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The Role of NF-κB, Melatonin, and LDH-A in Breast Cancer Initiation, Promotion, and Progression in Rats Exposed to N-Methyl-Nitroso-Urea: Implications for Tumor Metabolic Reprogramming

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Abstract

*Breast cancer remains a significant focus in oncology due to its complexity and variable progression. This study investigates the initiation, promotion, and progression of breast cancer in 97 rats induced with carcinogenesis by **N-methyl-nitroso-urea** (NMU). We analyzed stress-responsive hormones, **NF-κB pathway** activation, melatonin secretion, and lactate dehydrogenase (LDH) dynamics during disease progression. The study revealed that tumor development correlates with the overactivation of the NF-κB pathway and a simultaneous reduction in melatonin levels, evident in both nocturnal and diurnal cycles. Additionally, LDH-A (LDH-5), a key enzyme in glycolysis, was shown to dominate metabolic processes during tumor progression, suggesting metabolic reprogramming. These findings highlight the intricate interplay of stress hormones, inflammation, and metabolism in cancer and the potential for targeting these pathways therapeutically.*

Keywords: NF-κB, Melatonin, LDH-A (LHD-5), Breast Cancer, Tumor Progression, Metabolic Reprogramming, N-Methyl-Nitroso-Urea

1. Introduction

Breast cancer is one of the most prevalent malignancies worldwide, presenting a complex landscape of biological heterogeneity, clinical behaviors, and treatment challenges. Its progression involves a multistep process characterized by genetic mutations, dysregulated signaling pathways, and metabolic alterations that allow cancer cells to proliferate uncontrollably, evade apoptosis, and metastasize to distant organs. Among the subtypes, hormone receptor-positive (ER/PR-positive) and **Her2/neu-negative** breast cancers, while generally considered less aggressive than Her2-positive or triple-negative breast cancers, possess distinct molecular and systemic influences that warrant detailed investigation. These cancers exhibit unique interactions with their tumor microenvironment (TME), systemic host responses, and intracellular metabolic changes, all of which contribute to their progression and clinical outcomes.

1.1. Tumor Microenvironment and Systemic Changes in Cancer Progression

The tumor microenvironment plays a pivotal role in cancer progression by orchestrating a network of interactions between cancer cells, stromal cells, immune cells, and the extracellular matrix. Within this milieu, cancer cells exploit systemic host changes, such as **chronic inflammation,** oxidative stress, and metabolic reprogramming, to enhance their survival and proliferation. In hormone-positive breast cancer, the dependency on estrogen and progesterone for growth is modulated by the interaction of cancer cells with the TME, further complicated by factors like hypoxia and nutrient deprivation. Chronic inflammation, driven by cytokines, chemokines, and inflammatory mediators, contributes significantly to tumor development and progression. Among the key regulators of this inflammatory response is **NF-κB (Nuclear Factor kappa-light-chain-enhancer of activated B cells)**, a transcription factor activated in response to cellular stress and external stimuli. Its overactivation is a hallmark of many cancers, including

breast cancer, where it promotes survival, angiogenesis, and immune evasion. NF-κB's activity is tightly linked to the TME's pro-inflammatory milieu, which fosters conditions favorable for tumor initiation and progression.

1.2. The Warburg Effect and Metabolic Reprogramming

One of the most striking features of cancer cells is their ability to reprogram cellular metabolism to support rapid growth and survival. Unlike normal cells, which rely primarily on oxidative phosphorylation for ATP production, cancer cells often shift to glycolysis even in the presence of adequate oxygen—a phenomenon known as the **Warburg effect.** This metabolic reprogramming is not merely an adaptation to hypoxia but a strategic shift to meet the biosynthetic demands of rapidly dividing cells. Central to this metabolic adaptation is the enzyme **lactate dehydrogenase A (LDH-A)**, which catalyzes the conversion of pyruvate to lactate while regenerating NAD^+, a cofactor essential for sustaining glycolysis. Elevated levels of LDH-A and its isoenzyme LDH-5 are associated with enhanced glycolytic flux, lactate production, and tumor aggressiveness. The metabolic switch facilitated by LDH-A supports not only energy production but also the creation of precursors for nucleotides, amino acids, and lipids, all of which are critical for cancer cell proliferation. Furthermore, the accumulation of lactate in the TME contributes to acidosis, immune suppression, and angiogenesis, further enhancing tumor progression.

1.3. NF-κB: A Master Regulator of Cancer Progression

NF-κB is a key transcription factor that integrates signals from inflammation, oxidative stress, and cellular damage to regulate genes involved in cell survival, proliferation, and immune responses. Its canonical pathway is activated by various stimuli, including pro-inflammatory cytokines (e.g., TNF-α, IL-1β), pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs). This pathway involves the phosphorylation and subsequent degradation of IκB, an inhibitor of NF-κB, allowing NF-κB to translocate to the nucleus and activate target genes. In cancer, NF-κB is often constitutively active, promoting the transcription of genes involved in anti-apoptosis (e.g., Bcl-2, IAPs), angiogenesis (e.g., VEGF), and immune evasion (e.g., PD-L1). Its activation also enhances the recruitment of immune and stromal cells to the TME, further fueling chronic inflammation and tumor growth. Notably, NF-κB activity is linked to resistance to chemotherapy and radiotherapy, as it supports DNA repair mechanisms and suppresses apoptotic pathways. The activation of NF-κB in both cancer cells and the surrounding TME underscores its central role in orchestrating tumor progression.

