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Comparative Analysis of Human and Chicken Lymphocytes DNA Damage Exposed to Carbofuran

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ABSTRACT

Carbofuran, a highly toxic N-methyl carbamate insecticide banned in many regions due to ecological and health risks, exerts genotoxic effects through oxidative stress and DNA damage. This study evaluated and compared its time-dependent genotoxicity on human and chicken lymphocytes using the Alkaline Comet Assay, exposing isolated peripheral blood lymphocytes from both species to 50 µM carbofuran for 1 or 2 hours and quantifying DNA strand breaks using CASP software. Results revealed moderate DNA damage in human lymphocytes after 1 hour (Tail DNA% = 12.65%, TM = 0.51, OTM = 2.32), increasing moderately after 2 hours (Tail DNA% = 18.92%, TM = 1.14, OTM = 2.40). In stark contrast, chicken lymphocytes exhibited high DNA damage after 1 hour (Tail DNA% = 40.17%, TM = 17.68, OTM = 13.11), which significantly increased after 2 hours (Tail DNA% = 60.08%, TM = 29.44, OTM = 18.94). These findings demonstrate carbofuraninduced time-dependent DNA damage in both species but crucially reveal significantly higher susceptibility in chicken lymphocytes compared to human lymphocytes at the same concentration. This heightened vulnerability underscores carbofuran's extreme toxicity to birds and highlights the value of chicken lymphocytes as a sensitive model for Eco toxicological genotoxicity assessment.

INTRODUCTION

Lymphocytes play a fundamental role in the immune system by orchestrating targeted responses against infectious microorganisms, foreign substances, and tumors, while also mediating transplant rejection. They provide specific defense against pathogens protect against malignancies like multiple myeloma [1] and drive graft rejection by recognizing transplanted tissues as foreign invaders. These cells circulate in blood and lymph a colorless fluid within lymphatic vessels connecting lymph nodes throughout the body and reside in lymphoid organs including the thymus, lymph nodes, spleen, and human appendix [2].

Structurally, lymphocytes range from 7–20 µm in diameter. Microscopic examination using Wright's stain reveals a large, dark purple/blue nucleus with minimal eosinophilic cytoplasm. Larger variants display abundant cytoplasm containing reddish/purplish granular particles. In healthy states, the coarse nucleus approximates red blood cell size [3], with some lymphocytes exhibiting a clear perinuclear halo or lateral clear zone. Electron microscopy confirms prominent polyribosomes that facilitate high-volume synthesis of cytokines and immunoglobulins While T and B cells are indistinguishable in peripheral blood smears, flow cytometry enables specific lymphocyte population counts [4].

T Lymphocytes, central to cell-mediated immunity, mature from thymocytes in the thymus and express T-cell receptors (TCRs) that distinguish them from other lymphocytes. In humans, αβ T cells form through rearrangement of TCR alpha/beta chains for adaptive immunity [5], while ruminants possess γδ T cells with invariant TCRs for innate-like antigen presentation. T-cell development involves three critical thymic selection stages: beta selection (assembling functional β-chains at the DN stage and α-chains at the DP stage to form αβ TCRs), positive selection (DP thymocyte survival through MHC I/II engagement), and negative selection (elimination of autoimmune-prone thymocytes). T-cell activation requires dual signals: TCR binding to peptide-MHC complexes and co-stimulatory interactions (e.g., CD28 on T cells engaging B7 on antigen-presenting cells [6].

B lymphocytes (B cells) mediate humoral immunity through antibody production, while also functioning in antigen presentation and cytokine secretion. Their maturation occurs in the bone marrow in mammals

(Braspaiboon & Laokuldilok, 2024) and the bursa of Fabricius in birds. B cells express B-cell receptors (BCRs) on their surface, enabling specific antigen binding to initiate antibody responses [7].

Natural killer cells—classified as large granular lymphocytes (LGLs)—represent a third lineage derived from the common lymphoid progenitor (shared with B and T cells). These cells differentiate and mature in multiple sites, including the bone marrow, lymph nodes, spleen, tonsils, and thymus, before entering circulation [8]. NK cells provide rapid innate responses against virally infected cells and tumors uniquely recognizing stressed cells independently of antibodies or major histocompatibility complex (MHC) molecules to accelerate immune reactions [9].

