INTERNATIONAL JOURNAL OF MEDICAL SCIENCE AND DENTAL HEALTH
(ISSN: 2454-4191)
www.ijmsdh.org (2026)

Received: 13 Aug 2026
Revised: 26 Aug 2026
Accepted: 05 Sep 2026
Published: 18 Sep 2026

Volume 12 Issue 09, 2026
Page No - 136-148
DOI - 10.55640/ijmsdh-12-09-16

Article Citation: Khayriyeva Dilshoda Ulugbek kizi, & Bakhronov Jur'at Jurakulovich. (2026). Bcl-2 Immunoexpression In the Pancreas After Experimental 70% Acetic Acid Chemical Burn of The Digestive Tract: An Age-Stratified Quantitative Digital Immunohistochemical Study in Outbred Albino Rats. International Journal of Medical Science and Dental Health, 12(09), 136-148. https://doi.org/10.55640/ijmsdh-12-09-16

Bcl-2 Immunoexpression In the Pancreas After Experimental 70% Acetic Acid Chemical Burn of The Digestive Tract: An Age-Stratified Quantitative Digital Immunohistochemical Study in Outbred Albino Rats

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Abstract

Background. Concentrated acetic acid (vinegar essence, 70–80%) is the leading cause of corrosive ingestion across Russia, Eastern Europe and Central Asia, and produces not only local necrosis but a systemic phase of haemolysis, shock and multi-organ failure. Whether the pancreas — an organ outside the burn field - sustains injury at the level of apoptotic regulation has not been examined. Bcl-2 is the principal anti-apoptotic checkpoint of the mitochondrial cell-death pathway and is therefore the natural first marker to interrogate.

Objective. To quantify pancreatic Bcl-2 immunoexpression after a standardised 70% acetic acid burn of the digestive tract in rats of three ages, and to determine whether any change reflects true up-regulation in surviving cells or selective loss of Bcl-2-negative cells.

Methods. Ninety outbred albino rats aged 1, 3 and 6 months were assigned to intact control (n = 45) and chemical burn (n = 45), balanced across ages. Burn was produced by intra-oesophageal instillation of 0.5 mL of 70% acetic acid through a metal probe with 30–60 s exposure. Bcl-2 was demonstrated with DAB chromogen after citrate antigen retrieval; five fields per animal were scanned at ×200 and scored in QuPath 0.5.1. A total

of 9149 nuclei were classified. The labelling index was compared by χ^2 with Yates's correction, with odds ratios and Wilson 95% confidence intervals, and homogeneity of the effect across ages was tested by Woolf's Q. Because a labelling index is a ratio whose denominator may itself change with injury, positive-cell, negative-cell and total nuclear counts were additionally normalised to the analysed field area.

Results. The Bcl-2 labelling index was higher after burn at every age: 26.13% → 36.56% at 1 month, 29.20% → 35.68% at 3 months and 26.67% → 39.44% at 6 months (all $p < 0.001$). Pooled, it rose from 27.47% (1395/5078) to 37.39% (1522/4071); $\chi^2 = 101.8$, $p < 0.001$; odds ratio 1.58 (95% CI 1.44–1.72). The effect was not homogeneous across ages (Woolf Q = 7.61, df = 2, $p = 0.022$), so age-stratified reporting is required. Density analysis separated two distinct mechanisms. The density of Bcl-2-negative cells fell at every age (−36.5%, −30.6%, −16.7%), whereas the density of Bcl-2-positive cells was essentially unchanged at 1 and 3 months (+3.5%, −6.6%) and rose by 49.2% only at 6 months. The rise in labelling index at the two younger ages is therefore an arithmetic consequence of losing negative cells, while at 6 months it reflects genuine up-regulation. The pre-specified three-band grading scale classified every group, control and burned alike, as "moderate", and so detected none of this.

Conclusions. Caustic burn of the digestive tract measurably disturbs apoptotic regulation in the pancreas. Bcl-2-negative cells are selectively depleted at all ages, while a true anti-apoptotic response appears only in fully mature animals. A labelling index must not be reported without its denominator when the denominator is itself an outcome of the intervention — a caveat that applies well beyond this model.

Keywords: Bcl-2; apoptosis; immunohistochemistry; digital pathology; QuPath; pancreas; caustic ingestion; acetic acid; labelling index; rat.

1. Introduction

1.1. Clinical background

Corrosive ingestion is uncommon relative to other toxicological emergencies but disproportionately destructive [1, 2]. One agent dominates a specific region: acetic acid, sold at 2–6% as table vinegar almost everywhere, is a routine household preservative and cleaning agent at 70–80% ("vinegar essence") throughout Russia, Eastern Europe and Central Asia [3]. Ingestion above roughly 12% causes haemolysis, renal failure and shock; above 30% it reliably produces severe upper gastrointestinal injury together with intravascular haemolysis and multi-organ involvement [4]. In Russia acetic acid ingestion affects approximately 11.2 per 100 000 people annually, and the largest reported series — 400 consecutive patients poisoned with 70% acetic acid — recorded 21% mortality, haemolysis in 55% and renal injury in 35% [5].

It is that systemic phase which makes injury to organs outside the burn field plausible. Acute pancreatitis after acetic acid ingestion is documented [6], pancreatic involvement has required pancreatosplenectomy at emergency gastrectomy [7], and pancreatic oedema is a recognised finding on the computed tomography now used routinely for early risk stratification [8]. What has never been examined is whether the pancreas sustains injury at the level of apoptotic regulation.