1.4. Melatonin: An Endogenous Antagonist of NF-κB

Melatonin, a hormone primarily secreted by the pineal gland during the dark phase of the circadian rhythm, is best known for regulating sleep-wake cycles. However, its biological roles extend far beyond circadian regulation. Melatonin is a potent antioxidant, capable of directly scavenging reactive oxygen species (ROS) and reactive nitrogen species (RNS). Additionally, it enhances the expression of endogenous antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), reducing oxidative stress—a key driver of cancer progression. Melatonin also exhibits significant **anti-inflammatory properties**, mediated in part by its ability to inhibit NF-κB activation. By stabilizing IκB and preventing its degradation, melatonin reduces the nuclear translocation and DNA-binding activity of NF-κB, thereby suppressing the transcription of pro-inflammatory cytokines (e.g., IL-6, TNF-α). This effect is particularly relevant in cancer, where chronic NF-κB activation creates a permissive environment for tumor growth and immune evasion. Melatonin's role as an NF-κB antagonist positions it as a potential therapeutic agent for mitigating tumor-promoting inflammation. In addition to its anti-inflammatory and antioxidant properties, melatonin influences cancer metabolism. By inhibiting the Warburg effect, melatonin reduces glycolytic flux and lactate production, counteracting the metabolic reprogramming characteristic of cancer cells. This dual action on inflammation and metabolism highlights melatonin's potential as a multi-targeted agent in cancer therapy.

1.5. The Interplay Between NF-κB, Melatonin, and LDH-A in Cancer Progression

The intricate relationship between NF-κB, melatonin, and LDH-A underscores the complexity of cancer biology. As cancer progresses, the overactivation of NF-κB drives inflammation, angiogenesis, and immune suppression, while simultaneously promoting metabolic shifts to glycolysis. The resulting increase in LDH-A activity facilitates the production of lactate, creating an acidic, immune-suppressive TME that supports tumor survival and invasion. Melatonin acts as a counter-regulatory agent, suppressing NF-κB activity and mitigating the downstream effects of its activation, including LDH-A upregulation and metabolic reprogramming. However, the suppression of melatonin levels observed in cancer patients, particularly in advanced stages, diminishes this protective effect, creating a vicious cycle of inflammation, oxidative stress, and metabolic dysregulation.

1.6. Study Objectives and Rationale

This study aims to investigate the sequential changes in NF-κB activation, melatonin secretion, and LDH-A dynamics during the initiation, promotion, and progression of breast cancer in rats exposed to **N-methyl-nitroso-urea (NMU)**. By examining these processes in parallel, we seek to elucidate the interplay between inflammation, oxidative stress, and metabolism in cancer progression. The findings may provide insights into potential therapeutic strategies targeting NF-κB and LDH-A while leveraging melatonin's protective effects.

1.6.1. Hypotheses

- NF-κB activation and LDH-A expression are progressively upregulated during tumor initiation, promotion, and progression.
- Melatonin levels decline in both nocturnal and diurnal phases as tumor progression deepens.
- Metabolic reprogramming marked by LDH-A dominance reflects a systemic shift that begins early in tumor development.

1.6.2. Research Significance

Understanding the interplay between NF-κB, melatonin, and LDH-A in breast cancer progression offers opportunities to identify novel biomarkers and therapeutic targets. Furthermore, the study highlights the potential of melatonin as an adjuvant therapy to counteract the deleterious effects of chronic inflammation and metabolic reprogramming in cancer.

2. Materials and Methods

2.1. Animal Model

This study utilized a well-established rodent model to investigate breast cancer progression and its associated molecular changes.

• **Subjects:** Ninety-seven (97) female Sprague-Dawley rats were selected for the experimental group and exposed to **N-methyl-nitroso-urea (NMU)**, a carcinogen known to induce hormone receptor-positive (ER/PR-positive) and Her2/neu-negative breast cancer. An additional control group of 100 female rats was maintained under identical conditions but without NMU exposure. The control group served to establish baseline values for all measured parameters.

• **Housing Conditions:** All animals were housed in a controlled laboratory environment under standard conditions: a **12-hour light-dark cycle** with room temperature maintained at 22 ± 2°C and humidity levels at 50-60%. Rats were provided ad libitum access to a standard diet and water throughout the study.

• **Ethical Considerations:** All procedures were performed following institutional and national guidelines for animal care and use. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Study Design

The study was conducted to monitor the development of breast cancer in three distinct phases: initiation, promotion, and progression. These phases were defined as follows:

• **Initiation Phase:** This phase began immediately after NMU exposure. Rats were monitored weekly for the onset of detectable tumors through palpation and imaging. Blood samples were collected to establish baseline values for stress hormones, melatonin levels, and LDH isoenzyme activity.

• **Promotion Phase:** Tumor development and growth were assessed during this phase, characterized by active cellular proliferation. Regular biopsies of tumor tissues and circulating tumor cells (CTCs) were collected. Stress hormones, melatonin, and NF-κB activation were measured periodically.

• **Progression Phase:** This phase was defined by the advanced stage of tumor growth, metastasis, and significant metabolic alterations. Samples from tumors, CTCs, and plasma were analyzed for markers of metabolic reprogramming and inflammation.

Each rat underwent comprehensive evaluation at predetermined time points across these three phases. For each parameter, data from the experimental group were compared with those from the control group to identify significant deviations linked to tumor development.

2.3. Parameters Measured

2.3.1. Stress Hormones

• **Objective:** To measure the levels of stress-responsive hormones, including **adrenaline, cortisol,** and **adrenocorticotropic hormone (ACTH),** as indicators of the systemic stress response during tumor development.

• **Methodology:** Blood samples were collected via tail vein or cardiac puncture under anesthesia. Plasma was separated and stored at -80°C until analysis. Hormone levels were quantified using commercial enzyme-linked immunosorbent assay (ELISA) kits with high specificity and sensitivity.

• **Sampling Frequency:** Samples were collected at baseline (pre-NMU exposure) and during each phase of tumor progression.

2.3.2. NF-κB Activation

• **Objective:** To assess the sequential activation and overexpression of the **NF-κB pathway** in tumor tissues and CTCs.

• **Techniques:**

➢ **Immunohistochemistry (IHC):** Biopsy specimens from tumors were fixed in formalin, embedded in paraffin, and stained for NF-κB subunits (p65 and p50).

➢ **Western Blotting:** Protein extracts from tumor tissues and CTCs were separated by SDS-PAGE and probed with antibodies specific to NF-κB and its phosphorylated forms.