Carbofuran exposure induces significant, concentration-dependent DNA damage in human peripheral blood lymphocytes (PBLs) in vitro, as demonstrated by comet assay findings of increased tail length and tail moment indicative of single and double-strand breaks at concentrations ranging from 0.5 to 5.0 µM; notably, the damage observed at 5.0 µM was comparable to that caused by hydrogen peroxide, confirming oxidative stress as a primary mechanism of genotoxicity [10]. This damage extends beyond strand breaks to chromosomal level aberrations, including satellite associations, gaps, and breaks, with studies reporting an LD_{50} of 18 µM after 48 hours of exposure [11]. The genotoxicity of carbofuran is significantly exacerbated by its metabolic activation, where metabolites formed within biological systems exhibit enhanced DNA-damaging potential. Specifically, metabolites like 3-ketocarbofuran and the nitroso derivative nitrosocarbofuran demonstrate higher genotoxicity than the parent compound in mammalian systems. Nitrosocarbofuran, in particular, acts as a potent alkylating agent, causing direct base modifications in DNA that lead to strand breaks, as detected in mouse lymphocytes using micronucleus tests and comet assays [12]. Crucially, extendedterm exposure (14 days) to environmentally relevant, low concentrations of carbofuran (0.4–4.0 ng/mL) induces gene-specific structural instability. Using the sensitive FISH-comet assay, significant fragmentation has been documented in critical genomic loci, including the oncogene c-Myc and the tumor suppressor gene TP53 [13]. The disruption of these key genes, which are fundamental regulators of apoptosis and the cell cycle, represents a potential mechanism by which carbofuran exposure could initiate carcinogenic processes.

The present study aims to determine the effect of carbofuran on the DNA strands of both human and chicken lymphocytes. Specifically, it seeks to assess and compare the genotoxic potential of carbofuran in these two species using the Alkaline Comet assay as a sensitive biomarker of DNA strand breaks. Furthermore, the study aims to evaluate the time-dependent relationship and quantify the extent of carbofuran-induced DNA damage in human and chicken lymphocytes, thereby providing a comparative understanding of species-specific susceptibility to this pesticide at the cellular and molecular levels.

MATERIALS & METHODS

The present study was conducted at the Institute of Zoological Sciences, University of Peshawar, to assess DNA damage induced by carbofuran exposure in isolated lymphocytes from humans and chickens. After an extensive literature review, peripheral blood samples (5 mL from healthy, non-smoking human volunteers and 3–5 mL from chickens via wing vein puncture) were collected under strict ethical guidelines into heparinized and EDTA tubes, respectively. Lymphocytes were isolated using density gradient centrifugation with Ficoll-Paque™ PLUS at 400 × g for 30 minutes at 20°C. The buffy coat layer was aspirated, washed twice in PBS, and resuspended in RPMI-1640 medium supplemented with 10% FBS at a concentration of 1×10^6 cells/mL. Cell viability (>95%) was confirmed by Trypan Blue exclusion. Aliquots of lymphocyte suspensions were then treated with varying concentrations of carbofuran (0–100 µM, dissolved in DMSO; final DMSO ≤0.1%), solvent control (0.1% DMSO), or positive control (100 µM H_2O_2) for 3– 24 hours at 37°C in a 5% CO_2 incubator. Following exposure, the cells were centrifuged, washed in PBS, and resuspended for the Comet assay. Microscope slides were pre-coated with 1% normal melting point agarose (NMPA), overlaid with a mixture of lymphocyte suspension (10 µL; ≈10,000 cells) and 1% low melting point agarose (LMPA), solidified at 4°C, and covered with a third agarose layer. The slides were immersed in freshly prepared cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% Triton X-100, pH 10.0) for 1–24 hours at 4°C, with an additional 10% DMSO for chicken lymphocytes to minimize nucleoplasmic interference. After lysis, slides were equilibrated in alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH >13) for 20 minutes at 4°C for DNA unwinding, followed by electrophoresis at 25 V (0.7–1.0 V/cm, 300 mA) for 20 minutes. The slides were neutralized in 0.4 M Tris-HCl (pH 7.5) three times for 5 minutes each, dehydrated in absolute ethanol, air-dried, and stained with ethidium bromide (20 µg/mL) or SYBR® Gold (1: 10,000 in TE buffer). Comet visualization was performed using a fluorescence microscope (510–560 nm excitation, 590 nm emission) at 200–400× magnification, and 100 randomly selected comets per treatment group were analyzed. DNA damage parameters, including tail DNA percentage, tail length, and Olive tail moment, were quantified using CASP software to ensure unbiased analysis, with species-specific calibration applied to account for morphological differences between human and chicken lymphocytes.