1.2. Why Bcl-2

Cells under stress activate survival and death signalling simultaneously, and the two converge on the mitochondrion, where the balance is set by the pro- and anti-apoptotic members of the Bcl-2 family [9]. Bcl-2 itself is the prototypical anti-apoptotic member and the principal brake on mitochondrial outer membrane permeabilisation. In the pancreas it is constitutively expressed in acinar and ductal cells [10], and prosurvival Bcl-2 proteins stabilise pancreatic mitochondria and protect acinar cells against necrosis in experimental pancreatitis [11]. It is therefore the natural first marker with which to ask whether a distant chemical insult perturbs the survival–death equilibrium of pancreatic tissue.

The expected direction is downward. Reduced Bcl-2 with reciprocal induction of Bax and caspases is the standard finding in cerulein-induced acute pancreatitis, in chronic pancreatitis and in models of pancreatic fibrosis [11–13]. But that expectation is not universal. In end-stage heart failure, Bcl-2 and Bcl-xL rise alongside Bax, Bak and TUNEL positivity, and the authors read the increase as a concomitant compensatory anti-apoptotic mechanism operating in cells that are simultaneously dying [14]. Compensatory up-regulation of survival proteins in the cells that remain is a recognised pattern in acute injury more generally [15]. Which of these two patterns the pancreas follows after caustic burn is an empirical question.

1.3. A measurement problem that is rarely acknowledged

Immunohistochemical marker expression is almost universally reported as a labelling index: positive cells as a percentage of cells counted. That statistic is a ratio, and a ratio can move because its numerator changes, because its denominator changes, or both. In a tissue subjected to an injury that kills cells, the denominator is not a fixed reference — it is

itself an outcome. If an insult removes marker-negative cells preferentially, the labelling index rises even though no surviving cell has altered its expression by a single molecule. The two situations have opposite biological meanings and are indistinguishable from the index alone.

Digital pathology makes the distinction recoverable, because platforms such as QuPath [16] report the analysed field area alongside the counts, allowing positive, negative and total nuclei to be expressed as densities. This study reports both, and the difference between them turns out to carry the main biological finding.

1.4. The age question

Because corrosive ingestion is bimodal — accidental in children, deliberate in adults — an organ response that varies with developmental stage has direct clinical significance. The rat pancreas matures substantially over the first postnatal months, with acinar architecture, granule content and glucose-stimulated insulin secretion all changing, and a critical restructuring window around weaning [17, 18]. Anti-apoptotic set-point may well differ across that range, and the response to injury with it.

1.5. Aim

The aim was to quantify pancreatic Bcl-2 immunoexpression after a standardised 70% acetic acid burn of the digestive tract in rats aged 1, 3 and 6 months; to test whether the response is uniform across ages; and to determine, by density analysis, whether any change in the labelling index reflects true up-regulation in surviving cells or selective loss of Bcl-2-negative cells.

2. Methods

2.1. Ethical approval and reporting

The study was performed in the research laboratory of Abu Ali ibn Sino Bukhara State Medical Institute. All procedures followed the institutional "Rules for performing work using experimental animals" approved by the Institute's ethics committee (protocol No. 18 of 16 January 2018) and the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123, Strasbourg, 1986). Reporting follows the ARRIVE 2.0 guidelines [19].

2.2. Animals and design

Ninety outbred albino rats of both sexes, aged 1, 3 and 6 months, were bred in the Institute vivarium and maintained at 22–25 °C, 50–60% relative humidity and a 12–14 h light cycle with a balanced diet and free access to water. All animals were quarantined for seven days and admitted only after clinical examination excluded somatic and infectious disease. Allocation is shown in Table 1.

Table 1. Allocation of animals contributing to the immunohistochemical analysis.

Group	Description	1 month	3 months	6 months	Total
I	Intact control	15	15	15	45
II	Chemical burn (70% acetic acid)	15	15	15	45
	Total	30	30	30	90

2.3. Chemical burn model

Burn was produced by instilling 0.5 mL of 70% acetic acid into the oesophagus through a dedicated metal probe, with an exposure of 30–60 s, after which the tissues were irrigated with physiological saline. Control animals underwent identical handling and probe placement without acid. The concentration reflects the vinegar essence actually ingested in the region and used in the largest clinical series [3, 5]; injury above 30% is qualitatively different from injury below it, adding haemolysis and multi-organ involvement [4], and it is that systemic component which makes distant-organ injury plausible. Concentration also determines depth in a well-characterised way: 5% acetic acid produces necrosis confined to the mucosa, whereas 25% produces full-thickness necrosis of the wall [20], and models intended to yield deep, clinically representative lesions use 70–75% [21].

2.4. Tissue processing

Animals were fasted overnight with free access to water and killed by decapitation under ether anaesthesia. Pancreatic tissue was excised immediately and fixed in 10% neutral buffered formalin for 24–48 h, dehydrated through ascending ethanol concentrations, cleared in xylene and embedded in paraffin. Sections of 4–6 µm were cut on a rotary microtome and mounted on slides.

2.5. Immunohistochemistry

Immunohistochemical staining was performed on 4–6-µm-thick paraffin-embedded pancreatic tissue sections. The sections were deparaffinized in xylene and rehydrated through a graded series of ethanol concentrations and distilled water. Heat-induced antigen retrieval was performed by heating the sections in 0.01 M citrate buffer (pH 6.0) for 15–20 min. The sections were then allowed to cool to room temperature and rinsed with Tris-HCl buffer (pH 7.6).

Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide for 10 min at room temperature. After washing in Tris-HCl buffer, non-specific binding sites were blocked using an appropriate protein blocking solution. The sections were subsequently incubated with a primary antibody against B-cell lymphoma 2 (Bcl-2) at a working dilution of 1:100 overnight at 4°C.

Following incubation with the primary antibody, the sections were washed three times with Tris-HCl buffer and incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody/detection reagent according to the manufacturer's recommended procedure. Immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB) chromogen. The development of a distinct brown reaction product was considered indicative of positive Bcl-2 immunoreactivity. Following chromogen development, the sections were counterstained with Mayer's hematoxylin, dehydrated through ascending ethanol concentrations, cleared in xylene, and permanently mounted.

Bcl-2 immunoreactivity was evaluated based on the presence of distinct brown cytoplasmic staining in morphologically identifiable cells. Cells showing clear brown cytoplasmic immunoreactivity were considered Bcl-2-positive, whereas cells without specific brown staining were considered negative.

Appropriate positive and negative controls were included in each staining run. Lymphoid tissue was used as a positive control for Bcl-2 immunoreactivity, with strong staining expected in Bcl-2-expressing lymphoid cells. For the negative control, the primary antibody was omitted and replaced with the corresponding antibody diluent, while all other staining steps were performed identically. The absence of specific brown staining in the negative-control sections was used to assess non-specific background staining.

All tissue sections were processed under identical staining conditions. The stained sections were examined by light microscopy, and digital image acquisition and quantitative analysis were performed using QuPath software version 0.5.1.

2.6. Digital image analysis

Stained sections were imaged and analysed in QuPath version 0.5.1 [16]. Five fields of view per animal were captured at ×200. Within each field a region of interest was outlined over pancreatic tissue, cells were detected automatically, and each detected cell was classified as Bcl-2-positive or Bcl-2-negative. QuPath reported, for every image, the number of positive cells, the number of negative cells, the total number of detected cells and the area of the analysed region in square pixels. A total of 9149 cells were classified across the six group–age combinations.

Because the analysed area was recorded, three densities could be derived in addition to the labelling index: total nuclear density, Bcl-2-positive density and Bcl-2-negative density, each expressed per 10^5 px² of analysed field. These allow numerator and denominator to be examined separately.

2.7. Expression grading

A three-band ordinal scale was pre-specified: low (< 20% positive cells), moderate (20–60%) and high (> 60%). It is reported in Section 3.5 together with an assessment of whether it was fit for purpose.

2.8. Statistical analysis

Analysis was performed on cell counts rather than on per-animal percentages, since counts are what the platform returns. Labelling indices are given with Wilson score 95% confidence intervals, which are appropriate for binomial proportions and do not misbehave near the boundaries. Control and burn groups were compared at each age by the χ^2 test on the 2×2 table of positive and negative nuclei with Yates's continuity correction, and the effect expressed as an odds ratio with Woolf logit 95% confidence limits.

Because a single pooled estimate is only meaningful if the effect is consistent across strata, homogeneity of the three age-specific odds ratios was tested by Woolf's Q before pooling.

A trend in the labelling index across the three ages was tested within each group by the Cochran–Armitage test with scores 1, 3 and 6. Densities are descriptive and are reported as percentage change from the age-matched control. Two-sided $p < 0.05$ was taken as significant.

Limitation of the available data. QuPath output was retained as one pooled count per group–age combination rather than per animal, so between-animal variance cannot be estimated and the counts are treated as a single aggregated sample. This makes the confidence intervals narrower than a hierarchical analysis would give, because clustering within animals is not accounted for. Retaining per-animal counts in future work would permit a mixed-effects model with animal as a random effect, which is the statistically correct treatment; the direction and approximate magnitude of the effects reported here would not be expected to change, but their precision would be more honestly represented. This limitation is inherited from the source data and is stated wherever the intervals are used.

All calculations are reproduced as live formulas in the accompanying workbook.

3. Results

3.1. Distribution of Bcl-2 immunoreactivity

In control animals Bcl-2 appeared as a moderate, evenly distributed brown cytoplasmic reaction product. In the exocrine compartment it was present in acinar cells at consistent intensity across the lobule, with only minor field-to-field variation attributable to differences in local maturation at 1 month. In the endocrine compartment the reaction was more pronounced than in acinar tissue, and was most clearly expressed in the β cells occupying the islet core; α cells at the islet periphery showed moderate reactivity. Stromal fibroblasts and vascular endothelium were weakly or only episodically positive. The pattern was diffuse and homogeneous at all three ages, consistent with a controlled physiological balance between cell renewal and cell death, and neither focal over-expression nor abrupt loss was observed.

After burn the qualitative character of the staining changed as much as its quantity. The diffuse, even distribution seen in controls was replaced by a mosaic pattern in which strongly positive cells lay immediately adjacent to unstained ones. Intensity varied sharply between neighbouring acini, and some acini were almost devoid of reaction product. The cells retaining strong positivity were, as a rule, those whose morphology was best preserved; cells showing vacuolar dystrophy, nuclear pyknosis or karyorrhexis were typically negative. In the islets the uniform cytoplasmic reactivity of controls gave way to variable, patchy staining, most evident among β cells. Stromal and perivascular immunoreactivity became minimal or episodic, in the same fields that showed vascular dilatation and perivascular oedema.