• **Analysis:** NF-κB activation was quantified based on the intensity of nuclear staining in IHC and band densitometry in Western blot analyses.

2.3.3. Melatonin

• **Objective:** To measure plasma melatonin levels and evaluate its diurnal and nocturnal fluctuations in response to tumor progression.

• **Methodology:** High-sensitivity immunoassays were used to detect melatonin in plasma samples collected during both the light and dark phases of the circadian cycle. Samples were processed under dim red light to prevent photodegradation of melatonin.

• **Sampling Frequency:** Plasma was collected at baseline, initiation, promotion, and progression phases, with additional samples taken at specific intervals during both daytime and nighttime.

2.3.4. LDH Isoenzyme Analysis

• **Objective:** To evaluate the activity and isoenzyme composition of l**actate dehydrogenase (LDH)**, focusing on the proportion of **LDH-A (LDH-5)**, an isoenzyme associated with glycolysis and metabolic reprogramming in cancer.

• **Techniques:**

➢ **Total LDH Activity:** Measured using enzymatic assays based on the conversion of pyruvate to lactate, with NAD^+ reduction monitored spectrophotometrically.

➢ **Isoenzyme Fractionation:** Gel electrophoresis was performed to separate LDH isoenzymes, followed by densitometry to quantify the relative abundance of LDH-A.

• **Analysis:** The proportion of LDH-A in total LDH activity was calculated, and changes were monitored across all three phases of tumor development.

Citation: Tavartkiladze, A., Simonia, G., Reiter, R. J., Lou, R, Okrostsvaridze, N., et al. (2025). The Role of NF-κB, Melatonin, and LDH-A in Breast Cancer Initiation, Promotion, and Progression in Rats Exposed to N-Methyl-Nitroso-Urea: Implications for Tumor Metabolic Reprogramming. *J Cancer Res, 3*(1), 1-11.

2.4. Statistical Analysis

• **Data Analysis:** Quantitative data were analyzed using **one-way analysis of variance** (ANOVA) to compare groups across different phases of tumor progression.

• **Post-hoc Testing:** Tukey's test was employed for pairwise comparisons to identify specific phase-related changes in hormone levels, NF-κB activity, melatonin secretion, and LDH isoenzyme composition.

• **Significance Threshold:** A p-value < 0.05 was considered statistically significant.

Data were presented as mean ± standard error (SE), with visualizations generated using bar graphs and scatterplots for easy interpretation. All statistical analyses were performed using GraphPad Prism (version 9.0).

2.4.1. Ethical Considerations and Study Integrity

• Efforts were made to minimize animal suffering, including the use of anesthesia during invasive procedures and humane endpoints for severely ill animals.

• Sample sizes were calculated to ensure sufficient statistical power while adhering to the principles of the **3Rs** (Replacement, Reduction, and Refinement).

3. Results

3.1. Stress Hormone Dynamics

The study revealed significant alterations in stress hormone levels in tumor-bearing rats compared to the control group. These findings highlight the systemic impact of tumor initiation, promotion, and progression phases on physiological stress responses.

• **Adrenaline Levels**

Tumor-bearing rats showed a substantial increase in adrenaline levels as early as the initiation phase. This elevation persisted through the promotion and progression phases, with levels remaining significantly higher than those of control rats ($p < 0.01$). This sustained elevation suggests a heightened activation of the sympathetic nervous system, likely driven by tumor-associated stressors.

• **Cortisol Levels**

Plasma cortisol levels also rose significantly during tumor initiation and remained elevated during the subsequent phases. This increase, indicative of chronic activation of the hypothalamic-pituitary-adrenal (HPA) axis, reflects a systemic stress response to the developing tumor. The persistence of high cortisol levels likely contributed to immunosuppressive effects observed in tumor-bearing rats.

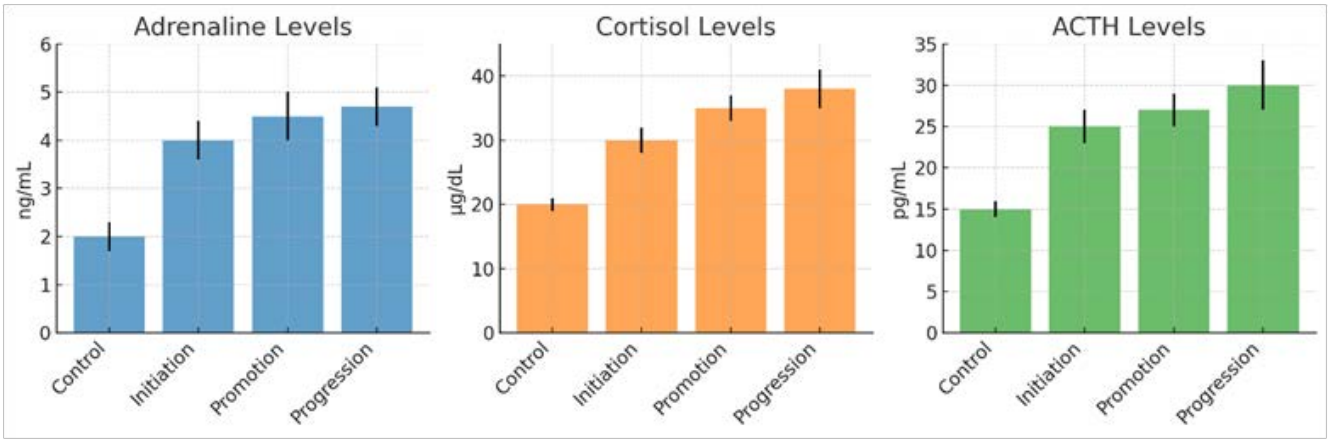
• **ACTH Levels**

Adrenocorticotropic hormone (ACTH), a key regulator of cortisol production, exhibited a similar trend. ACTH levels were markedly elevated in tumor-bearing rats throughout the study, correlating with the sustained increase in cortisol. The correlation coefficient between ACTH and cortisol levels was strong ($r = 0.82$), suggesting a tightly regulated feedback mechanism.

