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Silybum Marianum Oil Attenuates Carbon Monoxide-Induced Pancreatic Bcl-2 Overexpression: An Age-Dependent Immunohistochemical Study

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ABSTRACT: Chronic low-level carbon monoxide (CO) exposure is an escalating environmental hazard, yet its impact on the apoptotic balance of the exocrine and endocrine pancreas, and the modulating role of natural antioxidant oils, remain poorly defined. We evaluated the anti-apoptotic protein Bcl-2 in the rat pancreas after chronic CO exposure and after correction with Silybum marianum (milk thistle) seed oil, and how chronological age conditions this response. Outbred male rats aged 3 and 12 months were allocated to control, CO (0.01–0.05 mg/L in air, 2 months) and CO plus intragastric S. marianum oil (14 days) groups. Bcl-2 was detected by immunohistochemistry and quantified as the labelling index (percentage of positive-stained area) using QuPath digital image analysis (n=10 per subgroup). Data were analysed by two-way analysis of variance on log-transformed values with Tukey post-hoc tests and confirmed by non-parametric procedures. Chronic CO markedly increased pancreatic Bcl-2 positivity relative to age-matched controls (3-month: 5.12→23.91%, 4.7-fold; 12-month: 1.19→24.15%, 20.3-fold; $p<0.001$), indicating an intense but morphologically insufficient compensatory anti-apoptotic response. S. marianum oil significantly lowered Bcl-2 toward baseline (3-month: 11.08%; 12-month: 15.17%; $p<0.001$ versus CO), with a significant group×age interaction ($p<0.001$) reflecting less complete normalization in older animals. Pancreatic Bcl-2 over-expression tracks the

severity of CO-induced oxidative–hypoxic injury in an age-dependent manner, and S. marianum oil restores the apoptosis–survival balance, supporting its use as an adjunct pancreatoprotective agent.

1. INTRODUCTION

Carbon monoxide (CO) is a colourless, odourless product of the incomplete combustion of carbon-containing fuels and remains one of the most common causes of poisoning worldwide. According to the Global Burden of Disease Study 2021, unintentional CO poisoning accounted for an estimated 28,900 deaths globally in that year, and the true burden is underestimated because mild and chronic exposures are frequently misdiagnosed (Moberg et al., 2023; Mattiuzzi and Lippi, 2020). Beyond acute intoxication, progressive urbanization, motorization and the indoor use of solid fuels expose large populations to prolonged low-level CO, whose sub-lethal effects on internal organs are only partially characterized.

The classical mechanism of CO toxicity is the formation of carboxyhemoglobin: CO binds haemoglobin with roughly 200–250 times the affinity of oxygen, shifting the oxyhaemoglobin dissociation curve to the left and impairing tissue oxygen delivery, thereby producing haemic hypoxia. Contemporary evidence shows that CO toxicity extends well beyond hypoxia: CO inhibits mitochondrial cytochrome c oxidase, disrupts the electron-transport chain and oxidative phosphorylation, promotes the generation of reactive oxygen and nitrogen species, enhances lipid peroxidation and destabilizes cell membranes, and triggers endothelial dysfunction and inflammation (Reboul et al., 2017; Meyer et al., 2015). These converging insults lower ATP synthesis, disturb calcium homeostasis and ultimately activate necrotic and apoptotic cell-death programmes.

The organ-level consequences of CO have been documented for the brain, myocardium, coronary vasculature, lungs, liver, kidney and spleen (Andre et al., 2010; Reboul et al., 2017). The pancreas, a metabolically demanding gland with high oxygen consumption and limited antioxidant reserve, is a plausible but comparatively neglected target. Interestingly, CO exerts a dual, dose-dependent action on pancreatic tissue: at low, endogenous concentrations the heme-oxygenase/CO axis is cytoprotective and can protect β-cells from apoptosis (Günther et al., 2002), and CO directly modulates calcium and nitric-oxide signalling in pancreatic acinar cells through this pathway (Moustafa and Habara, 2014), whereas sustained exogenous exposure is expected to overwhelm these defences and to shift the balance toward cell death. The molecular readout of this balance is governed by the Bcl-2 family of proteins, which control mitochondrial outer-membrane permeabilization; the anti-apoptotic member Bcl-2 opposes the pro-apoptotic effectors and its expression is dynamically tuned by cellular stress (Singh et al., 2019; Qian et al., 2022).