This mosaicism is the visual counterpart of the quantitative findings that follow, and it is the reason the aggregate index required decomposition.

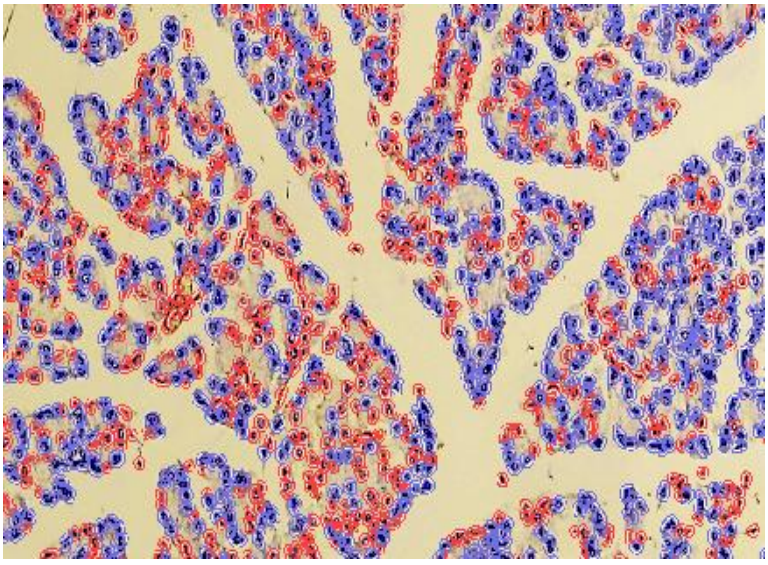

Figure 1. Bcl-2 immunoreactivity in the pancreas of a control rat aged 1 month. Moderate, evenly distributed cytoplasmic reaction product is present in acinar cells and is more pronounced in islet β cells; stromal and endothelial reactivity is low.

Labelling index 26.13% (375/1435 cells). Bcl-2, DAB chromogen, ×200. Scanned and scored in QuPath 0.5.1; cells classified as positive are rendered in red on the analysis overlay.

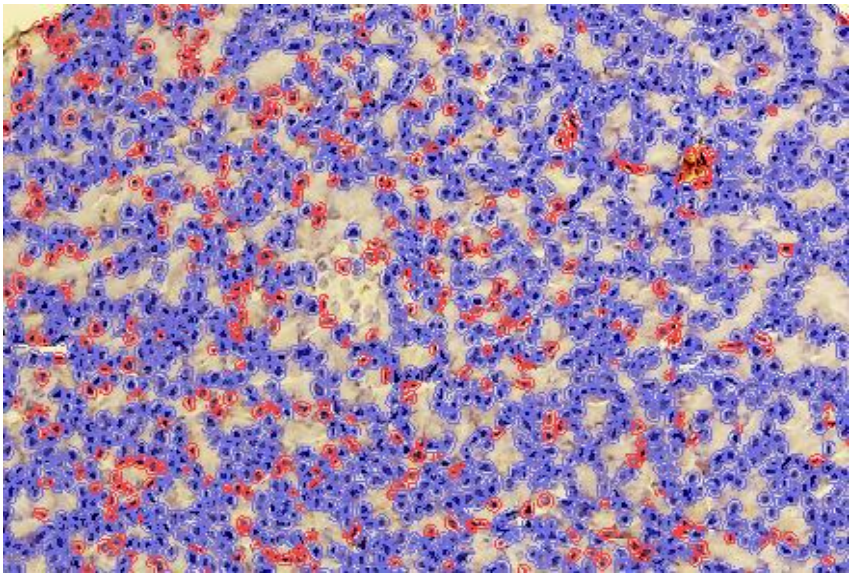

Figure 2. Bcl-2 immunoreactivity in the pancreas of a control rat aged 3 months. The diffuse, even distribution of reaction product across acinar structures indicates a stable anti-apoptotic set-point in the fully matured gland; islet β cells again stain more strongly than acinar cells. Labelling index 29.20% (560/1918 cells). Bcl-2, DAB chromogen, ×200; QuPath 0.5.1 overlay.

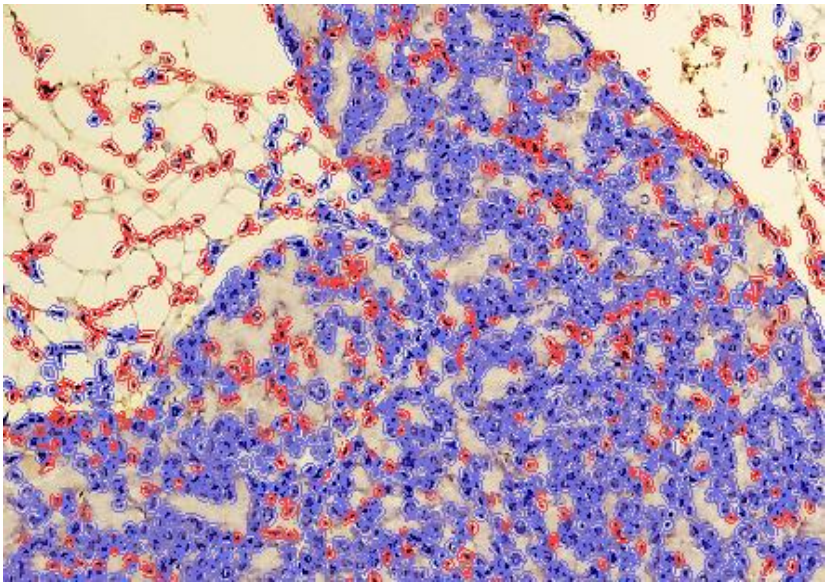

Figure 3. Bcl-2 immunoreactivity in the pancreas of a control rat aged 6 months. Uniform moderate cytoplasmic positivity is maintained in acinar cells, with distinct β-cell reactivity in the islets and low, focal stromal staining. Neither over-expression nor depletion is present. Labelling index 26.67% (460/1725 cells). Bcl-2, DAB chromogen, ×200; QuPath 0.5.1 overlay.

