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Zn^{64} aspartate modulates inflammation, tryptophan metabolism, and pancreatic function in diet-induced obesity

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Obesity-induced metabolic syndrome is characterized by chronic inflammation, hormonal imbalance, and altered neurotransmitter metabolism. Zinc plays a critical role in immune regulation and energy balance; however, the effects of isotopically modified chelate complexes in metabolic functions remain unexplored. This study investigated the therapeutic effects of isotopically enriched Zn^{64} Aspartate in a rat model of diet-induced obesity, focusing on inflammation, adipokine secretion, and tryptophan metabolism across serum, brain, duodenum, the pancreas, and adipose tissues. Male Wistar rats were fed a high-fat diet to induce obesity and treated with Zn^{64} Aspartate. Proinflammatory and anti-inflammatory cytokine levels, resistin, ghrelin, serotonin, tryptophan concentrations, and enzymatic activities of tryptophan hydroxylase (TPH), indoleamine 2,3-dioxygenase (IDO), and monoamine oxidase (MAO) were analyzed. Zn^{64} Aspartate significantly reduced pro-inflammatory cytokines and resistin levels while normalizing ghrelin. It enhanced serotonin levels and modulated TPH, IDO, and MAO activities in a tissue-specific manner, favoring serotonergic over kynurenine pathway metabolism. A decrease in pancreatic fibrosis, glycogen content, and an increase in the number of insulin-positive β-cells were observed in obese rats treated with Zn^{64} Aspartate, compared with the obese group. Conclusively, Zn^{64} Aspartate shows promise as a metabolic modulator by attenuating inflammation and restoring neuroendocrine balance in obesity. Further studies are warranted to validate its therapeutic potential.

Keywords Insulin, Fibrosis, Serotonergic system, Metabolic syndrome, Indoleamine 2,3-dioxygenase (IDO), Monoamine oxidase (MAO)

Zinc is a vital trace element involved in a broad spectrum of physiological processes including enzymatic catalysis, transcriptional regulation, immune function, and redox balance. In humans, total body zinc content ranges from approximately 1.5 to 3 g, with the majority (around 85%) distributed in skeletal muscle and bone, and the remainder found in the skin, liver, brain, pancreas, and other organs[1,2]. As a catalytic, structural, and regulatory cofactor in over 300 enzymes and 1000 transcription factors, zinc plays indispensable roles in maintaining cellular homeostasis. It regulates DNA synthesis, wound healing, growth, reproductive function, immune response, and oxidative stress management[3].

Among its most significant biological functions, zinc exerts potent anti-inflammatory and antioxidant effects, modulates cytokine signaling, and influences the activity of numerous hormone and neurotransmitter systems[4]. These properties have placed zinc at the forefront of interest in the pathophysiology and management of metabolic disorders, especially metabolic syndrome, a cluster of conditions including obesity, insulin resistance, hypertension, dyslipidemia, and systemic inflammation. Collectively, these components increase the risk of developing type 2 diabetes, cardiovascular disease, non-alcoholic fatty liver disease (NAFLD), and neurodegenerative disorders[5].

At the core of metabolic syndrome lies obesity, particularly visceral adiposity, which is now recognized not merely as an excess of energy stores but as a state of chronic low-grade inflammation and endocrine dysfunction.

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Adipose tissue secretes a broad array of bioactive substances (adipokines), including leptin, adiponectin, resistin, tumor necrosis factor-alpha (TNF-α), interleukins (IL-1β, IL-6), monocyte chemoattractant protein-1 (MCP-1), and plasminogen activator inhibitor-1 (PAI-1). These molecules modulate energy balance, glucose metabolism, insulin sensitivity, appetite, and inflammation[6].

In obesity, adipocyte hypertrophy and hyperplasia lead to increased secretion of pro-inflammatory cytokines and chemokines that recruit macrophages and other immune cells into adipose tissue. The resulting macrophage infiltration and polarization toward the M1 phenotype exacerbate local inflammation and oxidative stress. This inflammatory milieu impairs insulin signaling, increases lipolysis, and promotes hepatic gluconeogenesis, creating a vicious cycle of metabolic disruption[7].

Key cytokines such as TNF-α and IL-6 have been shown to inhibit insulin receptor signaling by inducing serine phosphorylation of insulin receptor substrate-1 (IRS-1) and reducing the expression of glucose transporter type 4 (GLUT4)[8]. Moreover, oxidative stress, driven by excessive production of reactive oxygen species (ROS) and insufficient antioxidant defenses, further impairs insulin sensitivity, damages lipids and proteins, and contributes to mitochondrial dysfunction[9].

Zinc plays an essential modulatory role in both immune response and metabolic signaling. It is a known regulator of nuclear factor kappa B (NF-κB), a master transcriptional regulator of inflammation. Zinc deficiency has been associated with elevated inflammatory markers, increased oxidative damage, and insulin resistance in both humans and animal models. Conversely, zinc supplementation has been found to lower levels of TNF-α, IL-1β, and IL-6, while reducing oxidative stress through the upregulation of metallothioneins, glutathione, and superoxide dismutase activity[10,11].

Zinc also influences the secretion and action of metabolic hormones such as insulin, leptin, ghrelin, and resistin. Resistin, predominantly secreted by macrophages in humans, has been implicated in insulin resistance by interfering with insulin receptor signaling and enhancing hepatic glucose production. Elevated serum resistin levels have been reported in obese individuals and correlate positively with inflammatory cytokines[12,13].

Ghrelin, produced mainly in the stomach, is another critical hormone affected in obesity. It stimulates appetite, promotes fat storage, and interacts with dopaminergic and serotonergic systems that regulate mood and energy balance[14]. In obesity, fasting ghrelin levels are paradoxically reduced, possibly as an adaptive mechanism to limit further energy intake; however, ghrelin resistance may develop, weakening its regulatory role[15].

In addition to adipokines, neurotransmitters and their precursors also play significant roles in metabolic homeostasis. The amino acid tryptophan is a precursor to serotonin, a neurotransmitter crucial for regulating satiety, mood, gastrointestinal function, and energy balance. Serotonin synthesis is mediated by the enzyme tryptophan hydroxylase (TPH) and degraded by monoamine oxidase (MAO)[16]. Another major metabolic route for tryptophan is the kynurenine pathway, which is activated during inflammation via the enzyme indoleamine 2,3-dioxygenase (IDO)[17–19].

Under inflammatory conditions, IDO is upregulated by cytokines such as IFN-γ, TNF-α, and IL-6, diverting tryptophan metabolism from serotonin synthesis toward kynurenine production. This not only decreases serotonin availability but also generates neuroactive metabolites such as quinolinic acid and kynurenic acid, which may impair cognitive and emotional regulation. Alterations in this balance have been linked to depression, anxiety, and abnormal feeding behavior in obesity and metabolic syndrome[20–22].

Given the central role of zinc in modulating both inflammatory and metabolic pathways, the use of isotopically enriched stable zinc, specifically Zn^{64}, in aspartate-bound form introduces a novel therapeutic approach. Zn^{64} is the most abundant stable isotope of zinc (~48.6% natural abundance), non-radioactive, and safe for biological applications.

While elemental zinc is commonly incorporated into nutritional supplements as sulfate, gluconate, or acetate salts, Zn^{64} aspartate chelate may provide superior bioavailability, enhanced cellular uptake, and potentially unique biological activity due to isotopic effects and the chelation properties of aspartate[23], which may act as an ionophore facilitating cellular membrane permeability.

Aspartate, an endogenous amino acid involved in the tricarboxylic acid (TCA) cycle, acts as a metabolic enhancer by fueling ATP production, promoting neurotransmitter synthesis (via glutamate and aspartate pathways), and supporting nitrogen balance. As a chelating agent, aspartate may stabilize Zn^{64} in the bloodstream, enhance its absorption, and facilitate targeted delivery to metabolically active tissues such as adipose tissue, brain, and gut[24].

Despite promising theoretical and preclinical foundations, the specific therapeutic effects of Zn^{64} Aspartate chelate on systemic and tissue-specific inflammation, metabolic hormones, and neurotransmitter pathways in obesity have not been systematically studied. Most prior research has focused on conventional zinc salts and dietary intake without distinguishing isotopic forms or considering zinc-amino acid complexes. There is also limited understanding of how isotopic composition in zinc formulations may differentially influence enzyme activities, hormonal balance, or neuromodulation in metabolically relevant tissues such as the brain, adipose tissue, gut, pancreas, and serum.

We hypothesized that administration of enriched Zn^{64} Aspartate in an obesity model could attenuate systemic and local inflammation, normalize resistin and ghrelin levels, and restore tryptophan metabolism through the modulation of TPH, IDO, and MAO enzyme activities. These effects were expected to improve the histophysiology of the pancreas, the central organ of insulin production, and to reflect improved metabolic integration between the immune, endocrine and nervous systems. In this study, we investigated the therapeutic potential of enriched Zn^{64} Aspartate as a novel metabolic modulator in obesity-related metabolic syndrome by exploring these parameters in serum, brain, duodenum, and adipose tissue. Furthermore, the absence of immunological changes in the control animals treated with Zn^{64} Aspartate suggests its safety and selective action in pathological conditions.