In parallel, there is growing interest in natural antioxidant oils as adjuncts that limit oxidative organ injury. *Silybum marianum* (milk thistle) seed oil is rich in the flavonolignan complex silymarin (silybin A/B, isosilybin, silychristin, silydianin), unsaturated fatty acids (predominantly linoleic and oleic acids), α-tocopherol, phytosterols and carotenoids, a composition associated with strong radical-scavenging and membrane-stabilizing activity and with the reduction of oxysterol-induced oxidative stress (Maaloul et al., 2024; Kamal et al., 2022; Wang et al., 2020). Silymarin and its constituents modulate apoptosis-regulating pathways, including the Bcl-2/Bax axis, in several toxic-injury models (Yassin et al., 2021; Fallah et al., 2021).

Research gap. Despite this background, three questions remain open. First, the apoptotic response of the pancreas to *chronic* low-level CO exposure has not been quantified with objective digital immunohistochemistry. Second, whether a natural antioxidant oil such as *S. marianum* can correct the CO-induced disturbance of the pancreatic anti-apoptotic programme is unknown. Third, because both baseline apoptotic tone and regenerative capacity change with age, it is unclear how chronological age conditions the injury and its correction. No previous study has addressed these questions together using standardized, reproducible quantification.

Hypothesis and aim. We hypothesized that chronic CO exposure induces an age-dependent compensatory up-regulation of pancreatic Bcl-2 that reflects the intensity of oxidative–hypoxic stress, and that *S. marianum* seed oil attenuates this response by relieving the underlying stress. Accordingly, the aim of this study was to perform a comparative, QuPath-based immunohistochemical analysis of Bcl-2 expression in the pancreas of 3- and 12-month-old rats under control conditions, after chronic CO exposure and after correction with *S. marianum* oil.

2. MATERIALS AND METHODS

2.1. Animals and ethical approval

The study used 210 outbred male albino rats of two age cohorts, 3 months (young adult) and 12 months (mature adult). All procedures complied with the Declaration of Helsinki principles for the use of animals, the ARRIVE 2.0 guidelines and international norms for laboratory-animal care (World Medical Association, 2013; Percie du Sert et al., 2020). The protocol was reviewed and approved by the Local Ethics Committee of Bukhara State Medical Institute named after Abu Ali ibn Sino (Protocol No. 12 of 1 April 2026; approval extract No. 6508 of 7 April 2026; electronic-signature document code XP78333173). Before the experiment all animals were quarantined for one week, screened for somatic and infectious disease, and maintained under standard vivarium conditions (12 h light/dark cycle, 22±2 °C, free access to standard chow and water). General condition, behaviour and body-weight dynamics were monitored throughout.

2.2. Experimental design and treatments

Animals were randomly allocated to three groups of 70 (35 per age): group I, control; group II, chronic CO exposure; group III, chronic CO exposure with *S. marianum* correction (Table 1). Chronic intoxication (groups II and III) was produced in purpose-built hermetic metal chambers with an air CO fraction of 0.01–0.05 mg/L for 2 months. In group III, after the exposure period, an ethanolic solution of *S. marianum* (milk thistle) seed oil (1:9 v/v) was administered intragastrically by metal gavage at 0.1 mL/animal once daily for 14 days. Control animals received 1 mL distilled water intragastrically for 14 days. Six animals died during CO exposure (two 3-month and two 12-month rats in group II; one 3-month and one 12-month rat in group III), leaving 204 animals for analysis. At the end of the protocol, rats were fasted overnight and euthanized in the morning under deep ether anaesthesia.

Table 1. Experimental groups and allocation of animals.

Group	Nature of the experiment	Age	Animals (n)	Deaths
I	Control (untreated)	3 months	35	—
		12 months	35	—
II	Chronic CO exposure (0.01–0.05 mg/L air, 2 months)	3 months	35	2
		12 months	35	2
III	Chronic CO exposure followed by intragastric *S. marianum* oil (1:9 v/v, 0.1 mL/day, 14 days)	3 months	35	1
		12 months	35	1
Total			**210 (204 analysed)**	**6**

Deaths occurred during CO exposure and are shown per subgroup.

