to 5 nucleobases in the 5'-end direction (Karwowski, 2021). The DNA repair capacity differed depending on the type of cdPu, its diastereomeric form (5'*S* or 5'*R*), and the interlesion distance. These features directly influenced the activity of proteins involved in DNA repair (Karwowski, 2019; Boguszewska *et al.*, 2021a; Szewczuk *et al.*, 2021; Kaźmierczak-Barańska *et al.*, 2021; Boguszewska *et al.*, 2021b). When eukaryotic DNA repair enzymes cannot form active complexes with DNA due to structural changes (e.g., resulting from cdPu occurrence), the DNA lesion remains in the cell unre-

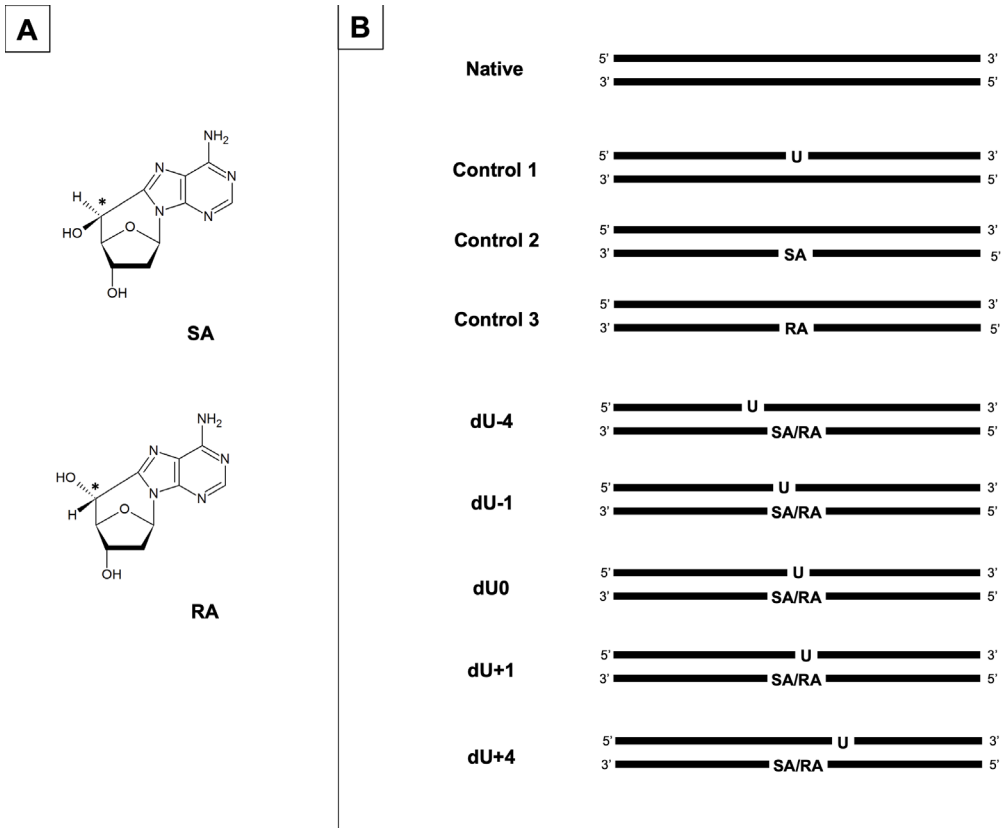

Figure 1. (A) The chemical structures of the (5′*S*)-5′,8-cyclo-2′-deoxyAdenosine (SA), (5′*R*)-5′,8-cyclo-2′-deoxyadenosine (RA); (B) The investigated ds-oligonucleotides (40 bp) with lesion positioning.
U – represents location of a 2′-deoxyUridine (dU); SA/RA – represents location of the (5′*S*) or (5′*R*) 5′,8-cyclo-2′-deoxyAdenosine; negative numbers – represent dU location 1–4 bp in 3′-end direction; positive numbers —represent dU location 1–4 bp in 5′-end direction.