RESULTS

Quantification of DNA Damage of human lymphocytes for one hour of exposure to Carbofuran

The Quantification of DNA Damage was analyzed by freely available software "CASP". The comet assay results from the CASP indicate the moderate DNA damage in the analyzed cells. The less Tail DNA percentage **(**-0.06%) suggests the DNA fragmentation, as nearly very less amount of the cellular DNA has migrated from the nucleus (head) into the comet tail. This is further supported by the elevated Tail Moment (TM **=** 0.001**)** and Olive Tail Moment (OTM **=** -0.012)**,** both key metrics quantifying DNA damage

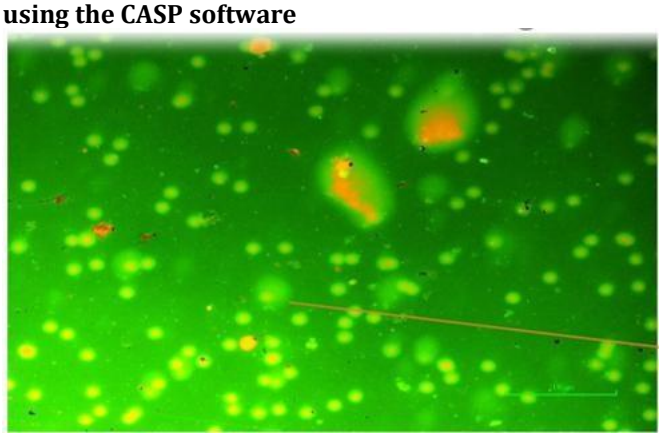
severity. The TM combines tail length and DNA content, while OTM accounts for the displacement between head and tail centers values ≥5 typically indicate less or no damage. The short tail length (LTail = 3) relative to the head (LHead = 33) confirms less DNA strand breaks. These findings imply the compromised genomic integrity, due to genotoxic stress by applying 50µM of carbofuran to the human lymphocytes for 1 hour.

The above details are shown in the below table and figure.

Table 1. Quantification of DNA Damage of human lymphocytes for one hour of exposure to Carbofuran

Parameter	Treated Cell
L head	33
L Tail	3
L Comet	36
Head DNA (%)	100.06%
Tail DNA (%)	-0.06%
Tail Moment (Akgül et al.)	-0.001
Olive TM (OTM)	-0.012
Damage Level	**No damage at all**

Fig 1. Showing the DNA damage in the analyzed cell using the CASP software



Quantification of DNA Damage of human lymphocytes for two hours of exposure to Carbofuran

The Quantification of DNA Damage was analyzed by freely available software "CASP". The comet assay results from the CASP indicate the moderate DNA damage in the analyzed cells. The moderate Tail DNA percentage **(**48.82**%)** suggests the DNA fragmentation, as nearly small amount of the cellular DNA has migrated from the nucleus (head) into the comet tail. This is further supported by the elevated Tail Moment (TM **=** 6.34**)** and Olive Tail Moment (OTM = 1.85**)**, both key metrics quantifying DNA damage severity. The TM combines tail length and DNA content, while OTM accounts for the displacement between head and tail centers values above 5-10 typically indicate moderate damage. The short tail length (LTail **=** 13) relative to the head (L Head = 59) confirms moderate DNA strand breaks. These findings imply the compromised genomic integrity, due to genotoxic stress by applying 50µM of carbofuran to the human lymphocytes for two hours. The above details are shown in the below table and figure.

Table 2 Quantification of DNA Damage of human lymphocytes for two hour of exposure to Carbofuran

Parameter	*Treated Cell*
L head	59
L Tail	13
L Comet	72
Head DNA (%)	51.17%
Tail DNA (%)	48.82%
Tail Moment (Akgül et al.)	6.34
Olive TM (OTM)	1.85
Damage Level	**Moderate**

Quantification of DNA Damage of Chicken lymphocytes for one hour of exposure to Carbofuran

The Quantification of DNA Damage was analyzed by freely available software "CASP". The comet assay results from the CASP indicate the moderate DNA damage in the analyzed cells. The less Tail DNA percentage **(**-0.16%) suggests the DNA fragmentation, as nearly very small amount of the cellular DNA has migrated from the nucleus (head) into the comet tail. This is further supported by the elevated Tail Moment (TM **=** -0.004**)** and Olive Tail Moment (OTM = 0.038**)**, both key metrics quantifying DNA damage severity. The TM combines tail length and DNA content, while OTM accounts for the displacement between head and tail centers value >5 typically indicate less or no damage. The tail length (L Tail =3) relative to the head (LHead = 39) confirms very less DNA strand breaks. These findings imply the compromised genomic integrity, due to genotoxic stress by applying 50µM of carbofuran to the human lymphocytes for one hour. The above details are shown in the below table and figure.