2.3. Tissue processing

Pancreata were excised, fixed in 10% neutral buffered formalin (tissue-to-fixative ratio 1:10) for 24–48 h, washed in running water for 1 h, dehydrated through graded ethanol, cleared and embedded in paraffin according to standard technique. Sections 4–6 μm thick were cut on an MC-2 rotary microtome and mounted on positively charged slides for immunohistochemistry.

2.4. Bcl-2 immunohistochemistry

Immunohistochemistry was performed using a standard heat-induced epitope retrieval and horseradish-peroxidase (HRP)

polymer detection protocol. Paraffin sections were deparaffinized in xylene and rehydrated through descending ethanol. Heat-induced epitope retrieval was carried out in 10 mM citrate buffer (pH 6.0) at 95–98 °C for 20 min, followed by cooling to room temperature. Endogenous peroxidase was quenched with 3% hydrogen peroxide (10 min) and non-specific binding was blocked with protein block (10 min). Sections were incubated with a rabbit monoclonal anti-Bcl-2 primary antibody (clone E17; Abcam, Cambridge, UK; catalogue ab32124) at 1:200 dilution for 60 min at room temperature (or overnight at 4 °C). After washing in Tris-buffered saline with 0.05% Tween-20, sections were incubated with an HRP-conjugated anti-rabbit polymer secondary reagent (EnVision+ System-HRP; Dako/Agilent, Santa Clara, CA, USA) for 30 min. The reaction was developed with 3,3'-diaminobenzidine (DAB) chromogen, counterstained with Mayer's haematoxylin, dehydrated and mounted. Positive controls (rat lymphoid follicles) and negative controls (primary antibody omitted) were run in every batch. Cytoplasmic/membranous brown staining was scored as Bcl-2-positive.

2.5. Digital image analysis (QuPath)

Stained sections were digitized and analysed with QuPath v0.4.0 open-source digital-pathology software (Bankhead et al., 2017). For each animal, multiple non-overlapping fields representative of the whole pancreatic section, encompassing both the exocrine parenchyma and the islets of Langerhans, were captured at ×400 magnification. Colour deconvolution separated the DAB and haematoxylin channels; automated cell detection with an intensity threshold on the DAB channel classified each nucleus-bearing cell as positive or negative. The primary outcome was the Bcl-2 labelling index, defined as the percentage of Bcl-2-positive area per animal averaged across fields; because staining was assessed over the whole section, this index reflects the combined exocrine and endocrine compartments rather than either compartment in isolation. In the analysis overlays, positive cells are outlined in red and negative cells in blue. Quantification was performed by an observer blinded to group allocation. For the quantitative comparison, a balanced subset of 10 animals per subgroup was drawn by computer-generated random numbers, blinded to staining outcome, yielding a 3 (group) × 2 (age) factorial design (total N=60); with the effect sizes subsequently observed, this sample provided greater than 90% statistical power at $\alpha=0.05$, and the balanced design maximizes the efficiency and robustness of the two-way analysis.

2.6. Statistical analysis

Statistical analysis was performed in IBM SPSS Statistics v26 with confirmatory computation in R. Data are expressed as mean ± standard deviation (SD) and, where appropriate, as median [interquartile range, IQR]. Normality was assessed by the Shapiro–Wilk test and homogeneity of variances by Levene's test. Because raw Bcl-2 percentages showed variance that increased with the mean (a characteristic of proportion data) and heteroscedasticity (Levene $p<0.05$), values were natural-log transformed, which satisfied the variance-homogeneity assumption (Levene $p=0.09$). The transformed data were analysed by two-way analysis of variance (ANOVA) with exposure group (three levels) and age (two levels) as fixed factors, including their interaction; partial eta-squared (η^2_p) is reported as the effect size. Pairwise comparisons used the Tukey honestly-significant-difference (HSD) test. As a robustness check consistent with the non-parametric framework of the parent study, an omnibus Kruskal–Wallis test with Dunn's post-hoc (Holm correction) and within-age Mann–Whitney U tests (with rank-biserial correlation as effect size) were also computed; these confirmed the parametric results. A two-tailed $p<0.05$ was considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Qualitative pattern of pancreatic Bcl-2 immunoexpression