paired, which can lead to mutations and finally to pathogenic states (e.g., carcinogenesis and/or accelerated aging). The goal of this preliminary study was to i) assess the general trends of CDL repair in the bacterial model, ii) to compare the impact of the distance between lesions within the cluster and the diastereomeric form of cdPu between bacterial model and previously studied eukaryotic models, and iii) to additionally verify and justify the use of *in vitro* eukaryotic models in CDL studies. This article has shown the biological consequences of complex CDL using a plasmid-based assay and a set of 40-mer ds-oligos containing dU (as a precursor of an AP site) and (5′*R*) or (5′*S*) cdA separated by 1-4 bp. The frequency of CDL mutation was evaluated and compared with the individual model lesion (dU). Furthermore, the influence of the distance between the lesions and the diastereomeric form of cdA has been assessed. The results presented show that CDL containing cdA and dU separated by 0-1 bp are highly mutagenic in *E. coli*.

MATERIALS AND METHODS

Preparation of oligonucleotides for the *E. coli* reporter system

Oligos used in this study (40 bp) contained ds-CDL: dU on one strand and cdA on the opposite strand distanced 0, 1, or 4 bases in both directions (Fig. 1, Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/). Oligos were obtained and characterized as previously described (Boguszewska *et al.*, 2021b). The mass spectra of the oligos are shown in the Supplementary Materials (Fig. S1, Table S2 at https://ojs.ptbioch.edu.pl/index.php/abp/). The single-stranded oligos were hybridized (90°C for 10 min, followed by slow cooling) with a 1.5-fold excess of the complementary strand in pure H_2O. The double-stranded oligos were phosphorylated at the 5'-end with 10 U T4 polynucleotide kinase (New England BioLabs, Ipswich, MA, USA) with 10 mM ATP in 1× T4 kinase reaction buffer at 37°C for 30 min. The duplexes were precipitated with cold ethanol (250 µL), placed on dry ice for 30 min, centrifuged (13 000 rpm, 4°C, 30 min), and dissolved in pure H_2O. The control oligos contained undamaged sequence (Native), single dU (Control 1), and single cdA (5'*S* and 5'*R* diastereomers, denoted Control 2 and 3, respectively) as shown in Fig. 1 and Table S1 at https://ojs.ptbioch.edu.pl/index.php/abp/.

Plasmid preparation and ligation

The plasmid pUC18 (5 µg, ThermoFisher Scientific, MA, USA) was linearized with 100 U SmaI (New England BioLabs, Ipswich, MA, USA) in 1× rCutSmart buffer at 25°C for 24 h followed by SmaI inactivation (65°C, 20 min). The linear plasmid was then incubated with 5 U calf intestinal alkaline phosphatase (New England BioLabs, Ipswich, MA, USA) at 37°C for 15 min followed by a second incubation at 55°C for 45 min. The linear dephosphorylated plasmid was purified with a Monarch® PCR & DNA Cleanup Kit (New England BioLabs, Ipswich, MA, USA) according to the manufacturer´s protocol. Aliquots of 200 fmol pUC18 plasmid DNA were ligated to 5 pmol of each oligo using the Quick Ligation Kit (New England BioLabs, Ipswich, MA, USA) at 25°C for 24 h.

Transformation of electrocompetent *E. coli*

The ligation products (50 ng) were added to 25 µl of electrocompetent *E. coli* bacteria (DH10β, New England BioLabs, Ipswich, MA, USA) and electroporated in an electroporation cuvette using Bio-Rad pulser (1.8 mV with a time constant ~5 ms). After electroporation, 975 µl of SOC was immediately added, followed by incubation at 37°C for 1 h with agitation (100 rpm). Transformants were selected using LB agar plates containing 100 µg/ml ampicillin at 37°C for 24 h. All transformations were performed three times.