Results

Effects of Zn^{64} aspartate on cytokine profile, resistin, and ghrelin levels in animal models of obesity

To investigate the immunomodulatory potential of isotopically modified Zn64 Aspartate in the context of obesity, we assessed the levels of key pro-inflammatory (IL-1β, IL-6, IL-12, IFN-γ) and anti-inflammatory (IL-4, IL-10, TGF-β) cytokines in adipose tissue and serum of experimental animals.

In animals subjected to a high-fat diet (HFD), a marked increase in all analyzed pro-inflammatory cytokines was observed in adipose tissue compared to the intact control group kept on a regular diet (Table 1). This cytokine elevation indicates the activation of a local chronic low-grade inflammatory process, characteristic of obesity-induced adipose tissue dysfunction. Notably, IL-1β, a central mediator of inflammation, was significantly elevated. The upregulation of IL-1β is known to disrupt insulin signaling by activating IκB kinase-β, which subsequently phosphorylates IRS-1 on serine residues, leading to insulin resistance. Furthermore, increased IL-1β promotes lipogenesis and the accumulation of triglycerides and free fatty acids in adipocytes, further amplifying metabolic dysregulation.

Similarly, IL-6 levels were significantly increased in the adipose tissue of obese animals. Given that adipocytes contribute approximately 35% of circulating IL-6, this elevation reflects both adipose tissue expansion and systemic inflammatory burden associated with obesity. IL-6 is also implicated in hepatic lipid metabolism dysregulation and has been associated with hepatic steatosis, insulin resistance, and cardiovascular complications through its role in stimulating C-reactive protein (CRP) production.

IL-12 and IFN-γ were also significantly elevated in the adipose tissue of obese animals. IL-12, upregulated by IFN-γ, plays a pivotal role in CD8+ lymphocyte recruitment and has been implicated in the pathogenesis of pancreatic inflammation and β-cell apoptosis. These findings collectively confirm a state of local pro-inflammatory activation in adipose tissue during obesity progression.

Conversely, a slight but consistent decrease in anti-inflammatory cytokines (IL-4, IL-10, and TGF-β) was detected in the adipose tissue of obese animals, suggesting insufficient counter-regulation of pro-inflammatory signaling.

Notably, administration of isotopically modified Zn64 Aspartate resulted in a significant attenuation of all measured pro-inflammatory cytokines in adipose tissue of obese animals. Simultaneously, levels of IL-4, IL-10, and TGF-β were significantly elevated compared to untreated obese animals, and in some cases exceeded those of the non-obese control group, indicating a strong immunomodulatory and anti-inflammatory effect of Zn64 Aspartate treatment. These data suggest that Zn64 Aspartate exerts its beneficial effects by restoring the balance between pro- and anti-inflammatory mediators within adipose tissue.

Importantly, administration of Zn64 Aspartate to control animals (non-obese, untreated) did not result in significant alterations in either pro- or anti-inflammatory cytokine levels, supporting the safety and conditional therapeutic action of Zn64 Aspartate under physiological conditions.

To further determine whether the inflammatory process was localized or systemic, serum levels of the same cytokines were measured (Table 2). Obese animals displayed significantly elevated serum levels of IL-1β, IL-6, IL-12, and IFN-γ, with a magnitude of increase exceeding those observed in adipose tissue. These findings are consistent with the hypothesis that obesity triggers a state of systemic inflammation, contributing to metabolic disturbances including insulin resistance and endothelial dysfunction.

Interestingly, no statistically significant changes were observed in serum IL-4 levels in obese animals compared with controls. However, IL-10 was modestly elevated, potentially reflecting a delayed or insufficient compensatory response to chronic systemic inflammation. TGF-β levels did not differ significantly in the serum of obese animals, further highlighting the imbalance between pro- and anti-inflammatory mediators.

Treatment with Zn64 Aspartate led to a marked decrease in systemic levels of IL-1β, IL-6, IL-12, and IFN-γ in obese animals. Concurrently, serum IL-4, IL-10, and TGF-β concentrations were significantly increased, surpassing even those observed in control animals. These data indicate that Zn64 Aspartate not only ameliorates local inflammation in adipose tissue but also exerts systemic anti-inflammatory effects.

Our findings demonstrate that treatment of obese animals with Zn^{64} Aspartate resulted in a significant normalization of cytokine expression patterns. Specifically, there was a notable decrease in pro-inflammatory cytokines (IL-1β, IL-6, IL-12, IFN-γ) and a concurrent increase in anti-inflammatory cytokines (IL-4, IL-10, TGF-β) in both serum and adipose tissue (see Tables 1 and 2). These changes suggest that the tested compound

Groups	Levels, AU/mg protein						
	Pro- inflammatory cytokines				Anti- inflammatory cytokines		
	IL-1	IL-6	IL-12	IFN-γ	IL-4	IL-10	TGF
C	5.6 ± 1.3	5.9 ± 0.7	1.2 ± 0.03	4.7 ± 1.2	4.8 ± 0.5	4.7 ± 0.7	4.5 ± 0.9
C + Zinc	4.9 ± 1.7	5.4 ± 0.3	1.0 ± 0.03	5.0 ± 0.8	5.0 ± 0.4	4.9 ± 0.2	5.1 ± 0.6
DIO	9.8 ± 2.8*	8.9 ± 0.7*	2.87 ± 0.08*	7.6 ± 1.2*	3.9 ± 0.2*	3.1 ± 0.6*	3.1 ± 0.1*
DIO + Zinc	5.8 ± 1.8#	6.1 ± 0.7#	1.99 ± 0.01#	4.9 ± 0.8#	5.1 ± 1.2#	5.8 ± 0.8#	5.2 ± 0.9#

Table 1. Cytokine profile in the adipose tissue of animals from experimental groups (M ± SEM, n = 10). *the difference is significant versus the control group; #the difference is significant versus the group of DIO. *C* control; *C + zinc* control on the background of administration of Zn^{64} Aspartate; *DIO* diet induced obesity; *DIO + zinc* diet induced obesity on the background of administration of Zn^{64} Aspartate.

Groups	Levels, AU/mg protein						
	Pro- inflammatory cytokines				Pro- inflammatory cytokines		
	IL-1	IL-6	IL-12	IFN-γ	IL-4	IL-10	TGF
C	3.4 ± 0.3	4.5 ± 0.3	0.5 ± 0.05	3.6 ± 0.8	5.1 ± 0.2	3.9 ± 0.4	3.8 ± 0.8
C + Zinc	3.5 ± 0.7	4.3 ± 0.2	0.3 ± 0.04	4.6 ± 0.6	4.6 ± 0.8	4.1 ± 0.5	4.1 ± 0.4
DIO	11.1 ± 2.0*	7.9 ± 0.5*	3.7 ± 0.07*	6.5 ± 0.8*	4.4 ± 0.9	4.1 ± 1.5	3.5 ± 1.3
DIO + Zinc	4.2 ± 0.4#	5.1 ± 0.4 #	2.4 ± 0.06*, #	4.1 ± 1.2	5.6 ± 1.6	6.8 ± 1.1*, #	5.7 ± 0.3*, #

Table 2. Cytokine profile in the serum of animals from experimental groups (M ± SEM, n = 10). *the difference is significant versus the control group; #the difference is significant versus the group of DIO. *C* control; *C + zinc* control on the background of administration of Zn^{64} Aspartate; *DIO* diet induced obesity; *DIO + zinc* diet induced obesity on the background of administration of Zn^{64} Aspartate.

Experimental groups	Resistin, AU/mg protein		Ghrelin, AU/mg protein
	Adipose tissue	Serum	Serum
C	0.26 ± 0.05	0.18 ± 0.05	0.019 ± 0.003
C + Zinc	0.25 ± 0.05	0.12 ± 0.05	0.016 ± 0.003
DIO	0.34 ± 0.05*	0.19 ± 0.05	0.027 ± 0.003*
DIO + Zinc	0.25 ± 0.05#	0.16 ± 0.05	0.019 ± 0.003#

Table 3. Resistin and ghrelin levels in adipose tissue and serum of animals from experimental groups (M ± SEM, n = 10). *the difference is significant versus the control group; #the difference is significant versus the group of DIO. *C* control; *C + zinc* control on the background of administration of Zn^{64} Aspartate; *DIO* diet induced obesity; *DIO + zinc* diet induced obesity on the background of administration of Zn^{64} Aspartate.

may exert systemic and local anti-inflammatory effects. Furthermore, the absence of immunological changes in the control animals treated with Zn^{64} Aspartate suggests its safety and selective action under pathological conditions.

This anti-inflammatory response could be mediated not only by direct inhibition of pro-inflammatory gene expression but also by a secondary upregulation of anti-inflammatory cytokines, creating a feedback mechanism that further suppresses inflammation. Given the well-established correlation between adipose tissue mass and cytokine production, it was reasonable to infer that the observed changes in the cytokine profile may also be partially attributed to reductions in adipose tissue mass, as supported by changes in adipokine levels[25].