In control animals, Bcl-2 immunoreactivity was sparse and confined mainly to scattered cells of the exocrine acini, occasional ductal epithelial cells and a few islet cells, appearing as faint cytoplasmic brown staining on a predominantly haematoxylin-blue background (Figure 1A, B; Figure 2A, B). Chronic CO exposure produced a striking, diffuse increase in the number and intensity of Bcl-2-positive cells throughout the exocrine parenchyma and the islets of Langerhans, with strong granular cytoplasmic staining (Figure 1C, D; Figure 2C, D). After correction with *Silybum marianum* oil, the density of Bcl-2-positive cells was visibly reduced relative to the CO group and approached, but did not fully reach, the control pattern (Figure 1E, F; Figure 2E, F). The QuPath classification overlays (Figure 2), in which positive cells are outlined in red and negative cells in blue, reproduced this gradient objectively and formed the basis of the quantitative analysis.

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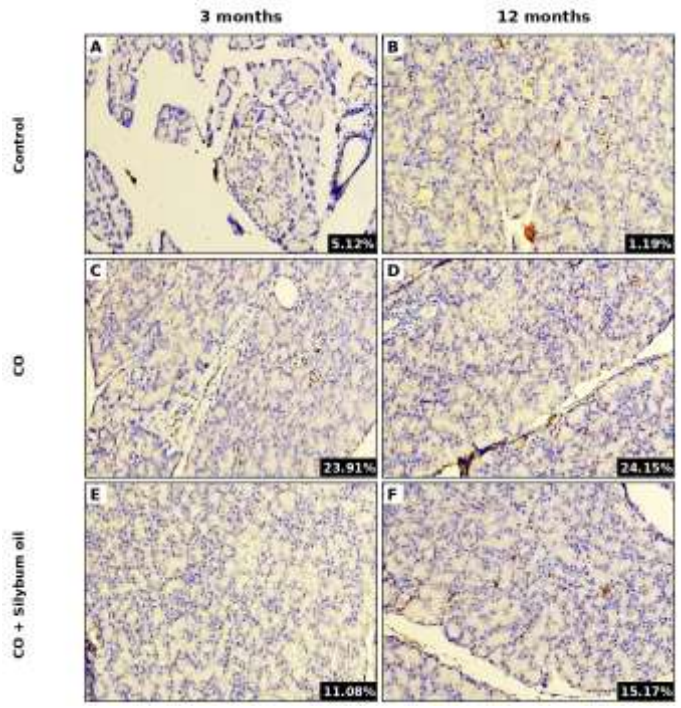

Figure 1. Representative immunohistochemical staining of Bcl-2 in the rat pancreas (DAB chromogen, Mayer's haematoxylin counterstain, ×400). Rows: control (A, B), chronic CO exposure (C, D) and CO + Silybum marianum oil (E, F). Columns: 3-month-old (A, C, E) and 12-month-old (B, D, F) animals. The mean percentage of Bcl-2-positive area for each subgroup is inset. Brown cytoplasmic/membranous signal denotes Bcl-2 positivity. Chronic CO exposure markedly increases the number and intensity of positive cells, which is attenuated after S. marianum oil correction.