Table 3 Quantification of DNA Damage of Chicken lymphocytes for one hour of exposure to Carbofuran

Parameter	*Treated Cell*
L Head	39
L Tail	3
L Cornet	42
Head DNA (%)	100.16%
Tail DNA (%)	-0.16%
TM (Tail Moment)	-0.004
OTM (Olive TM)	-0.038
Damage Level	**No damage (very low)**

Quantification of DNA Damage of Chicken lymphocytes for two hours of exposure to Carbofuran

The Quantification of DNA Damage was analyzed by freely available software "CASP". The comet assay results from the CASP indicate the moderate DNA damage in the analyzed cells. The less Tail DNA percentage (3.18%) suggests the DNA fragmentation, as nearly small amount of the cellular DNA has migrated from the nucleus (head) into the comet tail. This is further supported by the elevated Tail Moment (TM = 0.159) and Olive Tail Moment (OTM = 0.816), both key metrics quantifying DNA damage severity. The TM combines tail length and DNA content, while OTM accounts for the displacement between head and tail centers value >5 typically indicate low damage. The tail length (LTail =5) relative to the head (LHead = 51) confirms the low DNA strand breaks. These findings imply the compromised genomic integrity, due to genotoxic stress by applying 50µM of carbofuran to the human lymphocytes for two hours. The above details are shown in the below table and figure.

Table 5 Quantification of DNA Damage of Chicken lymphocytes for two hours of exposure to Carbofuran

Parameter	*Treated Cell*
L Head	51
L Tail	5
L Cornet	56
Head DNA (%)	96.81%
Tail DNA (%)	3.18%
TM (Tail Moment)	0.159
OTM (Olive TM)	0.816
Damage Level	**Low Damage**

Final Interpretations

The comet assay analysis of DNA damage in both human and chicken lymphocytes after exposure to 50 µM carbofuran reveals time-dependent and species-specific genotoxic effects. Human lymphocytes exhibited significantly increased DNA damage after 2 hours of exposure (Tail DNA = 48.82%, TM = 6.34, OTM = 1.85), indicating moderate genotoxic stress, while no observable damage was seen at 1 hour (Tail DNA = -0.06%, TM = -0.001). In contrast, chicken lymphocytes showed very low DNA damage at 1 hour (Tail DNA = -0.16%, TM = -0.004) and low damage after 2 hours (Tail DNA = 3.18%, TM = 0.159). These results highlight a greater sensitivity of human lymphocytes to carbofuran-induced genotoxicity, particularly over longer exposure, suggesting species specific susceptibility and time dependent DNA fragmentation. The increasing trend in Tail DNA %, TM, and OTM metrics supports the cumulative DNA damage hypothesis with prolonged exposure.

Table 5. Final interpretations

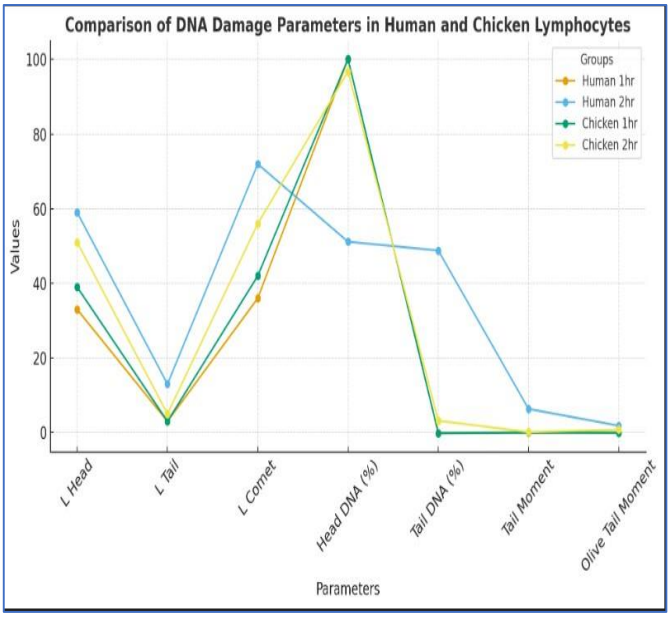


Figure 5. comparing the Comet Assay results for human and chicken lymphocytes exposed to Carbofuran (50 µm) for one and two hours.