To explore this hypothesis, we evaluated the levels of resistin, a pro-inflammatory adipokine that promotes adipogenesis and plays a critical role in the development of insulin resistance and metabolic dysfunction. In obese untreated animals, resistin levels were significantly elevated, especially in adipose tissue, indicating active adipocyte dysfunction and fatty mass expansion (Table 3). In contrast, resistin levels in the adipose tissue of obese animals treated with Zn^{64} Aspartate decreased to control values and were significantly different from those in the DIO group, suggesting that the compound either suppressed adipocyte maturation or limited fat accumulation. These data are consistent with the role of resistin as an autocrine and paracrine promoter of adipocyte differentiation and inflammation.

Since resistin is primarily secreted by preadipocytes and, to a lesser extent, by mature adipocytes (particularly in visceral fat depots) the normalization of its levels by Zn64 Aspartate suggests a downregulation of preadipocyte activation and adipogenesis, which also contributes to reduced pathological inflammation and overall metabolic improvement.

Further, we assessed ghrelin, a key regulator of appetite and energy homeostasis. In line with previous findings, a marked reduction in serum ghrelin levels was observed in obese animals, consistent with the known inverse correlation between ghrelin levels and body mass index (BMI), fat mass, and leptin concentrations[26]. Interestingly, treatment with Zn^{64} Aspartate normalized ghrelin levels in obese animals, bringing them back to values close to those of the non-obese control group[27].

This finding suggests that treatment with Zn64 Aspartate restores energy balance and appetite regulation by improving the functional status of adipocytes and reducing the pro-inflammatory signaling that disrupts metabolic hormones. Notably, administration of Zn64 Aspartate to healthy, non-obese animals led to a slight decrease in ghrelin levels, consistent with a shift toward reduced energy intake and enhanced satiety. Importantly, these changes were not accompanied by adverse effects, underscoring the safety profile of the test compound.

Effects of Zn^{64} aspartate on the serotonergic system in a rat model of obesity

To investigate the impact of the enriched Zn64 Aspartate on the serotonergic system, we measured the concentrations of serotonin and tryptophan, as well as the activities of key enzymes involved in serotonin metabolism: tryptophan hydroxylase (TPH), tryptophan decarboxylase (TDC), indoleamine 2,3-dioxygenase (IDO), and monoamine oxidase (MAO), in the serum, brain, and duodenum of obese and treated rats.

Experimental variable	Experimental groups			
	C	C + zinc	DIO	DIO + zinc
Tryptophan, μg/g	169.2 ± 12.6	154.7 ± 13.6	76.3 ± 9.74*	89.4 ± 8.99*
Serotonin, μg/g	6.56 ± 0.98	7.41 ± 0.54	14.6 ± 1.64*	9.73 ± 0.82*, #
Tryptophan hydroxylase (TPH) activity, AU/mg protein	398 ± 78	375 ± 61	210 ± 69*	253 ± 58*
Tryptophan decarboxylase (TDC) activity, AU/mg protein	0.81 ± 0.25	0.79 ± 0.22	0.65 ± 0.23*	0.78 ± 0.19
Monoamine oxidase (MAO) activity, AU/mg protein	3.33 ± 0.04	3.82 ± 0.07*	1.55 ± 0.07*	1.72 ± 0.03*, #
Indoleamine dioxygenase (IDO) activity, μmol/mg protein	2348 ± 301	2150 ± 231	1378 ± 305*	1768 ± 274*

Table 4. Peripheral serotonergic system activity in the duodenum (M ± SEM, n = 10). *the difference is significant versus the control group; #the difference is significant versus the group of DIO. *C* control; *C + zinc* control on the background of administration of Zn^{64} Aspartate; *DIO* diet induced obesity; *DIO + zinc* diet induced obesity on the background of administration of Zn^{64} Aspartate.

Experimental variable	Experimental groups			
	C	C + zinc	DIO	DIO + zinc
Tryptophan, μg/g	57.38 ± 5.73	56.12 ± 7.58	38.3 ± 6.72*	66.65 ± 9.62#
Serotonin, μg/g	9.53 ± 0.72	10.16 ± 0.62	3.88 ± 0.34*	6.33 ± 0.51*,#
Monoamine oxylase (MAO) activity, AU/mg protein	2.69 ± 0.06	2.52 ± 0.03	3.91 ± 0.04*	3.11 ± 0.08*,#

Table 5. Serum levels of tryptophan, serotonin and monoamine oxidase activity (M ± SEM, n = 10). *the difference is significant versus the control group; #the difference is significant versus the group of DIO. *C* control; *C + zinc* control on the background of administration of Zn^{64} Aspartate; *DIO* diet induced obesity; *DIO + zinc* diet induced obesity on the background of administration of Zn^{64} Aspartate.

In obese animals fed a high-fat diet, the activities of TPH, TDC, MAO and IDO in both the duodenum and serum were significantly reduced, indicating inhibition of serotonin biosynthesis in the peripheral system. This was accompanied by a decrease in tryptophan concentration in these tissues. Despite the suppressed enzymatic activity and low tryptophan levels, serotonin concentrations in the duodenum were significantly elevated compared to control animals (Table 4). These results align with the concept that the intestinal serotonin levels correlate with the development of obesity and are markedly increased in obese individuals[28]. Accumulation of serotonin in the intestine contributes to elevated serum glucose levels and the development of diabetes and obesity.

Administration of Zn64 Aspartate to obese rats led to increase in TDC activity to control levels. MAO activity was partially restored in the DIO+zinc group, exhibiting intermediate levels significantly different from both control and untreated DIO animals. This normalization was associated with a decrease in duodenal serotonin levels compared to the DIO group.

In the serum of DIO group, we observed a significant reduction in both tryptophan and serotonin concentrations, likely resulting from inhibition of serotonin synthesis reactions in enterochromaffin cells of the gastrointestinal mucosa (Table 5). An additional factor contributing to low circulating serotonin may by the activation of MAO, promoting serotonin degradation. Modulation of the peripheral serotoninergic system is considered a promising anti-obesity strategy, as it may reduce body weight and increase insulin sensitivity. Administration of Zn^{64} Aspartate to obese rats restores tryptophan levels to control values, partially increases serotonin concentrations (different from those in control and DIO groups), and decreases MAO activity to intermediate levels, indicating partial normalization of peripheral serotonergic metabolism.

In the brains of obese rats, tryptophan levels were significantly reduced (Table 6), likely due to impaired transport across the blood-brain barrier. Consequently, serotonin levels were also decreased, accompanied by suppressed TPH activity and increased activities of MAO and IDO. These changes indicate a shift of tryptophan metabolism away from serotonin synthesis and toward the kynurenine pathway. In obese rats treated with Zn^{64} Aspartate, partial restoration of both tryptophan and serotonin levels (different from those in the control and DIO groups) was observed. This effect was associated with an increase in TPH activity and a decrease in MAO activity to near-control levels.

In lean rats administered Zn64 Aspartate (C + Zinc group), no significant alterations were observed in either central or peripheral serotonergic parameters compared with the control group, with the exception of a marked increase in MAO activity in the duodenum. This elevation in duodenal MAO activity may indicate enhanced catabolism of serotonin and other biogenic amines within the gastrointestinal tract, possibly reflecting a zinc-induced modulation of intestinal oxidative enzymes or an adaptive response aimed at maintaining neurotransmitter homeostasis.

Histological assessment of pancreatic fibrosis, glycogen deposition, and insulin level

To evaluate the extent of pancreatic fibrosis in obese animals, Masson's trichrome-stained sections (Fig. 1A) were examined, as obesity-associated chronic low-grade inflammation is frequently accompanied by excessive

Experimental variable	Experimental groups			
	C	C + zinc	DIO	DIO + zinc
Tryptophan, μg/g	99.24 ± 8.53	87.71 ± 3.41	6.66 ± 1.08*	31.56 ± 2.14*, #
Serotonin, μg/g	27.26 ± 2.76	25.51 ± 2.71	7.4 ± 1.63*	20.1 ± 3.21*, #
Tryptophan hydroxylase (TPH) activity, AU/mg protein	281 ± 27	291 ± 29	245 ± 18*	286 ± 17#
Tryptophan decarboxylase (TDC) activity, AU/mg protein	1.32 ± 0.43	1.63 ± 0.41	2.19 ± 0.67	1.86 ± 0.37
Monoamine oxidase (MAO) activity, AU/mg protein	0.84 ± 0.08	0.91 ± 0.06	1.19 ± 0.07*	0.99 ± 0.08#
Indoleamine dioxygenase (IDO) activity, μmol/mg protein	1097 ± 285	1178 ± 267	2438 ± 290*	2007 ± 175*

Table 6. Central serotonergic system activity in the brain (M ± SEM, n = 10). *the difference is significant versus the control group; #the difference is significant versus the group of DIO. *C* control; *C + zinc* control on the background of administration of Zn^{64} Aspartate; *DIO* diet induced obesity; *DIO + zinc* diet induced obesity on the background of administration of Zn^{64} Aspartate.

extracellular matrix remodeling and collagen deposition. In the diet-induced obesity (DIO) group, prominent overgrowth of collagen fibers was observed, extending beyond the pancreatic capsule into the interstitial spaces of the parenchyma. Additionally, extensive fibrotic septa composed of dense collagen were evident, abnormally encasing clusters of white adipocytes that had infiltrated the pancreatic parenchyma, which is known as a hallmark of lipofibrotic remodeling in obesity.