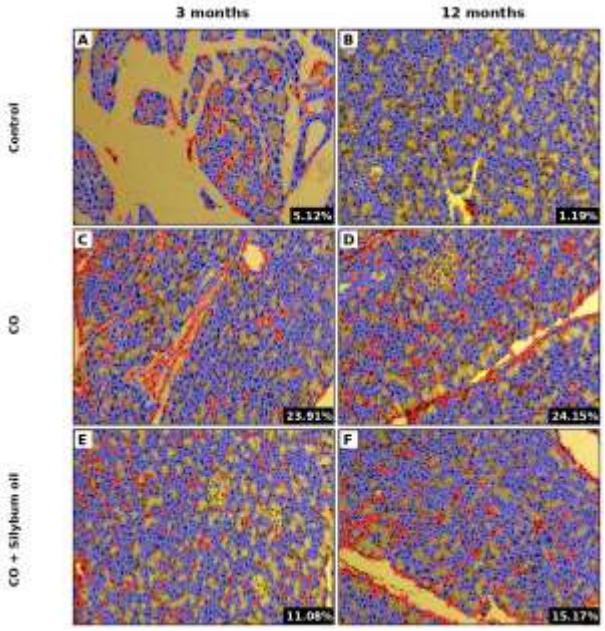

Figure 2. QuPath digital-pathology classification overlays corresponding to the fields in Figure 1 (×400). Positive cells are outlined in red and negative cells in blue after DAB/haematoxylin colour deconvolution and intensity thresholding. Rows: control (A, B), chronic CO exposure (C, D) and CO + Silybum marianum oil (E, F); columns: 3-month-old (A, C, E) and 12-month-old (B, D, F) animals. The overlays provide the objective, observer-independent basis for the percentage of Bcl-2-positive area.

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3.2. Quantitative digital immunohistochemistry

The percentage of Bcl-2-positive area for each subgroup is summarized in Table 2 and displayed in Figure 3. In the young-adult (3-month) cohort, Bcl-2 positivity rose from 5.12 ± 0.92% in controls to 23.91 ± 3.35% after chronic CO exposure, a 4.7-fold increase ($p<0.001$), and was reduced to 11.08 ± 1.86% after *S. marianum* oil correction, a 54% reduction relative to CO (2.16-fold lower; $p<0.001$). In the mature-adult (12-month) cohort, control positivity was lower (1.19 ± 0.38%), consistent with an age-related decline in baseline Bcl-2 tone, whereas CO exposure raised positivity to 24.15 ± 3.58%, a 20.3-fold increase ($p<0.001$); *S. marianum* oil lowered this value to 15.17 ± 2.41% (1.6-fold lower than CO; $p<0.001$). Thus, chronic CO drove pancreatic Bcl-2 to a similarly high plateau at both ages (~24%), while the protective effect of the oil was quantitatively greater and more complete in younger animals.

Table 2. Descriptive statistics of the percentage of Bcl-2-positive pancreatic area by exposure group and age (n = 10 per subgroup).

Group	Age	n	Mean ± SD (%)	Median [IQR] (%)	Fold vs control
Control	3 mo	10	5.12 ± 0.92	5.12 [4.59–5.65]	1.0 (ref.)
Control	12 mo	10	1.19 ± 0.38	1.19 [0.97–1.41]	1.0 (ref.)
CO	3 mo	10	23.91 ± 3.35	23.91 [21.97–25.85]	**4.7×**
CO	12 mo	10	24.15 ± 3.58	24.15 [22.08–26.22]	**20.3×**
CO + *S. marianum*	3 mo	10	11.08 ± 1.86	11.08 [10.00–12.16]	2.2×
CO + *S. marianum*	12 mo	10	15.17 ± 2.41	15.17 [13.78–16.56]	12.7×

SD, standard deviation; IQR, interquartile range. Fold change is expressed relative to the age-matched control. All CO and CO + S. marianum subgroups differ from their age-matched control at p<0.001 (Table 3).