Sequence analysis of mutated plasmid DNA

Single colonies grown on LB agar plates containing 100 µg/ml ampicillin and incubated at 37°C for 24 h were picked at random and used to inoculate separate aliquots of LB broth. The transformants were grown using 5 ml of LB broth containing 1 µg/ml ampicillin at 37°C for 16 h. The plasmid DNA was isolated from bacteria using the GeneMATRIX Plasmid Miniprep DNA Purification Kit (EURx, Gdańsk, Poland) according to the manufacturer´s protocol. The DNA of the bacterial plasmid was sequenced using the Applied Biosystems Big Dye sequencing kit (ThermoFisher Scientific, MA, USA) and the M13pUCr and M13pUCf primers that amplified the sequence across the inserted oligo with the damaged site. Sequencing data were analyzed using the Needleman-Wunsch global alignment tool online for nucleotide sequences (Fig. S2 at https://ojs.ptbioch.edu.pl/index.php/abp/). The mutation frequency was calculated by dividing the number of mutations found in the oligo by the total number of sequenced oligos. The frequency of individual mutations was calculated by dividing the number of times a mutation occurred by the total number of mutated sequences (Table 1).

Table 1. Mutations observed at the site of damage (± 3 bp) resulting from the bi-stranded clustered DNA lesions containing 5′,8-cyclo-2′-deoxyAdenosine (cdA) and 2′-deoxyUridine (dU) at different interlesion distances. The full sequences are shown in Supplementary materials (Fig. S2 at https://ojs.ptbioch.edu.pl/index.php/abp/).

Substrate oligonucleotide	Mutation frequency [%]	Type of mutation	Frequency of mutation type [%]
ScdA/dU0	45.5	ΔdU:ScdA	20.0 (1/5)
		ΔGC at +1 position	20.0 (1/5)
		ΔTA at +2 position	20.0 (1/5)
		25 bp deletion at dU:ScdA	20.0 (1/5)
		26 bp deletion at dU:ScdA	20.0 (1/5)
RcdA/dU0	18.2	ΔdU:RcdA	100.0 (2/2)
ScdA/dU-1	90.0	ΔT:ScdA	22.2 (2/9)
		ΔdU:G	33.3 (3/9)
		4 bp deletion at T:ScdA	11.1 (1/9)
		8 bp deletion at T:ScdA	11.1 (1/9)
		U:G to T:A	22.2 (2/9)
RcdA/dU-1	63.6	ΔdU:G	22.2 (2/9)
		ΔCG at +1 position	22.2 (2/9)
		ΔTA at +2 position	11.1 (1/9)
		6 bp deletion at T:RcdA	11.1 (1/9)
		22 bp deletion at T:RcdA	11.1 (1/9)
		ΔT at 0 and ΔG at -1 positions	11.1 (1/9)
		U:G to T:A	11.1 (1/9)
ScdA/dU+1	100.0	ΔT:ScdA	7.7 (1/13)
		ΔdU:G	30.8 (4/13)
		ΔTA at +2 position	7.7 (1/13)
		C:G to C:A transition at -1 position	7.7 (1/13)
		U:G to T:G transition at + 1 position	7.7 (1/13)
		5 bp deletion at T:ScdA	7.7 (1/13)
		28 bp deletion at T:ScdA	7.7 (1/13)
		U:G to T:A	23.1 (3/13)
RcdA/dU+1	90.9	ΔdU:G	27.3 (3/11)
		ΔTA at +2 position	9.1 (1/11)
		ΔC at +1 position	9.1 (1/11)
		U:G to T:A	36.4 (4/11)
		5 bp deletion at T:ScdA	9.1 (1/11)
		T:RcdA to C:G	9.1 (1/11)