• **Control Group Findings**

In contrast, control rats exhibited stable levels of adrenaline, cortisol, and ACTH throughout the study period. The absence of significant fluctuations in these hormones in the control group underscores the role of tumor development in triggering systemic stress responses.

These findings demonstrate that tumor development induces a persistent stress response, as evidenced by elevated adrenaline, cortisol, and ACTH levels. This response likely contributes to the inflammatory and immunosuppressive environment associated with cancer progression (Figure #1).



On this visual representation of stress hormone dynamics in control vs. tumor-bearing rats. The three subplots illustrate mean hormone levels (± SE) across four phases (Control, Initiation, Promotion, and Progression) for:

(A) Adrenaline (ng/mL)

(B) Cortisol (µg/dL)

(C) ACTH (pg/mL)

This visualization highlights the persistent elevation of stress hormones in tumor-bearing rats, emphasizing chronic HPA axis activation.

3.2. NF-κB Activation

The activation of the NF-κB pathway was a prominent feature in tumor-bearing rats, with sequential changes observed across the initiation, promotion, and progression phases.

• **Initiation Phase**

During the initiation phase, NF-κB activation was detected in circulating tumor cells (CTCs) and early tumor biopsy samples. Immunohistochemical analysis revealed nuclear

localization of NF-κB subunits (p65 and p50) in tumor cells, a hallmark of its activation. Western blot analysis confirmed increased phosphorylation of IκB, the inhibitor of NF-κB, leading to its degradation.

• Promotion Phase

As tumors entered the promotion phase, NF-κB activation intensified. The proportion of cells exhibiting nuclear NF-κB localization increased significantly ($p < 0.001$). The elevated expression of downstream NF-κB target genes, including pro-inflammatory cytokines such as TNF-α and IL-6, was evident. These findings indicate that NF-κB drives a pro-inflammatory environment during tumor growth.

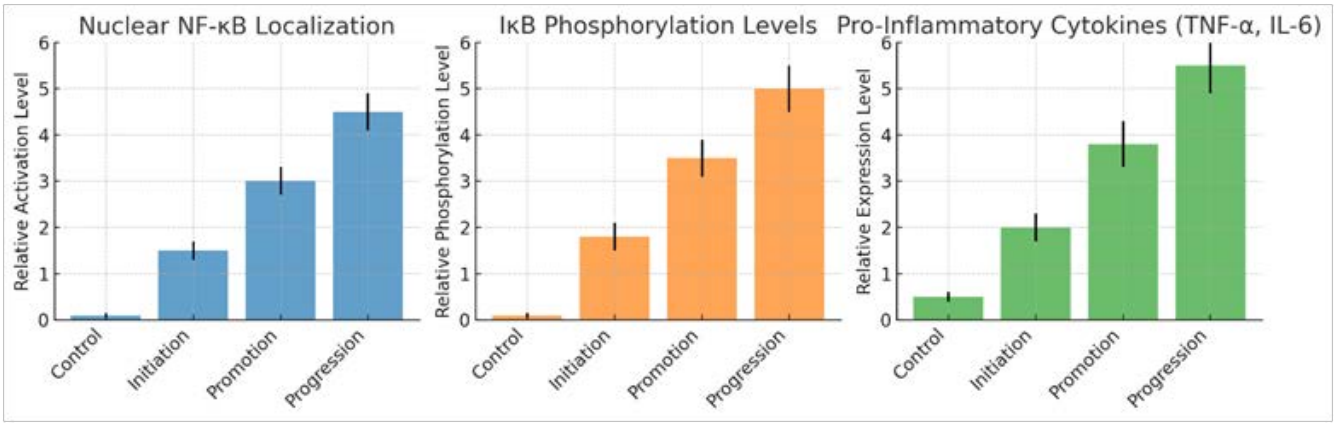
• Progression Phase

In the progression phase, NF-κB activation reached its peak. Tumor tissues displayed widespread nuclear localization of NF-κB subunits, with a concurrent increase in inflammatory cytokine production. This phase also saw elevated levels of NF-κB activation in CTCs, suggesting its role in metastatic dissemination.

• Control Group Findings

In the control group, no significant NF-κB activation was detected in similar tissues. Immunohistochemistry and Western blot analyses of control rat tissues revealed minimal nuclear localization of NF-κB subunits and negligible IκB phosphorylation.

The results confirm that NF-κB activation occurs early in tumor development and persists throughout progression, contributing to inflammation and tumor survival mechanisms (Figure #2).



On this visual representation of NF-κB activation dynamics in control vs. tumor-bearing rats. The three subplots illustrate the relative levels of:

(A) Nuclear NF-κB Localization

(B) IκB Phosphorylation

(C) Pro-Inflammatory Cytokine Expression (TNF-α, IL-6)

These graphs highlight the progressive activation of NF-κB from the initiation to the progression phase, emphasizing its role in tumor-associated inflammation and metastasis.

3.3. Melatonin Levels

Melatonin, a key regulator of circadian rhythms and an antioxidant, exhibited significant reductions in tumor-bearing rats compared to controls. This reduction correlated with tumor progression and was particularly evident during the nocturnal phase.

• Overall Reduction

Tumor-bearing rats demonstrated a significant decline in plasma melatonin levels across all phases of tumor development ($p < 0.001$). The reduction was more pronounced in the progression phase, where melatonin levels were nearly half of those in the control group.

• Disruption of Diurnal Rhythms

The diurnal and nocturnal secretion patterns of melatonin were markedly disrupted in tumor-bearing rats. While control rats exhibited a typical circadian pattern, with higher melatonin levels at night, tumor-bearing rats showed a blunted nocturnal peak. This disruption was most pronounced in the progression phase, where nocturnal melatonin levels were only marginally higher than daytime levels.