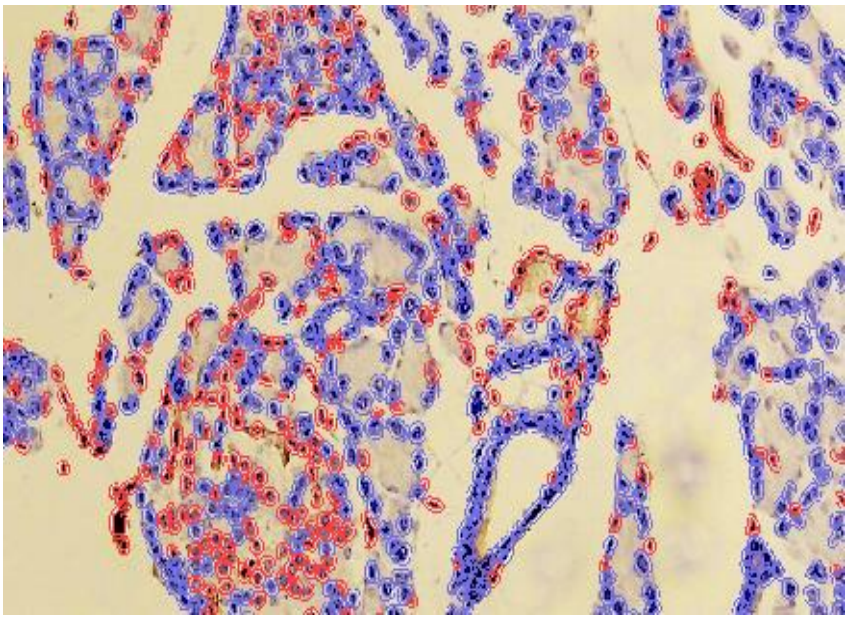

Figure 4. Bcl-2 immunoreactivity in the pancreas of a 1-month-old rat after chemical burn of the digestive tract with 70% acetic acid. Staining is mosaic rather than diffuse: strongly positive, morphologically preserved cells lie beside unstained cells showing vacuolar dystrophy and nuclear pyknosis. Labelling index 36.56% (366/1001 cells) — higher than control, although total nuclear density is 26.0% lower (Table 4). Bcl-2, DAB chromogen, ×200; QuPath 0.5.1 overlay.

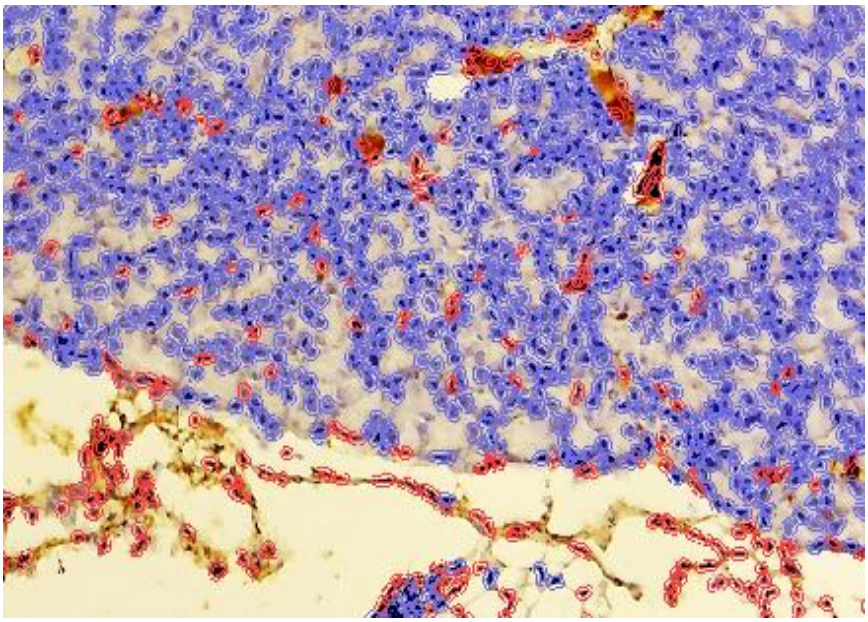

Figure 5. Bcl-2 immunoreactivity in the pancreas of a 3-month-old rat after chemical burn with 70% acetic acid. Reactivity is focal and unevenly distributed, with intensity differing sharply between adjacent acini; islet staining is patchy, most evidently among β cells. Labelling index 35.68% (521/1460 cells), with total nuclear density 23.6% below control. Bcl-2, DAB chromogen, ×200; QuPath 0.5.1 overlay.