Quantitative assessment of fibrosis included measurement of the relative area occupied by collagen fibers (stained blue) and the cumulative integrated density of collagen staining (Fig. 1B and C). Obesity induced a significant increase in both parameters compared to control animals, reflecting pronounced fibrotic remodeling. Administration of Zn^{64} Aspartate to obese rats resulted in a statistically significant reduction in cumulative integrated density of collagen fibers relative to the untreated DIO group, suggesting a partial reversal of fibrosis.

The collagen content remained higher than in controls, indicating incomplete normalization. Interestingly, the relative collagen-positive area in the DIO rats treated with Zn^{64} Aspartate showed an intermediate profile that did not differ significantly from the DIO, control, or the control treated with Zn^{64} Aspartate groups. Zn^{64} Aspartate treatment did not alter collagen deposition in lean rats, indicating a pathology-dependent therapeutic effect.

Periodic acid-Schiff (PAS) staining (Fig. 2A) was used to assess glycogen accumulation, which typically increases under insulin-resistant conditions due to hyperglycemia and compensatory glucose storage, including in pancreatic β-cells. In the control and the control treated with Zn^{64} Aspartate groups, pancreatic islets exhibited β-cells with pale-pink cytoplasm and a distinctive bright-raspberry staining of the basal membrane surrounding vessels, indicating normal glycogen levels.

In contrast, β-cells of DIO rats displayed diffuse and intense PAS-positive fine granules throughout the cytoplasm, consistent with excessive glycogen accumulation. Quantification of PAS-stained deposits by mean gray value (MGV) (Fig. 2B) confirmed a significant increase in glycogen deposition in the DIO group. The Zn^{64} Aspartate treatment in obese animals significantly reduced glycogen content to levels that were not statistically different from controls, suggesting an improvement in insulin sensitivity. No significant changes in glycogen content were observed in the control treated with Zn^{64} Aspartate animal group.

To further explore pancreatic β-cell function, insulin levels were assessed immunohistochemically. Histological evaluation of pancreatic islet morphology in the DIO group revealed a reduction in islet area, indicating progressive β-cell atrophy, which is typical for advanced insulin resistance. This finding contrasts with earlier stages of insulin resistance characterized by islet hypertrophy resulting from compensatory hyperplasia. The reduced islet size observed in our model suggests β-cell exhaustion and the onset of degenerative changes, including occasional amyloid-like deposits.

Insulin-stained sections and their deconvoluted images were analyzed to determine the cytoplasmic insulin content in islet β-cells using the MGV parameter (Fig. 3A). Strong insulin immunoreactivity was detected in β-cells of control, C + Zinc, and DIO + Zinc groups. In contrast, a marked reduction in insulin staining was observed in the DIO group, consistent with β-cell exhaustion due to chronic metabolic stress and insulin resistance. Quantitative analysis (Fig. 3B) revealed a significant decreased in insulin levels in obese rats compared to all other groups. Zn^{64} Aspartate administration in obese rats significantly increased insulin levels compared with untreated DIO animals, although they remained lower than those observed in the control group. Notably, insulin levels in the DIO + Zinc and C + Zinc groups did not differ significantly, further supporting the therapeutic potential of Zn^{64} Aspartate in restoring β-cell function. In lean animals, Zn^{64} Aspartate treatment did not influence insulin expression.

Discussion

Obesity remains one of the most serious global health challenges, contributing to the development of metabolic, cardiovascular, and endocrine disorders and thus representing a major medical and socioeconomic burden. The search for novel and safe therapeutic approaches capable of correcting the complex metabolic disturbances associated with obesity remains a high priority. Animal models of diet-induced obesity (DIO) are widely recognized as reliable tools for studying the mechanisms underlying obesity and for evaluating potential therapeutic strategies. The experimental model applied in the present study—high-calorie diet-induced obesity in rats—is a well-established and broadly validated approach that has been repeatedly used both by our group

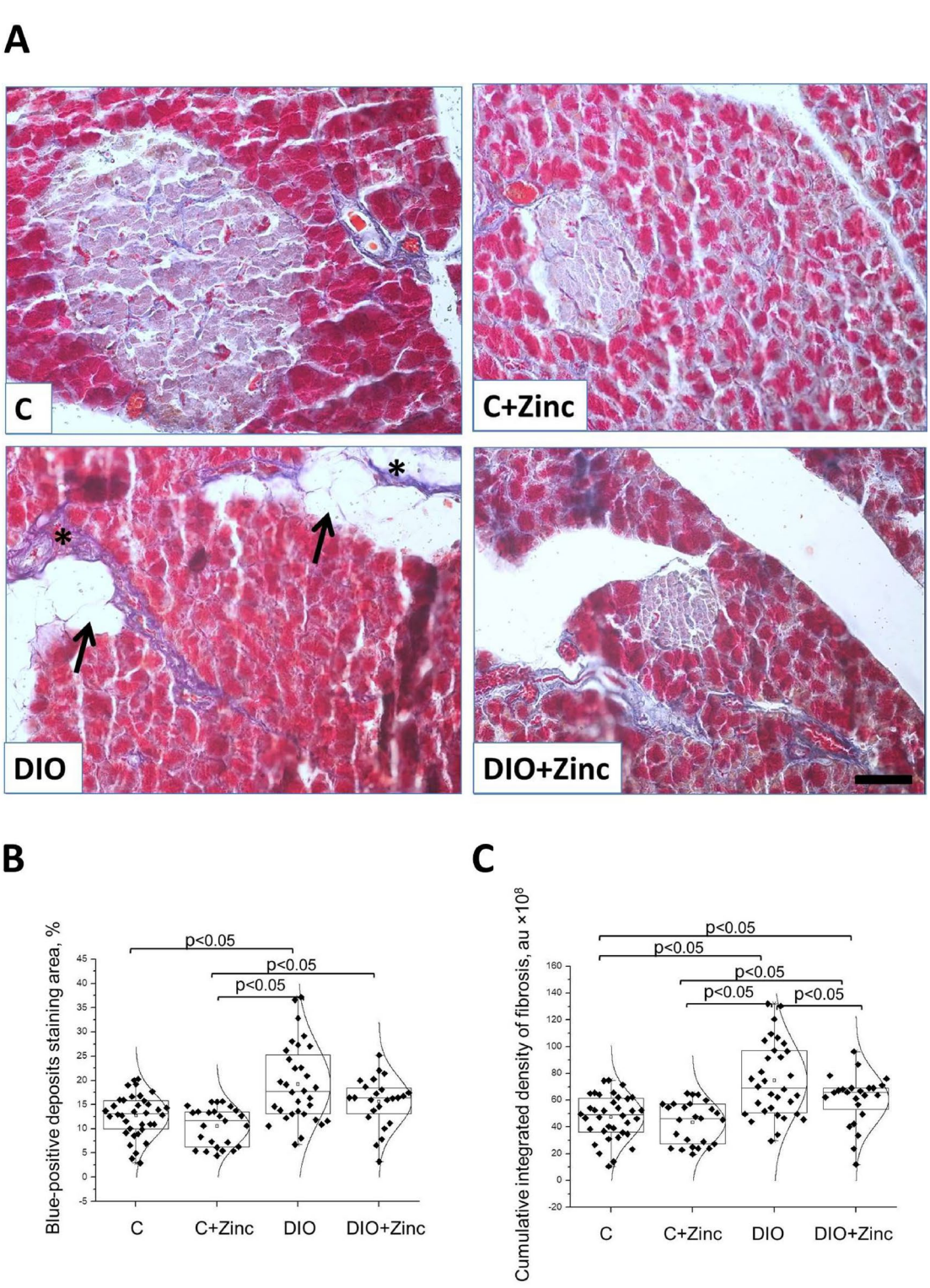


Fig. 1. (**A**) Representative Masson-stained pancreas sections from rats, scale bar: 50 µm. Collagen fiber marked with an asterisk; white adipocytes are marked with an arrow. (**B**) Quantification of fibrosis level as relative area of blue positive depositits and as C, cumulative integrated density of blue positive depositits; median, 25% and 75% percentile values. N = 10 rats per group, two-way ANOVA with Holm-Sidak post hoc multiple comparison tests. *NoteC* control; *C + zinc* control on the background of administration of Zn^{64} Aspartate; *DIO* diet induced obesity; *DIO + zinc* diet induced obesity on the background of administration of Zn^{64} Aspartate.