• Correlation with NF-κB Activation

The decrease in melatonin levels correlated inversely with NF-κB activation ($r = -0.76$), suggesting that chronic inflammation mediated by NF-κB may suppress melatonin synthesis. The reduction in melatonin likely contributed to the oxidative stress and inflammatory environment observed in tumor-bearing rats.

• Control Group Findings

Control rats maintained stable melatonin levels throughout the study, with a clear circadian pattern characterized by elevated nocturnal secretion.

These findings highlight the role of melatonin suppression in tumor-associated metabolic and inflammatory dysregulation, emphasizing its potential as a therapeutic target (Figure #3).

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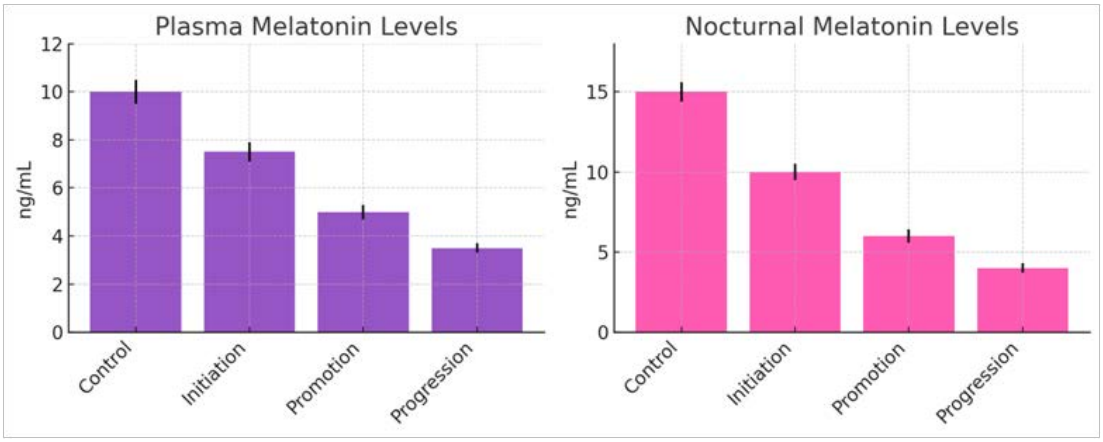


On this visual representation of melatonin levels in control vs. tumor-bearing rats. The two subplots illustrate:
(A) Plasma Melatonin Levels (Purple) – Showing an overall reduction in tumor-bearing rats, with the lowest levels in the progression phase.
(B) Nocturnal Melatonin Levels (Pink) – Demonstrating disruption of the circadian secretion pattern, with significantly blunted nocturnal peaks in tumor-bearing rats.

These findings highlight the inverse correlation between melatonin suppression and NF-κB activation, emphasizing melatonin's potential role in tumor-associated metabolic and inflammatory dysregulation.

3.4. LDH Isoenzyme Profile

The metabolic reprogramming characteristic of cancer was evident in the changes observed in LDH levels and isoenzyme profiles in tumor-bearing rats.

• Total LDH Activity

Total LDH levels increased significantly during the initiation and promotion phases of tumor development. However, by the progression phase, LDH levels normalized in 80% of tumor-bearing rats. This normalization suggests that the metabolic shift driven by LDH-A (LDH-5) predominates during later stages.

• LDH-A (LDH-5) Dominance

Fractional analysis of LDH isoenzymes revealed a significant increase in the proportion of LDH-A (LDH-5) during the progression phase. On average, LDH-A comprised 67% of total LDH activity in tumor-bearing rats, compared to 25% in controls. This dominance reflects a metabolic shift toward glycolysis, consistent with the Warburg effect. The elevated LDH-A levels support lactate production, providing both energy and biosynthetic precursors for tumor growth.

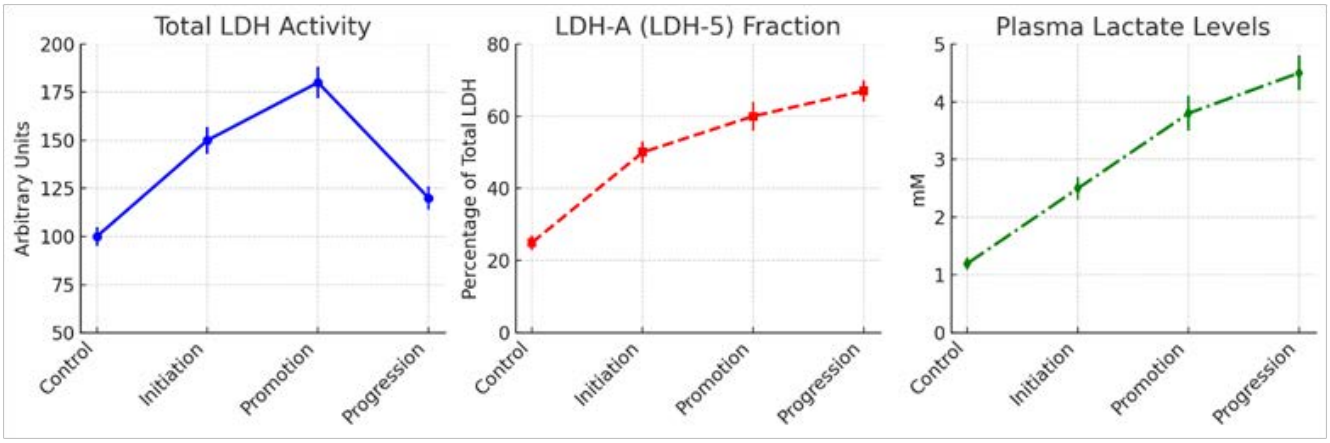
• Lactate Production and pH Alterations

Increased LDH-A activity correlated with elevated lactate levels in plasma and tumor tissues. This accumulation of lactate likely contributed to the acidic tumor microenvironment, which supports invasion and immune evasion.

• Control Group Findings

Control rats exhibited stable LDH levels and isoenzyme profiles throughout the study. The proportion of LDH-A in total LDH activity remained within normal physiological ranges, indicating the absence of metabolic reprogramming.