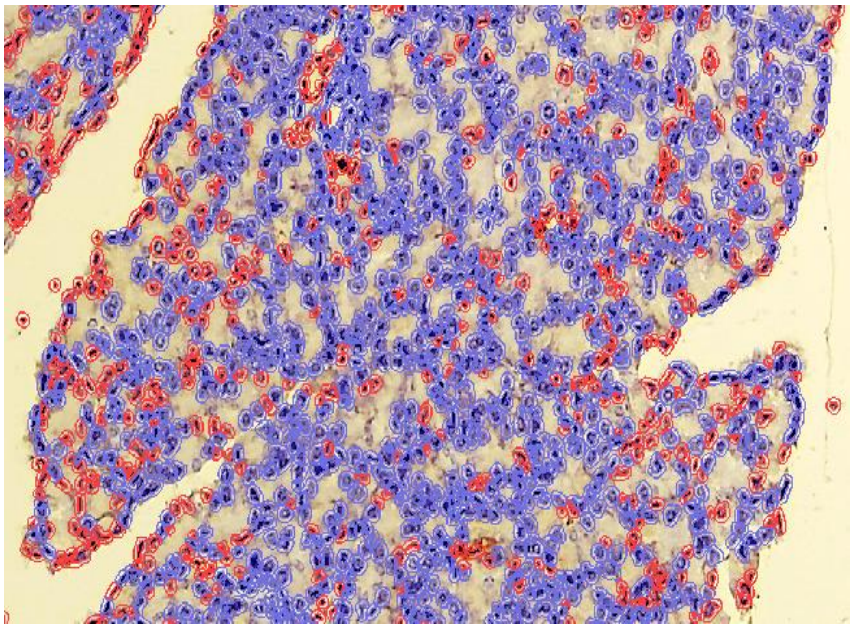

Figure 6. Bcl-2 immunoreactivity in the pancreas of a 6-month-old rat after chemical burn with 70% acetic acid. This is the only group in the series in which cellularity is preserved while immunoreactivity increases: the density of Bcl-2-positive cells is 49.2% above the age-matched control (Table 4), indicating genuine up-regulation in surviving cells rather than enrichment through loss of negative ones. Labelling index 39.44% (635/1610 cells). Bcl-2, DAB chromogen, ×200; QuPath 0.5.1 overlay.

3.2. Labelling index

The Bcl-2 labelling index was higher in burned animals than in age-matched controls at every age examined (Table 2). It rose from 26.13% to 36.56% at 1 month, from 29.20% to 35.68% at 3 months and from 26.67% to 39.44% at 6 months. In control animals the index was stable across the three ages, and the Cochran–Armitage test confirmed the absence of any age trend ($z = -0.05$, $p = 0.960$). In burned animals a weak upward trend with age did not reach significance ($z = +1.86$, $p = 0.063$).

All three comparisons were highly significant, with odds ratios between 1.35 and 1.79 (Table 3). Pooled across ages the index rose from 27.47% (1395 of 5078 nuclei) to 37.39% (1522 of 4071); $\chi^2 = 101.8$, $p < 0.001$, odds ratio 1.58 (95% CI 1.44–1.72).

Table 2. Bcl-2 labelling index by group and age, with Wilson score 95% confidence intervals.

Age	Control positive/total	Control LI (95% CI)	Burn positive/total	Burn LI (95% CI)	Difference (pp)	Analysed area (control / burn, px²)
1 mo	375 / 1435	26.13% (23.93–28.47)	366 / 1001	36.56% (33.64–39.59)	+10.43	758 244 / 714 825
3 mo	560 / 1918	29.20% (27.21–31.27)	521 / 1460	35.68% (33.27–38.18)	+6.49	750 148 / 747 286

Age	Control positive/total	Control LI (95% CI)	Burn positive/total	Burn LI (95% CI)	Difference (pp)	Analysed area (control / burn, px²)
6 mo	460 / 1725	26.67% (24.63–28.80)	635 / 1610	39.44% (37.08–41.85)	+12.77	770 224 / 712 707
Pooled	1395 / 5078	27.47% (26.26–28.72)	1522 / 4071	37.39% (35.91–38.88)	+9.92	2 278 616 / 2 174 818

LI = labelling index. pp = percentage points. Intervals do not account for clustering within animals (Section 2.8) and are therefore narrower than a hierarchical analysis would yield.

3.3. Effect size and its consistency across ages

Before pooling the three strata, homogeneity of the age-specific odds ratios was tested. Woolf's Q was 7.61 on 2 degrees of freedom (p = 0.022), rejecting homogeneity. The effect of burn on Bcl-2 expression is therefore not the same at every age, and the pooled odds ratio of 1.58, although statistically well determined, conceals a real difference between strata. Age-stratified reporting is required, and the pooled figure should be read as a summary rather than as a common effect.

The pattern behind the heterogeneity is that the effect was weakest at 3 months (OR 1.35) and strongest at 6 months (OR 1.79), with 1 month intermediate (OR 1.63). Section 3.4 shows that these three similar-looking odds ratios in fact arise from two entirely different cellular processes.

Table 3. Comparison of Bcl-2 expression between control and burned pancreas.

Age	χ^2	df	p	Odds ratio (95% CI)	Relative increase	Grade (pre-specified)
1 month	29.8	1	< 0.001	1.63 (1.37–1.94)	+39.9%	moderate → moderate
3 months	15.7	1	< 0.001	1.35 (1.16–1.56)	+22.2%	moderate → moderate
6 months	61.0	1	< 0.001	1.79 (1.55–2.07)	+47.9%	moderate → moderate
Pooled	101.8	1	< 0.001	1.58 (1.44–1.72)	+36.1%	moderate → moderate
Homogeneity of OR	7.61	2	0.022	rejected — effect differs by age	—	—

χ^2 with Yates's continuity correction on positive/negative nuclei; Woolf logit confidence limits. Homogeneity tested by Woolf's Q. "Relative increase" is the proportional rise in the labelling index over the age-matched control.