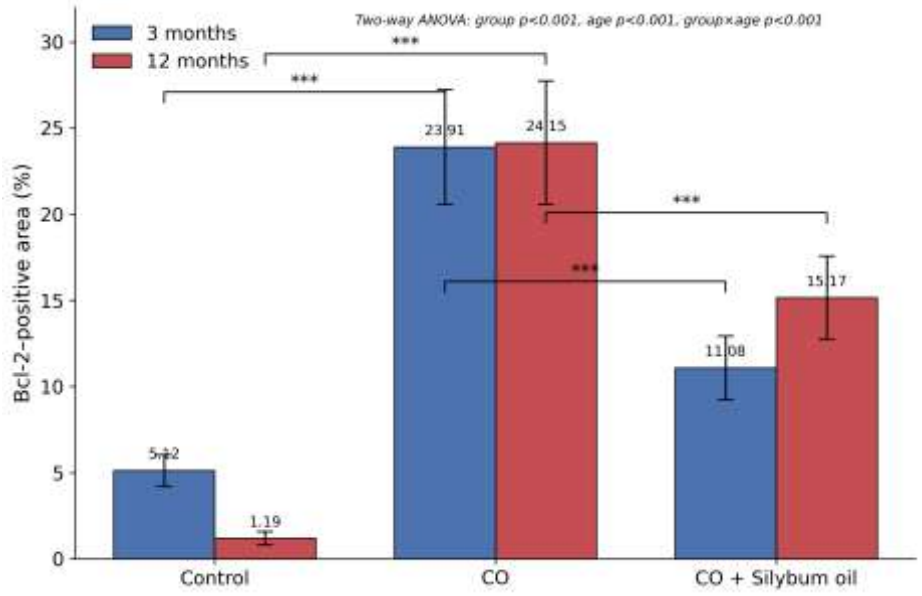

Figure 3. Percentage of Bcl-2-positive pancreatic area (mean ± SD, n = 10 per subgroup) by exposure group and age. Blue bars, 3-month-old animals; red bars, 12-month-old animals. Chronic CO exposure sharply increases Bcl-2 positivity at both ages, and Silybum marianum oil correction significantly lowers it toward control values. ***$p<0.001$ for the indicated contrasts (Tukey HSD on log-transformed data). The two-way ANOVA result is annotated above the plot.

3.3. Statistical modelling

Because the raw percentages were heteroscedastic (Levene p=0.001), the model was fitted on natural-log-transformed values, which restored variance homogeneity (Levene p=0.09) and approximate normality of residuals (Shapiro–Wilk p>0.8 in every cell). The two-way ANOVA (Table 3) revealed a very large main effect of exposure group ($F(2,54)=661.6$, $p<0.001$, $\eta^2_p=0.96$), a significant main effect of age ($F(1,54)=53.4$, $p<0.001$, $\eta^2_p=0.50$) and a strong group×age interaction ($F(2,54)=109.8$, $p<0.001$, $\eta^2_p=0.80$). The interaction reflects the divergent age behaviour of the three groups: baseline Bcl-2 fell significantly with age in controls (Tukey p<0.001), CO drove positivity to an equally high level at both ages (CO 3-month versus CO 12-month, p=1.00), and the residual positivity after correction was significantly higher in older than in younger animals (S. marianum 3-month versus 12-month, p=0.015). All within-age exposure contrasts were highly significant (control versus CO and CO versus CO + *S. marianum*, p<0.001). The non-parametric analysis mirroring the parent study fully confirmed these findings: the Kruskal–Wallis omnibus test was significant ($H=55.1$, $p=1.3\times10^{-10}$), and within-age Mann–Whitney U comparisons were all significant (p<0.001) with maximal rank-biserial effect sizes (r≥0.98); Cohen's d for the CO-versus-control contrast reached 7.7 (3-month) and 9.0 (12-month), and for the correction-versus-CO contrast −4.7 (3-month) and −2.9 (12-month), confirming very large, biologically meaningful effects.

Table 3. Two-way ANOVA on log-transformed Bcl-2 positivity and principal pairwise (Tukey HSD) comparisons.

Source / contrast	F / statistic	df	p-value	η^2_p
Exposure group	661.6	2, 54	<0.001	0.96
Age	53.4	1, 54	<0.001	0.50
Group × Age	109.8	2, 54	<0.001	0.80
Control vs CO (3 mo)	Tukey	—	<0.001	—
Control vs CO (12 mo)	Tukey	—	<0.001	—
CO vs CO + *S. marianum* (3 mo)	Tukey	—	<0.001	—
CO vs CO + *S. marianum* (12 mo)	Tukey	—	<0.001	—
Control 3 mo vs Control 12 mo	Tukey	—	<0.001	—
CO 3 mo vs CO 12 mo	Tukey	—	1.00 (ns)	—
S. marianum 3 mo vs 12 mo	Tukey	—	0.015	—

df, degrees of freedom; η^2_p, partial eta-squared; ns, not significant. Omnibus non-parametric confirmation: Kruskal–Wallis $H=55.1$, $p=1.3\times10^{-10}$; within-age Mann–Whitney U p<0.001 (r≥0.98).