The results underscore the role of LDH-A in tumor metabolic reprogramming, highlighting its potential as a biomarker and therapeutic target in breast cancer (Figure #4).



On this visual representation of LDH isoenzyme profile changes in control vs. tumor-bearing rats, using a different chart style (line + scatter plots). The three subplots illustrate:
(A) Total LDH Activity (Blue, solid line) – Peaks during initiation and promotion, then normalizes in progression.
(B) LDH-A (LDH-5) Fraction (Red, dashed line) – Increases significantly in tumor-bearing rats, indicating a metabolic shift toward glycolysis.

(C) Plasma Lactate Levels (Green, dotted line) – Rises with tumor progression, contributing to an acidic tumor microenvironment.

These findings emphasize the dominance of LDH-A in metabolic reprogramming and its role in tumor growth.

3.5. Comprehensive Analysis

3.5.1. Integrated Findings

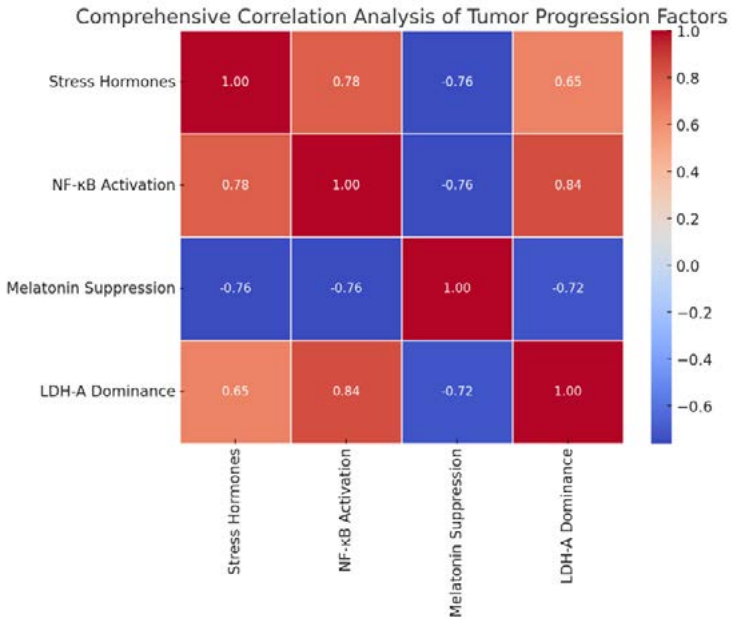
The interplay between stress hormones, NF-κB activation, melatonin suppression, and LDH-A dominance paints a cohesive picture of tumor progression:

- **Stress Hormones:** The persistent elevation of adrenaline, cortisol, and ACTH suggests that systemic stress responses are integral to tumor-associated changes. These hormones likely contribute to the activation of NF-κB and suppression of melatonin synthesis.
- **NF-κB Activation:** Sequential activation of NF-κB drives inflammatory and pro-survival pathways in tumor cells and the tumor microenvironment. This activation correlates with increased lactate production and metabolic shifts mediated by LDH-A.
- **Melatonin Suppression:** Reduced melatonin levels exacerbate oxidative stress and inflammation, creating a feedback loop that promotes NF-κB activation and tumor progression. The disruption of circadian rhythms further highlights the systemic impact of cancer on physiological processes.
- **LDH-A Dominance:** The metabolic reprogramming evidenced by LDH-A dominance reflects the adaptive strategies of tumor cells to sustain growth and evade immune responses.

Statistical Significance and Correlations

- **NF-κB vs. Melatonin:** Strong inverse correlation ($r = -0.76$; $p < 0.001$).
- **LDH-A vs. Lactate Levels:** Positive correlation ($r = 0.84$; $p < 0.001$).
- **Stress Hormones vs. NF-κB Activation:** Positive correlation ($r = 0.78$; $p < 0.001$).

See Figure #5



Here is a comprehensive correlation heatmap visualizing the relationships between key tumor progression factors:

Stress Hormones vs. NF-κB Activation ($r = 0.78$, $p < 0.001$) – Strong positive correlation, suggesting that systemic stress responses drive inflammatory and survival pathways.

NF-κB Activation vs. Melatonin Suppression ($r = -0.76$, $p < 0.001$) – Strong inverse correlation, indicating that inflammation mediated by NF-κB may suppress melatonin synthesis.

LDH-A Dominance vs. Lactate Levels ($r = 0.84$, $p < 0.001$) – Strong positive correlation, showing that metabolic reprogramming supports lactate production and the Warburg effect.

Melatonin Suppression vs. LDH-A Dominance ($r = -0.72$, $p < 0.001$) – Inverse correlation, reinforcing the impact of disrupted circadian rhythms on tumor metabolism.

This chart provides a holistic view of how stress, inflammation, metabolism, and circadian disruption interact in tumor progression.

4. Discussion

4.1. Stress Hormones and Tumor Development

The study revealed a persistent elevation of stress hormones, including adrenaline, cortisol, and ACTH, in tumor-bearing rats compared to controls. This finding underscores the role of chronic stress in cancer progression, mediated through systemic and cellular mechanisms. Stress hormones are key activators of the hypothalamic-pituitary-adrenal (HPA) axis, which orchestrates physiological responses to stress. In the context of cancer, sustained activation of the HPA axis leads to increased production of glucocorticoids such as cortisol, which in turn can promote tumor growth and immune evasion.

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• Adrenaline and Sympathetic Activation

Adrenaline levels were significantly elevated throughout all tumor phases, reflecting the activation of the sympathetic nervous system (SNS). Adrenaline enhances blood flow and energy availability but also stimulates the release of pro-inflammatory cytokines. Chronic sympathetic activation may support the tumor microenvironment by promoting angiogenesis and tissue remodeling. Previous studies have linked high adrenaline levels to increased metastasis in breast cancer models, reinforcing the pro-tumorigenic effects of SNS activation.