3.4. Decomposition of the index: numerator and denominator

The finding that the labelling index rose after an injury known to kill cells warranted examination of the denominator. Normalising the QuPath counts to the analysed field area separates the two components (Table 4), and the result is unambiguous.

Total nuclear density fell by 26.0% at 1 month and 23.6% at 3 months but was essentially unchanged at 6 months (+0.9%). The density of Bcl-2-negative cells fell at every age, and substantially: −36.5%, −30.6% and −16.7%. The density of Bcl-2-positive cells, by contrast, was almost unchanged at 1 and 3 months (+3.5% and −6.6%) and rose sharply — by 49.2% — only at 6 months.

Two distinct processes are therefore operating. At 1 and 3 months the tissue was being depleted of its Bcl-2-negative population while the positive population persisted at close to normal density; the rise in labelling index at those ages is an arithmetic consequence of that selective loss, not evidence that any surviving cell increased its expression. At 6 months the negative population was also depleted, but less so, and the positive population expanded by half — a genuine anti-apoptotic response in cells that survived. The three similar odds ratios in Table 3 thus have two different meanings, and no analysis confined to the labelling index could have distinguished them.

Table 4. Nuclear density decomposition (cells per 10^5 px² of analysed field).

Age	Total control	Total burn	Δ	Bcl-2⁺ control	Bcl-2⁺ burn	Δ	Bcl-2⁻ Δ
1 mo	189.25	140.03	−26.0%	49.46	51.20	+3.5%	−36.5%
3 mo	255.68	195.37	−23.6%	74.65	69.72	−6.6%	−30.6%
6 mo	223.96	225.90	+0.9%	59.72	89.10	+49.2%	−16.7%

Densities computed as (cell count / analysed area) × 10^5, using the areas listed in Table 2. Δ = percentage change from the age-matched control. The final column gives the change in Bcl-2-negative cell density.

3.5. Performance of the pre-specified grading scale

Applied as written, the three-band ordinal scale classified all six group–age combinations as "moderate expression", because every value between 26.13% and 39.44% falls inside the 20–60% band (Table 3, final column). The scale therefore recorded no difference between control and burned tissue at any age, while the underlying counts showed differences significant at $p < 0.001$ with odds ratios approaching 1.8.

This is worth reporting rather than quietly omitting. A scale whose middle band spans two-thirds of the possible range cannot resolve differences of ten percentage points, and for a marker whose physiological expression sits near the middle of that band the scale is uninformative by construction. Continuous reporting of the labelling index, with densities alongside, is the appropriate method here, and the ordinal scale is retained in this report only to document that it was pre-specified and found unfit for purpose.

4. Discussion

4.1. Principal findings

Three findings emerge. Chemical burn of the digestive tract measurably disturbed Bcl-2 expression in the pancreas, an organ outside the burn field, at every age tested. The effect was not uniform across ages, and formal testing rejected homogeneity of the odds ratios. And the change in labelling index arose from two mechanistically distinct processes — selective loss of Bcl-2-negative cells at 1 and 3 months, genuine up-regulation in survivors at 6 months — which the index alone could not have distinguished.

4.2. Direction of change and its reconciliation with the literature

That Bcl-2 labelling should rise after injury runs against the prevailing expectation. Down-regulation of Bcl-2 with reciprocal induction of Bax and caspases is the standard finding in cerulein-induced acute pancreatitis, in chronic pancreatitis and in models of pancreatic fibrosis [11–13], and it would be easy to read DAB slides in the light of that expectation and record a decrease. The counts do not support it.

The reconciliation lies in what is being measured. Those studies quantify Bcl-2 protein or mRNA in whole-tissue lysates, where a fall reflects the aggregate of the tissue including the cells that are dying. The present study counts cells one at a time, and a cell that has been destroyed contributes to neither numerator nor denominator. The two approaches can move in opposite directions in the same tissue without contradiction, and the density analysis in Section 3.4 shows exactly how: at 1 and 3 months, absolute Bcl-2-positive cell density did not increase at all. A whole-tissue assay of the same pancreas would very likely have shown a fall in total Bcl-2 content, because a quarter of the cells were gone.

The 6-month result is different in kind and cannot be explained this way, because cellularity was preserved while positive-cell density rose by half. Precedent for such a response exists. In end-stage heart failure, Bcl-2 and Bcl-xL are elevated alongside Bax, Bak and TUNEL positivity, and the increase is interpreted as a compensatory anti-apoptotic mechanism running concurrently with ongoing cell death [14]; compensatory induction of survival proteins in the cells that remain is a recognised pattern in acute injury more broadly [15]. In the pancreas specifically, prosurvival Bcl-2 proteins stabilise mitochondria and protect acinar cells against necrosis in experimental pancreatitis [11], so an induced increase is a coherent protective response rather than an anomaly.

4.3. Why age matters here

The heterogeneity of the odds ratios ($p = 0.022$) is a formal result, but the density data give it substance. Only the fully mature 6-month gland mounted a genuine anti-apoptotic response; the two younger groups lost cells without any measurable induction in those that survived. That is the opposite of the intuition that immature tissue should be the more adaptable, and it suggests that the capacity to up-regulate Bcl-2 under stress is itself a property acquired with maturity.