3.4. Interpretation and comparison with the literature

The central and most reproducible finding of this study is that chronic low-level CO exposure provokes a massive up-regulation of the anti-apoptotic protein Bcl-2 in the rat pancreas. At first sight an increase in a pro-survival protein might be read as protective; however, the magnitude and context of the response indicate that it is a *compensatory* reaction to severe oxidative–hypoxic stress rather than evidence of preserved tissue health. Chronic CO impairs oxygen delivery through carboxyhaemoglobin formation and simultaneously poisons mitochondrial cytochrome c oxidase, generating reactive oxygen species and lipid peroxidation (Reboul et al., 2017; Meyer et al., 2015). Under such stress, cells attempt to raise the anti-apoptotic threshold at the mitochondrial outer membrane by increasing Bcl-2, which sequesters pro-apoptotic effectors and delays mitochondrial outer-membrane permeabilization (Singh et al., 2019). A stress-driven rise in Bcl-2 is therefore best interpreted as a biomarker of injury intensity: the greater the insult, the greater the compensatory demand. This interpretation

is consistent with reports that Bcl-2 expression is dynamically increased in tissues subjected to oxidative and hypoxic injury and in tumour microenvironments as a survival adaptation (Qian et al., 2022).

The dual, dose-dependent nature of the CO–pancreas relationship helps to reconcile our findings with the protective effects of endogenous CO. At low physiological concentrations generated by heme oxygenase, CO is anti-apoptotic and can protect pancreatic β-cells from apoptosis and improve islet survival (Günther et al., 2002), and modulates acinar-cell calcium and nitric-oxide signalling (Moustafa and Habara, 2014). The present model, in contrast, applied sustained exogenous CO for two months, an exposure that overwhelms cytoprotective signalling and pushes the tissue into a state of chronic compensated stress, reflected here by saturating Bcl-2 positivity (~24% at both ages). The plateau reached at both 3 and 12 months (with a non-significant age difference within the CO group) suggests a ceiling to the compensatory response that is engaged maximally once a threshold of chronic injury is crossed.

Age materially shaped the baseline and the correction, producing the strong group×age interaction. Baseline pancreatic Bcl-2 was markedly lower in 12-month than in 3-month controls, in keeping with the recognized decline of anti-apoptotic tone and the shift toward a lower apoptotic threshold in the ageing pancreas (Riccillo et al., 2004). Consequently, the fold-increase driven by CO was far larger in older animals (20.3-fold) than in younger ones (4.7-fold), even though the absolute plateau was similar. This suggests that the ageing pancreas has less anti-apoptotic reserve at rest and must mount a proportionally greater compensatory response, a configuration that may render it more vulnerable to CO-related injury.

Correction with *Silybum marianum* seed oil significantly lowered pancreatic Bcl-2 toward, but not fully to, control levels. Within the compensatory framework, this downward normalization is the desired outcome: by relieving the upstream oxidative–hypoxic drive, the oil reduces the demand for compensatory Bcl-2 and restores the apoptosis–survival balance rather than abolishing protection. The bioactivity is attributable to the silymarin flavonolignan complex together with the oil's unsaturated fatty acids, α-tocopherol and phytosterols, which scavenge radicals, stabilize membranes and reduce oxysterol-driven oxidative stress (Maaloul et al., 2024; Kamal et al., 2022; Wang et al., 2020). Silymarin and its constituents are known to modulate the Bcl-2/Bax axis and to attenuate apoptosis in several models of chemical and metabolic organ injury (Yassin et al., 2021). The less complete normalization observed in 12-month animals (15.17% versus 11.08% in the young) indicates that the ageing pancreas responds more slowly or incompletely to antioxidant correction, an age effect with clear translational relevance for older, more exposed populations.

These findings carry particular relevance for the endocrine pancreas. The islets of Langerhans depend on a finely tuned apoptotic threshold to maintain β-cell mass, and endogenous heme-oxygenase-derived CO normally supports β-cell survival (Günther et al., 2002); a chronic, saturating perturbation of the Bcl-2 set-point of the kind documented here is therefore likely to compromise islet homeostasis. Because our labelling index was measured over the whole section, it captures both the exocrine parenchyma and the islets, and the marked, age-amplified disturbance suggests that chronic environmental CO may constitute an under-recognized metabolic stressor with potential implications for glucose regulation in exposed populations. The normalization achieved with *Silybum marianum* oil raises the possibility of endocrine as well as exocrine protection, a hypothesis that warrants dedicated islet-level and functional (glycaemic) evaluation.