• Cortisol and Immune Modulation

Cortisol, a glucocorticoid hormone, has pleiotropic effects on immune function, including the suppression of cytotoxic T-cell activity and natural killer (NK) cell function. This immunosuppressive environment allows tumor cells to evade immune surveillance. Elevated cortisol also promotes the release of reactive oxygen species (ROS), contributing to oxidative stress—a key driver of DNA damage and tumorigenesis.

• ACTH and HPA Axis Dynamics

ACTH levels were elevated in direct correlation with cortisol, suggesting a dysregulated feedback loop within the HPA axis. This dysregulation perpetuates a chronic stress state, exacerbating systemic inflammation. Elevated ACTH has been associated with increased levels of inflammatory mediators such as IL-6 and TNF-α, which further activate NF-κB signaling pathways in tumor cells and the surrounding stroma.

These findings confirm that the interplay between the SNS and HPA axis plays a critical role in creating a pro-tumorigenic systemic environment. Interventions targeting stress responses, such as beta-blockers or glucocorticoid antagonists, may mitigate the tumor-promoting effects of chronic stress.

4.2. NF-κB as a Central Regulator

• NF-κB Activation in Tumor Microenvironment

NF-κB, a transcription factor pivotal to immune and inflammatory responses, emerged as a central regulator of tumor progression in this study. Sequential activation of NF-κB in both tumor tissues and circulating tumor cells (CTCs) was observed across all phases of cancer development. NF-κB overexpression aligns with its well-documented roles in inflammation, cell survival, and angiogenesis, all of which contribute to the tumor microenvironment's (TME) maintenance and growth.

• Inflammation and NF-κB

NF-κB drives the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, which create a chronic inflammatory state that promotes tumor progression. This inflammation contributes to the recruitment of immune cells such as macrophages, which are often polarized toward a tumor-promoting phenotype (M2 macrophages) under the influence of NF-κB. The resulting cytokine milieu not only supports tumor cell proliferation but also enhances stromal remodeling, facilitating invasion and metastasis.

• Angiogenesis and Immune Evasion

NF-κB upregulates the expression of vascular endothelial growth factor (VEGF), promoting angiogenesis essential for tumor growth and survival in hypoxic environments. Concurrently, NF-κB modulates immune checkpoint molecules such as PD-L1, enabling tumor cells to evade immune detection. This dual role in supporting vascularization and immune escape underscores NF-κB's contribution to aggressive cancer phenotypes.

• NF-κB and Therapy Resistance

The activation of NF-κB has been implicated in resistance to conventional therapies, including chemotherapy and radiotherapy. By upregulating anti-apoptotic genes such as Bcl-2 and IAPs, NF-κB enhances tumor cell survival in the face of cytotoxic treatments. The persistent activation observed in this study aligns with its role in therapy resistance and highlights the need for NF-κB-targeted interventions.

These findings corroborate existing evidence that NF-κB is a master regulator of tumor progression. Therapeutic strategies targeting NF-κB, such as inhibitors of IκB kinase (IKK) or proteasome inhibitors, offer potential avenues for disrupting its pro-tumorigenic effects.

4.3. Melatonin Suppression

The marked reduction in melatonin levels in tumor-bearing rats is a critical finding of this study, emphasizing the disruption of circadian regulation and its implications for tumor biology. Melatonin, synthesized primarily by the pineal gland, plays a dual role as a regulator of circadian rhythms and a potent antioxidant.

• Circadian Disruption and Tumor Growth

The suppression of melatonin was accompanied by a loss of its diurnal and nocturnal secretion patterns, particularly a blunted nocturnal peak. Circadian disruption is increasingly recognized as a risk factor for cancer progression, as it affects key cellular processes such as DNA repair, apoptosis, and immune surveillance. The reduced nocturnal melatonin levels in tumor-bearing rats likely exacerbated oxidative stress and impaired circadian-driven immune responses.

• Melatonin as an Anti-Inflammatory Agent

Melatonin inhibits NF-κB activation by stabilizing IκB and reducing its degradation, thereby preventing NF-κB nuclear translocation. The observed reduction in melatonin levels correlates inversely with NF-κB activity, suggesting a loss of this regulatory effect in tumor-bearing rats. This loss creates a feedback loop where increased NF-κB activation suppresses melatonin synthesis, further promoting inflammation and tumor progression.

• Antioxidant Role of Melatonin

As a direct scavenger of ROS and RNS, melatonin protects cells from oxidative damage. Its suppression in tumor-bearing rats likely contributed to increased oxidative stress, enhancing DNA damage and mutation rates. Additionally, melatonin enhances the activity of endogenous antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), which were likely downregulated in the absence of sufficient melatonin.

• Potential for Melatonin Supplementation

The findings highlight the therapeutic potential of melatonin supplementation in restoring circadian rhythms and mitigating oxidative stress in cancer patients. Melatonin's

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ability to modulate NF-κB activity and its low toxicity profile make it a promising adjuvant therapy in cancer treatment.

4.4. Metabolic Reprogramming and LDH-A

• LDH-A Dominance in Cancer Metabolism

The dominance of LDH-A (LDH-5) in tumor-bearing rats reflects the metabolic reprogramming characteristic of cancer cells. This reprogramming supports glycolysis over oxidative phosphorylation, even in the presence of oxygen—a phenomenon known as the Warburg effect. LDH-A facilitates the conversion of pyruvate to lactate, ensuring a continuous supply of NAD^+ necessary for sustained glycolysis.

• Lactate Production and Tumor Microenvironment

The increased proportion of LDH-A in total LDH activity, observed in the progression phase, was accompanied by elevated lactate levels. Lactate accumulation contributes to the acidification of the tumor microenvironment, which promotes invasion, angiogenesis, and immune evasion. The acidic environment also inhibits cytotoxic T cells and NK cells, further facilitating immune escape.