RESULTS AND DISCUSSION

IR (e.g., ultraviolet, X-ray) can lead to the formation of characteristic lesion clusters which may decrease DNA repair efficiency (Sage & Harrison, 2011). This study evaluated the mutagenic potential of bistranded CDL (containing cdA and dU at different positions, Fig. 1) using a plasmid-based assay and an *E. coli* bacterial model (Cunniffe *et al.*, 2014). Mutations resulting from the presence of investigated CDL were characterized by sequence analysis. The results obtained have shown that the mutation frequency was much higher for investigated CDL than for the oligo with an undamaged sequence and oligos containing only dU or cdA lesions (Fig. S2 at https://ojs.ptbioch.edu.pl/index.php/abp/). The sequence of the undamaged oligonucleotide was intact after recovery of the *E. coli* plasmid. Furthermore, no mutations were found for oligos containing isolated lesions (Control 1–3, Fig. 1, Table S1 and Fig. S2 at https://ojs.ptbioch.edu.pl/index.php/abp/). Therefore, isolated lesions (dU and cdA) were effectively removed by the bacterial DNA repair systems and the correct genetic information was recovered. The situation became more complicated in the case of double-stranded CDL containing (5'*S*) 5',8-cyclo-2'-deoxyAdenosine (ScdA) or (5'*R*) 5',8-cyclo-2'-deoxyAdenosine (RcdA) on one strand and dU on the complementary strand distanced up to 4 bp (Fig. 1, Fig. S2 at https://ojs.ptbioch.edu.pl/index.php/abp/). The presence of RcdA has been shown to lead to a lower frequency of mutation formation than the presence of ScdA (Fig. 2). This observation was in good agreement with our previous, recently published studies of complex CDL in which RcdA was more easily repaired than ScdA by nuclear and mitochondrial extracts from eukaryotic cells (Boguszewska *et al.*, 2021a; Kaźmierczak-Barańska *et al.*, 2021; Boguszewska *et al.*, 2021b). RcdA bends the DNA double helix to a greater extent than ScdA; therefore, DNA repair enzymes are more likely to recognize and remove it from the plasmid sequence (Karwowski, 2013). On the other hand, previous studies have shown that the repair of lesions within the cluster occurs in a certain order to minimize the formation of potentially lethal double-strand breaks (DSB) (Cunniffe *et al.*, 2014; Lomax *et al.*, 2004; Eccles *et al.*, 2009). The results presented may also indicate hierarchical repair (Eccles *et al.*, 2009). The results of the *E. coli*-based assay show that the 4 bp distance between the lesions within a cluster is sufficient for undisturbed removal of dU and cdA (Fig. 2). Oligos denoted as dU+4 and dU-4 for both diastereomers have shown no changes in the sequence (comparing to native, undamaged ds-oligo, Fig. S2 at https://ojs.ptbioch.edu.pl/index.php/abp/). The only exception occurred for the oligo denoted ScdA/dU-4, since a case of 22 bp deletion was detected at the ScdA site (Fig. 2 and Fig. S2 at https://ojs.ptbioch.edu.pl/index.php/abp/). It is noteworthy that in our previous *in vitro* studies on the influence of cdPu on the BER mechanism, dU+4 presented numerous significant difficulties during repair, *i.e.,* disturbed strand elongation by polymerases, no strand ligation, and strand degradation. However, it seemed that this mutual placement of the dU+4 lesion is not problematic in the case of a living organism, such as the discussed *E. coli* bacterial model.

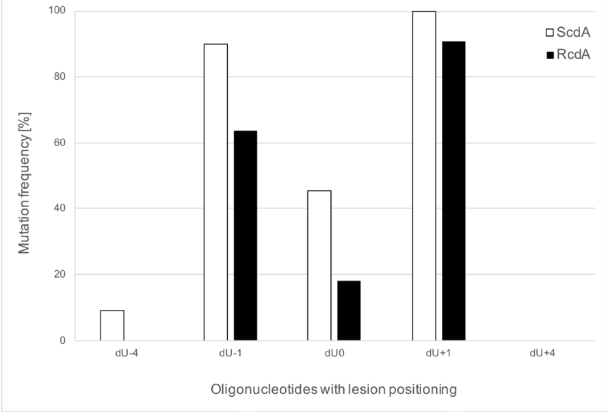

Figure 2. Mutation frequency caused by bi-stranded clustered DNA lesions containing (5′*S*)-5′,8-cyclo-2′-deoxyAdenosine (ScdA) or (5′*R*)-5′,8-cyclo-2′-deoxyadenosine (RcdA) and 2′-deoxyUridine (dU) separated by 0-4 bp.
Mutation frequency was calculated by dividing the number of mutations found in the single sample sequence by the total number of samples sequenced. The frequency of individual mutations was calculated by dividing the number of times a mutation occurred by the total number of mutated sequences.