Methodologically, the use of QuPath for whole-section, observer-independent quantification (Bankhead et al., 2017) strengthens the reliability of these results relative to manual, semi-quantitative scoring, and the concordance between the parametric (log-ANOVA) and non-parametric analyses indicates that the conclusions are robust to distributional assumptions. Taken together, the data identify pancreatic Bcl-2 as a sensitive, quantifiable index of chronic CO injury and its correction.

Limitations. This study quantified a single anti-apoptotic marker; a fuller picture of the apoptotic balance would require paired assessment of pro-apoptotic effectors (e.g. Bax, cleaved caspase-3) and of the Bcl-2/Bax ratio, as well as oxidative-stress and functional (glycaemic, enzymatic) end-points. The Bcl-2 labelling index was measured over the whole pancreatic section; compartment-specific analysis separating the exocrine parenchyma from the islets of Langerhans was not performed and would refine the localization of the response. The exposure model reproduces chronic environmental CO but does not capture intermittent human exposure patterns; blood carboxyhaemoglobin was not measured, so internal CO dose was inferred from the controlled chamber concentration rather than confirmed biochemically, and only male rats of two ages were studied. These considerations define clear directions for further work rather than caveats to the present, internally consistent findings.

4. CONCLUSIONS

Chronic low-level carbon monoxide exposure induces a large, age-dependent compensatory over-expression of the anti-apoptotic protein Bcl-2 in the rat pancreas, the magnitude of which indexes the severity of oxidative–hypoxic injury and saturates at both studied ages. Correction with *Silybum marianum* seed oil significantly normalizes pancreatic Bcl-2 toward control values by relieving the underlying stress, with a more complete effect in younger animals, as demonstrated by objective QuPath-based digital immunohistochemistry and confirmed by both parametric and non-parametric statistics. These findings identify pancreatic Bcl-2 as a sensitive quantitative marker of chronic CO injury and support *S. marianum* oil as a promising natural adjunct for mitigating CO-related pancreatic damage, particularly warranting evaluation in ageing, more susceptible populations.

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Author Contributions

S.R. Adizov: conceptualization, methodology, investigation, immunohistochemistry, digital image analysis, writing – original draft. N.E. Sulaymanova: formal (statistical) analysis, epidemiological framing, data curation, writing – review and editing. M. Abdullaeva: conceptualization, supervision, validation, histopathological interpretation, writing – review and editing. B.K. Badridinova: endocrinological interpretation, resources, writing – review and editing. M. Kudratova: investigation, tissue processing and data curation. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Statement

All experimental procedures involving animals were conducted in accordance with the ethical principles of the Declaration of Helsinki for the use of animals, the ARRIVE 2.0 guidelines and international norms for the care and use of laboratory animals. The study protocol was reviewed and approved by the Local Ethics Committee of Bukhara State Medical Institute named after Abu Ali ibn Sino, Ministry of Health of the Republic of Uzbekistan. As recorded in the extract from Protocol No. 12 of the Local Ethics Committee meeting of 1 April 2026 (approval extract No. 6508 of 7 April 2026; electronic-signature document code XP78333173, verified through the IJRO.GOV.UZ system), the committee reviewed the doctoral research of the independent researcher S.R. Adizov entitled "Comparative characterization of the morphometric indices of the pancreas under the chronic effect of carbon monoxide" (specialty 14.00.02 – Morphology; scientific supervisor DSc, Associate Professor M. Abdullaeva) and recommended it for preclinical investigation. The approval was confirmed by the Vice-Rector for Science and Innovation, M.M. Amonov, and certified by the committee secretary, S.Z. Nasirova, PhD.

Data Availability

The datasets generated and analysed during the current study, including per-animal QuPath measurements and the statistical output, are available from the corresponding author on reasonable request.

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