An alternative reading deserves stating. The younger animals may simply have been sampled at a different point on the same trajectory — induction may occur later, or may have occurred and resolved, in tissue whose turnover is faster. A single time point cannot distinguish a developmental difference in capacity from a difference in kinetics, and this is the principal reason a time course is the most valuable next experiment.

4.4. The methodological point, and its generality

The clearest transferable lesson concerns measurement rather than pancreatic biology. A labelling index is a ratio whose denominator, in any injury model that kills cells, is itself an outcome of the intervention. Where a treatment removes marker-negative cells preferentially, the index rises with no change whatever in the surviving cells — precisely what happened here at 1 and 3 months. Reporting the index alone would have supported a confident and entirely wrong conclusion that burn induces Bcl-2 at all ages.

The remedy is inexpensive. Digital pathology platforms already report the analysed area alongside the counts [16]; deriving positive, negative and total densities requires no extra staining, no extra slides and no extra animals, only that the area figure be retained and used. This should arguably be routine wherever a marker index is reported in a cytotoxic injury model.

4.5. Mechanisms by which the pancreas is reached

Four routes are individually plausible and not mutually exclusive: direct chemical injury transmitted through the duodenal wall and the pyloro-duodenal region, where pyloric spasm causes acid to pool and contact time to be longest [1, 7]; portal absorption of the agent and its metabolites; splanchnic hypoperfusion during the shock phase, which is common at this concentration and accompanies the haemolysis seen in over half of clinical cases [5]; and the systemic oxidative and inflammatory cascade characteristic of burn injury of any origin [22]. The reduced stromal and perivascular immunoreactivity observed in fields showing vascular dilatation and perivascular oedema is consistent with the third and fourth of these.

4.6. Limitations

- Single marker. Bcl-2 is one member of a family whose net effect is set by the balance between pro- and anti-apoptotic members. The Bax/Bcl-2 ratio, not Bcl-2 alone, is the informative quantity [11], and a TUNEL assay or cleaved caspase-3 would establish the apoptotic index directly. The present data show that the survival–death equilibrium is disturbed, but not the net direction of cell death.

- Ki-67 not reported. The protocol specified Ki-67 alongside Bcl-2 to assess proliferation, but no Ki-67 results exist in the source material. Proliferation and apoptosis are the two arms of tissue turnover, and the interpretation offered here — particularly the 6-month result — would be considerably strengthened by knowing whether proliferative activity rose in parallel.

- Counts pooled across animals. QuPath output was retained per group rather than per animal, so between-animal variance cannot be estimated and clustering is unaccounted for; the confidence intervals are consequently narrower than they

should be. A mixed-effects model with animal as a random effect is the correct analysis and requires only that per-animal counts be retained.

- Antibody and controls undocumented. Clone, supplier, dilution, incubation conditions, detection system and the positive and negative controls are not recorded (Section 2.5), which prevents independent replication.

- Blinding not stated. Whether the observer was blinded to group allocation is unrecorded. Automated classification in QuPath reduces but does not eliminate the scope for bias, since region selection remains a manual step.

- Single time point. The trajectory of Bcl-2 expression, and whether the 6-month induction is a difference in capacity or in timing, cannot be determined from one endpoint.

- Density normalised to field area, not to tissue area. QuPath reports the area of the outlined region, which includes interstitial space. Since burn expands the stroma, part of the fall in nuclear density may reflect that expansion rather than cell loss alone. Normalising to segmented parenchymal area would resolve this and is achievable from the existing images.

- Sex not analysed. Animals of both sexes were used but results were not disaggregated.

5. Conclusions

1. Chemical burn of the digestive tract with 70% acetic acid measurably disturbs apoptotic regulation in the pancreas, an organ lying outside the burn field. The Bcl-2 labelling index was significantly higher in burned animals at every age (pooled 27.47% → 37.39%; $\chi^2 = 101.8$, $p < 0.001$; OR 1.58, 95% CI 1.44–1.72).

2. The effect is not uniform across ages. Woolf's Q rejected homogeneity of the age-specific odds ratios ($Q = 7.61$, $df = 2$, $p = 0.022$), so the pooled estimate is a summary rather than a common effect and age-stratified reporting is required.

3. Density decomposition shows two distinct processes behind one apparent finding. Bcl-2-negative cell density fell at every age (−36.5%, −30.6%, −16.7%), while Bcl-2-positive cell density was unchanged at 1 and 3 months (+3.5%, −6.6%) and rose by 49.2% only at 6 months. The rise in labelling index in the younger animals reflects selective loss of negative cells; genuine up-regulation occurs only in the fully mature gland.

4. A labelling index must not be reported without its denominator in any model where the intervention kills cells. Reporting positive, negative and total nuclear densities alongside the index costs nothing beyond retaining the analysed field area, and here it reversed the biological interpretation at two of three ages.

5. The pre-specified three-band grading scale (< 20% / 20–60% / > 60%) classified every group as "moderate" and detected none of the differences the counts established at $p < 0.001$. Ordinal scales with a middle band spanning two-thirds of the range are unfit for markers whose physiological expression lies near their centre; continuous reporting should be used instead.

6. The natural next steps are a pro-apoptotic partner marker (Bax, with the Bax/Bcl-2 ratio) together with TUNEL or cleaved caspase-3, the Ki-67 data specified but never reported, per-animal counts permitting a mixed-effects analysis, and a time course to establish whether the 6-month induction reflects a difference in capacity or in kinetics.

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