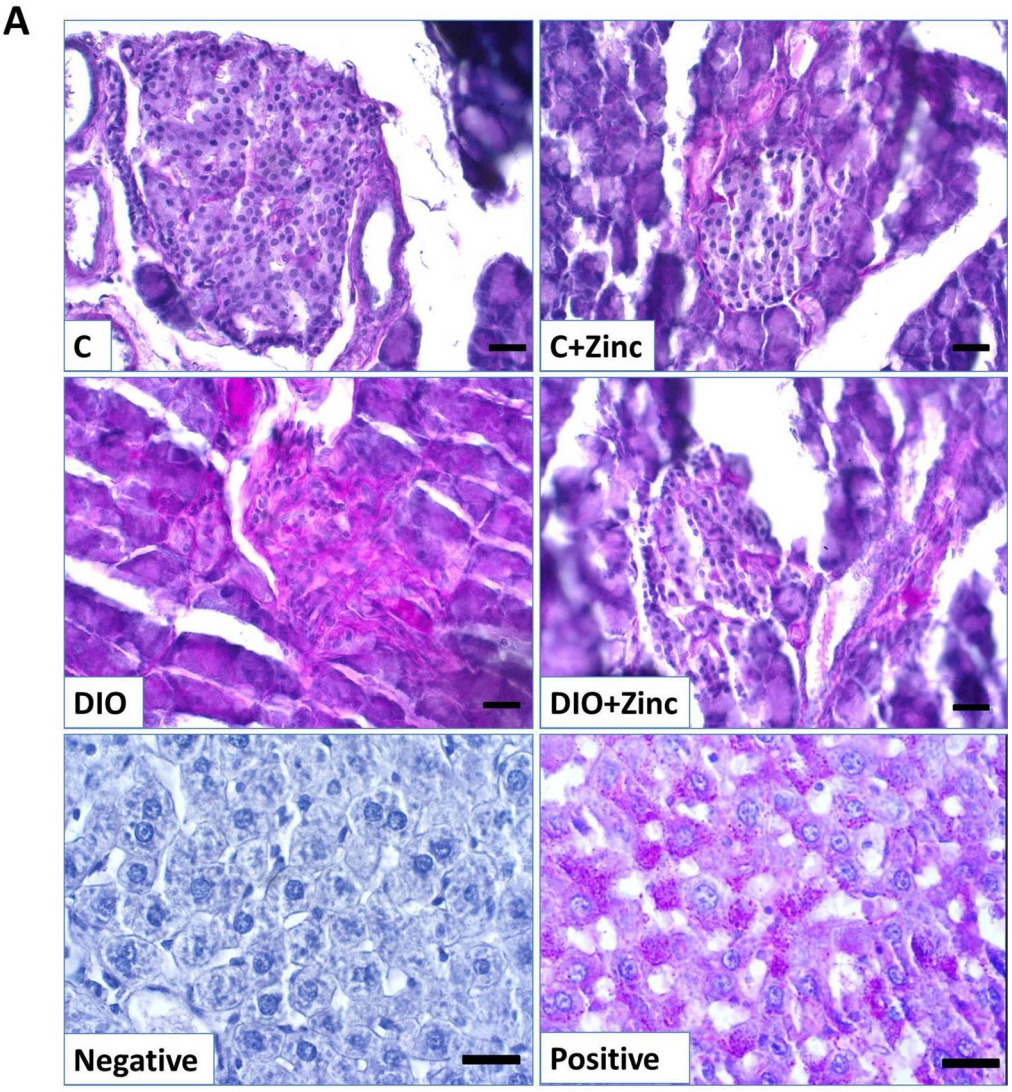


Fig. 2. (**A**) Representative PAS-stained pancreas sections from rats with negative and positive control of histochemical glycogen detection in liver sections, scale bar: 10 µm. B, Quantification of glycogen contain as mean grey value of PAS positive deposits in pancreatic islands; median, 25% and 75% percentile values. N = 10, two-way ANOVA with Holm-Sidak post hoc multiple comparison test. *NoteC* control; *C + zinc* control on the background of administration of Zn^{64} Aspartate; *DIO* diet induced obesity; *DIO + zinc* diet induced obesity on the background of administration of Zn^{64} Aspartate.

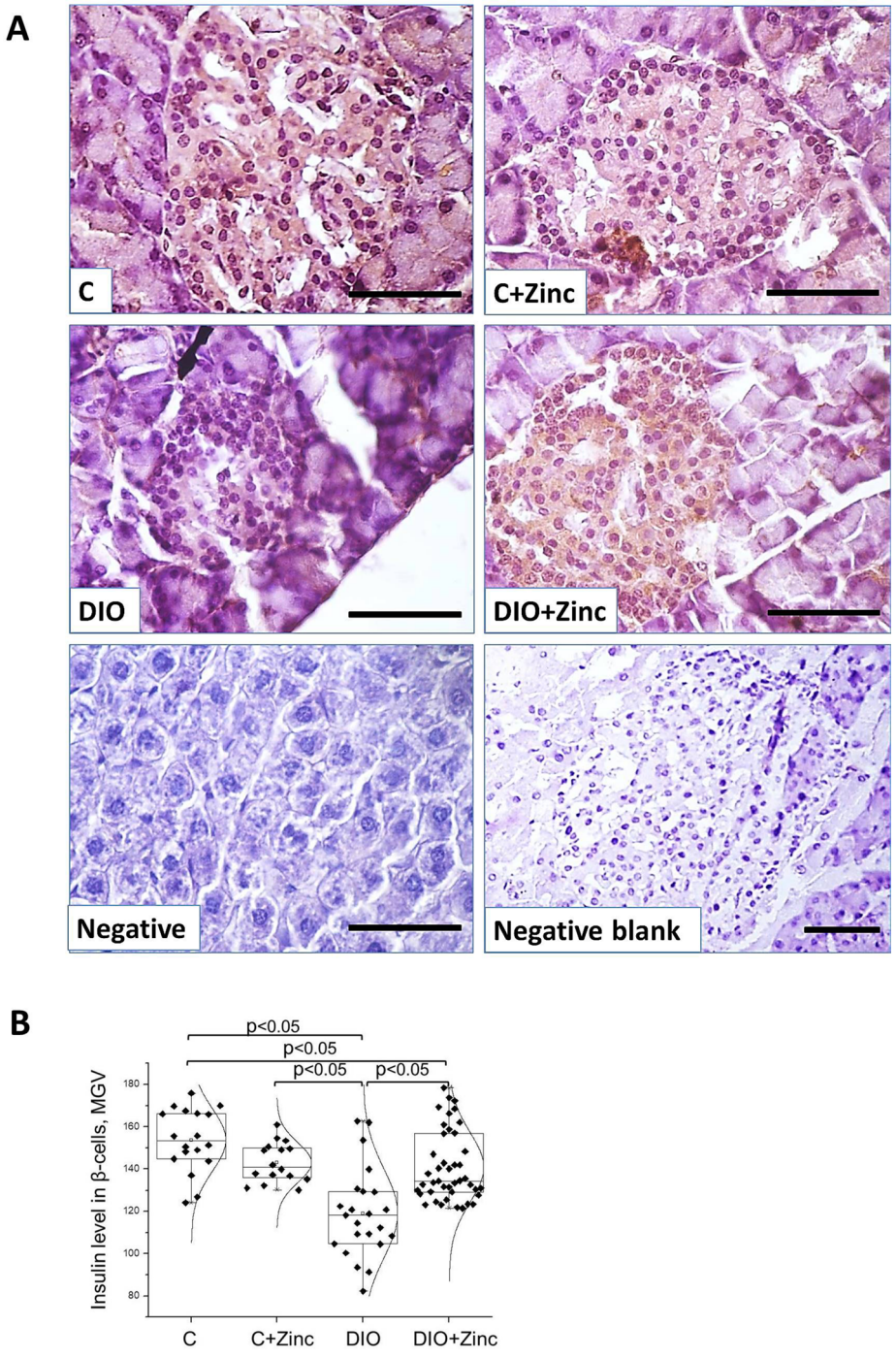


Fig. 3. (**A**) Representative insulin-stained pancreas sections from rats with negative (liver section) and negative blank (without primarily antibody) control of IHC insulin detection, scale bar: 50 µm. (**B**) Quantification of insulin level in the β-cells as mean grey value of DAB-positive islands; median, 25% and 75% percentile values. N = 10, two-way ANOVA with Holm-Sidak post hoc multiple comparison tests. *NoteC* control; *C + zinc* control on the background of administration of Zn^{64} Aspartate; DIO –diet induced obesity; *DIO + zinc* diet induced obesity on the background of administration of Zn^{64} Aspartate.

and by other investigators to assess metabolic, endocrine, and oxidative alterations characteristic of obesity[29,30]. In this model, prolonged consumption of a high-fat, energy-dense diet leads to a marked increase in body weight, adipose tissue mass, and body mass index, along with dyslipidemia, hyperglycemia, and oxidative stress, thereby closely reproducing the metabolic features of human obesity. Using this model, in our previous study, we demonstrated that zinc-containing compounds exerted beneficial metabolic effects in rats with diet-induced obesity, including a reduction in body weight gain and body mass index, normalization of serum glucose and insulin levels, restoration of lipid metabolism, and improvement of pancreatic and hepatic morphology

following high-calorie diet feeding[27]. These findings confirmed the therapeutic potential of zinc-based isotopic interventions in correcting obesity-associated metabolic and structural alterations.