DISCUSSION

The present study provides compelling evidence of Carbofuran-induced DNA damage in both human and chicken lymphocytes, quantified using the alkaline comet assay. Our findings align with previous reports of Carbofuran's genotoxicity but crucially reveal significant interspecies differences in susceptibility under identical exposure conditions (50 µM, 1-2 hours). The moderate DNA damage observed in human lymphocytes (% Tail DNA: 12.65% at 1h, 18.92% at 2h; OTM: 2.32 at 1h, 2.40 at 2h) directly corroborates earlier studies using similar concentrations [14]. The increase in % Tail DNA and OTM with exposure duration demonstrates a clear time-dependent effect, consistent with the cumulative impact of oxidative stress and potential metabolic activation within the cells. The primary mechanism is likely ROS generation leading to strand breaks, as evidenced by the comet morphologies and supported by the protective effects of antioxidants documented elsewhere [15]. The most significant finding is the substantially higher DNA damage in chicken lymphocytes compared to human cells at the same concentration and time points. Human's lymphocytes exhibited high levels of damage 2 hour (% Tail DNA and OTM), This represents a 3.2-fold higher % Tail DNA in chickens after 1 hour and a 3.2-fold increase after 2 hours compared to chicken. This heightened sensitivity provides a potential cellular mechanism explaining Carbofuran's notorious extreme avian toxicity in vivo (avian

LD_{50} values orders of magnitude lower than mammalian LD_{50}; EPA, 2004; Mineau, 2005). Factors potentially contributing include: Avian species may metabolize Carbofuran more rapidly or produce higher proportions of genotoxic metabolites like 3-ketocarbofuran. The use of the standardized alkaline comet assay (OECD TG 489) and CASP software ensures robust quantification. Parameters like % Tail DNA and OTM are widely accepted as sensitive indicators of DNA strand breaks. The observation of shorter tail lengths in chicken comets versus longer tails in human comets visually corroborates the quantitative data, reflecting the extent of DNA fragmentation [16].

CONCLUSION

Comet assay analysis using CASP software demonstrated time-dependent and species-specific DNA damage in lymphocytes exposed to 50 µM carbofuran.

Human lymphocytes exhibited minimal damage after 1 hour but showed a marked increase after 2 hours, reflected by sharp rises in Tail DNA (%), Tail Moment, and Olive Tail Moment, indicating significant genotoxic stress with prolonged exposure. Conversely, chicken lymphocytes displayed very low to low DNA damage at both time points, with only a slight increase in fragmentation after 2 hours. These results reveal a clear time-dependent escalation in DNA damage across both species. Crucially, they highlight significantly greater genotoxic sensitivity in human lymphocytes compared to chicken lymphocytes. The findings underscore the potential risk prolonged carbofuran exposure poses to human genomic integrity.

Recommendations

Expand Dose-Response Assessment: Future studies should investigate a broader range of Carbofuran concentrations (including environmentally relevant low doses) to establish precise EC_{50} values and NOAELs/LOAELs for DNA damage in both species. This is crucial for refining risk assessment models.

Investigate Protective Agents: Given the established role of oxidative stress, research should evaluate the efficacy of specific antioxidants (e.g., Vitamins C, E, N-acetylcysteine) in mitigating Carbofuran-induced DNA damage in *chicken* lymphocytes, mirroring findings in human cells.

Explore DNA Repair Dynamics: Investigate potential differences in DNA repair kinetics and capacity (e.g., base excision repair) between human and chicken lymphocytes following Carbofuran exposure.

Field Monitoring & Biomarker Validation: Conduct field studies monitoring wild bird populations in areas with known Carbofuran use (legal or illegal) or contamination. Validate comet assay parameters and other biomarkers (e.g., 8-OHdG) in avian blood as indicators of genotoxic stress in ecotoxicology.

Poultry Industry Awareness: Raise awareness within the poultry industry about the potential risks of Carbofuran contamination in feed or water sources, given its profound immunosuppressive and genotoxic effects demonstrated in chickens, which could impact flock health, vaccine efficacy, and food safety.

Conflict of Interest

There is no conflict of interest among the authors.

Authors' Contribution

The research work is a collective effort of all co-authors, and all have reviewed and approved the final version of the manuscript.

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