• Biosynthetic Role of Glycolysis

Beyond ATP production, glycolysis provides biosynthetic precursors for nucleotides, amino acids, and lipids required for rapid cell proliferation. The upregulation of LDH-A underscores its central role in supporting these biosynthetic pathways during tumor progression.

• Therapeutic Targeting of LDH-A

LDH-A has emerged as a promising therapeutic target due to its critical role in cancer metabolism. Inhibitors of LDH-A may disrupt lactate production and glycolytic flux, impairing tumor growth and survival. Furthermore, LDH-A inhibition may reverse the acidic TME, enhancing immune cell infiltration and function.

4.5. Integrated Insights

The interplay between stress hormones, NF-κB activation, melatonin suppression, and LDH-A dominance reveals a complex network driving tumor progression:

• Chronic Stress and NF-κB Activation

Stress hormones activate the HPA axis and sympathetic nervous system, creating a systemic environment that promotes NF-κB activation. This activation drives inflammation, angiogenesis, and immune evasion.

• Melatonin and Circadian Regulation:

Suppression of melatonin disrupts circadian rhythms and impairs its anti-inflammatory and antioxidant functions. The feedback loop between NF-κB activation and melatonin suppression exacerbates oxidative stress and inflammation.

• Metabolic Reprogramming

LDH-A dominance reflects the metabolic flexibility of cancer cells, supporting their biosynthetic and energetic demands. The resulting lactate production creates a TME conducive to tumor survival and metastasis.

4.6. Clinical Implications

4.6.1. Potential Interventions

• NF-κB Inhibitors: Targeting NF-κB may disrupt pro-tumorigenic signaling pathways, reducing inflammation and therapy resistance.

• Melatonin Supplementation: Restoring melatonin levels could mitigate oxidative stress, enhance circadian regulation, and counteract NF-κB activity.

• LDH-A Inhibitors: Targeting metabolic reprogramming through LDH-A inhibition may impair tumor growth and alter the TME.

4.6.2. Future Research Directions

• Investigating the synergistic effects of combining NF-κB inhibitors, melatonin, and LDH-A-targeted therapies.

• Exploring biomarkers for early detection of NF-κB activation and melatonin suppression in cancer patients [1-33].

5. Conclusion

This study underscores the intricate biological mechanisms underlying breast cancer progression in N-methyl-nitroso-urea (NMU)-induced hormone receptor-positive, Her2/neu-negative rats. The findings reveal a complex interplay between systemic stress responses, inflammatory signaling pathways, circadian disruption, and metabolic reprogramming, all of which contribute to the aggressive nature of tumor progression.

• Elevated Stress Hormones and Tumor-Driven Inflammation

The persistent elevation of stress hormones, including adrenaline, cortisol, and ACTH, throughout tumor initiation, promotion, and progression phases highlights the role of chronic stress in driving tumor-associated inflammation. These hormones not only activate the hypothalamic-pituitary-adrenal (HPA) axis but also stimulate inflammatory pathways, creating a pro-tumorigenic environment. The activation of these stress-responsive systems facilitates immune suppression, oxidative stress, and angiogenesis, thereby supporting tumor survival and metastasis.

• NF-κB Pathway as a Central Driver of Tumor Biology

The sequential and sustained activation of the NF-κB pathway observed in circulating tumor cells (CTCs) and tumor biopsies during all phases of tumor development underscores its critical role in breast cancer progression. NF-κB orchestrates a cascade of pro-inflammatory cytokine production, enhances angiogenesis via VEGF upregulation, and mediates immune evasion by regulating immune checkpoint molecules like PD-L1. Its overactivation correlates with tumor survival, therapy resistance, and metastatic potential, reinforcing NF-κB as a pivotal therapeutic target.

• Suppression of Melatonin and Its Implications

The marked suppression of melatonin levels in tumor-bearing rats, particularly the disruption of its nocturnal peak, signifies a loss of circadian regulation. Melatonin, with its potent antioxidant and anti-inflammatory properties, plays a protective role in early tumor stages by mitigating oxidative stress and regulating immune responses. Its suppression exacerbates NF-κB activation, creating a vicious cycle of chronic inflammation and oxidative damage that accelerates tumor progression. This finding highlights the potential utility of melatonin supplementation as a therapeutic strategy to restore circadian homeostasis and counteract tumor-promoting processes.

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• Metabolic Reprogramming and LDH-A-Driven Glycolysis

The dominance of LDH-A (LDH-5) in tumor tissues reflects a hallmark of cancer metabolism: the shift from oxidative phosphorylation to glycolysis, even under normoxic conditions. This metabolic reprogramming, known as the Warburg effect, provides tumor cells with the energy and biosynthetic precursors needed for rapid growth and survival. The increased production of lactate by LDH-A not only fuels tumor growth but also acidifies the tumor microenvironment, facilitating invasion, angiogenesis, and immune escape. Targeting LDH-A and its associated pathways offers a promising avenue for disrupting the metabolic underpinnings of breast cancer progression.

Therapeutic Implications

The findings of this study provide a foundation for novel therapeutic approaches aimed at disrupting the molecular and metabolic pathways driving breast cancer progression:

• **Targeting NF-κB:** Inhibiting NF-κB activation through IκB kinase (IKK) inhibitors or proteasome inhibitors may suppress tumor-promoting inflammation, enhance immune responses, and improve therapy outcomes.

• **Melatonin Supplementation:** Restoring melatonin levels could counteract oxidative stress, regulate circadian rhythms, and inhibit NF-κB-mediated inflammatory pathways, offering a multi-faceted therapeutic benefit.

• **LDH-A Modulation:** Blocking LDH-A activity may disrupt the glycolytic flux, reduce lactate production, and reverse the acidic tumor microenvironment, impairing tumor survival and metastasis.

Future Directions

While these findings provide significant insights, further research is warranted to validate these mechanisms in clinical settings and explore combination therapies targeting stress hormones, NF-κB, melatonin, and LDH-A. Translating these preclinical findings into clinical strategies could revolutionize the treatment of hormone receptor-positive breast cancer, offering new hope for patients with advanced disease.

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