The most mutagenic variants among the investigated ds-oligos comprised dU0, dU-1, and dU+1 (Fig. 2, Table 1). The mutation frequency increased in the following order: RcdA/dU0<ScdA/dU0<RcdA/dU-1<ScdA/dU-1<RcdA/dU+1<ScdA/dU+1. The fact that clusters containing dU+1 (for both diastereomers of cdA) have the highest mutagenic potential corresponded to previous studies in eukaryotic models (Pearson *et al.*, 2004; Cunniffe *et al.*, 2014; Karwowski, 2019; Boguszewska *et al.*, 2021a; Kaźmierczak-Barańska *et al.*, 2021; Boguszewska *et al.*, 2021b; Karwowski *et al.*, 2014). It was found that the most abundant mutation in the case of ScdA/dU+1 was dU:G deletion (30.8%). The second most frequent change was U:G → T:A transition (Table 1). Interestingly, for RcdA/dU+1 the opposite results were observed. The most repeated mutation was U:G→T:A transition (36.4%) while deletion of the dU:G pair was the second most frequent in the structure of the clustered lesion area (Table 1). In the case of dU-1, the most common mutation was dU:G deletion for ScdA. In the case of RcdA/dU-1 dU:G deletion and C:G deletion located at position +1 to cdPu showed the same mutation frequency (22.2%, Table 1). Lesions located opposite each other within the cluster (position dU0) resulted in deletions at the damage site. When the (5′*R*) isomer was present within CDL, only the dU:RcdA pair was removed. On the other hand, oligos containing the ScdA/dU0 cluster resulted in point deletions at 0, +1, and +2 positions and a larger deletion of 26 bp (Table 1 and Fig. S2 at https://ojs.ptbioch.edu.pl/index.php/abp/). The results obtained and presented in this article of an *E. coli*-based assay suggest that the bacteria were more prone to remove ´problematic´ base pairs from the DNA sequence that cause deletions than to replace them with correct or incorrect bases. This observation was made concerning a dU distanced 0 or 1 bp from the discussed CDL containing ScdA or RcdA. The deletions occurred most frequently at the location of dU, most probably due to disturbed processes of repair and/or replication.

In conclusion, it has been shown that cdA separated by 0 or 1 nucleobase from the other lesion located on the opposite strand within the cluster causes numerous mutations. The increased frequency of mutations may indicate impaired *E. coli* DNA repair mechanisms by CDL containing cdPus. On the other hand, the distance of 4 bp between cdA and dU within the cluster seemed to provide sufficient "space" for the repair enzymes to operate properly; therefore, the lesions were removed from the DNA sequence and the correct genetic information

was regained. In addition, the ScdA lesion seemed to be efficiently managed by bacterial repair mechanisms. This phenomenon is beneficial due to the more frequent incidence of ScdA isomer than RcdA in dsDNA. Additionally, transition mutations resulting from this complex case of CDL are likely to impair protein structures if they are located within a gene sequence. This study shed some new light on the repair machinery of complex CDL including cdA and dU by the entire living organism. The discussed results of the *E. coli*-based assay have confirmed some of our previous observations from *in vitro* experiments with cellular extracts of eukaryotic cells (xrs5, BJ, and XPC). Interestingly, the results have shown that despite the model (eukaryotic or bacterial) the distance between lesions within the cluster and the diastereomeric form of cdPu present similar trends of DNA repair disruptions. From our point of view, it provided additional verification of the previously presented trends that describe how the distance between cdPu and dU impacts the eukaryotic DNA repair processes and provided additional justification for performing CDL studies with *in vitro* models, which is common in the field. This study has shown for the first time the differences between the impact of diastereomeric forms of cdA on the repair of the second lesion in the *E. coli* model. This article presented preliminary and initial results that require further studies in the context of recognition of DNA repair mechanisms involved in the repair of CDL and tandem lesions induced by IR.

Declarations

Author Contributions: Conceptualization and methodology B.K..; validation, K.B., J.K-B., and B.K.; formal analysis, K.B, and B.K..; investigation, K.B., and B.K.; resources, B.K.; data curation, B.K.; writing – original draft preparation, K.B., and B.K.; writing – review and editing, K.B., J.K-B., and B.K.; visualization, K.B.; supervision, B.K.; project administration, B.K; funding acquisition, B.K. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Compliance with Ethical Standards: This article does not contain descriptions of studies performed by the authors with the participation of humans or using animals as objects.

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