Building upon these findings, the present investigation was designed to explore the broader regulatory potential of Zn64 Aspartate, a bioavailable isotopically enriched zinc complex, in obesity-induced metabolic dysfunction. Specifically, we evaluated whether administration of the isotopically enriched zinc complex could modulate systemic and adipose-tissue inflammation, normalize adipokine secretion (resistin and ghrelin) as well as restore the balance between tryptophan and serotonin metabolism through the regulation of key enzymes involved in these pathways. In addition, we examined whether these biochemical effects were associated with improvements in pancreatic histophysiology, including reductions in fibrosis and glycogen accumulation and restoration of insulin-producing β-cells.

Our results demonstrated that Zn64 Aspartate supplementation normalized the resistin/ghrelin ratio, promoted favorable shifts in tryptophan and serotonin levels, and was accompanied by improved pancreatic histoarchitecture, reduced fibrosis, and restoration of β-cell integrity. These outcomes indicate that Zn64 Aspartate acts not only as a metabolic modulator but also as a regulator of neuroendocrine and inflammatory homeostasis in diet-induced obesity. Therefore, this study extends our previous work by providing new insight into the immunometabolic and neuromodulatory mechanisms through which isotopically enriched zinc mitigates obesity-associated pathophysiological alterations.

There are several potential mechanisms that may explain the observed modulatory effects of Zn64 Aspartate. First, the compound may enhance the synthesis of zinc-dependent enzymes and transcription factors, for which zinc serves as a structural and catalytic component. The increased availability of intracellular zinc under conditions of intensified free radical production and oxidative stress promotes the upregulation of key antioxidant enzymes, thereby maintaining an adequate antioxidant reserve. Importantly, zinc is capable of inducing the synthesis of various antioxidants, anti-inflammatory cytokines, and factors that regulate intracellular signaling cascades and fundamental metabolic processes.

According to the literature, levels of zinc-α2-glycoprotein (ZAG), which reduces fat accumulation by stimulating lipolysis in adipocytes, are significantly decreased in obesity[31]. Furthermore, the differentiation of adipocytes, particularly within brown adipose tissue, is tightly controlled by a group of transcription factors, many of which depend on the intracellular availability of zinc[32].

One of the primary mechanisms through which zinc may influence the cytokine profile involves the modulation of transcription factors that are sensitive to oxidative stress. Zinc is known to inhibit the activation of nuclear factor-kappa B (NF-κB) and other redox-sensitive transcription factors, thereby reducing the expression of pro-inflammatory genes such as IL-6 and IL-8[33]. This mechanism may explain the observed cytokine-modulating and pancreatic fibrosis remodeling effects of the Zn^{64} Aspartate in our experimental model.

Our findings demonstrate that administration of isotopically modified Zn^{64} Aspartate significantly modulates the cytokine profile in obese animal models, attenuating pro-inflammatory markers while enhancing anti-inflammatory mediators. These results align with growing evidence supporting the immunomodulatory potential of zinc-based compounds in metabolic disorders[34].

Obesity is characterized by chronic low-grade inflammation, marked by elevated levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, and IFN-γ in both adipose tissue and systemic circulation[35]. These cytokines contribute to insulin resistance and β-cell dysfunction, thereby exacerbating metabolic dysregulation[36]. In our study, treatment with Zn^{64} Aspartate led to a pronounced reduction of these pro-inflammatory cytokines, suggesting its role in mitigating obesity-induced inflammation. Concomitantly, Zn^{64} Aspartate treatment elevated anti-inflammatory cytokines, including IL-4, IL-10, and TGF-β, surpassing their levels observed in control animals. IL-10, in particular, is known for its ability to suppress pro-inflammatory cytokine synthesis and promote insulin sensitivity. The upregulation of IL-10 following Zn^{64} Aspartate administration highlights its potential to restore immune balance in obesity.

These immunomodulatory effects may be attributed to enhanced bioavailability and cellular uptake of the Zn^{64} isotope when complexed with aspartate, facilitating its integration into enzymatic systems that regulate oxidative stress and inflammation. The unique properties of Zn^{64} Aspartate, including its high isotopic purity and stable chelation with aspartate, may potentiate its biological activity compared to conventional zinc supplements[37].

Importantly, the absence of significant changes in cytokine levels in non-obese control animals treated with Zn64 Aspartate indicates a favorable safety profile and a high degree of specificity under pathological conditions. This specificity is crucial for therapeutic applications, minimizing potential adverse effects in normophysiological states.

Overall, the comprehensive analysis of Zn^{64} Aspartate effects on the pathogenesis of obesity allow us to consider enriched Zn^{64} as a promising bioactive therapeutic agent for developing compounds aimed not only at treating chronic inflammatory conditions, antioxidant depletion, and serotonergic dysfunction but also at preventing diseases associated with systemic metabolic disorders[38–40].

The current study demonstrates that diet-induced obesity causes a profound dysregulation of the serotonergic system at both the peripheral and central levels. In the peripheral tissues, particularly in the duodenum and serum, obesity was associated with reduced activity of serotonin biosynthetic enzymes and depletion of the tryptophan pool. Nonetheless, duodenal serotonin levels were paradoxically increased, a finding consistent with reports of elevated intestinal serotonin in obesity[41]. This increase in peripheral serotonin may contribute to hyperglycemia, insulin resistance, and metabolic disturbances, as serotonin plays a role in regulating glucose metabolism, vasoconstriction, and gastrointestinal motility[42]. Meanwhile, reduced serum serotonin and tryptophan levels, in the context of increased MAO activity, further indicate accelerated serotonin degradation, thereby compromising its physiological functions.

In the central nervous system, the reduction in tryptophan availability, potentially due to competition with branched-chain amino acids across the blood-brain barrier[43,44], resulted in decreased serotonin synthesis.

Increased MAO and IDO activities further contributed to serotonin depletion and shunted tryptophan metabolism toward the kynurenine pathway, generating neurotoxic metabolites. Such dysregulation is known to impair mood and satiety signaling, potentially promoting hyperphagia, depressive states, and obesity[45,46].

Treatment with Zn^{64} Aspartate significantly ameliorated these disturbances. The observed restoration of tryptophan levels, normalization of TPH activity, and reduced IDO and MAO activity across all examined tissues suggest that Zn^{64} Aspartate has a multi-level modulatory effect on serotonin metabolism. By enhancing serotonin biosynthesis and reducing degradation, Zn^{64} Aspartate appears to rebalance serotonergic function, which could, in turn, improve metabolic and behavioral outcomes[47].

These findings are in line with accumulating evidence that zinc plays a key neuromodulatory role in the serotonergic system through multiple mechanisms. Experimental studies have shown that $Zn2^{+}$ directly modulates serotonin uptake and receptor activity. García-Colunga et al.[48] demonstrated that zinc enhances 5-HT uptake in rat brain tissue, suggesting facilitation of serotonin reuptake mechanisms, while Satała et al.[49] reported a concentration-dependent dual mode of Zn action at 5-HT_1A receptors, potentiating agonist binding at sub-micromolar levels and inhibiting it at higher concentrations. Chronic zinc administration has also been shown to induce adaptive changes in the density of hippocampal and cortical 5-HT_1A/5-HT_2A receptors[50], indicating that zinc availability can fine-tune serotonergic receptor signaling in both pre- and postsynaptic neurons. Moreover, zinc ions act as allosteric inhibitors at 5-HT_7 receptors[51], providing further evidence for their capacity to modulate serotonergic tone through receptor-level interactions.

In the context of our study, the normalization of systemic serotonin under Zn^{64} Aspartate supplementation is most plausibly linked to peripheral (non-neuronal) serotonin pools, primarily derived from platelets and enterochromaffin cells, rather than to direct central neuronal effects. This interpretation is supported by clinical observations that dietary zinc intake positively correlates with serum serotonin levels in humans[52]. Thus, the beneficial effects of Zn^{64} Aspartate likely reflect the restoration of zinc-dependent enzymatic and receptor functions that regulate both serotonin metabolism and its peripheral bioavailability.

Taken together, these results align with emerging evidence that zinc influences serotonergic signaling not through isotope-specific mechanisms but via maintenance of optimal zinc homeostasis, receptor sensitivity, and redox balance[53,54]. The dual impact of Zn^{64} Aspartate on both central and peripheral serotonin systems underscores its therapeutic potential in addressing the neurohormonal dysregulation underlying obesity.

In summary, our results suggest that the treatment with Zn64 Aspartate exerts multifaceted beneficial effects in obesity, likely through:

- Downregulation of pro-inflammatory cytokines, both locally and systemically,
- Upregulation of anti-inflammatory cytokines,
- Normalization of resistin levels, indicating improved adipocyte functionality,
- Restoration of ghrelin homeostasis, suggesting a positive effect on energy metabolism.
- In the brain, restoration of tryptophan and serotonin levels, accompanied by elevated TPH activity and reduced IDO and MAO activity,
- In the duodenum, reduced serotonin accumulation and increased biosynthetic enzyme activity,
- In serum, normalization of tryptophan and serotonin concentrations with decreased MAO activity.

Collectively, these findings suggest a broad restorative effect of Zn^{64} Aspartate on serotonin metabolism and inflammatory regulation across central and peripheral compartments, highlighting its potential as a modulator of obesity-associated inflammation and metabolic dysfunction. Further research, including clinical trials, is warranted to evaluate its efficacy and safety in human populations and to elucidate the molecular mechanisms underlying its therapeutic action.

Methods

Preparation of Zn^{64} aspartate solution

The chemical compound used was zinc;(2S)-2-amino-4-hydroxy-4-oxobutanoate, with a molecular formula of $C_8H_{12}N_2O_8Zn^{64}$ and a molar mass of 328 g/mol. The solution contained 5 mg of Zn^{64} (stable isotope form), 20.8 mg of L-aspartic acid (100% dry matter basis), and sterile water for injection up to 1 mL (Fig. 4). The $^{64}Zn(HAsp)_2$ chelate complex was synthesized using ^{64}Zn-enriched zinc oxide and L-aspartic acid via the following reaction: $^{64}ZnO\downarrow + 2H_2Asp = {}^{64}Zn(HAsp)_2 + H_2O$.



Fig. 4. Chemical and structural formulas of the prepared Zn^{64} Aspartate solution.

A 50.024 g sample of Zn^{64} oxide was dissolved in 1.5 L of water by adding 166.47 g of aspartic acid and stirring at 60–70 °C. The reaction was considered complete when the solution became fully transparent, indicating total dissolution of ^{64}ZnO. If a solid compound was required, the solution was concentrated via rotary evaporation at 70 °C under 0.1 atm, resulting in a solid glassy $^{64}Zn(HAsp)_2$ product containing approximately 10% water by mass. The final aqueous solution was hydrophilic (log P = − 3.89) and chemically identical to standard zinc aspartate[55].

The production of the Zn^{64} L-aspartate solution is based on a well-established coordination chemistry approach, utilizing the known ability of amino acids to form stable salts in aqueous media. This process involves the formation of coordination bonds between the electron-donating α- and β-carboxyl groups and the amino group of aspartic acid with divalent zinc ions, resulting in the formation of a chelate complex. Consequently, the final preparation contains a single active pharmaceutical ingredient (Zn^{64} L-aspartate) fully solubilized in an aqueous medium.

The compatibility of the formulation components is primarily determined by evaluating their behavior in solution, including susceptibility to degradation under various environmental conditions, and optimizing the pH to preserve the functional integrity and physicochemical stability of the Zn^{64} Aspartate complex. Through systematic pharmaceutical development, the composition of the Zn^{64} Aspartate solution was refined and optimized to ensure long-term stability.

Stability testing over a five-year monitoring period confirmed that the formulation, containing 5 mg of Zn^{64} per 1 mL of solution, maintained its physicochemical properties and active content without significant degradation, demonstrating56obust stability under recommended storage conditions.

Toxicological and pharmacokinetic assessment of Zn^{64} Aspartate in rats confirmed its favorable safety profile and predictable biodistribution. In acute experiments, the compound showed relatively high tolerance, with lethal doses exceeding several tens of milligrams per kilogram depending on the route of administration, and males generally less sensitive than females. Under repeated administration, no behavioral, clinical, or histopathological abnormalities were observed at therapeutically relevant doses, and the no-observed-adverse-effect level (NOAEL) was established within the 8–17 mg/kg range. Pharmacokinetic evaluation demonstrated rapid systemic absorption, proportional dose-exposure relationships, and predominant renal elimination, with physiological accumulation mainly in the liver, kidneys, and pancreas. Altogether, these findings indicate efficient clearance and a wide margin of safety for Zn^{64} Aspartate in experimental rats.

Animals and experimental design

Forty adult male Wistar rats (initial weight 200 ± 5 g) were used. All animals were obtained and maintained under standard laboratory conditions in an accredited vivarium at the Educational and Scientific Centre "Institute of Biology and Medicine," Taras Shevchenko National University of Kyiv, Ukraine. All experimental procedures were conducted in accordance with the European Directive 2010/63/EU, Ukrainian legislation on animal protection, and approved by the institutional Ethics Committee. Reporting adhered to the ARRIVE guidelines[56]. The general body weight changes (BMI (g/cm2) and Weight gain as of the end of the experiment (%)) are described in previous paper[27]

To induce diet-induced obesity (DIO), animals were fed a high-fat diet (HFD) for 4 weeks. The HFD consisted of standard chow (60%), lard (10%), chicken eggs (10%), sucrose (9%), peanuts (5%), dry milk (5%), and sunflower oil (1%)[7]. After 4 weeks, animals were randomly assigned into four experimental groups (n = 10 per group) (Fig. 5):

1. Control (C) – standard chow and water ad libitum for 10 weeks.
2. Control + Zn^{64} Aspartate (C + Zinc) – standard chow and water ad libitum; received Zn^{64} Aspartate every third day (4.5 mg in 2 mL by oral gavage).
3. DIO – continued high-fat diet and water ad libitum for an additional 6 weeks.
4. DIO + Zn^{64} Aspartate (DIO + Zinc) – continued high-fat diet and water ad libitum; received Zn^{64} Aspartate every third day (4.5 mg in 2 mL by oral gavage) for 6 weeks.

Body weight was measured weekly after an overnight fast and daily food intake was monitored. At the end of the 10-week experimental period, rats were euthanized 24 h after the final Zn^{64} Aspartate administration by decapitation using the guillotine method. This procedure was selected in order to obtain unaltered blood samples for biochemical and hormonal analysis, as the use of chemical anesthetics may interfere with serum metabolic parameters. The method was performed by trained personnel in accordance with the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123, "Strasbourg Bioethics Convention") and was approved by the bioethical commission of the Educational and Scientific Centre "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv. Blood and tissue samples were collected immediately for subsequent analysis.

Determination of interleukins, resistin, and ghrelin in serum and adipose tissue

Quantification of interleukins (IL-1β (sc-52012), IL-6 (sc-32296), IL-10 (sc-32815), IL-12 (sc-365389), IFN-γ (sc-74106), IL-4 (sc-53084), TGF-β (sc-374433), resistin (sc-16117), and ghrelin (sc-517596), Santa Cruz Biotechnology, USA) was performed using enzyme-linked immunosorbent assay (ELISA) according to established manufacturer protocols. Briefly, 96-well microplates with high protein-binding capacity were coated with antigen (10 μg/mL) diluted in 0.1 M $NaHCO_3$ buffer (pH 9.6) and incubated overnight at 4 °C. Unbound antigen was removed by triple washing with Tris-buffered saline (TBS) containing 0.05% Tween-20, followed by washes without detergent. Non-specific binding was blocked by incubating wells with 1% bovine serum albumin (BSA) in TBS for 1 h at 37 °C. After another washing step, primary antibodies, diluted in TBS per manufacturer's

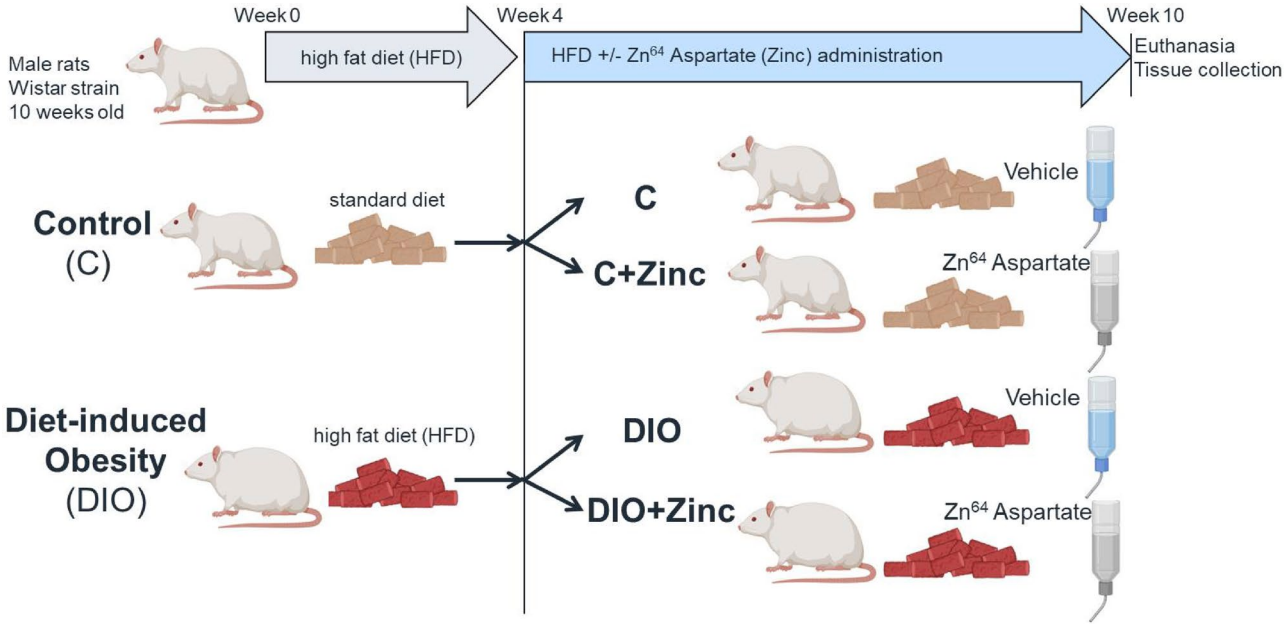


Fig. 5. A graphical illustration of the study design. *NoteC* control; *C + zinc* control on the background of administration of Zn^{64} Aspartate; *DIO* diet induced obesity; *DIO + zinc* diet induced obesity on the background of administration of Zn^{64} Aspartate.

instructions) were applied for 1 h at 37 °C. Following a second round of washes, horseradish peroxidase (HRP)-conjugated secondary antibodies: bovine anti-goat antibodies (sc-2350) and mouse anti-goat IgG-HRP (sc-2354, Santa Cruz Biotechnology, USA) were added and incubated for 1 h at 37 °C. Detection was performed using o-phenylenediamine dihydrochloride (OPD) as the substrate in phosphate-citrate buffer (0.05 M) with 0.3% H_2O_2. After 10 min, the enzymatic reaction was terminated with 2.5 N sulfuric acid. Absorbance was read at 492 nm using a μQuant spectrophotometer (BioTek Instruments, USA).

Quantification of serotonin and tryptophan in serum, brain, and duodenal homogenates

To measure serotonin and tryptophan concentrations, 1:5 (v/v) aliquots of serum or tissue homogenates were deproteinized with 0.4 M perchloric acid and incubated at 4 °C for 60 min. Samples were then centrifuged at 800 × g for 5 min at 4 °C. The resulting supernatant was neutralized to pH 5–6 using 2 M KOH and subjected to a second centrifugation. Supernatants were loaded onto KM-Sepharose columns equilibrated with 0.01 M sodium phosphate buffer (pH 6.2). Elution was carried out sequentially with buffer 1 (0.01 M sodium phosphate, pH 6.2) for tryptophan and buffer 2 (0.03 M sodium phosphate, pH 6.2) for serotonin. Tryptophan levels were measured with a spectrofluorometer at an excitation wavelength of 295 nm and an absorption wavelength of 550 nm compared to a blank sample that contained an appropriate volume of distilled water instead of the test sample. Serotonin levels were measured with a spectrofluorometer at an excitation wavelength of 359 nm and an absorption wavelength of 485 nm compared to a blank sample, which contained an appropriate volume of distilled water instead of the test sample[57].

Measurement of tryptophan hydroxylase (TPH) activity

TPH activity in brain and duodenal homogenates was assessed following the method of Kuhn et al.[58]. Homogenates were centrifuged at 12,000 × g for 30 min at 4 °C. The supernatant was added to an incubation mixture containing 500 mM Tris–HCl (pH 7.4), 20 mM dithiothreitol (DTT), 1 mM $CaCl_2$, 4 mM L-tryptophan, and 50 μg catalase. Incubation was conducted at 37 °C for 15 min, after which the reaction was terminated by addition of 6 M $HClO_4$. Following protein precipitation, samples were centrifuged at 600 × g for 5 min. Optical density was measured using a spectrofluorometer at excitation/emission wavelengths of 295/540 nm. Control samples contained incubation buffer and distilled water.

Determination of indoleamine-2,3-dioxygenase (IDO) activity

IDO activity was evaluated according to the method described by Kudo et al.[59]. After thawing and centrifugation of tissue homogenates at 12,000 × g for 30 min, supernatants were incubated in potassium phosphate buffer (100 mM, pH 7.5) containing 5 mM L-tryptophan, 10 mM ascorbic acid, 0.2 mM methylene blue, and 50 μg catalase at 37 °C for 30 min. The reaction was halted by adding 10% trichloroacetic acid (TCA) and centrifuged at 600 × g for 5 min. The supernatant was neutralized with 1 M Tris–HCl (pH 7.0), and the absorbance was measured at 360 nm using a spectrofluorometer versus a blank sample that contained the incubation medium and distilled water.

Assessment of monoamine oxidase (MAO) activity in serum

Serum monoamine oxidase activity was determined using a colorimetric method [60]. The method consists in the formation of benzaldehyde from benzylamine hydrochloride under the action of MAO. Benzaldehyde interacts with 2,3-dinitrophenylhydrazine and forms insoluble hydrazone which can be precipitated by centrifugation. The hydrazone precipitate, in turn, forms a stable compound of raspberry color in an alkaline medium, the content of which can be determined spectrophotometrically.

MAO activity was measured based on benzylamine substrate conversion]. The reaction mixture included 0.2 M phosphate buffer (pH 7.4), distilled water, and 1% benzylamine hydrochloride. Blank controls excluded benzylamine. The reaction was initiated by adding serum aliquots and incubated at 37 °C for 3 h. Termination was achieved by 10% TCA precipitation followed by centrifugation at 600 × g for 5 min. Supernatants were reacted with 0.1% 2,3-dinitrophenylhydrazine in 2 M HCl for 25 min at room temperature to form hydrazone derivatives. After hydrazone precipitation and centrifugation, pellets were resuspended in 3 M NaOH and 96% ethanol to develop a raspberry-colored complex. Absorbance was measured at 460 nm using a spectrofluorometer. The optical density of the samples was measured using a spectrofluorometer with excitation at 460 nm versus ethanol.

Histopathological analysis of pancreatic fibrosis, glycogen content, and β-cell insulin expression

For histological evaluation, pancreatic tissues were fixed in 4% neutral buffered paraformaldehyde (Sigma-Aldrich, Merck) for 72 h at room temperature. After fixation, samples were dehydrated through a graded ethanol series, embedded in paraffin, and sectioned at a thickness of 8 µm using a rotary microtome. Histological and immunohistochemical analyses were performed on serial sections.

All stained slides were visualized using an Olympus BX41 light microscope (Tokyo, Japan) equipped with an Olympus DP20 digital camera. Images were captured and processed using QuickPHOTO MICRO software (Promicra, Prague, Czech Republic). Morphometric analyses were performed with FIJI/ImageJ software (NIH, Bethesda, MD, USA).

Assessment of pancreatic fibrosis

Fibrotic changes were evaluated on sections stained with Masson's trichrome. Blue-stained collagen fibers were quantified as a percentage of the total tissue area using the "Masson Trichrome" vector in the Color Deconvolution plugin of ImageJ. The relative area of blue deposits is estimated using a threshold value and area fraction measurement. To calculate the cumulative integral density of the fibrosis parameter, the area fraction of blue deposits is multiplied by its measured integral density[61,62].

Determination of glycogen content

Glycogen content was assessed using periodic acid–Schiff (PAS) staining. Liver sections were used as positive and negative controls (with and without periodic acid treatment, respectively). After staining, digital image analysis was performed using the H-PAS vector in the Color Deconvolution plugin. The intensity of PAS-positive staining was quantified by measuring the mean gray value (MGV) of the PAS-positive areas, representing relative glycogen content[63,64].

Immunohistochemical evaluation of β-cell insulin level

Immunohistochemistry for insulin was performed to assess β-cell insulin expression. After deparaffinization and rehydration, antigen retrieval was carried out by heat treatment in citrate buffer (pH 6.0). Sections were incubated overnight at 4 °C in a humidified chamber with a rabbit monoclonal anti-insulin antibody [EPR17359] (ab181547, Abcam) at a 1:1000 dilution. The following day, sections were incubated at room temperature for 1 h with an HRP-conjugated secondary antibody (Goat anti-Rabbit IgG, Fc-specific, AP156P, Lot No. 3407309; EMD Millipore, USA) diluted 1:200. Visualization was achieved using 3,3′-diaminobenzidine (DAB), followed by hematoxylin counterstaining (Merck, Germany). Image analysis was conducted using the H-DAB vector in the Color Deconvolution plugin, and the insulin expression level was quantified by measuring the mean gray value (MGV) of DAB-positive β-cells to whole pancreatic islets[65].

Statistical analysis

For each staining method, five representative sections per animal were analyzed. Data distribution normality was assessed using the Kolmogorov–Smirnov test. Statistical significance was evaluated using two-way ANOVA with Holm–Sidak post hoc correction for parametric data. For non-normally distributed datasets, the Kruskal–Wallis test for independent samples was employed. A p-value < 0.05 was considered statistically significant. All statistical analyses were conducted using OriginPro 8 (OriginLab Corporation, USA). Results are reported as mean ± standard error of the mean (SEM) in tabular format, and as median with interquartile range (25th–75th percentile), minimum, and maximum values in box plots.

Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

O.K., T.V., T.H., N.R., performed the experiments and wrote the paper; R.B. performed the experiments; O.K., M.T., S.G., L.M., A.B., R.B., O.S. interpreted the data; S.G., L.M. provided editorial review. O.S. supervised the project, performed the experiments, interpreted data, and wrote the paper; All authors read and approved the final manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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